TREATMENT OF PETROLEUM HYDROCARBONS IN OIL-BASED DRILL CUTTING MUD USING BIOWISH^{TM} BIOAUGMENTATION PRODUCTS

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ABSTRACT

Treatment of Petroleum Hydrocarbons in Oil-Based Drill Cutting Mud using BiOWiSH™ Bioaugmentation Products

Diego Jose Zepeda

The efficacy of BiOWiSH™-Thai Aqua, a commercially discontinued microbial product, in remediating oil based drill-cutting mud (DCM) was researched in this study. Experimentation was performed directly on DCM and on sand contaminated with oil extracted from DCM. A gas chromatograph-mass spectrometrer and a respirometer were used for analysis of total petroleum hydrocarbons (TPH) and CO_2 production respectively. Five experiments were analyzed by TPH extraction and analysis; four experiments were analyzed by respirometric analysis. The specific microcosm conditions tested in the experiments were control, nutrient-only control, and bioaugmentation product.

This study concluded that there might be potential for bioaugmentation of TPH using BiOWiSH™-Thai Aqua. However, a more extensive study including multiple replicates of samples over a longer sampling time period is required to make a conclusion. TPH analysis from the Sand Microcosm Experiment suggested that in seven days, the addition of BiOWiSH™-Thai Aqua improved TPH removal relative to the control by 89% while the nutrient-only control improved by 58%. Respirometric analysis suggested CO_2 respiration of glucose overshadowed CO_2 respiration from biodegradation. Thus, major conclusions could not be made from the respirometric analysis.

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CHAPTER 1. INTRODUCTION

According to the Energy Information Administration, in recent years the United States has increased its oil and natural gas production, creating a large increase in number of oil drilling sites (U.S. crude oil and natural gas production). Oil-based drill-cutting mud (DCM) also referred to as drilling mud, is created during oil and gas exploration and production (E&P). The EPA lists DCM as one of the E&P wastes exempt from federal hazardous waste regulations (EPA 2002). These muds are produced in large volumes and contain high levels of petroleum hydrocarbons along with other contaminants (Leonard & Stegemann, 2010). If not disposed of properly, DCM may create major environmental hazards and become a long-term liability of its generators. It has been recommended that drill-cutting mud be properly treated before disposal in secured landfills or re-used as raw materials in production plants or land restoration projects (Eghele, Gbadebo, & Taiwo, 2010). Physical/chemical treatment technologies include solvent extraction, thermal treatment, and incineration (Orszulik, 2008). Bioremediation is a treatment technology that utilizes microorganisms to degrade organic contaminants such as petroleum hydrocarbons. This method offers a more economical and ecological way to treat contaminants than physical/chemical treatment methods (Hung, Lee Tay, Tay, & Wang, 2010). Introducing bacteria with genetic potential to degrade the wide range of total petroleum hydrocarbons (TPH) compounds in DCM is a form of bioremediation known as bioaugmentation (Al-Shdaifat, Ammari, Goussous, & Tahhan, 2011). Bioaugmentation is not necessary, but its incorporation may accelerate the

degradation process. The soils present in DCM may not have been historically exposed to petroleum and thus may be lacking in petroleum-degrading microorganisms, making bioaugmentation a useful treatment technology.

This study tested the efficacy of BiOWiSH^{TM} bioaugmentation products. The primary focus of this study was testing BiOWiSH^{TM} - Thai Aqua, a commercially discontinued microbial product, in biodegrading petroleum hydrocarbons. This product serves as a basis for formulation of newer products created by BiOWiSH^{TM} Technologies Inc. The product was tested using microcosm experimentation in a controlled environment. Samples drawn from the microcosms were subject to analysis. Analytical methods used to analyze the effectiveness of BiOWiSH^{TM}-Thai Aqua included measurement of TPH concentration by extraction and gas chromatography-mass spectrometry (GC/MS) analysis and measurement of microbial CO_2 production using a MicroOxymax^{TM} respirometer. The initial experiment microcosms contained DCM and the final experiment microcosms contained sand contaminated with petroleum extracted from the original DCM source. The final experiment was conducted to attain more conclusive results from TPH analysis using a more extractable media compared to DCM. The effect of glucose and other nutrients was also assessed during the experiments. Glucose was added to determine the effects of adding an additional carbon substrate to the microcosms. The objective of this study was to determine if the BiOWiSH^{TM}-Thai Aqua is a successful bioaugmentation strategy for the remediation of oil based drill-cutting mud.

CHAPTER 2. BACKGROUND

2.1. Drill Cutting Mud Impact on Environment

Drilling fluids are essential and used in the process of drilling during oil exploration and production. They serve several purposes: cooling and lubrication of the drill bit, stabilization of the well bore, controlling of subsurface pressures, and transporting the drill cuttings to the surface (Ball, Schliephake, & Stewart, 2012). When the fluid and cuttings mixture reaches the surface it is separated via shale shakers (Figure 2.1). The removed fluid is re-circulated in the operation and the drill cuttings are stored in the mud pit for additional management.

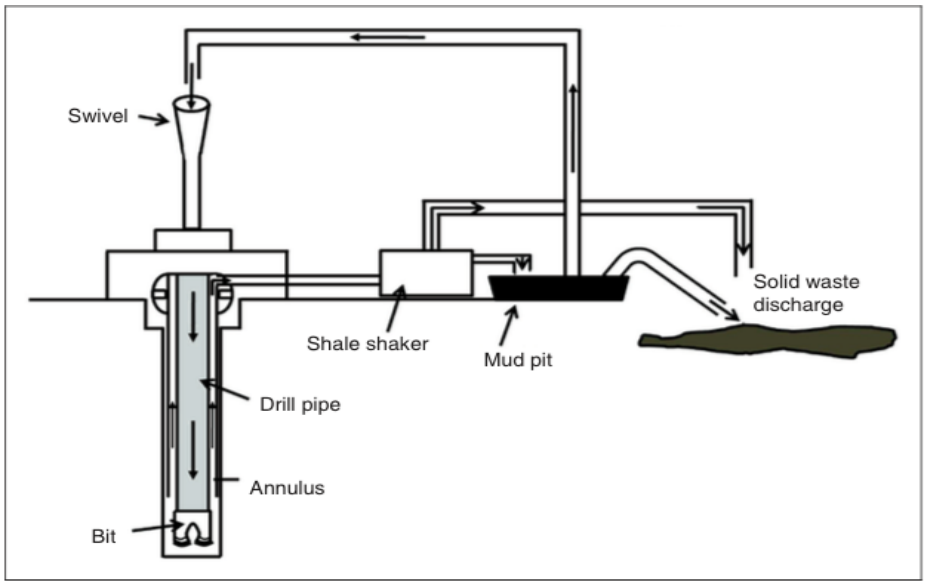

Figure 2.1 Schematic of drilling process

Limitations of the separation process lead to a solid waste stream containing drill cuttings and residual drilling fluids known as DCM. Typically, DCM is a mixture of oil-rich silts and clay particles and is biologically deficient (Ball et al., 2012). Because the soils in DCM are not typically exposed to petroleum prior to becoming DCM, they often lack microbial populations capable of degrading

petroleum compounds. This waste contains both aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) (Leonard & Stegemann, 2010). Field studies show that on average, drilling one meter of borehole yields 0.6 m^3 of DCM (Steliga & Uliasz, 2014). In general, boreholes depths range from 1,500 m to 3,500 m and generate between 900 m^3 and 2,100 m^3 of DCM (Shadizadeh & Zoveidavianpoor, 2012). These numbers magnified across drilling processes around the world indicate an extremely high volume of DCM waste production. Environmental management policies worldwide have a preference for recycling as the primary treatment of DCM (Steliga & Uliasz, 2014).

Treatment by bioremediation is one way the reuse of DCM can be achieved. Biodegradation is feasible in an optimized environment with the proper addition of nutrients, aeration, and moisture (Fernández-Linares, et al, 2007). Petroleum hydrocarbons can be detoxified and mineralized when utilized as a carbon source during microbial processes (Hung et al., 2010). Aliphatic hydrocarbons are non-aromatic compounds, which can be linked by single bonds, double bonds, or triple bonds named alkanes, alkenes, and alkynes respectively. PAHs are formed of multiple aromatic rings. Of the aliphatic hydrocarbons, the single chain alkanes are metabolized more quickly than the double and triple bond alkenes and alkynes. Due to their complexity and toxicity, PAH and PCB compounds are recalcitrant and cannot be efficiently degraded (Hung et al., 2007)

Environmental factors, including nutrient levels, temperature, pH, and moisture levels affect the efficiency of microbial kinetics (Becker & Seagren,

2009). Controlling and or modifying these factors may be necessary to achieve successful bioremediation. During aerobic degradation, organic compounds are oxidized and serve as metabolic electron donors. A continuous supply of oxygen, or another electron acceptor, is necessary for biodegradation to occur (Becker & Seagren, 2009). Bioremediation effectiveness can be improved in some cases when bioaugmentation is coupled with biostimulation (enhancing microbial populations by addition of nutrients) (Paliwal, Puranik, & Purohit, 2012). Paliwal et al also report that recalcitrant compounds, like PAHs, may be successfully biodegraded by using a consortium of multiple microbial strains that target contaminant intermediates.

2.2. Bioaugmentation Treatment Effectivenes

Many research studies of *in-situ* and lab-scale treatments of petroleum hydrocarbon contaminants have been performed to assess the usefulness of the bioaugmentation strategy. Successful bioaugmentation of petroleum compounds can be achieved by isolating bacteria from environments where contamination had been present for decades. Microorganisms capable of surviving in contaminated soil are strong candidates for successful bioaugmentation. (Mrozik & Piotrowska-Seget, 2010).

A field pilot test by Dutta, et al (2003) explored the effectiveness of treating petroleum-contaminated soil from a crude oil spill site in a reactor. The reactor augmented with naturally adapted consortia, nutrients, and aeration experienced 75% TPH removal in one year. A study by Gunsch and Ikuma (2013) tested the effectiveness of genetically engineered microorganisms as a

bioaugmentation strategy. The *in-situ* experiment was performed using a soil-column method at a petroleum-contaminated site. Table 2.1 lists the experimental conditions tested in the experiment.

Table 2.1 Contents of soil-columns in experiment

Soil Column	Genetic Bioaugmentation	Glucose Addition
1	No	Yes
2	Yes	No
3	Yes	Yes

Results of the study concluded that soil columns containing genetically engineered microorganisms experienced significantly higher degradation rates than the soil columns without. The bioaugmentation treatment with genetically engineered microorganisms showed limited long-term impacts on the native soil microorganism community. Gunsch and Ikuma (2013) suggested that bioaugmentation with genetically engineered microorganisms could be an effective and environmentally sustainable strategy for remediating environmental petroleum contamination. Barbaferi, et al (2001) tested the bioaugmentation effects on PAH degradation under slurry phase conditions. A reactor controlled the temperature and airflow into a chamber containing the PAH contaminated soil with added macronutrients and indigenous PAH degrading bacteria. Macronutrients were added to achieve a C:N:P ratio of 100:10:1 by weight. Barbafieri et al. (2001) concluded that high molecular weight PAH compound degradation was enhanced with the addition of PAH degrading bacteria and macronutrients. After 34 days of treatment, there was 65-90% PAH removal.

Selecting microorganisms capable of treating specific compounds of concern is critical in accomplishing treatment of petroleum hydrocarbons. Table 2.2 lists a summary of microorganisms found in literature to successfully treat petroleum hydrocarbons (Mrozik & Piotrowska-Seget, 2010). According to the table, aliphatic and PAH compounds are normally treated with a single strain or consortia of microorganisms.

Table 2.2 List of microorganisms, their type, and treatable contaminant(s)

Microorganism(s)	Type	Contaminant(s) Treated	References
Comamonas testosteroni, BR60	S.S.	Crude oil, PAHs	Gentry et al. (2001)
Pseudomonas aeruginosa, WatG	S.S.	Diesel oil	Ueno et al. (2006)
Rhodococcus sp., Acinetobacter sp., Pseudomonas sp.	C	PAHs	Yu et al. (2005)
Bacillus subtilis DM-04, Pseudomonas aeruginosa M and NM	C	Crude petroleum-oil hydrocarbons	Das and Mukherjee (2007)

Abbreviations: S.S. – single strain, C – consortia

There are limitations to the bioremediation/bioaugmentation strategies. It is difficult to distinguish whether microorganisms added to a contaminated body are responsible for all, some, or none of the degradation (Hung et al., 2010). Sometimes processes such as natural attenuation are responsible for degradation. Contamination in the environment is rarely under ideal conditions such as homogenous distribution of the contaminants to determine biodegradation effectiveness. Recalcitrant compounds have been biodegraded under controlled lab conditions that may not be possible in the environment. Under natural conditions, these compounds are highly stable and chemically

inert. These limitations must be taken into account when considering bioremediation/bioaugmentation as a treatment alternative for petroleum hydrocarbons. Future research on bioaugmentation is necessary to have a better understanding of its restrictions. A more systematic method should be adopted to understand the exact function of inoculated bacteria in the removal of contaminants (Kuhad, Parmar, and Sing, 2011). This would provide better control of the biodegradation process, even in unpredictable conditions.

2.3. Bioaugmentation of TPH Biodegradation using BiOWiSH™ Products

BiOWiSH™ Thai Aqua is a commercially discontinued product created by BiOWiSH™ Technologies, Inc. Per the product's online website (http://www.biowishtechnologies.com/us/), it is a natural biocatalyst containing a consortium of bacteria, enzymes, nutrients and co-factors. The bacterial contents are primarily lactobacillus (*P. acidilactic, P. pentosaceus*) and Bacillus (*subtilis*) with a total plate count of more than $1x10^5$ CFU/gram. Its recommended uses are municipal wastewater treatment plants, surface water systems, and industrial wastewater treatment systems. BiOWiSH™-Thai Aqua's benefits are enhancement of biological hydrolysis and oxidization of complex organic material, sludge yield reduction, and noxious odor generation reduction. BiOWiSH™ Technologies, Inc. suggests its product may be a candidate for the bioremediation of soil containing petroleum hydrocarbons.

A study conducted by Cal Poly graduate Michael Lehrer (2012), using a similar BiOWiSH™ Technologies Inc. product, named BiOWiSH™-Aqua FOG, tested the product's ability to biodegrade motor oil and diesel oil hydrocarbons in

soil. The main difference being BiOWiSH^{TM}-Aqua FOG has a key usage in breaking down fats, oils, and greases. The study concluded the product was successful in significantly improving biodegradation of the motor oil contaminants, but unsuccessful in biodegrading diesel compounds. Results indicated 45% more biodegradation of TPH in microcosms containing 500 parts per million (ppm) of BiOWiSH^{TM}-Aqua FOG than the control microcosms.

Other BiOWiSH^{TM} products tested in this study include BioCure Microbial Technologies (BMT) Remediate, Microbial Discovery Group (MDG Petro), Osprey Biotechnics BPB, and Osprey Biotechnics MPB. Table 2.3 lists the bacterial species and composition of the products.

Table 2.3 Additional BiOWiSH^{TM} bioaugmentation products tested

Product Name	Bacterial Species	Composition
BMT Remediate	Mix of *Rhodococcus* and *Arthobacter*	Undisclosed
MDG PETRO Petro	Mix of *Bacillus* and *Pseudomonas*	*Bacillus* spores, vegetative bacteria, soluble diluent
Osprey BPB	*Pseudomonas*	Undisclosed
Osprey MPB	Mix of *Bacillus* and *Pseudomonas*	*Bacillus* spores, vegetative bacteria, soluble diluent

CHAPTER 3. METHODS

3.1. DCM Microcosm Experiments

The first phase of experiments were used to test the BiOWiSH™ bioaugmentation products on DCM collected from a site in San Antonio, Texas. The site provided two buckets of five-gallon volume containing two different DCM samples. Midwest Laboratories in Omaha, Nebraska analyzed the samples for chemical constituents. The results of the analysis are listed in Table 3.1. The moisture content was 4.44 and 4.47 mg/Kg for sample 1 and sample 2 respectively.

Table 3.1 List of chemicals found in DCM

Analyte	Sample 1 (mg/Kg)	Sample 2 (mg/Kg)
Barium (total)	2,886	2,840
Chloride	2,314	1,913
Chromium	15.6	14.6
Lead (total)	10	13.5
Arsenic	14.9	14.3
Benzene	N.D.	N.D.
TPH as Gasoline	N.D.	N.D.
TPH as Diesel	64,500	54,200
TPH as Waste Oil	N.D.	N.D.

Abbreviations: N.D. – non-detectable concentrations

3.1.1. DCM Microcosm Experiments Preparation

A total of five DCM microcosm experiments were conducted in this study. Microcosms for all seven DCM experiments were prepared at lab scale using 1,000 mL Erlenmeyer flasks. AirOtop seals equipped with 0.2 µm filters covered flasks to prevent cross contamination while maintaining a high air exchange rate. As a base, all microcosms contained 600 g of de-ionized water and 300 g of DCM to mimic the 2:1 ratio of water to soil used on site in San Antonio, Texas. A

photograph of the microcosm setup is shown in Figure 3.1. Depending on the microcosm condition carried out by the respective microcosm, amendments were added to the microcosms. The three microcosm conditions used for the experiments were control, nutrient-only control, and bioaugmentation product. The control condition did not receive additional amendments. The nutrient-only control condition was used to test the effects of fertilizer and glucose addition. The bioaugmentation product condition was used to test the effects of fertilizer, glucose, and bioaugmentation product addition. Water-soluble Grow More fertilizer with an N:P:K ratio of 20:20:20 and Fisher Scientific lab grade Dextrose Anhydrous were used. Bioaugmentation products tested in addition to BiOWiSH^{TM}-Thai Aqua included Osprey BPB, Osprey MPB, MDG Petro, and BMT Remediate. Table 3.2 lists the tests of all six DCM experiments in chronological order with details of constituents added, sampling times, and objective of each experiment.

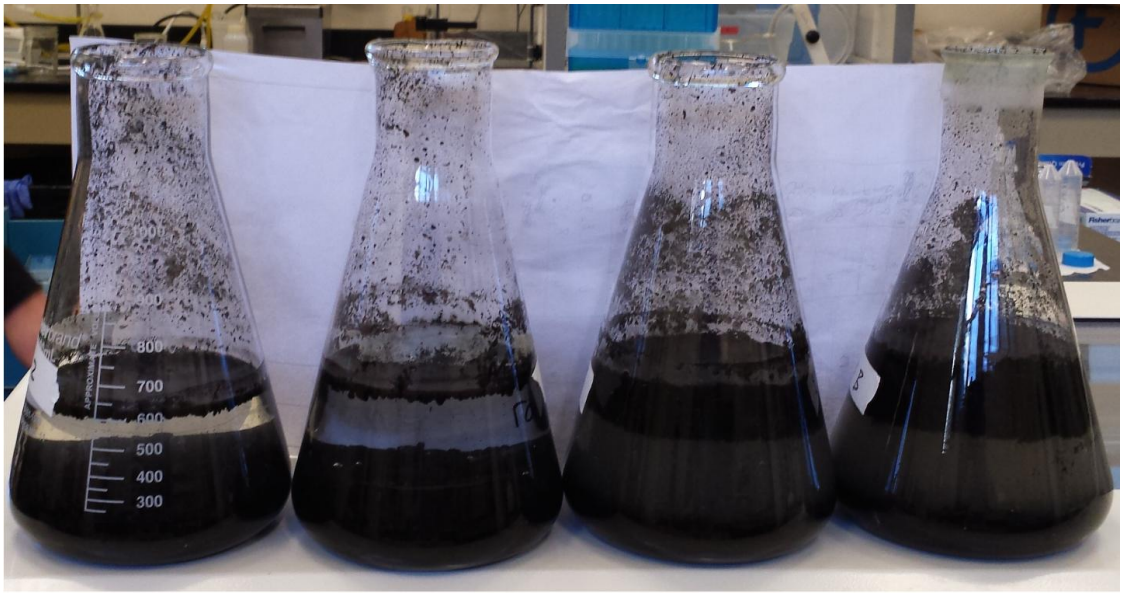

Figure 3.1 Photograph of DCM microcosm experiment microcosms

Table 3.2 List of DCM Microcosm Experiments and contents in chronological order

Experiment Name	DCM Sample No.	Microcosm Condition	Bioaugmentation Product[1]	Fertilizer	Glucose	Sample Times (days)	Objective
Microcosm Amendment Dosage Experiment	1	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	0.25, 0.75, 1, 1.25, 1.75, 2, 2.25, 2.75, 3, 7, 10, 13	Determine which amendment concentrations are optimal for use in remainder of the experiments.
		Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (119 mg/Kg$_{soil}$)	54.4 mg/Kg$_{soil}$	--		
		Nutrient-Only Control	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Nutrient-Only Control	--	54.4 mg/Kg$_{soil}$	--		
Initial Bioaugmentation Experiment	1	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	4, 6, 9, 31	Determine effectiveness of multiple products' ability to remediate TPH
		Bioaugmentation Product	Osprey BPB	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Bioaugmentation Product	Osprey BPB w/ Soy Lecithin[2]	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Bioaugmentation Product	Osprey MPB	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Bioaugmentation Product	Osprey MPB w/ Soy Lecithin	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Bioaugmentation Product	MDG Petro	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Bioaugmentation Product	MDG Petro w/ Soy Lecithin	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		

Concentration (unless stated otherwise in table)
[1]Bioaugmentation Product – 500 mg/Kg$_{soil}$
[2]Soy lecithin – 10 g/Kg$_{soil}$

Short Term Experiment	1	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	0, 2, 3, 12	Determine effectiveness of multiple product's ability to remediate TPH
		Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Bioaugmentation Product	BMT Remediate	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Bioaugmentation Product	BMT Remediate	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
DI Water Addition Experiment	1	Control	--	--	--	1, 2, 3.5	Determine effects of water addition to DCM
		Control	--	--	--		
Effect of Sodium Sulfate Addition on TPH Analysis Experiment	2	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	0.33, 1, 2, 3	Determine if addition of Na_2SO_4 improves TPH extraction efficiency
		Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Control	--	--	--		

Concentration (unless stated otherwise in table)
[1]Bioaugmentation Product – 500 mg/Kg_{soil}
[2]Soy lecithin – 10 g/Kg_{soil}

As recommended on the product description, the BiOWiSH^{TM}-Thai Aqua product was activated for 24 hours prior to inoculation. The activation protocol consisted of adding 250 mg of BiOWiSH^{TM}- Thai Aqua, 1 g of fertilizer, and 5 g of glucose to 1,000 mL of de-ionized water to attain the desired concentrations. Contents were stored in an incubator shaker set to 25 °C and 75 rpm. After 24 hours, 600 g of the activated mixture were added to 300 g of DCM. The microcosms were vigorously shaken to achieve homogeneity and placed in the incubator shaker throughout the experiment duration. Activation was not used for the other microbial products.

3.2. Sand Microcosm Experiment

The final experiment consisted of testing on sand media donated by construction companies. The sand was chosen for experimentation to attain a higher extraction efficiency using a more extractable media. Results from the DCM microcosm experiments suggested the DCM was a complicated media to efficiently extract. Microcosms in the Sand Microcosm Experiment contained 100 g of sand sifted using a no. 40 sieve (420 µm) and a no. 100 sieve (149 µm). The no. 40 sieve removed large particles and the no. 100 sieve removed fine clay particles. The sieved sand was rinsed thoroughly with de-ionized water to remove excess dust particles. The sand was then sterilized in a Tuttnauer Brinkman 2540E autoclave under 121°C and 16 psi conditions for 60 minutes. Oil extracted directly from the DCM used in the DCM experiments was added to the sterilized sand for the experiment to attain a concentration of approximately 800

mg/Kg. A GC/MS was used to analyze the sand experiment for TPH concentrations over time.

3.3. Sand Microcosm Experiment Preparation

Three microcosms were prepared for the final experiment. Microcosms were prepared using 250 mL Erlenmeyer flasks with AirOtop seals added as in the first phase experiments. Each microcosm received 100 g of the contaminated sand and 20 g of de-ionized water to attain 17% moisture content. The moisture level between 15-20% was chosen based on the study by Chang et al. (2009), which successfully treated diesel-contaminated soils. A photograph of the microcosm setup is displayed in Figure 3.2. Table 3.4 lists the microcosm conditions tested. The three environmental conditions in this experiment were similar to the DCM Microcosm experiments. For simplicity, the sand was assumed to have the density of water.

Figure 3.2 Photograph of sand microcosm experiment microcosms

Table 3.4 Contents of sand microcosm experiment

Microcosm Condition	Bioaugmentation Product	Fertilizer	Glucose	Sample Times (days)
Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (500 mg/$Kg_{soil+water}$)	2 g/$Kg_{soil+water}$	10 g/$Kg_{soil+water}$	0, 1, 2, 3, 7
Nutrient-Only Control	--	2 g/$Kg_{soil+water}$	10 g/$Kg_{soil+water}$	
Control	--	--	--	

The BiOWiSH^{TM} – Thai Aqua product was activated for 24 hours. The activation protocol consisted of thoroughly mixing 200 mg of BiOWiSH^{TM}-Thai Aqua, 800-mg of fertilizer, and 4,000 mg of glucose in 100 mL of de-ionized water. Contents were stored in an incubator shaker set to 25 °C and 75-rpm. After 24 hours, 20 g of the activated mixture was added to 100 g of contaminated sand. The sand was stirred with a sterilized spoon to achieve homogeneity. All three microcosms were placed in an incubator shaker set to 25 °C and 75 rpm throughout the seven days of experiment duration.

3.4. Sampling Protocol

For each sample, 15 g of DCM or contaminated sand were removed from the microcosms for TPH analysis. To prevent cross contamination, each microcosm was assigned a sampling spoon that was labeled and wrapped in aluminum foil. Every sample was collected in an autoclaved 50 mL beaker and covered with tin foil. Labels with the sample date/time, experiment name, and sampler initials were placed on the beakers for identification. The samples were placed in the freezer until it was time for extraction and analysis. As recommended by Lehrer (2012), to prevent TPH losses, the samples were never kept in the freezer for longer than one week.

3.5. TPH Extraction and Analysis

Prior to extraction, the samples were thawed and dried overnight at 50 °C to remove excess water. According to Dutta et al. (2003) samples may be dried at oven temperatures below 60 °C without experiencing TPH losses. The sample beakers were re-weighed after drying to determine dry weight of sample. The protocol for TPH extraction was derived from EPA Standard Method #3550. Fifteen mL of MeCl was added to the 50 mL beaker containing the sample. The contents were sonicated for 3 minutes at 60,000 Hz using a Sonifier 250 (Branson Ultrasonics Corp., Danbury, Connecticut). For excess water removal, 20 g of anhydrous sodium sulfate (Na_2SO_4) was added to a 24 cm diameter Fluted Grade Whatman 802 filter mounted in a glass funnel. Five grams of Na_2SO_4 were also added directly to the sample beaker. The aqueous portion was decanted through the fluted filter. The samples were filtered through a 0.22 µm EMD Millipore filter mounted on a vacuum pump filtering apparatus coupled with a tube to obtain the sample. A second 15 mL of MeCl was added to the sample beaker and the process was repeated yielding a total extract volume of 30 mL.

All extracted samples were placed in GC vials and analyzed with an Agilent Technologies 6890N Gas Chromatograph (splitless inlet) coupled with an Agilent 5975B inert Mass Selective Detector. An Agilent 7683B Series Injector with a capacity of eight GC vials automatically loaded the samples. A 10 µL syringe injected the samples at a volume of 2 µL, and was automatically rinsed twice with MeCl in between sample loadings to prevent cross contamination. After a sample was loaded, the front inlet was pressurized to 12.26 psi at a

temperature of 200- °C. The oven temperature rose from 45 °C to 275 °C at a rate of 12 °C per minute. The temperature was held at 275 °C for the remainder of the 34-minute sample run time. The GC/MS was programmed to run all samples in quadruplets. Chromatograms were integrated across the entire run time. The samples' quadruplet peak areas were averaged to obtain a representative peak area. The resulting peak area was used to calculate the TPH concentration of the samples. Calibration curve equations were derived from standards of known concentrations of diesel oil for the DCM microcosm experiments and oil extracted from the DCM for the sand microcosm experiment.

3.5.1. Calibration Curve Preparation

Two standards curves were prepared, one for each of the two experiment phases. Both curves were necessary due to the high TPH concentrations in the DCM and the low TPH concentrations in the sand. Standards for DCM microcosm experiments were prepared using diesel to represent the high TPH as diesel present in the DCM samples. 5.09 g of diesel was weighed in a 100 mL volumetric flask. The flask was filled to volume with MeCl for a final standard TPH concentration of 50,900 mg/L. Dilutions were prepared from the stock solution by pouring 50 mL of the stock solution into a 100 mL volumetric flask and filling the flask to volume with MeCl. A total of 4 serial dilutions were prepared by repeating this process. Each standard was placed in a 2 mL GC vial and run in quadruplets through the GC/MS. The peak areas of the resulting chromatograms were plotted with their corresponding standard concentration in Figure 3.3.

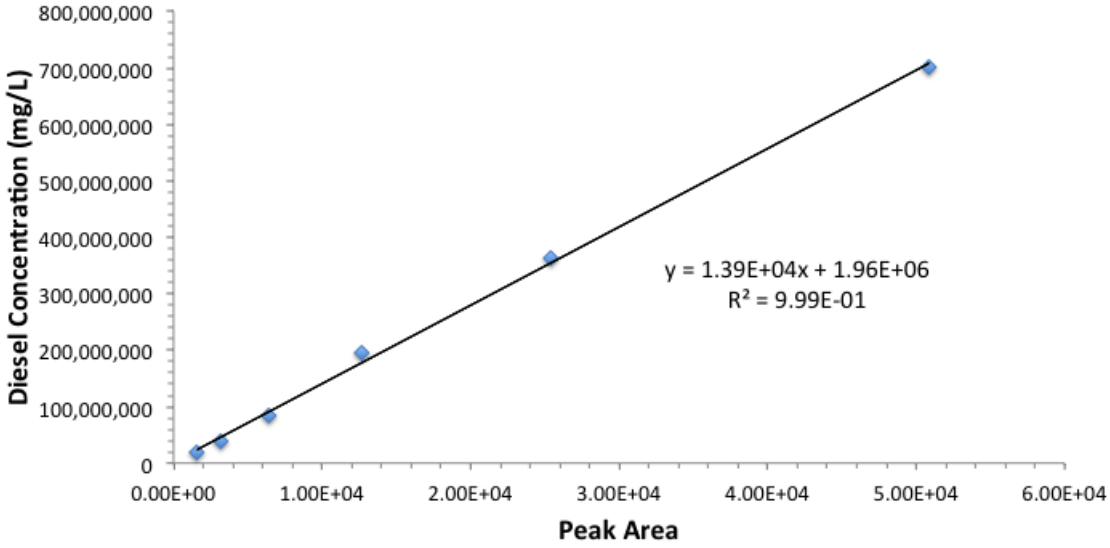

Figure 3.3 Calibration Curve for the DCM microcosm experiments

Standards for sand experiments were prepared similar to the DCM experiment standards. Oil extracted directly from the DCM was used instead of diesel oil. The oil in the DCM was extracted as described in section 3.6. The filtered oil and MeCl solution was poured into a 1,000 mL beaker and placed in a fume hood to allow the MeCl to evaporate overnight. Once the MeCl was evaporated, the leftover 2.3 g of DCM oil was left in a beaker in the fume hood for an additional three days to allow volatile compounds to evaporate. Over the course of three days, the DCM oil reduced in mass by 0.06 g indicating a total of 2.5% volatilization losses. 0.209 g of the final oil product were weighed in a 100 mL volumetric flask which was filled to volume with MeCl for a final standard TPH concentration of 2,090 mg/L. Standard dilutions were prepared by pouring 50 mL from the stock solution into a 100 mL volumetric flask and bringing the flask to volume. The process was repeated to prepare 4 dilutions, and all five standards were run in quadruplets with the GC/MS. The peak areas of the resulting

chromatograms were plotted with their corresponding standard concentration in Figure 3.4.

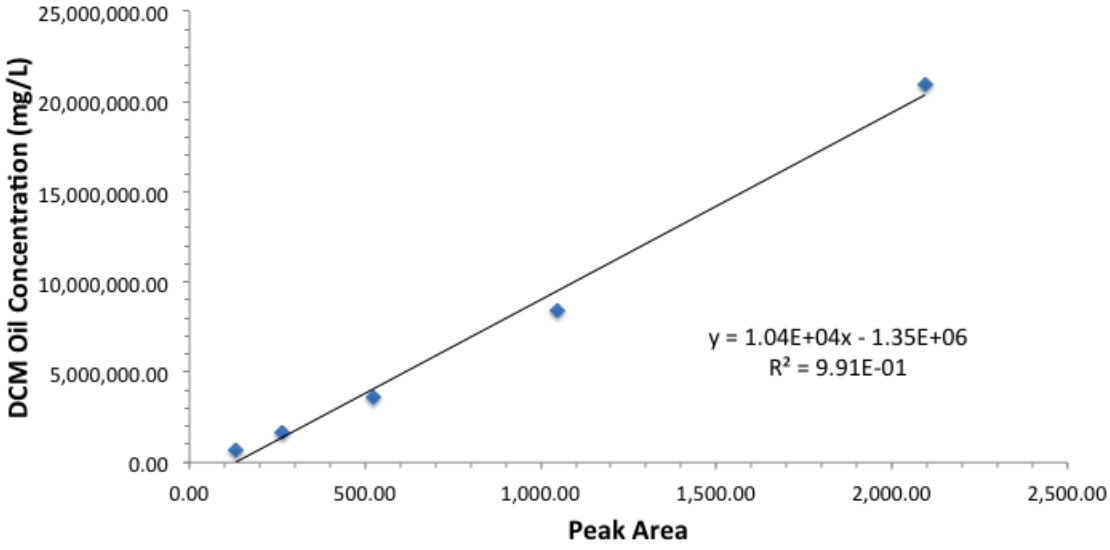

Figure 3.4 Calibration Curve for the sand microcosm experiment

3.6. TPH Extraction Method Efficiency Analysis

An analytical experiment was conducted to test the accuracy of the TPH extraction protocol used in this study. Four samples of unknown TPH concentration provided by BiOWiSH^{TM} Technologies, Inc. were extracted and analyzed using the method described in the previous section of this study. The same samples were extracted and analyzed simultaneously by Midwest Laboratories and A & B Labs in Houston, Texas. The four samples contained different concentrations of DCM diluted with caliche. It is important to note that these samples were prepared from fresh DCM and not DCM collected from microcosms containing a 2:1 ratio of water to soil.

3.7. DCM Microcosm Respirometric Analysis

A total of four respirometric experiments were conducted using DCM microcosms to provide secondary analysis to the DCM microcosm experiments in section 3.1 of this study. Thus, the microcosm conditions were identical to the DCM microcosm experiments. As a base, all microcosms contained 66 g of DCM and 132 g of de-ionized water. A Micro-Oxymax^{TM} Respirometer (Columbus Instruments; Columbus, Ohio) with a CO_2 and a methane sensor was used for respirometric analysis. The respirometer had a 10-channel expansion interface equipped with a condensing air drier. Columbus Instruments calibrated the CO_2 and methane sensors and the re-circulating pump. The 10-channels were connected to 250 mL threaded Pyrex^{TM} bottles via screw cap. To ensure conditions were similar to the DCM experiments, the bottles were placed in a water bath to maintain 25 °C temperature throughout the experiment. Diagnostics (basic operations, valves and sensors, and expansion unit) were run prior to beginning every experiment for quality assurance purposes. The Micro-Oxymax^{TM} program recorded the time (hours), channel temperature (°C), and CO_2 accumulation (µl) for every channel on a .dat file.

3.7.1. DCM Microcosm Respirometric Analysis Preparation

Table 3.3 lists the respirometric experiments in chronological order. Activation of BiOWiSH^{TM}-Thai Aqua was completed using the same procedure as previously described for the DCM experiments; the Osprey products were not activated. Following the 24-hour activation, 132 g of activated liquid mixture were

added to 66 g of DCM. The microcosm bottles were shaken vigorously to achieve homogeneity and placed in the water bath for the duration of the experiments.

Table 3.3 List of DCM microcosm respirometric analysis experiments

Experiment Name	DCM Sample No.	Microcosm Condition	Bioaugmentation Product[1]	Fertilizer	Glucose	Experiment Duration (days)	Objective
Microcosm Amendment Dosage Experiment	1	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	3	Provide secondary analysis for the Microcosm Amendment Dosage Experiment 1
		Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (119 mg/Kg$_{soil}$)	54.4 mg/Kg$_{soil}$	--		
		Nutrient-Only Control	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Nutrient-Only Control	--	54.4 mg/Kg$_{soil}$	--		
Autoclaved DCM Experiment	1	Bioaugmentation Product (autoclaved DCM)	BiOWiSH^{TM} – Thai Aqua	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	7	Determine the effects of using autoclaved DCM
		Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Control (autoclaved DCM)	--	--	--		
		Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Nutrient-Only Control	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$		
		Control	--	--	--		

Concentration (unless stated otherwise in table)
[1]Bioaugmentation Product – 500 mg/Kg$_{soil}$

Re-Dose Experiment 1	2	Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg_{soil}	10 g/Kg_{soil}	7	Observe effects of nutrient addition on DCM prior to re-inoculating with microbial products
		Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
Re-Dose Experiment 2	2	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	6	Observe effects of re-inoculating DCM with microbial products
		Bioaugmentation Product	Osprey MPB	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Bioaugmentation Product	Osprey MPB	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control (autoclaved DCM)	--	2 g/Kg_{soil}	10 g/Kg_{soil}		
		Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}		

Concentration (unless stated otherwise in table)
[1]Bioaugmentation Product – 500 mg/Kg_{soil}

CHAPTER 4. RESULTS AND DISCUSSION

4.1. DCM Microcosm Experiment Results

The results of the five DCM microcosm experiments provide tables with the experimental setups of each experiment, graphical representation of the TPH analysis from samples, and an analysis of the results. It is important to note that the DCM did not contain a significant amount of volatiles as indicated in section 2.7.1 of this study. DCM oil extracted from the DCM experienced 2.5% volatilization losses over the course of three days. The observed rapid TPH concentration decreases in the following results are not likely due to volatilization of TPH. Tables containing the data for the experiments in this section can be found in Appendix A.

4.1.1. Microcosm Amendment Dosage Experiment

This microcosm experiment was conducted to observe the treatment differences using four microcosms containing different amendment dosages. The experimental setup is listed on Table 4.1, and Figure 4.1 is a graphical representation of the TPH analysis results. Two different microcosm conditions were tested using two different concentration amounts for the bioaugmentation product, fertilizer and glucose amendments. From the figure, all four microcosms exhibited similar TPH results over time. The microcosms decreased from the initial concentration of approximately 60,000 mg/Kg to approximately 20,000 mg/Kg in 13 days. The observed decrease in TPH concentration of 67% after thirteen days is significantly more rapid than the 75% TPH removal after one year in the test performed by Dutta, et al (20003). It is important to note that there are

fluctuating concentrations throughout the experiment duration although the general trend of the TPH concentrations is steadily decreasing. Conclusions cannot be made regarding the differences in the two treatments due to the rapid decrease and fluctuating concentrations indicating there is some experimental error. The higher amendment dosage concentrations for the bioaugmentation product and Nutrient-Only Control microcosm conditions, however, were used in all of the following DCM microcosm experiments. The higher concentrations were assumed to provide a more optimal environment for TPH biodegradation.

Table 4.1 Experimental setup and objective for the Microcosm Amendment Dosage Experiment

Experiment Name	Name in Figure 4.2	Microcosm Condition	Bioaugmentation Product	Fertilizer	Glucose	Objective
Microcosm Amendment Dosage Experiment	BiOWiSH^{TM} – Thai Aqua 1	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (500 mg/Kg$_{soil}$)	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	Determine which amendment concentrations are optimal for use in remainder of the experiments.
	BiOWiSH^{TM} – Thai Aqua 2	Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (119 mg/Kg$_{soil}$)	54.4 mg/Kg$_{soil}$	--	
	Nutrient-Only Control 1	Nutrient-Only Control	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$	
	Nutrient-Only Control 2	Nutrient-Only Control	--	54.4 mg/Kg$_{soil}$	--	

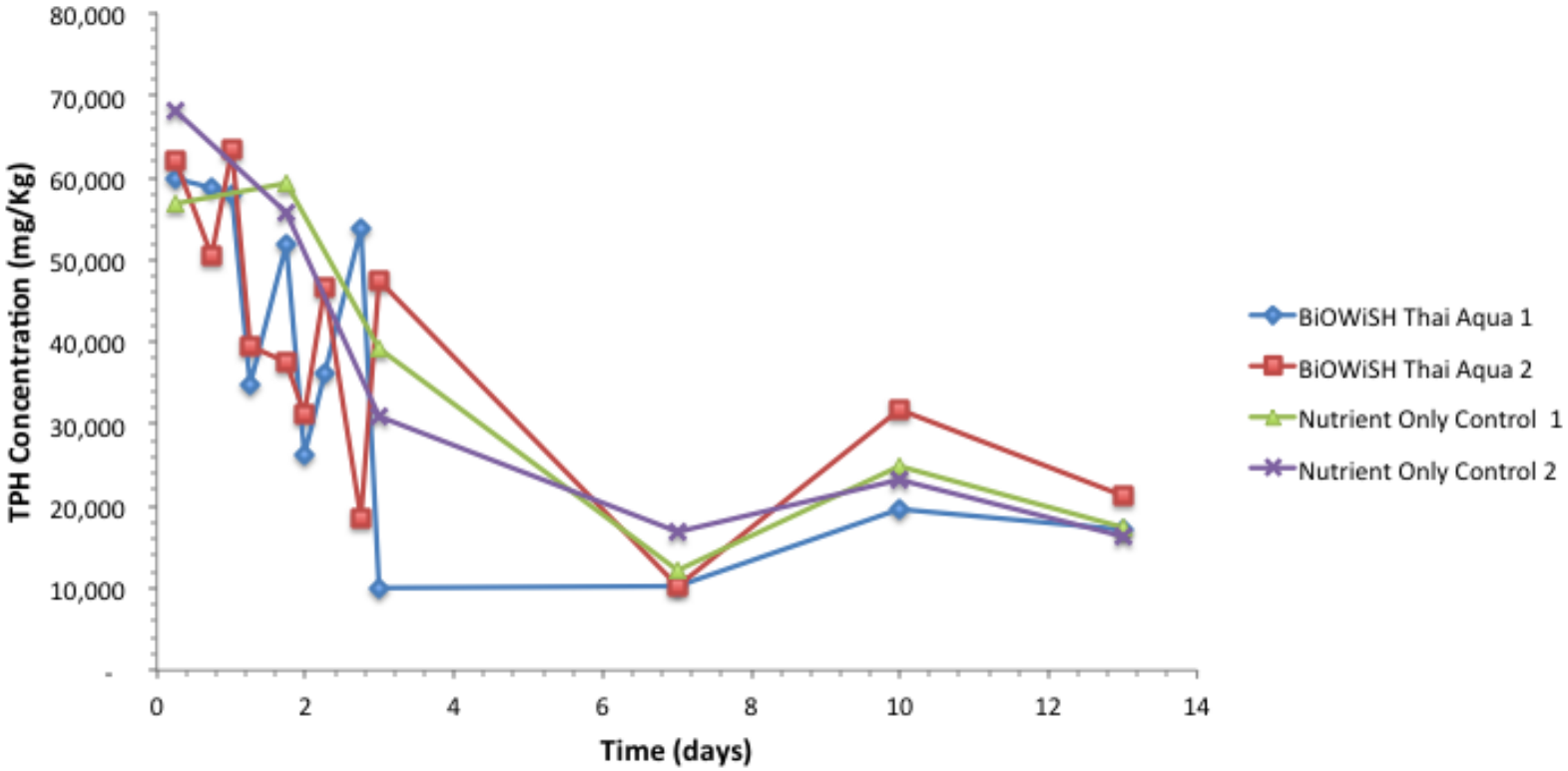

Figure 4.1 TPH concentration over time in the Microcosm Amendment Dosage Experiment

4.1.2. Initial Bioaugmentation Experiment

This microcosm experiment was conducted to compare the effects of bioaugmentation using four different microbial products. The surfactant soy lecithin was also added during microcosm setups to explore the effects of surfactant addition to bioaugmented DCM microcosms. The experimental setup is listed on Table 4.2, and Figure 4.2 is a graphical representation of the TPH analysis results. There were missing data points for Osprey MPB at time 4 days, MDG Petro at times 4 and 6 days, and MDG Petro w/ soy lecithin at 4 and 6 days. This was due to the accidental breakage of vials containing the samples. The measured TPH concentrations of the DCM in the microcosms after 4 days were significantly higher than the concentrations of fresh DCM Midwest Laboratories provided (Table 3.1). There were also fluctuating concentration measurements over time. The Osprey BPB, Osprey MPB, and MDG Petro w/ soy lecithin samples displayed higher concentration in samples following previous lower concentration samples. This indicates experimental error during TPH extraction. Due to the experimental errors, conclusions cannot be made from these data. Future experiments aimed to achieve higher quality results by improving the TPH extraction and analysis method and prevention of sample vial breakage.

Table 4.2 Experimental Setup and objective for the Initial Bioaugmentation Experiment

Experiment Name	Microcosm Condition	Bioaugmentation Product[1]	Fertilizer	Glucose	Objective
Initial Bioaugmentation Experiment	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	Determine effectiveness of multiple products' ability to remediate on a long term basis
	Bioaugmentation Product	Osprey BPB	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	Osprey BPB w/ Soy Lecithin[2]	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	Osprey MPB	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	Osprey MPB w/ Soy Lecithin	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	MDG Petro	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	MDG Petro w/ Soy Lecithin	2 g/Kg_{soil}	10 g/Kg_{soil}	

Concentration
[1]Bioaugmentation Product – 500 mg/Kg_{soil}
[2]Soy Lecithin – 10 g/Kg_{soil}

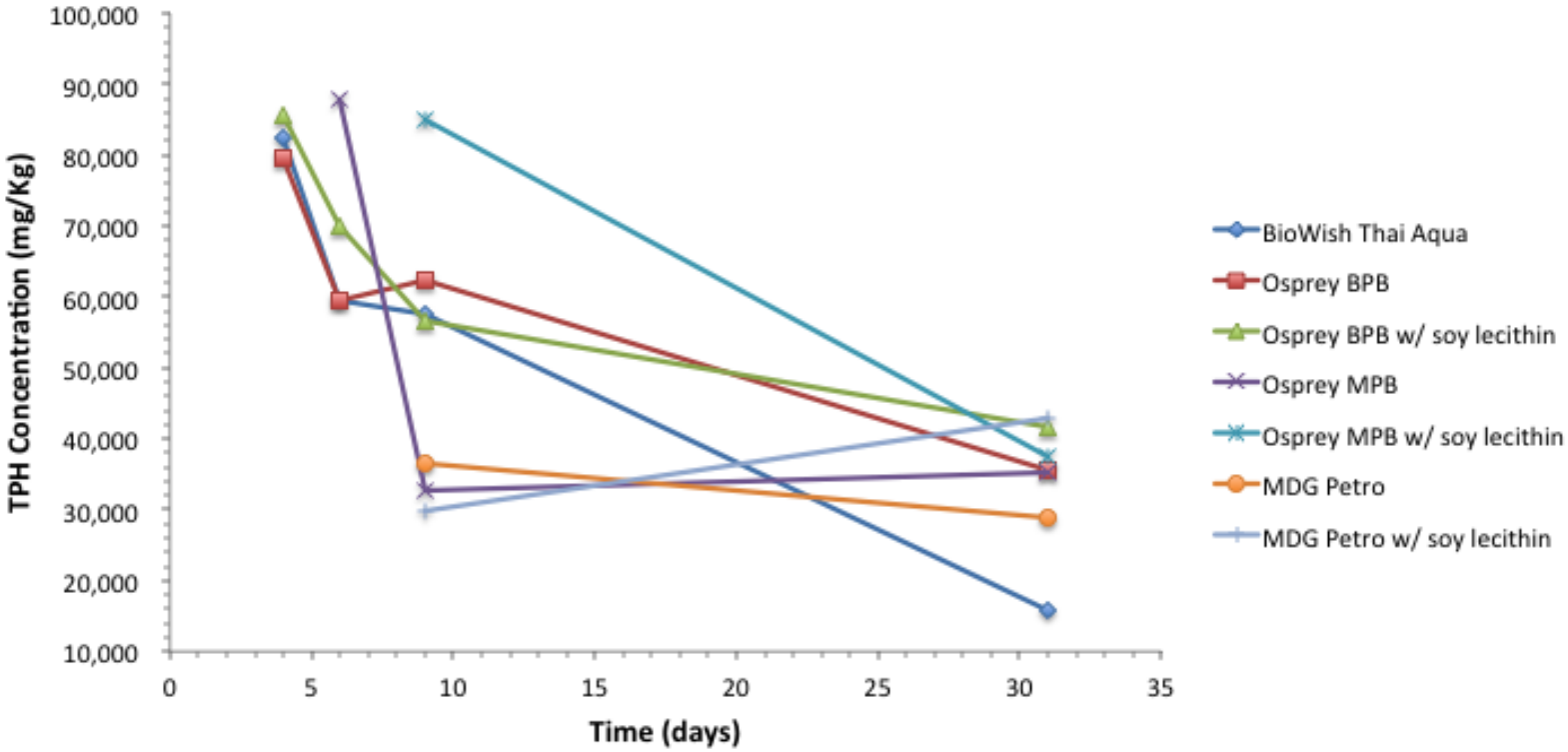

Figure 4.2 TPH concentrations over time in the Initial Bioaugmentation Experiment

4.1.3. Secondary Bioaugmentation Experiment

This microcosm experiment was conducted to compare three different treatments using three different microcosm setups containing the BiOWiSH^{TM} – Thai Aqua, BMT Remediate, and nutrient-only control conditions. The contents of the experimental setup are listed on Table 4.3, and Figure 4.3 is a graphical representation of the TPH analysis results. The three microcosm treatments were set up in duplicates. The results in Figure 4.3 show the averaged TPH concentrations of duplicates of each of the three treatments. After 12 days of incubation, the BMT Remediate and nutrient-only control exhibited the lowest observed TPH reduction of approximately 15,000 mg/Kg. The final observed TPH concentration using the BiOWiSH^{TM} – Thai Aqua treatment was approximately 21,000 mg/Kg. It is important to note that after three days the BiOWiSH^{TM} – Thai Aqua TPH concentration was 17,500 mg/Kg. The three treatments displayed approximately 30,000 mg/Kg observed TPH reduction in 12 days. The results of this experiment suggested the microcosms experienced abnormally rapid TPH concentration reduction and fluctuations in concentration over time. The following experiment aimed to identify the cause for the observed effects by introducing a microcosm condition containing only de-ionized water.

Table 4.3 Experimental setup and objective for the Short Term Experiment

Experiment Name	Microcosm Condition	Bioaugmentation Product[1]	Fertilizer	Glucose	Objective
Secondary Bioaugmentation Experiment	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	Determine effectiveness of multiple product's ability to remediate on a short term basis
	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	BMT Remediate	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Bioaugmentation Product	BMT Remediate	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}	

Concentration
[1]Bioaugmentation Product – 500 mg/Kg_{soil}

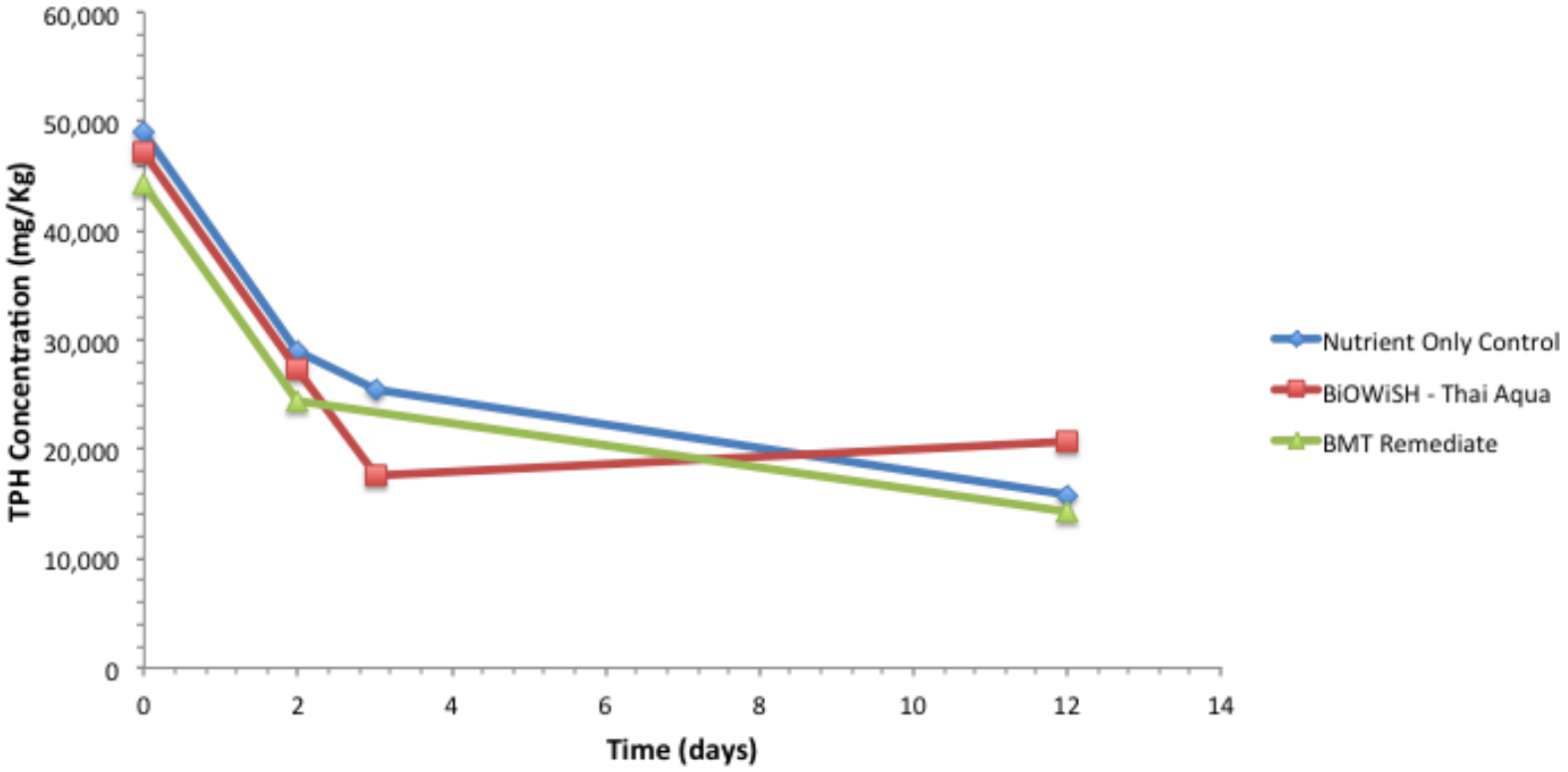

Figure 4.3 TPH concentrations over time in the Short Term Experiment

4.1.4. DI Water Addition Experiment

The microcosm control condition was introduced and tested in duplicate microcosms in this experiment to observe the effects of de-ionized water only addition to microcosms. The contents of the experimental setup are listed on Table 4.4, and Figure 4.4 is a graphical representation of the TPH analysis results. Both replicates experienced a decrease in TPH concentration of approximately 40,000 mg/Kg in 3.5 days. The following equations were used to calculate the first order rate constants (k). The first equation is the first order rate constant equation where [A] is the final concentration and $[A]_0$ is the initial concentration. The time from the initial to final concentration is t, and the first order rate constant is k. The second equation is the first equation re arranged to solve for the first order rate constant.

$$[A] = [A]_0 \, e^{-kt}$$

$$k = -\frac{\ln\frac{[A]}{[A]_0}}{t}$$

The average first order rate constant of the controls was 0.35/day. First order rate constants from biodegradation of weather drilling wastes in literature range from 0.005-0.02/day (Jakubowicz, Kapusta, & Steliga, 2010). Thus the average rate constant in this experiment was approximately 18-70 times greater than constants found in literature.

BiOWiSH™ Technologies, Inc. provided results from a TPH analysis of the aqueous layer from a study similar to this one. The aqueous layer from

microcosms containing a 2:1 ratio of water to DCM was sampled over the course of 72 hours. Results indicate that the aqueous samples contained a maximum of 1,660 mg/L of TPH as Diesel (Midwest Laboratories, Inc. 2014). The transport of TPH from the DCM to the aqueous layer is negligible and was not an explanation for the high levels of TPH removal in the DCM.

Table 4.5 summarizes the results of further DCM characteristics analysis performed during this time by Midwest Laboratories, Inc. (2014). Results indicate that the DCM contained fatty acids and fibers. Midwest Laboratories, Inc. (2014) confirmed the fatty acids and fibers, which contain carbon chains, may have been falsely characterized as hydrocarbons during TPH analysis. A possible explanation for the significantly large decrease of 40,000 mg/Kg in 3.5 days may be that the easy to degrade fatty acids and fibers were biodegraded by the microorganisms and resulted in false TPH reductions in the samples. It is also possible that the addition of water to DCM complicated the extraction process. According to Solutions on Site Environmental, Inc. (2011), addition of Na_2SO_4 will successfully disintegrate clay structures, allowing oils to be extracted. The following experiment explored this possibility.

Table 4.4 Experimental setup and objective for the DI Water Addition Experiment

Experiment Name	Microcosm Condition	Bioaugmentation Product	Fertilizer	Glucose	Objective
DI Water Addition Experiment	Control	--	--	--	Determine effects of water addition to DCM
	Control	--	--	--	

Table 4.5 Results for percent composition from the secondary DCM analysis provided by Midwest Laboratories

Analyte	Percent Composition
Fat (acid hydrolysis)	9.79
Fiber (neutral detergent)	16.0

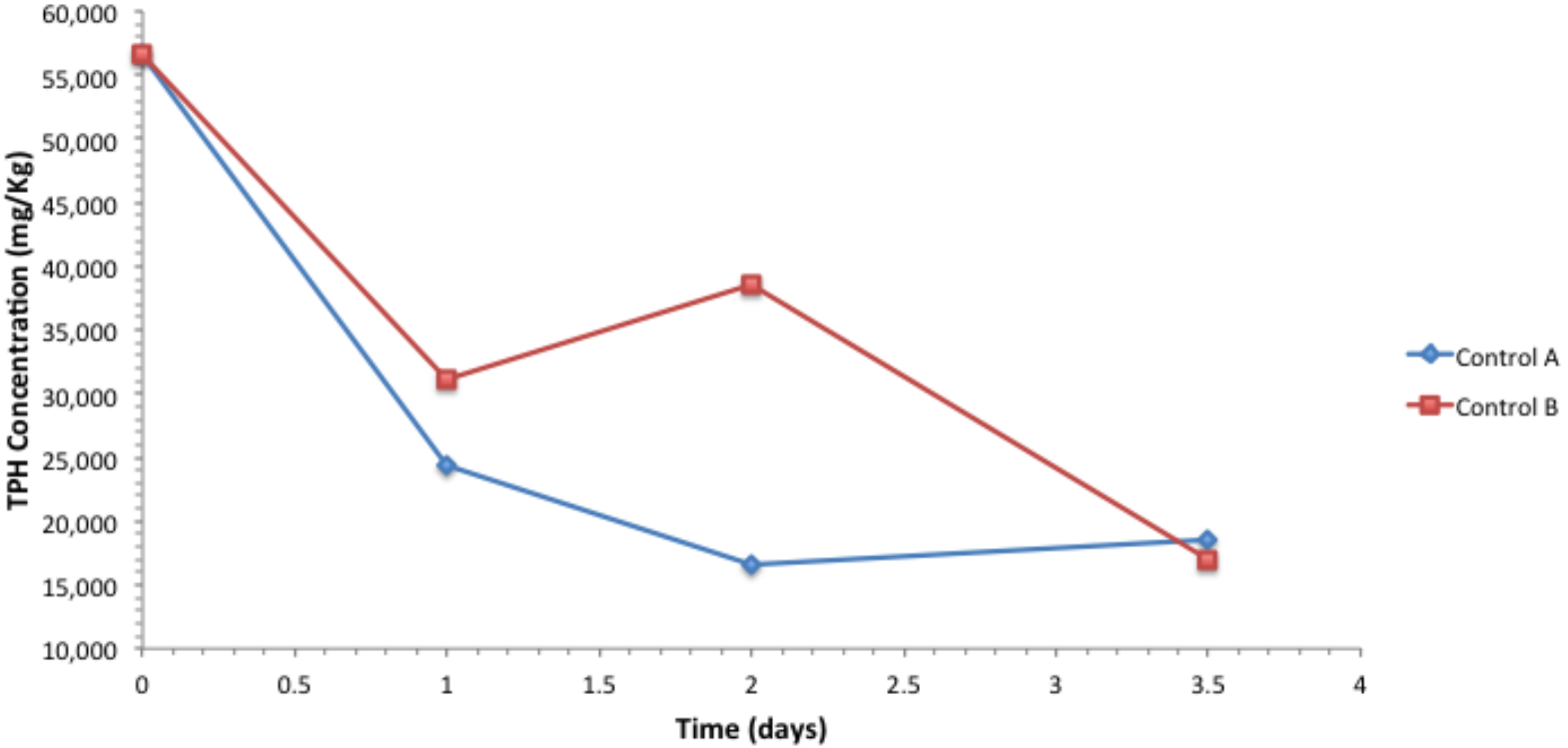

Figure 4.4 TPH concentration over time in the DI Water Addition Experiment

4.1.5. Effect of Sodium Sulfate on TPH Analysis Experiment

In this microcosm experiment, Na_2SO_4 was mixed with de-ionized water and added to samples prior to extraction to achieve a 1:1:1 ratio by weight of DCM, de-ionized water, and Na_2SO_4. Contents were placed in an incubator shaker at 25 °C and 75-rpm for one hour to ensure proper mixing. Samples were dried in oven at 50 °C to remove excess water and obtain dry weights. The contents of the experimental setup are listed on Table 4.6, and Figure 4.5 is a graphical representation of the TPH analysis results. Results indicate there was a slight improvement in TPH extraction from the DCM samples over time. The control microcosm condition showed a final TPH concentration of 42,500-mg/Kg after 3 days compared to 18,000-mg/Kg after 3.5 days in the control microcosm condition of the previous Experiment. This suggests water addition to DCM did complicate the extraction process. Despite the improvements in extraction efficiency, the overall large decrease (approx. 20,000 mg/Kg) in TPH concentration exhibited by the control microcosm condition was still a significantly more rapid decrease than reported in literature. The fluctuating concentrations were also still present from one sample time to the next in all three of the tests in this experiment. The TPH extraction method used in this study was tested for accuracy in the following section.

Table 4.6 Experimental setup and objective for the Effect of Sodium Sulfate Addition on TPH Analysis Experiment

Experiment Name	Microcosm Condition	Bioaugmentation Product[1]	Fertilizer	Glucose	Objective
Effect of Sodium Sulfate Addition on TPH Analysis Experiment	Bioaugmentation Product	BiOWiSH™ – Thai Aqua	2 g/Kg_{soil}	10 g/Kg_{soil}	Determine if addition of Na_2SO_4 improves TPH extraction efficiency
	Nutrient-Only Control	--	2 g/Kg_{soil}	10 g/Kg_{soil}	
	Control	--	--	--	

Concentration
[1]Bioaugmentation Product – 500 mg/Kg_{soil}

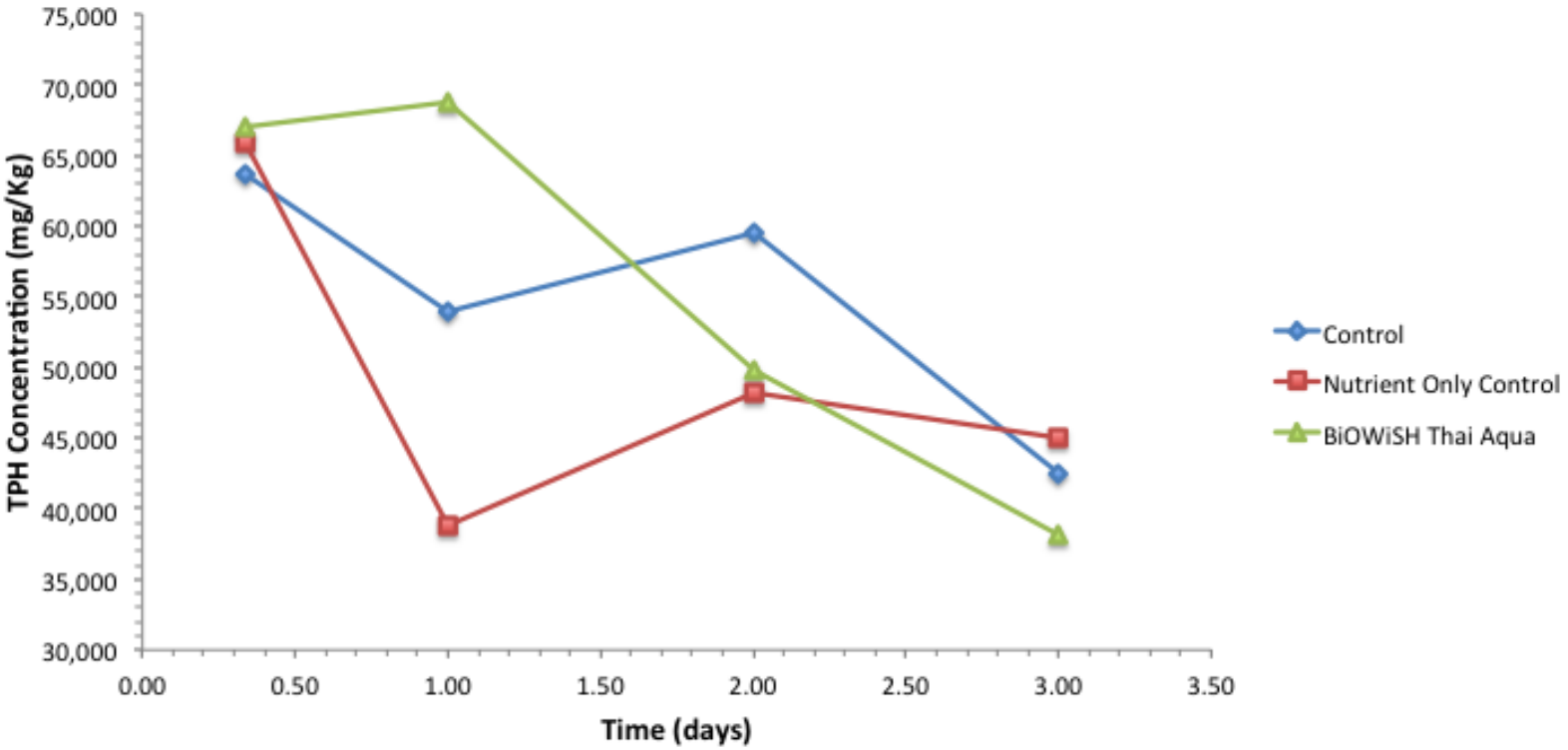

Figure 4.5 TPH concentrations over time in the Effect of Sodium Sulfate Addition on TPH Analysis Experiment

4.2. TPH Extraction Method Efficiency Analysis

This analysis tested the accuracy of the TPH extraction protocol used in previous experiments. BiOWiSHTM Technologies, Inc. provided four samples of unknown TPH concentration. The four samples contained different concentrations of DCM diluted with caliche to provide a range of TPH concentrations. It is important to note that these samples were prepared from fresh DCM and not DCM collected from microcosms containing a 2:1 ratio of water to soil. Midwest Laboratories and AB Labs simultaneously performed TPH extraction and analysis on the same soil samples. Figure 4.6 provides the results provided of all three labs.

AB Labs provided results significantly different from Midwest Laboratories and Cal Poly. The extraction method used by AB Labs used the solvent Pentane rather than MeCl used by both Midwest Laboratories and Cal Poly. The lower concentrations present in the AB Labs samples indicated Pentane was unable to extract as many hydrocarbons and provided lower extraction efficiency. Midwest Laboratories and Cal Poly provided similar results. The results of this experiment conclude the TPH extraction and analysis methods in this study are reproducible and may be used for the Sand Microcosm Experiment in the next section. Because the diluted samples containing fresh DCM provided more accurate results, it was also concluded that fatty acids and fibers are likely being biodegraded in microcosms.

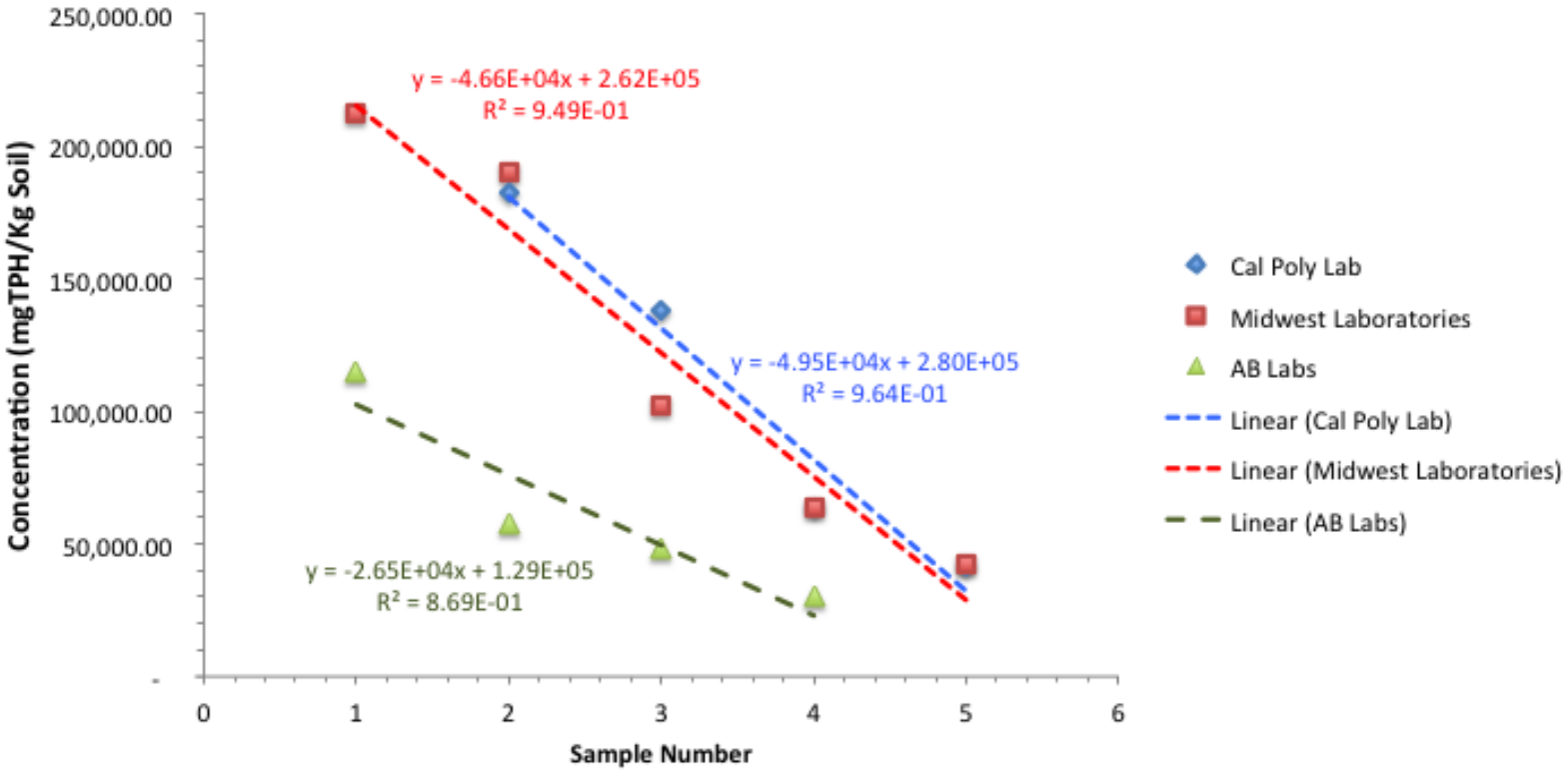

Figure 4.6 Graphical representation of the TPH concentrations in the TPH Extraction Method Efficiency Analytical Experiment

4.3. Sand Microcosm Experiment Results

Sand was chosen as the medium to provide a more extractable soil matrix. The study by Lehrer (2012) provided accurate TPH extraction results when using sand media. The contents of the experimental setup are listed on Table 4.7, and Figure 4.7 is a graphical representation of the TPH analysis results. The percent TPH removal, percent change, and first order rate constants with respect to the microcosm condition are given in Table 4.8. The following equations were used to calculate the TPH removal and change in TPH removal:

$$TPH\ Removal(\%) = \frac{Initial\ TPH\ Conc. - Final\ TPH\ Conc.}{Initial\ TPH\ Conc.}\ x\ 100\%$$

$$Change\ in\ TPH\ Removal(\%) = \frac{TPH\ Removal\ (\%) - TPH\ Removal(\%)_{water\ only\ control}}{TPH\ Removal\ (\%)_{water\ only\ control}} x100\%$$

The microcosm preparation described in section 3.2.1 of this study did not achieve a homogenous sand medium. The Day 0 concentrations vary for the three microcosms as shown on Figure 4.7. From Day 0 to Day 3, the TPH concentrations in the control microcosm remain relatively steady, and the nutrient-only control microcosm and BiOWiSH^{TM}-Thai Aqua microcosm steadily decrease. By Day 7 the control microcosm had a TPH concentration decrease of 25% while the nutrient-only control and BiOWiSH^{TM}-Thai Aqua had a TPH removal of 39.6% and 47.2% respectively. The percent change relative to the control was 58.9% in the nutrient-only control and 89.3% in the BiOWiSH^{TM}-Thai Aqua. The first order rate constants were calculated similar to the first order rate

constants in the DI Water Addition Experiment. The control, nutrient-only control, and BiOWiSH^{TM} – Thai Aqua had first order rate constants of 0.04, 0.07, and 0.09/day respectively. These values are closer to the 0.005-0.02/day found in literature compared to the first order rate constant of 0.35/day in the DI Water Addition Experiment (Jakubowicz, Kapusta, & Steliga, 2010). Despite the highest removable percentage observed in the BiOWiSH^{TM} –Thai Aqua microcosm, replicates are necessary to confirm the bioaugmentation product was responsible for the TPH concentration decrease. Tables containing the data for this experiment can be found in Appendix A.

Table 4.7 Experimental setup for the Sand Microcosm Experiment

Microcosm Condition	Bioaugmentation Product	Fertilizer	Glucose
Bioaugmentation Product	BiOWiSH^{TM} – Thai Aqua (500 mg/Kg$_{soil}$)	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$
Nutrient-Only Control	--	2 g/Kg$_{soil}$	10 g/Kg$_{soil}$
Control	--	--	--

Table 4.8 TPH Removal, change in TPH removal, and the first order rate constants in the microcosms of the Sand Microcosm Experiment

Microcosm Condition	TPH Removal (%)	Change in TPH Removal (%)	First Order Rate Constant (/day)
Control	25.0	--	0.04
Nutrient-Only Control	39.6	58.9	0.07
Bioaugmentation Product	47.2	89.3	0.09

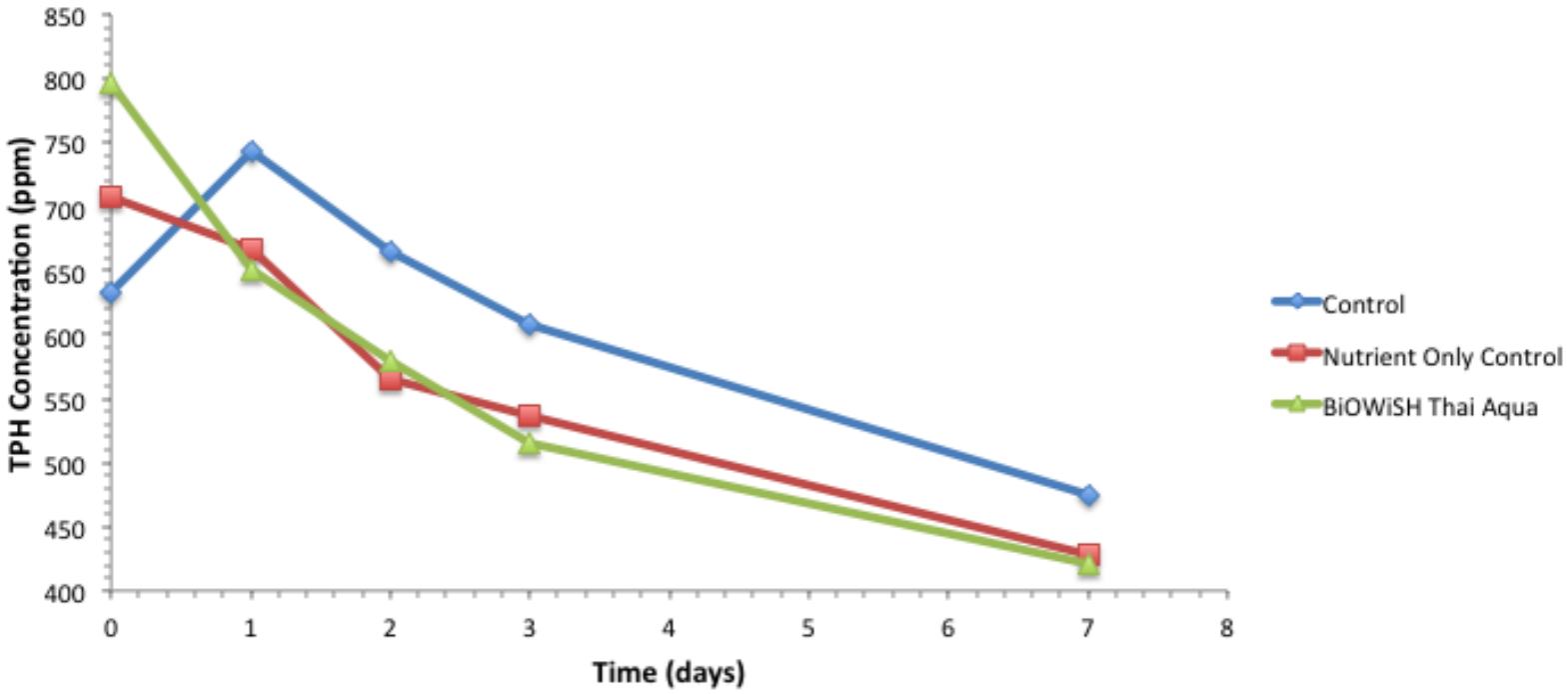

Figure 4.7 TPH concentrations over time in the Sand Microcosm Experiment

4.3.1. Sand Microcosm Experiment Chromatograms

Figures 4.8, 4.9, and 4.10 are side-by-side chromatograms of the control, nutrient-only control, and BiOWiSH^{TM}-Thai Aqua samples at times 0 and 7 days in the Sand Experiment. The alkane peaks were observed in the three figures between 10 and 22 minutes. There was similar visible reduction in the peaks for all three treatments.

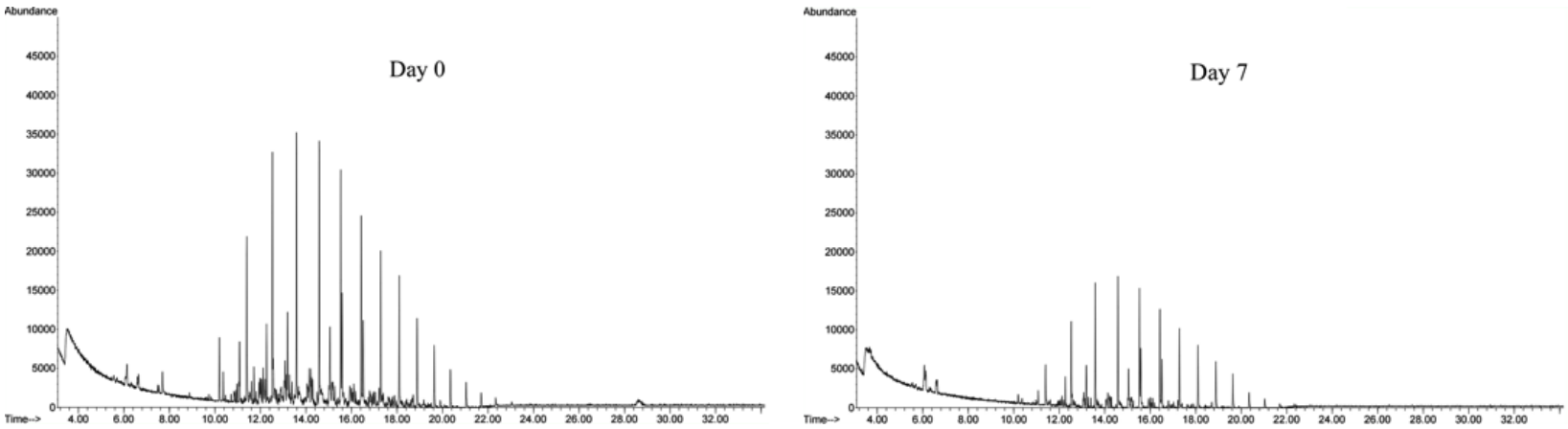

Figure 4.8 Chromatograms for the control microcosm at times 0 and 7 days

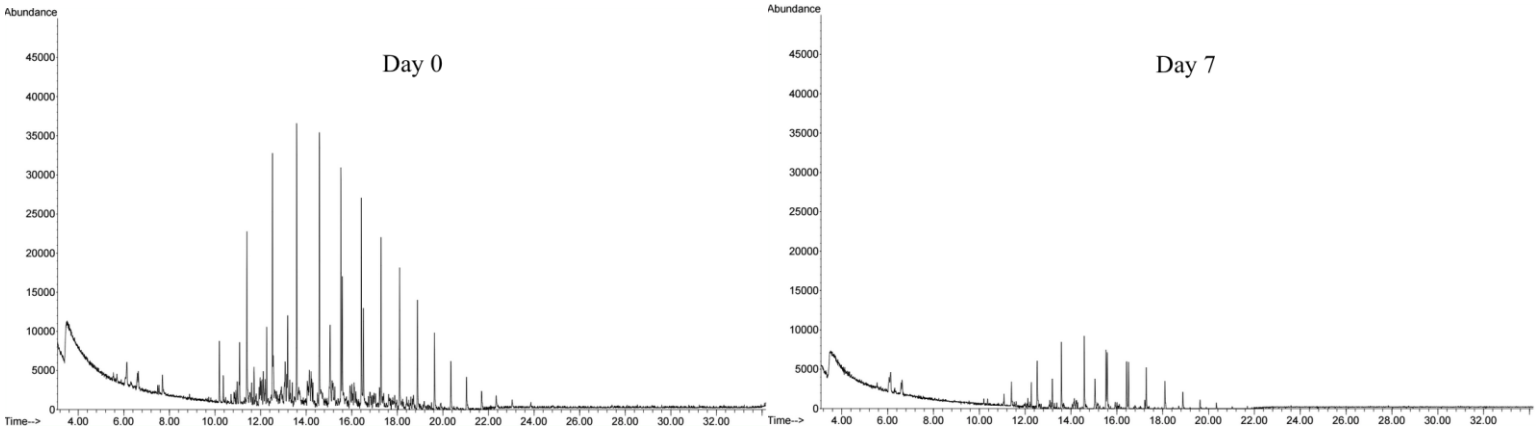

Figure 4.9 Chromatograms for the nutrient-only control microcosm at times 0 and 7 days

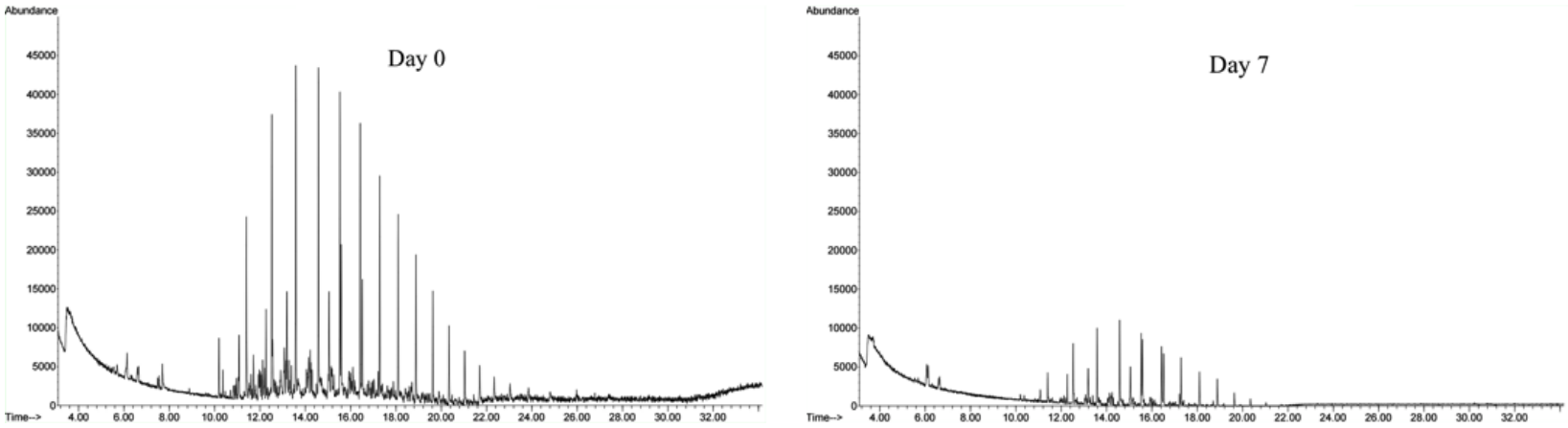

Figure 4.10 Chromatograms for the BiOWiSH^{TM} - Thai Aqua microcosm at times 0 and 7 days

4.4. DCM Microcosm Respirometric Analysis

Results from the four respirometric analysis experiments suggested CO_2 respiration of glucose overshadowed CO_2 respiration from biodegradation. Thus conclusions cannot be made from the four DCM Microcosm Respirometric Analysis experiments. The addition of BiOWiSH^{TM} bioaugmentation products reduced the lag phase for glucose degradation and displayed more rapid CO_2 activity compared to the microcosms containing nutrient addition. Results of the respirometric analysis are located in Appendix C.

CHAPTER 5. CONCLUSIONS

The first five DCM Microcosm Experiments were inconclusive but the Sand Microcosm Experiment suggested that there is potential for bioaugmentation improvement using BiOWiSH^{TM} – Thai Aqua. However, multiple replicates of the experiment microcosms in the Sand Microcosm Experiment are required to provide more conclusive results. The results of the Sand Microcosm Experiment indicated that after 7 days, the nutrient-only control and BiOWiSH^{TM}-Thai Aqua microcosms achieved higher TPH removal than the control microcosm. The nutrient-only control microcosm achieved approximate 85% of the TPH removal achieved by the BiOWiSH^{TM}-Thai Aqua microcosm. However, The change in TPH removal relative to the control was approximately 30% higher in the BiOWiSH^{TM}-Thai Aqua than in the nutrient-only control.

Experiment analysis suggested DCM is a complicated media to use in experimental analysis. The fatty acids and fibers, containing short carbon chains, present in the DCM likely showed up as petroleum hydrocarbons during TPH analysis. These easily degraded compounds were likely the primary target of added and indigenous microorganisms. The rapid TPH decrease demonstrated by the experiments containing DCM may have been due to the degradation of the fatty acids and fibers. The addition of water to the DCM also created a complicated medium for extraction. The Effect of Sodium Sulfate Addition on TPH Analysis Experiment suggested Na_2SO_4 addition to samples prior to extraction slightly improved this matter. Additional tests must be conducted to

further evaluate the BiOWiSH^{TM}-Thai Aqua product's ability to biodegrade the oil-based DCM in this study.

CHAPTER 6. RECOMMENDATIONS FOR FUTURE WORK

It is recommended that a 6-month experimental study be conducted to understand the long-term effects of BiOWiSH^{TM} Technologies, Inc. bioaugmentation products. A minimum of five replicates of all of the microcosms is recommended to attain conclusive results with statistical analysis. More experimentation with surfactant addition to DCM may also be assessed to observe if TPH compounds become more bioavailable. TPH extraction methods capable of extracting TPH compounds DCM sampled from microcosms containing water would prove beneficial to the objective of this study. The isolation of organisms at different times throughout experimentation would provide insight on what species in the BiOWiSH^{TM}-Thai Aqua successfully biodegrade the TPH compounds in the DCM

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APPENDICES

A. TPH concentration data tables for all experiments

Table A2. TPH concentrations in the microcosms of Microcosm Amendment Dosage Experiment

Time (Days)	0.25	0.75	1	1.25	1.75	2	2.25	2.75	3	7	10	13
	TPH Concentration (mg/Kg)											
BiOWiSH™-Thai Aqua 1	59,829	58,762	57,948	34,826	51,869	26,297	36,112	53,825	10,042	10,213	19,516	17,113
BiOWiSH™-Thai Aqua 2	62,207	50,373	63,490	39,370	37,581	31,173	46,597	18,524	47,590	10,183	31,771	21,247
Nutrient-only control 1	56,807	N.A.	N.A.	N.A.	59,200	N.A.	N.A.	N.A.	39,211	12,162	24,914	17,517
Nutrient-only control 1	68,193	N.A.	N.A.	N.A.	55,760	N.A.	N.A.	N.A.	31,053	16,781	23,084	16,215

Table A2. TPH concentrations in the microcosms of the Initial Bioaugmentation Experiment

Time (Days)	4	6	9	31
	TPH Concentration (mg/Kg)			
BiOWiSH™-Thai Aqua	82,539	59,645	57,560	15,698
Osprey BPB	79,625	59,359	62,226	35,533
Osprey BPB w/ Soy Lecithin	85,587	69,953	56,680	41,664
Osprey MPB	N.A.	87,953	32,574	35,223
Osprey MPB w/ Soy Lecithin	N.A.	N.A.	85,009	37,464
MDG Petro	N.A.	N.A.	36,648	28,877
MDG Petro w/ Soy Lecithin	N.A.	N.A.	29,795	42,760

Table A3. TPH concentrations in the microcosms of the Secondary Bioaugmentation Experiment

Time (Days)	0	0.25	1	2	3	12
	TPH Concentration (mg/Kg)					
Nutrient-Only Control A	56,634	35,684	49,893	26,234	28,676	12,038
Nutrient-Only Control B	56,634	62,325	32,817	31,846	22,094	19,672
BiOWiSH™-Thai Aqua A	56,634	39,032	35,977	29,756	27,642	20,614
BiOWiSH™-Thai Aqua B	56,634	51,786	43,570	25,065	7,493	20,972
BMT Remediate A	56,634	44,086	34,941	28,779	N.A.	12,149
BMT Remediate B	56,634	48,438	24,776	20,117	N.A.	16,337

Table A4. TPH concentrations in the microcosms of the DI Water Addition Experiment

Time (Days)	0	1	2	3.5
	TPH Concentration (mg/Kg)			
Control A	56,634	24,424	16,536	18,587
Control B	56,634	31,051	38,508	17,029

Table A5. TPH concentrations in the microcosms of the Effect of Sodium Sulfate on TPH Analysis Experiment

Time (Days)	0.33	1	2	3
	TPH Concentration (mg/Kg)			
Control	63,621	54,025	59,525	42,546
Nutrient-only control	65,933	38,809	48,221	45,015
BiOWiSH™-Thai Aqua	66,954	68,854	49,780	38,099

Table A6. TPH concentrations in the microcosms of the Sand Microcosm Experiment

Time (Days)	0	1	2	3	7
	TPH Concentration (mg/Kg)				
Control	633	743	665	608	475
Nutrient-only control	708	667	565	537	428
BiOWiSH™-Thai Aqua	796	650	580	516	421

B. GC/MS Method

```
OVEN
 Initial temp: 45 'C (On)                    Maximum temp: 325 'C
 Initial time: 3.00 min                      Equilibration time: 0.50 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


FRONT INLET (SPLIT/SPLITLESS)                BACK INLET (UNKNOWN)
 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


COLUMN 1                                     COLUMN 2
 Capillary Column                            (not installed)
 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


FRONT DETECTOR (NO DET)                      BACK DETECTOR (NO DET)


SIGNAL 1                                     SIGNAL 2
 Data rate: 20 Hz                            Data rate: 20 Hz
 Type: test plot                             Type: test plot
 Save Data: Off                              Save Data: Off
 Zero: 0.0 (Off)                             Zero: 0.0 (Off)
 Range: 0                                    Range: 0
 Fast Peaks: Off                             Fast Peaks: Off
 Attenuation: 0                              Attenuation: 0


COLUMN COMP 1                                COLUMN COMP 2
 (No Detectors Installed)                    (No Detectors Installed)
```

(Lehrer, 2012)

C. Graphs for DCM Microcosm Respirometric Analysis

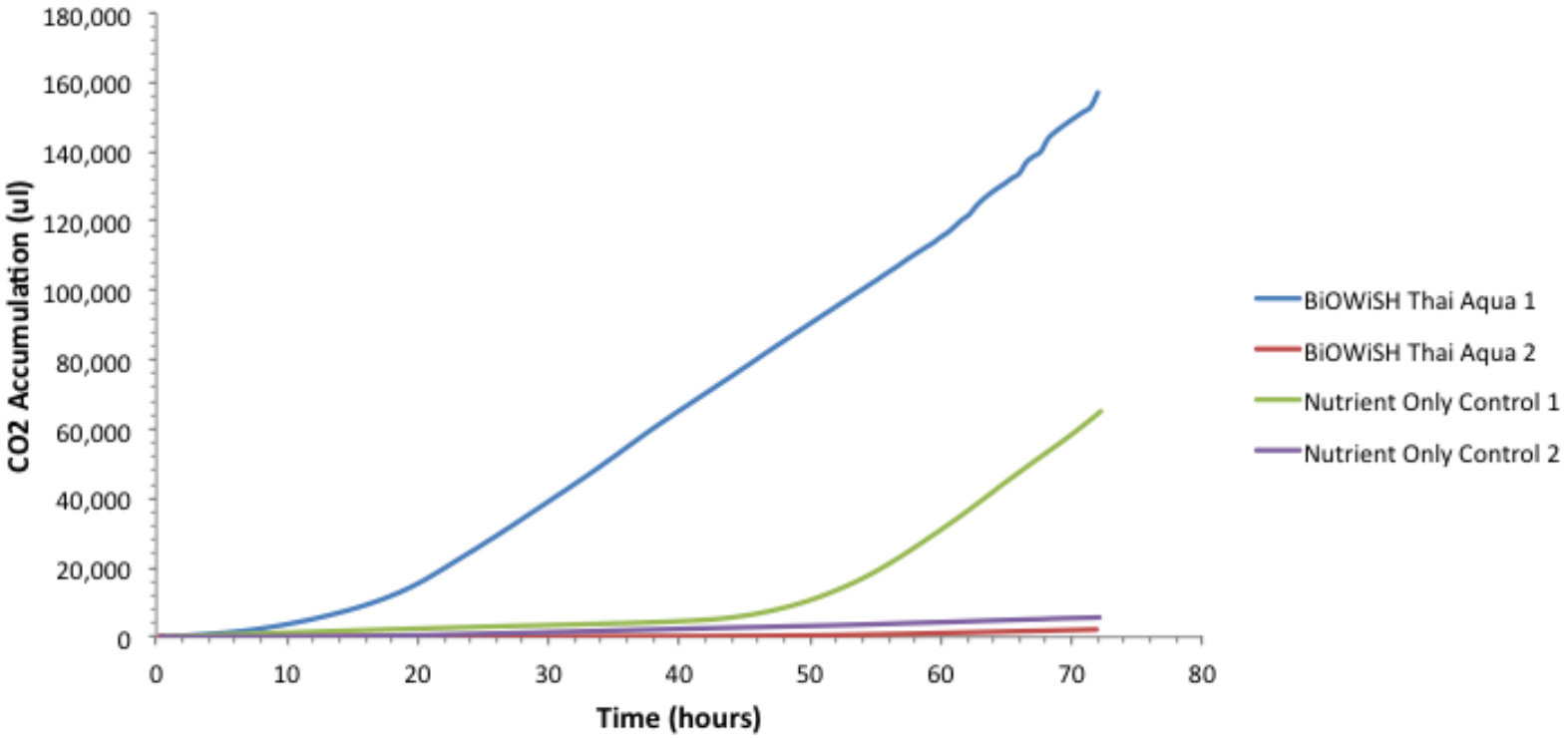

Figure C1. CO_2 accumulation across time in the Microcosm Amendment Dosage Experiment

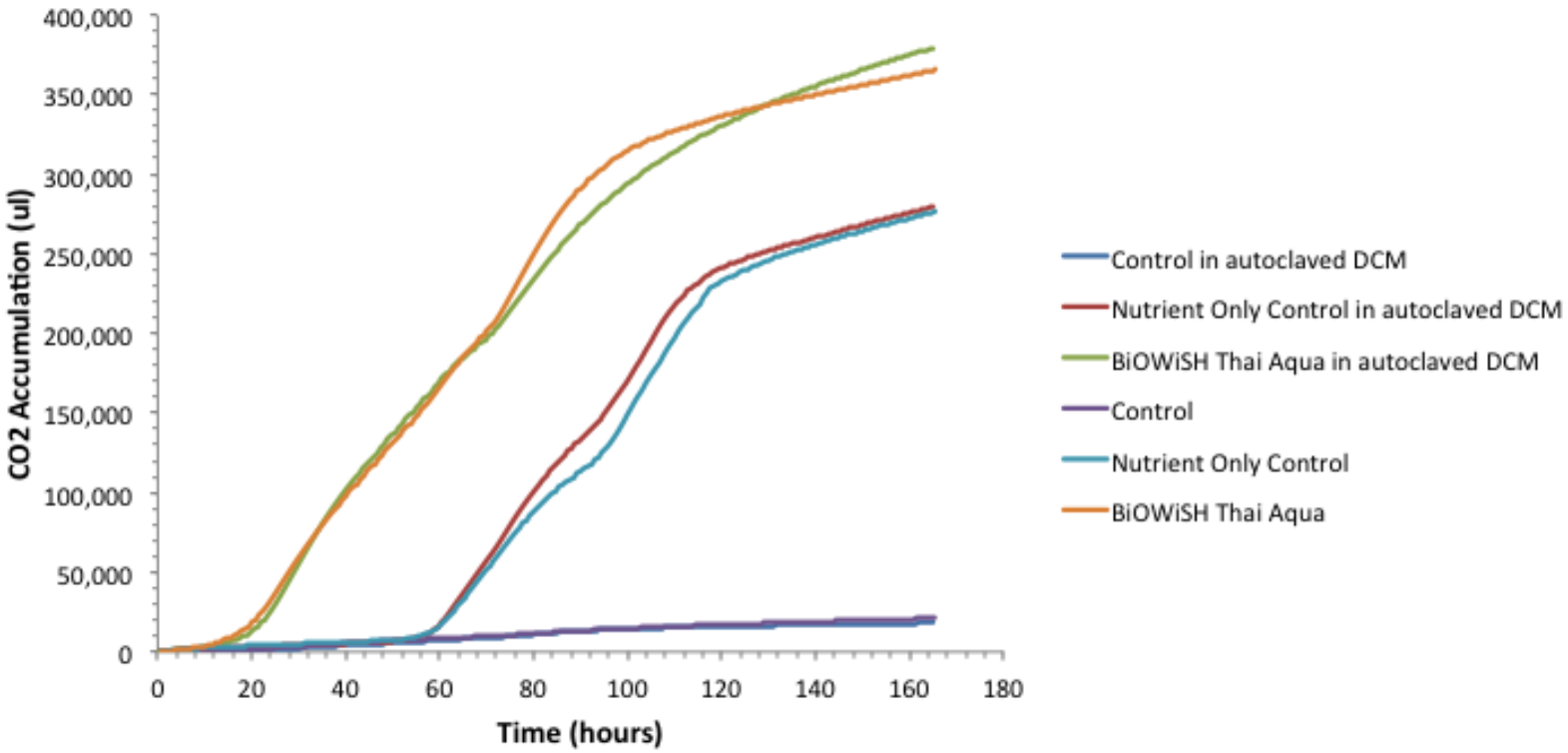

Figure C2. CO_2 accumulation across time in the Autoclaved DCM Experiment

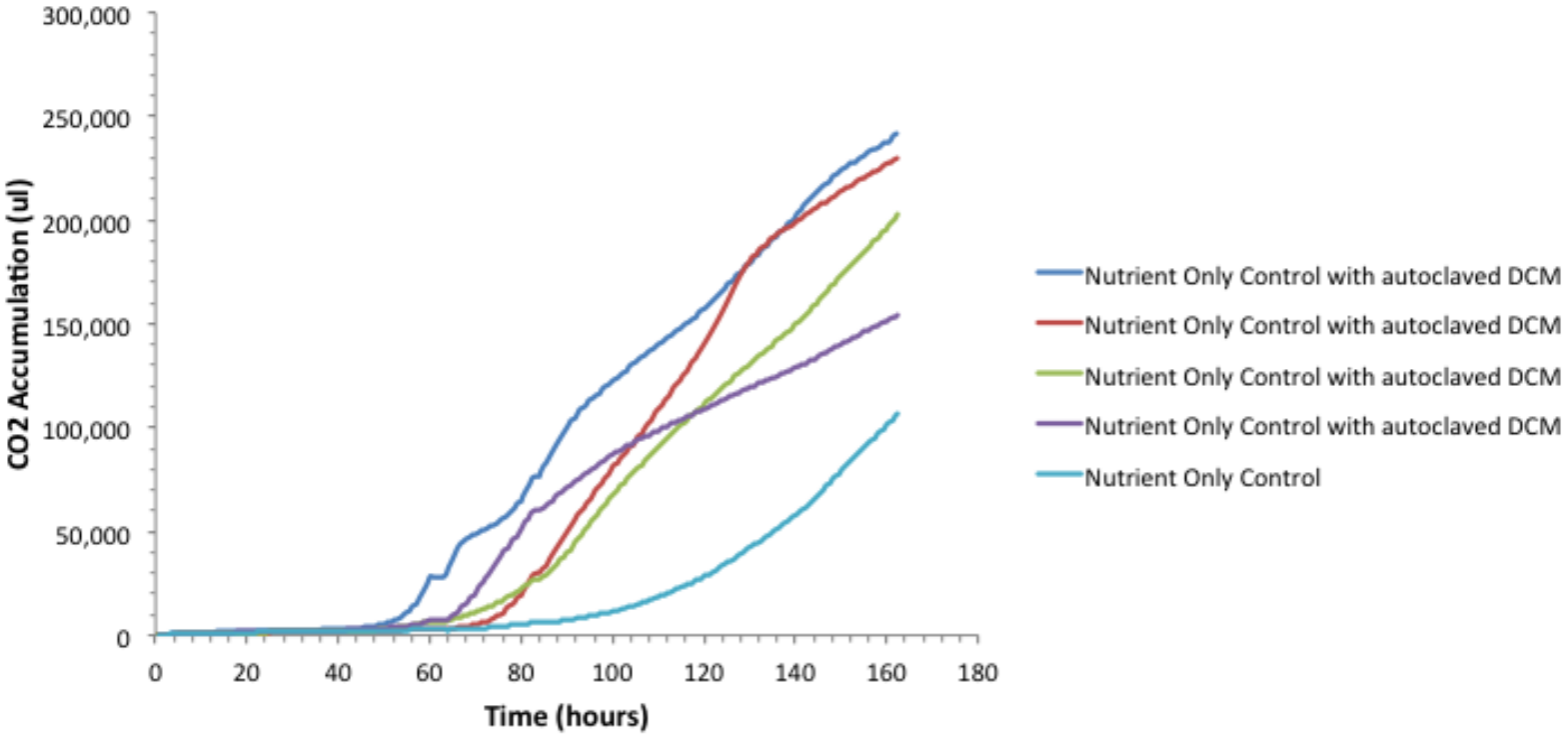

Figure C3. CO_2 accumulation across time in the Re-Dose Experiment 1

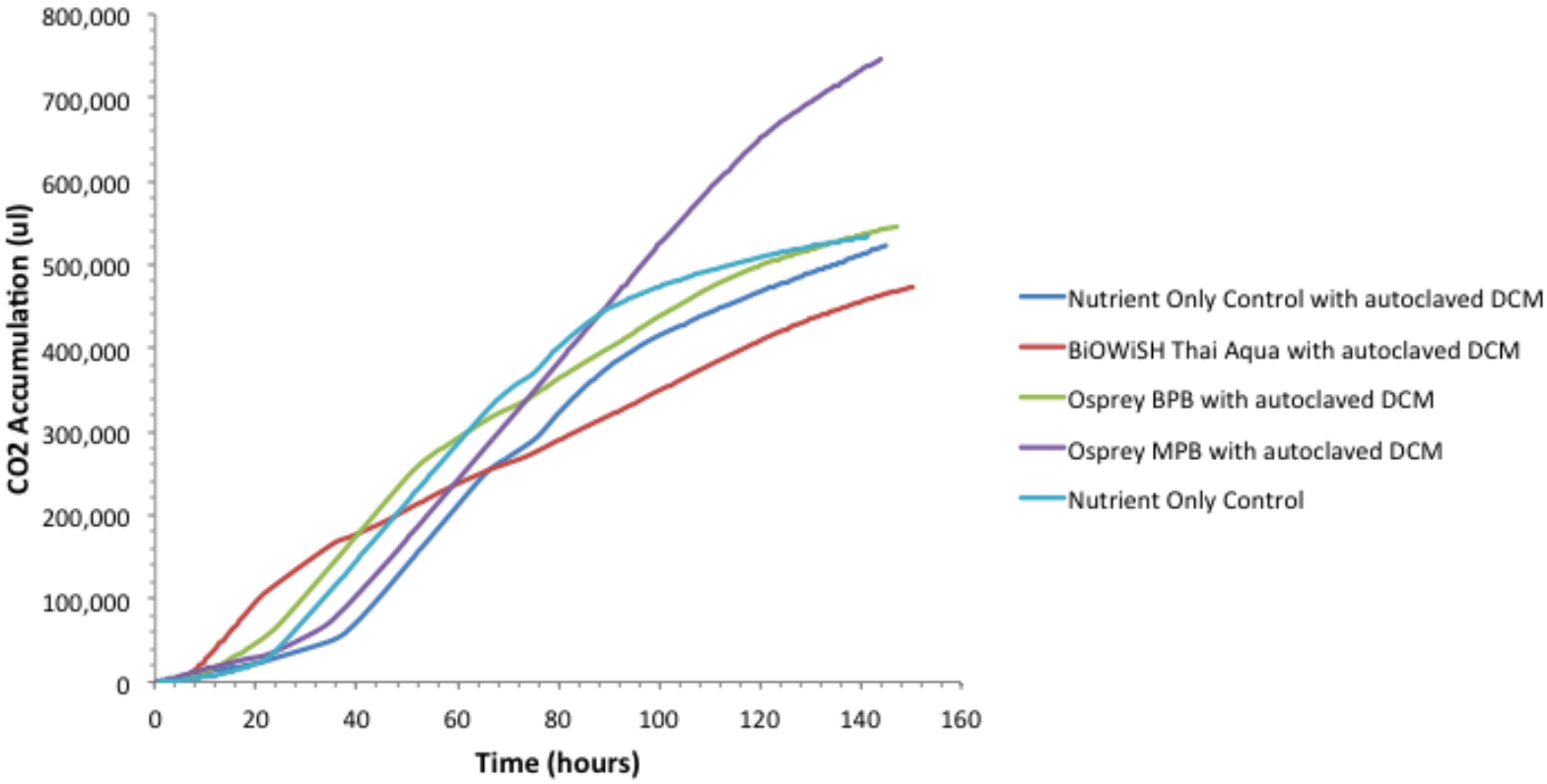

Figure C4. CO_2 accumulation across time in the Re-Dose Experiment 2