SOLID PEROXIDE STIMULATED PHENANTHRENE REMOVAL FROM CONTAMINATED RIVER SEDIMENT

By

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A thesis submitted in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE IN ENVIRONMENTAL ENGINEERING

WASHINGTON STATE UNIVERSITY
Department of Civil and Environmental Engineering

DECEMBER 2009

To the faculty of Washington State University:

The members of the Committee appointed to examine the thesis of KATHERINE SCHAFFNIT find it satisfactory and recommend that it be accepted.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Jeremy Rentz for his guidance and support in both the laboratory as I have conducted research, and in academia as I have had the opportunity to begin my work in the field of environmental engineering. I would also like to thank my committee, Dr. Richard Watts and Dr. Jeffrey Ullman, for their support and expertise throughout the writing process.

I am forever indebted to Haley Falconer and Thomas Leake, the seasoned engineers in our office, who spent countless hours explaining the ins and outs of wastewater treatment to a biologist-recently-turned-engineer. Also, if it were not for Haley, I would probably still be in the lab (months later) meticulously placing peroxide blocks in jars with tweezers and topping them off with an icing bag of pungent river sediment.

SOLID PEROXIDE STIMULATED PHENANTHRENE REMOVAL FROM CONTAMINATED RIVER SEDIMENT

Abstract

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December 2009

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While extensive data are available regarding polycyclic aromatic hydrocarbon (PAH) contamination in soils, far fewer studies have been dedicated to PAH contaminated sediments. Because current treatment options for PAH contaminated sediments are expensive and invasive, bioremediation provides a more cost effective treatment strategy. A variety of naturally occurring soil bacteria are capable of degrading PAHs either through metabolism or cometabolism (Ri-He, Ai-Sheng et al. 2008), but their activities are largely limited by electron acceptor availability. Current research has indicated that both oxygen and nitrate addition are capable of enhancing in situ bioremediation, and the objective of this laboratory experiment was to stimulate aerobic PAH degradation in contaminated sediment through the addition of either solid oxygen release compounds (ORC®) or potassium nitrate crystals. Phenanthrene biodegradation in laboratory-contaminated sediment by aerobic microbes was significantly enhanced through solid magnesium peroxide cube (ORC ®) addition to both low organic content (LOC) and high organic content (HOC) sediments, eventually reaching 70% removal in LOC sediments and 51% in HOC sediments. Waters overlying LOC sediments were supersaturated

with oxygen, with dissolved oxygen (DO) levels reaching 13.6 mg/L, while waters overlying HOC sediments maintained relatively low DO levels. pH in overlying waters was not significantly influenced in LOC sediments and slight, but still insignificant, increases were observed in HOC sediment microcosms. Nitrate treatment was only examined in HOC sediments and failed to enhance phenanthrene remediation. DO levels were significantly raised, possibly due to microbial inhibition, and pH remained constant. PCR analysis of sediment samples for dioxygenase presence confirmed the existence of PAH degraders in both oxygen amended and control microcosms. ORC® treated samples also showed increasing PAH degrader presence over time, which corresponded to observed increases in phenanthrene degradation. Despite high DO levels in nitrate treated microcosms, dioxygenase levels suggested that PAH degrading populations did not grow throughout the course of the experiment. These data showed high potential for solid peroxide-stimulated biodegradation as a sediment remediation technique, and warrant further research both in the laboratory and in the field. Conversely, nitrate did not appear to be an effective remediation tool for the soils tested, and was even suspected to have inhibited microbial activity.

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1.0 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants and, due to their prevalence, toxicity, and recalcitrance, the CERCLA Priority List of Hazardous Substances has ranked PAHs at number 8 (ATSDR-PAH 2007), and the United States Environmental Protection Agency (USEPA) has listed 16 PAHs as Priority Pollutants for monitoring (Samanta, Singh et al. 2002). While extensive data are available regarding PAH contamination in soils, far fewer studies have been dedicated to PAH contaminated sediments. Low molecular weight PAHs released into the environment are often volatilized (Watts 1998), but, due to their hydrophobicity, PAHs reaching terrestrial and aquatic environments adsorb readily to available organic matter, drastically decreasing their mobility and leading to recalcitrance (Hughes, Beckles et al. 1997). The 2004 National Sediment Quality Survey conducted by the EPA reported that 75% of examined sites were contaminated to a level that presented possible (and sometimes probable) health risks, due in part to PAH contamination. Furthermore, approximately 24% of EPA designated Tier 1 Superfund sites are PAH contaminated (USEPA 2005).

Current treatment options for PAH contaminated sediments, such as in situ capping and dredging, are expensive and invasive. In situ capping has been used at about 15 Superfund sites nationwide (USEPA 2005). This treatment method involves placing a clean material layer (i.e., sand) at the sediment-water interface to create a barrier between the contaminated material and the overlying water (Himmelheber, Taillefert et al. 2007). While aquatic organisms are no longer in contact with contaminants, natural attenuation can be negatively effected. Himmelheber et al. (2007) showed that this ecosystem alteration influenced the sediment redox conditions which are the very basis of biologically mediated contaminant transformations. Oxygen profiles, which penetrate only centimeters into the sediment, are shifted upward out of

the contaminated substrate after sand caps are applied (Figure 1). The fastest PAH biodegradation rates are carried out by aerobic organisms; therefore, oxygen disappearance from contaminated sediments can inhibit or eliminate natural microbial activities and thereby, biodegradation. Dredging has been used at over 100 Superfund Sites (USEPA 2005), and presents a serious disruption to natural ecosystems. Dredging is also disadvantageous due to requirements for special equipment and techniques, such as dewatering pretreatment (USEPA 1998), and designated storage and remediation sites.

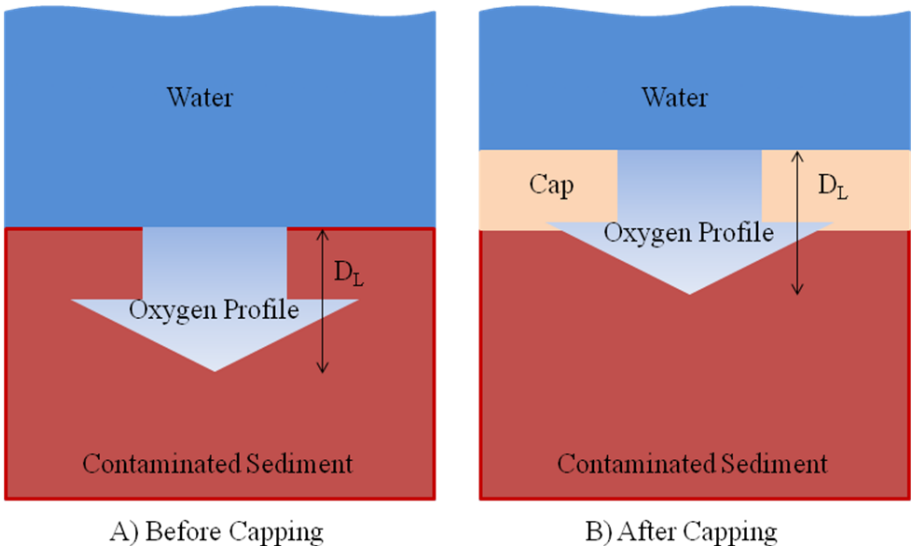

Figure 1: Decreased potential for aerobic contaminant biodegradation to occur are observed after cap application. After capping, oxygen penetration profiles are shifted upwards away from contaminated sediments, decreasing oxygen availability to sediment microbes. D_L indicates the depth to which oxygen will penetrate.

A variety of naturally occurring soil bacteria are capable of degrading PAHs either through metabolism or cometabolism (Ri-He, Ai-Sheng et al. 2008), but their activities are largely limited by electron acceptor availability. Oxygen is the most efficient terminal electron acceptor and its presence is required both for aerobic bacterial respiration and by the characteristic dioxygenase enzyme carried by aerobic PAH-oxidizing bacteria (Ri-He, Ai-Sheng

et al. 2008). Oxygen, however, may not be readily available in river sediments, leading to limited microbial growth and degradation activity. Electron acceptor addition may, therefore, mitigate this limitation and enhance microbe facilitated biodegradation. Though not as efficient as oxygen, nitrate is also a common limiting electron acceptor.

Current research has indicated that oxygen is capable of enhancing in situ bioremediation, as this electron acceptor is often the limiting factor in microbial activity. The addition of such compounds, therefore, has the potential to circumvent this natural limitation. In a study of contaminated river sediment by Quantin et al. (2005), it was noted that 78% of PAHs were degraded under aerobic conditions, while anaerobic PAH removal was negligible. This percent removal fell within the 50 to 80% removal range measured by Lei et al. (2004) in an examination of three- and four-ring PAHs from aged contaminated sediment under aerobic conditions (Lei, Khodadoust et al. 2004). It has also been reported that aerobic degradation of three-ring and four-ring PAHs proceeded up to nearly four times more rapidly than under anaerobic conditions (McNally, Mihelcic et al. 1998). Finally, Boyd et al. (2005) linked growth of PAH degrading bacterial colonies and increased degradation to increased dissolved oxygen. These data indicate that oxygen amendments may be effective bioremediation enhancements.

PAH biodegradation under denitrifying conditions has also been examined, though not as extensively, and investigations into its efficiency have produced mixed results. Lei et al. (2004) concluded that PAHs were not amenable to biodegradation under denitrifying conditions, as insignificant degradation was reported after 25 weeks. A second experiment, however, revealed 41% phenanthrene removal when nitrocellulose was added to release nitrate as an electron acceptor under anaerobic conditions (Tang, Carpenter et al. 2005). This electron acceptor addition generated an increase in anaerobic phenanthrene degradation rate by a factor of two

(Tang, Carpenter et al. 2005). McNally et al. (1998) also detected PAH degradation under denitrifying conditions, although, as noted previously, it was up to four times slower than aerobic degradation.

Oxygen Release Compound (ORC®) is composed of intercalated magnesium peroxides capable of releasing oxygen over extended periods of time. Since its appearance in 1994, ORC® has been employed at over 6 500 sites in 50 states and 11 countries (Koeningsberg 2001) as a time-release electron acceptor for accelerating natural attenuation of aerobically degradable compounds. Koeningsberg (2001) reported that ORC® has been used to treat BTEX (Benzene, Toluene, Ethylbenzene and Xylenes) and PAHs in aquifer systems around North America with up to 99% removal efficiency. Based on these results, as well as promising results of enhanced PAH degradation under aerobic conditions, we investigated ORC® application to stimulate bioremediation in phenanthrene contaminated river sediment.

Specific research objectives were to:

1. Stimulate and expedite aerobic and anoxic PAH degradation in laboratory contaminated sediment.
    a. Evaluate the potential of solid oxygen release compound (ORC®) as a biodegradation stimulant (aerobic).
    b. Evaluate the potential of nitrate addition as a biodegradation stimulant (anoxic).

2.0 EXPERIMENTAL DESIGN AND METHODS

2.1 Sediment Collection

River sediment was collected from Paradise Creek, Pullman, WA on February 12, 2009 (Experiment 1: Low organic content (LOC) river sediment) and May 13, 2009 (Experiment 2: High organic content (HOC) river sediment). LOC sediment was shoveled into a large cooler in excess of 24 kg. The sample was transported and stored outdoors in the cooler until it was contaminated and microcosms were set up. HOC sediment was collected from the bank opposite the original location with a shovel. Sediment was collected, transported and stored in two 10 gallon plastic buckets until contaminated and microcosms were set up.

2.2 Chemicals

Phenanthrene (95% purity) was acquired from Sigma-Aldrich (St. Louis, MO). The solid peroxide used in this study was a mixture of magnesium peroxide (MgO_2), magnesium oxide (MgO) and magnesium hydroxide ($Mg(OH)_2$) (Oxygen Release Compound (ORC®), Regenesis Bioremediation Products, San Clemente, CA). The solid nitrate used in this study was potassium nitrate (KNO_3) crystals acquired from JT Baker (Phillipsburg, NJ). Ammonium chloride was ordered from Fluka BioChemika (Buchs, Switzerland). HPLC-grade acetone, dichloromethane, and methanol were purchased from JT Baker (Phillipsburg, NJ). Nitrogen gas had a minimum purity of 99%.

2.3 Experimental Design

2.3.1 Experiment 1: Phenanthrene Degradation with Magnesium Peroxide Addition in Low Organic Content River Sediment

Paradise Creek sediment was spiked with phenanthrene using a shell coating method modified from Ke et al. (2003). Three grams of phenanthrene were dissolved in 200 mL of acetone in a 500 mL glass bottle. The phenanthrene/acetone mixture was poured over 1 L clean, dry sand and mixed with a stainless steel spatula in an aluminum pan. The sand was left under a

hood for two hours to allow the acetone to evaporate, mixed one last time and poured into a 4 L amber bottle. The bottle was left uncapped under the hood overnight to ensure all acetone had volatilized. Sand was then transferred to a 1 L amber plastic Nalgene® bottle and stored in the freezer until the time of use. 24 kg of Paradise Creek sediment were measured into a 5 gallon bucket. The dry sand was poured into the sediment and homogenized for 20 min using an electric paint mixer. Homogenized sediment was left in the bucket for 24 hours after mixing to allow phenanthrene adsorption to the soil organic matter. Estimated phenanthrene concentration was 117 mg/kg. Levels detected after HPLC analysis were approximately 67.5 mg/kg. This may have been due to soil organic content, a connection which will be discussed with experimental results.

Solid magnesium peroxide cubes were created by mixing 150 g of ORC® powder with 81 mL of deionized water in a glass beaker with a stainless steel spatula. The resulting slurry was approximately 65% ORC®, within the range recommended by the manufacturer. The slurry was poured into plastic ice cube trays (90 cubes, 0.5x0.5x0.5 in each, per tray) and set out overnight to dry. Once dry, the trays were inverted to release the cubes. The cubes were sorted to ensure they were of roughly equal size.

Sixty four microcosms were constructed in 250 ml amber glass bottles for an eight week bioremediation study. Half the microcosms (32) were treated with ORC blocks. Four blocks were placed in the bottom of each bottle and weighed on average 7.02 g (standard deviation = 0.51 g) collectively (Figure 2). The blocks were covered with approximately 150 g of contaminated sediment using a plastic frosting bag. The bottles were filled with 180 mL of tap-water to minimize air-filled headspace and sealed with a lid (a plastic bucket was filled with fast running hot tap water and left uncovered in the lab one hour to decrease Cl^- concentration).

Thirty-two control microcosms were also established without ORC treatment blocks. The bottles contained approximately 150 g of contaminated sediment and 190 mL of water. Initial soil samples were taken after every tenth microcosm to establish the starting sediment phenanthrene concentration. Microcosms were incubated in boxes and stored at room temperature until they were destructively sampled (see Section 2.4).

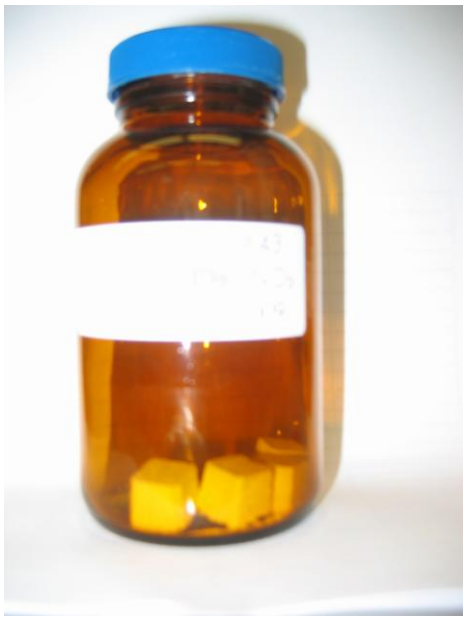

Figure 2: 250mL amber glass jar containing four ORC® blocks, prior to sediment addition.

2.3.2 Experiment 2: Phenanthrene Degradation with Magnesium Peroxide and Potassium Nitrate Addition in High Organic Content Sediment

Paradise Creek sediment was spiked with phenanthrene using methods described for Experiment 1 (26 days prior to microcosm establishment). Ammonium chloride was subsequently added to the phenanthrene-contaminated soil at a concentration of 0.1 mg ammonia/kg sediment six days prior to microcosm establishment (5.1625 g NH_4Cl dissolved in 100 mL H_2O and added to 17.4 kg of Paradise Creek sediment). Ammonium chloride was added to ensure that adequate nitrogen was available to the microbes, and that potassium nitrate was utilized as an electron acceptor rather than as a nitrogen source. The sediment was homogenized

with an electric paint mixer for 15 min after ammonium chloride addition. The estimated phenanthrene concentration was 116 mg/kg. Levels detected after HPLC analysis were approximately 49.3 mg/kg. This may have been due to soil organic content, a connection which will be discussed with experimental results. The estimated ammonium concentration was 0.1 g/kg.

Seventy-two microcosms were constructed for an eight week bioremediation study in the same manner described for Experiment 1. One third of the microcosms (24) were treated with ORC blocks weighing an average of 6.77 g (standard deviation = 0.52 g). (Solid magnesium peroxide cubes were created using the same methods described for Experiment 1.) One third of the microcosms (24) were treated with potassium nitrate crystals weighing approximately 7 g (standard deviation = 0.025 g) and placed in the bottom of each microcosm in a manner similar to block placement described for Experiment 1 (Figure 3). Twenty-four control microcosms were also established without ORC or nitrate treatment. Initial soil samples were taken after every ten microcosms. Microcosms were incubated in boxes and stored at room temperature until they were destructively sampled (see Section 2.4). This experimental setup was used to simulate environmental conditions.

Figure 3: 250 mL amber glass jar containing 7g potassium nitrate crystals, prior to sediment addition.

2.4 Experimental Methods

2.4.1 Microcosm Data Collection

Triplicate samples were taken for analysis on days 7, 14, 21, 35, 42, 49, and 56 for Experiment 1, and days 7, 14, 21, 28, 35, 49, and 56 for Experiment 2. Temperature and DO were measured using a YSI 52 DO probe calibrated to 91.7% with water bubbled with air for two hours for Experiment 1. Experiment 2 employed the YSI 55 DO probe. pH was determined using a Denver Instrument UltraBasic pH/mv meter. Water was pipetted from each microcosm after measurements were taken and refrigerated at 4°C in amber glass bottles for future analysis. Between 0.25 and 0.5 g of soil were taken from each microcosm and DNA was extracted using PowerSoil Kit #12888-100. Remaining soil samples were frozen at -20 °C for future analysis.

2.4.2 ASE Extraction (Experiment 1)

Phenanthrene was extracted from sediments using a Dionex ASE 200 Accelerated Solvent Extractor (ASE) (Dionex Corporation, Sunnyvale, CA). Prior to extraction, frozen microcosms were set out to thaw two hours before sediment samples were to be collected for

ASE procedures. Approximately 20 g of sediment were collected from each microcosm using an acetone rinsed stainless steel spatula and placed in a labeled aluminum tray for drying. 20 g samples were dried for 18 hours in a 105°C drying oven. Once dry, samples were ground to a fine powder using an acetone-rinsed mortar and pestle and weighed again (samples lost up to 6 g of water-weight in the drying process). Ground samples were added to 22 mL acetone rinsed stainless steel extraction cells and cells were filled with clean sand (baked two hours at 400°C to ensure cleanliness) to minimize solvent waste. Sediment and sand were poured out, mixed to homogenize, and placed back into the extraction tubes. Extraction was performed according to the manufacturer's instructions at the recommended system conditions (Table 1). Extracts were collected in clean 60 mL amber volatile organic analysis (VOA) vials.

Table 1: ASE 200 Extraction Conditions

System Pressure	10 MPa (1500 psi)
Oven Temperature	100 °C
Oven Heat-up Time	5 min
Static Time	5 min
Static Cycles	4
Solvent	50/50 Dichloromethane/Acetone
Flush Volume	60% of extraction cell volume
Nitrogen Purge	1 MPa (150 psi) for 30 sec
Total Extraction Time	25 min

2.4.3 Dichloromethane Extraction (Experiment 2)

Phenanthrene was extracted from sediments using dichloromethane. Prior to extraction, frozen microcosms were set out to thaw three hours before sediment samples were to be collected for extraction. Approximately 20 g of sediment were collected from each microcosm using an acetone rinsed stainless steel spatula and placed in a labeled aluminum tray for drying. 20 g samples were dried overnight in a 105°C drying oven. Once dry, samples were ground to a fine powder using an acetone-rinsed mortar and pestle and weighed again (samples lost up to 6 g

of water-weight in the drying process). Ground samples were added to clean 60 mL amber volatile organic analysis (VOA) vials. 45 mL of HPLC-grade dichloromethane were added to the pulverized sediment. Vials were sealed and shaken by hand and then vortexed for five seconds, and left to settle overnight at room temperature. Vials were shaken and vortexed again the following day, and left to sit overnight again. 20 mL of sediment-free dichloromethane were pulled off from each vial and transferred to a second amber VOA vial on the third day. Extracted samples were refrigerated until further analysis.

2.4.4 Exchange to Methanol

Prior to HPLC analysis, ASE extracts dissolved in 50/50 dichloromethane/acetone or 100% dichloromethane were exchanged to methanol. VOA vials were placed in 50°C water bath and samples were evaporated until dry under a gentle stream of nitrogen gas (1 hr). Methanol (20 mL) was added to the dried extract for analysis by high performance liquid chromatography (HPLC). The tubes were returned to the 4°C refrigerator for further analysis.

2.4.5 HPLC Analysis

Phenanthrene was analyzed using an Agilent HP 1100 HPLC equipped with Agilent 1100 fluorescence and diode array detectors after EPA method 610. Compound separation was achieved using a Supelco Supelcosil LC-PAH 5-µm column (15 cm x 4.6 mm x 5 µm; Sigma-Aldrich, St. Louis, MO), a 26°C column temperature, a 1 mL/min flow rate and a 50 µL injection volume. A Supelco LC-18 Supelguard (Sigma-Aldrich, St. Louis, MO) protected the column and detectors from strongly sorbing compounds and matrix particles. The mobile phase was 80% methanol and 20% water. FLD parameters were set at 244 excitation and 360 emission, and DAD parameters were set at 254 nm absorbance. Chemstation Software Revision A.09.03 (Agilent Technologies, Santa Clara, CA) integrated peak areas using standard preset parameters.

Phenanthrene was identified and quantified by comparing retention times and integrated peak areas to known standards. Methods for standard preparation are described in Appendix B.

2.4.6 Microbial Techniques

DNA extraction allowed characterization of microbial communities. DNA was extracted from each microcosm using the PowerSoil ™ DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), following the manufacturers instructions. DNA extracts were stored at -80°C for further analysis.

DNA extracts were analyzed for gram negative dioxygenase presence, targeting the PAH-RHD_{α} gene, using the StepOne ™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Samples were run in duplicate according to run methods summarized in Appendix A. A standard curve was generated using *Pseudomonas putida* diluted at a ratio of 1:5 (Figure 16).

3.0 RESULTS AND DISCUSSION

3.1 Experiment 1: Phenanthrene Degradation with Magnesium Peroxide Addition in Low Organic Content River Sediment

Phenanthrene biodegradation in low organic content Paradise Creek sediment by aerobic microbes was significantly enhanced through solid magnesium peroxide cube (ORC ®) addition (Figure 4). Statistically significant removal was determined using t-tests and observed after 28 days of ORC® treatment in the course of the eight week study. After a full eight weeks of incubation, treatment yielded approximately 70% phenanthrene removal while control microcosms degraded only 10% of the added phenanthrene (Figure 4). Initial phenanthrene concentrations of approximately 67.5 mg/kg were reduced to about 20.7 mg/kg by the treated microcosms, but remained much higher, about 61.3 mg/kg, in untreated microcosms. Degradation in the microcosms was equally well modeled by zero-order kinetics (a linear equation; $y=mx+b$) ($R^2 = 0.838$), and first order kinetics ($y=y_0*e^{kt}$), ($R^2 = 0.838$). A linear decay equation suggested that phenanthrene in the sediment was available in excess of what the microbial population present was capable of degrading. A first order equation, by contrast, indicated that substrate and not microbial population was limiting degradation. Because R^2 values were equal and less than 0.9, this indicated that mixed kinetics were occurring in these systems. There was very little overlap between the control and treatment data, again showcasing the statistical difference observed between treatments.

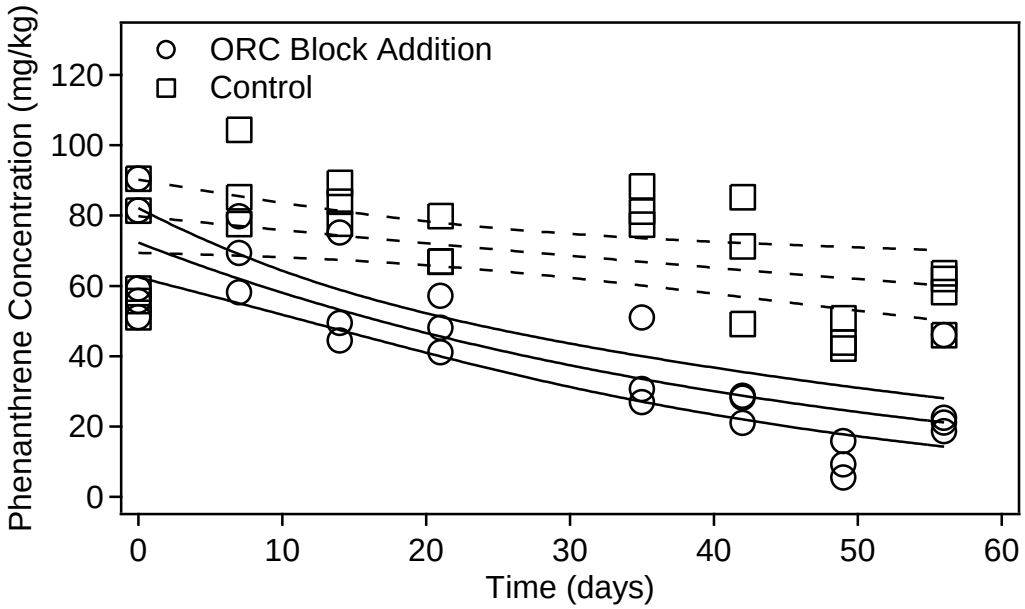

Figure 4: Enhanced phenanthrene removal with ORC® treatment in low organic content river sediment. Phenanthrene concentrations statistically lower than initial contamination levels (day 0) appeared at day 35. Degradation was significantly higher in treatments than the controls at days 35, 49 and 56.

While degradation reached 70% completion within eight weeks in the newly spiked sediments used in these experiments, Lei et al (2004) noted that the degradation rates achieved in their experiments using aged contaminated sediment were much lower than previously recorded in freshly spiked sediment. These findings imply that further laboratory testing may be required with aged samples before this biodegradation enhancement can be transferred to the field. These fresh samples may be better indicators of the extent to which bioremediation is possible because fresh samples may not have allowed phenanthrene adequate time to fully adsorb to organic matter in the sediment.

Exposure to air during storage and homogenization may have allowed oxygen infiltration into sediments, augmenting bioremediation rates in treatment microcosms and accounting for the 10% degradation observed in control microcosms. After sediment collection and just prior to homogenization, Lei et al (2004) discarded the top layer of sediment from each bucket as it had

been in contact with headspace, and thereby exposed to oxygen. Further investigations may seek to employ such techniques to negate the possible consequences of oxygen infiltration beyond what was knowingly applied through ORC® addition.

ORC® treatment increased dissolved oxygen (DO) levels in waters overlying contaminated sediments (Figure 5). ORC® addition raised DO levels significantly after 21 days of treatment, reaching a high value of 13.62 mg/L, while DO levels in control treatments failed to change significantly throughout the course of the experiment, reaching a high of only 7.45 mg/L. With the exception of microcosms sampled at days 35, 119, and 175, DO levels remained significantly higher than levels recorded at week one (day seven) for ORC® treated microcosms. The discrepancy at day 35 appears to be an anomaly, falling in the midst of steadily increasing DO concentrations. The latter deviations, however, could be explained by ORC® exhaustion over time. Continued aerobic microbial activity (and therefore oxygen consumption) and diminished or negligible oxygen input as ORC® release potential begins to drain would cause oxygen concentration to decrease over time. Treatment cells were also found to have significantly higher DO concentrations than controls in four out of eight sampling events.

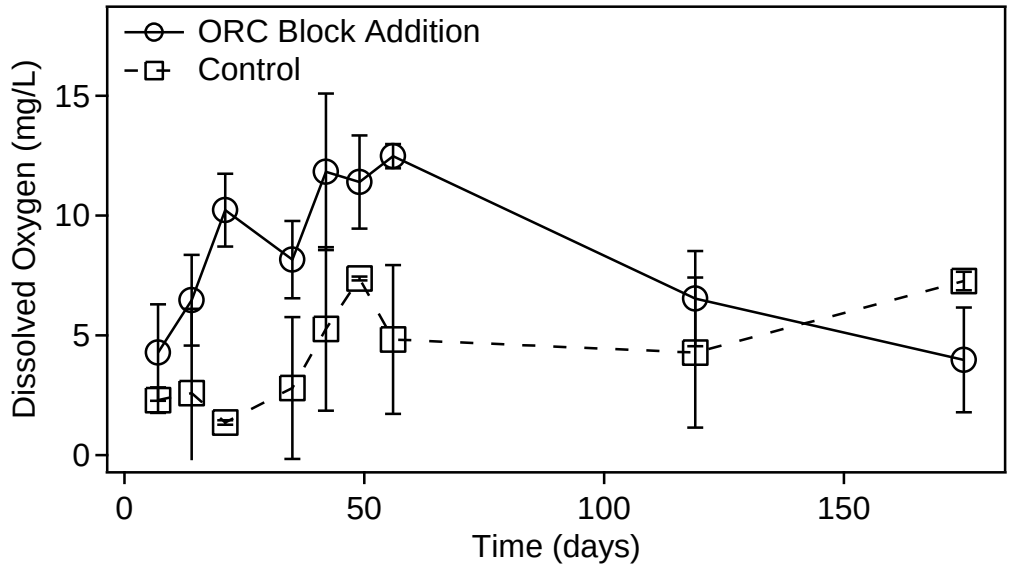

Figure 5: Average dissolved oxygen concentration over time in water overlying low organic content river sediment showed a dramatic increase in ORC® treated microcosms. Error bars indicate standard deviation of three replicates.

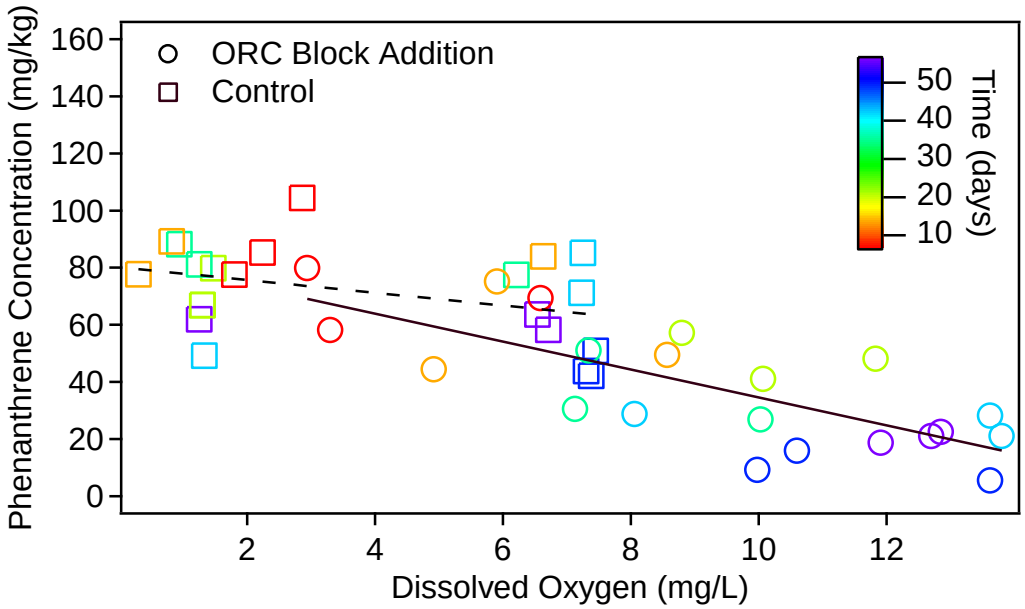

Figure 6: Phenanthrene concentration versus dissolved oxygen in low organic content river sediment microcosms. ORC® treatment corresponded to increased dissolved oxygen concentration and lowered phenanthrene levels, indicating that its addition stimulated phenanthrene biodegradation. Color scale indicates sampling day.

Equation 1: ORC Block Addition: $y = (83.485 \pm 9.44) - (4.88 \pm 0.961)x$; $R^2 = 0.5767$

Equation 2: Control: $y = (880.138 \pm 5.69) - (2.235 \pm 1.21)x$; $R^2 = 0.5767$

Enhanced oxygen concentrations in overlying water correlated with increased phenanthrene biodegradation, indicating that aerobic microbial activity was stimulated. Assuming a saturated air value of 8.5 mg/L (Moore, Stanitski et al. 2005), ORC® treatment supplied oxygen well beyond saturation values (up to 13.62 mg/L), presumably increasing oxygen availability to microbes in the sediment and enhancing aerobic phenanthrene biodegradation. As can be seen in Figure 6, relating DO concentration and phenanthrene concentration for each microcosm, ORC® treatment is correlated with both elevated DO levels in the overlying water and increased phenanthrene degradation when compared to control treatments. As is evident in Equation 1 and Equation 2, and is also visible in the slope of best fit lines (Figure 6), there was a dramatic difference in the relationship between phenanthrene concentration and DO in treatment and control microcosms (m = -4.88 (ORC® Treatment), m = -2.235 (Control)). Because bioremediation is slow when oxidizers (electron acceptors) are present in small quantities (Tang, Carpenter et al. 2005), the increase in terminal electron acceptor availability provided by ORC® improved growth conditions and increased microbial activities and degradation.

Improper sealing of control microcosms may have led to slight increases in dissolved oxygen concentrations. Although not supplemented with oxygen sources as were the treatments, control microcosms showed a slight increase in DO over time. Throughout the sampling process, it was noted that a few caps had cracked during storage and others were no longer sealed tightly. Such observations could indicate that oxygen was allowed to diffuse into microcosm water, thus elevating DO levels measured at sampling.

Real-time-PCR analysis confirmed increased dioxygenase gene presence in ORC®-treated, phenanthrene-contaminated microcosms (Figure 7), indicating that ORC® treatment

stimulated microbial PAH biodegradation. Dioxygenase mediated oxidation is the first step in microbial PAH degradation, and gene presence in DNA extracted from soil samples indicated that soil microorganisms capable of degrading PAHs were present. Microbes known to carry the gene include members of the genera *Sphingomonas*, *Alcaligenes*, and *Pseudomonas*, (Samanta, Singh et al. 2002), but these experiments did not aim to identify the active bacteria, and only sought to confirm that activity potential existed and was stimulated under treatment conditions. The noticeable increase in dioxygenase presence between treated (ORC®) and untreated samples (Figure 8) indicated that applied ORC® treatments did, in fact, act to increase the aerobic PAH degrading population. This in turn would have lead to the increased phenanthrene degradation rates discussed previously. This correlation between ORC® treatment and increased dioxygenase concentration supported microbial biodegradation as a mechanism of decay, indicating that degradation was not solely due to chemical oxidation.

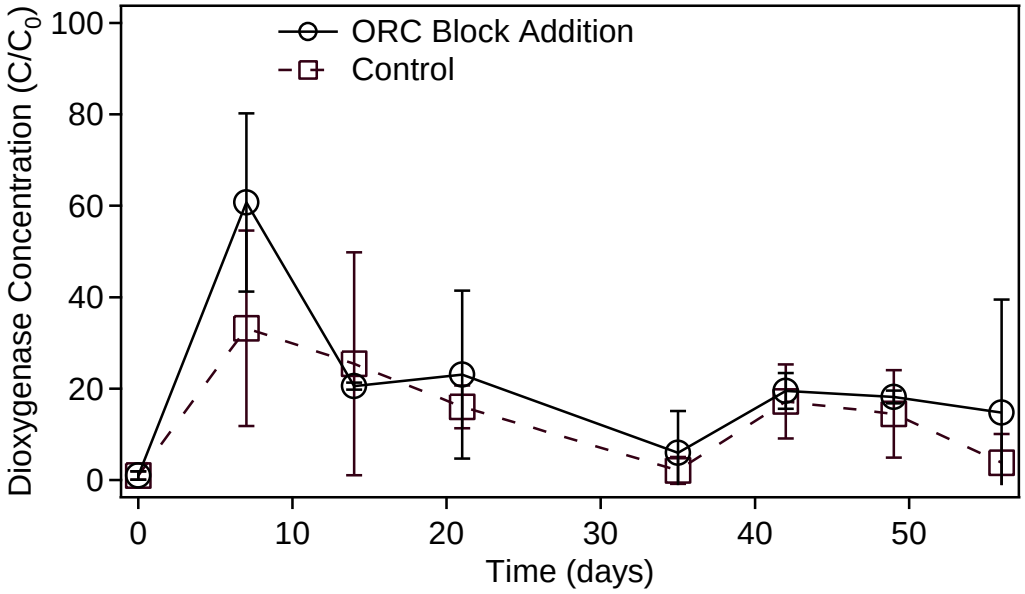

Figure 7: Real-Time PCR indicated elevated dioxygenase concentrations associated with ORC® treatment, which in turn would be expected to correlate with increased phenanthrene degradation.

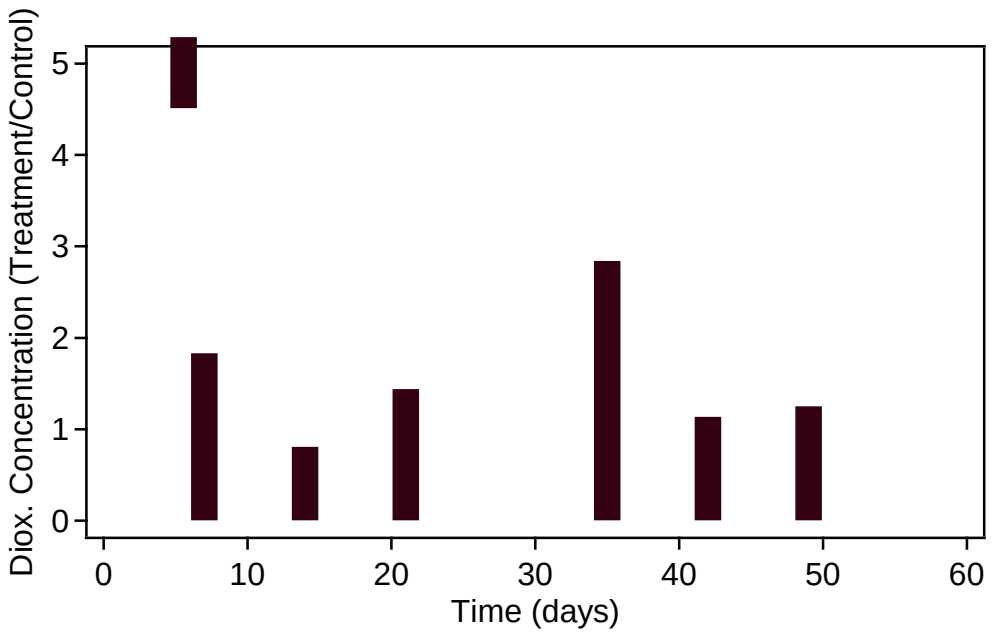

Figure 8: Ratios between the dioxygenase concentrations in ORC® treated and control microcosms showed that the dioxygenase concentrations were consistently higher in ORC® treated microcosms than in controls. Days 7, 35, and 56 show concentrations two, three, and nearly four times the recorded control values, respectively.

The PAH-degrading microbial population increased with phenanthrene addition, a trend also noted by Ri-He et al. (2008). They reported that the PAH degrading community increased noticeably at days 21 and 45 when compared to the heterotrophic community, and comprised 100% of the microbial population at 135 days (when not provided with supplemental nutrients). These data concurred with our PCR findings regarding exposure-mediated bacterial adaptation and evolution toward PAH degradation. While a certain amount of fluctuation existed, dioxygenase numbers in treated microcosms were consistently higher than values ascertained at day 0 (Figure 7). At week 56 of the experiment, dioxygenase concentrations in ORC® treated microcosms were 14.8 times greater than the values recorded at week 0. Though not as dramatic, dioxygenase concentrations in control microcosms also tended to increase after day 0 (eventually reaching 3.79 times the initial values) indicating that bacteria were adapting to phenanthrene presence at a slower rate. These data suggested that phenanthrene presence in an

aerobic environment stimulated the survival and evolution of bacteria capable of oxidizing phenanthrene.

ORC® treatment did not appear to significantly influence pH levels in experimental microcosms (Figure 9), a change which would be of considerable concern to benthic organisms. pH variation was not statistically significant when comparing week one (day seven) to subsequent weeks. On days 35 and 56, pH was significantly lower in treated microcosms than in controls, but the remaining six sampling days were statistically similar.

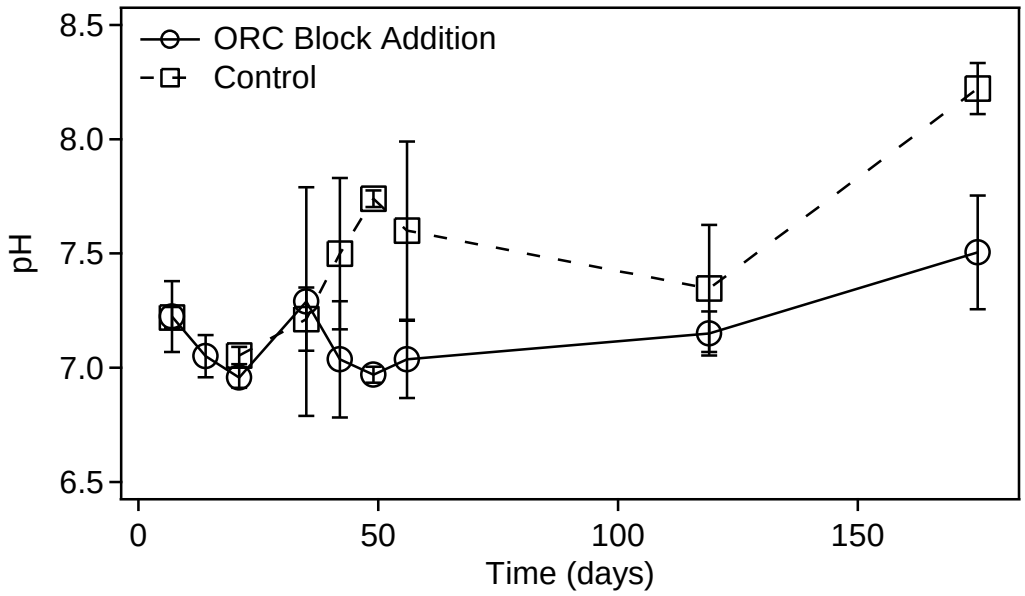

Figure 9: Average pH versus time in water overlying low organic content river sediment. ORC® treatment did not significantly alter pH, and hydrogen ion consumption by methanogens may account for increasing pH in control microcosms. Error bars indicate standard deviation of three replicates.

Equation 3: $MgO_2 + H_2O \rightarrow ½\ O_2 + Mg(OH)_2$

Equation 4: $Mg(OH)_2 \rightarrow Mg^{2+} + (OH^-)_2$ $pK_a = 9.3$

Although ORC® breakdown (described by Equation 3 and Equation 4) provided the potential to raise the pH of surrounding environments through OH^- production, soil buffering

capacity and lack of mass transfer may help explain the moderate pH maintained in treatment cells. While treatment pH failed to rise above 7.5, $Mg(OH)_2$ is known to achieve equilibrium at pH 9.3 (Waite, Bonner et al. 1999), suggesting that pH buffering was involved. Supporting this finding is a 2003 Technical Bulletin published by Regenesis reporting that soil buffering capacity maintained moderate pH levels throughout ORC® application. Similar experiments reported that any elevation in pH remained close to the application site due to the insoluble nature of the solid compound, but that pH did not rise above 8 in buffered soil systems (Regenesis 2003). Other studies have also reported the buffering capacity of soils which neutralized this pH shift (Hambrick, DeLaune et al. 1980; Barcelona and Holm 2002).

High pH in control microcosms when compared to treatment cells (Figure 9) may have been due to hydrogen ion consumption by methanogens in non-aerated environments. Methanogenesis is one of the final steps in organic matter decay, and occurs ubiquitously in anaerobic environments (Zeikus 1977). Methanogens can utilize hydrogen ions as electron acceptors during the oxidation of propionate to acetate and carbon dioxide prior to methane generation (Boone 1987). The ensuing depletion of hydrogen ions may explain the elevated and increasing pH seen for control microcosms in Figure 9.

Organic content from the Paradise Creek sediment used in this experiment was determined to be approximately 13.0 g/kg, or 1.3%, and may account for the fact that initial phenanthrene quantities extracted were far below what was expected. Soil organic content is directly related to contaminant adsorption, which inhibits biodegradation. Based on its octanol-water partition coefficient, phenanthrene is considered to be highly hydrophobic ($\log K_{OW} = 4.52$; Watts 1998) indicating low water solubility and a tendency to adsorb to organic material in soils and sediment. The K_D value (soil partition coefficient) for phenanthrene at this organic content is

263.4, indicating that phenanthrene is 263.4 times more likely to partition into soils, as opposed to the aqueous phase (calculations based on Watts 1998). This suggests, therefore, that phenanthrene should be strongly tied to the soil matrix and unavailable to biota. Along with explaining decreased bioavailability, these numbers and their implications also indicate that extraction efficiency may have been negatively affected by high sorption. This may help explain why phenanthrene concentrations detected in initial samples were half the expected values based on the quantity of contaminant added (117.3 mg/kg expected, 67.5 mg/kg detected).

3.2 Experiment 2: Phenanthrene Degradation with Magnesium Peroxide and Potassium Nitrate Addition in High Organic Content Sediment

Phenanthrene biodegradation in high organic content Paradise Creek sediment by aerobic microbes was significantly enhanced after eight weeks of solid magnesium peroxide cube (ORC ®) treatment (Figure 10). Statistically significant removal was seen after five weeks of the eight week remediation study. After a full eight weeks of incubation, treatment yielded approximately 51% phenanthrene removal while control microcosms degraded only 20% of the added phenanthrene. Initial phenanthrene concentrations of approximately 49.3 mg/kg were reduced to about 23.7 mg/kg in treated microcosms, but remained around 39.2 mg/kg in control microcosms.

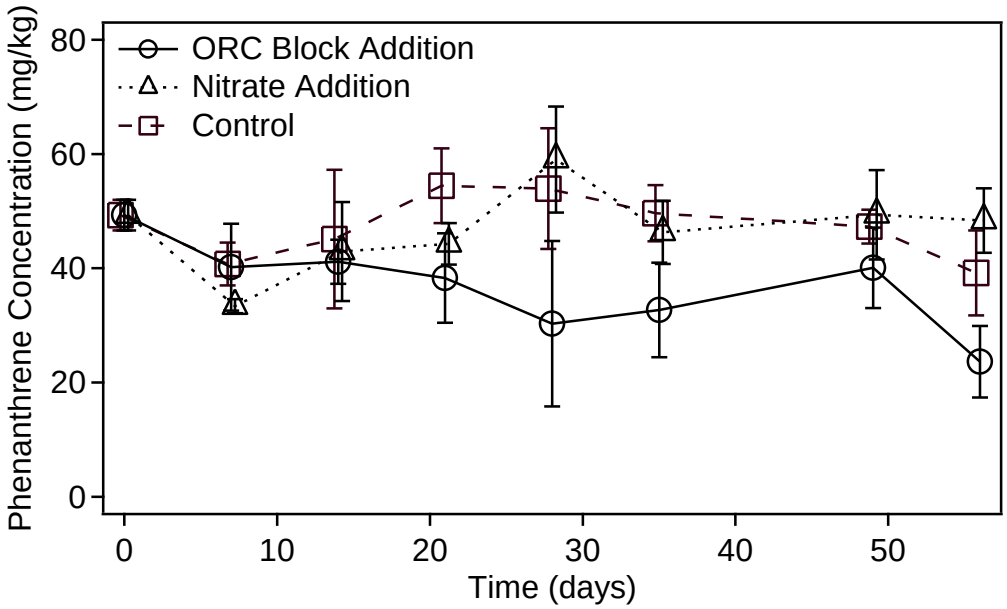

Figure 10: Phenanthrene degradation was enhanced by ORC® treatment in high organic content river sediment.

Linear modeling revealed that ORC® enhanced degradation proceeded more rapidly in LOC sediment than HOC sediment. Linear fitting generated a slope of -0.301 mg/kg*day for experiments in HOC sediments, and a slope of -0.938 mg/kg*day for experiments in LOC sediments. This indicated that degradation proceeded over three times more slowly at higher organic concentrations. This could be explained by either increased sorption to the increased organic carbon content, or that bacteria will preferentially utilize soil organic matter over phenanthrene as a carbon source when the option is available.

Both the degree of degradation and extraction efficiency decreased in HOC sediment as compared to results gathered from LOC sediments. High organic content sediments produced 51% phenanthrene degradation, about 19% less than in LOC for which removal was calculated to be approximately 70%. Likewise, HOC and LOC batches of Paradise Creek sediment were contaminated with about 116 mg/kg and 117 mg/kg phenanthrene, respectively. Maximum extraction from HOC sediment was recorded at 69.3 mg/kg (60%) and from LOC sediment was

recorded at 90.5 mg/kg (77%). The differences between degradation and extraction efficiencies can be explained by varying degrees of sorption related to the differences in organic content. Sorption is directly proportional to the fraction of organic matter found in soils (Watts 1998), and as such it would follow that HOC sediments would bind phenanthrene more effectively. It has also been noted that desorption is almost always the limiting factor in the rate of contaminant remediation (Watts 1998). High sorption would indicate little availability, and thereby little treatment. This discrepancy could also be accounted for by the extraction methods used. As discussed previously, phenanthrene was extracted from LOC sediments using the ASE, while extractions from HOC sediments were performed using a manual dichloromethane method. It could have been that the latter method did not extract as efficiently as the ASE. Differing extractions methods may also have affected the ability to compare experiments. While trends should not have been affected, absolute values may not be comparable.

Phenanthrene biodegradation in HOC Paradise Creek sediment by denitrifying microbes was not significantly enhanced by potassium nitrate crystal addition, and in fact, performed worse than control microcosms, indicating that nitrate treatment may have inhibited microbial activity (Figure 10). Phenanthrene concentration in nitrate amended cells did not differ statistically from controls, but, after eight weeks of incubation, nitrate treated cells yielded only 1.9% degradation compared to 20% in controls and 51% in ORC® amended cells. Similar findings were reported by Lei et al (2004), who cited that, no phenanthrene degradation occurred when sediments were amended with $NaNO_3$, but that degradation of readily degradable co-substrate (i.e., organics) was noted in association with denitrification. Although the latter was not noted in this case, the research cited here applied 12 times more nitrogen than applied by Lei et al (2005) which may have been responsible the disparate results.

Ammonium and nitrate addition may have inhibited phenanthrene biodegradation. Low phenanthrene degradation was observed in nitrate treated and control microcosms, both of which were ammonium amended (Figure 10). These results correspond with data collected by both Johnson et al. (1999) and Philips et al. (2000), which suggested that phenanthrene mineralization rates were decreased with nitrogen addition. When phenanthrene concentrations in control microcosms are compared between LOC and HOC experiments, ammonium addition to HOC sediments seems to have inhibited phenanthrene biodegradation (Figure 4, Figure 10).

Contrary to results seen previously in LOC sediments, ORC® treatment failed to significantly alter dissolved oxygen levels in water overlying high organic content sediments (Figure 11). DO levels in ORC® treated microcosms did not change significantly when compared to values recorded at day seven, nor did DO levels change significantly when compared to values recorded in control microcosms sampled the same day (i.e., day 21 versus day 21). While ORC® treated microcosms reached a high of 1.16 mg/L DO, this value appeared to be an anomaly, as the next highest value recorded was 0.87, and most readings fell between 0.2 and 0.5 mg/L. The control microcosms reached a high of 0.66 mg/L, and increased significantly at day 49 (compared to day seven). This increase lasted only one week, and also appears to have been an anomaly. The failure of ORC® treatment to appreciably increase DO levels could have been due to the soil type.

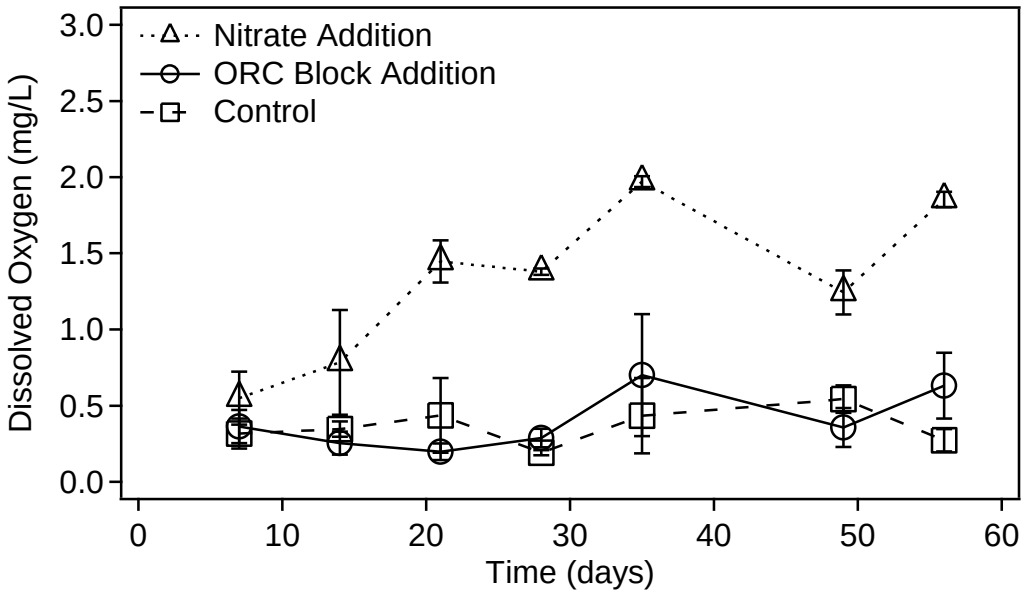

Figure 11: Average dissolved oxygen concentration versus time in water overlying high organic content river sediment. Error bars indicate standard deviation of three replicates.

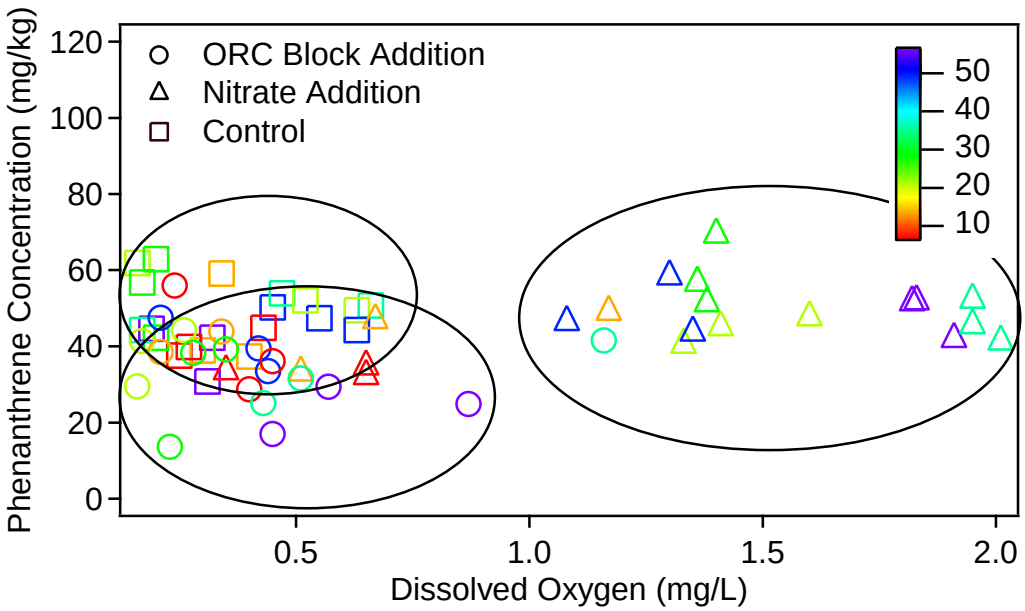

Figure 12: Dissolved oxygen in overlying water versus phenanthrene concentration in high organic content river sediment. ORC® treatment enhanced phenanthrene degradation despite its ability to successfully enhance DO levels.

The failure to observe increasing DO levels in experiments utilizing HOC sediments could also have been due to the sampling equipment used. While LOC sediment experiments employed a YSI 52 DO probe, high organic sediment experiments utilized a YSI 55. While it may be expected that trends would remain the same regardless of sampling equipment, there did appear to be a significant difference between instruments when reading the same sample. Although trends may be accurate, the DO numbers could not directly be compared from one experiment to the next, and it must be considered that even trends were altered by changing equipment.

High sediment organic content may have increased sediment microbial activity, leading to the maintenance of a constant dissolved oxygen level with ORC® addition. As noted previously, Experiment 1 involved sediment with comparatively low organic content (1.3%), while the results reported here were obtained using sediments containing much higher organic content (3.4%, discussed later). This increase in organic content may have allowed organic degrading microbes to proliferate along with the phenanthrene degrading microbes that were previously shown to multiply under similar conditions. This would have increased overall sediment microbial activity in comparison to LOC sediments, which in turn would have resulted in an increase in oxygen utilization. With increased oxygen utilization, oxygen buildup in overlying waters may have been inhibited. If oxygen diffusion rates out of the sediment were equal to microbial utilization rates, this would also explain the relatively constant DO levels.

Nitrate treatment enhanced dissolved oxygen levels in water overlying HOC contaminated sediment (Figure 11). DO levels increased significantly after 21 days of treatment and remained statistically higher than day seven for the remainder of the eight week study. When compared to both control microcosms and ORC® treatments, DO levels were again

statistically higher beginning on day 21 and remained so for the duration of the study. As stated previously, control microcosms reached a high of only 0.66 mg/L, whereas, nitrate treatments soared to 2.01 mg/L. Increasing DO in nitrate treated microcosms may indicate microbial inhibition under these microcosm conditions. With no microbial activity, leaky caps may have allowed adequate oxygen to penetrate the systems to increase DO levels over time.

While oxygen concentrations were not augmented by ORC® treatment in HOC sediment microcosms as they were in LOC microcosms, results showed enhanced phenanthrene degradation in these microcosms indicating that the treatment successfully stimulated aerobic microbial activity (Figure 12). As discussed previously, the failure to measure increases in DO may have been due to an overall increase in microbial activity, and thereby an increase in oxygen consumption. This could indicate that phenanthrene degraders were still adequately supplied with electron acceptors, and able to degrade phenanthrene successfully. While oxygen was not supplied in excess as it was in LOC sediments, it was supplied in sufficient quantity to maintain PAH biodegradation.

Real-time-PCR analysis failed to reveal increased dioxygenase gene presence in HOC phenanthrene contaminated sediments treated with ORC® (Figure 13). These details contrasted with findings using LOC sediments and indicated that ORC® treatment failed to stimulate growth of PAH degrading bacteria. Increased degradation rates in ORC® treated samples, in this instance, can not be explained by an increase aerobic PAH degrading population as hypothesized earlier. The lack of PAH degrading population growth may be in part explained by the failure for ORC® treatment to significantly raise DO levels.

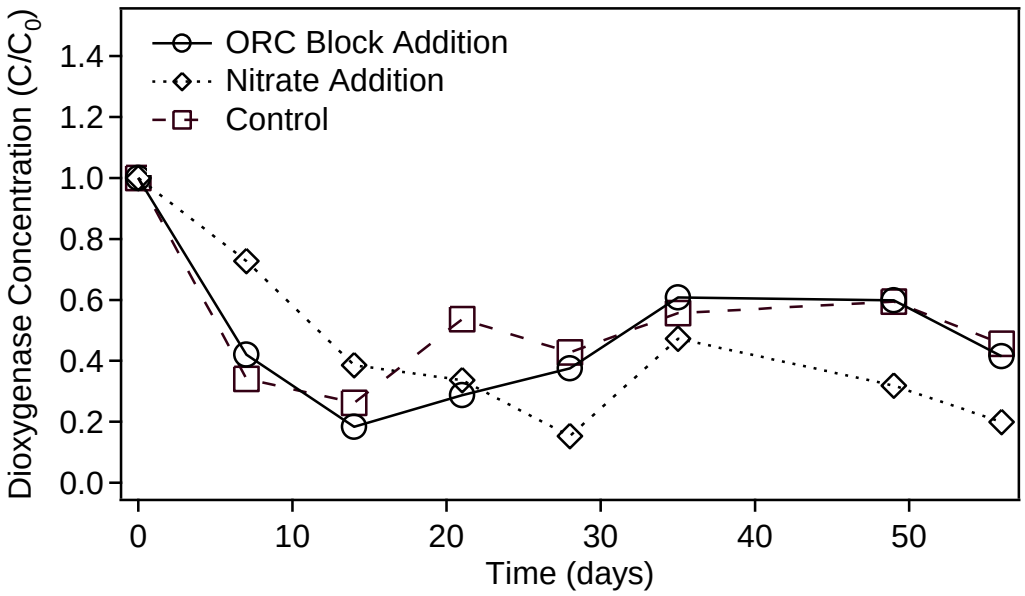

Figure 13: Real-Time PCR indicated that ORC® treatment failed to elevate dioxygenase concentrations, and could not be used to explain increased phenanthrene degradation. Nitrate treatment microcosms showed the lowest dioxygenase concentrations as time progressed.

Dioxygenase concentration dropped dramatically in the first 15 days of observation, a phenomenon perhaps explained by the experimental set up. Whereas LOC sediments were contaminated one day prior to use, HOC sediments were contaminated 26 days in advance. The 26 day period during which the high organic content sediments incubated prior to use, may have allowed phenanthrene degrading communities to develop before the experiment was run. This is supported by the initial dioxygenase concentrations: LOC sediments revealed initial concentrations around 0.035 (relative to arbitrary concentrations assigned to the standard curve), while HOC sediments contained concentrations closer to 0.560. The dioxygenase decrease that occurred during the first 15 days may be explained by declining oxygen availability in sediments. The subsequent upswing may be due to the reintroduction of oxygen during the time microcosms were established, as well as ORC® addition in the case of oxygen amended microcosms, which would both create favorable conditions for dioxygenase development.

Dioxygenase concentration in nitrate amended microcosms tended to remain below levels seen in control microcosms, and remained significantly below those seen in ORC® treated cells. Because dioxygenase activity depends on the presence of molecular oxygen, which was not provided during nitrate treatment, this may have been expected. It was also noted, however, and discussed previously, that nitrate treated cells were characterized by comparatively high DO levels (Figure 11). This discrepancy could again be explained by an overall inhibition of microbial activity by nitrate addition. Despite adequate oxygen to act as an electron acceptor and carbon sources (both soil organics and phenanthrene), no microbial activity was observed (as either change in pH, oxygen consumption, phenanthrene or organic degradation). If microbial growth were inhibited, even in the presence of oxygen, it would not be expected to see development of dioxygenase genes and activity.

Many PAH-degrading bacteria are oligotrophs (Johnson, Scow 1999), and excessive nitrogen addition (both ammonium and nitrate, in this case) may have stunted microbial growth, explaining the low dioxygenase concentrations seen across the board. Ammonium was added to phenanthrene contaminated sediment two days prior to microcosm establishment. This addition corresponds to the initial drop in dioxygenase concentration observed in all three treatments (Figure 13). While dioxygenase levels began to recover in both control and ORC® treatments after 14 days, nitrate treatments maintained low enzyme levels. This trend was likely due to higher nitrogen input (ammonium plus nitrate) to these microcosms.

ORC® treatment did not significantly influence pH levels in experimental microcosms, and in fact, roughly mirrored the pH trends seen in control microcosms (Figure 14). Whereas previous studies in LOC sediments revealed a pH increase in control microcosms when compared to relatively constant pH in ORC® treated cells, this was not the case in HOC

sediments. It was hypothesized earlier that the pH increase seen in control microcosms may have been due to hydrogen ion consumption by organic degrading methanogens. Were the HOC to have increased the total sediment microbe count as previously proposed, this would also suggest an increase in methanogenic species. Methanogen presence in ORC® treated cells could account for parallel increases in pH between oxygen amended and control microcosms. The HOC sediment may also have been characterized by decreased soil buffering capacity, leading to slight pH increases.

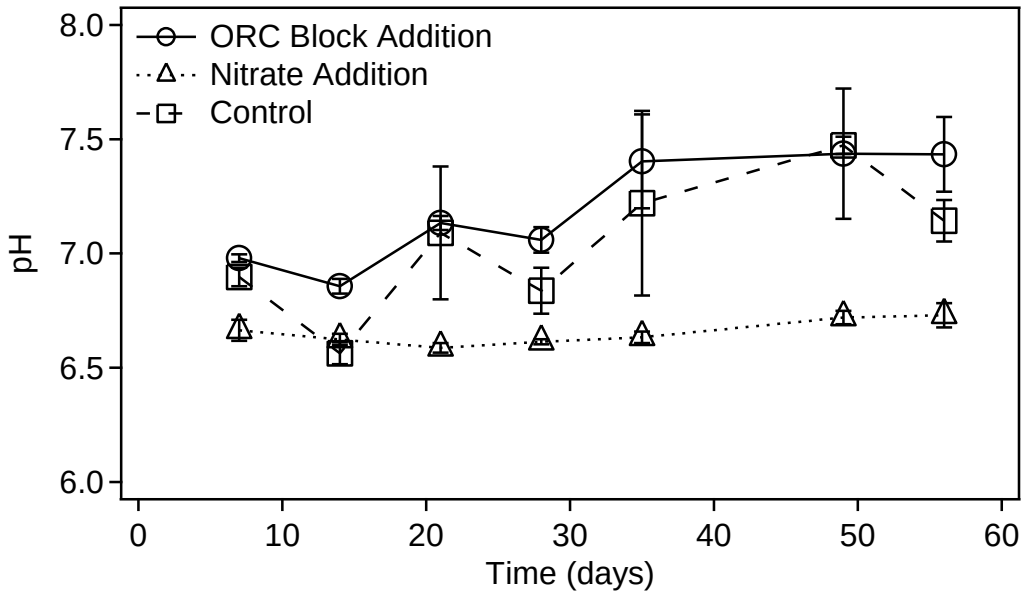

Figure 14: Average pH versus time in high organic content river sediment. Error bars indicate standard deviation of three replicates.

Nitrate treatment did not cause pH to fluctuate, and values remained significantly below those recorded for both controls and ORC® treatments, possibly indicating a lack of microbial activity (Figure 14). As hypothesized in light of the failure for oxygen to disappear in nitrate treated microcosms, nitrate treatment may have had an inhibiting effect on microbial growth, damping microbial utilization of both oxygen and carbon sources. Were methanogens not

present to consume hydrogen, pH drift caused by microbial activity would not have been evident, and pH data would appear much as seen here.

Organic content from the Paradise Creek sediment used in this experiment was determined to be approximately 34.2 g/kg, or 3.4%, and may, in combination with differing extraction methods, account for the fact that initial phenanthrene quantities extracted were far below what was expected. The K_D value (soil partition coefficient) for phenanthrene at 3.4% organic content is 698.3, 2.6 times higher than the K_D calculated for LCO sediments (calculations based on Watts, 1998). The increased tendency to adsorb to soils may help explain why phenanthrene concentrations detected in initial samples were less than half the expected values based on the quantity of contaminant added (116.3 mg/kg expected, 49.3 mg/kg detected).

While both ORC® treated and control microcosms showed significant organic degradation after eight weeks of incubation, organic content in nitrate treated microcosms remained roughly the same from beginning to end, supporting microbial inhibition by nitrate addition. As proposed previously, the high organic content of sediments used in the second experiment may have allowed for the proliferation of organic-degrading bacteria alongside phenanthrene-degrading bacteria. Analysis of organic content at week eight revealed that after initial organic content was determined to be approximately 3.4%, control and ORC® were reduced significantly to 2.9% and 3.0% organics, respectively. Nitrate treated microcosms, however, maintained an organic content of 3.3%, revealing a lack of organic-degrading microbial activity. Supporting this conclusion are the following findings: 1) phenanthrene failed to disappear from nitrate treated microcosms, 2) oxygen was not consumed in nitrate treated microcosms and, in fact, increased over time, 3) dioxygenase concentrations remained low,

despite relatively high dissolved oxygen concentrations, and 4) the maintenance of such a constant pH suggests little to no microbial activity was occurring.

4.0 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Phenanthrene removal was significantly enhanced by solid peroxide (ORC®) addition in both HOC and LOC sediments; an expected result due to its low molecular weight and widely reported microbial degradation under aerobic conditions. The correlations drawn between DO, RT-PCR, and phenanthrene concentration data gathered from LOC sediments suggested that the increased degradation observed in treated microcosms was due at least in part to increased microbial activity. RT-PCR and DO data gathered from experiments using HOC sediments failed to support biodegradation as a primary remediation mechanism, and may warrant investigation into ORC® enhancement of other degradation mechanisms such as chemical oxidation.

Nitrate treatment at the levels applied here failed to enhance phenanthrene biodegradation, and actually appeared to inhibit microbial activity of any kind. Neither phenanthrene nor organics were degraded, oxygen was not consumed, and pH was not altered significantly. Similar studies applying lower nitrate concentrations revealed increased organic degradation via denitrification, but phenanthrene removal was still not observed (Lei, Khodadoust et al. 2004). This suggested that it was the high concentrations that caused overall microbial inhibition, but that nitrate still may not be an effective PAH remediation strategy. Nitrate addition, therefore, would not be a recommended treatment option based on the results of this experiment.

Soil organic content and its ability to adsorb hydrophobic organic contaminants such as phenanthrene had a notable effect on both degradation rates and extraction efficiency, and may be a determining factor in the application of bioremediation strategies such as those tested here. Decreased extraction efficiency indicated that phenanthrene was strongly adsorbed to HOC sediments and would therefore be unavailable to degradation by microbes. Though removal was

still observed, decreased efficiency in HOC sediments may indicate that ORC® treatment is a more viable treatment strategy in LOC sediments.

Due to the high cost and invasive nature of current sediment remediation strategies, the success of this comparatively low cost, non-invasive method has positive implications for the field. Whereas capping and dredging require much disturbance to natural water systems, ORC® insertion beneath contaminated sediment layers would be relatively simple, and the ecosystem would be able to recover comparatively quickly.

The maintenance of a relatively constant moderate pH under ORC® treatment conditions supports the non-invasive nature of this treatment strategy. pH fluctuation presents a threat to aquatic organisms, and showing that ORC® is safe where pH is concerned is further support for promotion of its use in the environment.

Based on the success of the experiments presented here, recommendations could be made to continue investigation of such techniques. As noted by Lei et al (2004), laboratory- and naturally-contaminated sediments react differently to treatment, so further research will be required to validate solid peroxide use at the field scale. Future laboratory scale testing may seek to incorporate both a negative and positive controls. A negative control in which microbes have been rendered ineffective (heat killed or poisoned) may further confirm the role of microbes in the observed degradation. Likewise, a positive control in which oxygen was delivered in excess to maintain aerobic conditions could shed light onto the potential microbial activity under truly aerobic conditions.

Further experiments may also seek to find a balance between stimulant addition and degradation increase. The conditions achieved in this experiment were those of excess. ORC® addition to LOC sediments resulted in oxygen supersaturation, meaning that oxygen was

produced faster than it was utilized by bacteria, ultimately leading to oxygen accumulation. These details suggest that the bacteria were not limited by electron acceptors (oxygen). Subsequent research may seek to meet, without exceeding, microbial oxygen requirements and achieve similar degradation results at lower ORC® doses. While results have shown that these conditions successfully stimulated microbial phenanthrene degradation, future researchers may strive to find a balance between enhanced degradation and excess material additives.

5.0 APPENDIX A: R-PCR METHODS AND STANDARD CURVES

R-PCR Universal Assay – Target: Gram Negative Dioxygenase gene (detects all bacteria and archaea)

Methods

Primers:

PAH GN For	5'- GAG ATG CAT ACC ACG TKG GTT GGA -3'
PAH GN Rev	5'- AGC TGT TGT TCG GGA AGA YWG TGC MGT T -3'

Assay components (25µL reaction):

Power SYBR® Green PCR Master Mix	1x	Applied Biosystems
PAH GN For	54.10 nM	IDT
PAH GN Rev	61.40 nM	IDT
Sample/Standard/Blank H_2O	2 µL	

Reaction Temperature Profile:

Step 1	95.0 °C	10 min	1 cycle
Step 2	95.0 °C	30 sec	
	57.0 °C	30 sec	
	72.0 °C	30 sec	
	80.0 °C	10 sec	40 cycles
Step 3	72.0 °C	07 min	1 cycle

Melt curve from 51.0°C to 95.0°C in +0.1°C increments

Standard Used:

Pseudomonas putida 6/16/08

Additional information:

Each sample/standard/blank is tested in duplicate to check precision.

Results

The standard was created by diluting 2 µL of *Pseudomonas putida* DNA extract (6/16/08) with 18 µL of Nuclease free water. Subsequent 1:10 serial dilutions were made. The first dilution has an arbitrary 100 unit concentration; all dilution concentrations are in reference to the extract. Concentrations 100, 0.8, 0.16, and 0.032 were used to create the standard curve for the experiment run.

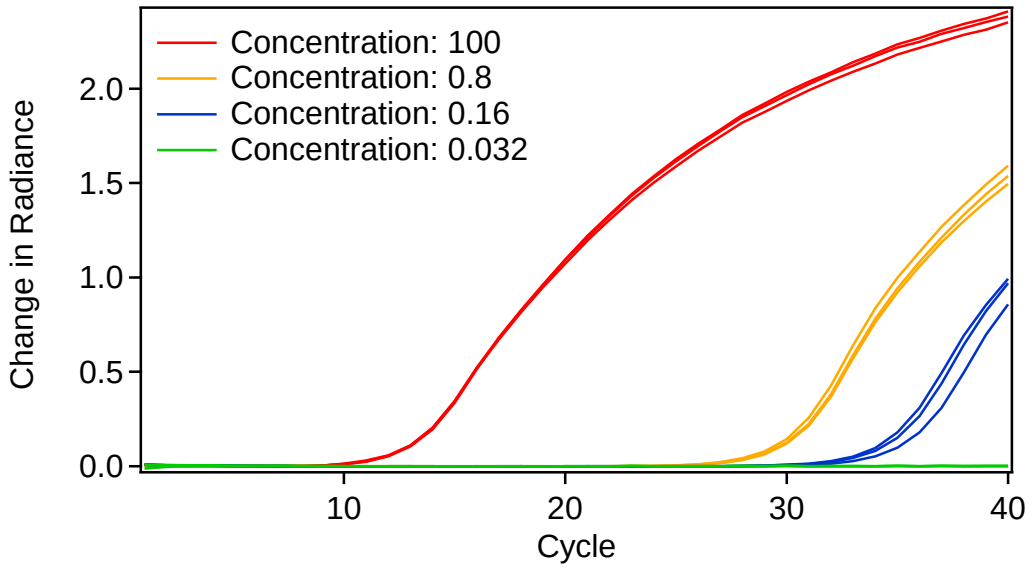

Figure 15: Amplification plot for *Pseudomonas putida* dilutions. Parallel lines indicate good amplification.

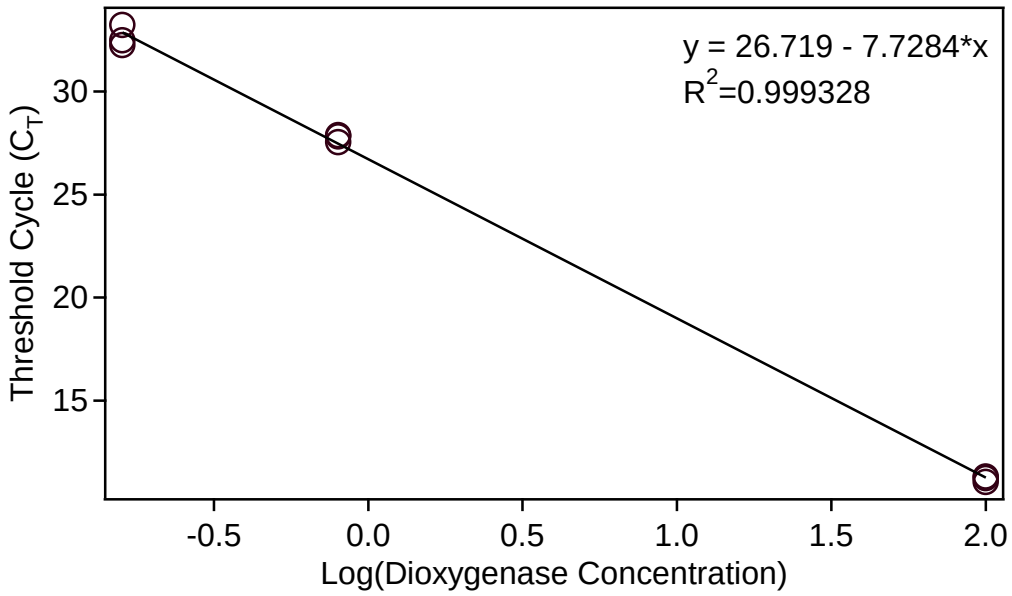

Figure 16: Standard curve created by *Pseudomonas putida* dilutions using the GN PAH-RHD$_\alpha$ gene. The R^2 value indicates a good fit. The threshold value was 0.02938.

6.0 APPENDIX B: HPLC STANDARD CURVES

Phenanthrene standards were created in the following concentrations: 100, 50, 10, 5 and 1 mg/L phenanthrene in methanol. A 2500 mg/L stock solution was created in a 50 mL volumetric flask by adding 0.125 g Phenanthrene and adding methanol to the fill line. Dilutions were performed in 25 mL volumetric flasks using this 2500 mg/L solution to achieve the desired concentrations (Table 2). These standards were used to generate a standard curve on the HPLC to be used in phenanthrene concentration analysis.

Table 2: Dilutions used in 25 mL volumetric flasks to generate standards.

$$\begin{array}{l} 100\mu L \\ 50\mu L \\ 10\mu L \\ 5\mu L \\ 1\mu L \end{array} + \left[\begin{array}{l} methanol \\ added \\ to \\ fill-line \end{array}\right] = \begin{array}{r} 100mg/L \\ 50mg/L \\ 10mg/L \\ 5mg/L \\ 1mg/L \end{array}$$

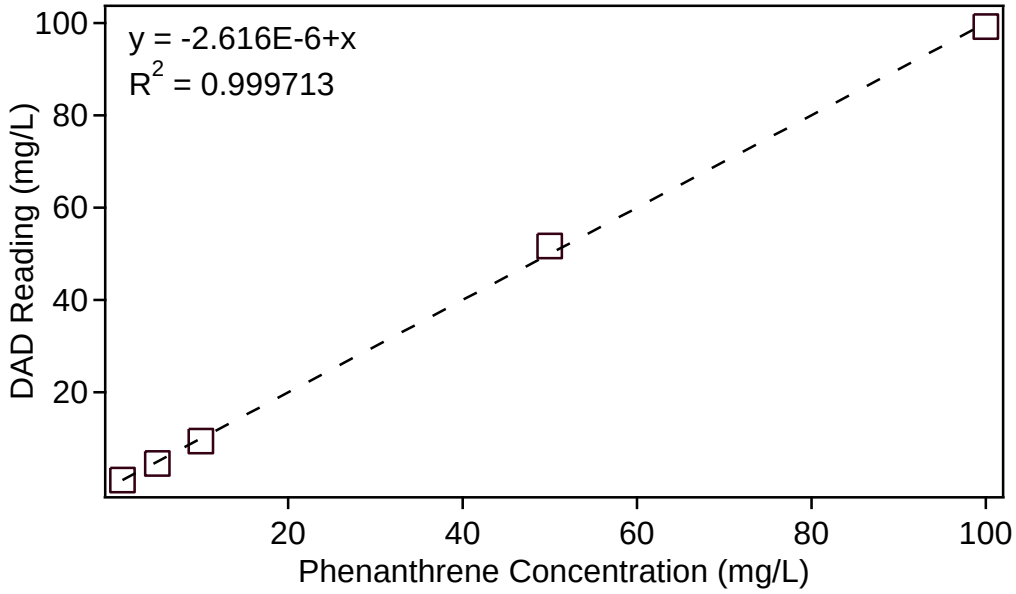

Figure 17: Standard curve generated during HPLC analysis of phenanthrene in high organic content sediment. The R^2 value indicates a good fit.

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