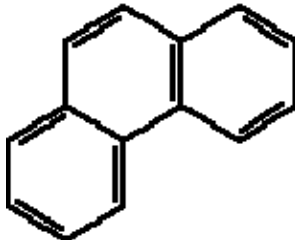


### Bioremediation of Phenanthrene Contaminated Soils

Bioremediation is a process that uses microorganisms to change toxic pollutants into non-toxic compounds. Naturally occurring populations of microorganisms, called consortiums, work together to break down organic matter. When a consortium's environment becomes polluted with a toxic compound, the microorganisms capable of degrading the toxic compound will thrive and metabolize the compound.



Phenanthrene (PHE) is a non-volatile, polycyclic aromatic hydrocarbon (PAH). It is found in diesel fuel and is a common pollutant of soil, estuarine water, and sediment. It is used in the industry to make dyes, plastics, pesticides, explosives, and drugs. It is a carcinogen and it can be very harmful to natural ecosystems.

There are several species of microorganisms that can degrade PHE. It has been shown that some PHE degraders are easy to culture from PHE contaminated environmental samples (Bogardt & Hemmingsen, 1992). *Pseudomonas spp.* and *Arhrobacter spp.* are the most commonly found and cultured microorganisms that can degrade PHE. An example of a specific type of *Pseudomonas* species that can degrade PHE is *Pseudomonas aeruginosa* and *Pseudomonas* strain R. (Dean, et. al 2001).

Phenanthrene is eventually broken down into CO<sub>2</sub> and H<sub>2</sub>O by a consortium of microorganisms. There are several steps and several organisms involved in the degradation of PHE to CO<sub>2</sub>. The PHE degradation pathway can be found at the following website:  
[http://umbbd.msi.umn.edu/pha/pha\\_map.html](http://umbbd.msi.umn.edu/pha/pha_map.html).

#### Microcosm Lab

In this lab, you will study the bioremediation of PHE by conducting two experiments. One experiment will involve creating a microcosm containing PHE contaminated soil and soil microorganisms. A microcosm is defined as a miniature model of something, in this case a soil ecosystem that has been contaminated with PHE. These microcosms will be seeded with more PHE and incubated for several weeks to allow the soil microorganisms to degrade the PHE. You will analyze the biodegradation of the PHE over time and compare these microcosms to controls that prevent the biodegradation (incubated at -80° C). You will analyze the breakdown of PHE and the metabolic intermediates produced during the degradation process using Gas Chromatography and Mass Spectroscopy (GC-MS). The GC separates the components of the mixture and the MS characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals (Gas Chromatography-Mass Spectroscopy Background, 1998). With this instrument, you are able to separate the individual components of a mixture and identify them. It is also possible to determine the concentrations of each of the components. Therefore, when a sample of contaminated soil is injected into the GCMS one can experimentally determine what products are present in the mixture and estimate the relative abundance of each of these components. We hypothesize that the GC-MS results

will show a lower concentration of PHE in the experimental microcosm compared to the control microcosm, where we expect a high concentration of PHE because it is not being broken down. We expect the presence of metabolic intermediates of PHE degradation in the experimental microcosm, whereas none should be present in the control.

### Protocol

Each group will receive a sample of contaminated soil. Each group will build 2 microcosms and incubate them for several weeks. One microcosm will be incubated at room temperature to allow the PHE degraders to grow and metabolize the PHE. The other will be incubated at  $-80^{\circ}\text{C}$  to prevent the PHE degraders from growing (control). A sample of each microcosm will then be given to the CHEM 2412 lab, who will run each sample through the GC-MS and provide the results to our lab for analysis.

Components of the Microcosms

Microcosm	Contaminated Soil Sample (25g)	NP stock solution* (2mL)	Ethanol solution of PHE (2mL)	H <sub>2</sub> O (18mL)	Incubation Temperature ( $^{\circ}\text{C}$ )
Control	×	×	×	×	-80
Experimental	×	×	×	×	25

\*NP is a nutrient solution of nitrogen and phosphorous which are nutrients necessary for the growth of PHE degraders.

\*Both microcosms contain the same components, however, the control will be incubated at  $-80^{\circ}\text{C}$  to prevent degradation from occurring, giving us an initial concentration of PHE in the soil. Whereas, the experimental microcosm will be incubated at room temperature to allow degradation thereby allowing us to view the amount of PHE after degradation has occurred.

1. Each group should obtain (2) 125 mL Erlenmeyer flasks, (1) 50 mL beaker, (2) 2 mL pipettes, (2) 25 mL pipettes, NP stock solution, water and ethanolic PHE solution. Label the flasks with your initials, the date, and microcosm description (Control or Experimental)
2. Weigh out 25 grams of soil and place the sample in the bottom of the 125 mL Erlenmeyer flask labeled Experimental Microcosm
3. Using the 2mL pipette, pipette 2 mL of the NP stock solution into a 50 mL beaker
4. Using the 25 mL pipette, pipette 18 mL of H<sub>2</sub>O in the same 50 mL beaker as the NP solution
5. Using the other 2 mL pipette, place 2 mL of the ethanolic PHE solution into the 50 mL beaker and gently swirl. Add this solution to the experimental microcosm.
6. Repeat steps 2-5 for the Control Microcosm.
7. Place a square of parafilm over each microcosm flask and place Experimental Microcosm on the shaking incubator and incubate at  $25^{\circ}\text{C}$  in the dark.

8. Place Control Microcosm in the bucket in the front of the room. Your instructor will place all of these microcosms in the -80°C freezer.

9. The microcosms will be incubated for 3-4 weeks. You should periodically check your microcosms to ensure that it maintains a “slurry” consistency and to make observations. If the soil appears dry or does not flow freely in the flask add 2 mL of water at a time.

10. Record Results weekly in your lab journal. Answer the following questions each week.

Are there any visible fungi or bacteria growing on the surface of the soil?

Are there any noticeable color changes?

What is the consistency of each? Has it changed over time?

11. After you receive the data from the CHEM 2412 class analyze the mass spectrometer data. Did you see a PHE peak in the experimental? How about the control? If so, was the relative abundance of PHE more or less in the control as compared to the experimental? Did this support your hypothesis? Why or why not? Did you detect any metabolic intermediates of PHE degradation in the experimental sample?

### The Plate Count Experiment

It has been previously shown that less than 0.1% of the microbial community in unpolluted soils consists of organisms capable of metabolizing polycyclic aromatic hydrocarbons. When soil is contaminated with PAHs up to 100% of the microbial community is capable of metabolizing PAHs (Zytner, Salb, & Stiver, 2006).

The purpose of this experiment is to determine the amount of PHE degrading microorganisms present in PHE contaminated soil samples vs. uncontaminated soil samples. This will be accomplished by conducting a serial dilution, followed by plating the microorganisms. PHE will be added to the plate and after 5 days of incubation we will be able to determine which plates contain microorganisms that degrade PHE by observing the level of fluorescence and the amount of PHE crystals on each plate. PHE crystallizes out of solution when it is applied to the plates. As it is degraded the crystals disappear. Thus the plates containing fewer crystals after several days of incubation have more PHE degraders on them. PHE also fluoresces under UV light. A plate containing a lot of PHE will fluoresce brightly under UV light while a plate that has less PHE will fluoresce less brightly. Thus, plates that fluoresce brightly after a few days of incubation will contain fewer PHE degraders than those that do not fluoresce as brightly.

We hypothesize that the contaminated soil will contain more PHE degrading microorganisms due to the selective pressure introduced by the contaminant. Plates produced from contaminated soil will fluoresce less brightly and contain fewer crystals after several days of incubation as compared to plates produced from non contaminated soil. There will be microorganisms present in the

uncontaminated soil, however they will not be able to degrade the PHE as well as the microorganisms in the contaminated soil.

### Protocol

Each group will be given a 1 gram of a soil sample, either contaminated with PHE, or uncontaminated.

1. Place the 1 g soil sample in an autoclaved glass bottle w/ cap containing 24 mL of saline solution. Shake the soil solution gently for five minutes. You should make every attempt to avoid the solution touching the cap (which is not glass). **NOTE: PHE is absorbed by plastic.**
2. After shaking for five minutes, use a glass pipette and pipette 10 mL of 24 mL sample into an empty test tube with cap. In this step, be careful and try to gather as much of the liquid that settles on top – try not to get clumps of dirt in your test tube. After transfer label test tube with group number and 1/25 (g/mL)
3. Each group should gather 5 additional test tubes (with caps) that contain 9 mL of sterile saline. Label each of these test tubes with the group number and  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$
4. Carry out a serial dilution beginning with the test tube previously labeled 1/25. If you need help remembering how to carry out a series dilution refer back to your previous labs or ask the instructor
5. After the dilution series has been carried out you will be plating the  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  test tubes only. Gather nine nutrient agar plates. Each test tube will have three replicates. Label each agar plate with the dilution number, replicate number, date, and group number
6. Obtain a plate spreader and a petri dish containing ethanol. Using the plate spreading technique, pipette a 0.1 mL sample of the  $10^{-3}$  test tube onto the correctly labeled plate and spread the sample evenly with the plate spreader. Repeat this process for each addition replicate
7. Repeat step 6 for the  $10^{-4}$  and  $10^{-5}$  samples
8. Once each sample has been plated, gather your plates, and place in a room temperature incubator.
9. After five days in the incubator, check your plates.
10. Find the dilution that has a countable number of colonies. Count the colonies on the 3 plates of this dilution. Average the number of colonies and determine the number of cells/gram of soil. Ask your instructor if you need help. Record your data in table 1 and 3.
11. Now observe your plates at each dilution and look for crystal formation and fluorescence. Score the plates based on the following scheme:

- a. 1 - Little to no degradation is occurring. Still see plate covered with crystals. When placed under UV light lots of fluorescence present
- b. 2 - Some degradation occurring. Plate has more crystals than clearing areas. Most of the plate fluoresces under UV light
- c. 3 - Significant degradation occurring. Plate has more clearing areas than crystals. Less than half the plate fluoresces under UV light
- d. 4 – Definite degradation occurring. Plate has very few crystals and composed of mostly clear areas around colonies. Little fluorescence under UV light

Below are some photos that may help you determine how to classify your plates. If you are unsure about your plate use the UV light and classify according to the amount of fluorescence present on the plate.

CLASSIFYING W/OUT UV LIGHT:

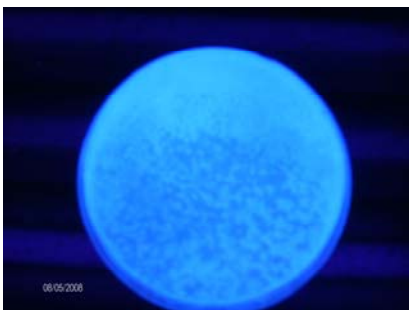


Classified as a ONE – Crystals clearly visible on surface of agar; little degradation occurring because no clear zones

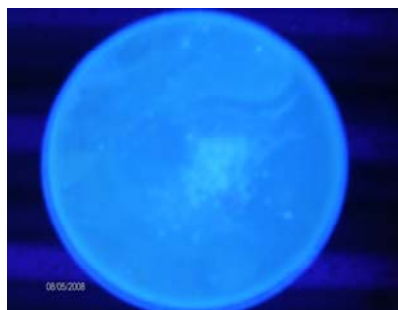


Classified as a FOUR – Majority of plate is clear of crystals; degradation is occurring in areas w/out crystals

CLASSIFYING WITH UV LIGHT:



Classified as a ONE – Majority of plate fluoresces under UV light



Classified as a FOUR – Very little of the plate fluoresces under UV light

12. Score your group data in Table 2. Your instructor will let the class know which dilution from which to collect the average PHE degradation data for comparison amongst the class. You should collect this data in Table 4.

**GROUP DATA (data from your own group)**

Table 1. Colony Counts

	<b>Countable Dilution_____</b>	<b>Colonies/amount plated=Plating conc.</b> <b>Work:</b>
Plate 1		<b>Plating conc./dilution=Tube 1 conc.</b>
Plate 2		<b>Work:</b>
Plate 3		<b>Tube 1 conc./(1gram soil/25ml)</b>
<b>Average</b>		<b>Work:</b> <b>Average cells/gram=</b>

Table 2. PHE Degradation

	<b>10<sup>-3</sup></b>	<b>10<sup>-4</sup></b>	<b>10<sup>-5</sup></b>
Plate 1			
Plate 2			
Plate 3			
<b>Average and SD</b>			

**CLASS DATA**

Table 3. Group Cell Enumeration Data

	<b>Average Cells/Gram Contaminated soil</b>		<b>Average Cells/Gram Uncontaminated soil</b>
Group 1		Group 4	
Group 2		Group 5	
Group 3		Group 6	
<b><u>Average and SD</u></b>		<b><u>Average and SD</u></b>	



3. How can this experiment be improved?
4. Describe any possible sources of error in the experiment.

**Things to Think About After Collecting Results from Series Dilution Experiment**

1. Which dilution would you expect should contain the most plates with a classification number of 3 or 4? Which of your dilutions actually contained the most number of plates that were classified as a 3 or 4 (hint: look at table 2)? Explain any discrepancies.
1. Did the results support the hypothesis (hint: look at table 4)?
2. Which samples (contaminated or uncontaminated soil) had the most colonies/gram? Explain these results (hint: look at table 3).
3. How can these experiments be improved?

4. Describe any error you detected in your experiment.

#### Works Cited

Agency for Toxic Substances and Disease Registry. (1996, September). *Polycyclic Aromatic Hydrocarbons (PAHs)*. Retrieved August 5, 2008, from Agency for Toxic Substances and Disease Registry: <http://www.atsdr.cdc.gov/tfacts69.pdf>

Bogardt, A. H., & Hemmingsen, B. B. (1992). Enumeration of Phenanthrene-Degrading Bacteria by an Overlayer technique and Its Use in Evaluation of Petroleum-Contaminated sites. *Applied and Environmental Microbiology*, 2579-2582.

Dean, S. M. (2001). Bioremediation and Biodegradation. *Journal of Environmental Quality*, 1126-1133.

*Gas Chromatography-Mass Spectroscopy Background*. (1998, January 20). Retrieved April 2008, 4, from GMU: <http://www.gmu.edu/departments/SRIF/tutorial/gcd/gc-ms2.htm>

Leahy, J. G., & Colwell, R. R. (1990). Microbial Degradation of Hydrocarbons in the Environment. *Microbiological Reviews*, 305-315.

Zytner, R., Salb, A., & Stiver, W. (2006). Bioremediation of Diesel Contaminated Soil : Comparison of Individual Compounds to Complex Mixtures. *Soil and Sediment Contamination*, 277-297.