Degradation of PAHs by Indigenous Microbes in Contaminated River Sediment

by

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Abstract

Sediment from a polycyclic aromatic hydrocarbon (PAH) contaminated site on the Mahoning River was incubated for 0 and 4 days with the addition of PAHs in 0, 3, 6, and 12 μg/ml concentrations. PAH concentrations, microbial activity, and viable heterotrophic counts were performed at Day 0 and Day 4. PAHs were extracted and analyzed using GC/MS. Samples with PAH concentrations of 0, 3, and 6 μg/ml decreased after incubating 4 days while samples with 12 μg/ml concentrations were found to inhibit degradation after incubating 4 days. Microbial activity, measured using 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction, showed similar activity in all samples initially and a decrease in activity after 4 days. Viable heterotrophic counts were higher for Day 4 incubations suggesting that microorganisms in sediment were able to grow and reproduce, but eventually became carbon limited due to lowered activity measurements. Results suggest that microorganisms in sediment are able to degrade PAHs up to a 6 μg/ml concentration, but may eventually become carbon limited.

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Introduction

The Mahoning River

The Mahoning River is 108 miles long and runs through northeastern Ohio into western Pennsylvania (Figure 1). Beginning around 1900 and continuing for nearly three-quarters of a century, the lower Mahoning River supported one of the most intensely industrialized steel-producing regions in the world (U.S. Army Corps of Engineers, 1999). The steel mills and other factories began to close by the 1970s. In 1980, the Environmental Protection Agency began studying water quality and discovered significant levels of polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and various metals (Ohio EPA, 1996). Although industrial activity has slowed down drastically over the past several years and water quality has improved, hazardous levels of contaminants remain in the sediments and a dermal contact advisory remains in effect for the lower 34 miles of the river from Warren, OH to the Ohio-Pennsylvania border (Ohio Department of Health, 2000).

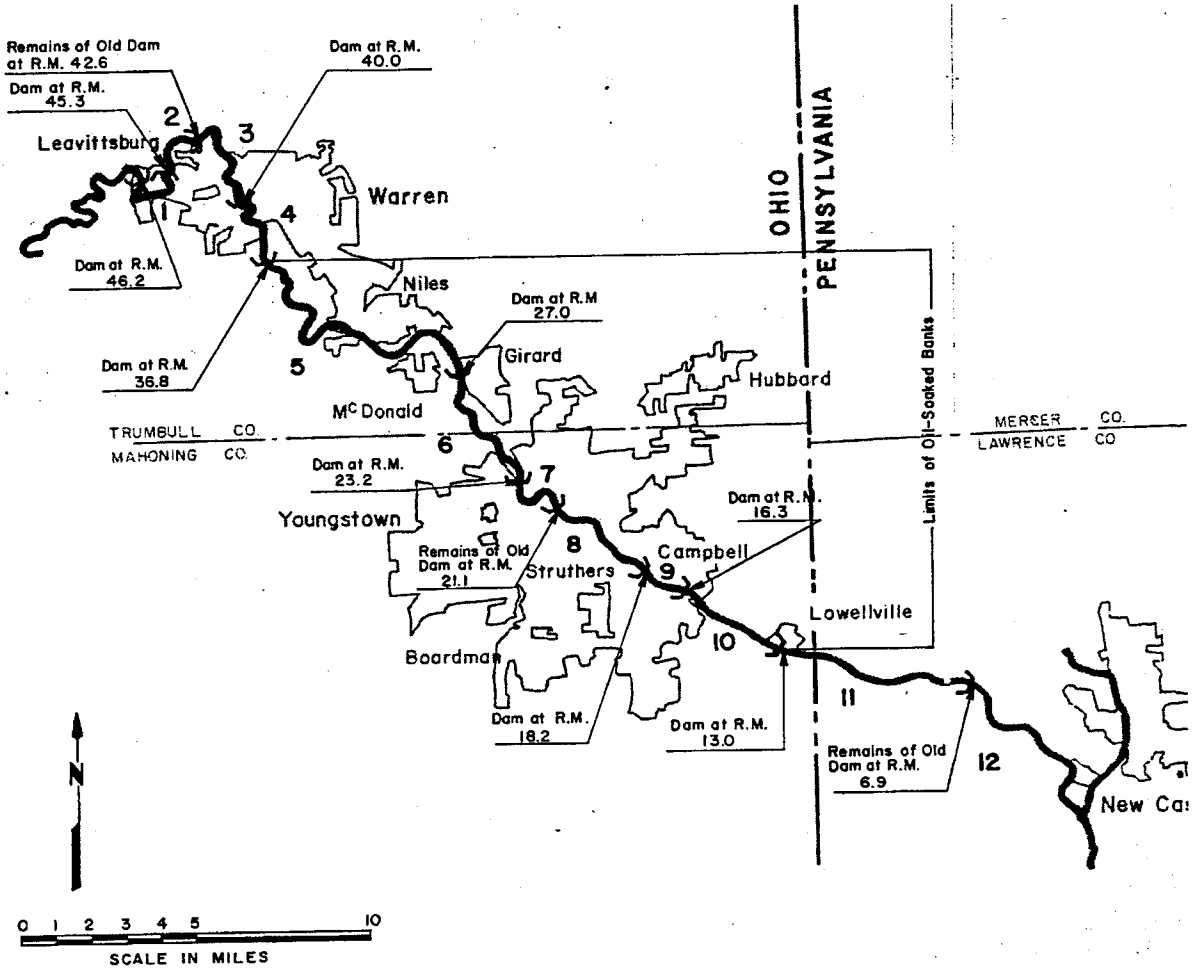

Figure 1: Map depicting Lower Mahoning River.

Polycyclic Aromatic Hydrocarbons

PAHs constitute a large and diverse class of organic compounds that are described as molecules consisting of three or more fused aromatic rings in various structural configurations (Blumer, 1976). PAHs are common environmental pollutants often found in fossil fuels (Menzie et al. 1992; Kilbane, 1998). They are universal combustion products of organic matter and are prevalent in various industrial processes including the petroleum industry and coal-refining processes (Samantha et al. 1999). As fossil fuels were extracted and burned during the industrial revolution, the production rate of PAHs greatly increased, especially in localized areas such as rivers, estuaries, and harbors where the rate of accumulation exceeded the rate of environmental degradation (Hites, 1980). PAHs are hydrophobic and adsorb tightly to sediment particles (Johnson and Ghosh, 1998). Accumulation of PAHs in aquatic systems can therefore be attributed to the sequestering of hydrophobic organic contaminants by suspended and bed sediments, resulting in sediment contamination (Verrhiest et al., 2001). PAHs are known carcinogens and are potentially toxic, genotoxic, and mutagenic (Luthy et al.; Menzie et al. 1992). Their ubiquitous distribution, persistence in the environment, and deleterious effects on human health increase the need for a safe and effective way of removing PAHs from sediments (Kanaly and Harayama, 2000; Luthy et al. 1994).

Biodegradation

Dredging is the physical removal of contaminated sediments and is one of the most common methods for removing PAHs and other pollutants from the environment. Dredging, however, disturbs environmental surroundings and can release contaminants back into the environment (Herbich, 1992). Biodegradation can be a less disruptive and

more cost effective method of contamination removal but is currently less used than other methods. Biodegradation is the ability of microorganisms to metabolize organic compounds in order to capture chemical energy for growth. Degradation is important for natural and industrial cycling of environmental chemicals and its occurrence in nature is derived from the collective metabolism of many bacteria (Wackett and Ellis, 1999). Bacteria such as *Acaligenes denitrificans* and *Pseudomonas (Sphingomonas) paucimobilis* have been found to degrade fluoranthene and several other PAHs (Weissenfels et al. 1990; Mueller et al. 1990). The degradation of a PAH molecule by bacteria is dependent in part upon both the number of aromatic rings and the pattern of ring linkage (Kanaly and Harayama, 2000). According to van der Meer et al., "the initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of the PAH to a dihydrodiol by a multicomponent enzyme system. The dihydroxylated intermediates may then be processed through either an *ortho* cleavage or a *meta* cleavage type of pathway, leading to central intermediates such as protocatechuates and catechols, which are further converted to tricarboxylic acid cycle intermediates" (van der Meer et al., 1992). Biodegradation of PAHs can occur under aerobic and anaerobic conditions but it is suggested that PAHs are poorly biodegraded in the absence of oxygen (Grishchenkov et al. 2000; Leduc et al., 1992).

Goals/Objectives

The goal of this research is to understand how PAHs persist and are degraded in the environment. The objectives are to measure PAH degradation, microbial activity, and viable heterotrophic counts in aerobic incubations of contaminated river sediment.

Methods

Sample Collection

Sediments were collected with a manual auger from the bank of the Mahoning River in Lowellville, Ohio on January 31, 2004. Lowellville was chosen as a collection site due to high levels of PAH contamination (Moser, 2002). Sediment was obtained from approximately 2 m into the bank, and then stored in airtight containers. Samples were refrigerated immediately upon returning to the laboratory.

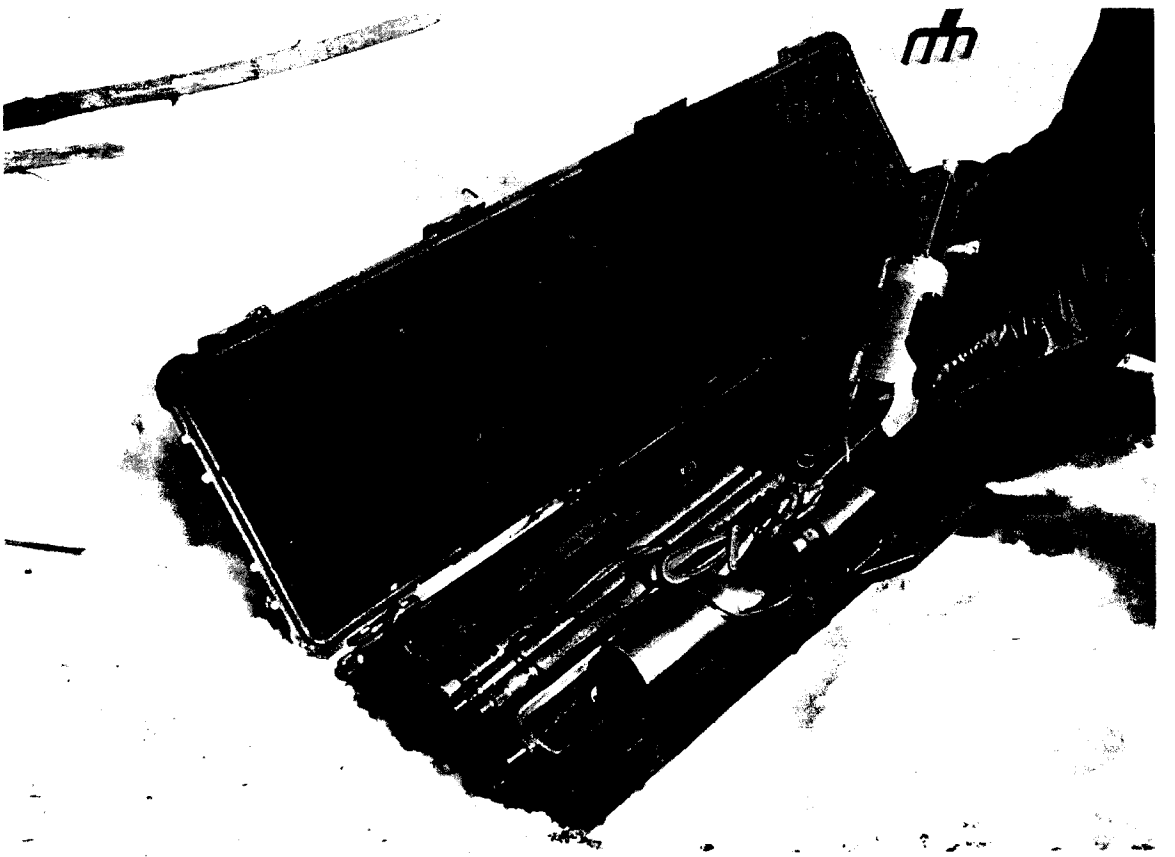

Figure 2: Manual auger used to obtain sediment samples.

Figure 3: Sample collection site on bank of Mahoning River in Lowellville, OH.

Figure 4: Drilling into riverbank using manual auger.

Method Development

Sterile Sediment Controls

Autoclave-only controls were made using 3g of sediment and 6 ml of BSM (Appendix 1). Autoclave/sodium-azide controls were made using 3 g of sediment and 6 ml of BSM with the addition of sodium-azide (Appendix 1). Sodium-azide was used in addition to autoclaving because it has been found to be a respiratory inhibitor capable of stopping microbial respiration at the cellular level (Ning et al. 1996; Johnston and Kipphut, 1988). Initial microbial activity and viable heterotrophic counts were performed immediately after preparing controls. Both autoclave-only controls and autoclave/sodium-azide controls were next autoclaved and left to incubate at 30°C for 24 hours. Microbial activity and viable heterotrophic counts were again performed after the 24-hour incubation period. All controls were autoclaved and incubated two more times with microbial activity and viable heterotrophic counts performed after each 24-hour incubation period.

Microbial Activity Time Curve

Incubations were made with 1 g of sediment and 2 ml of BSM (Appendix 1) with the addition of PAH stock solution (Appendix 2) in the amounts of 0, 3, 6, and 12 µg per milliliter of incubation volume. Approximately .2 ml of each sample and .3 ml of BSM were added to a 15 ml tube and allowed to incubate at room temperature for 30 minutes. Next .5 ml of 1.08 mM INT (Appendix 2) was added to each sample and incubated for 0, 5, 10, 20, 40, and 80 minutes at room temperature. INT formazan was extracted with 6.0 ml of acetonitrile at room temperature for 10 minutes. Samples were filtered using a 9 cm Büchner funnel and filter paper (70 mm in diameter). Sediment was then washed with 6.0

ml of acetonitrile, and the filtrate was read in a Biomate UV-Visible spectrophotometer at 490 nm. This procedure was repeated after allowing samples to incubate for 4 days at 30°C. Viable heterotrophic counts were also performed initially and after 4 days.

Sediment Incubation

Incubations were made in triplicate using 1 g of sediment and 2 ml of BSM (Appendix 1). PAH stock solution (Appendix 2) was added in the amounts of 0, 3, 6, and 12 μg per milliliter of incubation volume. Initial PAH concentrations, microbial activity, and viable heterotrophic counts were performed. Measurements were repeated after incubating for 4 days at 30°C.

Killed controls were made using 1 g of sediment and 2 ml of BSM. After autoclaving 3 times, PAH stock solution was added in concentrations of 0, 3, 6, and 12 μg per milliliter of incubation volume. After extracting PAHs to determine initial concentrations, activity and viable heterotrophic counts were performed. Controls were next incubated at 30°C for 4 days before autoclaving, and measurements were again performed.

PAH Extraction and Analysis

PAHs were extracted using a modified extraction method for PAHs and lipids from sediment (Fang and Findlay, 1996). Surrogate solution in the amount of 50 μl was added to each sample to measure extraction efficiency. The surrogate solution (Restek) contained 1 μg/μl each of 2-flurobiphenyl, *p*-terphenyl-d14, and nitrobenzene-d5. Next, 7.5 ml of dichloromethane (DCM), 15.0 ml of methanol, and 2.5 ml of 100 mM phosphate buffer (Appendix 3) were added to the 50 ml sample tubes containing 1.0 g of sediment and 2 ml BSM (Appendix 1). The tubes were covered, shaken, and left to stand overnight in a cold room at approximately 10°C. The next day, 7.5 ml of DCM and 7.5 ml of DI water were added to split phases and the samples were again shaken and left to stand in a cold room overnight.

Collecting and processing extracted sample

The upper water/methanol phases were removed with a clean Pasteur pipette connected to an aspirator. Organic phases from the samples were then transferred to sodium sulfate (Na_2SO_4) columns (Appendix 3). DCM in the amount of 1 ml was added to the sample tubes and mixed. The organic phases were again transferred to the Na_2SO_4 columns. DCM was added twice more without vortexing or centrifuging, and added to the columns. Finally the columns were rinsed with two 1 ml aliquots of DCM and pulled to dryness.

Partition Bligh and Dyer extracted lipids into PAH fraction

The samples were transferred from DCM into hexane using solvent exchange. (Appendix 3). To collect the PAH fraction of the samples, clean conical tubes were placed in the column apparatus to collect hexane. The samples in 200 µl hexane were doped with 1 drop of chloroform, vortexed, and transferred to silica columns (Appendix 3). After drawing the samples into the columns, the previous step was repeated 3 times using 100 µl of hexane. The PAH fractions were then washed from the columns using 5 ml of hexane in 1, 2, and 2 ml aliquots. The last ½ ml of the fractions was allowed to drip by gravity.

Cleanup of PAH fractions on Aminopropyl columns

PAH fractions were cleaned on aminopropyl (NH_2) columns (Appendix 3) by first drying to 100 µl then bringing volume to 200 µl with hexane in conical tubes. The tubes were doped with 1 drop of chloroform before vortexing and adding to the columns. A total of 300 µl of hexane in three 100 µl aliquots was added before washing the PAH

fractions from the column using 5 ml hexane in 1, 2, and 2 ml aliquots. The columns were then let go to dryness.

The PAH fractions were analyzed using a Hewlett Packard 5890 Gas Chromatograph/5970B Mass Spectrometer in accordance to USEPA method 8270C (USEPA, 1996b). The GC/MS was fitted with a Supelco SPB-5 column measuring 30 M, 0.32 mm ID, and .25 μm film. The mass spectrometer was equipped with a Burle Channeltron electron multiplier #5772. The injection port and transfer line temperatures were 250°C, the detector temperature 280°C. The samples (1.0 μl) were injected using an autosampler. The temperature of the column was held at 45°C for 2 minutes, then was ramped at 20°C per minute until 310°C was reached. The final temperature was held for 5.5 minutes. The total run time was 20.75 minutes. Responses were taken from the GC/MS software and used to determine final concentrations of PAHs (Appendix 4). A standard curve was performed with the GC/MS using 2.0, 10.0, 20.0, 40.0, and 80.0 μg/ml PAH standards.

Microbial Activity

Microbial activity was determined by measuring reduction of INT to INTF (Moser et al., 2003; Crane, et al. 1991; Altmann, 1969). Dehydrogenases are oxidoreductase enzymes that take part in respiration by oxidizing organic compounds through the transfer of electron pairs from a substrate to nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$). The tetrazolium salt, 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), competes with NAD^+ and $NADP^+$ for electrons and is reduced to a red colored formazan (INTF) as it accepts

electrons. This reduction can then be analyzed colormetrically using a spectrophotometer, providing an accurate measure of microbial activity.

To measure microbial activity, approximately .2 ml of sample and .3 ml of BSM (Appendix 1) were added to 15 ml test tubes. The samples were incubated for 30 minutes at room temperature. Next, .5 ml of 1.08 mM INT (Appendix 5) was added and mixed. The samples were allowed to incubate for 5 minutes at room temperature. The INT formazan was then extracted with 6.0 ml of acetonitrile at room temperature for 10 minutes. Samples were filtered and the sediment washed with 6.0 ml of acetonitrile. The filtrate was then read in a Biomate UV-Visible spectrophotometer at 490 nm.

Control samples were killed with 6.0 ml of acetonitrile prior to the addition of 0.5 ml of 1.08 mM INT to prevent reduction. Control samples were then allowed to extract for 10 minutes after the addition of INT before filtering.

Viable Heterotrophic Counts

Viable heterotrophic counts, measured in colony forming units (CFUs), were performed by adding .1 ml from each sample to 9.9 ml of sterile BSM. Serial dilutions were performed ranging from 10^{-2} to 10^{-7}. Each sample (.1 ml) was then plated onto nutrient agar (Appendix 6). After incubating the plates at 30°C for 48 hours, colonies were counted and recorded for each dilution.

Results and Discussion

Method Development

Sterile Sediment Controls

Initial microbial activity was lower in the autoclave-only controls than in the autoclave/sodium-azide controls. Activity of the autoclave-only controls continued to be lower after autoclaving one time. They appeared higher than controls with sodium-azide, however, after autoclaving two or more times. Viable heterotrophic counts showed initial growth before autoclaving for both autoclave-only controls as well as autoclave/sodium-azide controls, but there was no growth observed in either control after autoclaving one time. These results suggest that autoclaving three times is sufficient to limit activity and growth of microorganisms in sediment. Autoclaving with the addition of sodium-azide, however, is slightly more efficient.

Table 1: Microbial activity (average absorbance at 490 nm ± standard deviation) measured by INT reduction.

	Autoclave-only controls	Autoclave/sodium-azide controls
Initial activity	0.369 ± .078	0.475 ± .140
Autoclave 1x	0.199 ± .057	0.267± .050
Autoclave 2x	0.142 ± .075	0.083 ± .055
Autoclave 3x	0.044 ± .029	0.019 ± .007
Autoclave 4x	0.023 ± .001	0.014 ± .008

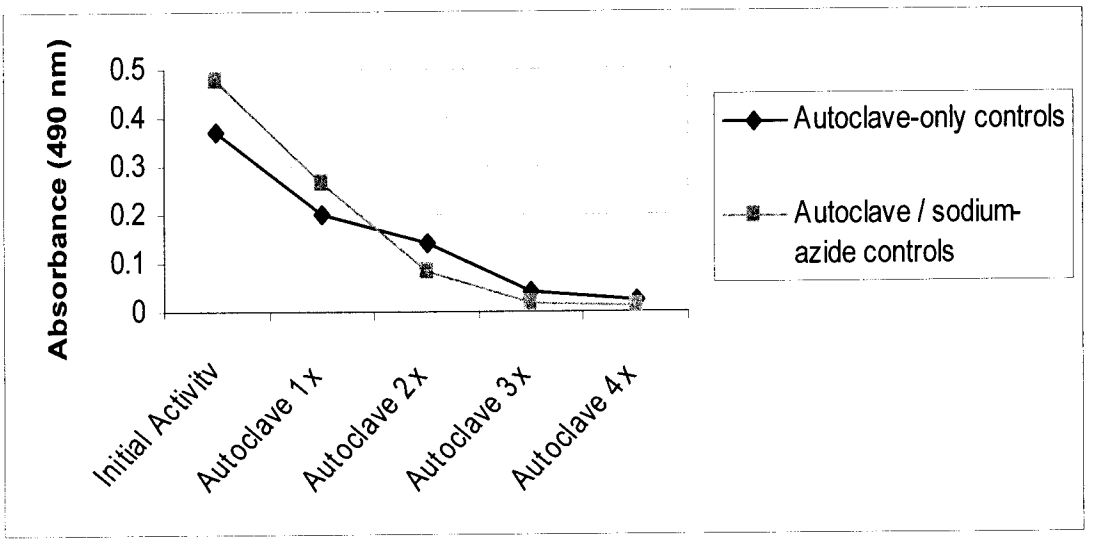

Figure 5: Microbial activity (average absorbance at 490 nm) in sterile sediment controls measured by INT reduction.

Table 2: Average CFUs for autoclave-only and autoclave/sodium-azide controls before and after autoclaving.

	Autoclave-only controls	Autoclave/sodium-azide controls
Initial	1.9×10^6	2.6×10^6
Autoclave 1x	0	0
Autoclave 2x	0	0
Autoclave 3x	0	0
Autoclave 4x	0	0

Activity Time Curve

Although initial microbial activity measurements (Day 0) showed an increase in activity for all samples from 5 to 80 minutes of INT incubation, results showed that a 5 minute incubation time is sufficient when performing activity measurements. When samples were allowed to incubate at 30°C for 4 days prior to measurements (Day 4), activity increased up to 5 minutes of INT incubation. Activities were much more variable, however, for all concentrations after a 5 minute incubation period. Overall, activity measurements in Day 4 samples were lower in Day 0. Although, viable heterotrophic counts showed growth for both Day 0 and Day 4 measurements, growth was higher in the 4-day cultures. These results suggest that after 4 days of incubation at 30°C prior to testing, carbon sources may have become limited allowing an increase in growth, but eventually slowing the activity of microorganisms in the sediment.

Table 3: Initial microbial activity measurements (Day 0) with 0 to 80 minute INT incubation time.

Added PAH concentration	0 min.	5 min.	10 min.	20 min.	40 min.	80 min.
0 µg/ml	0.278	0.559	0.713	0.808	0.876	0.923
3 µg/ml	0.249	0.634	0.755	0.855	0.898	0.845
6 µg/ml	0.298	0.483	0.707	0.793	0.876	0.911
12 ug/ml	0.285	0.650	0.824	0.834	0.972	1.068

Table 4: Microbial activity measurements (Day 4) with 0 to 80 minute INT incubation time.

Added PAH concentration	0 min.	5 min.	10 min.	20 min.	40 min.	80 min.
0 µg/ml	0.113	0.218	0.272	0.291	0.363	0.452
3 µg/ml	0.114	0.185	0.161	0.225	0.269	0.228
6 µg/ml	0.122	0.142	0.189	0.168	0.194	0.199
12 µg/ml	0.112	0.181	0.149	0.415	0.208	0.234

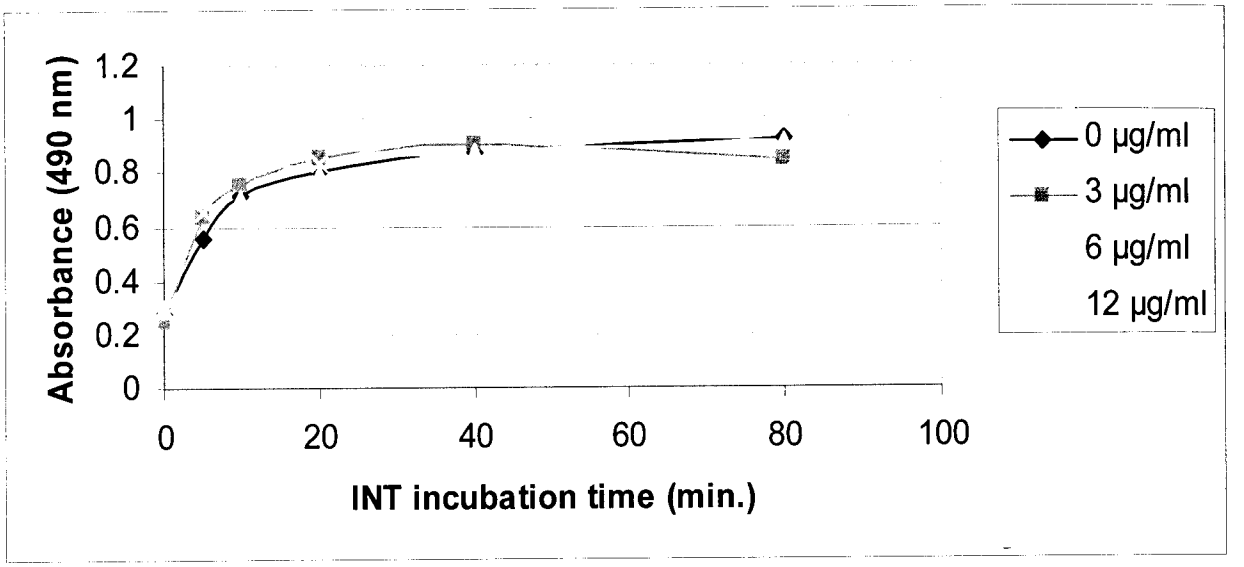

Figure 6: Microbial activity measurements (Day 0) after incubating with INT for 0 to 80 minutes.

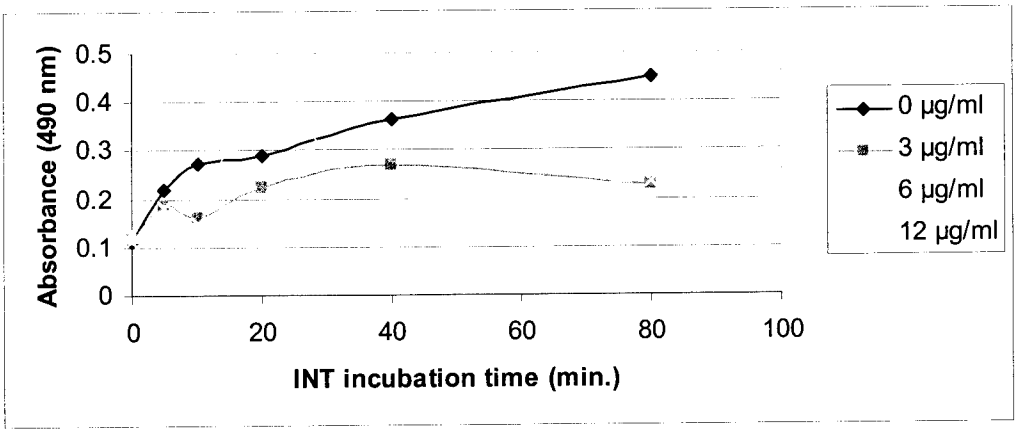

Figure 7: Microbial activity measurements (Day 4) after incubating with INT for 0 to 80 minutes.

Table 5: Average CFUs for Day 0 and Day 4 time curve measurements.

Concentration of added PAHs	Day 0	Day 4
0 µg/ml	**7.0×10^6**	**$> 10^8$**
3 µg/ml	**4.6×10^6**	**$> 10^8$**
6 µg/ml	**4.9×10^6**	**$> 10^8$**
12 µg/ml	**3.0×10^6**	**1.5×10^8**

Sediment Incubation

PAH concentrations decreased in incubations with PAH additions of 0, 3, and 6 μg/ml. Incubations with PAH additions of 12 μg/ml, showed a higher concentration at Day 4 than at Day 0 indicating that the higher PAH concentration may inhibit degradation. Overall, the highest rate of degradation occurred in the 6μg/ml concentrations suggesting that this concentration was optimal for degradation.

Initial activity measurements (Day 0) were fairly consistent across PAH concentrations. The activity was higher overall in Day 4 samples. Viable heterotrophic counts, however, were higher in the Day 4 samples suggesting that microorganisms grew but may have eventually become carbon limited as expressed by lowered activity.

Killed controls showed considerably higher concentrations of PAHs at Day 4 than at Day 0 indicating that microorganisms were inhibited, therefore lowering degradation rates and showing higher concentrations of PAHs. In the killed controls, the microbial activity was low in both the Day 0 and Day 4 controls. The greatest amount of activity was seen in the initial control at the 3 μg/ml concentration, but overall the activity was still relatively low. No growth was seen in any of the samples at any concentration when viable heterotrophic counts were performed. These results show that autoclaving incubations of varying PAH concentrations three times will successfully inhibit sediment microorganisms and is effective as a killed control.

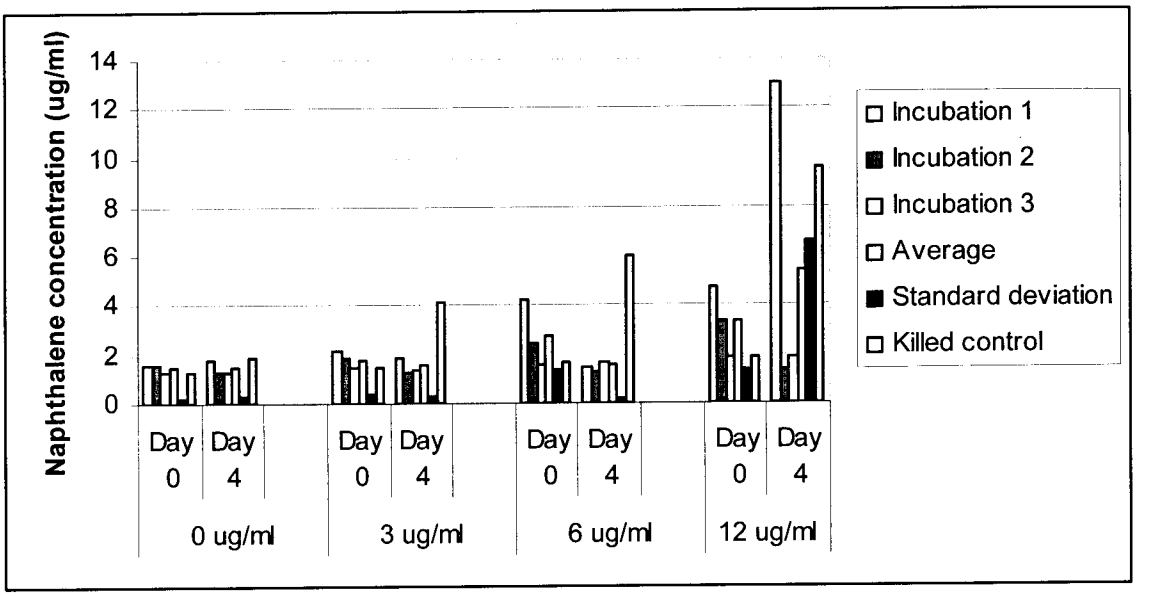

Figure 8: Naphthalene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

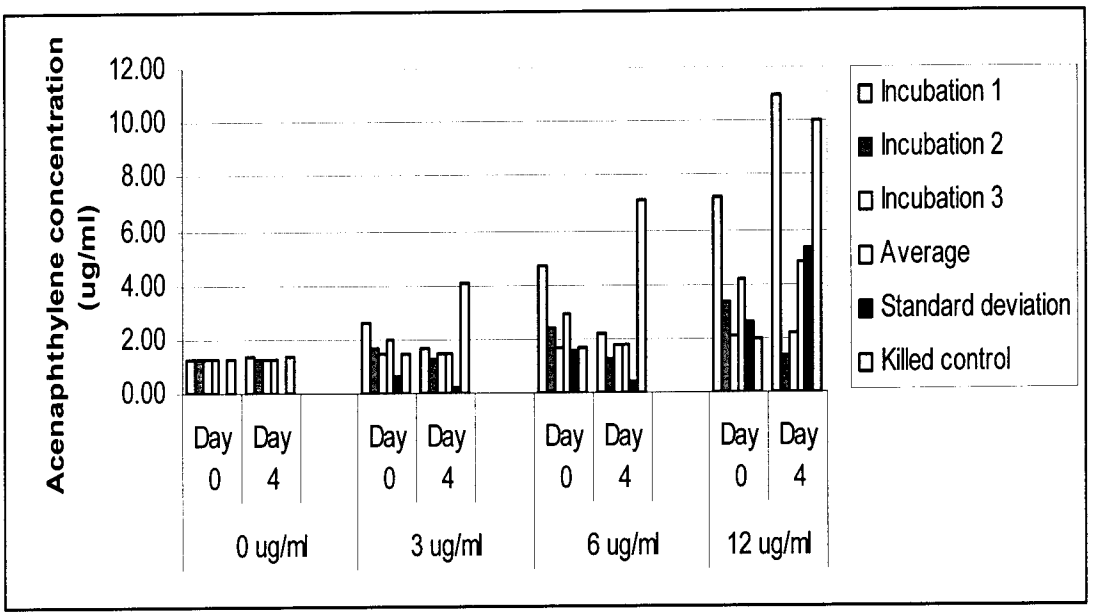

Figure 9: Acenaphthylene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 μg/ml of added PAHs.

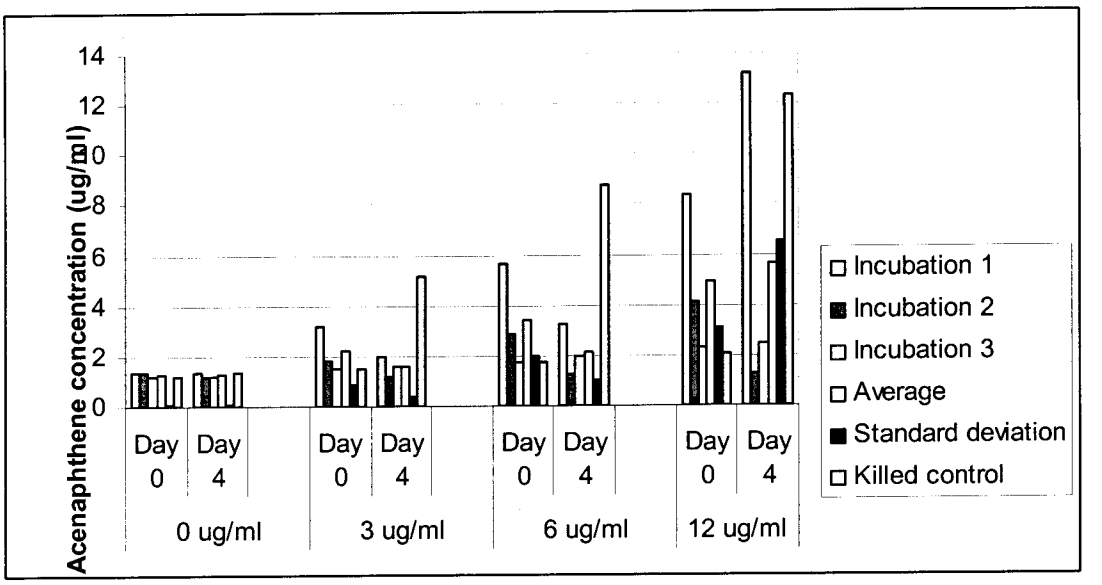

Figure 10: Acenaphthene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

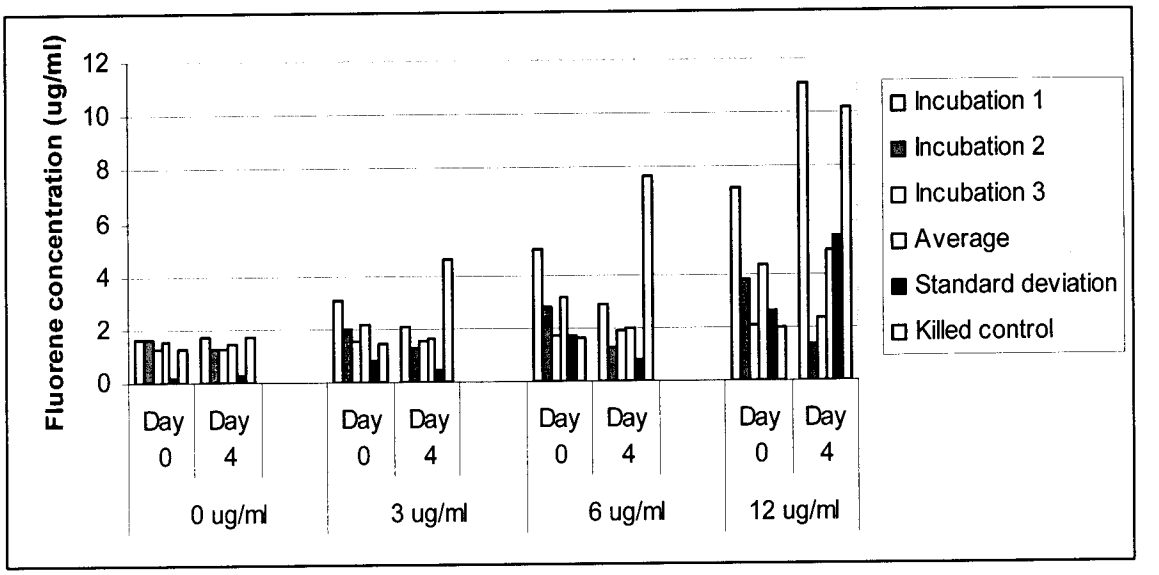

Figure 11: Fluorene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

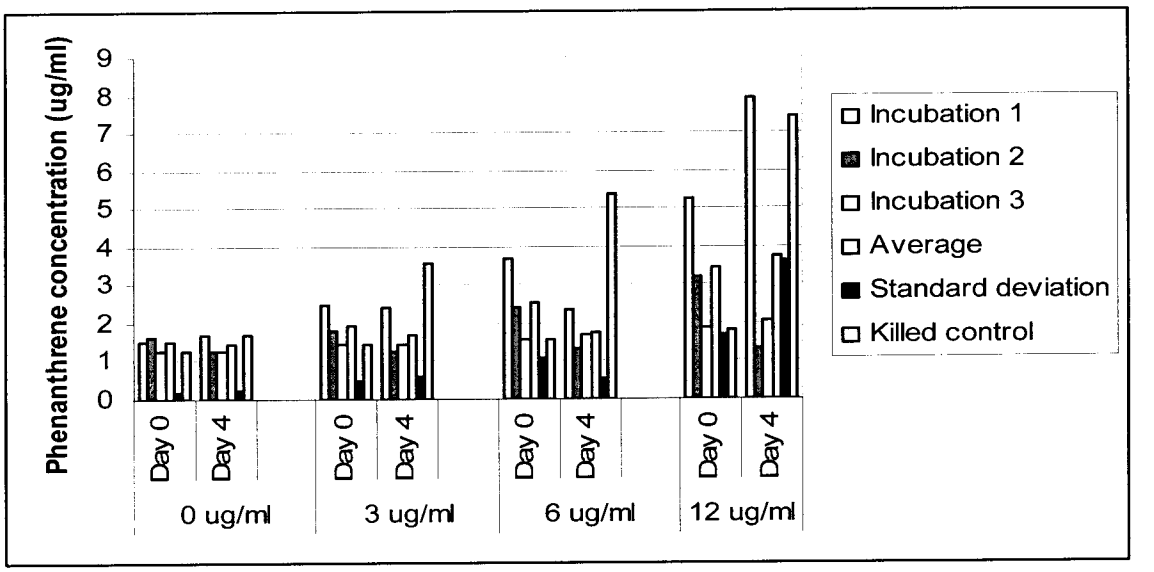

Figure 12: Phenanthrene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

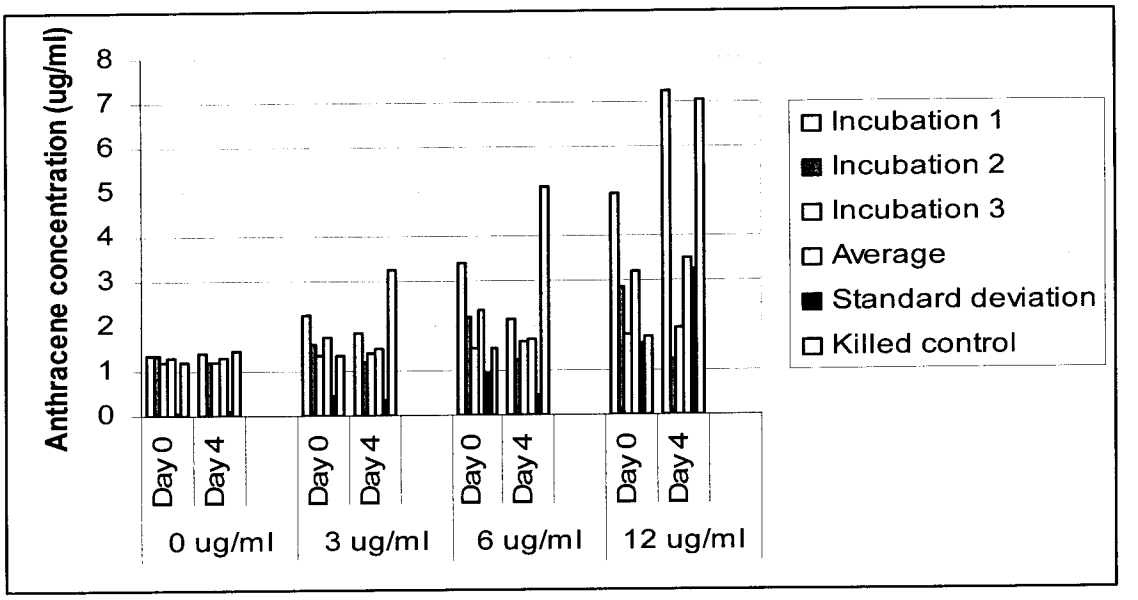

Figure 13: Anthracene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 μg/ml of added PAHs.

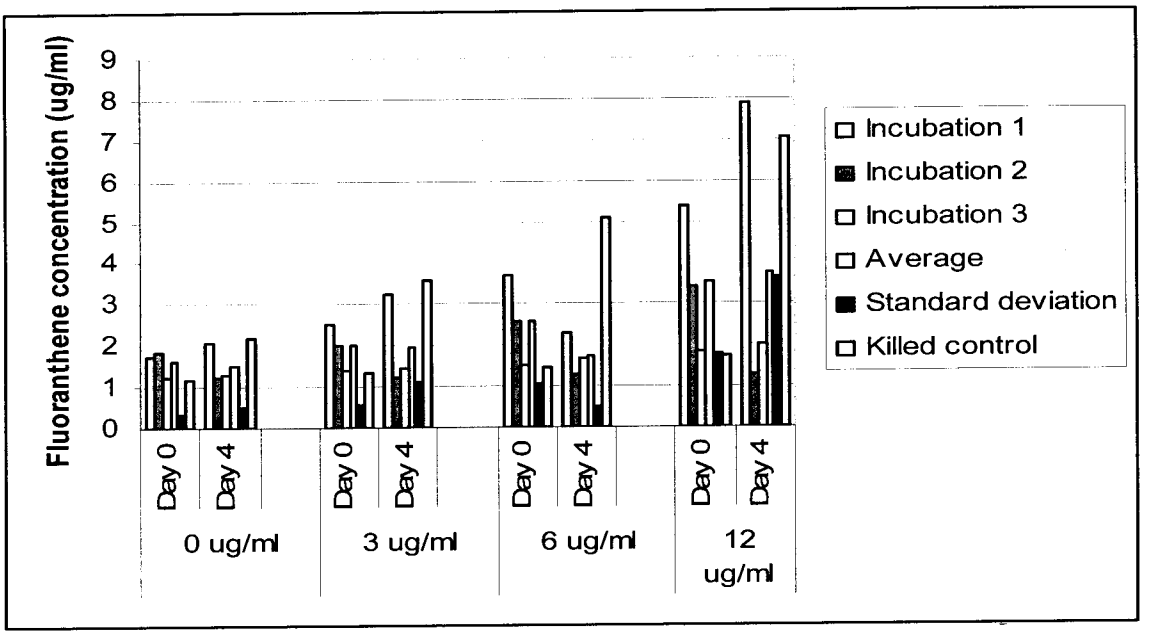

Figure 14: Fluoranthene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

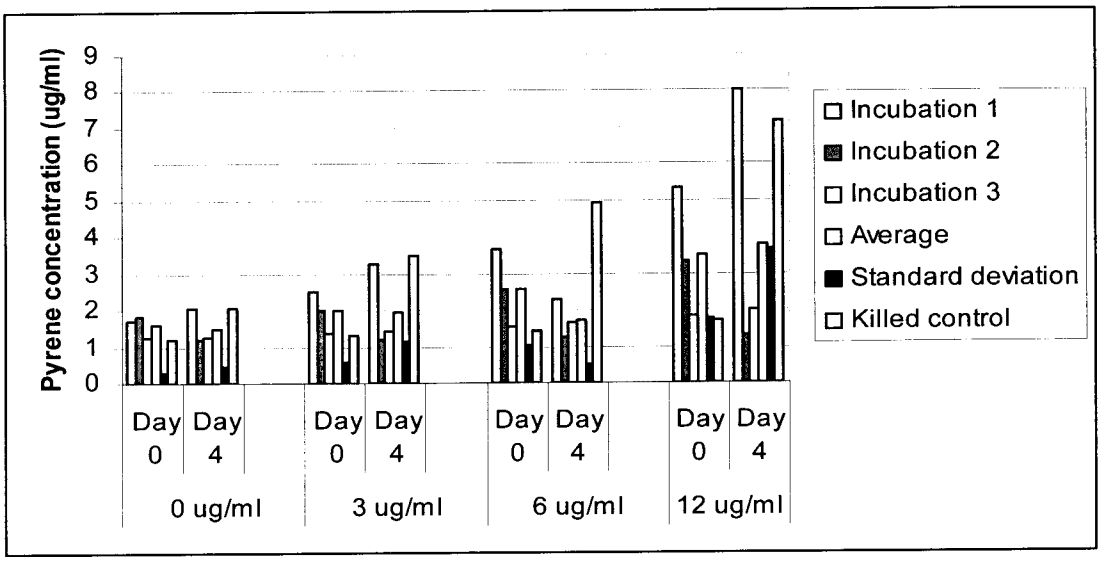

Figure 15: Pyrene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 μg/ml of added PAHs.

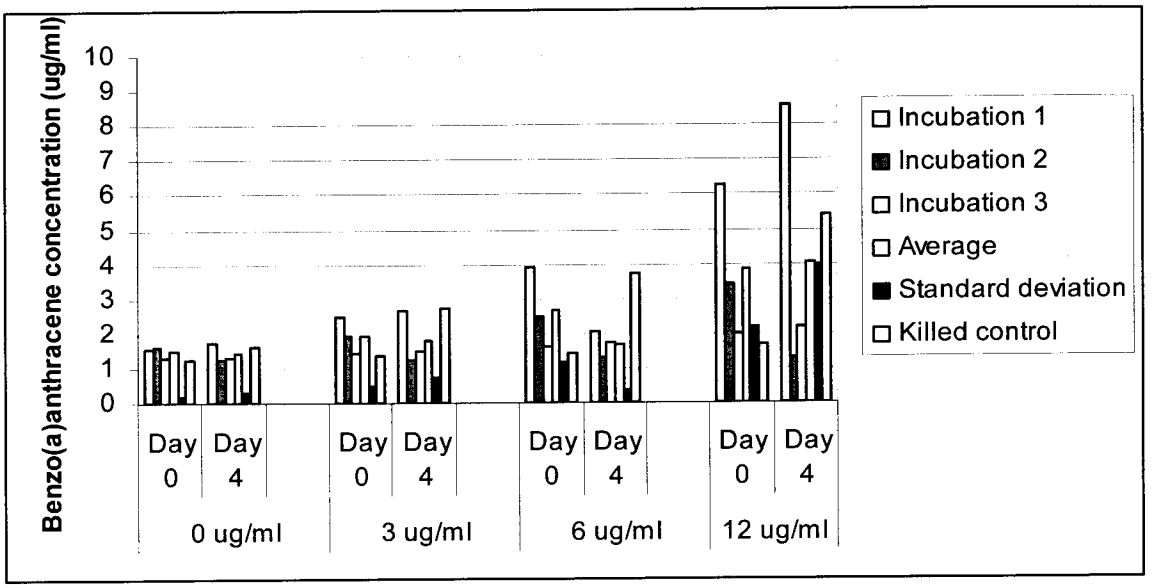

Figure 16: Benzo(a)anthracene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 μg/ml of added PAHs.

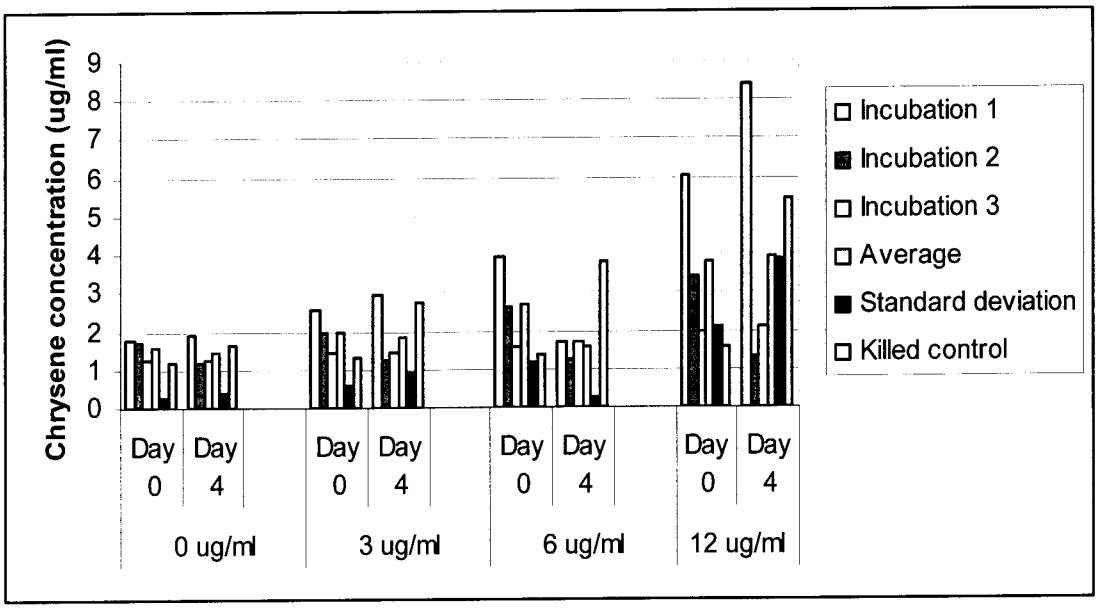

Figure 17: Chrysene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 μg/ml of added PAHs.

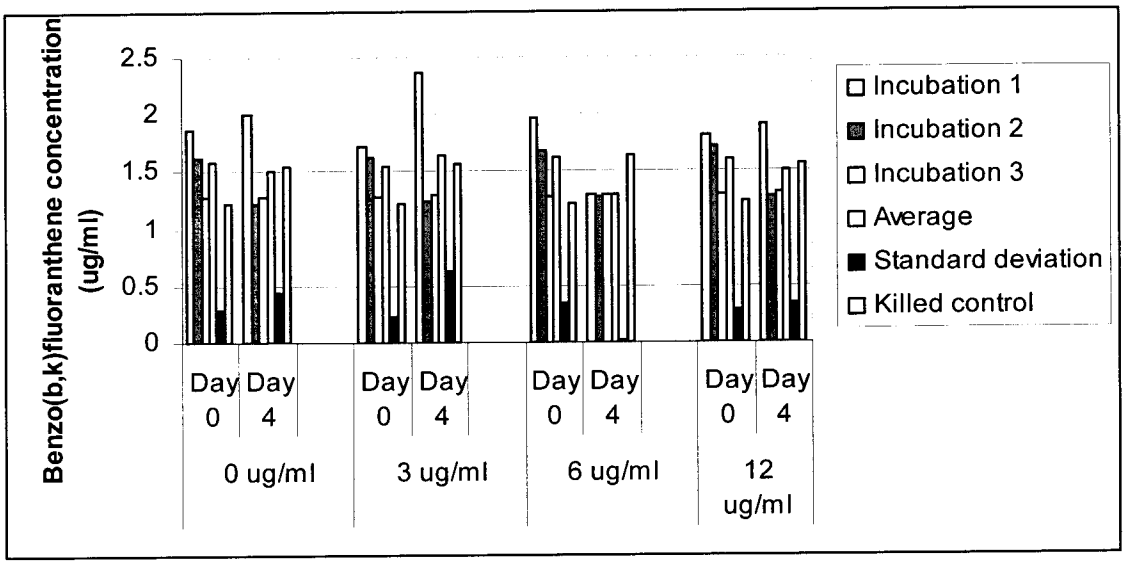

Figure 18: Benzo(b,k)fluoranthene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

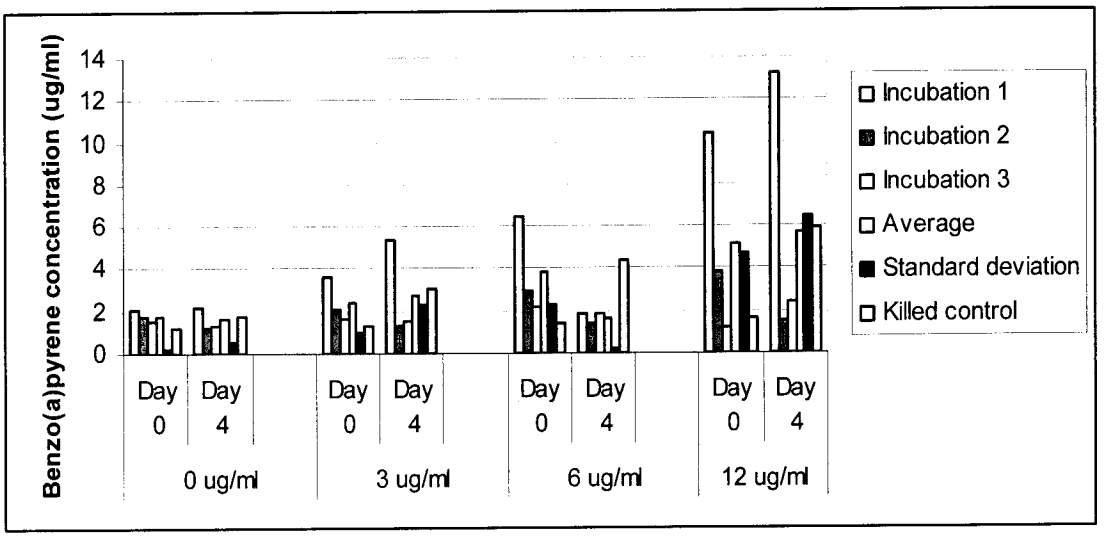

Figure 19: Benzo(a)pyrene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

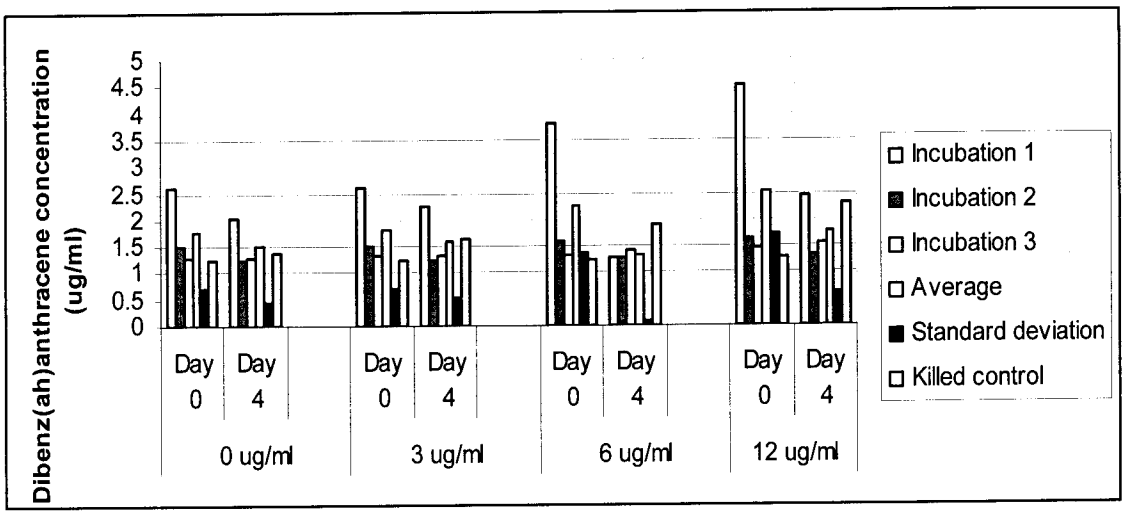

Figure 20: Dibenz(ah)anthracene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

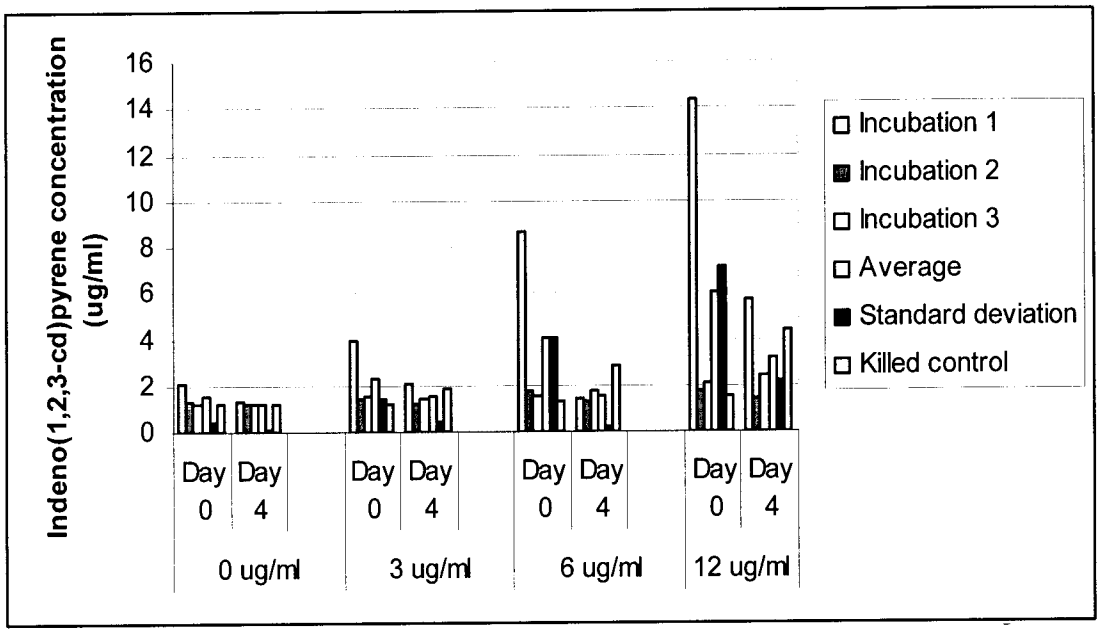

Figure 21: Indeno(1,2,3-cd)pyrene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

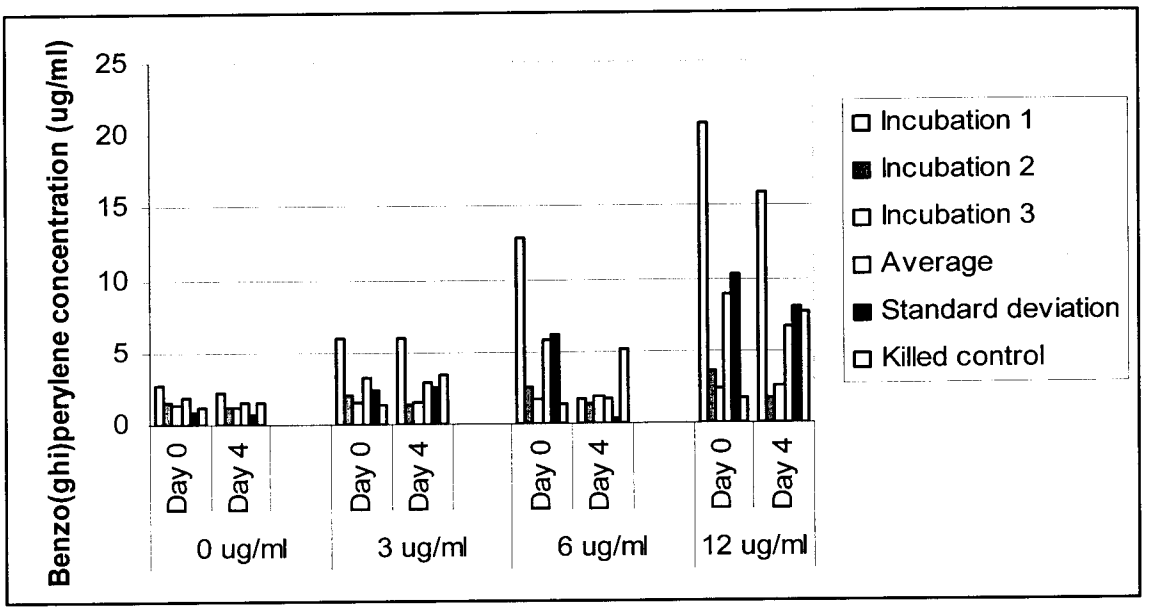

Figure 22: Benzo(ghi)perylene concentrations at Day 0 and Day 4 in triplicate sediment incubations with 0, 3, 6, and 12 µg/ml of added PAHs.

Table 6: Average microbial activity ± standard deviations for sediment incubations at Day 0 and Day 4.

Added PAH concentration	Day 0	Day 4
0 µg/ml	0.132 ± .047	0.031 ±. 023
3 µg/ml	0.138 ±. 079	0.032 ± .006
6 µg/ml	0.148 ±. 028	0.017 ± .024
12 µg/ml	0.133 ± .065	0.020 ±.020

Table 7: Average CFUs for sediment incubations at Day 0 and Day 4.

Added PAH concentration	Day 0 averages	Day 4 averages
0 µg/ml	3.9×10^6	1.5×10^7
3 µg/ml	8.6×10^5	7.4×10^7
6 µg/ml	2.6×10^6	1.6×10^8
12 µg/ml	5.2×10^6	$> 10^{8*}$

* Highly variable - Results from the 3 incubations were 4.0×10^7, too numerous to count (TNTC), and 1.8×10^5

Table 8: Microbial activity (absorbance at 490 nm) of Day 0 and Day 4 killed controls.

Added PAH concentration	Day 0	Day 4
0 µg/ml	-0.011	-0.002
3 µg/ml	0.023	0.054
6 µg/ml	-0.01	0.008
12 µg/ml	0.011	-0.006

References

Altmann, F.P. 1969. The use of eight different tetrazolium salts for a quantitative study of pentose shunt dehydrogenation. Histochemie. 19:363-374.

Blumer, M. 1976. Polycyclic aromatic hydrocarbons in nature. Sci. Am. 234:35-45.

Crane, F.L., I.L. Sun. R. Barr and H. Low. 1991. Electron and proton transport across the plasma membrane. Journal of Bioenergetics and Biomembranes. 23:773-803.

Fang, J. and R.H. Findlay. 1996. The use of a classic lipid extraction method for simultaneous recovery of organic pollutants and microbial lipids from sediments. Journal of Microbiological Methods. 27:63-71.

Grishchenkov, V.G., R.T. Townsend, T.J. McDonald, R.L. Autenrieth, J.S. Bonner and A.M. Boronin. 2000. Degradation of petroleum hydrocarbons by facultative anaerobic bacteria under aerobic and anaerobic conditions. Process Biochemistry. 35:889-896.

Herbich, J.B. (ed). 1992. Handbook of Dredging Engineering. McGraw-Hill, New York.

Hites, R.A. 1980. Polycyclic aromatic hydrocarbons in an anoxic sediment core from the Pettaquamscutt River. Geochim. Cosmochim. 44:873

Johnson, K. and S. Ghosh. 1998. Feasibility of anaerobic biodegradation of PAHs in dredged river sediments. Water Science Technology. 38:41-48.

Kanaly, R. A. and S. Harayama. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. Journal of Bacteriology. 182:2059-2067.

Kilbane, J.J. 1998. Extractability and subsequent biodegradation of PAHs from contaminated soil. Water, Air, and Soil Pollution. 104:285-304.

Luthy, R.G., A. Ramaswami, S. Ghoshal and W. Merkel. 1993. Environ. Sci. Technol. 27:2914

Menzie, C., B.B. Potocki and J. Santodonato. (1992. Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26:1278-1284.

Mosher, J.J. 2002. A biological and chemical comparison of impacted subsurface sediments of the lower Mahoning River. Masters Thesis. Youngstown State University. 82 pp.

Moser, J.J., B.S. Levison and CG. Johnston. 2003. A simplified dehydrogenase enzyme assay in contaminated sediment using 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride. Journal of Microbiological Methods. 53:411-415.

Mueller, J.G., P.J. Chapman, B.O. Blattmann and P.H. Pritchard. 1990. Isolation and characterization of a fluoranthene-utilizing strain of Pseudomonas paucimobilis. Appl. Environ. Microbiol. 56:1079-1086.

Ning, L., K.J. Kennedy and L. Fernandes. 1996. Biosorption of 2,4-dichlorophenol by live and chemically inactivated anaerobic granules. Water Resources. 30:2039-2044.

Samantha, S.K., A.K. Chadraborti and R.K. Jain. 1999. Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the f formation 1-naphthol. Appl. Microbial. Biotechnol. 53:98-107.

State of Ohio Department of Health. 2000. Ohio Sport Fish Consumption Advisory. Ohio Department of Health Year 2000 Advisory. 2000:1-8.

State of Ohio Environmental Protection Agency. 1996. Biological and water quality study of the Mahoning River Basin. OEPA Technical Report MAS/1995-12-14:1-249.

United States Army Corps of Engineers. 1999. Mahoning River Environmental Dredging Study (Ohio). Energy and Water Development Appropriations Act of 1999 (Public law 104-303).

United States Environmental Protection Agency. 1996b. Semivolatile organic compounds by gas chromatography/mass spectrometry (GC/MS). Method 8270C Revision 3:1-54.

van der Meer, J.R., W.M. de Vos, S. Harayama and A.J.B. Zehnder. 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56:677-694.

Verrhiest, G.J., B. Clement, B. Volat, B. Montuelle and Y. Perrodin. 2002. Interactions between a polycyclic aromatic hydrocarbon mixture and the microbial communities in a natural freshwater sediment. Chemosphere. 46:187-196.

Wackett, L.P. and L.B.M. Ellis. 1999. Predicting Biodegradation. Environmental Microbiology. 1:119-124.

Weissenfels, W.D., M. Beyer and J. Klein. 1990. Degradation of phenanthrene, fluorine and fluoranthene by pure bacterial cultures. Appl. Microbiol. Biotechnol. 32:479-484.

Appendix 1: Solutions for Sterile Sediment Controls

Basal salts media: weighed 1.0 g of diammonium phosphate and 0.0125 g of magnesium sulfate. Added trace elements in the following amounts:
Boric acid 4.64 g
Zinc sulfate 3.48
Cobaltous sulfate 1.92 g
Cupric Sulfate 0.16 g
Manganous sulfate 0.12 g
Ferric ammonium sulfate 2.98 g
Ammonium molybdate 0.44 g

Sodium-azide: Added 18 mg of sodium-azide to 100 ml BSM for sterile sediment controls and .054 mg of sodium-azide to 100 ml BSM for sediment incubation killed controls

Appendix 2: Solutions for Microbial Activity Time Curve

PAH stock solution: 10 mg of the following PAHs were added to 50 ml of acetone:
Acenapthene
Acenapthylene
Anthracene
1,2-Benzanthracene
Benzo(a)pyrene
Benzo(ghi)perylene
Chrysene
Dibenz(a,h)anthracene
Fluoranthene
Fluorene
Naphthalene
Phenanthrene
Pyrene

1.08 mM iodonitrotetrazolium chloride solution: weighed 0.03 g of INT and added 0.100 ml of N,N-dimethylformadine, mixing well with a glass rod. Brought to volume with deionized water in a 50 ml volumetric flask and sonicated with light heat for twenty minutes.

Appendix 3: Solutions for PAH Extraction and Analysis

100 mM Phosphate buffer: added 8.7 g of K_2HPO_4 to 500 ml of deionized water. Adjusted pH to 7.4 with 1N hydrochloric acid.

Preparation of sodium sulfate (Na_2SO_4) columns: Preparation of Na_2SO_4 columns to collect and process extracted samples involved the addition of 1 g of dry Na_2SO_4 to clean 6 ml glass columns. The columns were then packed with 2 ml of DCM without drying.

Solvent exchange: samples in DCM were dried down to 100 µl using a nitrogen evaporator. Hexane in the amount of 1 ml was added and again samples were dried to 100 µl. This was repeated two more times.

Preparation of Silica columns: The columns were prepared by rinsing with 2 ml of chloroform and 2 ml of hexane. Copper filings were added to the column after cleaning in 2 rinses of 1 N HCL, methanol, DCM, and hexane before drying under nitrogen.

Aminopropyl (NH_2) column construction and conditioning: column construction and conditioning first involved the addition of 1 ml optima grade chloroform then another 2 ml before pressurizing and leaving to drip. Hexane in the amount of 2 ml was added and pulled through at 1 drop per second, but not dried.

Appendix 4: Equations for PAH Extraction and Analysis

Day 0 calculations: Averaged the triplicate runs for each concentration of each compound to give an average of each compound and all concentrations for incubations 1, 2, and 3 (X_1, X_2, X_3)

Ex: Naphthalene (Incubation 1)

Conc.	Run 1	Run 2	Run 3	Avg. (X_1)
0 µg/ml	1818	1754	1866	**1813**
40 µg/ml	4976	4910	5150	**5012**
80 µg/ml	16408	16528	16969	**16635**
160 µg/ml	19208	18916	19644	**19256**

Determined concentration for each internal standard before injection:

$$\frac{\text{Avg. of internal standard (for all concentrations)}}{\text{Avg. of internal standard in std. curve (for all concentrations)}} \times 40\ \mu g/ml = \text{Conc. of IS before inj.}$$

Ex: Naphthalene-d8

Conc.	Avg. of 3 runs	Conc. (std. curve)	Avg. of 3 runs
0 µg/ml	69011	2 µg/ml	30172
40 µg/ml	66593	10 µg/ml	30240
80 µg/ml	66205	20 µg/ml	30126
160 µg/ml	65567	40 µg/ml	30492
		80 µg/ml	28833
Total Avg.	**66844**	**Total Avg.**	**29973**

$$\frac{66844}{29973} \times 40 = 89 \text{ (conc. of IS before inj.)}$$

Correlated internal standards with compounds according to EPA method 8270:

Internal Standandards	Correlating PAHs and surrogates
Naphthalene-d8	Nitrobenzene-d5 (surrogate) Naphthalene
Acenaphthene-d10	2-fluorobiphenyl (surrogate) Acenaphthylene Acenaphthene Fluorene
Phenanthrene-d10	Phenanthrene Anthracene Fluoranthene Pyrene
Chrysene-d12	Terephenyl-d14 (surrogate) Benzo(a)anthracene Chrysene Benzo(b,k)fluoranthene Benzo(a)pyrene
Perylene-d12	Dibenz(ah)anthracene Indeno (1,2,3-cd)pyrene Benzo(ghi)perylene

Used equation to find final response of each compound:

$$(X_1) \times \frac{\text{Conc. of IS before inj.}}{40} = \text{Final response of compound}$$

Ex: Naphthalene $1813 \times \frac{89}{40} = 4033$ (Final response)

Used $y = mx + b$ from standard curve for each compound and solved for x to give final concentration:

Where: y = final response of compound
x = final concentration of compound

Ex: Naphthalene $y = 12436x - 15995$

$$\frac{4033 + 15995}{12436} = x$$

$$x = 1.61$$

Appendix 5: Solutions for Microbial Activities

1.08 mM iodonitrotetrazolium chloride solution: weighed 0.03 g of INT and added 0.100 ml of N,N-dimethylformadine, mixing well with a glass rod. Brought to volume with deionized water in a 50 ml volumetric flask and sonicated with light heat for twenty minutes.

Appendix 6: Solutions for Viable Heterotrophic Counts

Nutrient agar plates: nutrient agar was made with the following:
Agar 15.0 g
Peptone 5.0 g
NaCl 5.0 g
Yeast extract 2.0 g
Beef extract 1.0 g