EFFECT OF INCUBATION CONDITIONS ON THE ENRICHMENT OF PYRENE-DEGRADING BACTERIA IDENTIFIED BY STABLE ISOTOPE PROBING IN A PAH-CONTAMINATED SOIL

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ABSTRACT

MAIYSHA D. JONES: Effect of Incubation Conditions on the Enrichment of Pyrene-Degrading Bacteria Identified by Stable Isotope Probing in a PAH-Contaminated Soil (Under the direction of Dr. Frederic K. Pfaender)

Many high molecular weight polycyclic aromatic hydrocarbons (PAH) are known or suspected carcinogens and as ubiquitous environmental pollutants, their remediation is necessary to reduce human and environmental health risk. Since PAH are biodegradable, bioremediation offers potential for site clean-up. Bioremediation techniques include *in situ* methods, or excavation followed by reactor treatment. To determine whether bacterial community diversity depends on treatment method, two incubation conditions were examined by stable isotope probing of pyrene-degrading bacteria in an aged PAH-contaminated soil. Microcosms of continuously mixed soil slurry or static, field-wet soil were spiked with [U-^{13}C] pyrene and incubated in the dark at room temperature for 28 days. Recovered ^{13}C-enriched DNA was analyzed by denaturing-gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries. The minimal diversity observed between DGGE profiles suggests that pyrene-degrading bacterial community diversity may be independent of treatment method, though slurry libraries were slightly more diverse than field wet libraries.

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LIST OF ABBREVIATIONS

ATSDR	**Agency for Toxic Substances and Disease Registry**
CERCLA	**Comprehensive Environmental Response, Compensation, and Liability Act**
CMN	**Contaminated Minnesota Soil**
DGGE	**Denaturing Gradient Gel Electrophoresis**
dNTPs	**Deoxynucleotide Triphosphates**
EPA	**United States Environmental Protection Agency**
FW	**Field Wet**
FW+N	**Field Wet with Nutrient Amendment**
FW-N	**Field Wet without Nutrient Amendment**
HPLC	**High-Performance Liquid Chromatography**
LSC	**Liquid Scintillation Counting**
OTU	**Operational Taxonomic Unit**
PAH	**Polycyclic Aromatic Hydrocarbons**
PCR	**Polymerase Chain Reaction**
PYR	**Pyrene**
rRNA	**Ribosomal RNA**
SARA	**Superfund Amendments and Reauthorization Act**
S+N	**Slurry with Nutrient Amendment**
S-N	**Slurry without Nutrient Amendment**
WHC	**Water Holding Capacity**

CHAPTER ONE

INTRODUCTION

1.1. STATEMENT OF THE PROBLEM

The Love Canal disaster of 1978 resulted in residents of this Niagara Falls, New York neighborhood being tormented by unexplained cancers and birth defects. The neighborhood had been built on land previously used as a dumping ground for hazardous industrial waste since the 1920s. Amidst growing national concern regarding the release of hazardous waste from contaminated sites, the United States Congress enacted the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) in 1980. Commonly called Superfund, this legislation made liable those responsible for hazardous waste release, established a trust fund for remediation when responsible parties could not be named, and gave the United States Environmental Protection Agency (EPA) administrative control. With the Superfund Amendments and Reauthorization Act (SARA) of 1986, the Superfund Basic Research Program was created under the management of the National Institute of Environmental Health Sciences. This network of federally funded grants is designated for research on many aspects of the science associated with hazardous chemicals, including the development and improvement of hazardous waste remediation technologies.

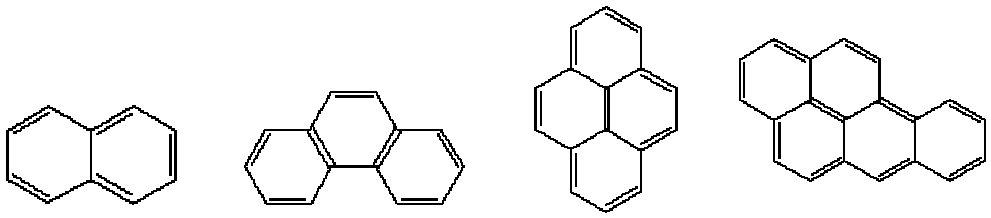
Origins and Characteristics of PAH

Polycyclic aromatic hydrocarbons (PAH) are one group of compounds regulated by the EPA as hazardous substances and several are in the top ten hazardous chemicals of the Agency for Toxic Substances and Disease Registry (ATSDR). Generally existing as complex mixtures, PAH result from the incomplete combustion of both natural and anthropogenic organic compounds. They are released into the air in cigarette smoke, engine exhaust and after certain cooking practices, and can enter water and soil systems in stormwater run-off or in waste released from industrial processes (2, 40, 62, 71).

Both physical and chemical characteristics of PAH affect their bioremediation in the soil environment. With very stable structures composed of two or more fused benzene rings, they are inherently hydrophobic such that high molecular weight PAH are increasingly less water soluble and therefore less bioavailable to microorganisms that utilize substrates in the aqueous phase (29)(Table 1). Their tendency to adsorb onto soil organic matter increases with molecular weight, leads to sequestration in micro- and nanopores within the soil matrix, and also contributes to decreased bioavailability, especially over time (3, 9, 12, 15, 23, 24, 28, 37, 64).

Bioavailability is not the only hurdle in PAH bioremediation. Metabolites resulting from the transformation of one PAH may inhibit the transformation of another in the same system, allowing this second PAH to persist (30). Likewise, the absence of a suitable co-substrate may limit the biodegradation of an otherwise bioavailable compound (25). Despite the challenge these characteristics of PAH pose to bioremediation, failing to remediate PAH-contaminated soil can lead to groundwater contamination, PAH accumulation in plants and animals, and increased human and environmental health risk (2, 45).

Table 1. A selection of PAH, molecular weights and water solubilities.



PAH	Naphthalene	Phenanthrene	Pyrene	Benzo(a)pyrene
MW	128.17	178.23	202.26	252.32
Solubility	31 mg/L	1.6 mg/L	0.135 mg/L	0.0016 mg/L
Log K_{ow}	3.30	4.57	4.88	5.97

Remediation Strategies

Current strategies employed to remediate soil contamination include both *in situ* and *ex situ* methods. Natural attenuation is the least invasive and least expensive *in situ* method for soil remediation. Through this process, contaminated soil is allowed to remain in place such that naturally occurring physical, chemical, and biological processes dominate contaminant transformation and degradation. Bioaugmentation and biostimulation are methods of *in situ* bioremediation where either a culture of microorganisms acclimated for specific substrate removal is added to the soil system or the growth of indigenous organisms is encouraged, respectively. Though bioremediation usually refers to the use of microorganisms, phytoremediation employs plants to enhance the removal of contaminants from soil and has gained recognition in recent years as a relatively low cost option for *in situ* soil remediation (6, 44, 63). More expensive, *ex situ* methods include excavation and replacement and excavation and treatment. Excavation and replacement involves separating the contaminated

soil from the negatively impacted population, moving it to a designated waste disposal site, and replacing the excavated soil with clean soil (38). Excavation and treatment involves removing the contaminated soil for physical (incineration), chemical (solvent extraction), or biological (slurry reactor) treatment (38).

It has been shown that slurry bioreactors are effective in PAH-contaminated soil remediation (25, 33, 36, 55, 60) and there are both advantages and disadvantages to this method of biological treatment. One advantage is that the investigator can inoculate the reactor with their microbial community of choice based on the specific substrate to be degraded. Because the selected culture of microorganisms is presumably acclimated for the degradation of the specific substrate, little to no lag time is expected before significant substrate removal takes place. The culture will contain the enzymes necessary to perform the biochemical pathways needed to degrade the substrate. Some lag time would be seen, however if the production of these enzymes is induced by the presence of an unfamiliar substrate. One disadvantage of slurry bioreactors is that there can be great expense incurred in the start-up and maintenance of the biological treatment facility (55). *In situ* treatment is an alternate remediation strategy of interest aimed at decreasing the financial burden incurred by *ex situ*, bioreactor treatment.

In summary, many high molecular weight PAH are known or suspected carcinogens. They are ubiquitous environmental pollutants and their remediation is necessary to reduce human and environmental health risk. There are technologies currently in place to remediate PAH contamination in soils, but in order to define lower cost, less invasive strategies, further investigation into the differences between these technologies is warranted.

1.2. RESEACH OBJECTIVES

In the context of *in situ* bioremediation, the most important aspect of the system being remediated is the nature of the microbial community in residence. It has been shown that less than 1% of microbial life has been identified, isolated, and cultivated (5). With the advent and subsequent standard use of molecular methods to isolate, sequence, and manipulate 16S rRNA genes, our ability to dissect microbial diversity has been greatly enhanced. Microbial ecologists can now not only discern phylogenetic relationships between and among both cultured and uncultured microorganisms, but we can also identify microorganisms without the need for traditional isolation and cultivation techniques (26, 41, 51, 70).

Stable isotope probing (SIP) is one cultivation-independent technique that has been considered for application in bioremediation studies (34, 68). This technique utilizes ^{13}C-labeled substrates to identify microorganisms capable of degrading the labeled substrate (49). Using compounds synthesized from a stable isotope of an atom affords investigators the ability to isolate, by density-gradient ultracentrifugation, ^{13}C-enriched ("heavy") DNA derived from only those organisms that incorporated the ^{13}C-labeled carbon source into its cellular structures. In this way, we can be confident that the organisms identified are in fact those that participated in labeled substrate transformation and/or degradation. Various techniques have been employed in attempts to link function with identity and to ultimately determine phylogenetic relationships (10, 34), but DNA-based SIP has been adopted in most recent studies of complex systems (42, 48, 50, 60).

The purpose of this study was to determine whether different bacterial communities would be enriched from the same aged, PAH-contaminated soil treated by two different

biological methods. Pyrene served as the model PAH in comparing a simulated slurry bioreactor system (*ex situ*) to a simulated biostimulation system (*in situ*). Bacteria responsible for pyrene transformation and degradation were identified by SIP.

The first objective to be accomplished was to optimize incubation conditions under which the SIP investigation would take place. It seemed reasonable to assume that the addition of inorganic nutrients would stimulate the growth of any resident microbes and that this would in turn increase the rate of pyrene degradation. The literature, however, is not clear as to whether the addition of inorganic nutrients enhances the growth of the microbial populations (66) or its ability to enhance degradation of a given substrate (11, 27, 47, 56, 66). This is most likely a soil-specific issue such that the effect of nutrient addition was determined for this particular soil. Microcosm treatments included continuously mixed slurry and static field wet incubations, with and without nutrient addition, resulting in four incubation conditions. Nutrient effects on bacterial community diversity and pyrene mineralization were assessed via denaturing gradient gel electrophoresis (DGGE) and liquid scintillation counting (LSC), respectively.

The second objective of this project was to identify those microorganisms active in pyrene transformation and degradation and determine whether a particular treatment method had any effect on the pyrene-degrading community enriched. This was accomplished using a new set of microcosm treatments chosen from the results of the nutrient effects study. Phylogenetic relationships were determined and the effects of treatment conditions on community diversity were compared via 16S rRNA gene clone libraries.

CHAPTER TWO

MATERIALS AND METHODS

2.1. CHEMICALS AND GLASSWARE

All chemicals, solvents, reagents, and media used in this study were of the highest quality commercially available. ^{12}C Pyrene was obtained from Sigma Chemical Company (St Louis, MO) and stored at room temperature. [U-^{13}C] Pyrene was synthesized from [U-^{13}C] naphthalene (60). ^{14}C Pyrene was also obtained from Sigma, diluted in methanol and stored at -20ºC until use (23). Nuclease free water was obtained from Promega Corporation (Madison, WI) and used for PCR and reconstituting primers. Water used to make buffer solutions was deionized and carbon filtered prior to use. All glassware was acid and solvent washed and then autoclaved prior to use.

2.2. PAH CONTAMINATED SOIL

Soil samples were obtained from the Reilly Tar and Chemical Corporation's St. Louis Park Plant (St. Louis Park, MN), a former coal tar distilling and wood preserving facility. Physical characteristics of this creosote contaminated soil include a moisture content of 5.49 ± 0.85% (University of Wisconsin-Madison Soil and Plant Analysis Laboratory, Madison, WI), pH=7.5 (UW), total PAH 4390 ± 690 mg/kg, and pyrene concentration 463 ± 100 mg/kg. Total PAH measurement was performed by Eno River Laboratories (Durham, NC) using EPA method 8270 on triplicate samples and refers to the following 16 EPA-listed priority

compounds: acenaphthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, benzo[a]pyrene, chrysene, dibenzo[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene. This soil is known to harbor a pyrene-degrading microbial community (23). Samples were stored in the dark at 4ºC until needed. Subsamples were sieved (2mm) and likewise stored for experimental use.

2.3. FIELD CAPACITY APPROXIMATION

The Field or Water Holding Capacity (WHC) of the contaminated Minnesota soil (CMN) was approximated by a modified container capacity procedure (13). Three 5 g, aliquots of CMN were dried at 105ºC for 24 hours. Each dried soil aliquot was added to a separate container, saturated with water, covered loosely with aluminum foil and left to drain for 2 hours at room temperature. Each container consisted of a 6 inch diameter water-saturated filter paper (Whatman No. 6, 0.2 μm pore-size) shaped to fit a glass funnel held by a 250 mL glass beaker. The average approximate WHC was 2.71 g water/g dry CMN.

2.4. PRIMER SELECTION FOR CONVENTIONAL PCR

The 16S ribosomal RNA (rRNA) is the most common small subunit rRNA molecule used for phylogenetic analysis in molecular ecology (5, 70). The specific amplification targets in this study were conserved regions of the eubacterial 16S rRNA gene. The primers 27f (specific to *Bacteria*: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (universal: 5'-ACGGCTACCTTGTTACGACTT-3') (32) were used to estimate DNA yield after extraction. Primers P63f (5'-CAGGCCTAACACATGCAAGTC-3') with a 5'-GC clamp and P518r (5'-

ATTACCGCGGCTGCTGG-3') (20) were used to visualize community profiles by denaturing gradient gel electrophoresis (DGGE). The *Escherichia coli* K-12 screen was performed with primers ECP79f (5'-GAAGCTTGCTTCTTTGCT-3') and ECR620r (5'-GAGCCCGGGGATTTCACAT-3') (57). Primers 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') (19) and 1492r were used in preparation for cloning.

2.5. CONDITIONS FOR CONVENTIONAL PCR

Each reaction was carried out in a Bio-Rad Gene Cycler with the following component concentrations and temperature programs. 27f/1492r and P63f/P518r: Each 25 μL reaction contained 2 mM $MgCl_2$ 1:10 dilution of 10X PCR buffer (100mM Tris-HCl, pH=8.3, 500 nM KCl), 250 μM each dNTP, 500 nM each primer, 2.5 U *Taq* polymerase, 0.5 μL template DNA, and nuclease free water. The 2 hour temperature program consisted of an initial 1 min denaturation at 95ºC followed by 30 cycles of 30 sec at 90ºC, 45 sec at 55ºC, and 1 min at 72ºC, and a final elongation of 5 min at 72ºC. ECP79f/ECR620r: Each 25 μL reaction contained the same set of components and concentrations. The 3 hour temperature program consisted of an initial 5 min denaturation of at 94ºC followed by 25 cycles of 1 min at 94ºC, 1 min at 55ºC, and 3 min at 72ºC, and a final elongation at 72ºC for 15 minutes. 8f/1492r: Each 50 μL reaction contained the same set of components and concentrations substituting 3 μL template DNA. The 3 hour temperature program consisted of an initial 5 min denaturation at 94ºC followed by 25 cycles of 1 min at 94ºC, 1 min at 50ºC, and 3 min at 72ºC, and a final elongation at 72ºC for 10 minutes.

2.6. PYRENE MINERALIZATION

2.6.1. Soil Spiking Protocol

Thirty-five grams of CMN were air dried in a fume hood for 24 hours and then separated into 4g, 17.5g and 8g portions. This was sufficient soil to allow for loss of moisture and still have desired soil masses in microcosm vessels. Following a triplicate liquid scintillation count (LSC) of the activity of 1µL ^{14}C pyrene in methanol (Packard TriCarb 1900TR, Packard Instruments, Meriden, CT), it was determined that 53.5µL of this solution was needed to reach 20,000dpm/g soil in 17.5g air dried CMN. A final concentration of 1000mg ^{12}C pyrene/kg soil was desired such that 17.5mg ^{12}C pyrene was dissolved in 1mL acetone. Pyrene stock solutions were prepared immediately prior to being spiked into the soil.

The 53.5µL ^{14}C pyrene in methanol was dispensed into a clean 250mL beaker and placed in a fume hood to completely volatilize the solvent. Two thirds of the ^{12}C pyrene in acetone and 20% (3.5g) of the 17.5g air-dried soil portion were then added to that same beaker. The contents were mixed manually for one minute using a clean glass rod (39, 54). In a second 250mL beaker, the remaining third of the acetone solution was mixed manually for one minute with 20% (1.6g) of the 8g air-dried soil portion using a second clean glass rod (39, 54). Both beakers were covered loosely with aluminum foil and placed in a fume hood for 24 hours to allow the acetone to evaporate (39). The remaining air-dried soil portions were then mixed into their respective beakers in ~20% increments for 30s per increment until all 17.5g and 8g had been incorporated (39, 54).

2.6.2. Buffer Solutions

Slurry Buffer without Nutrient Amendment

Two liters of 10mM phosphate buffer system were prepared from 1 part monobasic (3.33 mM KH_2PO_4) and 2 parts dibasic (6.67 mM Na_2HPO_4) solutions. The pH was adjusted to soil pH=7.5 using NaOH or HCl as necessary.

Field Wet Buffer without Nutrient Amendment

Two liters of 75mM phosphate buffer system were prepared and adjusted as above using 25 mM KH_2PO_4 and 50 mM Na_2HPO_4 solutions.

Nutrient Amended Slurry Buffer

A final concentration of 500mg Nitrogen per kg soil was desired. For a 1 to 3 soil to liquid slurry, 650mg NH_4Cl were added to a 1L volumetric flask and brought to volume with 10mM phosphate buffer.

Nutrient Amended Field Wet Buffer

To achieve 500mg N/kg soil, 4.78g NH_4Cl were brought to 1L in a volumetric flask with 75mM phosphate buffer.

2.6.3. Microcosms

Each of the four experimental microcosms—slurry with nutrient amendment (S+N), slurry without nutrient amendment (S-N), field wet with nutrient amendment (FW+N), field wet without nutrient amendment (FW-N)—were run in triplicate with ^{14}C pyrene and in

duplicate with ^{12}C pyrene as parallel incubations. Controls consisted of duplicate volatilization controls, duplicate slurry and duplicate FW incubations without nutrient amendment or pyrene addition, one slurry killed control, and one FW killed control. Killed controls were achieved with the addition of phosphoric acid to pH<2. Each microcosm consisted of 1g CMN in a 40mL glass EPA vial. In addition, slurry vials contained 3mL of the corresponding buffer solution and FW vials were adjusted to a moisture content (MC) of 70% WHC with the corresponding buffer solution. All ^{14}C pyrene microcosms were nested with a 13mm disposable glass culture tube containing a piece of filter paper roughly 2.54cm^2 in size just saturated with 2N KOH (~75µL) as a $^{14}CO_2$ trap. All vials were capped with Teflon-coated silicon disk-lined screw caps. Slurry microcosms were placed in a wire basket covered with a black plastic bag to minimize exposure to light and then on an orbital shaker at 180 rpm. FW microcosms were placed in a wire basket in a cabinet and remained static. All microcosms were incubated in the dark at room temperature for 49 days.

2.6.4. Monitoring Mineralization

Inoculating the spiked soil portions with the remaining air-dried soil marked Day 0 of this experiment. Pyrene mineralization was monitored by measuring $^{14}CO_2$ evolution by LSC of each $^{14}CO_2$ trap in 10mL scintillation cocktail (ScintiSafe Plus, Fischer Chemical) at day 3 and then weekly until the cumulative percent of initial [^{14}C] as $^{14}CO_2$ neared an asymptote. The $^{14}CO_2$ trap was replaced at each sampling point.

2.6.5. DNA Extraction

All microcosms were sacrificed after 7 weeks when the cumulative percent of initial [^{14}C] as $^{14}CO_2$ began to reach an asymptote in the S+N microcosm. Total community DNA was extracted in 0.5 g portions from the duplicate parallel microcosms containing ^{12}C pyrene using the Ultraclean Soil DNA Kit (MoBio Laboratories, Solano Beach, CA). FW microcosm samples were extracted directly. Slurry microcosm samples were pelleted in autoclaved, screw-top microcentrifuge tubes before extraction. Bead beating tubes containing 0.5g soil portions were shaken horizontally on a flatbed vortexer and the manufacturer's spin column protocol was followed to recover DNA.

2.6.6. Bacterial Community Profile

Representative profiles for each incubation condition were compared by DGGE analysis. Total community DNA extracts from each condition were amplified with primers P63f and P518r by PCR. Fifteen microliters of the resulting amplicons were loaded onto a 6% polyacrylamide gel with a 30 to 60% urea-formamide denaturing gradient. The gel was run in 1X TAE buffer overnight at 60ºC and 1000 volt-hours on a Bio-Rad Dcode system and post-stained with ethidium bromide for visualization. Differences in the banding patterns resulting from the various incubation conditions were used to determine which conditions would be used for the SIP investigation.

2.7. STABLE ISOTOPE PROBING

2.7.1. Soil Spiking Protocol

Twenty grams of CMN were air dried in a fume hood for 24 hours and separated into 4 g and 14 g portions. For experimental microcosms, methanol was added to 4 mg of [U-^{13}C] pyrene crystals until all the pyrene dissolved. ^{12}C pyrene stock solutions of 500, 250, 100, and 50 mg/L were made in methanol and used to create a standard curve from which the concentration of [U-^{13}C] pyrene in methanol was measured by high-performance liquid chromatography (HPLC). The resulting concentration was 355 mg/L. A final concentration of 1000 mg [U-^{13}C] pyrene/kg soil was desired such that 11.27 mL solution was needed to spike 4 g CMN. For parallel and control incubations the same final concentration of ^{12}C pyrene was desired such that 14 mg ^{12}C pyrene was dissolved in 1 mL acetone immediately prior to being spiked into the soil.

The 11.27 mL [U-^{13}C] pyrene in methanol was added to a clean 100 mL beaker containing 20% (0.8 g) of the 4 g soil portion. The pyrene in acetone solution was added to a clean 250 mL beaker containing 20% (2.8 g) of the 14 g soil portion. Each mixture was stirred manually with a separate glass rod for one minute. Both beakers were covered loosely with aluminum foil and placed in a fume hood for 24 hours to allow the solvent to evaporate. The remaining air-dried soil portions were then mixed into their respective beakers in ~20% increments for 30 sec per increment until the remaining soil had been incorporated.

2.7.2. Microcosms

Each of the two experimental incubation conditions—slurry with nutrient amendment (S+N) and FW with nutrient amendment (FW+N)—was run in duplicate with [U-^{13}C] pyrene and in duplicate with ^{12}C pyrene as parallel incubations. Controls consisted of one slurry killed control, one FW killed control, and duplicates of each incubation type with no pyrene addition. Killed controls were achieved with the addition of phosphoric acid to pH<2. Each microcosm consisted of 1 g soil in a 40 mL glass EPA vial. In addition, slurry vials contained 3 mL of the corresponding buffer solution and FW vials were adjusted to a MC of 70% WHC with the corresponding buffer solution. Slurry microcosms were placed in a wire basket covered with a black plastic bag to minimize exposure to light and then on an orbital shaker at 180 rpm. FW microcosms were placed in a wire basket in a cabinet and remained static. All microcosms were incubated in the dark at room temperature for 28 days.

2.7.3. Monitoring Pyrene Concentration

Inoculating the spiked soil portions with the remaining air dried soil marked Day 0 of this experiment. Pyrene disappearance was monitored by extracting 100 mg FW (wet weight) and 1 mL slurry aliquots with 500 and 1000 µL of ethyl acetate, respectively. Aliquots were taken from the parallel incubations with ^{12}C pyrene reserved for this purpose. Each aliquot was vortexed with ethyl acetate in a conical glass centrifuge tube and then centrifuged at 4000 rpm for 10 minutes (Marathon 21K/R, Fisher Scientific). The organic layer of the resulting supernatant was filtered through a 0.2 µm nylon filter using a glass syringe and stored in crimp-sealed amber gas chromatography vials at -20ºC until pyrene

concentration was determined by HPLC. Extracts were taken at day 3 and then weekly until the incubation vessels were sacrificed.

2.7.4. DNA Extraction

All incubations were sacrificed at the day 28 endpoint as determined from the ^{14}C pyrene mineralization data. Total community DNA was extracted from the experimental microcosms incubated with [U-^{13}C] pyrene, the duplicate controls incubated without additional pyrene, and the ^{12}C pyrene parallel microcosms as stated in section 2.6.5 with the exception of autoclaved 1X Tris EDTA (10mM Tris HCl, 1mM EDTA; pH=8.0) replacing Solution 5 provided in the MoBio kit. Extracts originating from the same 1g sample were combined prior to further manipulation.

2.7.5. *Escherichia coli* K-12 Screens

An initial screen for the 16S rRNA gene of *E. coli* K-12 was performed on the total community DNA from each [U-^{13}C] pyrene microcosm prior to separation and fractionation to determine whether this organism's DNA was appropriate for use as an indicator of separation efficiency. A second *E. coli* K-12 screen was later performed on all recovered fractions to delineate the heavy fraction from the light fraction (Section 2.7.9). Both screens were performed with primer set ECP79f/ECR620r under the corresponding PCR conditions.

2.7.6. DNA Separation

Unlabeled and ^{13}C-labeled DNA were separated by cesium chloride density gradient ultracentrifugation in a Sorvall OTD60B ultracentrifuge with a TV-1665 vertical rotor and

corresponding ultracentrifuge tubes (50). In addition to 1 g CsCl/ml DNA solution and 100 µL of 10 mg/mL ethidium bromide, 1.25 µL unlabeled *Escherichia coli* K-12 DNA was added to each tube to indicate separation efficiency. Centrifugation occurred at 265,000 g and 20-23ºC for 16-18 hours (50).

2.7.7. DNA Isolation

^{13}C-labeled DNA was isolated by fractionation of each ultracentrifuge sample and DNA recovery from those fractions. Three needles were carefully set into each ultracentrifuge tube and fractions were collected from the bottom of the tube. The first needle vented the top of the tube, the second needle was placed in the bottom of the tube perpendicular to the work surface, and the third needle, placed near the top of the tube, allowed water to displace the gradient from the top of the tube using a syringe pump (60). Fractions were collected in 2 mL eppendorf tubes in 400 µL increments every 30 seconds. Twelve fractions were collected from each ultracentrifuge tube. Tubes not being fractioned at that moment remained on the bench top to minimize disruption of gradient by repeated movement and were kept covered to prevent light from damaging the DNA. Collected fractions were held in the dark at 4ºC until DNA recovery. DNA was recovered from each fraction by butanol and ethanol extraction to remove ethidium bromide and cesium chloride, respectively, the same day fractions were collected (59). During cesium chloride removal, fractions were stored at 4ºC overnight before dissolving pellets in 75µL autoclaved 1X TE.

2.7.8. PCR Amplification of Isolated DNA Fractions

The DNA in each of the twelve fractions from each ultracentrifuge tube was amplified by PCR with primer set 27f/1492r under the corresponding PCR conditions and visualized on 1% agarose gel containing ethidium bromide. Those fractions that yielded PCR product (fractions 3-8) were further analyzed by DGGE (primers P63f/P518r). The resulting bacterial community profiles were used to determine which fractions would be designated "heavy" and "light" (i.e. those fractions containing primarily ^{13}C-labeled and unlabeled DNA, respectively).

2.7.9. Delineating Heavy and Light Fractions

The heavy fraction in a given sample was determined by it being the lowest fraction collected from the ultracentrifuge tube that yielded a PCR product, and which did not contain any *E. coli* K-12 DNA. The light fraction in a given sample was determined by it being the highest fraction collected from the ultracentrifuge tube that yielded a PCR product, and which did contain *E. coli* K-12 DNA. Presence or absence of *E. coli* K-12 DNA was determined by a second screen of each fraction as stated in section 2.7.5.

2.7.10. Construction of 16S rRNA Gene Clone Libraries

A 16S rRNA gene clone library was constructed from the heavy fraction of each of the duplicate [U-^{13}C] pyrene microcosms, resulting in 4 libraries. Primer set 8f/1492r was used to amplify the 16S rRNA genes in each heavy fraction. The resulting amplicons served as the insert for the ligation reaction with the pCR®2.1 plasmid vector that was then transformed into competent *E. coli* cells (Invitrogen TA Cloning Kit, Carlsbad, CA). A

blue/white patch plating screen was performed on randomly selected clones to identify those that had successfully been transformed. White, transformed colonies were grown overnight in Luria-Bertani broth with 50 μg/mL kanamycin and 15% glycerol stocks of each clone were made for storage at -20ºC. Each library consisted of 23 to 25 clones for which 200 μL of the respective glycerol stock solution was sent to SeqWright DNA Technology Services (Houston, TX) for sequencing with primer 8f in a 96-well plate. A neighbor-joining phylogenetic tree of 16S rRNA genes was constructed from the resulting 71 good sequences (34 slurry and 37 FW) using ClustalX (65) and bootstrapped 1000 times without considering gaps (43). Good sequences were compared to public sequence databases using BLASTN (4) to identify relative sequences. Chimeras were resolved using the CHIMERA_CHECK tool within Ribosomal Database Project II (RDP) (16).

CHAPTER THREE

RESULTS

3.1. PYRENE MINERALIZATION

3.1.1. Monitoring Mineralization

Pyrene mineralization was monitored in each ^{14}C pyrene-containing microcosm over the 49 day incubation period. The corresponding data are presented in Figure 1 and Table 2. All conditions showed a lag time of at least three days. The S-N and both FW conditions each showed a lag time of 7 days. Between 7 and 14 days a mineralization rate of less than 0.75% per day was seen for these incubations while during the same time period, the S+N condition showed more than 3% mineralization per day. By day 28, the S+N condition showed successive decreases in mineralization rate. It was decided that this would be the point at which incubations for the ensuing SIP investigation would be sacrificed to minimize the chance of cross-feeding (incorporation of label by secondary consumers)(60). The pyrene mineralization experiment was allowed to proceed beyond 28 days to determine whether the extent of mineralization in the other incubations would approach that observed in the S+N incubations, though this was not the case after 21 days of additional incubation.

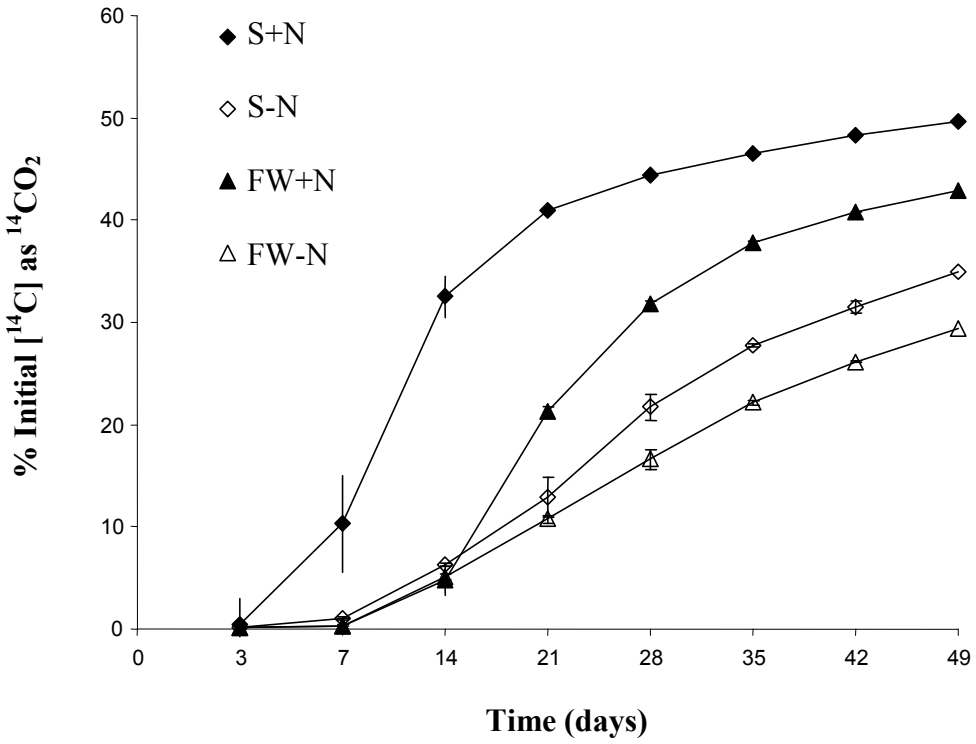


Figure 1. Pyrene mineralization in each microcosm over 49 days. The mean of triplicate samples is plotted for each incubation condition as the percent [^{14}C] evolved as $^{14}CO_2$ ± sample standard deviation.

Table 2. Pyrene mineralization rates and cumulative percent of mineralization in each microcosm after 28 days[a].

Microcosm Condition	k_o[b]	k_{max}[c]	28d Total[d]
Slurry + Nutrients	**1.47**	**3.20***	**44.4 ± 0.4**
Slurry - Nutrients	**0.15**	**1.25****	**21.7 ± 2.0**
FW + Nutrients	**0.05**	**2.36*****	**31.8 ± 0.6**
FW - Nutrients	**0.05**	**0.83****	**16.6 ± 0.4**

a. All rates are reported as % of initial [^{14}C] evolved as $^{14}CO_2$ d^{-1}.
b. Initial mineralization rate, k_o (0-7 days)
*c. Maximum mineralization rate, k_{max}. (*7-14, **21-28, ***14-21 days)*
d. Cumulative % of initial [^{14}C] evolved as $^{14}CO_2$ after 28 days of incubation ± sample standard deviation.

The S+N condition had the fastest initial mineralization rate (0-7 days) while all others were negligible over the same time period. Treatments with nutrients had higher maximum mineralization rates than their no-nutrient counterparts, though the FW+N microcosm took 7 days beyond S+N to achieve its maximum. These maximum rates were more than two and a half times those without nutrients within the same treatment and took 14 more days compared to S+N to reach this state.

A significant increase ($p < 0.001$) in the extent of mineralization was seen in each microcosm where additional nutrients were provided compared to those without nutrient amendment. Pyrene was mineralized to a significantly greater extent ($p < 0.001$) in slurry microcosms compared to FW microcosms whether additional nutrients were present or not, suggesting that the extent of mineralization was influenced by physical treatment and not the availability of additional nutrients alone.

3.1.2. Bacterial Community Profiles

Parallel FW microcosms containing unlabeled pyrene yielded more concentrated total community DNA extracts than slurry microcosms. A PCR for DGGE was performed using these extracts as template. The resulting bacterial community profiles shown in Figure 2 compare the different nutrient conditions for both slurry and FW microcosms with their respective controls and provide a qualitative representation of community diversity. Upon visual inspection, there is little difference in community diversity regardless of the presence or absence of nutrients or additional pyrene. The lowest band in lanes 7 and 8, however, appears to be unique. Another notable difference across all lanes is in relative band intensity. The more starting material that is present, the more copies can be made such that more concentrated DNA extracts provide more template for PCR. This can result in more intense bands when amplicons are resolved by DGGE. Relative DGGE band intensity has been used as a measure of population density (14, 61), where more intense bands indicate the presence of a greater concentration of 16S rRNA genes belonging to the organisms represented by that band, however extraction and PCR-related biases can influence relative intensities (8, 67).

In a remediation context, whether *in situ* or *ex situ*, supplemental inorganic nutrients are likely to be added to stimulate the growth of the microbial population. The growth stimulated by nutrient addition is essential as SIP relies on microbial growth on the labeled compound for labeled atoms to be incorporated into cellular structures. For these reasons and because there appeared to be a greater concentration of genes in the treatments with nutrient amendment, inorganic nutrients were added to microcosms assembled for SIP with [U-^{13}C] pyrene.

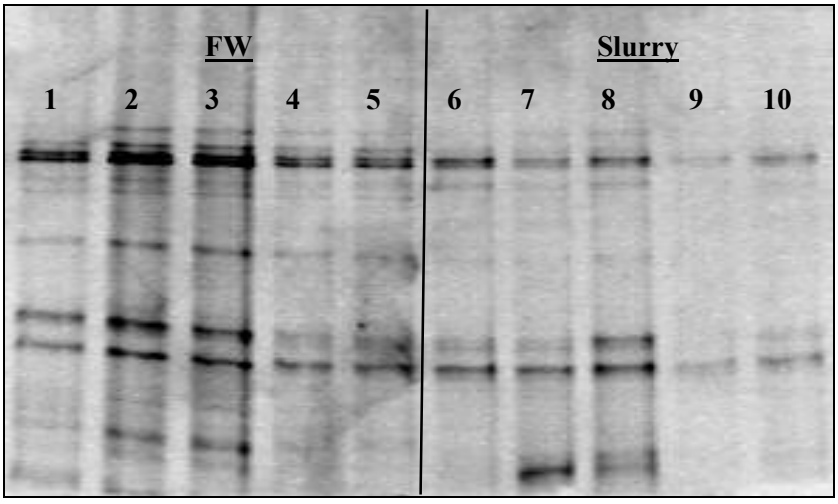


Figure 2. Negative image of DGGE gel resulting from nutrient condition comparison. Lanes 1 and 6 represent FW and slurry microcosms containing their respective buffer solutions without nutrient amendment or pyrene addition (controls). Lanes 2-3 and 7-8 represent microcosms containing ^{12}C pyrene and their respective buffer solutions with nutrient amendment. Lanes 4-5 and 9-10 represent microcosms containing ^{12}C pyrene and their respective buffer solutions without nutrient amendment.

3.2. STABLE ISOTOPE PROBING

3.2.1. Monitoring Pyrene Disappearance

Pyrene disappearance was monitored weekly by HPLC in microcosms containing unlabeled pyrene incubated in parallel to those containing [U-^{13}C] pyrene. The resulting data showed no identifiable trend (data not shown), as there was no meaningful change in measured pyrene concentration over the length of this experiment in any of the microcosms. Conclusions drawn regarding the extent of pyrene disappearance were based, therefore, on Figure 1.

3.2.2. Delineating Heavy and Light Fractions

Heavy and light fractions were chosen based on their location in the ultracentrifuge tube, amplification of PCR product (primers P63f/P518r, Figure 3), and the absence or presence of *E.coli* K-12 DNA, respectively. Fraction six of each duplicate slurry and FW tube contained *E.coli* K-12 DNA as evidenced by PCR product resulting from this fraction being used as template in a PCR with primers ECP79f/ECR620r. No product was amplified when any other fraction was used as template with these primers. Fraction 6 was therefore designated the light fraction for all four incubations. Fractions 3 and 4 were both candidates for heavy fraction designation based on their location in the ultracentrifuge tube and production of PCR product. The lowest fractions from which adequate PCR product resulted were slurry fraction 4 and FW fraction 3 (Figure 3). Figure 4 shows the profiles resulting from DGGE of slurry and FW fractions 3, 4, and 6. Fraction 4 was designated the heavy fraction in slurry incubations and fraction 3 was designated the heavy fraction for FW incubations. Slurry fraction 3 and FW fraction 4 were run for consistency purposes, and do not represent heavy or light fractions specifically, however looking from FW fraction 6 to 4 to 3 (light fraction to heavy fraction) demonstrates how the community transitions as pyrene degraders are enriched.

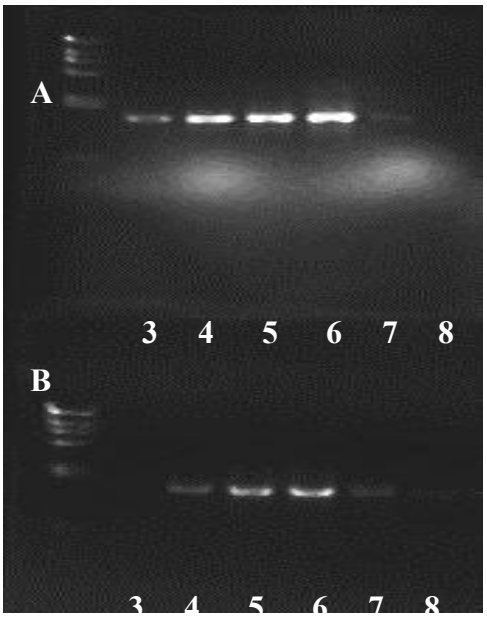


Figure 3. Positive image of recovered DNA fractions. Lowest numbered lanes correspond to fractions collected from the bottom of the respective ultracentrifuge tube. (A) Representative recovered DNA fractions from FW+N incubations. (B) Representative recovered DNA fractions from S+N incubations.

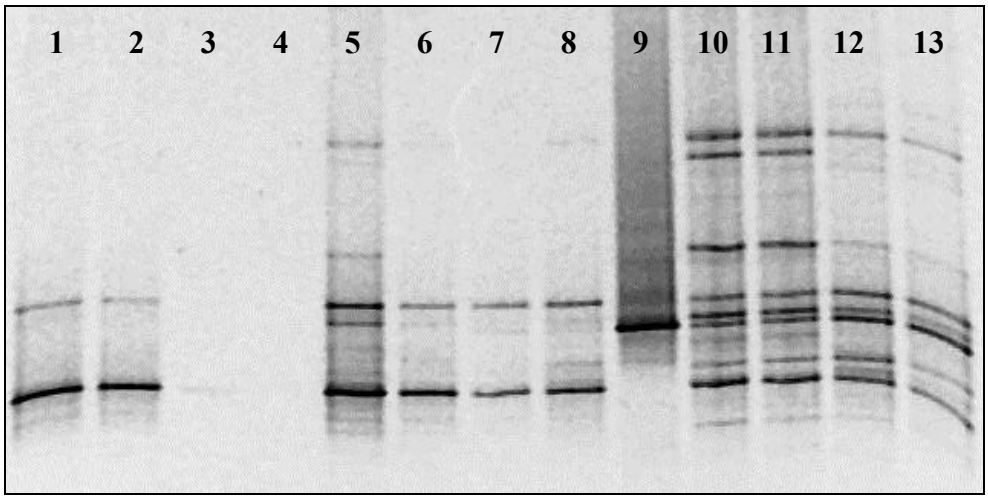


Figure 4. Negative image of DGGE gel delineating heavy and light fractions. Lanes 1-4, 5-8, and 10-13 represent fractions 3, 4, and 6 respectively. Lane 9 contains E. coli K-12 amplicon. Other lanes correspond to the incubation condition of origin and the fraction from the ultracentrifuge tube: (1-2) FW heavy fraction, (3-4) slurry fraction 3, (5-6) FW fraction 4, (7-8) slurry heavy fraction, (10-11) FW light fraction, (12-13) slurry light fraction.

3.2.3. Phylogenetic Analysis of 16S rRNA Gene Clone Libraries

A neighbor-joining phylogenetic tree of 16S rRNA genes was constructed from 16S rRNA gene sequences greater than 400 base pairs in length obtained from SeqWright after sequences were compared to public databases (Figure 5). This tree displays those 16S rRNA gene sequences most closely related to sequences recovered from this study. It also includes related 16S rRNA genes from cultured and as yet uncultured bacteria implicated in PAH degradation. A summary of closely related sequences and their sources is given in Table 3. Two chimeras were resolved using the CHIMERA_CHECK program within RDP II (16) and were excluded from phylogenetic analyses.

All of the sequences from the field wet microcosms (37/37) and the majority of sequences from the slurry microcosms (30/34) were γ-Proteobacterial. Six operational taxonomic units (OTUs) were resolved when all 71 sequences were compared to one another with minimum match percentage of 97% (22). OTU PG2-97a represents 1 slurry and 2 FW sequences while PG2-97b represents 29 slurry and 35 FW sequences. Collectively, the 67 partial 16S rRNA gene sequences represented by these two OTUs were 96% similar to one another and 98% similar to the 16S rRNA gene sequence of an uncultured bacterium recovered from oil-polluted soil in Romania (unpublished, GenBank accession number DQ378229). Both OTUs were 97% and 98% similar to uncultured soil bacterium clones PYR10d3 and PYR10d11, respectively, identified by SIP with pyrene in a different PAH-contaminated soil (Singleton, et al. submitted). *Thioalkalivibrio* is the genus most closely related to these 16S rRNA sequences with only 89% similarity.

The four remaining sequences were α-Proteobacterial and represent themselves in four OTUs containing one sequence each. These sequences only appeared in slurry incubations.

Slurry clone number 08 from replicate 1 (slurry 1-08) was 99% similar to the family Bradyrhizobiaceae. Slurry clones 2-03, 2-20, and 2-22 were each ≥99% similar to the genus *Caulobacter*. Slurry clones 2-03 and 2-22 were 83% similar to one another and 100% similar *Caulobacter leidyi*.

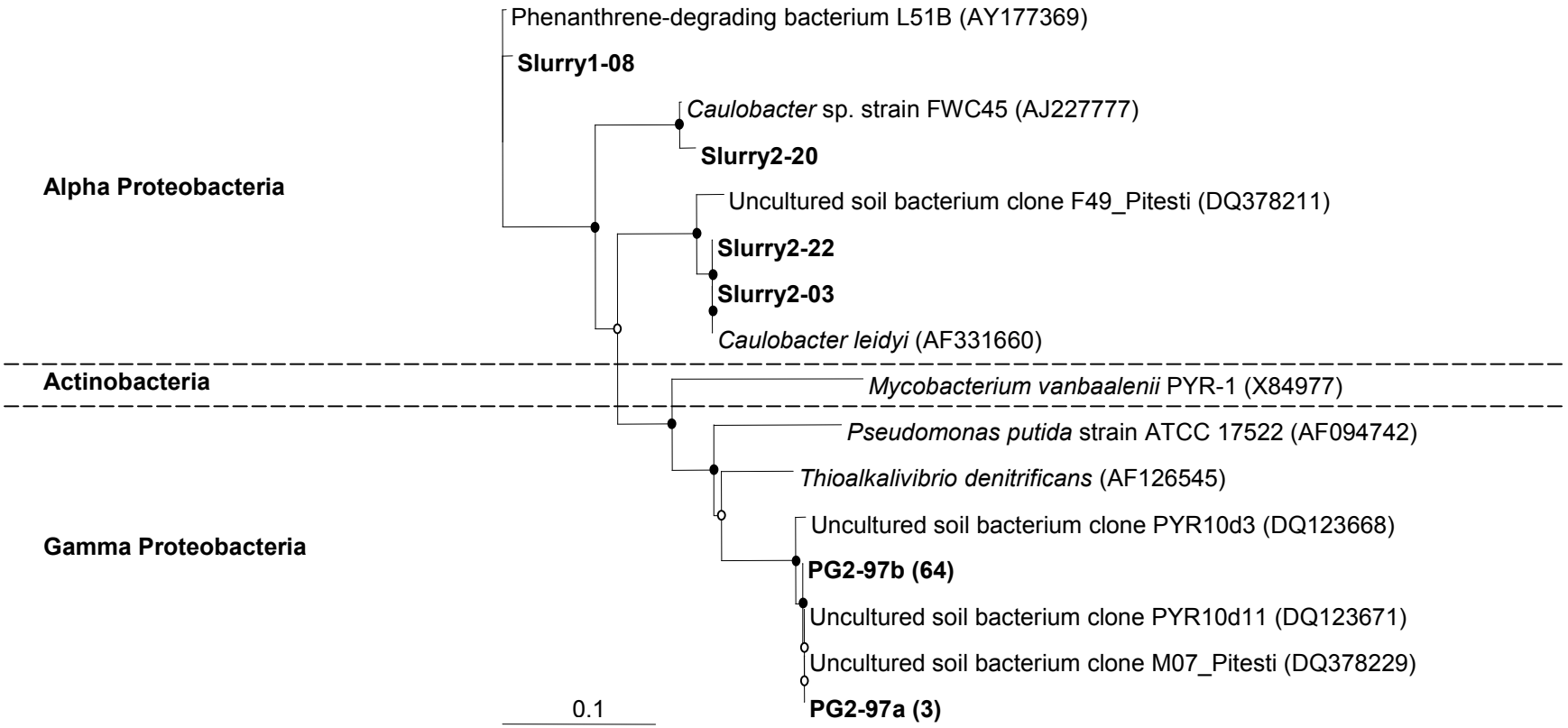


Figure 5. Phylogenetic tree of 16S ribosomal RNA genes. Filled and open circles at nodes indicate greater than 99% and greater than 50% bootstrap support, respectively. Clonal sequences recovered from "heavy" DNA in this study are identified in bold text followed by the number of clones represented by an OTU in parentheses where necessary. GenBank accession numbers (68) are also in parentheses.

Table 3. BLASTN search results of closely related and PAH-implicated 16S rRNA gene sequences. Related sequences are identified by their GenBank accession numbers in parentheses. Clones from this study are indexed by the number of bases used in comparisons and the percent similarity to related sequences.

Closely Related Sequence (accession number)	OTU	Source
α-Proteobacteria		
*Phenanthrene-degrading bacterium L51B (AY177369)	Slurry 1-08 (818; 98)	Soil column system
Caulobacter leidyi (AF331660)	Slurry 2-03 (638; 100) Slurry 2-22 (638; 100)	Mine tailings
Caulobacter sp. strain FWC45 (AJ227777)	Slurry 2-20 (607; 99)	Stream water, Burnaby, BC, Canada
γ-Proteobacteria		
*Uncultured soil bacterium clone PYR10d11 (DQ123671)	PG2-97a (949; 98) PG2-97b (949; 98)	Bioreactor treating PAH-contaminated soil
*Uncultured soil bacterium clone M07_Pitesti (DQ378229)	PG2-97a (942; 98) PG2-97b (942; 98)	Oil-polluted soil from Romania

*Have been implicated in PAH-degradation.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

Slurry bioreactors are commonly used for *ex situ* biological treatment of PAH contaminated soil, but *in situ* treatment methods are much less costly. The goal of this study was to determine whether bacterial community diversity depends on the method of biological treatment employed for remediation. *Ex situ*, continuously mixed, slurry treatment was compared to *in situ*, static, field wet treatment in a PAH-contaminated soil known to contain an active pyrene-degrading microbial community. [U-^{13}C] pyrene was used in a stable isotope probing investigation to identify members of the pyrene-degrading communities resulting from each treatment condition.

The pyrene disappearance that occurred in this study was attributed to the resident soil bacteria, but it is important to note that these represent only a portion of the possible types of microorganisms that could be present. Archaeal 16S rRNA gene sequences have been recovered from CMN by others in this research group, and fungi have not only been implicated in pyrene biodegradation (17, 52, 58), but soil drying and rewetting has been shown to promote fungal dominance (7). No archaeal or fungal 16Sr RNA genes were recovered from the heavy DNA fractions in this study.

Effects of Nutrient Amendment and Treatment Method on Pyrene Mineralization

The literature was unclear as to the utility of adding inorganic nutrients to enhance biodegradation (11, 27, 47, 56, 66). Inorganic nutrient addition usually encourages the growth of resident microorganisms, but whether this leads to increased contaminant degradation may be a matter of the specific physiochemical properties of each individual soil matrix. In this soil, nutrient addition was indicated for greater enrichment of resident organisms as seen by more intense bands in those profiles representing microcosms with nutrient amendment (Figure 2) and further by the greater extent of degradation in those microcosms containing nutrients (Table 2). This is consistent with the work of Potter, et al (47) who studied this same soil and added inorganic nutrients in the same ratio as was done here (Oxygen Demand:N:P=100:5:1). Though the extent of pyrene mineralization was greatest in the S+N microcosms, there appeared to be a greater density of bacteria in the FW+N microcosms as evidenced by relative band intensity (Figure 2). This lack of correlation between extent of mineralization and community density is consistent with previous studies indicating that nutrient or treatment status alone may not be predictive of rates or extents of substrate removal (11, 27, 47, 66).

From a bioremediation perspective, the variables responsible for the initial (k_o) and maximum (k_{max}) rates of mineralization, as well as those responsible for the ultimate extent of mineralization should be determined as the effects of these variables can be helpful in remediation strategy design. In this study, the higher initial rate of pyrene mineralization in S+N could be attributed to nutrient addition as S-N underwent the same physical treatment, but did not demonstrate a similar initial rate. In FW incubations, no trend is seen as the initial mineralization rates of both FW+N and FW-N incubations are equal and negligible.

Oppositely, when nutrient conditions are held constant treatment method appears to be responsible for the increase in k_o of S+N. Considering both k_{max} and extent of pyrene mineralization, both nutrient and treatment status influence each parameter such that the responsible variable cannot be elucidated from data presented here.

Reasons for the greater rates and extent of pyrene mineralization in slurry microcosms are notable. Constant agitation simulated the continuous mixing that would occur in a laboratory or full-scale bioreactor. This agitation increased the contact of soil aggregates, resident bacteria, and pyrene in the aqueous phase. Though not highly soluble, the ability for soil-sorbed pyrene to be in contact with the aqueous phase increases the bacterial community's ability to metabolize it. Agitation also increases the system's dissolved oxygen concentration, which can promote both biomass growth and metabolic activity.

Though nutrient addition may not lead to microbial population growth or correspond to increased removal rates in all cases (47, 66), it was the case in this work that nutrient addition led to increased community density for both treatments types and that nutrient addition coupled with static treatment led to the greatest community density. The slurry treatment method had a less dense resident community but coupled with nutrient addition resulted in the greatest extent and highest initial and maximum rates of pyrene mineralization.

In summary, though nutrient addition and physical treatment proved important to pyrene mineralization in this study, the enigmatic relationship between biomass growth and rate and extent of PAH mineralization needs further investigation. Complex environmental systems and the microbial communities they contain are fastidious in their nutritional and

physical demands as we encourage them to do as we desire. The rate and extent of degradation of a compound are both important, but there must be some balance in order for timeline, cost, and policy needs of a particular bioremediation venture to be met. Ideally the fastest rates will lead to the greatest extents of substrate removal in the shortest amount of time, but in complex environmental systems this may not always be practically achievable.

Effects of Treatment Method on Pyrene-degrading Bacterial Community Diversity

Phylogenetic analysis of heavy DNA fractions of ^{13}C-enriched DNA from PAH-contaminated soil microcosms treated by two different biological methods resulted in nearly identical communities of pyrene degraders identified by stable isotope probing. Uncultured γ-proteobacteria appear to be the dominant degraders in both slurry and field wet systems (67/71 sequences), however two different families of α-proteobacteria were also represented (4/71 sequences).

There was little diversity in the sequences recovered from the field wet treatment as they were all 96% identical to each other (37/37). They associated most closely with uncultured γ-proteobacteria. The slurry treatment enriched a slightly more diverse community as both α- and γ-proteobacterial sequences were recovered from these microcosms. Though the four α-proteobacterial sequences account for nearly 12% of the slurry sequences recovered, they only account for less than 6% of total sequences. The resulting communities enriched by the individual treatment methods are not highly diverse, but α-proteobacteria here seem to prefer the high moisture content of the slurry environment (1, 46).

The γ-proteobacterial sequences recovered were similar to other uncultured bacteria from environmental sources but none were very similar to any organisms that have been cultivated. Various γ-proteobacteria (21, 29) and Actinobacteria (18, 31, 35, 53, 69) have been identified as using pyrene as the sole carbon and/or energy source, but no sequences representing these organisms were recovered here. Many of these cultivated isolates resulted from the use of traditional microbial ecology techniques, which can be biased in not selecting more fastidious organisms or those that may only grow in consortia as is often the case in environmental systems. Not recovering sequences of known pyrene degraders supports the notion that most microorganisms remain unknown and suggests that many of the bacteria responsible for pyrene degradation have yet to be named.

The four α-proteobacterial sequences recovered were similar to both cultured and uncultured bacteria from environmental sources and three were very similar to *Caulobacter* species. Various analyses, including 16S rRNA gene sequencing, have suggested that *Caulobacter leidyi* is really a Sphingomonad (51), such that organisms represented by sequences designated Slurry 2-03 and 2-22 may also be Sphingomonads. This group has been implicated in pyrene transformation (30) and its close phylogenetic association with *Caulobacter leidyi* is apparent in Figure 5.

In summary, continuously mixed slurry bioreactor treatment has been the traditional route of *ex situ* remediation of contaminated soils, but *in situ* methods can provide a lower cost remediation alternative. Whether increased bacterial population density leads to greater substrate removal has yet to be definitively determined, but bacterial community diversity may also play a role. The γ-proteobacteria recovered here seem to be the dominant pyrene degraders in both slurry and field wet treated microcosms however α-proteobacteria also

contribute to pyrene transformation and degradation. It cannot be said conclusively that treatment method is the ultimate determinant of bacterial community diversity although it seemed to be a factor in this study.

Quantifying Represented Groups

The α- and γ-proteobacterial sequences recovered in this study are similar to those recovered in another pyrene SIP investigation with a different PAH-contaminated soil, but all represent uncultivated species. In that study, sequence majority was inversely associated with relative gene abundance in the bioreactor community (Singleton, et al. submitted). Though γ-proteobacteria were the majority of the sequences recovered here, they may not represent the greatest abundance of genes in the CMN community. This question can be answered using real-time quantitative PCR to quantify the abundance of pyrene-degrader 16S rRNA genes relative to those of the community as a whole. The next steps for the heavy fractions recovered in this study should include quantification of the relative abundance of 16S rRNA genes from pyrene-degrading bacteria identified by SIP in the contaminated Minnesota soil. A primer set was specifically designed to amplify the 16S rRNA genes of the majority group of sequences represented in the clone libraries, but qPCR runs thus far are inconclusive such that a primer redesign may be necessary.

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