ENHANCED HYDROCARBON BIODEGRADATION USING BIOAUGMENTATION WITH BIOWISH™-AQUA FOG

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Committee Membership

**TITLE: ENHANCED HYDROCARBON BIODEGRADATION USING
BIOAUGMENTATION WITH BIOWISH™-AQUA FOG**

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Abstract

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This study was done to determine the effectiveness of a commercially available bioaugmentation product, BiOWiSH™-Aqua FOG, for remediating petroleum-contaminated sandy soil. Biodegradation enhancement by BiOWiSH™-Aqua FOG was evaluated in laboratory microcosms by directly measuring total petroleum hydrocarbon (TPH) and indirectly using respirometry. Attempts were made to enrich hydrocarbon-degrading bacteria in BiOWiSH™-Aqua FOG, and the resulting enrichment cultures were screened using respirometry as well. Potential hydrocarbon-degrading bacteria in BiOWiSH™-Aqua FOG were isolated. Experiments were performed at bench-scale using microcosm bottles containing sand contaminated with either motor oil or No. 2 diesel fuel. The microcosms were incubated at 25°C under aerobic conditions. TPH measurements of soil in the microcosms at 0, 25 and 56 days indicated that the addition of 500-ppm BiOWiSH™-Aqua FOG improved biodegradation of the motor oil-contaminated soil by 45%. However, BiOWiSH™-Aqua FOG did not have a measurable effect on biodegradation in the diesel-contaminated soil.

In the respirometry experiments, BiOWiSH™-Aqua FOG and two hydrocarbon-enriched BiOWiSH™-Aqua FOG cultures were evaluated indirectly by the measurement of microbial carbon dioxide production and oxygen uptake using a

MicroOxymaxTM respirometer. The respirometry experiments showed that in the six-day period following motor oil-contamination of soil, the addition of BiOWiSHTM-Aqua FOG substantially improves biodegradation rates. The added organisms in the product out-performed the indigenous organisms in the 5-6 days following contamination of the soil. The CO₂ production observed in the BiOWiSHTM microcosms contaminated with motor oil was much greater than CO₂ production without motor oil, which confirms that the observed metabolism can be attributed to motor oil biodegradation rather than metabolism of other organic material in the soil. Enriched consortia consistently generated far less CO₂ than microcosms with the 500 ppm BiOWiSHTM-Aqua FOG. Stoichiometric calculations suggested that BiOWiSHTM-Aqua FOG removed approximately 1400 ppm TPH (14%) from the soil in 6.5 days, while an enrichment culture of BiOWiSHTM-Aqua FOG only reduced TPH levels by 459 ppm (5%). This result suggests that increased biodegradation rate in bioaugmented soil is aided by biodiversity in the augmenting inoculum. A potential hydrocarbon-degrading candidate organism was isolated from the product and cultured on Bushnell-Haas agar and plate-count agar (PCA). While at least two distinct colony types were successfully grown on media with motor oil, these same colonies appeared on Bushnell-Haas agar with no apparent carbon source, and survived repeated transfers onto this same medium. Therefore, their status as hydrocarbon-degraders is inconclusive. More thorough enrichment work could be pursued, especially using soil samples collected from petroleum-contaminated sites.

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Introduction

Petroleum compounds constitute a major threat to environmental quality (Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, & Naidu, 2011). Soil can become contaminated from leaky underground storage tanks and industrial facilities involved in the production and distribution of petroleum products (Mariano, de Angelis, Pirollo, Contiero, & Bonotto, 2009). Offshore oil spills release crude oil onto beaches and into estuaries (Jernelov, 2010). Damage to the environment persists even after crude oil concentrations have dropped below lethal levels (Peterson et al., 2003). Petroleum is a complex mixture of hydrocarbon chains and ring structures of various molecular weights, and its levels in the environment are measured as total petroleum hydrocarbon (TPH). Bioaugmentation of the contaminated site with added organisms can enhance the rate at which the contaminating petroleum compounds are broken down (Atlas, 1995). This study was done to determine the effectiveness of a commercially available product, BiOWiSH™-Aqua FOG, in remediating petroleum-contaminated soil. BiOWiSH™-Aqua FOG was evaluated by two methods: direct TPH measurement by extraction and GC/MS analysis and indirectly by the measurement of microbial carbon dioxide production and oxygen uptake using the MicroOxymax™ Respirometer. These experiments were performed at the bench-scale in sealed 1-gallon Pyrex™ microcosm bottles containing sand contaminated with motor oil and diesel fuel. The product was also enriched for hydrocarbon degrading organisms, and a hydrocarbon-degrading candidate organism was isolated from the product and cultured independently on both Bushnell-Haas agar and Plate Count Agar.

Background

Hydrocarbon Contamination of Environment

Soil can become contaminated from leaky underground storage tanks (LUST) and industrial facilities involved in the production and distribution of petroleum products (Mariano, et al., 2009). Offshore oil spills, such as the *Exxon-Valdez* and the *Deepwater Horizon*, can contaminate beaches and estuaries with crude oil (Jernelov, 2010). The *Exxon-Valdez* spill caused damage to the environment that persisted even after crude oil concentrations had dropped below lethal levels (Peterson, et al., 2003). Much of the light crude spilled by the *Deepwater Horizon* explosion was deposited on the shoreline, and will have dire implications for those ecosystems (Kostka et al., 2011). In these situations, it was necessary that the contaminants biodegrade as quickly as possible to minimize danger to public health and to prevent environmental damage. One approach is bioaugmentation of the indigenous microbial population with added organisms to enhance biodegradation rates (Atlas, 1995). The composition of the petroleum hydrocarbon mixture will determine the ease or difficulty with which the mixture can be biodegraded (Atlas, 1981). Environmental hydrocarbon contaminants come in two main varieties, aliphatics and aromatics. The aliphatics can be saturated (alkanes), unsaturated (alkenes, alkynes) or may form cyclic ring structures. Aromatic hydrocarbons are compounds that contain one or more aromatic ring structures. Some contaminants are more readily degraded by the indigenous microbes than others (Tyagi, da Fonseca, & de Carvalho, 2011). Alkanes tend to be quickly metabolized, while polynuclear aromatic hydrocarbons (PAH) are extremely recalcitrant (Van Hamme, Singh, & Ward, 2003). The levels of nitrogen and phosphorous can also limit the amount of petroleum that can be degraded, as can the availability of oxygen in the

soil (Atlas, 1981). Petroleum compounds are non-polar and hydrophobic, and thus do not disperse easily in aqueous environments, limiting their availability to microbes, which live in the presence of water.

Bioremediation is the biodegradation of environmental contaminants by living organisms. In some cases, this process can be aided by the addition of a cultured strain or consortium of microbes foreign to the contaminated site. These foreign organisms would be ideally suited to metabolizing the contaminant. They might be obtained from another contaminated site, or be a culture of indigenous organisms enriched using the contaminant. In the case of a fresh spill, the advantage of adding microorganisms to a contaminated site would be that the inoculum can have the ability to rapidly degrade the contaminant, whereas more time may be required for the indigenous microbes to adjust their metabolisms and begin biodegradation (da Silva, Alvarex, & Timmis (ed.), 2010). In the case of a contaminated site that may be decades old, the indigenous population may not have been able to degrade the more recalcitrant chemical species, and adding competent organisms could improve overall contaminant removal. Another attractive feature of this approach is that detrimental environmental effects of the bioremediation/bioaugmentation treatment are negligible or even nonexistent (Atlas, 1995).

Fertilizing chemicals such as bioavailable nitrogen and phosphorous compounds are introduced to the contaminated site (Tyagi, et al., 2011). This aspect of remediation is termed “biostimulation.” Additional measures become necessary when natural attenuation of contaminants is limited by the chemical properties of the contaminants,

nutrient concentrations, contaminant bioavailability, and the suitability of the indigenous organisms to metabolize the contaminant. A comprehensive bioremediation strategy addresses all of the limitations of natural attenuation of the contaminants. Adding nitrates, phosphates and ammonia, prevents these nutrients from limiting the metabolism of the petroleum contaminants. Repeated tilling of the soil throughout the treatment process aerates the soil, replenishing oxygen (Mishra, Jyot, Kuhad, & Lal, 2001). Surfactants can also be used to disperse the petroleum compounds, increasing their availability to the microorganisms. If bioaugmentation is used, the added organisms would be able to aid in the metabolism of the contaminants if the indigenous microbes are not sufficiently effective.

Studies of the Effectiveness of the Bioaugmentation Approach

A number of *in situ* and laboratory studies have been conducted to determine the effectiveness of bioaugmentation of petroleum hydrocarbon contaminants. In one study, indigenous organisms were enriched and isolated from a contaminated site and then re-inoculated to augment the site's microbial population (Rosenberg et al., 1992). After approximately one month, bioaugmentation resulted in 88% removal of hydrocarbons, while natural attenuation in the control plots resulted in only 15% degradation. One study found that a commercially available bioaugmentation product, Terrazyme®, was effective at remediating crude oil-contaminants, reducing the coverage of oil residue on beach-side rocks (Tsutsumi et al., 2000). A closely related study found that adding organisms using the Terrazyme® commercial product dramatically reduced the petroleum hydrocarbons present after 3 weeks had elapsed (Hozumi, Tsutsumi, & Kono, 2000). An added microbial inoculum including

Acinetobacter baumannii, *Burkholderia cepacia*, and *Pseudomonas sp.* provided a dramatic increase in biodegradation of oily sludge at an old oil refinery: the two plots of soil to which microbes were added achieved 90% degradation in 120 days, while the control plot featured 14% degradation (Mishra, et al., 2001). The effectiveness of bioaugmentation was demonstrated when used with nutrient amendments to the soil (Rahman et al., 2003). The addition of biosurfactants increased contaminant bioavailability. The study found that the addition of a hydrocarbon-degrading bacterial consortium improved degradation of n-alkanes in the 12-40 equivalent carbon range. Bioaugmentation was effective in treating soil from a 65 year-old superfund site (Popile, Inc.) contaminated with polynuclear aromatic hydrocarbons (PAH), with 87% removal after 11 months, significantly greater than the 34% removal accomplished by biostimulation alone (Straube et al., 2003). One study found that an added inoculum used in the bioaugmentation approach is best obtained from the site to be treated, enriched and then re-injected to bolster the number of competent organisms in the soil (Bento, Camargo, Okeke, & Frankenberger, 2005). Bento, et al., enriched a consortium of indigenous hydrocarbon-degrading *Bacillus cereus*, *Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus pumilus*, *Acinetobacter junii* and *Pseudomonas spp.* from soil obtained from Long Beach, CA. Malina and Zawierucha (2007) found that when KMnO_4 was used to oxygenate soil obtained from an abandoned air field contaminated with oil, respiration rates increased 71-97% for bioaugmentation compared to biostimulation alone. Bioaugmentation can be an effective strategy when toxic co-contaminants (in this case heavy metals) inhibit biodegradation rates. By the end of 42 days, increased respiration rates and a 75% reduction in total hydrocarbons were

observed when bioaugmentation of soil contaminated over an 90 year period (Buondonno, Ermice, Buondonno, Murolo, & Pugliano, 1998) was compared to the controls (Alisi et al., 2009). A bench scale study (Tahhan, Ammari, Goussous, & Al-Shdaifat, 2011) found that adding two bacterial consortia comprised of *Pseudomonas spp.*, *Micrococcus spp.*, and *Bacillus spp.* to oily sludge increased total petroleum hydrocarbon removal rates by over 30% and that degradation can be enhanced by multiple injections of the augmenting organisms over time. A four-phase remediation approach (Chien, Kao, Surampalli, Huang, & Hou, 2011): flushing with a surfactant, flushing with water, oxidation of the soil with KMnO_4 , and inoculation of petroleum-degrading *Pseudomonas spp.*, caused 77% removal while natural attenuation resulted in only 41% removal.

Under some conditions, simple biostimulation with nutrients and oxygen are sufficient to accomplish biodegradation of the contaminants and an added consortium will either have no effect or be detrimental to the overall degradation rate. A bench scale study in aerobic bioreactors measured the degradation of spent motor oil in top soil that had been contaminated for 40 years. Natural attenuation resulted in 50% contaminant removal, bioaugmentation caused 66% removal, and biostimulation was found to be the most effective, removing 75% of the oil (Abdulsalam, Bugaje, Adefila, & Ibrahim, 2011). In this case, bioaugmentation failed to be the most effective approach for contaminant removal, possibly due to the long history of contamination in the soil used in the experiment. Bioaugmentation with *Staphylococcus hominis* and *Kocuria palustris* failed to cause any improvement in the degradation of diesel fuel in soil from three gas stations (Mariano, et al., 2009). Bioaugmentation was ineffective and possibly

deleterious to diesel biodegradation in sand (Demque, Biggar, & Heroux, 1997). A pilot study was conducted on twenty soil plots representing ten different treatment conditions and the various contributions of bioaugmentation, biostimulation, aeration (tilling), were determined by measuring TPH and BTEX levels. It was found that bioaugmentation was not successful in improving the removal rate of diesel. Venosa et al., (1996) compared biostimulation/bioaugmentation of soil plots contaminated with light crude oil to untreated contaminated plots. The results indicated a significant difference in the hydrocarbon degradation rates between the bioaugmented/biostimulated plots and the control plots. However, there was not a significant difference between the bioaugmented plots and the biostimulated plots.

Table 1 below provides a summary of the petroleum hydrocarbon degradation/bioaugmentation studies described above in chronological order, from oldest to newest. Papers are listed by first author, the year the study was published, the contaminant, the bioaugmenting inoculum, the scale of the experiments and treatments, and the outcome. Despite the numerous studies characterizing the advantages, limitations, and effectiveness of bioaugmentation as a technique for remediating environmental contaminants, there continue to be challenges associated with implementation of the technique. Da Silva and Alvarez point out that it cannot always be proven that bioaugmentation worked properly, since it is very challenging to determine whether the augmenting organisms are actually responsible for the removal of contaminants. Proper controls must be used to distinguish the possible factors that contribute to degradation rates, and this is not always practical in pilot or full-scale studies.

Table 1. Summary of bioaugmentation/biodegradation of petroleum compounds in soil.

Study	Year	Contaminant	Inoculum	Scale	Contamination History	Outcome
(Rosenberg, et al.)	1992	Oil Spill	Enriched indigenous organisms	Pilot	Not specified	Success
(Venosa, et al.)	1996	Light Crude Oil	Enriched indigenous organisms	Pilot	Naïve	Failure
(Demque, et al.)	1997	Diesel Fuel	Enriched indigenous organisms	Pilot	Unknown	Failure
(Tsutsumi, et al.)	2000	Crude Oil	<i>Terrazyme®</i> Commercial product	Pilot	3 years	Success
(Hozumi, et al.)	2000	Crude Oil	<i>Terrazyme®</i> Commercial product	Bench	3 years	Success
(Mishra, et al.)	2001	Oily Sludge	<i>Acinetobacter baumannii</i> , <i>Burkholderia cepacia</i> , <i>Pseudomonas</i> from a previously contaminated site	Pilot, Full	Not specified	Success
(Rahman, et al.)	2003	Oily Sludge	<i>Bacillus</i> , <i>Pseudomonas</i> , <i>Micrococcus</i> hydrocarbon-degrading isolates	Bench	Naïve	Success
(Straube, et al.)	2003	PAH	<i>Pseudomonas aeruginosa</i> Strain 64	Bench	65 years	Success
(Bento, et al.)	2004	Diesel Oil	Indigenous Long Beach, CA HC-degrading <i>Bacillus cereus</i> , <i>Bacillus sphaericus</i> , <i>Bacillus fusiformis</i> , <i>Bacillus pumilus</i> , <i>Acinetobacter junii</i> and <i>Pseudomonas spp.</i>	Bench	Not specified	Success
(Malina and Zawierucha)	2007	Oil	Not specified	Bench	~60 years	Success
(Alisi, et al.)	2009	Diesel Oil, Heavy Metals	Heavy metal resistant bacterial strains	Bench	90 years	Success
(Mariano, et al.)	2009	Diesel Fuel	<i>Staphylococcus Hominis</i> , <i>Kocuria palustris</i>	Bench	10 years	Failure
(Tahhan, et al.)	2011	Oily Sludge	<i>Pseudomonas spp.</i> , <i>Micrococcus spp.</i> , and <i>Bacillus spp.</i>	Bench	Not specified	Success
(Abdulsalam, et al.)	2011	Spent Motor Oil	<i>Bacillus subtilis</i> , <i>P. aeruginosa</i>	Bench	40 years	Failure
(Chien, et al.)	2011	Diesel Oil	Enriched indigenous <i>Pseudomonas spp.</i>	Bench	Not specified	Success

Despite the numerous studies characterizing the advantages, limitations, and effectiveness of bioaugmentation as a technique for remediating environmental contaminants, there continue to be challenges associated with implementation of the technique. Da Silva and Alvarez point out that it cannot always be proven that bioaugmentation worked properly, since it is very challenging to determine whether the augmenting organisms are actually responsible for the removal of contaminants. Proper controls must be used to distinguish the possible factors that contribute to degradation rates, and this is not always practical in pilot or full-scale studies. Failure of bioaugmentation to increase biodegradation rates can be due to several causes (Goldstein, Mallory, & Alexander, 1985). If the inoculated microbes are subjected to predation by other organisms on site, and are removed faster than they can multiply, then the added population will not survive. The added organisms will not remove the contaminant if it prefers to metabolize other substrates. Some kind of chemical may be present on-site that inhibits growth of the added microorganisms. If the contaminant concentration is too low, and the added organisms require it for growth, they will not thrive. Finally, the added organisms may not be able to come into physical contact with pollutant. These complicating issues can render bioaugmentation treatment ineffective, and makes proper site characterization necessary to confirm that bioaugmentation will have a decent chance of being successful.

More research is needed to determine how best to apply added microbes to the site in question and how the microbes will move through the soil once they have been introduced to the site. Each contaminated site has a unique set of properties, all of which affect the natural rate of attenuation of contaminants and the suitability of

bioaugmentation as a remediation strategy. In some cases, these properties can conspire to render the bioaugmentation approach ineffective, and the added microbes can even hinder natural attenuation (da Silva, Alvarez and Timmis 2010).

To develop a bioaugmentation strategy that has the greatest chance of success, the structure, coordination, interaction, and the microorganisms (foreign and indigenous) need to be studied (Boon, Verstraete, & Timmis (ed.), 2010). The substrates and their respective availabilities and concentration gradients will affect the metabolism of the microbial community. The growth of the organisms will be determined by the intercellular signaling that cause biofilms to form and sets limits on population size. Not only will cellular growth be impacted by intercellular coordination, but also by interactions with the environment and chemical species present within it. The organisms, foreign and indigenous, will affect the outcome of any bioaugmentation strategy, and should also be characterized. In short, the microbial ecology of the contaminated site should be evaluated as fully as possible in order for bioaugmentation to have best possible chance of having a positive outcome. Because of the complexity of the array of variables and factors that influence the outcome, bioaugmentation does not succeed in all situations, and therefore its appropriateness must be evaluated on a case-by-case basis (Tyagi, et al., 2011; vanVeen, vanOverbeek, & vanElsas, 1997).

BiOWiSH™-Aqua FOG as a Bioaugmentation Strategy for Petroleum Contamination

BiOWiSH™-Aqua FOG is a consumer product developed by BiOWiSH™ Technologies, Inc. It consists of a proprietary blend of dormant soil bacteria and fungi, as well as carbon and nitrogen sources, which provide the nutrient component of the product. It also contains the surfactant saponin, which has the ability to disperse insoluble material. The manufacturer recommends the product for use on municipal sewage, private sewage, and industrial wastewater. BiOWiSH™ Technologies, Inc. also believes that the product may be useful in remediation soil of contaminated with hydrocarbons. According to the product's online documentation (www.biowishtechnologies.com May 3, 2012), BiOWiSH-Aqua™ FOG has the following benefits.

- “Reduces sludge production and handling (up to 60%)
- Increases plant capacity by reducing contact time (capital avoidance)
- Eliminates odorous emissions
- Delivers substantial energy savings due to less aeration (30 to 50%)
- Lessens the need for chemical additives
- Stabilizes and improves plant operations
- Removes hydrogen sulphide, ammonia and nitrates
- Pre-treats influent in collection systems
- 100% natural and non-toxic”

The mechanism and effectiveness by which this product will remediate oil and grease (hydrocarbons) may be due to contributions of the bacterial, nutrient, and surfactant components. A photograph of the product label is shown in Figure 1 below.

Figure 1. The BiOWiSH™-Aqua FOG bioaugmentation product.

Methods

For the remediation experiments, soil microcosms were prepared from sand obtained from Los Osos, California. Three hundred-gram soil microcosms were prepared for the first experiment. Smaller 200-g sand microcosms were used in the second experiment. The sand was fully characterized by measuring its bulk density, porosity, organic content, moisture content, and cation exchange capacity (fertility).

Microcosm Sand Characterization

Sand obtained from Los Osos, California, was passed through a no. 40 sieve (420 microns) to remove large rocks and plant material. The sand was then sieved using a no. 100 sieve (149 micron) to remove fine clay particles.

Bulk Density

Five 10-mL samples of the sand obtained above approximately were used to determine the bulk density. Each sample was transferred to a container and placed in an oven at 105 °C to evaporate all water content. Each sample was weighed, and each weight divided by the volume to determine the bulk densities of each sample.

Porosity

Five sand samples of the Los Osos sand were transferred heated in an oven at 105 °C for 15 min. The samples were then placed in 15-mL beakers. Water was pipetted into the beakers until sand became saturated. The amount of water added was recorded and the saturated sand was weighed.

pH

The method used to measure pH was adapted from EPA Standard Method #9045d. Five samples of the Los Osos sand were dried in an oven at 105 °C for 15 min. 10-mL

volumes of DI water were added to each sand sample. The soil-water mixture was stirred for 30 seconds every 3 minutes for a total of three stirring/waiting cycles. The mixture was allowed to settle until a clear liquid formed above the settled soil. A calibrated pH probe was immersed in each water/sand mixture and the pH was recorded. The electrode reading was verified by using narrow range pH paper (6.0-6.5, Fischer Scientific). Three samples of wet soil with Miracle-Gro™ All-Purpose Plant Food were also measured as described above to determine the effect of the addition of the fertilizer on the soil pH.

Moisture content

Five samples of the Los Osos sand were obtained and weighed. Each sample was transferred to a container and placed in an oven at 105 °C for 15 min to evaporate all water content and weighed a second time.

Organic Content (Volatile Solids)

The dried sand from the moisture content determination described above was placed in a 550°C oven for 15 min and each sample was weighed.

Cation Exchange Capacity (CEC)

Five 5-g samples of the Los Osos sand were each placed in 50-mL centrifuge tubes. 20 mL of 1 M ammonium acetate was added to each centrifuge tube. The tubes were placed on a shaker for 30 minutes. The tubes were then spun at 2000 rpm for 5 minutes in a centrifuge. The supernatant was poured off into a beaker and set aside. Another 20 mL of ammonium acetate was added to each centrifuge tube, placed onto a

vortex mixer, shaken, and centrifuged. The supernatant was added to the previous supernatant and then diluted to 50 mL using a volumetric flask.

1:11 dilutions were prepared of each extract by adding 1 mL of the extract to 10 mL of 1 M ammonium acetate. The original extracts and the dilutions were analyzed for calcium, magnesium, sodium, and potassium using an atomic absorption spectrophotometer (AAS). The AAS was configured and calibrated by Dr. Chip Appel using a four-point calibration and initial and final calibration verifications using standards.

Microcosm Preparation

A total of 24 soil microcosms were prepared in 1-gallon glass jars with TeflonTM-lined lids. 12 microcosms were contaminated with 10,000 ppm No. 2 Diesel fuel, and 12 microcosms were contaminated with 10,000 ppm SAE 30 motor oil. Three of the diesel-contaminated microcosms were inoculated with 10 ppm of the BiOWiSHTM, three were inoculated with 100 ppm, and three were inoculated with 500 ppm. Three microcosms were not inoculated. This procedure was repeated for the motor oil-contaminated microcosms as well.

To prepare the motor-oil contaminated soil, 2.988 kg of the sieved Los Osos sand was combined with 36 g of Castrol® HD SAE 30 motor oil in a 6 gallon steel trash can for a target concentration of 10,000 ppm motor oil. To prepare the diesel-contaminated soil, 3.276 kg of the sieved Los Osos sand was combined with 39 g of No. 2 diesel fuel in a 6 gallon steel trash can for a target concentration of 10,000 ppm diesel.

The microcosms needed to be biostimulated with enough nitrogen and phosphorous to ensure that hydrocarbon metabolism of the contaminants was not nutrient-limited. The microbial metabolism can be reduced to the following chemical equations, the first representing bacterial production of biomass and the second representing respiration. Based on published C:N ratios the composition of the biomass was assumed to be $C_5H_7O_2N$ (Rittmann & McCarty, 2001). Octane was assumed to be representative of the hydrocarbon mixture.

The doses of nitrogen fertilizer were determined as follows. There were 3.00 g of petroleum (motor oil or diesel) in each microcosm bottle. From the simple calculation shown below, it can be determined that there are 26.3 mmol octane in each microcosm.

$$3 \text{ g } C_8H_{18} \left(\frac{1 \text{ mol}}{114.08 \text{ g}} \right) \left(\frac{1000 \text{ mmol}}{1 \text{ mol}} \right) = 26.3 \text{ mmol } C_8H_{18}$$

A minimum of 40% of the carbon is used for biomass production (Rittman, et al. 2001) and the rest of the carbon is converted to carbon dioxide. The number of moles of nitrogen needed so that the octane is converted to biomass is given by stoichiometry based on the balanced chemical reaction for the production of biomass given above.

$$40\% \times 26.30 \text{ mmol } C_8H_{18} \left(\frac{8 \text{ mmol } C_5H_7O_2N}{5 \text{ mmol } C_8H_{18}} \right) \times$$

$$\left(\frac{1 \text{ mmol } N}{1 \text{ mmol } C_5H_7O_2N} \right) \left(\frac{1 \text{ mol } N}{1000 \text{ mmol } N} \right) = 0.0168 \text{ mol } N$$

The composition of Miracle-Gro™ All-Purpose Plant Food is 24-8-16 NPK, or 24% nitrogen compounds (3.5% ammonia, 20.5% urea), 8% phosphorous compounds, and 16% potassium compounds. This means that in each gram of Miracle-Gro™, there are 35 mg of NH₃ and 205 mg of CH₄N₂O.

$$0.035 \text{ g NH}_3 \left(\frac{1 \text{ mol NH}_3}{17 \text{ g NH}_3} \right) \left(\frac{1 \text{ mol N}}{1 \text{ mol NH}_3} \right) = 0.00206 \text{ mol N}$$

$$0.205 \text{ g CH}_4\text{N}_2\text{O} \left(\frac{1 \text{ mol CH}_4\text{N}_2\text{O}}{60.06 \text{ g CH}_4\text{N}_2\text{O}} \right) \left(\frac{2 \text{ mol N}}{1 \text{ mol CH}_4\text{N}_2\text{O}} \right) = 0.00683 \text{ mol N}$$

Therefore there are 0.00889 moles of N for each gram of Miracle-Gro™ All Purpose Plant Food. The volume of 125 g/L Miracle-Gro™ stock solution to add was determined by the following computation.

$$\begin{aligned} 0.0168 \text{ mol N Needed} & \left(\frac{1 \text{ g Miracle Gro}}{0.00889 \text{ mol N}} \right) \left(\frac{\text{L Stock solution}}{125 \text{ g Miracle Gro}} \right) \left(\frac{1000 \text{ mL}}{1 \text{ L}} \right) \\ & = 15.1 \text{ mL Miracle Gro Stock solution Needed} \end{aligned}$$

Therefore, 15 mL of the 125 g/L stock solution was added to each microcosm to ensure that metabolism of the petroleum compounds was not limited by nitrogen availability. Table 2 below summarizes the experimental design. When all components were combined, the total mass of each microcosm was 300 g. Each microcosm was prepared in triplicate.

Table 2. Experimental Design

Contaminant	BiOWiSH™-Aqua FOG Dose (ppm)
Diesel Fuel	0 (Control)
	10
	100
	500
Motor Oil	0 (Control)
	10
	100
	500
Replications: 3 for each condition	
Incubation: Aerobic, Temperature = 25°C in the Dark	
Nutrients: 15.0 mL Miracle-Gro™ All Purpose Plant Food (9.4 g/kg soil)	
Sampling: At 0, 25, and 56 Days	

Table 3. Microcosm Recipes.

Final BiOWiSH™ Concentration	Water (mL)	5000 ppm BiOWiSH™ (mL)	125 g/L Miracle-Gro™ (mL)	Sand/Petroluem mix (g)
0 ppm	30.0	0.0	15	255
10 ppm	29.4	0.6		
100 ppm	24.0	6.0		
500 ppm	0.0	30.0		

The composition of the sand microcosms is given in Table 3. Since the microcosms were prepared in triplicate, the water, Miracle-Gro™ solution, and BiOWiSH™-Aqua FOG were mixed together for all three replicates for a final volume of 135 mL. The 135-mL volume was then apportioned equally among replicates. For example, the 10 ppm

inoculum microcosms were prepared as follows: 1.8 mL of 5000 ppm BiOWiSH solution was added to 88.2 mL of H₂O and 45 mL of 125 g/L Miracle-Gro™ using a 1000 µL micropipette. The Miracle-Gro™ and water were measured out using a 100 mL graduated cylinder. This yielded a final solution 135 mL in volume. As with the control microcosms, the 135 mL solution was divided equally among three dry microcosms. The oil/soil mixture and the diesel/soil was combined with water and Miracle-Gro™ solution so that there was 30 mL of water and 15 mL of 125 g/L Miracle-Gro™ solution for every 255 g of soil, or close to saturation. This moisture level of 15% was chosen based on previous work with hydrocarbon degradation in oily sludge (Chokshi, 2003).

Five 25-g samples were removed from the diesel/sand mixture and five 25-g samples were removed from the motor oil/sand mixture prior to microcosm preparation. These samples were subjected to extraction and TPH analysis as described below.

Three 25-g samples were removed from each microcosm at 25 and 56 days. The samples were then placed in a freezer since the TPH measurement process was time consuming and had to be performed over the subsequent weeks. The total amount of time elapsed between freezing and GC/MS analysis was recorded.

Extraction and TPH Analysis

The TPH extraction protocol adapted from EPA Standard Method #3550 is as follows. 25-g samples of each soil mixture were removed from the microcosm and placed in a 100-mL sample bottle. 2 mL of 5-g/L hexacosane in MeCl was added to the sample to serve as an internal recovery standard. 25 mL of MeCl was added to the sample bottle containing the soil and sonicated for 3 min at 60,000 Hz using a Sonifier 250 (Branson

Ultrasonics Corp., Danbury, Connecticut). Sodium sulfate (Na_2SO_4) was added to a 24 cm (diameter) 802 Fluted Grade Whatman Inc. filter mounted in a glass funnel to remove water from the extracts. Na_2SO_4 was also added directly to the sample bottle. The extract was poured through the fluted filter and then through a Millipore API 04200 glass fiber filter into a test tube. Another 25 mL of MeCl was added to the soil sample. This additional solvent was then sonicated and filtered as described above and added to the previous 25 mL of MeCl for a total extract volume of 50 mL.

Samples from each extract were run through an Agilent Technologies 6890N Gas Chromatograph (splitless inlet) with an Agilent 5975B inert Mass Selective Detector. A 50-m fused silica column 250 microns in diameter was used (Agilent Catalog #19091S-433) in the chromatograph. Samples were automatically loaded using an Agilent 7683B Series Injector capable of holding eight GC vials, two solvent vials, and a waste vial. The sample injection volume was 2 μL from a 10- μL syringe. To ensure no samples were cross-contaminated, the Agilent Injector was programmed to rinse the syringe twice with methylene chloride before taking sample extract from the GC vial. The temperature ramped from 45°C to 275°C at a rate of 12°C per minute and was then held at 275°C for the remainder of the 34 minute run time. The front inlet where the samples were injected was pressurized to 12.26 psi at a temperature of 200°C. Helium was used as the carrier gas.

The chromatogram was integrated over the entire run-time, and the resulting area was used to calculate the TPH concentration in the solvent extract using a calibration curve derived from known standards. Standards were run sporadically along with samples and their peak areas compared with the original standard curves to detect any drift in the GC

signal. All samples and standards were run in triplicate and the mean of the three peak areas was used in subsequent calculations.

Preparation of Standard Curves

Separate calibration curves were prepared for diesel and motor oil. For the motor oil standards, 0.0250 g of Castrol® HD SAE 30 motor oil was weighed out in a small beaker and rinsed into a 50-mL volumetric flask using MeCl. The flask was then filled to volume with methylene chloride, for a final solution with 5000-mg/L motor oil in MeCl. The following standard dilutions were then prepared from the 5000-mg/L stock solution.

Table 4. Motor Oil Standard Preparation

Number	Dilution	Motor Oil Concentration (mg/L)
1	None	5000
2	1:2	2500
3	1:4	1250
4	1:8	625
5	1:16	313
6	1:32	156
7	1:64	78
8	1:128	39

The standard dilutions were prepared from the 5000-mg/L stock solution by pouring 25 mL of the stock solution into a 50 mL volumetric flask and bringing the contents up to volume with pure MeCl. This process was repeated six additional times to obtain a range of motor oil concentrations. Samples of each dilution were placed in 2-mL crimp-

top vials and run in duplicate through the GC/MS. The chromatograms generated by the GC/MS were integrated and the peak areas tabulated.

Figure 2. Calibration curve for motor oil. GC peak area response is given as a function of motor oil concentration. Error bars are 95% confidence intervals (n=4). Note that the curve displays a slightly non-linear response despite having an R^2 value of 0.98.

The diesel standard curve was prepared in a manner similar to that the of the motor oil calibration curve, and was also used to generate a standard curve for hexacosane as well. 0.500 g of diesel was weighed out in a small beaker and rinsed into a 100 mL volumetric flask using methylene chloride (MeCl). Twenty milligrams of hexacosane was also weighed out and rinsed into the same 100-mL volumetric flask. The flask was then filled to volume with methylene chloride, for a final solution with 5000 mg/L diesel and

125 mg/L hexacosane. The following standard dilutions were then prepared from the 5000 mg/L stock solution as shown in Table 5.

Table 5. Diesel and hexacosane standard preparation.

Number	Dilution	Diesel Concentration (mg/L)	Hexacosane Concentration (mg/L)
1	None	5000	200
2	1:2	2500	100
3	1:4	1250	50
4	1:8	625	25
5	1:16	313	12.5
6	1:32	156	6.3
7	1:64	78	3.1
8	1:128	39	1.6

The standard dilutions were prepared from the 5000-mg/L stock solution by pouring 50 mL of the stock solution into a 100 mL volumetric flask and bringing the contents up to volume with pure MeCl. This process was repeated six additional times to obtain a range of diesel and hexacosane concentrations. Samples of each dilution were placed in 2-mL crimp-top vials and run with four replicates through the GC/MS using Method TPH.M (Huang, 2006). The chromatograms generated by the GC/MS were integrated and the peak areas tabulated.

Figure 3. Diesel calibration curve. GC response peak area is given as a function of diesel concentration in solvent. Error bars are 95% confidence intervals (n=4).

Figure 4. Hexacosane calibration curve. GC response peak area is given as a function of hexacosane concentration in methylene chloride solvent. Error bars are 95% confidence intervals (n=4).

Enrichment of BiOWiSH™-Aqua FOG for Hydrocarbon Degrading Microbes

A hydrocarbon-degrading consortium was enriched on solid media by the following method. A 5000-ppm solution of BiOWiSH™-Aqua FOG was activated for 48 hours with shaking at 25 °C. A loop full of the solution was then streaked onto basal Bushnell-Haas agar (BHA), a basal medium used in the cultivation of hydrocarbon degrading organisms (Venkateswaran et al., 1993). The agar was prepared with a suspension of motor oil droplets (Castrol® HD SAE 30). The plates were incubated at 25°C for 1 week. Colonies from this plate were re-plated onto the same media and incubated for an additional week. A colony was picked from the second plate onto plate-count agar (PCA) and incubated at 25°C until visible colonies formed. This was then transferred to a PCA slant and stored at 4°C. This enrichment culture will be subsequently referred to as the Agar Enrichment Culture (AEC).

BiOWiSH™-Aqua FOG was also enriched for hydrocarbon degraders using liquid media. 100 mL of BH broth with 0.1% yeast extract (0.10 g in 100 mL) was prepared. 2 drops of non-sterile motor oil was added to the flask for a concentration of approximately 750 mg/L. BiOWiSH™-Aqua FOG that had been activated for 96 hours in the presence of motor oil was inoculated in the media and placed in an incubator/shaker at 25°C and 60 rpm. After 24 hours had elapsed, 100 mL of BH broth with 0.1% yeast extract was prepared with 2 drops of non-sterile motor oil for a concentration again of approximately 750 mg/L and was inoculated with 20 µL of the culture from the preceding day and placed in an incubator/shaker at 25°C and 60 rpm. All media was sterilized at 121°C and 16 psi for 15 minutes in a Tuttnauer Brinkman

2540E autoclave. This enrichment culture will be subsequently referred to as the Broth Enrichment Culture (BEC).

Preparation of Microbial Inoculum Cultures

The cultures used in the respirometry experiment were derived from the cultures prepared above. The BiOWiSH™-Aqua FOG inoculum was prepared in a 15,000 ppm suspension. The broth enrichment culture of BiOWiSH™-Aqua FOG (BEC) was inoculated into Bushnell-Haas broth containing 0.05% yeast extract and 750 mg/L SAE 30 motor oil. A loop full of the agar enrichment culture of BiOWiSH™-Aqua FOG (AEC) was taken from a refrigerated slant (4 °C) and inoculated into Bushnell-Haas broth containing 0.05% yeast extract. All three inocula were incubated with shaking for 24 hours at 60 rpm and 25 °C.

Microcosm Preparation for Respirometry

Respirometry Experiment #1

A total of eight 200-g microcosms were prepared for the first respirometry experiment in 2-L Pyrex media bottles. A total of four experimental conditions were tested in duplicate: BiOWiSH™-Aqua FOG, BEC, AEC, and no inoculum (the biotic control). All microcosms contained motor oil.

Table 6. Microcosm Recipes for the first respirometry experiment.

Microcosm (Respirometer Channel)	Test Conditions	Inoculum Dose
1,2	Biotic Control	None
3,10	BiOWiSH™	24 hour activated 500 ppm BiOWiSH™
6,7	BEC	6.7 mL of 24 hour incubated culture
8,9	AEC	6.7 mL of 24 hour incubated culture
4,5	Background	None

To prepare the microcosms, 178 g of the sieved Los Osos sand was weighed out. 2 g of SAE 30 motor oil was then added to achieve an approximate hydrocarbon concentration of 10,000 ppm. For the biotic controls (Microcosms 1 and 2), 15 mL DI water was added using a 10-mL pipet. For Microcosms 3, 6, 7, 8, 9, and 10, 8.3 mL DI water was added. Microcosms 3 and 10 were inoculated with 6.7 mL of 15,000 ppm BiOWiSH™-Aqua FOG. Microcosms 6 and 7 were inoculated with the BEC prepared previously (Figure 5).

Figure 5. Preparation of the broth enrichment culture (BEC) inoculum from BiOWiSH™-Aqua FOG used in the respirometry experiments.

Microcosms 8 and 9 were inoculated with the 6.7 mL of the AEC prepared previously (Figure 6).

Figure 6. Preparation of the agar enrichment culture (AEC) inoculum from BiOWiSH™-Aqua FOG used in the respirometry experiments.

All inoculations were performed using 10-mL sterile pipets. Since the respirometry experiment would proceed for only one week, only 5 mL of a 125-g/L solution of Miracle-Gro™ All-Purpose Plant Food was deemed to be necessary to prevent nutrient limitation of hydrocarbon degradation. The final moisture content of each microcosm was 15%. Microcosm 4 consisted of an empty 250-mL media bottle and Microcosm 5 consisted of an empty 2-L media bottle.

Respirometry Experiment #2

A total of ten 200-g microcosms were prepared for the second respirometry experiment in 2-L Pyrex media bottles. Five experimental conditions were tested in duplicate:

BiOWiSH™-Aqua FOG with and without motor oil, and the AEC with and without motor oil, and a biotic control with no inoculum. As shown in Table 7, Microcosms 1 and 2 were inoculated with the AEC, contaminated with 10,000 ppm SAE 30 motor oil. Microcosms 3 and 10 were also inoculated with the AEC, but no oil was added. Microcosms 6 and 7 were inoculated with 48 hour-activated 15,000 ppm BiOWiSH™-Aqua FOG and motor oil. Microcosms 8 and 9 were inoculated with the same BiOWiSH™-Aqua FOG solution, but no oil was added. Microcosms 4 and 5 were contaminated with motor oil, but no augmenting inoculum was added. De-ionized water was added to all microcosms so that the total moisture content was 10%. Five milliliters of 125-g/L Miracle-Gro™ solution was added to ensure there was sufficient nitrogen for hydrocarbon degradation.

Table 7. Microcosm Recipes for the second respirometry experiment.

Microcosm (Respirometer Channel)	Test Conditions	Inoculum Dose	Motor Oil
1,2	AEC with Oil	6.7 mL of 48 hour incubated culture	Present
3,10	AEC without Oil	6.7 mL of 48 hour incubated culture	None
6,7	BiOWiSH™ with Oil	48 hour activated 500 ppm BiOWiSH™	Present
8,9	BiOWiSH™ without Oil	48 hour activated 500 ppm BiOWiSH™	None
4,5	Biotic Control without Oil	None	None

Respirometry

The microcosm bottles prepared in the previous section were connected to the expansion interface of a Micro-Oxymax™ respirometer (Columbus Instruments; Columbus, Ohio) equipped with carbon dioxide, methane, and oxygen sensors, a 10-channel expansion interface and a condensing air drier.

The respirometer was calibrated using the injection method. A sealed 500-mL bottle was connected to the respirometer and purged with pure nitrogen. The CO₂ sensor gain was then adjusted to zero. Pure carbon dioxide (4.6 mL) was injected using a 10-mL Hamilton GASTIGHT™ syringe through a septum in the cap of the 500-mL bottle to achieve a concentration of 0.5% CO₂ inside the 500-mL bottle. The span of the CO₂ sensor was then adjusted to 0.5%. The oxygen sensor and methane sensor were calibrated by Columbus Instruments (Columbus, Ohio) two weeks before operation. Calibration and diagnostic output is given in Appendix C. The oxygen concentration, oxygen consumption, cumulative oxygen consumption rate, percent carbon dioxide concentration, carbon dioxide production rate and cumulative carbon dioxide produced were measured by the sensors and recorded electronically. The respirometer, water bath, re-circulating pump, and gas tanks are shown in the photograph in Figure 7.

Figure 7. The MicroOxymax Respirometer, bottled gas, water bath, and recirculating pump.

Isolation of Hydrocarbon-Degrading Organisms from BiOWiSH™-Aqua FOG.

24-hour-activated BiOWiSH™-Aqua FOG was streaked onto each of three Bushnell-Haas agar plates and one drop of non-sterile motor oil was distributed evenly over the streaks using a sterile glass spreader. For comparison, 24-hour-activated BiOWiSH™-Aqua FOG was streaked onto Bushnell-Haas agar with no motor oil in triplicate, incubated at 25 °C for four days and inspected for growth. Several colonies were streaked onto Bushnell-Haas agar with motor oil (BHMA) in triplicate and plain Bushnell-Haas agar (BHA) without motor oil. A BHMA control plate was used to determine if non-

sterilized motor oil contained culturable organisms. The plain BHA was used to control for growth not attributable to the motor oil spread over the surface of the agar. The plates were incubated for an additional 17 days, inspected for growth, and three different colonies were transferred to plate count agar (PCA). The PCA plates were allowed to incubate for 5 days, and then colonies were picked individually, streaked onto PCA and incubated for 1 day. This procedure (see Figure 8 below) isolated two colonies potentially capable of hydrocarbon metabolism.

Figure 8. Isolation of Hydrocarbon-degrading organisms from BiOWiSHTM-Aqua FOG.

Results and Discussion

Soil Properties

Table 8 below provides a summary of the results of the soil characterization analysis of the Los Osos sand used for the microcosms in both the TPH analysis experiment and the respirometry experiments. The raw data are given in Appendix B.

Table 8. Soil Properties.

Soil Characteristics	Value	Error (n = 5, p = 0.05)
Bulk Density (g/mL)	1.493	0.017
% Moisture Content	0.295	0.016
% Moisture Content (Dry Basis)	0.296	0.016
% Organic Content	0.95	0.33
pH	6.80	0.05
pH with Miracle-Gro™	5.53	0.04
Porosity	0.372	0.015
Cation Exchange Capacity, CEC (cmol _c /kg)*	3.09	0.21

*cmol_c/kg is centimol equivalents of cations per kilogram.

TPH Analysis of Diesel and Motor Oil-Contaminated Microcosms

Motor Oil-Contaminated Microcosms

Average TPH measurements in the motor oil-contaminated microcosms are given in Table 9, and represented graphically in Figure 9. At 25 days, there was little difference between the degradation observed at each of the BiOWiSH™ doses in the motor oil-contaminated microcosms (Figure 9). In fact, the mean TPH measurements were higher in the 100-ppm and 500-ppm BiOWiSH™-Aqua FOG microcosms than in the 10-ppm and control microcosms. After 56 days, there was a significant correlation between biodegradation and BiOWiSH™ dose, with the mean TPH concentration decreasing with increasing inoculum concentration (Figure 9).

Table 9. Average TPH measurements for the microcosms contaminated with motor oil. Uncertainties shown are standard deviations among three replicates.

Days Elapsed	TPH Concentration (ppm)		
	0	25	56
Motor Oil Control	8769 ± 1157	6035 ± 875	6288 ± 141
Motor Oil 10 PPM		5862 ± 891	6155 ± 1074
Motor Oil 100 PPM		7057 ± 268	5873 ± 764
Motor Oil 500 PPM		6471 ± 1189	5177 ± 570

The percent change in the average TPH removal was calculated as follows: first the percent TPH removal was calculated as:

$$\%TPH \text{ Removal} = \frac{[Initial \text{ TPH}] - [TPH \text{ at } 56 \text{ days}]}{[Initial \text{ TPH}]} \times 100\%$$

Then the percent change in TPH removal was then calculated as shown below:

$$\%Change\ in\ TPH\ Removal = \frac{\%Removal - \%Removal\ in\ Control}{\%Removal\ in\ Control} \times 100\%$$

By this calculation, as shown in Table 10, the average percent change in TPH removal was marginal in the 10-ppm microcosms, being close to a 5% improvement. Improvement in TPH removal *due to bioaugmentation* was close to 15% in the 100-ppm microcosms, and nearly 45% in the 500-ppm microcosms.

Table 10. Increase in the percent removal of TPH in the motor oil-contaminated microcosms.

Inoculum Dose (ppm)	Average % TPH Removal	Average %Change in TPH Removal
0	28.3	-
10	29.8	5.4
100	33.0	16.7
500	41.0	44.8

Figure 9. TPH levels in the motor oil-contaminated microcosms. Error bars are the standard deviations among three replicates.

Diesel-Contaminated Microcosms

BiOWiSH™-Aqua FOG did not have an impact on TPH removal for the diesel-contaminated soil. The initial TPH measurement of the diesel-contaminated soil was 5900 ppm, after 25 days the average TPH fell by ~900 ppm and after 56 days final TPH concentrations were ~4400 ppm (Figure 10). After 56 days, the average percent change in TPH removal in the 10 ppm and 100 ppm microcosms were 25% and 32% respectively, while the average percent change in TPH removal in the 500 ppm microcosms was essentially zero. A statistical investigation of the TPH levels after 56 day in the diesel-contaminated microcosms is given below.

Table 11. Average TPH measurements for the diesel-fuel contaminated microcosms. Uncertainties are standard deviations among three replicates.

Days Elapsed	TPH Concentration(ppm)		
	0	25	56
Diesel Control	5865 ± 497	5006 ± 268	4634 ± 487
Diesel 10 PPM		5150 ± 139	4321 ± 376
Diesel 100 PPM		5098 ± 473	4238 ± 987
Diesel 500 PPM		4964 ± 163	4645 ± 390

Table 12. Increase in the percent removal of TPH in the diesel-contaminated microcosms due to various BiOWiSH™ doses.

Inoculum Dose (ppm)	Average %TPH Removal	Average %Change in TPH Removal
0	21.0	-
10	26.3	25.4
100	27.7	32.2
500	20.8	-0.9

Figure 10. TPH levels in the diesel-contaminated soil microcosms. Error bars are the standard deviations among three replicates.

Statistical Analysis of TPH Measurements

A statistical analysis of the data taken from analysis of the 56 day samples was carried out. The experimental design was a 3-factor completely randomized design with three replicates. As summarized in Table 13, the three factors were two quantitative predictors (analysis time and inoculum dose) and one categorical predictor (contaminant). Making the contaminant a predictor allowed the 56 day data to be analyzed together. Otherwise, separate statistical analyses would have had to be done for the motor and diesel separately. The categorical predictor had two levels, diesel and motor oil. Therefore, the appropriate statistical analysis was determined to be multiple linear regression.

Table 13. Predictor variables and response variable for multiple linear regression of 56 day TPH measurements.

Predictor Variable	Units	Description	Type	Response Variable -TPH concentration after 56 days in parts per million (ppm)
Analysis Time	Days	The maximum number of days elapsed between removing each of the three soil samples from each microcosm and GC analysis of the extract	Quantitative	
Contaminant	N/A	No. 2 Diesel or SAE 30 Motor Oil	Qualitative	
Inoculum Dose	ppm	Initial concentration of inoculum in the microcosm at the start of the experiment	Quantitative	

The maximum number of days that elapsed between freezing the soil samples and GC analysis (analysis time) was included as a predictor. The analysis time is the sum of the number of days the soil samples stayed in the freezer until being extracted and the number of days the methylene chloride extracts stayed in the freezer before being

analyzed by the GC/MS. In doing so, any non-random effects of sample storage in the freezer were taken into account. A plot of the TPH measurements after 56 days versus analysis time is shown in Figure 11 below. The values of R^2 and equations for the regression lines in Figure 11 are not shown, because effects of the other predictor variables on TPH have not been removed. However, there is a negative correlation between TPH and analysis time.

Figure 11. Effect of Analysis Time on TPH measurements. Lines shown are linear regressions of each series.

For multiple linear regression to be valid, the data must be normal, feature equal variance, be linear, and be independent. From the Minitab output in Appendix A, there are no unusual observations, so high leverage points and influential points are not an issue. Based on the normal probability plot and the histogram in Appendix A, Figure 30,

the points fall close to the diagonal of the normal probability plot and the histogram features a bell-shape. The deleted residual plots (Figure 31 and Figure 32) all feature random scatter across the range of the data, indicating that the linearity and equal variance assumptions are valid. The independence assumption is valid since the TPH response in one microcosm could not affect the response of another microcosm. To determine whether or not the model can be simplified, a partial F tests were used to eliminate insignificant predictors. The interactions between analysis time and inoculum, and analysis time and contaminant were tested for significance with partial F-tests. The result of the test was that these interactions were not significant ($p = 0.05$). Therefore, all the model assumptions are justified. The final multiple linear regression model is:

$$\text{Diesel TPH} = 5314 - 0.65 \times \text{Inoculum Dose} - 71.9 \text{ Analysis Time}$$

$$\text{Motor Oil TPH} = 7810 - 2.73 \times \text{Inoculum Dose} - 71.9 \text{ Analysis Time}$$

After adjusting for the other predictors, there was a decrease of between 121 and 425 ppm TPH for each additional 100 ppm of BiOWiSHTM-Aqua FOG inoculated into the soil for the motor oil-contaminated microcosms (p -value = 0.05). These finding are opposed to the results obtain by Abdulsalam and his co-workers who found that biostimulation of motor oil degradation with nitrogen and phosphate compounds resulted in TPH reductions of 75%, while bioaugmentation/biostimulation resulted in 66% removal (Abdulsalam, et al., 2011) This discrepancy can be explained by noting that the soil used in Abdulsalam's study was not naïve, but had been contaminated with the spent

motor oil for forty years. Therefore, the organisms present had already has ample opportunity to acclimate, and an added consortium would have no advantage.

After adjusting for the other predictors, there was an effect of between an increase of 2.93 and a decrease of 4.24 ppm TPH for each additional ppm of BiOWiSHTM-Aqua FOG inoculated into the soil for the diesel-contaminated microcosms (p-value = 0.05). Therefore, it cannot be said for certain that inoculum dose has a measurable effect on TPH degradation in soil contaminated with diesel, since the confidence interval includes zero. This result is consistent with the finding of other researchers who found that bioaugmentation was not effective for diesel fuel (Demque, et al., 1997; Mariano, et al., 2009). It is important to note that for both diesel and motor oil-contaminated samples, there was a mean decrease of between 16.8 and 127 ppm TPH for each additional day elapsed between sampling and GC/MS analysis (p-value = 0.05). Therefore, storage of samples had the effect of lowering the measured TPH and thus samples should not have been kept in the freezer for more than one week. This effect was removed in the statistical analysis, and therefore the effect of bioaugmentation with motor oil is statistically valid.

Diesel Chromatograms

The output of GC analysis of extract from diesel-contaminated soil used at the start of the experiment is shown in Figure 12. A “hump” is observed between 6 and 22 minutes, indicating an unresolved complex mixture typical of petroleum distillates. Alkanes appear as regular spikes and the hexacosane peak (internal standard) eluted at 22.4 minutes. The identities of these peaks were determined using the Agilent mass spectrometer and software. After 25 days, the alkane peaks and “hump” were approximately the same as in the chromatogram at 0 days in Figure 13. However, by 56 days, there was a noticeable decrease in the alkane peaks in the chromatogram in Figure 14. From these observations, at 25 days, there was no evidence of preferential degradation of alkanes, but by 56 days there was clearly more alkanes degraded than other petroleum compounds.

Figure 12. Gas Chromatograph of extract from diesel contaminated soil at the start of the experiment.

Figure 13. Chromatogram from the 25 day extraction of a soil sample from Microcosm 1 inoculated with 500 ppm initial BiOWiSH™. Note that except for the hexacosane peak, the chromatogram appears nearly identical to the chromatogram taken at the start of the experiment.

Figure 14. Chromatogram from the 56 day extraction of a soil sample from Microcosm 1 inoculated with 500 ppm initial BiOWiSH™. Note that the alkane peaks have been reduced in size compared to the 25 day chromatogram.

Motor Oil Chromatograms

The output of GC analysis of extract from motor oil-contaminated soil used at the start of the experiment is shown in Figure 15. The majority of the motor oil petroleum compounds eluted between 17 and 26 minutes. As in the diesel chromatograms, the hexacosane peak is a tall spike with a retention time of 22.4 minutes. After 56 days (Figure 16), the chromatogram looks qualitatively similar, but slightly smaller. Like the diesel chromatograms, the “hump” represents a complex unresolved mixture of hydrocarbons.

Figure 15. Chromatogram of an extract of sand contaminated with motor oil used in the TPH measurement experiments.

Figure 16. Chromatogram of an extract of motor oil-contaminated soil sample after 56 days elapsed. The soil sample was taken from a microcosm inoculated with 500 ppm BiOWiSH™-AQUA FOG.

Respirometry

Respirometry Experiment #1 Results

In the first respirometry experiment, the overall metabolic activity (carbon dioxide production) in bioaugmented soil microcosms was compared to soil microcosms without bioaugmentation. All of the microcosms were biostimulated with nutrients. The short-term effects of bioaugmentation can be seen in the data depicted in Figure 17, Figure 18, Figure 19, and Figure 20, which show cumulative CO₂ production with respect to time, CO₂ production rate over time, cumulative O₂ consumption for each of the microcosms, and cumulative CO₂ production after 160 hours. Microcosms spiked with 500 ppm BiOWiSH™-Aqua FOG had the greatest initial CO₂ production rate (Figure 17). This is further exemplified in Figure 19 which shows a 25% greater CO₂ production for microcosms inoculated with 500 ppm BiOWiSH™ than the biotic control without BiOWiSH™. Comparing Figure 17 and Figure 19, the O₂ uptake mirrors the CO₂ production in each microcosm, as would be expected.

During the first 100 hours, CO₂ production was slightly greater for the two enrichment cultures compared to the biotic control (Figure 20). However, by the end of the experiment (170 hours), the cumulative CO₂ production by the Agar Enrichment Culture (AEC) was about the same as the biotic control, and the Broth Enrichment Culture (BEC) produced slightly less CO₂ than and the biotic control.

Figure 17. Cumulative carbon dioxide production in the first respirometry experiment.

Figure 18. Carbon dioxide production rate (ppm/hour) in the first respirometry experiment. Error bars are standard error (n = 2).

Figure 19. Cumulative oxygen uptake in the first respirometry experiment. Error bars are standard error (n = 2).

Figure 20. Cumulative CO₂ production after 160 hours (ppmV) in the first respirometry experiment. Error bars are the standard error (n=2).

Respirometry Experiment #2 Results

In the second respirometry experiment, BiOWiSH™-Aqua FOG was tested with and without added motor oil so that CO₂ production from motor oil biodegradation could be discerned from possible CO₂ production from metabolism of natural organic material in the soil or in the BiOWiSH™ product. The AEC enrichment was tested in the same way. The greatest cumulative CO₂ production was observed in the BiOWiSH™ microcosms contaminated with motor oil throughout the 160-hour experiment (Figure 24). The BiOWiSH™ microcosms with motor oil featured dramatically more CO₂ production, than their oil-free counterparts, indicating utilization of the motor oil hydrocarbons stimulated additional microbial growth above and beyond that supported by the soil's other organic content and the organic matter supplied by the BiOWiSH™ inoculation. The BiOWiSH™ without motor oil had essentially the same level of CO₂ production as the biotic control without motor oil. The AEC with motor oil also produced more CO₂ than the AEC without oil, but much less than the microcosms with motor oil and BiOWiSH™. The AEC without oil produced the least CO₂, which was less than half of that produced in the presence of oil. Motor oil therefore was also metabolized by the AEC-augmented microbial community. The CO₂ production by the contaminant-free biotic control was lower than and the motor oil-free BiOWiSH™ inoculated microcosms.

Overall CO₂ production was about 5 times greater in the second respirometry experiment (compare Figure 21 and Figure 24). This is likely due to the lower moisture content in the second respirometry experiment, which was only 10% compared to 15% in the first experiment. This 33% reduction in moisture, improved aeration, allowing for greater aerobic respiration rates (Chokshi, 2003). The agar enrichment culture

produced 60,000 ppmV CO₂ at 10% moisture content compared to only 25,000 ppmV at 15%. Because of this, the two experiments cannot be compared directly.

CO₂ production rose sharply in the first 60 hours in the 500 ppm BiOWiSH™ microcosms with motor oil (#6 and #7) and then declined for the remainder of the experiment (Figure 22). In contrast, the 500-ppm BiOWiSH™ microcosms without motor oil (#8 and #9) had far less metabolic activity, with initial spikes at 25 and 55 hours, and then a rapid decline to a relatively low carbon dioxide production rate from 70 hours onward. Carbon dioxide production rates varied significantly, especially in the second half of the experiment (Figure 22). This was due to the respirometer drawing fresh air with low CO₂ levels into the microcosm, an operation termed “refresh,” in response to rapid changes in measured carbon dioxide levels. The biotic controls without motor oil (#4 and #5), maintained relatively constant low CO₂ production levels until 90 and 145 hours respectively, at which point their behavior began to feature the fluctuations described above, indicating increases in metabolic activity at those points.

The uptake of oxygen (Figure 23) showed essentially the same metabolic patterns as Figure 21. BiOWiSH™ in the presence of motor oil featured the greatest cumulative oxygen uptake over the entirety of the 160 hour experiment, the isolate with motor oil featured the second greatest O₂ uptake, and the microcosms without oil had the least O₂ uptake. These data clearly indicate that hydrocarbons are being utilized within the first week of biodegradation and stimulate dramatic increases in metabolic rate fueled by hydrocarbons.

Figure 21. Cumulative CO₂ Production in the second respirometry experiment.

Figure 22. Carbon dioxide production (ppm/hour) in the second respirometry experiment.

Figure 23. Cumulative oxygen uptake over the course of the second respirometry experiment.

Figure 24. Final Cumulative CO₂ production after 160 hours in the second respirometry experiment.

The CO₂ production can be used to estimate the TPH removal in the microcosms with using theoretical metabolic stoichiometry. For this calculation it is assumed that CO₂ behaves as an ideal gas at 25°C (298 K) and there are 0.8 grams of carbon per gram of TPH. It can be approximated that 60% of the carbon used goes to CO₂ production and 40% goes to biomass production (Rittmann & McCarty, 2001). The average CO₂ production in the 48 hour-activated BiOWiSH™-Aqua FOG microcosms *without* motor oil was 70,000 µL, and with motor oil the CO₂ production was 336,000 µL. The difference of 266,000 µL (0.266 L) CO₂ could therefore be attributed to motor oil biodegradation. The ideal gas law allows the molar specific volume to be calculated.

$$\begin{aligned}
 pv &= RT \rightarrow v = \frac{RT}{p} \\
 &= \frac{\left(8.314 \frac{J}{kmol \cdot K}\right) (298 \text{ K})}{101,330 \text{ Pa}} \\
 &= 24.5 \frac{m^3}{kmol} \times \frac{1 \text{ kmol}}{1000 \text{ mol}} \times \frac{1000 \text{ L}}{1 \text{ m}^3} \\
 &= \mathbf{24.5 \text{ L/mol}}
 \end{aligned}$$

This value is used in the calculation below, along with the yield coefficient, the molar mass of carbon, and the weight percent of carbon in petroleum hydrocarbon, to find the mass of TPH used to generate 0.266 L of CO₂.

$$\begin{aligned}
 &0.266 \text{ L CO}_2 \left(\frac{\text{mol CO}_2}{24.5 \text{ L}} \right) \left(\frac{12 \text{ g C}}{\text{mol C}} \right) \\
 &\times \left(\frac{1 \text{ g C taken up}}{0.60 \text{ g C used for respiration}} \right) \times \left(\frac{1 \text{ g TPH}}{0.8 \text{ g C}} \right) \left(\frac{1000 \text{ mg TPH}}{1 \text{ g TPH}} \right) = \mathbf{270 \text{ mg TPH}}
 \end{aligned}$$

Since the total mass of soil in the microcosm was 0.2 kg, the estimated TPH removal is 1360 ppm TPH or 14% over the course of 6.7 days.

The TPH degradation in the AEC-inoculated microcosms in the second respirometry experiment can be calculated in a similar manner. The average difference between the AEC with oil and the AEC without oil was 90,000 μL or 0.09 L

$$0.090 \text{ L } CO_2 \left(\frac{\text{mol } CO_2}{24.5 \text{ L}} \right) \left(\frac{12 \text{ g C}}{\text{mol C}} \right) \\ \times \left(\frac{1 \text{ g C taken up}}{0.60 \text{ g C used for respiration}} \right) \times \left(\frac{1 \text{ g TPH}}{0.8 \text{ g C}} \right) \left(\frac{1000 \text{ mg TPH}}{1 \text{ g TPH}} \right) = \mathbf{92 \text{ mg TPH}}$$

This value corresponds to a TPH removal of 459 ppm or 5% of the initial 10,000 ppm.

The BiOWiSH™-Aqua FOG consortium therefore proved to be nearly three times as effective as the isolate.

Hydrocarbon-degrading Organisms Isolated from BiOWiSH™-Aqua FOG

Based on the results of the respirometry experiment, there are organisms capable of hydrocarbon degradation in BiOWiSH™ –Aqua FOG. An attempt was made to culture these organisms on Bushnell-Haas agar. After 21 days, Bushnell-Haas agar with motor oil (BHMA) plates (21 day incubation) showed scattered colonies (Figure 25). Colonies appeared in the spaces between streak lines, because the motor oil was spread over the surface of the agar after streaking colonies. However, many colonies remained on the streak lines, however. The colony morphology was white, circular, entire, and less than 1 mm diameter. There were also scattered pink colonies, also less than 1mm diameter. These were not contaminants, since they were present only on the streak lines.

Figure 25. 24 hour-activated BiOWiSH™-Aqua FOG grown on BHMA for 21 days.

Bushnell-Haas agar without motor oil (BHA) plates (Figure 26) showed colonies on the streak lines. Colony morphology was the same as the BHMA plates. Again, the pink colonies were no contaminants, since they grew only on the streak lines.

Figure 26. 24 hour-activated BiOWiSH™ grown on BHA for 21 days.

After 17 days, the re-plated colonies on BHMA and BHA gave rise to white colonies (Figure 27). Note the pink colonies did not appear on this set of plates. Growth on BHMA plates was more robust than the growth on the BHA plates. The motor oil test plate had only one mold contaminant on the edge of the agar, indicating that the non-sterile motor oil does not contain organisms culturable on BHA. The re-plating yielded

more robust growth, even without the benefit of residual carbon from BiOWiSH™-Aqua FOG.

Figure 27. Re-plated BiOWiSH™-Aqua FOG on BHMA after 17 days incubation.

Two organisms re-plated from the second set of BHMA plates onto PCA and then isolated on PCA are shown in Figure 28. One colony appeared beige-colored, circular and entire. The other colony was yellow, circular, and entire.

Figure 28. Beige (Left panel) and yellow (right panel) colonies isolated from BiOWiSH™-Aqua FOG after 24 hours on PCA.

The beige colony stained Gram-positive and from the left-hand panel of Figure 29 appears to be rod-shape (bacillus). The yellow colony stained Gram-negative and from the right-hand panel appears to be a bacillus as well. The ability of these organisms to survive on a medium that contains only hydrocarbons as carbon sources supports the hypothesis that BiOWiSH™-Aqua FOG contains organisms capable of accelerating hydrocarbon degradation.

Figure 29. Gram stain of beige colony (Left panel) and yellow colony (Right panel) isolated from BiOWiSH™-Aqua FOG. Magnification was 1000X.

Conclusions

In carefully-controlled microcosm experiments with TPH measurements at 0, 25 and 56 days, the addition of BiOWiSH™-Aqua FOG significantly improved biodegradation of motor oil-contaminated soil. However, BiOWiSH™-Aqua FOG did not have a measurable effect on diesel-contaminated soil. After 56 days, the average change in TPH concentration in the motor oil-contaminated microcosms inoculated with 500 ppm BiOWiSH™-Aqua FOG was 45% greater than that in the control microcosms. The motor oil-contaminated microcosms with the low dosing rates of 10 and 100 ppm exhibited final average TPH reduction rates 5.4% and 17% greater than the controls at 56 days, respectively. A statistical analysis (p -value = 0.05) of the TPH levels in the motor oil-contaminated microcosms indicated that the effect of BiOWiSH™ was statistically significant, with a mean decrease of 273 ± 152 ppm TPH was observed for each additional 100 ppm BiOWiSH™-Aqua FOG inoculated into the soil for the motor oil-contaminated microcosms. Given the positive correlation between inoculum dose and final TPH concentration, it is possible that increasing the inoculum dose to 1000 ppm might improve degradation rates further. From the statistical analysis of the microcosms contaminated with No. 2 diesel fuel, inoculum dose did not have a significant effect on TPH degradation in soil contaminated with diesel.

The ability of BiOWiSH™-Aqua FOG to improve biodegradation of motor oil and not diesel fuel could be attributed to several factors. Diesel fuel has been shown to contain compounds toxic to microorganisms, and inhibits microbial growth in soil at concentrations above 3000 ppm (Lapinskiene, Martinkus, & Rebzdaite, 2006). Thus,

the toxic properties of diesel fuel may have prevented the growth of the BiOWiSH™ organisms. Also, alkane-degraders are ubiquitous (Berthe-Corti & Fetzner, 2002), and therefore bioaugmentation would be expected to have less effect on biodegradation rates of petroleum distillates high in alkanes. It appears therefore that BiOWiSH™-Aqua FOG provided organisms to degrade more complex hydrocarbons in motor oil, while the indigenous organisms were entirely competent to degrade the alkane fraction of the diesel fuel. Furthermore, engine motor oils contains dispersant and detergent additives to prevent oil films from forming inside motor vehicle engines (Yamaguchi, Roby, Francisco, Ruelas, & Godfrey, 2002), and these surfactants may have made the motor oil more bioavailable.

Sample storage time before GC analysis also had a significant effect on final TPH concentrations, but since this effect was removed in the statistical analysis, this study still yielded good statistics for the effect of BiOWiSH™. Nonetheless, such storage times should be minimized in future experiments.

The respirometry experiments gave insight into the biodegradation dynamics in the first week following petroleum hydrocarbon contamination of soil. These experiments showed that in the initial week following motor oil-contamination of soil, the addition of BiOWiSH™-Aqua FOG substantially improves biodegradation rates, with the added organisms out-performing the indigenous organisms in the 5-6 days following the introduction of contaminant and augmenting organisms to the soil. The greatest CO₂ production was observed in the BiOWiSH™ microcosms contaminated with motor oil throughout the entirety of the 160 hour experiment. The microcosms augmented with 500 ppm BiOWiSH™-Aqua FOG produced 68 mL (33,000 ppm) of CO₂ compared to 56

mL (25,000 ppm). Towards the end of the first respirometry experiment, the indigenous organisms showed signs of acclimating to the contaminant, with measured CO₂ production rates surpassing that of the 500 ppm BiOWiSH™-Aqua FOG microcosms (although the cumulative CO₂ production was still much greater with BiOWiSH™ than without). One drawback of respirometry is that it shows respiration from all substrates in the sample and not just the contaminant of interest. However, by running samples with and without motor oil the respiration of the motor oil contaminant can be discerned. This suggests that respirometry is a valuable tool for screening, and evaluating and optimizing bioaugmentation products.

The respirometry experiments also showed that moisture content in the soil has a significant effect on biodegradation rates. Motor oil biodegradation rates were about 5 times greater with 10% moisture compared to 15% moisture. Since the first microcosm experiments were done with 15% moisture, this suggests that much better biodegradation rates could have easily been obtained by using lower moisture content. Results like these demonstrate the importance of providing users of bioaugmentation products with clear instructions for optimal biodegradation conditions.

Initial attempts to improve the product by enriching for hydrocarbon-degrading organisms in BiOWiSH™-Aqua FOG were unsuccessful. Enriched consortia consistently generated far less carbon dioxide than 500 ppm BiOWiSH™-Aqua FOG. BiOWiSH™-Aqua FOG removed approximately 1400 ppm TPH (14%) from the soil in 6.5 days, while an enrichment culture of BiOWiSH™-Aqua FOG only reduced TPH levels by 459 ppm (5%). This result suggests that the efficacy BiOWiSH™-Aqua FOG bioaugmentation is aided by its biodiversity.

Additional tests are needed to identify the hydrocarbon-degrading bacteria in the BiOWiSHTM-Aqua FOG product. While at least two distinct colony types were successfully grown on media with motor oil, these same colonies appear on Bushnell-Haas agar with no apparent carbon source, and survived repeated transfers onto this same medium. Therefore, their status as hydrocarbon-degraders is inconclusive, and further tests will be required to confirm their ability to metabolize petroleum compounds. Enrichments could also be made from petroleum-contaminated soils, and these enrichments could be used to further improve bioaugmentation products.

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Appendix A – Statistical Analysis

Minitab Regression Output

General Regression Analysis: TPH versus Inoculum, Analysis Time, Contaminant

Regression Equation

Contaminant

Diesel TPH = 5314.01 - 0.652824 Inoculum - 71.8876 Analysis Time

Motor Oil TPH = 7809.99 - 2.72759 Inoculum - 71.8876 Analysis Time

Coefficients

Term	Coef	SE Coef	T	P	95% CI
Constant	7809.99	612.923	12.7422	0.000	(6527.12, 9092.85)
Inoculum	-2.73	0.725	-3.7614	0.001	(-4.25, -1.21)
Contaminant					
Diesel	-2495.98	353.344	-7.0639	0.000	(-3235.54, -1756.42)
Analysis Time	-71.89	26.322	-2.7311	0.013	(-126.98, -16.80)
Inoculum*Contaminant					
Diesel	2.07	0.988	2.0991	0.049	(0.01, 4.14)

Term	VIF
Constant	
Inoculum	2.21477
Contaminant	
Diesel	3.14743
Analysis Time	3.35492
Inoculum*Contaminant	
Diesel	2.63022

Summary of Model

S = 487.860 R-Sq = 78.26% R-Sq(adj) = 73.68%
 PRESS = 7395344 R-Sq(pred) = 64.44%

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	16274512	16274512	4068628	17.0945	0.000004
Inoculum	1	720786	3367391	3367391	14.1483	0.001321
Contaminant	1	12179574	11876176	11876176	49.8983	0.000001
Analysis Time	1	2325468	1775279	1775279	7.4589	0.013267
Inoculum*Contaminant	1	1048685	1048685	1048685	4.4061	0.049405
Error	19	4522142	4522142	238007		
Lack-of-Fit	17	4191817	4191817	246577	1.4929	0.475200
Pure Error	2	330325	330325	165162		
Total	23	20796654				

Fits and Diagnostics for Unusual Observations
 No unusual observations

Figure 30. Four-in-one plot for the final linear regression model of the 56 day TPH measurements, Model 3.

Figure 31. Deleted Residuals vs. Analysis Time for the final model, Model 3.

Figure 32. Deleted Residuals vs. Inoculum dose for the final model, Model 3.

Appendix B – Soil Characterization

Table 14. Bulk density, porosity, pH, organic content, and moisture content raw data.

	Sample					Statistics		
	1	2	3	4	5	Average	Standard Deviation	Error (95% confidence t-value = 2.776)
Bulk Density								
volume of sand (L)	0.01	0.01	0.01	0.01	0.01	1.49	0.014	0.017
beaker (g)	8.34	8.84	8.90	8.90	8.83			
weight sand + beaker (g)	23.26	23.87	23.61	23.96	23.75			
weight of sand (g)	14.91	15.03	14.71	15.07	14.92			
density of sand (g/mL)	1.491	1.503	1.471	1.507	1.492			
Moisture Content						0.295	0.013	0.016
weight before drying (g)	15.30	15.64	15.47	15.64	15.50			
weight after drying (g)	15.26	15.59	15.42	15.60	15.46			
weight of water (g)	0.044	0.049	0.046	0.045	0.043			
% moisture content	0.290	0.315	0.300	0.290	0.281			
% moisture content (dry basis)	0.290	0.315	0.301	0.290	0.281	0.296	0.013	0.016
Organic Content						0.953	0.262	0.325
Dry Weight before burn (g)	15.26	15.59	15.42	15.60	15.46			
Weight after burn (g)	15.04	15.47	15.29	15.47	15.32			
% organic carbon	1.421	0.821	0.820	0.842	0.862	0.953	0.262	0.325
pH						6.300	0.274	0.340
weight of sand (g)	10.0004	10.0095	10.0097	10.0067	10.0331			
pH (paper)	6.5	6.0	6.0	6.5	6.5			
pH (probe)	6.77	6.78	6.87	6.80	6.78	6.800	0.041	0.050
pH with Miracle-Gro™	5.58	5.55	5.53			5.55	0.025	0.04
Porosity						0.372	0.012	0.015
Dry Weight (g)	41.68	44.18	44.24	41.68	45.46			
Saturated Weight (g)	45.28	47.86	47.80	45.35	49.32			
Volume water added (mL)	3.62	3.70	3.60	3.80	3.88			
Volume of Soil (mL)	10.0	10.0	10.0	10.0	10.0			
Porosity	0.362	0.370	0.360	0.380	0.388	0.372	0.012	0.015

Calculation of Ion Concentrations and CEC

The calculation of Ion Concentrations and CEC in cmol_c/kg for Sample #2 is shown below.

$$\frac{47.08 \text{ mg } \text{Ca}^{2+}}{\text{L}} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{1 \text{ mol}}{40.078 \text{ g}} \times \frac{100 \text{ cmol}}{1 \text{ mol}} \times \frac{0.050 \text{ L}}{5.0004 \text{ g}} \times \frac{1000 \text{ g}}{1 \text{ kg}} \times \frac{2 \text{ cmol}_c}{\text{cmol}} \\ = \mathbf{2.35 \text{ cmol}_c/\text{kg}}$$

$$\frac{6.73 \text{ mg } \text{Mg}^{2+}}{\text{L}} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{1 \text{ mol}}{24.305 \text{ g}} \times \frac{100 \text{ cmol}}{1 \text{ mol}} \times \frac{0.050 \text{ L}}{5.0004 \text{ g}} \times \frac{1000 \text{ g}}{1 \text{ kg}} \times \frac{2 \text{ cmol}_c}{\text{cmol}} \\ = \mathbf{0.553 \text{ cmol}_c/\text{kg}}$$

$$\frac{5.17 \text{ mg } \text{K}^{+}}{\text{L}} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{1 \text{ mol}}{39.0983 \text{ g}} \times \frac{100 \text{ cmol}}{1 \text{ mol}} \times \frac{0.050 \text{ L}}{5.0004 \text{ g}} \times \frac{1000 \text{ g}}{1 \text{ kg}} \times \frac{1 \text{ cmol}_c}{\text{cmol}} \\ = \mathbf{0.132 \text{ cmol}_c/\text{kg}}$$

$$\frac{2.04 \text{ mg } \text{Na}^{+}}{\text{L}} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{1 \text{ mol}}{22.98977 \text{ g}} \times \frac{100 \text{ cmol}}{1 \text{ mol}} \times \frac{0.050 \text{ L}}{5.0004 \text{ g}} \times \frac{1000 \text{ g}}{1 \text{ kg}} \times \frac{1 \text{ cmol}_c}{\text{cmol}} \\ = \mathbf{0.0886 \text{ cmol}_c/\text{kg}}$$

Table 15. Ion concentrations and CEC determination.

Sample #	Mass (g)	Extract Volume (L)	Dilution Factor				Concentration in Test Solution (mg/L)			
			Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺	Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺
1	5.0090	0.100	1.000	1.000	1.000	1.000	20.67	3.27	2.68	0.70
2	5.0004	0.050	0.091	1.000	0.091	1.000	4.28	6.73	0.47	2.04
3	4.9952	0.050	0.091	1.000	0.091	1.000	4.27	7.10	0.46	2.09
4	4.9998	0.050	0.091	1.000	0.091	1.000	4.30	7.14	0.47	2.30
5	5.0002	0.050	0.091	1.000	0.091	0.091	4.25	7.07	0.46	0.33

Concentration in Extract (mg/L)				Concentration in Extract (cmol _c /kg)				Total CEC (cmol _c /kg)	Error (95% Confidence, t-value = 2.776)
Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺	Ca ²⁺	Mg ²⁺	K ⁺	Na ⁺		
20.67	3.27	2.68	0.70	2.0593	0.5372	0.1368	0.0608	2.7941	
47.08	6.73	5.17	2.04	2.3452	0.5528	0.1320	0.0886	3.1186	
46.97	7.10	5.06	2.09	2.3397	0.5832	0.1292	0.0907	3.1428	
47.30	7.14	5.17	2.30	2.3562	0.5865	0.1320	0.0999	3.1745	
46.75	7.07	5.06	3.63	2.3288	0.5807	0.1292	0.1576	3.1963	
Average				2.2858	0.5681	0.1318	0.0995	3.0853	0.2054

Appendix C – Micro-Oxymax™ Respirometer Diagnostic output

Diagnostic Output for the First Respirometry Experiment

Micro-Oxymax Diagnostic Log File

Software Version: 2.1.1

System started on: 11:39:19: Mar Fri 2012

Valves and Sensors Test Results

Tested Logged on: 13:57:42: Mar 23 2012

Test	Drier 1	Drier 2	Drier 1	Drier 2	Drier 1
Drier 2					
-	Restriction	Restriction	Volume	Volume	Leakage
Leakage					
1	2.71 mmHg		307 ml		0.08 ml/min
2	2.79 mmHg		303 ml		0.08 ml/min
3	2.78 mmHg		300 ml		0.09 ml/min

Calibration Gas Pressurization 800.78 mmHg PASS

Nitrogen Gas Pressurization 800.68 mmHg PASS

Sensor Type	Reading	Min:	Max:	S.Dev.	Pass/Fail
O2	20.615	20.564	20.615	0.052	PASS
CO2	0.030	0.030	0.033	0.003	PASS
CH4	0.495	0.478	0.534	0.056	PASS

Basic Operations Test Results

Tested Logged on: 13:58:44: Mar 23 2012

Software Version: 2.1.1

Barometric Pressure	756.05 mmHg	+/- 0.0001	Pass
Reference Temperature	29.8 C	+/- 0.0000	Pass
Primary Temperature	25.9 C	+/- 0.0010	Pass
Auxillary Temperature	27.1 C	+/- 0.0009	Pass
Sensor Pressure Setpoint	800.78 mmHg	+/- 0.0001	Pass
Stabilization Time	1 sec		

Expansion Unit Diagnostics Test Results

Tested Logged on: 17:24:04: Mar 23 2012

Chan #	Restriction	Volume (ml)	Leak (ml/min)
Chan 1	23.91 mmHg	2139 ml	-0.67 ml/min
Chan 2	34.62 mmHg	2109 ml	-0.82 ml/min
Chan 3	33.17 mmHg	2134 ml	-0.78 ml/min
Chan 4	31.43 mmHg	378 ml	-0.12 ml/min
Chan 5	25.25 mmHg	2306 ml	-6.57 ml/min
Chan 6	33.14 mmHg	2113 ml	-0.33 ml/min
Chan 7	34.72 mmHg	2149 ml	-0.75 ml/min
Chan 8	32.00 mmHg	2132 ml	-0.58 ml/min
Chan 9	32.09 mmHg	2141 ml	-0.56 ml/min
Chan 10	33.23 mmHg	2164 ml	-0.76 ml/min

Diagnostic Output for the Second Respirometry Experiment

Basic Operations Test Results

Tested Logged on: 14:05:57: Apr 02 2012

Software Version: 2.1.1

Barometric Pressure	756.84 mmHg	+/- 0.0001	Pass
Reference Temperature	31.0 C	+/- 0.0008	Pass
Primary Temperature	25.6 C	+/- 0.0009	Pass
Auxillary Temperature	27.1 C	+/- 0.0009	Pass
Sensor Pressure Setpoint	800.78 mmHg	+/- 0.0002	Pass
Stabilization Time	1 sec		

Valves and Sensors Test Results

Tested Logged on: 14:21:36: Apr 02 2012

Test	Drier 1	Drier 2	Drier 1	Drier 2	Drier 1
Drier 2					
-	Restriction	Restriction	Volume	Volume	Leakage
Leakage					
1	2.69 mmHg		296 ml		0.07 ml/min
2	2.71 mmHg		295 ml		0.06 ml/min
3	2.74 mmHg		299 ml		0.07 ml/min

Calibration Gas Pressurization 800.68 mmHg PASS

Nitrogen Gas Pressurization 800.68 mmHg PASS

Sensor Type	Reading	Min:	Max:	S.Dev.	Pass/Fail
O2	20.769	20.765	20.774	0.008	PASS
CO2	0.008	0.007	0.011	0.004	PASS
CH4	0.551	0.502	0.578	0.076	PASS

Expansion Unit Diagnostics Test Results

Tested Logged on: 14:49:51: Apr 02 2012

Chan #	Restriction	Volume (ml)	Leak (ml/min)
Chan 1	24.13 mmHg	2206 ml	-1.15 ml/min
Chan 2	35.71 mmHg	2155 ml	-0.92 ml/min
Chan 3	38.47 mmHg	2142 ml	-0.61 ml/min
Chan 4	32.96 mmHg	2208 ml	-0.23 ml/min
Chan 5	34.17 mmHg	2206 ml	-1.46 ml/min
Chan 6	37.25 mmHg	2181 ml	-0.45 ml/min
Chan 7	36.17 mmHg	2170 ml	-0.82 ml/min
Chan 8	36.40 mmHg	2166 ml	-0.51 ml/min
Chan 9	35.37 mmHg	2157 ml	-0.50 ml/min
Chan 10	32.26 mmHg	2166 ml	-0.56 ml/min

Appendix D - Gas Chromatography Method

OVEN

Initial temp: 45 'C (On)
Initial time: 3.00 min
Ramps:
Rate Final temp Final time
1 12.00 275 12.00
2 0.0(Off)
Post temp: 0 'C
Post time: 0.00 min
Run time: 34.17 min

Maximum temp: 325 'C
Equilibration time: 0.50 min

FRONT INLET (SPLIT/SPLITLESS)

Mode: Splitless
Initial temp: 200 'C (On)
Pressure: 12.26 psi (On)
Purge flow: 50.0 mL/min
Purge time: 0.50 min
Total flow: 54.4 mL/min
Gas saver: On
Saver flow: 20.0 mL/min
Saver time: 2.00 min
Gas type: Helium

BACK INLET (UNKNOWN)

COLUMN 1

Capillary Column
Nominal length: 30.0 m
Nominal diameter: 250.00 um
Nominal film thickness: 0.25 um
Mode: constant flow
Initial flow: 1.5 mL/min
Nominal init pressure: 12.27 psi
Average velocity: 44 cm/sec
Inlet: Front Inlet
Outlet: MSD
Outlet pressure: vacuum

COLUMN 2

(not installed)

FRONT DETECTOR (NO DET)

SIGNAL 1

Data rate: 20 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

BACK DETECTOR (NO DET)

SIGNAL 2

Data rate: 20 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

COLUMN COMP 1

(No Detectors Installed)

COLUMN COMP 2

(No Detectors Installed)