Hazard Control Technologies, Inc.

Clemson Final Report

FINAL REPORT: TIWET PROJECT - 09440

A STUDY OF THE PHYSICAL PROPERTIES, BIOLOGICAL EFFECTS AND POSSIBLE USAGES OF F-500 IN ENVIRONMENTAL PROTECTION AND RESTORATION

Submitted to:

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PREFACE[1]

When this study began, the company was known as FSI Laboratories and the product was known as Fuelbuster.

FSI Laboratories was later incorporated and became known as Fuel Buster Laboratories, Inc. In 1997, Fuel Buster Laboratories, Inc. was merged into Fire Buster International, Inc. and the surviving company's name was changed to Hazard Control Technologies, Inc.

Shortly after the merger and company name change, Hazard Control Technologies, Inc. changed the name of their product from Fuelbuster to F-500 for marketing purposes.

MESSAGE TO THE READER FROM HAZARD CONTROL TECHNOLOGIES, INC.:

Hazard Control Technologies, Inc. (HCT) only received permission to change references to the name of the product tested (Fuelbuster to F-500) and the name of the company (from FSI Laboratories to Hazard Control Technologies, Inc.).

HCT did not receive permission to make grammatical changes (spelling, grammar, etc.) to the report; therefore, we did not make any grammatical or content changes from the original published report.

F-500 is a "Water Additive", not a "foam"; however, there are circumstances in this report where F-500 is referred to as "foam." The Clemson Final Report is a scientific study and was not necessarily authored by personnel with an intimate, working knowledge of fire suppression terminology.

Michael T. Greiner
Vice President/Marketing and Technical Services

[1] This preface was not part of the original TIWET Project - 09440 document.

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A STUDY OF THE PHYSICAL PROPERTIES, BIOLOGICAL EFFECTS AND POSSIBLE USAGES OF F-500 IN ENVIRONMENTAL PROTECTION AND RESTORATION

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INTRODUCTION

F-500 products have unique attributes that suggest possible applications in environmental protection and/or restoration. The primary application for F-500 is fire fighting. This surfactant is made by Hazard Control Technologies, Inc. and is composed of nontoxic chemicals. The non-toxic formulation should also provide environmental protection in areas receiving runoff from fire fighting activities. Effluent control and treatment are also a possibilities. The formulation disperses organic liquids in water, removes organic liquids from soils, and inhibits volatilization of organic liquids. These properties should provide important applications for industries as a nontoxic means of controlling spills, fires, and emission of hazardous organic compounds. Additionally, F-500 may aid waste site remediation by increasing water solubility or bioavailability of organic contaminants.

CORROSIVITY

Corrosivity Methods

Corrosivity of F-500 to carbon steel was performed as described in the SW-846 Manual of EPA Methods. The specific method was SW-846 1110. Four steel coupons were weighed individually. Three of the steel coupons were placed on a Teflon sleeve affixed to a rubber stopper. The fourth steel coupon was used as a control and was not subjected to the test chemical. Test chemical was poured into a one liter flask and placed on a hot stir plate. The temperature was monitored to ensure a reading of 55 Celsius. Three steel coupons were lowered into the test chemical and allowed to remain for 24 hours. Steel coupons were then removed from the test chemical. The control coupon along with the three treated coupons were subjected to the test chemical were rinsed with acetone. All four steel coupons were then scrubbed using a bristle brush and rinse with acetone. Steel coupons were weighed once again to determine the weight variation. The following calculation was used to determine the Corrosion Rate in units of micrometers per year (μmpy). Weight loss was measured in milligrams, steel coupon surface area in square centimeters, and time in years.

$$\text{Corrosion Rate (}\mu\text{mpy)} = \frac{1000 \times \text{weight loss (mg)} \times 11.145}{\text{surface area (cm}^2\text{)} \times \text{time (hrs)}}$$

Corrosivity Results and Discussion

Data from 24-hour tests at 55 C show that corrosion was 7±4 μm/yr for F-500 and 53±3 μm/yr for F-500 Foam (Figure 1). These values are well below the threshold limit of 6.35 mm/yr established by the USEPA for corrosive materials (EPA 1986). In comparison, corrosivity data for water are 8 μm/yr for carbon steel, and salt water produces corrosivity of 75-500 μm/yr, with an average of approximately 200 μm/yr.

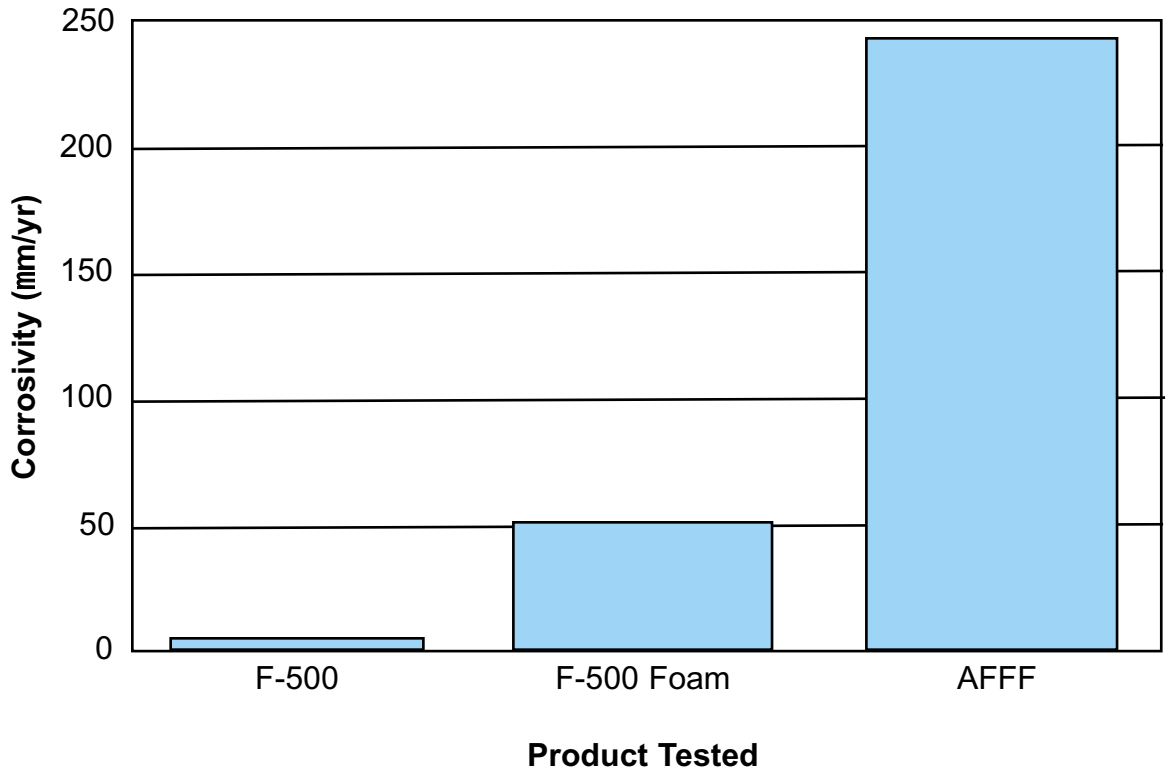

Figure 1. Corrosivity of carbon steel exposed to two formulations of F-500 and AFFF foam under laboratory conditions.

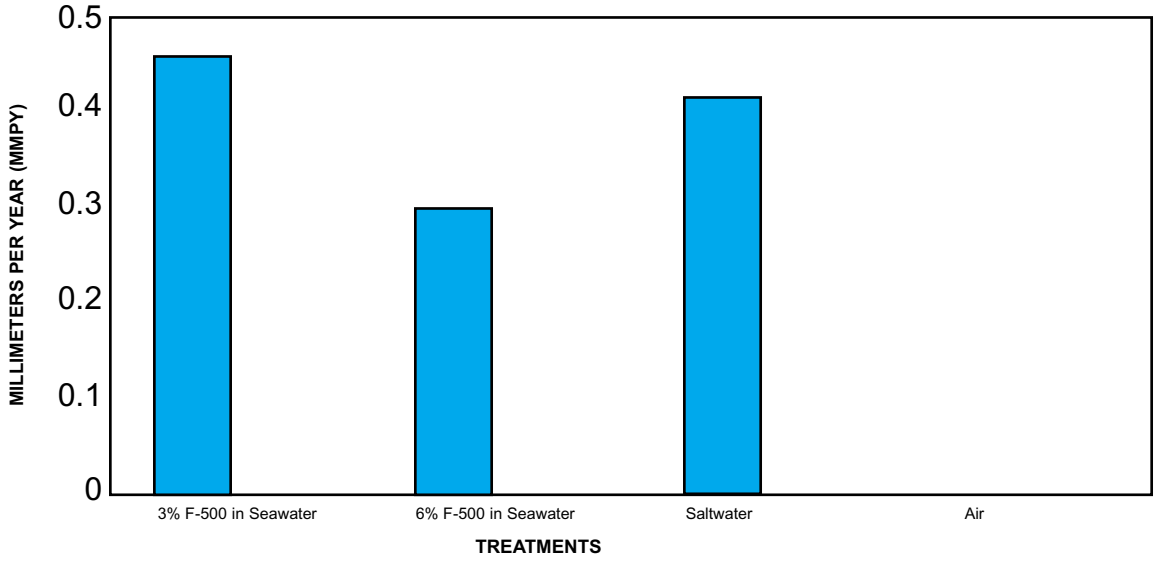

Figure 2. Corrosion of carbon steel in seawater and in two solutions of F-500 dissolved into seawater.

Acids at concentrations of 1 M corrode carbon steel at a rate of 20,000 μm/yr. Through tests performed under the same conditions as those for F-500, we have determined the corrosivity of 6% AFFF foam to be 243±33 μm/yr.

Data show that F-500 corrosivity is comparable to that of water and 10-30 times less than its primary competitor. Such corrosion rates suggest that equipment exposed to F-500 would experience little more corrosion than would equipment exposed to water.

Further testing was conducted with salt water collected at high tide from the Atlantic Ocean just offshore at Folly Beach, SC. The corrosivities of sea water, 3% F-500 in seawater, and 6% F-500 in seawater were 414±52, 462±53, and 300±45 μm/yr, respectively (Figure 2). Our measured corrosivity for seawater is on the high end of the worldwide salinity gradient, but the data support the validity of our test methods. Sea water and 3% F-500 in seawater displayed similar corrosivities ($p=0.23$), whereas the 6% F-500 was less corrosive than was seawater ($p=0.058$). These data indicate that 6% F-500 in seawater may prove less corrosive to metals in real world situations than would seawater alone. F-500 in freshwater was 59 times less corrosive than was seawater tested under the same conditions. The difference is highly significant ($p<0.001$).

SOIL LEACHING

Soil Leaching Methods

Soil columns were prepared using clean surface soils from the SC Agricultural Experiment Station, Clemson, SC. Soil columns were 5 cm in diameter and 1.2 m in length. Columns were packed while filled with water and flushed with five pore volumes of water. Twenty five milliliters of diesel fuel was added to the head of the column and allowed to soak into the column. Diesel was then flushed through the soil with purified water. This approach was taken to approximate a fuel leak from underground storage

tanks into aquifers or vadose zone soils. Separate experiments were conducted by mixing diesel fuel directly with soils before addition water. This provided a situation more representative of diesel fuel spilled directly onto soil. In these tests, water was collected after 200 or 500 ml of water had eluted from the respective columns. Collected eluent volumes were generally 100 to 200 ml during this interval. Water saturated with the appropriate fuel was placed in two containers, of the same type used for pore volume collection. Analyses of these samples served to correct for volatile losses during the experiment.

Concurrently, columns were tested by adding 50 ml of diesel fuel to soil columns, as described above, and eluting columns with 25 ml of 3% F-500 in purified water. Purified water was used as the eluent during the balance of each experiment. Fuel components were analyzed by extracting eluent with methylene chloride, evaporating the methylene chloride to 5 ml, and quantifying the fuel components with gas chromatography-flame ionization detection.

Soil Leaching Results and Discussion

Three columns were treated with diesel alone (control) and three were treated with diesel + F-500 (treatment) all columns were prepared from the same soil, and soil columns were matched by flow rate. Three pairs of columns were used to determine the shape of diesel elution profiles from the soil (Figures 3-5). Occasional oil globules were released from glass wool at the base of the soil columns. This caused free standing diesel on the surface of water isolated in several fractions. When replicate samples showed no diesel release in similar elution volumes, anomalously high diesel concentrations in isolated elutions were removed from data comparisons.

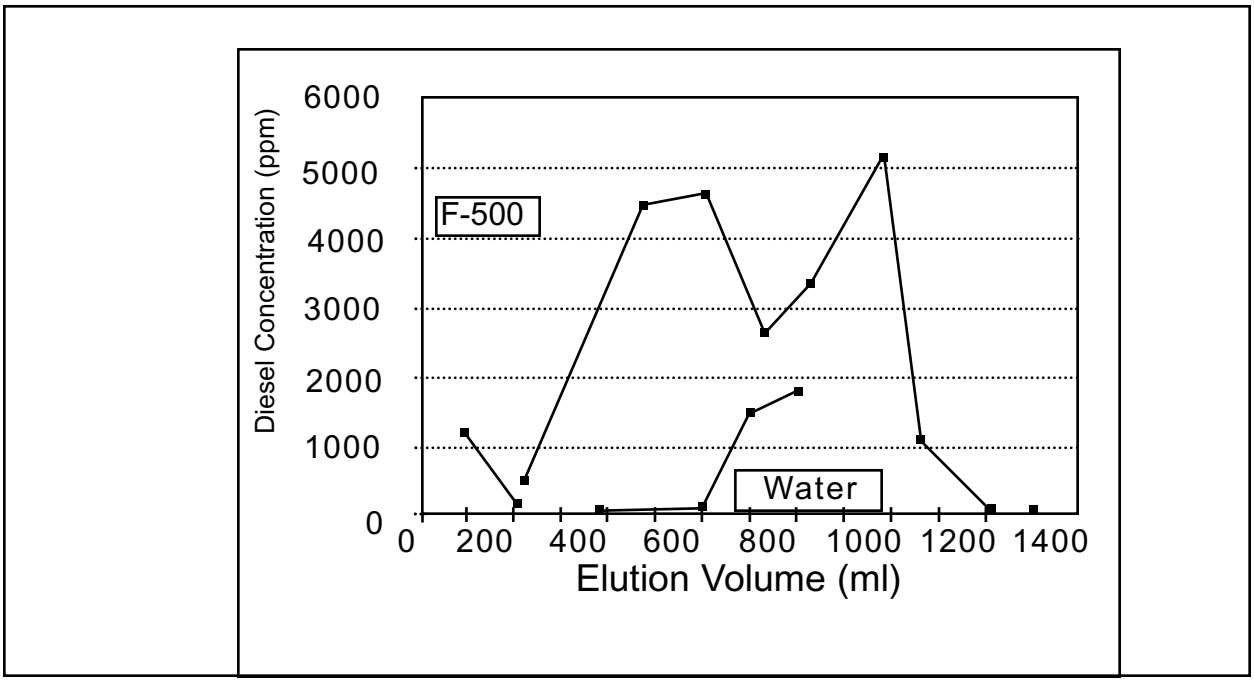

Figure 3. Elution of diesel fuel from 600 g clay loam soil columns loaded with 200 ml of diesel. Fifty ml of 3% F-500 was added to one column before elution with water.

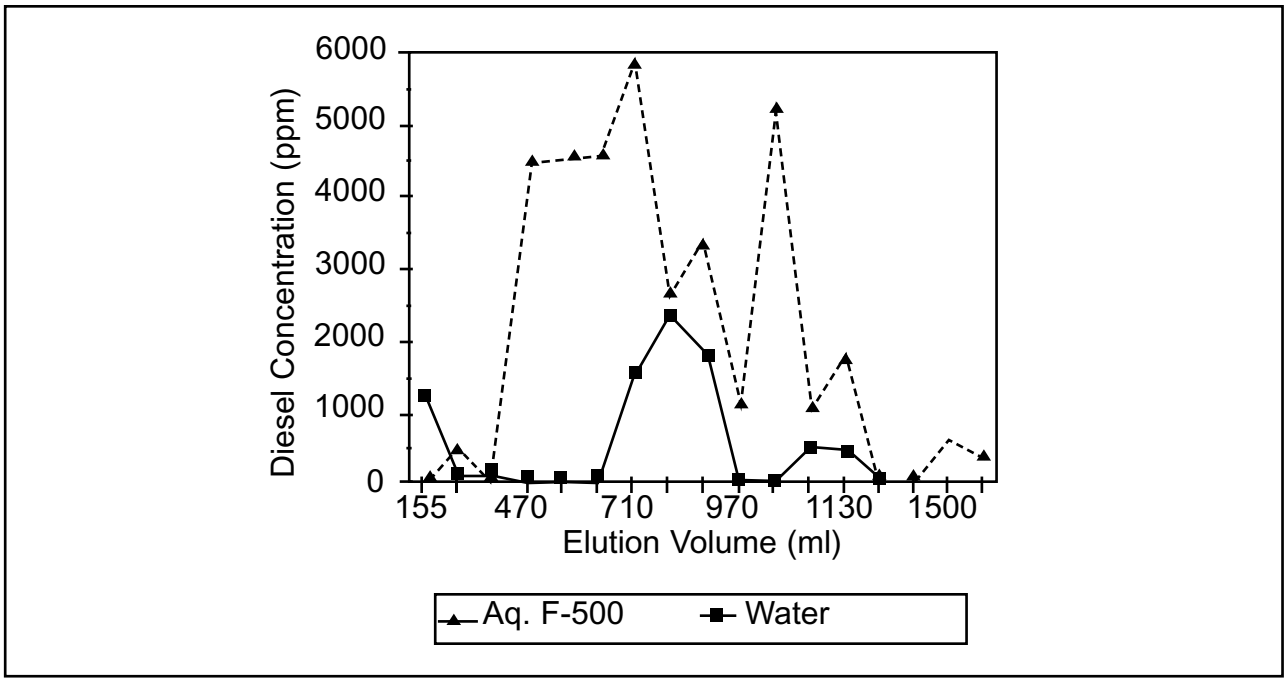

Figure 4. Average elution of diesel fuel from 600 g clay loam soil columns loaded with 200 ml of diesel fuel. Fifty ml of 3% F-500 was added to one column before elution with water.

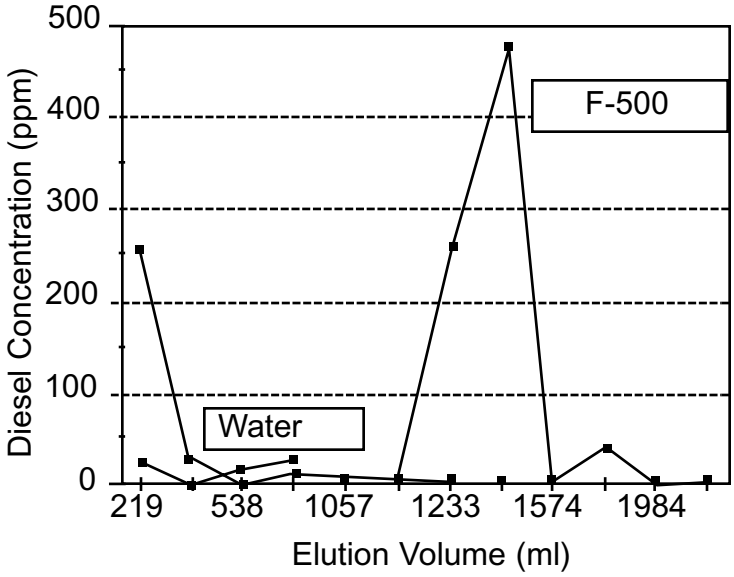

Figure 5. Elution of diesel fuel from 600 g clay loam soil columns loaded with 50 ml of diesel fuel. Fifty ml of 3% F-500 was added to one column before elution with water.

Oil products may require 50-80 pore volumes of solution flow to elute from sandy material. Lower volumes should be required when surfactant is added and the surface tension of water in lowest. In fact, 16 pore volumes (1300 ml) of F-500 and water lowered diesel concentrations in eluent from >4000 ppm to <100 ppm. Following addition of 50 ml of 3% F-500, aqueous flushing of the soil columns yielded maximum diesel fuel elution in the 400 to 600 ml aqueous fractions which contain the 4^{th}-8^{th} pore volumes (Figure 3). Diesel concentrations in these fractions were approximately 4500 ppm. A second maxima occurred at the 10^{th} pore volume. This fraction contained >5000 ppm diesel, but returned to <1000 ppm by the next pore volume. Diesel elution remained below 100 ppm for the balance of the experiment (Figure 3).

Control soils, eluted with water only, released fuels in a fraction containing pore volumes 7-9 with a smaller release occurring in the 10^{th}-12^{th} pore volumes. Diesel concentrations were 2400 ppm and 400 ppm in the respective maxima. Similar elution patterns were noted for replicate soil columns (Figure 4). In control soil columns with low fuel loading, diesel concentration in eluting water remained constant at 25-50 ppm through 14 pore volumes (Figure 5) with the exception of one fuel globule release. Diesel elution appeared at the 12^{th} pore volume for F-500 treated soils, but lasted only two pore volumes. These columns appear to have retained insufficient diesel to release measurable quantities for an appreciable duration of time.

In the region of the two maxima, relative differences in diesel release by 1) 50 ml F-500 followed by water and 2) water alone are 2.2 (5300:2400) in the pore volumes 6-9 and 6.2 (2500:400) in pore volumes 10-12. Ratios were calculated using average concentration occurring in F-500 eluent and water eluent near the respective maxima.

Enhanced elution of diesel that is more tightly bound to soils is consistent with the proposed mechanism of fuel removal by F-500. This surfactant allows better aqueous solvation of nonpolar organics. Thus surfactant enhancement of fuel removal (relative to removal by water alone) should correlate with the strength of fuel-soil interaction. This means as the absolute difficulty of removing fuel from soil with water alone increases, the ratio of fuel released by F-500/water to fuel released by water alone will increase. When fuel concentrations have not exceeded soil binding capacities, fuel partitioning into water is more difficult than in instances where fuel has exceeded binding capacity.

Fewer pore volumes were required to elute fuels when less than one pore volume of 3% aqueous F-500 was added before elution with water. When high concentrations of diesel were present in soils, fuel elution occurred earlier and was sustained for several pore volumes (Figures 3 and 4). It is important to note that F-500 addition to soil columns totaled only 50% of the pore volume for the columns. Overall, F-500 eluted 8 times more diesel from soil columns and sped initial elution by a factor of two (Figure 5). These data suggest a quicker and more thorough removal of fuels from soils using F-500 as compared to water.

CRITICAL MICELLAR CONCENTRATION

Critical Micellar Concentration Method

To determine the concentration of surfactant that could be used in soil and aqueous remediation of anthracene, the Critical Micellar Concentration (CMC) had to be determined. A surface tension method using the Cahn Microbalance, and the resulting measurements were plotted versus the logarithm of the F-500 concentration. The

intersection of the two linear portions of the plot is defined as the Critical Micellar Concentration of a surfactant.

Critical Micellar Concentration Results and Discussion

The CMC of F-500 was calculated to be 3.98×10^{-3}, which is relatively higher than other commercial nonionic surfactants (Appendix 1). This can be due to a difference in the analyzing instruments or, F-500 may contain high CMC surfactant..

SMOKE REDUCTION

Smoke Reduction Methods

Tests were conducted using containers of burning toluene placed beneath an inverted glass funnel. Above this funnel was a Teflon housing that contained a centered glass thimble packed with florisil. A high volume air sampler (Anderson Prod.) was used to draw smoke from the toluene fire through the funnel and into the thimble where smoke. Other combustion products were trapped on the florisil. Separate toluene containers, glass funnels and collection thimbles were used for each burn. Toluene smoke from each trial was timed at between one and two minutes. Nine burns were performed. Three burns were allowed to proceed normally. Water was sprayed through the smoke plume during three burns and F-500 was sprayed during three others. Toluene was ignited and allowed to burn for 15 seconds before placing them beneath their respective funnel/thimble. Quantitative assessments of smoke reduction were performed by weighing toluene smoke that deposited on glass funnels during the two minute test periods.

Florisil sorbents were then exhaustively extracted and concentrated for toxicity determinations. H4IIE rat hepatoma cell assays were used to determine carcinogenicity.

Toxicity evaluations were performed with portions of soot extracts. The solvent from a 1 ml extract aliquot was exchanged with dimethylsulfoxide (DMSO) and diluted to 1 ml with DMSO. Rat hepatoma cell lines (H4IIE) were grown to uniform coverage in a growth media and exposed to each extract and to a 2,3,7,8 tetrachloro dibenzo-p-dioxin control. Toxic responses were measured as induction of ethoxyresorufin-o-deethylase, one of the P450 mixed function oxidases.

Light transmittance: Interference with light transmission was measured in a glass chimney two inches in diameter. Perpendicular to the main cavity of the chimney a viewing portal was constructed with 1 inch glass tubing that extended 2 inches and quartz windows were mounted at the end of both tubing extensions. This viewing portal was placed one foot from the bottom of the glass chimney. Wide spectrum light transmission was determined using a spectrophotometer with a fiber optic cable mounted at each window of the chimney. The fiber optic cable and spectrometer were constructed in the Department of Chemistry at Clemson University. Five spectra were acquired for each experiment.

Smoke Reduction/Visibility Results and Discussion

Visual observations showed that less soot was deposited on funnels when the smoke was treated by F-500. This visual observation was confirmed by weighing the amount of soot collected on each funnel. Mean and standard error values for soot collected above the toluene fire was 1.5±0.3 mg/sec, 0.67±0.13 mg/sec and 0.24±0.13 mg/sec for control, water and F-500 treatments, respectively. F-500 and water introduction into the smoke plume reduced soot/smoke significantly ($p<0.009$). Furthermore, F-500 reduced soot/smoke significantly compared to water ($p=0.016$). These data are significant since surfactant treatment was to the smoke plume not the fire

itself. This information along with vapor reduction experiments suggest an interruption of vapor phase combustion possibly by inhibiting radical coalescence into soot particles.

Smoke Toxicity

Many products of incomplete combustion were found as would be expected for the sooty smoke produced. Known toxicants were found in all smoke samples. For example, toluene, pentanone, phenol, methylphenol, indene derivatives, biphenyl, azulene, fluorene derivatives, anthracene derivatives phenanthrene derivatives, chrysene, benzopyrenes, benzoperlyene, and indenopyrene were identified in both samples. Smoke treated with F-500 contained a smaller quantity of many toxicants than did other treatments. This was the case for compounds such as: benzo(a)pyrene, terphenyls, fluorene derivatives, cyclohexanols, indene derivatives, and several higher molecular weight polynuclear aromatic hydrocarbons. Several compounds found during F-500 treatment of the flame were unique to that sample. Cleavage of constituents in the fire fighting formulation produced such compounds as: dibenzofuran, hexanoic acid derivatives, heptadecane, octadecanate esters, hexadecanate esters, and ethoxy benzenes.

Toluene fires, with or without suppressant addition, produced contaminants that were vastly less toxic than TCDD. This was expected, but was used as a verification of relative enzyme induction. The toluene fire extinguished by F-500 produced smoke and combustion by-products that were 98.6% less toxic than compounds released from untreated fires (Table 1). This is only one measure of toxicity. However, the H4IIE assay provides an indication of carcinogenic potential of tested compounds and responds well to most products of incomplete combustion. Further tests are needed to establish confidence limits around the observed toxicity reduction.

Table 1. Toxicity of smoke from toluene fires as determined through H4IIE cell culture assays.

Extinguishment Method	ED50	TCDD Equivalents
None	$4x10^{-4}$	$7.0x10^{-9}$
F-500	$5.6x10^{-2}$	$5.4x10^{-11}$

Reduced smoke quantity and smoke toxicity are important safety considerations for those trying to escape or extinguish fires. Smoke inhalation often causes fatalities during fires. Moreover, repeated exposure to toxicants in smoke is hazardous. Reducing such exposures to fire fighting professionals should reduce long term health effects of repeated smoke inhalation.

Light Transmission: Visibility across the constructed chimney was not totally obscured by smoke rising from the small piece of burning rubber. The smoke produced was sprayed with aqueous F-500 and visible light transmission was increased by 68±3%. Assuming similar molar extinction coefficients for the smoke components before and after F-500 treatment, this translates into a 97% reduction in products of incomplete combustion.

TEMPERATURE DETERMINATIONS

Temperature Determination Methods

The first of these tests were done with diesel/gasoline fires inside a pan of 4 inch diameter. Heat produced during the experiment was determined with a hand held K-type thermocouple. Data were recorded manually at proscribed time intervals during the test. Each fire was ignited and burned until the flame temperature was constant as measured

one foot above the fuel surface. The fire was then extinguished and the temperature measured. Extinguishing agents were AFFF, carbon dioxide, dry powder, F-500, and Halon. In all cases, the fire was extinguished within 3 seconds. Temperature monitoring lasted for one minute in all experiments. Each experiment was conducted five times.

In a second set of experiments, metal heated in a fire was covered with 6% AFFF and 3% F-500. Again the temperature of the metal was monitored with a hand held K-type thermocouple. Temperature monitoring was done prior to covering the metal and for five minutes thereafter. This process was conducted seven times for each extinguishing agent.

Temperature Determination Results and Discussion

Heat produced from flames was shown to decrease rapidly with all extinguishment methods (Table 2). F-500 and AFFF seemed to produce approximately the same drop in flame temperature largely due to the heat capacity of the water carrier used for both applications. Heat was dissipated from flames within 10 to 30 seconds.

Following this test the ability for F-500 and AFFF to dissipate heat from heated metal was investigated. After the initial radiant cooling phase, the foam blanket appeared to act as an insulator, retaining the heat in the metal. After one minutes the temperature of the metal covered with AFFF was above 300 C. F-500 addition to the metal produced temperature decreases to less than 100 C within seconds (Table 3). This data shows the ability of F-500 to rapidly dissipate heat from flames and heated surfaces.

ELECTRICAL TESTS

Electrical Test Method

A hand held electrical multi meter was used to measure resistance, voltage, and amperage across a circuit containing a normal 115 V electrical outlet, a multimeter, and a networked ground. The voltmeter was capable of measuring 100 MΩ, 0.1 V and 0.1 A. An empty aerosol can was placed in the circuit between the voltmeter and the electrical outlet to insure that constant voltage and low resistance were maintained. The circuit was then broken between the aerosol can and the electrical outlet. A full aerosol can of F-500 was then connected to the voltmeter and placed twelve inches from the electrical outlet. The F-500 was dispensed into the electrical outlet, and electrical measurements were made for five seconds and recorded manually. Each measurement was done in triplicate. The experimenter wore fire resistant gloves and rubber boots to minimize potential hazards from electrical shock.

Electrical Test Results and Discussion

The voltage measured at the outlet was 118.6 V and the amperage across the circuit without the F-500 Aerosol Can fire extinguisher was 5.5 A. Resistance across the same circuit was not measurable. Reference voltages and resistances from the laboratory bench top through the voltmeter to ground was 54.8 MΩ. Spray directed into the electrical socket produced 0.0 A and 0.46$\pm$0.07V. The accompanying resistance was 32.3$\pm$10 MΩ. This resistance is extremely high and approaches that between an epoxy resin bench top and ground. Measured electrical data demonstrate that electric conductance should not occur between household electrical outlets and normally operated F-500 Aerosol Cans. This test was intended to determine the potential for electrical shock in the course of extinguishing a grease of other nonelectrically fire.

Table 2. Temperature reduction of metal surfaces engulfed in flames and extinguished by several processes.

	Temperature (F) during the experimental time course				
Substances	Before Extinguish	10 Sec After	30 Sec After	45 Sec After	60 Sec After
F-500 1	1211	311	194	NA	NA
F-500 2	1299	360	208	NA	NA
F-500 3	1274	235	131	NA	NA
F-500 4	1270	333	189	NA	NA
F-500 5	1312	392	210	NA	NA
MEAN	1273	326	186	NA	NA
STD.DEV.	67	85	61	NA	NA
Carbon Dioxide 1	1254	577	315	221	178
Carbon Dioxide 2	1256	523	288	217	194
Carbon Dioxide 3	1339	577	266	201	NA
Carbon Dioxide 4	1276	549	273	214	183
Carbon Dioxide 5	1333	626	280	203	NA
MEAN	1292	571	284	211	185
STD.DEV.	37	34	17	8	7
Halon 1	1380	712	477	334	208
Halon 2	1369	500	338	244	183
Halon 3	1308	563	316	284	237
Halon 4	1339	680	316	262	199
Halon 5	1308	648	340	262	210
MEAN	1341	621	357	278	208
STD.DEV.	30	78	60	31	18
Dry Powder 1	1022	430	239	167	NA
Dry Powder 2	1333	444	244	183	NA
Dry Powder 3	1342	500	219	158	NA
Dry Powder 4	1360	534	234	180	NA
Dry Powder 5	1306	491	239	165	NA
MEAN	1273	480	235	171	NA
STD.DEV.	127	38	9	9	NA
AFFF 1	1269	338	171	NA	NA
AFFF 2	1267	320	154	NA	NA
AFFF 3	1285	374	165	NA	NA
AFFF 4	1337	500	228	162	NA
AFFF 5	1308	446	219	162	NA
MEAN	1293	396	188	162	NA
STD.DEV.	27	68	30	NA	NA

Table 3. Temperature profiles of heated metal for one minute following treatment with fire suppressants.

Substances	Before Extinguish	1 Seconds After	10 Seconds After	20 Seconds After	30 Seconds After	40 Seconds After	50 Seconds After	60 Seconds After
F-500 1	1071	126	118	90	77	75	75	72
F-500 2	1164	140	97	88	81	81	77	72
F-500 3	1134	127	138	102	100	86	84	79
F-500 4	1146	117	151	104	88	91	88	86
F-500 5	1112	131	140	111	93	91	90	88
F-500 6	1164	120	158	100	99	99	91	93
F-500 7	1326	120	162	109	104	102	102	99
F-500 8	1148	131	158	106	106	97	99	99
MEAN	1158	127	140	101	93	90	88	86
STD.DEV.	70	7	21	8	10	9	9	10
AFFF 1	1630	948	752	671	604	572	210	210
AFFF 2	1238	554	748	720	619	540	448	374
AFFF 3	1220	966	729	734	874	775	466	406
AFFF 4	1256	966	941	729	925	892	775	774
AFFF 5	1045	1094	973	806	873	907	887	577
AFFF 6	1022	882	741	1006	1024	921	928	892
AFFF 7	1216	1067	1036	1123	1044	1051	1024	775
MEAN	1233	925	846	827	852	808	677	573
STD.DEV.	185	166	122	158	164	176	281	234

The experiment did not determine the ability of such an extinguisher to extinguish an electrical fire without risk of electrical shock.

VAPOR REDUCTION

Vapor Reduction Methods

Approximately 1 ml of hexane was added to a 250 ml round bottom flask which was being flushed with helium gas at 50 ml/min. The system was open to the atmosphere. Hexane vapors were measured from the headspace of the flask using a 10 µl syringe. Collected vapors were analyzed using a capillary column gas chromatograph with flame ionization detection. This system was capable of measuring hexane vapors at 2.5 minute time intervals. The experiment was repeated four times to determine the behavior of hexane in the system. In four additional analyses, 1 ml of F-500 was sprayed into the headspace of the flask. This addition occurred 3 minutes following hexane introduction into the system.

Acetone was tested in a similar situation. The exception being that acetone was analyzed every 3 minutes, beginning 1.0 minutes after introducing the acetone into the system. This procedure was repeated four times to evaluate the behavior of acetone in the test system. In four additional analyses, 1.6 ml of F-500 was sprayed into the headspace of the flask. This addition occurred 0.5 minutes after acetone introduction.

Vapor Reduction Results and Discussion

Hexane vapors were found to persist for nearly 10 minutes in the experimental system utilized. High concentrations were maintained for 5 minutes. F-500 addition to these hexane vapors lowered concentrations by approximately 38% in ten seconds and by more than 96% within two and one half minutes of addition (Figure 6). This reduction

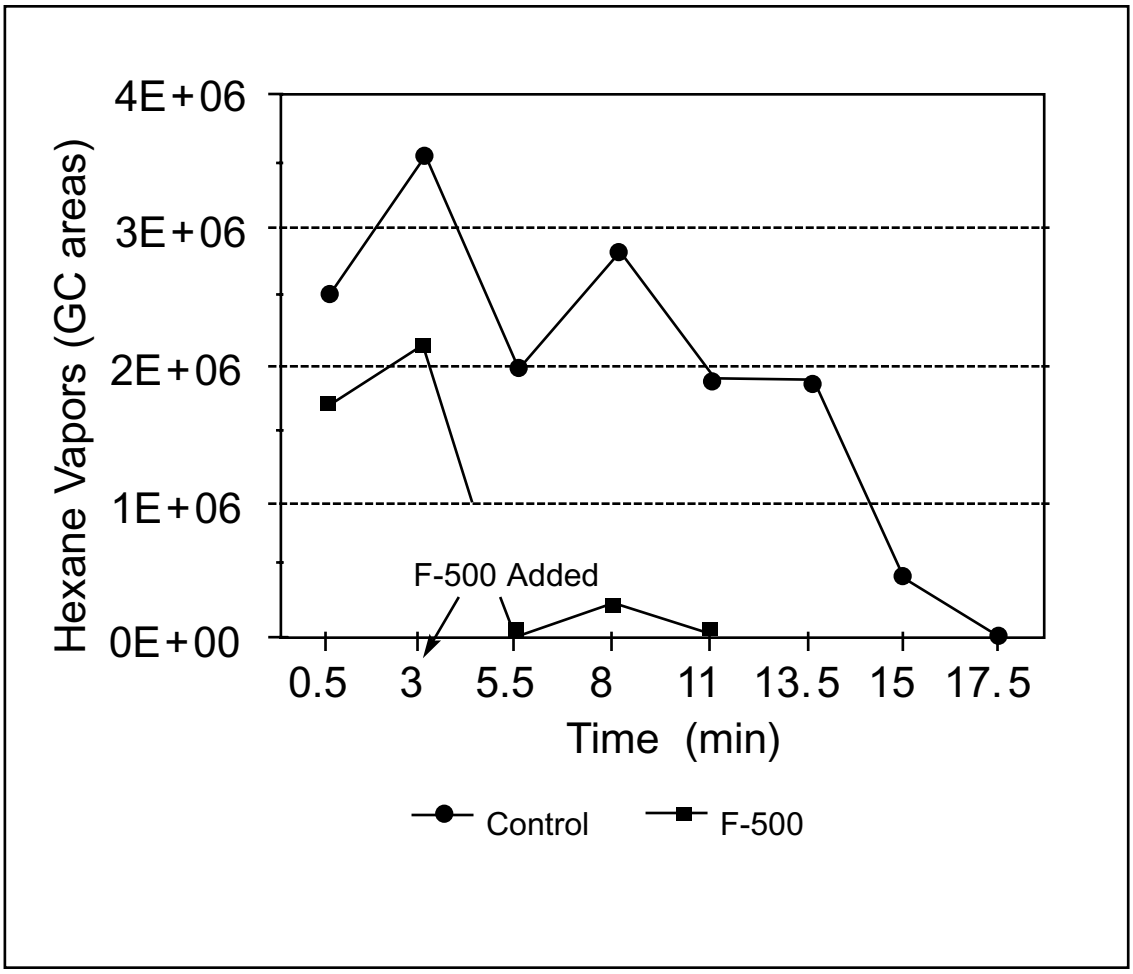

Figure 6. Effect of F-500 on vapors from 1 ml of hexane within a 250 ml round bottom flask receiving 50 ml/minute of inert gas

was maintained for the duration of the hexane residence time in the flask. These data indicate application of aqueous F-500 to flammable liquids or to their vapors can eliminate flammability concerns which arise from hydrocarbons. Acetone vapors were similarly reduced following treatment with F-500 (Figure 7). The time required for reduction of acetone was approximately 30 seconds. In this time interval, acetone vapors were reduced by 61%. The overall vapor reduction of acetone over the duration of the experiment was greater than 85%. Data were generated as fast as the gas chromatograph could reliably produce data.

PHYSICAL CHARACTERISTICS DISCUSSION

There are two primary benefits that should be obtained when treating oils with F-500. Both benefits are achieved as F-500 dissolves oils/fuels into water. Oils/Fuels dissolved into water are dispersed and the vapors from the fuel are not as concentrated. These two characteristics of F-500 have been determined in our laboratories and in some instances by government agencies in the United States of America.

Vapor reduction has been found by the State of Georgia, USA. Tanker truck spills of gasoline in Georgia, USA have been handled with F-500 to insure the safety of Emergency Response Teams. Following one accident, hydrocarbon vapors far exceeded the lower explosive limit (LEL). Vapor concentrations were reduced to <1% of the LEL within minutes of treatment. Vapor concentrations of combustible materials are always measured during fuel spills to insure worker safety, and in all instances where F-500 was used, combustible vapors were significantly reduced. F-500 also reduces the amount and toxicity of smoke from hydrocarbon fires.

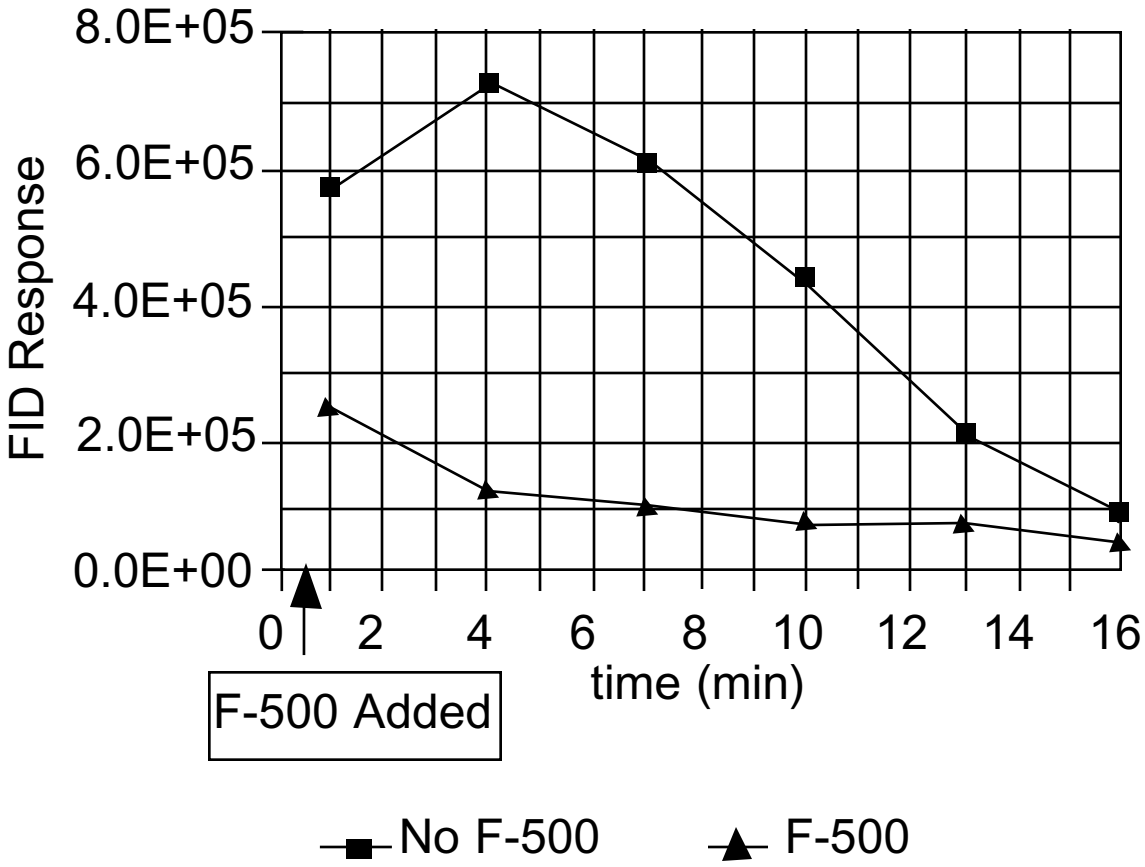

Figure 7. Effect of F-500 on vapors from 1 ml of acetone in a 250 ml round bottom flask receiving 50 ml/min inert gas.

Minimal corrosivity is also a benefit of using F-500. Corrosion of steel is much slower in F-500 products than in AFFF. Addition of F-500 products to water does not significantly increase the corrosion of carbon steel. This is true in fresh water and in sea water. This indicates no significant change in the ionic strength of water following the introduction of F-500. This hypothesis is confirmed by the fact that aerosol dispensers of F-500 did not conduct electricity even when dispensed directly into operational electric outlets.

BIODEGRADATION

Biodegradation Method

Biodegradation of diesel fuel with and without surfactant products was determined. Naturally occurring microbes from pesticide treated soil was collected from the SC Coastal Experiment Station in Charleston, SC. Experiments were conducted using 15 L cylindrical test chambers with appropriate humidified air inlets and outlets. Six containers received 3 kg of soil and 50 ml of diesel fuel. F-500 was added to four containers and two were left with diesel only. Degraded organic compounds were sampled through collection of head space gases on carbon traps and by extraction of diesel components from soil. Hydrocarbons were determined by GC-FID. Follow up research concerning anthracene degradation was performed and is provided in the attached thesis (Appendix 1: Pattanayek, 1996).

Enhanced Bioremediation:

Soil microbes have been characterized in soils from the SC Coastal Experiment Station. These soils have suitable microorganisms at population densities of 10,000/g soil. Microbial degradation studies were conducted using these soils. After the initial equilibration period of 12 hours, a significant decrease in vapors was observed in the

headspace of control chambers for twelve hours (t=12 hrs to t=24 hrs) in treatment and control chambers. During this time period, vapors from the headspace of F-500 treatments were also significantly lower than the vapors from the control chambers. Vapor release became more sporadic during the next 24 hours (t=Day 1 to t=Day 2), and no differences were noted in diesel vapors during that time interval. Overall we have seen equivalent diesel vapor generation for 8 days from the two treatments (Figure 8). It is interesting to note that diesel vapors from the F-500 treatments were lower than those from controls on each day except Day 2. However, this single datum eliminates any significance to the overall observations for the experiment.

Minimal vapor reduction is a reasonable finding in this instance as there was a great excess (30X) of fuel compared to F-500 in this study and the head space of the chambers contained significant amounts of vapors prior to F-500 addition. Normal ratios of Fuel to F-500 during spill clean-up are 1:1. Our goal in this experiment was to measure effects manifested through addition of minimal amounts of F-500 to fuel laden soils. Other tests in this report have determined significant vapor reduction following fuel treatment with F-500.

The chemical composition of F-500 indicates enhanced chemical availability to microbes may be possible through addition of F-500 to contaminated sludge or soils. During 8 days of testing, significant differences were not observed (Figure 9). However, hydrocarbon degradations by native microbes were enhanced in later dates of the evaluation. This result is reasonable as enhanced chemical degradation by naturally occurring microbes usually requires one to two weeks of experiments of this type (Scholtz et al., 1988). Recently, two days of seminars described possibilities of surfactant assisted chemical remediation of organic contaminants (ACS, 1994).

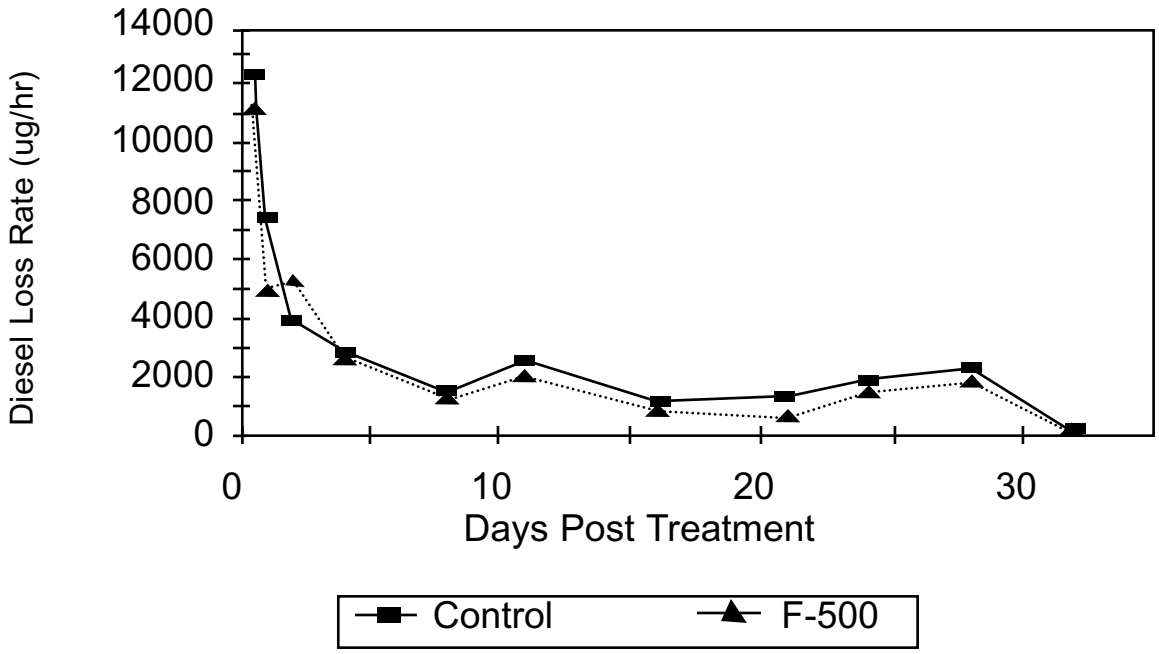

Figure 8. Diesel vapor released from clay loam soil with and without F-500 addition

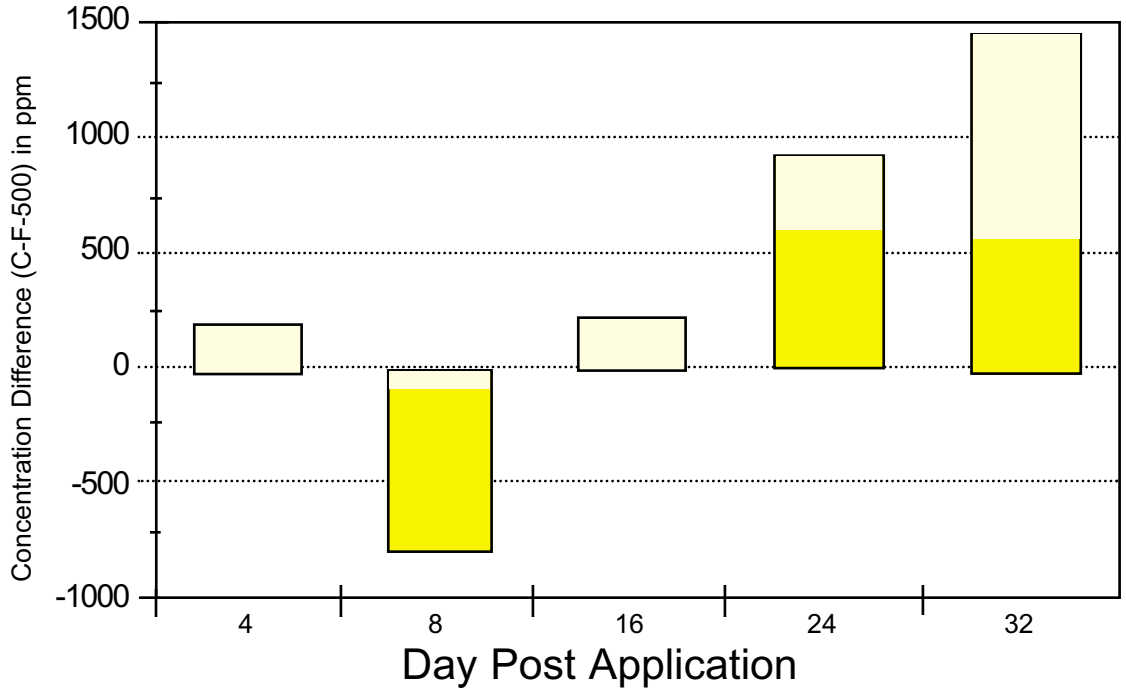

Figure 9. Effect of F-500 application on the natural degradation of #2 diesel in clay loam soils during controlled laboratory studies.

Presentations indicated a major role for surfactants in future hazardous waste management strategies.

During anthracene degradation studies, small but significant amounts of anthracene were mineralized (Appendix 1). A low concentration of F-500 was chosen to test the effect of F-500 on remediation. The concentration of F-500 was less than its; CMC which enhances microbial degradation (Aronstein and Alexander, 1992), Aronstein et al., 1991). The microbial population used in this study was 1.25×10^6 cfu/ml, which was on the lower end of the recommended scale. Thus, a very high surfactant concentration was not used in order to minimize the inhibition of anthracene degraders. Using F-500 at low concentrations my have minimized the possibility of competition between the surfactant and the contaminant as a nutrient source by microorganisms (the concentrations of anthracene added in the F-500-treatment bioreactor are approximately 1.19×10^{-2} M in soil and 4.82×10^{-2} M in aqueous media) this effect was observed in Tiehm's study (1994), where PAH degradation was inhibited because anionic surfactant sodium dodecyl sulfate (SDS) was preferred as a growth substrate. However, the F-500 concentration used was less than the CMC which would also minimize micelle formation.

MICROBIAL TOXICITY

Data presented here represent a condensed version of work by Pattanayek (1996). Further details can be obtained therein (Appendix 1).

Microbial Toxicity Method

Anthracene (0.05 g : 99+% *Sigma Chemical*) as a solution in 10 ml methylene chloride was place into a sterile 250 ml Erlenmeyer flask of culture flask and the solution was evaporated to dryness using nitrogen gas. To this culture flask was added salts

media which was prepared according to Gray et al. (1994). Soil (3.5 g) was then inoculated into the culture flask, plugged with a cotton plug and shaken in an orbit shaker (Lab-Line) at 200 rpm for 7 days at room temperature. The soil-salts media mixture was then allowed to settle and the supernatant (5 ml) containing soil microorganisms was transferred into fresh salts media (100 ml) and further cultures were maintained by periodic transfer into fresh media every 3 weeks where anthracene was the sole carbon source. Colony counts where monitored by serial dilution and viable plate count technique (Collins and Lyne, 1985).

Microbial Toxicity Results and Discussion

The respiration inhibition tests on activated sludge microorganisms using F-500 revealed the effective concentration range of F-500 in inhibit 50% (EC50) of the rate of respiration when compared to control (no F-500) was between 2000 and 3000 mg/L. These concentration corresponds to 0.012 and 0.017 M.

Enriched soil microorganism biomass could not be brought up to levels comparable to activated sludge in order to determine toxicity of F-500. Therefore, the EC_{50} range of F-500 for activated sludge microorganisms was presume to be the EC_{50} range for the soil microorganisms. Toxicity tests on microorganism are also performed by plate count or spectrophotometric or tubidometric methods, but it has been suggested that these methods may not be adequate in determining toxicity. Remediation is mostly concerned with oxidation capacity of microorganisms rather than viability because degradation and respiration both involve the utilization of oxygen to produce energy.

F-500 concentrations used in both Bioremediation studies (BAS and BAA) caused respiration inhibition of approximately 7% of the microbial population. Some growth of damage to cellular components may also have occurred. However, the inhibitory effect

of surfactants on microorganisms does depend on specific environmental conditions (Talmage, 1994), the type of acclimation history (Talmage, 1994) of bacteria, and the presence of other nutrient factors. If F-500 is used for remediation purposes in the field, the other external factors also come in to play. Rain, sunlight and weathering actions can also alter the effect of surfactant toxicity. F-500 if compared to general nonionic surfactants, would most likely undergo rapid primary and ultimate biodegradation under aerobic conditions. According to Swisher (1987), surfactant biodegradation under anaerobic conditions relatively slow. Therefore, use of nonionic surfactants in biodegradation under aerobic conditions should be an agent of choice in future remediation studies, where nonionic surfactants would serve the purpose of cleaning up contaminated sites, without causing any degenerative effect themselves.

RAT TOXICITY TEST

Data presented in this section represent an overview of the work by Hunt (1995). Greater detail can be obtained from the report in its entirety (Appendix 2).

Rat Toxicity Test Method

A single dose of 5 g/kg of compound was administered as required by FDA Toxicity Limit Tests for compounds to be used in items to be consumed by humans. F-500 was administered by oral gavage at a rate of 5 ml/kg. Since the density of the compound was 1 g/ml, the compound was administered neat according to the weight of the animal. The vehicle control was water. Rats were fasted overnight (>16 hrs) prior to test substance administration.

Rats were monitored closely the first two hours post-dose, and then at least two more times the day of dose. Thereafter, each animal was observed at least once daily to

determine general health and survival. Cage-side observations following dosing included an evaluation of the animal's skin, fur, eyes, fecal droppings, activity, and general behavior at that point in time. Particular attention was given to observation of possible symptoms of exposure including tremors, convulsions, lethargy, salivation, and diarrhea. Rats were handled, as required, to evaluate their physical condition.

Results and Discussion

Administration of the test substance was found to cause no compound-related mortality, no abnormal behavioral manifestations, no differences in mean weights between treated and control groups, and no gross pathologic changes upon necropsy. Symptoms of compound associated diarrhea disappeared by 48 hours post-dose in all affected animals, and observed respiratory symptoms were most likely a result of aspiration of a small amount of the test substance into the lungs rather than a toxic response. Based on the results of survival, cage-side observations, and gross necropsies, the limit test was met and no further LD50 testing is necessary.

These tests indicate that F-500 could be used as an ingredient in items to be consumed by humans. Although this is not the intended use of F-500, it does represent a worst case acute exposure scenario and indicates that F-500 does not cause mammalian mortality.

Aquatic Toxicity Tests

Introduction

Aquatic toxicity tests are desirable for newly formulated chemical products in order to insure protection of aquatic resources when the chemicals are released into aquatic ecosystems. Chemicals released into water bodies may effect vertebrate or invertebrate fauna. Thus both types of organisms should be tested. Tests should be performed with species for which large data bases are available for comparative toxicology evaluation. Commonly used test species are *Daphnia sp., Chironomus sp., Oncorhynchus mykiss* and *Pimephales promelas*.

Methods

Static toxicity tests were performed over 96 hour periods to determine the LC50 of F-500 to juvenile fathead minnows (*Pimephales promelas*) and daphnia. Both species were maintained in reconstituted moderately hard water as specified by USEPA. Tests were maintained at 22±1 C and a neutral pH with a 12 hour light/12 hour dark cycle. Test organisms were fed and then acclimated to the test system for 24 hours before being exposed to varying concentrations of aqueous F-500. No food was provided to test organisms for the remainder of the test. A control and five dose groups were tested. Each minnow test utilized 10 fish per treatment and each individual was placed into a separate test chamber for a total of 10 fish per dose group. For each daphnia treatment group, five individuals were placed into each of five test containers for a total of 25 organisms per dose group. Toxicant addition to each chamber within a dose group was made from a common solution. Four day old minnows were obtained from a commercial source and were used because this life stage of the minnow has been determined to be

the most sensitive for this and similar fish species. One to two day old daphnia were used as they are most sensitive life stages for such aquatic invertebrates.

F-500 was added to water as a concentrate. HPLC with UV detection was calibrated with at least 5 solutions containing F-500 spanning the concentration range 50 to 500 ppm. The calibrated HPLC system was used to determine F-500 concentrations in stock solutions from which individual dosages were serially diluted. Concentrations reported are based on the mass of concentrate added per volume of water. During the tests the number of living organisms was determined daily. Daily mortality records allows us to determine 24, 48, 72, and 96 hour LC50s during a single experiment. Shorter term LC50s are important to know as the use pattern of F-500 and the frequency of use would indicate short residence time in natural waters. Data were transformed to log probit units for LC50 determination.

Results and Discussion

Four day old fat head minnows exhibited LC50s that ranged from 920 to 1490 µg/L (Table 4), which are considered slightly toxic. These data indicate short exposures to F-500 produce lower toxicity to these sensitive life stages than do longer exposures. Our results are interesting in view of previous data concerning F-500 toxicity to adult mummichogs. In these tests, F-500 was not toxic until concentrations reached near percent concentrations. There are two possible explanations for the large differences in the fathead and mummichog. First and most importantly, the life stages are different and the adult fish would be expected to be more resistant to toxicant effects. The minnows we used were tested during their transition from endogenous to exogenous feeding, and the fish were not fed during the test. During the transition in caloral use, fish undergo additional stress which makes them most susceptible to toxicants. This is partially

because the fish are attempting for the first time to acquire for food that was not already a part of their body. Second, the fathead and mummichog are adapted to waters of different salinities which causes fish to use different osmoregulatory strategies and thus toxicants will effect salt water and fresh water species differently. Moreover, more salinity and high pH of the water used for the mummichog evaluation may have altered the charge distribution or molecular conformation of the F-500 rendering it less toxic to fish.

Table 4. LC50 data for fathead minnows (*Pimephales promelas*) exposed to F-500 in moderately hard water.

TRT (mg/L)	24 hour Mortality	% Mort	48 hour Mortality	% Mort	72 hour Mortality	% Mort	96 hour Mortality	% Mort
0	1	0.1	1	0.1	1	0.1	1	0.1
100	0	0	0	0	0	0	0	0
1000	1	0.1	3	0.3	4	0.4	4	0.4
1,670	6	0.6	8	0.8	9	0.9	10	1
2,780	7	0.7	9	0.9	9	0.9	9	0.9
4,630	10	1	10	1	10	1	10	1
LC50	1490 µg/L		1200 µg/L		1110 µg/L		920 µg/L	

Toxicity tests using daphnia neonates produced interesting results. Concentrations used were spanned a range of 0.036 to 0.28 µg/L. The highest observer mortality was 56% and this occurred at 96 hours in the lowest concentration treatment. Decreasing mortality occurred in the higher dose groups. This led to the assumption that treatments were mislabelled. In data analysis the treatment designations were reversed and the control remained as originally designated. This data analysis produced a good LC50 curve (r2=0.81) which predicted an LC50 of 0.25 µg/L. This estimate indicates

daphnia neonates were succeptable to F-500 at lower concentrations than were juvenile minnows (Table 4). It appears that F-500 is 4 times more toxic to daphnia neonates than to the juvenile fatheads. Such relative toxicities are well known for surfactants. Surfactants efficiently weaken or dissolve the chitin exoskeletons and of aquatic invertebrates thereby disrupting osmoregulation. This process is most pronounced when insects have poorly developed exoskeletons which occurs during molting and just after hatching. Having provided these comparisons of toxicities, we must suggest that additional studies be conducted to better define the 96 hour LC50 of F-500 to *daphnia magna*.

Toxicity tests presented in this document were designed to produce toxicity at the lowest possible concentration. Juvenile life stages that are just changing from indogenous to exogenous feeding are extremely succeptable to xenobiotic toxicants. This is generally true because protective enzyme systems are not fully developed. Also, test organisms were not fed following exposure to F-500. Caloral stress experience by test organisms for the four day test duration increases the adverse effects of xenobiotics. It may seem that such caloral stress is unnecessary and should be eliminated from test protocols. However, the toxicants are likely to adsorb to food from the water. This may increase exposure if all food is consumed. Conversely, if toxicants produce avoidance of the food or if all food is simply not consumed, toxicant exposure would be decreased. Thus withholding food during tests is the best means of normalizing exposures to the numerous types of chemicals that must be tested with these protocols.

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APPENDIX A

BIOREMEDIATION OF ANTHRACENE CONTAMINATED SOIL USING A NONIONIC SURFACTANT F-500Ò

A Thesis

Presented to

the Graduate School of

Clemson University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Environmental Toxicology

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by

Mala Pattanayek

December 1996

ABSTRACT

Surfactants enable the mass transfer of nonpolar compounds from hydrophobic phases to hydrophilic phases, thereby increasing the bioavailability of these compounds to microorganisms for degradation. Low concentrations of nonionic surfactants can enhance biodegradation of polycyclic aromatic hydrocarbons (PAHs) by increasing their solubility. A nonionic surfactant, F-500®, was combined with enriched indigenous soil microorganisms to bioremediate soil containing the anthracene. F-500 was used at concentrations below its critical micellar concentration (CMC) which was determined by surface tension method. This study comprised a control group which accounted for abiotic degradation of anthracene, and two treatment groups, one, with microorganisms, and the other with microorganisms and F-500. F-500 enhanced the mineralization of anthracene in soil by 50% compared to treatment without F-500 and by 96% compared to control. However, the total amount of anthracene remediated from soil was less than 1%. The effect of soil on the bioavailability of anthracene was also determined by measuring the amount of anthracene minearlized in aqueous media. F-500 did not enhance mineralization of anthracene in aqueous media compared to control or treatment without F-500. The total amount of anthracene mineralized in aqueous media was approximately 2% which was still low, but significantly greater than the amount mineralized in soil. Mineralization rates and half-life of anthracene in soil and aqueous media were also determined which revealed that anthracene bound to soil and was rendered less bioavailable for microbial degradation. Enhanced mineralization of anthracene from soil indicated that F-500 aided the desorption of anthracene from the soil matrix. The toxic range of F-500 on microorganisms was also determined by OECD method using activated sludge as the test organisms. The EC_{50} range of F-500 was

calculated between 2000 and 3000 mg/L. The concentration of F-500 used in the remediation studies was significantly below the EC_{50} range.

DEDICATION

I dedicate my thesis to the following people who made everything seem possible; my parents Sandhya and Nimai Pattanayek, for having faith in me all along and, for giving me this rare opportunity to come to the other side of the globe for higher education; my sister Nupur Pattanayek, who gave me extraordinary encouragement in many ways and last but not least, Rochna Dhand who supported, encouraged and most of all put up with my various mood changes throughout my entire graduate life at Clemson.

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CHAPTER I

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are incomplete combustion products of fossil fuels (Tiehm, 1994), toxic in nature and may be hazardous to the environment. Significant quantities of PAHs can enter the environment through various modes including, accidental gasoline spills, fires, and domestic and industrial waste effluent. PAH containing compounds like petroleum, can migrate through soil columns reaching groundwater where they can reside for long periods of time (Robinson et al., 1990). PAHs are non-polar in nature and bind to the soil matrix, making their complete physical removal extremely difficult (Robinson et al., 1990).

Various biological (Gray et al., 1994) and physical techniques have been developed to decontaminate polluted soils and groundwater. These techniques include incineration, solidification and solvent extraction (Comeau et al., 1993). Bioremediation, a biological method of decontamination, is considered highly efficient and cost-effective (Cutright and Lee, 1994). Bioremediation of soils contaminated with PAHs is an alternative method of detoxifying hazardous waste, which may replace traditional methods such as incineration (Aronstein et al., 1991, Cerniglia 1992). In order to validate bioremediation as a viable decontamination method, we need to develop a system which can optimize degradation rates of contaminants.

It has been suggested that only PAHs in a soluble form can be utilized by microorganisms as an energy source (Aronstein et al., 1991). Bacteria are capable of synthesizing surfactants, commonly known as bio surfactants (West and Harwell, 1992) which act as emulsifying agents (Thomas et al., 1986), to increase the solubility of non-polar compounds. Primary classes of bio surfactants include glycolipids, amino-acid containing lipids, phospholipids, fatty acids and peptides. However, a large amount of

energy is expended by microorganisms to disperse (Thomas et al., 1986) non-polar compounds.

Surfactants, being amphiphilic in nature, mediate between immiscible phases and are known to increase concentrations of hydrophobic compounds in the aqueous phase (Tiehm, 1994). At high concentrations, surfactants have been reported to solubilize non-polar organic compounds in soil, or soil water mixtures (Aronstein and Alexander, 1992) making them available for degradation by microorganisms. However, use of high concentrations of surfactants is expensive (Doscher, 1977) and can also inhibit the viability of microorganisms (Cserháti et al., 1991). Surfactants can disrupt the cellular membrane of microorganisms by interacting with the lipid components of the cell (phospholipid bilayer) and by reacting with enzyme and cellular proteins required for proper functioning of microbes (Swisher, 1987). A study conducted by Aronstein and Alexander (1992) reported that non-ionic surfactants used at concentrations slightly above or slightly below the critical micellar concentration (CMC) enhanced biodegradation of aromatic hydrocarbons in soil.

The fate of a contaminant is also determined by the presence of soil (Walton and Anderson, 1988) in a system. Hydrocarbons can partition into the organic matter of soil (Wu and Gschwend, 1986) and be rendered non-bioavailable to the microorganisms for degradation. If hydrocarbons can bind to soil, then one would expect hydrocarbon mineralization half-life to be greater in the presence of soil than in the absence of soil (e.g. in aqueous media).

Nonionic surfactants are more susceptible to microbial degradation and show less antibacterial activity than cationic and anionic surfactants (Laha and Luthy, 1992). Toxicity of surfactants to microorganisms can be determined by measuring the effect of surfactants on their respiration rate (Kleèka et al., 1985). The ability of microorganisms to

degrade organic compounds and utilize them as an energy source depends on the viability of the microbial population. The viability of microbial populations can be determined by measruing their oxygen consumption or respiration rates (Kleèka et al., 1985).

A study was conducted in order to test the ability of nonionic surfactant, F-500, to enhance remediation of soil containing anthracene. The effect of soil on the bioavailability of anthracene to microorganisms was also investigated by remediating aqueous media containing anthracene. By comparing percent mineralization and mineralization half-life of anthracene in soil and aqueous media, the effect of soil in bioavailability was determined. The toxic range of F-500 on microorganisms was determined by respiration inhibition tests using activated sludge as test organisms.

CHAPTER II

RESEARCH OBJECTIVES

The objectives of this research were to:

1. To determine whether nonionic surfactant, F-500, enhanced the bioremediation of soil containing anthracene.

2. To determine the effect of soil on bioavailability of anthracene for microbial degradation.

3. To determine the toxic range of F-500 on microorganisms.

CHAPTER III

LITERATURE REVIEW

Anthracene

Anthracene is a tricyclic aromatic hydrocarbon (PAH) with a molecular of weight of 178.22 g/mole and a relatively low water solubility, ranging from 0.041 to 0.075 mg/L at 25° C (May and Wasik, 1978). Table I lists other physical properties of anthracene. Anthracene is a product of incomplete combustion of organic matters, fossil fuel, crude oil, fire, oil spills and waste incinerations. When pure, anthracene is colorless with violet fluorescence; when impure, it is yellow with green fluorescence (Boudavari et al., 1989).

Table I

Physical Properties of Anthracene

Anthracene	Mol. Wt. (g/mole)	Specific Density d^{27}_4	Melting Point (mp)	Boiling Point (bp_{760})	Absorption spectra (nm)
$C_{14}H_{10}$	178.22	1.25	218° C	342° C	506

(Boudavari et al., 1989)

As combustion products, PAHs are ubiquitous (Means et al., 1980) in nature and thus are found widely distributed in various contaminated sites. Pollution by PAHs is mainly associated with obtaining, transporting, processing, combusting and disposing of fossil fuels. Anthracene enters the environment via the atmosphere, terrestrial runoff and various industrial sources, where rapid association with particulate matter and suspended sediments occurs (Helmstetter and Alden, 1994). The low water solubility of anthracene and its high affinity for organic particulate material causes its accumulation and persistence in the benthos. The resonance energy of anthracene along with a large

hydrophobic molecular structure results in its thermodynamic stability (Weissenfels et al., 1990), thus hindering its degradation.

Degradation of Anthracene

The fate of PAHs are a major environmental concern as they or their transformation products are toxic in nature and are considered as potential carcinogens and mutagens (Pahlmann and Pelkonen, 1987). Anthracene itself is not carcinogenic (Williams and Weisburger, 1991) but is phototoxic (Holst and Geisy, 1989). Anthracene exhibits median levels of photo-induced, acute toxicity to aquatic organisms relative to other PAHs (Oris and Geisy, 1986). A structure-toxicity relationship has been developed which suggests that anthracene is excited to a triplet state by absorbing light energy. This excited anthracene undergoes photochemical reactions returning to ground state by transferring energy to molecular oxygen (Oris and Geisy, 1986). Singlet oxygen may be formed in this manner which has been hypothesized to cause respiratory membrane damage (Oris and Geisy, 1985).

PAHs which can become ultimate carcinogens are derived from an angular benz[a]anthracene skeleton (Williams and Weisburger, 1991, Mahaffey et al., 1988) because molecules with a bay region are preferentially oxidized to bay region epoxide diols (Jerina et al., 1984). Carcinogenesis results due to electrophilic intermediates formed during PAH metabolism (Thakker et al., 1985) which then form adducts with DNA through intercalation between adjacent base pairs (Zylstra et al., 1994, dell'Omo and Lauwreys, 1993).

PAHs are susceptible to biodegradation in the environment (Oost et al., 1990). The biodegradability of PAHs in the environment depends on their physical and chemical properties, their concentration and diffusion rates in soil or water, and their bioavailability

to microorganisms (Sutherland et al. 1995). The aromaticities of PAHs are characterized by high resonance energy which make them recalcitrant to microbial attack in the environment. However, there are many microorganisms known to metabolize lower molecular weight PAHs, such as naphthalene, phenanthrene and anthracene (Sutherland et al. 1995) by cleaving the benzene ring (Sims and Overcash, 1983). The rates of degradation of PAHs are inversely proportional to the number of rings in the PAH molecule (Sutherland et al., 1995). PAHs may undergo photolysis and chemical oxidation, but the major decomposition process is microbial degradation in soil (Weissenfels et al, 1990). The half-life of anthracene ranges from 1200 to 11040 hours in soil, 0.58 to 1.7 hours in surface water and 2400 to 22080 hours in groundwater (Howard et al., 1991). The recalcitrance for microbial attack increases directly with octanol:water partitioning coefficient (Kow) and inversely with aqueous solubility as desorption of PAHs with high Kow values are relatively slow and less available for microbial uptake (Aronstein et al. 1991).

In animals, oxidation products of PAHs include arene oxides catalyzed by multiple forms of cytochrome P450 monooxygenases (Cerniglia, 1992). In microorganisms, the principle aerobic metabolic pathway involves oxidation of the aromatic rings by dioxygenases to form dihydrodiols (Cerniglia, 1992) which are then transformed to diphenols and further cleaved by other dioxygenases. According to Figure 1 (Evans et al., 1965), anthracene is metabolized to anthracene cis-1,2-dihydrodiol, 1,2-dihydroxyanthracene, cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid and 2-hydroxy-3naphthaldehyde, 2-hydroxy-3-naphthoic acid, and 2,3-dihydroxynaphthalene. The intermediate, 2,3-dihydroxynaphthalene is degraded to salicylic acid and catechol and ultimately to carbon dioxide and water (Dagley and Gibson, 1965). Evans et al., (1965) proposed this mechanism of anthracene

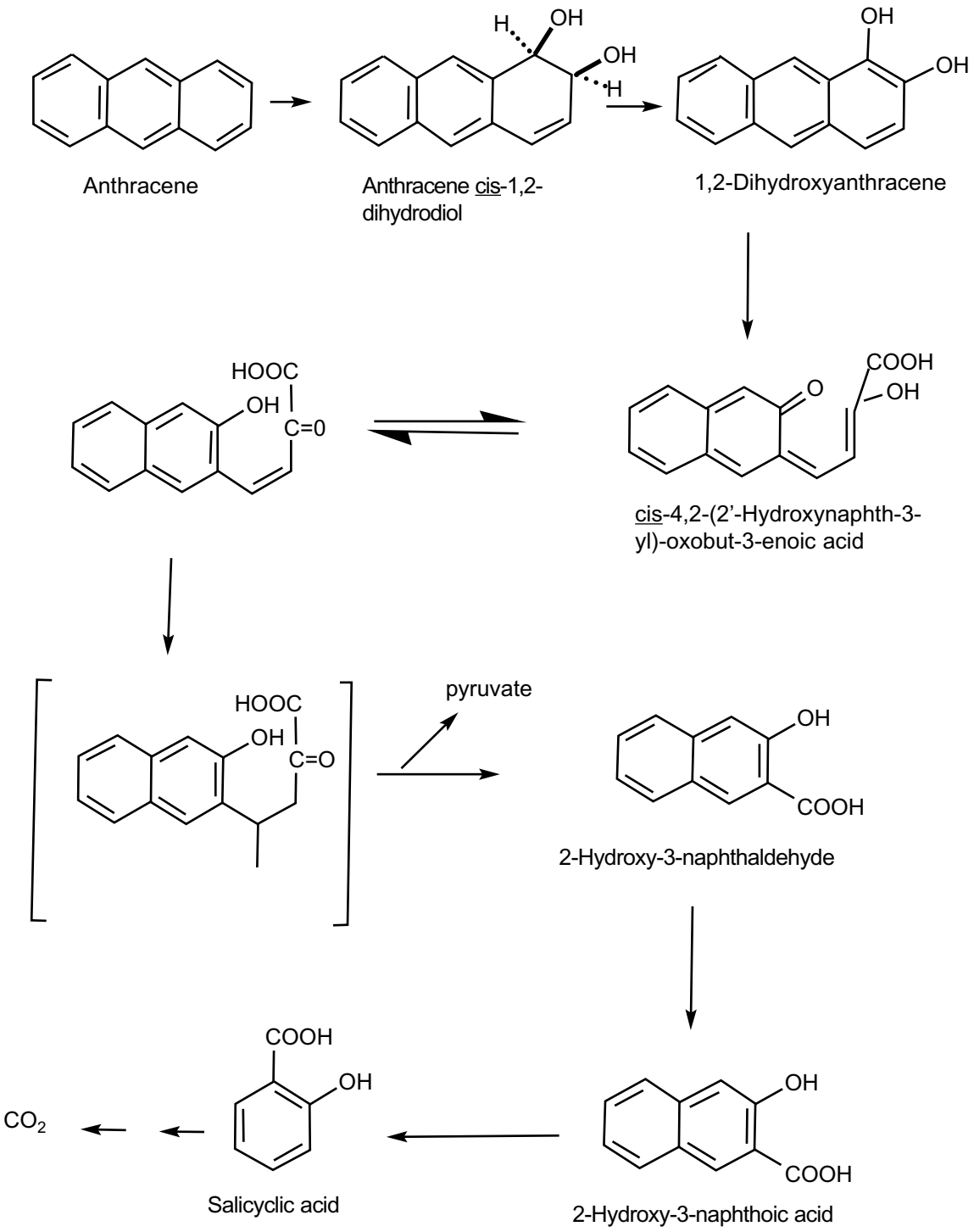

Figure 1. Pathway for Bacterial Metabolism of Anthracene by *Pseudomonas aeruginosa.* (Adapted from Evans et al.)

degradation by *Pseudomonas aeruginosa*. Some fungi, bacteria and cyanobacteria which produce cytochrome P450 monooxygenases convert PAHs to arene oxides which are then hydrated by epoxide hydrolase to form trans-dihydrodiols or rearranged enzymatically to form phenols (Sutherland, 1995). Anthracene is metabolized to trans-1,2-dihydrodiol by *Cunninghamella elegans* and *Rhizoctonia solani*. *Cunninghamella elegans*, then conjugates trans-1,2-dihydrodiol to 1-anthryl sulfate (Cerniglia 1982) and *Rhizoctonia solani* conjugates the product to three different forms of xylosides (Sutherland et al 1992). Some white-rot fungi which decay lignin and cellulose, can metabolize PAHs to quinones. The metabolism of anthracene by *Phanerochaete chrysosporium* to carbon dioxide via anthraquinone and phthalic acid (Hammel et al., 1991) is shown in Figure 2. *Bjerkandera* species and other white-rot fungi metabolize anthracene to 9,10-anthraquinone as a final product (Field et al 1992). Other microbial species that degrade anthracene are listed in Table II.

Table II

Anthracene Degrading Microorganisms

Bacteria	Fungi
Beijerinckia sp.	*Bjerkandera* sp.
Pseudomonas putida	*Cunninghamella elegans*
Pseudomonas paucimobilis	*Phanaerochaete chrysosporium*
Pseudomonas cepacia	*Ramaria* sp.
Rhodococcus sp.	*Rhizoctonia soloni*
Flavobacterium sp.	*Trametes versicolor*
Arthrobacter sp.	

(Cerniglia, 1992)

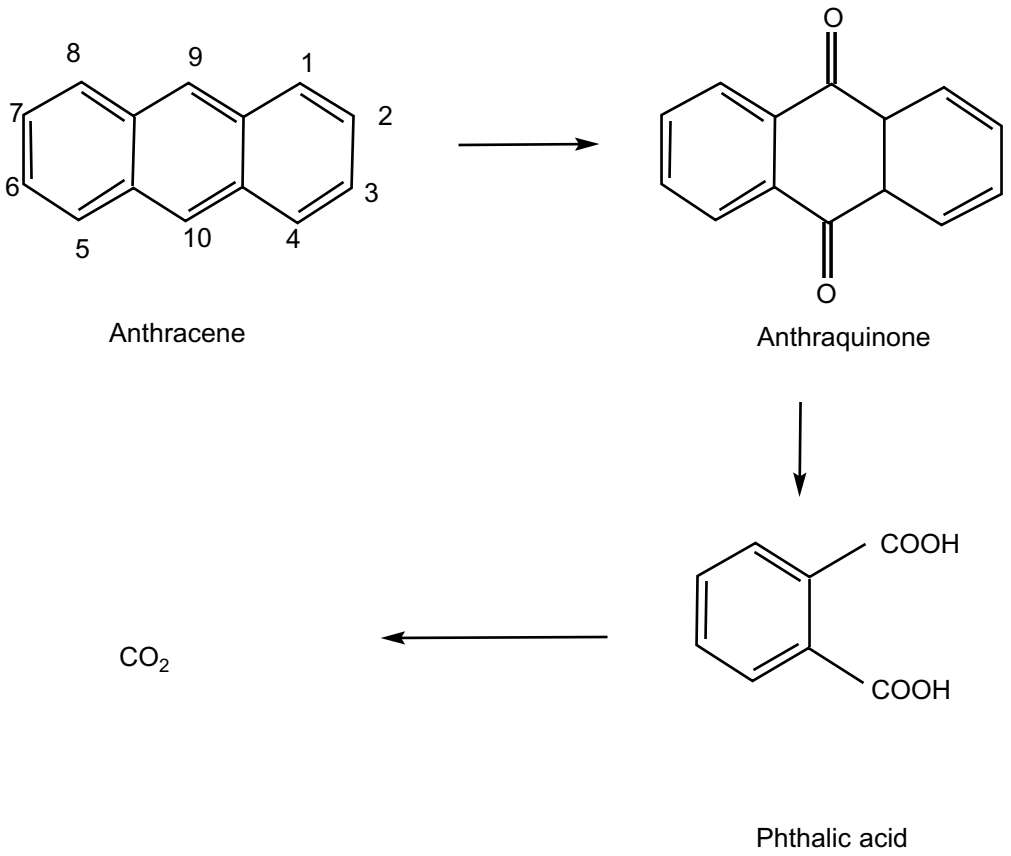

Figure 2. Oxidation of Anthracene by *Phanerochaete chrysosporium.* (Adapted from Sutherland et al, 1955).

Very little information exists on anaerobic metabolism of PAHs in soil by microorganisms (Sutherland et al., 1995). However anaerobic biodegradation of anthracene is significantly slower (4800 to 44160 hours : Howard et al., 1991) than aerobic biodegradation (1200 to 11040 hours : Howard et al., 1991).

Surfactants

Surfactants are also known as surface-active agents because they concentrate at interfacial regions like air-water, oil-water, and solid-liquid interfaces (Cross, 1987). The surface activity of surfactants is due to their amphiphilic nature. Such molecules contain one soluble and one insoluble moiety (West and Harwell, 1992). Surfactants may dissolve in water as a monomer, adsorb at an interface, or be incorporated with other surfactant molecules as a part of a micelle (Mihelcic and Luthy, 1991). In aqueous systems, a surfactant has a polar hydrophilic moiety and a non-polar hydrophobic moiety, referred as the head and tail groups, respectively (West and Harwell, 1992). The ability of surfactants to act as wetting and solubilizing agents has made them especially applicable in various industrial and commercial products such as household detergents, stabilizers for drilling mud, and emulsifiers in paints, pesticides and some foods (Donovan, 1992).

Surfactants are classified according to the nature of the hydrophilic (or head) portion of the molecule. If the head carries a negative charge, the surfactant is termed anionic. Surfactants with positively charged heads are termed cationic. Those surfactants with both positive and negative charges on their heads are termed zwitterionic and surfactants which carry no charge on their heads are termed non-ionic (West and Harwell, 1992). Prominent chemical differences between surfactants are due to their head groups (West and Harwell, 1992).

A feature unique to surfactants is the ability to aggregate into dynamic clusters, called micelles, in aqueous media (Tsomides et al., 1995). Surfactants usually exist in their monomeric form at concentrations less than a compound specific threshold value, referred to as the critical micellar concentration (CMC) (Laha and Luthy, 1992). Below this concentration some fraction of the surfactant adsorbs at system interfaces (Laha and Luthy, 1992). Micelle formation of surfactant monomers occurs above the CMC, which is specific for individual surfactants. Table III indicates that observed CMCs are different for each surfactant. The CMC represents a narrow concentration range over which the partial derivatives of many solution properties (i.e. surface tension) display abrupt changes in value with respect to surfactant concentration (Laha and Luthy, 1992). Solubilization of hydrophobic compounds commences at the CMC and is a linear function of surfactant concentration (Laha and Luthy, 1992). In a micelle, individual monomers are oriented with their hydrophilic moieties in contact with the aqueous phase and their hydrophobic moieties oriented toward the interior of the aggregate. These nonpolar moieties spontaneously associate with each other in the process of micellization to form various geometrical volumes including spheres and spheroids.

Table III

Critical Micellar Concentration (CMC) of Commercial Nonionic Surfactants
Determined at 25° C using Fisher Tensiometer

Surfactant	**CMC (mols/L)**
Brij 30	2.3×10^{-5}
Igepal CA-720	2.3×10^{-4}
Tergitol NP-10	5.4×10^{-5}
Triton X-100	1.7×10^{-4}

Note: Determined by Laha and Luthy (1991).

Under conditions where the mass transfer of the contaminant controls bioconversion, surfactants may be added to soil to enhance bioconversion. The amphiphilic nature of surfactants allows mass transfer of compounds from hydrophobic to hydrophilic phases, reducing interfacial tension and increasing dissolution rates by solubilization and emulsification (Tiehm, 1994). These effects increase chemical mobility and bioavailability to microorganisms in the soil. Transfer of anthracene (or any PAH) from the surfactant micelles to the microorganisms may occur by uptake from the water phase after diffusing from the micelles or by close contact with the microbial cell wall, perhaps accompanied by membrane fusion (Miller and Bartha, 1989).

In terms of remediation, hydrophilic heads groups of cationic surfactants adsorb strongly onto negatively charged surfaces rendering them less favorable than other classes of surfactants especially in soil and aquifer remediation. Cationic surfactants are also toxic and resistant to biodegradation (Swisher, 1987). Anionic surfactants also adsorb onto soils, but to a lesser extent than cationic surfactants. The negatively charged heads orient themselves away from the surface and extend into the solution, and as the surfactant concentration increases, the surface of the soil particle may become covered with surfactant inhibiting the surfactant from orienting flat on the soil surface. The hydrophobic moieties of the surfactants in solution then associate their hydrophobes with those already adsorbed forming a hemi-micelle. A hydrophobic organic compound can thus partition into this hydrocarbon-core of the hemi-micelle leading to increased sorption of the contaminant and limiting its availability for degradation. Nonionic surfactants sorb to a lesser extent than anionic and cationic surfactants. They are mostly non-toxic and biodegradable (Donovan, 1992), rendering them as the surfactant of choice in enhancing bioremediation. Nonionic surfactants sorb with either end oriented towards the surface. The hydrophobic moiety can sorb if a

hydrogen bond can be established with soil. Nonionic surfactants are usually comprised of a polyoxyethylene chain consisting of various quantities of ethylene oxide molecules. These surfactants are prepared by addition of ethylene oxide to compounds containing one or more active hydrogen atoms, such as alkylphenols, fatty alcohols, fatty acids, and fatty amines, rendering them polar active in nature (Donovan, 1992). Nonionic surfactants may interact with soil organics in various ways, including: hydrophobic surface interactions between the hydrocarbon chains of the surfactant and the hydrophobic regions of humic matter; by hydrogen bonding between surfactant ethoxylate groups and polar groups of humic matter; or by partitioning of the nonionic surfactant into bulk organic matter (Laha and Luthy, 1992).

Toxicity of Nonionic Surfactants

Although toxicity of nonionic surfactants are compound and species specific, a few generalizations can be made based on structure-activity relationships. Toxicity generally decreases with increasing hydrophilicity (Talmage, 1994) i.e. increasing ethylene oxide chain length. Increased ethylene oxide chain length would make the compound more polar and less fat soluble, therefore decreasing emulsification and penetration into the membrane of organisms. Branched alkyl chains are less toxic than linear alkyl chains (Talmage, 1994). In a study conducted by Tiehm (1994), alkylethoxylate surfactants with ethoxylate chains ranging from 9 to 12 were toxic to PAH degrading *Mycobacterium* spp.

Surfactants can also be toxic to higher level organisms. According to a study conducted by Dorn et al (1996), fathead minnow (*Pimephales promelas*) reproduction was significantly reduced when exposed to nonionic surfactant-$C_{14\text{-}15}$ linear alcohol ethoxylate (LAE), at concentrations > 280 μg/L, and larval survival decreased significantly

above 330 μg/L. The acute LC_{50} to bluegill sunfish (*Lepomis macrochirus*) was found to be 700 μg/L (Talmage, 1994) and the 10 day LC_{50} was found to be 900 μg/L (Dorn et al., 1996).

Nonionic surfactants at high concentrations (> 10 mg/L) have little effect on the growth of higher plants (Talmage, 1994). Bishop and Perry (1981) observed the 7 day EC_{50} for duckweed (*Lemna gibba*) was 21 mg/L. In a study conducted by Ernst et al (1971), on orchid seedlings (*Phalaenopsis* sp.) using various nonionic surfactants, growth and development was inhibited at concentrations above 1000 mg/L.

Toxic effects on microorganisms and higher organisms are a result of surfactant interaction with proteins, changing the shape and activity of enzymes and solubilizing structural proteins which result in changes in cell permeability (Swisher, 1987). In fish and invertebrates, surfactants act both chemically and physically on respiratory organs, by penetrating gill membranes and interfering with gas and ion exchange (Talmage, 1994) and organisms die of suffocation (Wildish, 1974). In higher plants, surfactants alter the ultrastructure of cells by emulsifying membranes (Healy et al; 1971).

In laboratory animals such as rats and rabbits, nonionic surfactants exhibit a low order of acute toxicity by oral, dermal and inhalational routes of exposure (Talmage, 1994). Subchronic exposures lasting up to 91 days, oral doses of up to 500 mg/kg/day and dermal exposures of 50 mg/kg/day (Talmage, 1994) did not produce any significant toxic effect. This class of surfactants are not carcinogenic (Talmage, 1994). Triton DF-16, a nonionic surfactant was found to be weakly teratogenic to frogs (*Xenopus*) (Dawson et al. 1989), but Talmage (1994) reported that nonionic surfactants are not teratogenic to rats and pups. In mammals, nonionic surfactants are rapidly absorbed by the gastrointestinal system and eliminated (Talmage, 1994, Hunt et al., 1995). In

humans, nonionic surfactants have been reported to be mildly irritating to the skin (Talmage, 1994).

Bioremediation

Regulators, treatment firms and customers are looking for cost-effective technologies that can meet cleanup standards and provide permanent treatment of hazardous wastes. Bioremediation is classified as one such technology (Thayer 1991). Bioremediation may be defined as the biological process of correcting an ecosystem which has been contaminated by anthropogenic means. The biological agents most frequently used for remediation are microorganisms, such as bacteria and fungi, that can degrade pollutants. These microorganisms can be indigenous to the contaminated site. The microorganisms could also be extracted from the site, isolated, enriched elsewhere and then brought back to the contaminated area. Microorganisms are being genetically engineered to degrade specific contaminants in certain locales (Atlas and Bartha, 1972). Bioremediation of toxic organics in soil is a promising technology and an alternative to mechanical means (Comeau et al. 1993). It is a more cost effective method (Laha and Luthy, 1992) compared to physical and chemical methods (Comeau et al., 1993). The microbial capability to reduce toxic levels to concentrations that present no hazard to human health or the environment can be considered as a positive outcome of bioremediation using natural means.

Bioremediation is an extension of the biological treatment processes used to treat wastes, such as composting, sewage treatment and landfills (Atlas and Bartha, 1972). Traditional waste treatment methods include physical, chemical, biological or thermal means. Physical methods such as adsorption, filtration and extraction are effective for many wastes, but additional treatment is required if the waste is not destroyed. Chemical treatment can leave hazardous by-products or residual sludge. Incineration is

a very effective method for reducing the volume of wastes and for completely destroying them but is a very expensive treatment process. Furthermore the gaseous emissions and ash residues require further treatment (Thayer 1991, Wark and Warner, 1981).

Bioremediation can be classified into three broad categories - land treatment, bioreactors, and *in situ* treatment (Thayer, 1991). Land treatment is the least expensive method, but requires large land space for long periods of time. Traditionally land treatment required the incorporation of hydrocarbon waste into the soil, but recent land regulations have banned this practice (Bossert and Compeau, 1995). Modern land treatment techniques involve excavation and transfer of contaminated soil to the treatment area (Bossert and Compeau, 1995). This treatment is essentially bioremediation because the treatment conditions are optimized for indigenous microbial degradation by the addition of nutrients (Bossert and Compeau, 1995). Bioreactors are more efficient for hazardous waste removal and are usually designed to handle groundwater (Thayer 1991). However, this operation requires materials to be pumped into bioreactors and separated. If a batch method is used, only limited waste can be remediated at a time, which could lead to high maintenance and operating expenses. *In situ* bioremediation is a suitable alternative which avoids the handling of hazardous wastes for treatment (Thayer 1991). There are essentially two approaches to bioremediation *in situ*, stimulation of indigenous bacteria, and bioaugmentation or introduction of degradative bacteria (Ensley and DeFlaun, 1995). Stimulation of indigenous microbial activity employs the growth of these aerobic contaminant degrading microorganisms by incorporating nutrients, optimizing pH, temperature and oxygen content by circulating groundwater through pumping and reinjection (Thayer, 1991). This *in situ* treatment was successfully undertaken following the *Exxon-Valdez* oil spill in Alaska, which occurred on March 25th 1989 (Pritchard and Costa, 1991). Oil soaked

rocks along the shores of Prince William Sound, Alaska were treated with the fertilizer Inipol EAP-22. Results showed that biodegradation of oil was enhanced two- to three-fold in comparison with untreated oil-soaked rocks with no adverse ecological effects (Pritchard and Costa, 1991).

Bioaugmentation has been successful in remediating soil contaminated with pentachlorophenol. In this case, *Flavobacterium* (Crawford and Mohn, 1985) and *Arthrobacter* ATCC 33790 (Edgehill et al; 1983) were added to the soil. The contaminant, 2,4,5-trichlorophenoxyacetic acid was remediated from soil using *Pseudomonas cepacia* (Chaterjee et al., 1982). Though bioaugmentation is acceptable from a regulatory stand point, the presence of insufficient population of degrading bacteria may limit the method. Bioaugmentation can also enhance the rate of decontamination (Comeau et al., 1993), but a major drawback is that nonindigenous bacteria may not be able to compete with the native population. However, according to Ensley and DeFlaun (1995), long term survival of these bacteria may not be an issue. With the addition of sufficient active microbial population, remediation may be achieved before the population declines. The probability that the non-indigenous bacterial population might exceed the native bacterial population is very slight. The native bacterial population will far exceed the added bacterial population because they would be more acclimated to the conditions of the contaminated site unless perpetual optimal conditions are provided for the added bacteria.

Researchers have shown that organic compounds may be stabilized against rapid degradation through sorption on clays (Harter and Slotzky, 1971) and humic materials (Martin et al., 1978). Compounds that are bound to soil surfaces are more resistant to biodegradation (Ogram et al., 1985) and less available to microorganisms for degradation (Robinson et al., 1990). However, Remberger et al. (1986) have

demonstrated that substrates that are tightly bound to sediments can remain accessible to biological transformation.

The physiochemical factors governing the rates of bioremediation are still not thoroughly understood, especially in the case of non-polar organics in the soil water system. Under conditions where mass transfer of a limiting nutrient controls bioconversion, surfactants may be added to soil to enhance bioconversion. Surfactants allow mass transfer of compounds from hydrophobic to hydrophilic phases, reducing interfacial tension (Laha and Luthy, 1992). They increase dissolution rates by solubilization and emulsification (Tiehm, 1994), resulting in increased mobility and bioavailability of nutrients to microorganisms in the soil for degradation.

CHAPTER IV

MATERIALS AND METHODS

Experimental Plan

In order to conduct bioremediation in this study firstly, the CMC of F-500 was determined by surface tension method. F-500 concentration below its CMC was used to enhance remediation in soil and aqueous media. Secondly, soil was collected from the study site. The indigenous microorganisms from this soil were enriched over three months. A soil bioremediation experiment was set up with control, non-F-500 treatment and F-500 treatment bioreactors. Enriched microorganisms were used to remediate soil containing anthracene by aerobic degradation over 91 days. Thirdly, aqueous media containing anthracene was remediated in order to investigate the effect of soil on anthracene bioavailability to microorganisms for degradation. The aqueous media remediation was set up similar to the soil remediation and aerobically degraded over 41 days. Remediation in both soil and aqueous media was monitored using radiolabeled anthracene and analyzing the end product $^{14}CO_2$. Lastly the toxic range of F-500 to microorganisms was determined by calculating the EC_{50} range by conducting respiration inhibition tests following OECD guidelines.

The amount of anthracene mineralized in soil and aqueous media was quantified and plotted. The effect of F-500 on enhancement of mineralization was determined by comparing the different treatments in both soil and aqueous remediation studies. The effect of soil on anthracene bioavailability was determined from mineralization rates and mineralization half-life of anthracene in the presence and the absence of soil.

Soil Collection

Three, five gallon aluminum buckets were washed with soap, rinsed with water and dried. These buckets and lined with plastic bags before being used as collecting vessels for soil. The study site was a coal depositing area of Clemson University, SC. Soil from the natural drainage area was collected with shovels to approximately two inches in depth, transferred into the aluminum buckets, and transported to the laboratory for testing. Organic content, pH, mineral content, moisture and texture of soil were determined at the Agricultural Service Center and Soil Laboratory (Clemson University).

Critical Micellar Concentration Determination

In order to determine the concentration of surfactant that could be used in soil and aqueous remediation of antharcene in this study, the CMC had to be determined. This was conducted by the surface tension method using the Cahn Microbalance, and the resulting measurements were plotted versus the logarithm of the F-500 concentration. The intersection of the two linear portions of the plot is defined as the CMC of a surfactant.

Microbial Culture - Enrichment, Growth and Maintenance

Anthracene (0.05 g : 99+% *Sigma Chemicals*) as a solution in 10 ml methylene chloride was placed into a sterile 250 ml Erlenmeyer flask or culture flask and the solution was blown to dryness using nitrogen gas. To this culture flask was added salts media which was prepared according to Gray et al. (1994). This media consisted of 1.33 g/L KH_2PO_4, 2.67 g/L K_2HPO_4, 1g/L NH_4Cl, 2 g/L Na_2SO_4, 2 g/L KNO_3, 0.05 g/L $FeSO_4.7H_2O$ and 1 ml trace metal solution (Pfening et al. 1966) - 200 mg/L $FeSO_4.7H_2O$, 10 mg/L $ZnSO_4.7H_2O$, 3 mg/L $MnCl_2.4H_2O$, 30 mg/L H_3BO_3, 20 mg/L $CoCl_2.6H_2O$, 1mg/L $CuCl_2.2H_2O$, 2 mg/L $NiCl_2.6H_2O$, and 3 mg/L $Na_2MoO_4.2H_2O$ and

0.2 g/L $MgSO_4.7H_2O$ (all *Sigma Chemicals*). Salts media was autoclaved for 20 minutes at 121° C before use.

Soil (3.5 g) was then inoculated into the culture flask, plugged with a cotton plug and shaken in an orbit shaker (Lab-Line) at 200 rpm for 7 days at room temperature. The soil-salts media mixture was then allowed to settle and the supernatant (5 ml) containing soil microorganisms was transferred into fresh salts media (100 ml) and further cultures were maintained by periodic transfer into fresh media every 3 weeks where anthracene was the sole carbon source. Colony counts were monitored by serial dilution and viable plate count technique (Collins and Lyne, 1985).

Radiolabelled Anthracene

[9^{-14}C] anthracene (anthracene labelled at the 9th carbon position) with specific activity 55.7 mCi/mmol, concentration 1.09 mCi/ml in benzene and total volume 2 ml i.e. 3.48 mg/ml (*Chemsyn Science Laboratories*) was used in this study. Benzene was allowed to evaporate and the compound was then diluted to a volume of 10 ml with methylene chloride which brought the final concentration to 0.696 mg/ml. The purity of [9^{-14}C] anthracene was 99.7% determined by thin layer radiochromatography (*Chemsyn Science Laboratories*).

A set of standard concentrations of this radiolabeled anthracene (3.91×10^{-6} M, 3.91×10^{-5}M, 3.91×10^{-4}M, 3.91×10^{-3} M, 3.91×10^{-2} M and 3.91×10^{-1} M) was prepared in order to determine a standard plot, Figure 3, from which the anthracene mineralization was quantified in soil and aqueous media.

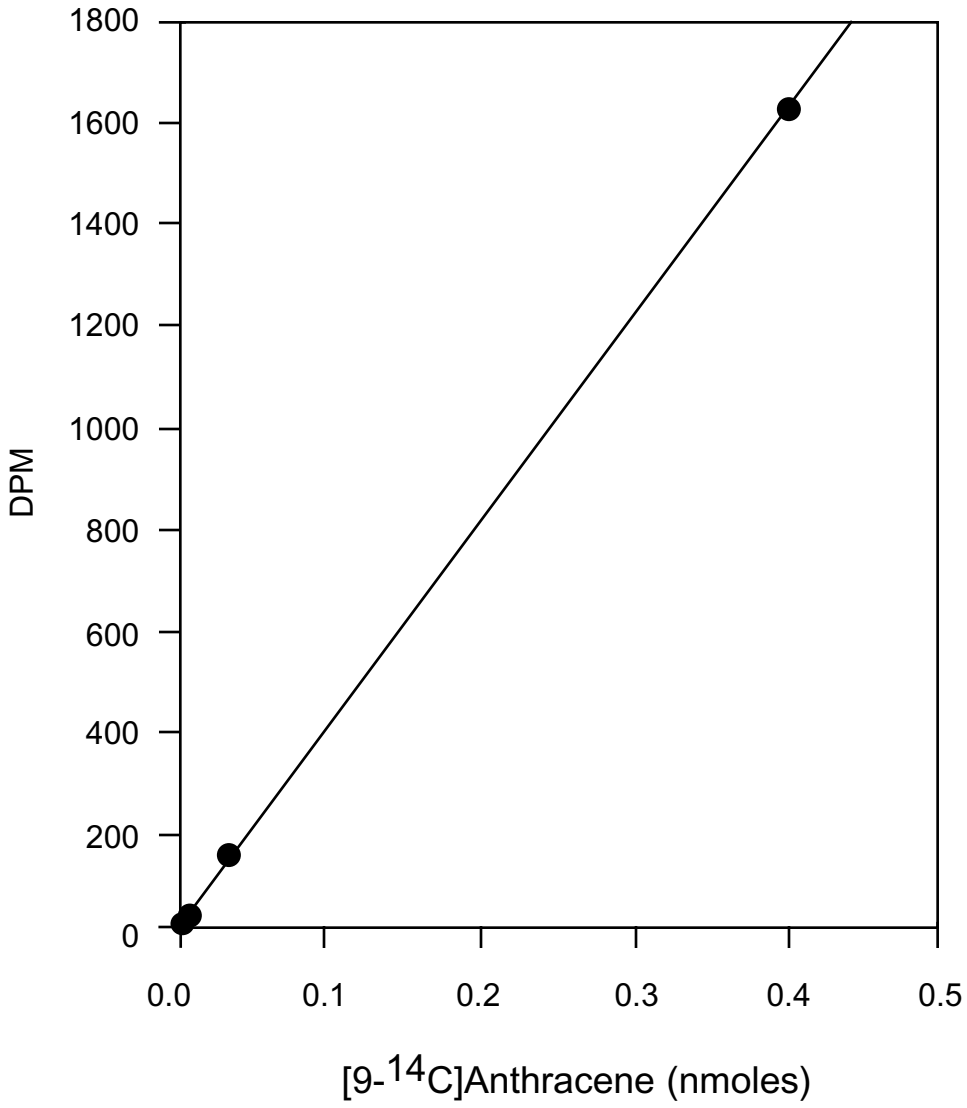

Figure 3. Standard Plot for the Radioactivity of [9-^{14}C] Anthracene. The regression equation for this plot was y = 4134.62 *x* + 1.7998 with R^2 = 0.997.

Cell Harvest

Enriched microbial culture was harvested by centrifuging in batches of 5 ml culture at 2500 rpm for 5 minutes at 25° C. The microbial pellet obtained was washed three times with sterile salts media at 2500 rpm and 25° C and finally suspended in 5 ml salts media. The enriched microbial cell population was determined by plate count (Malik, 1991).

Bioremediation of Anthracene in Soil (BAS)

Bioreactor Set-Up: Each bioreactor was set up according to Figure 4, and it comprised of a reaction chamber (250 ml Erlenmeyer flasks) which was fitted with an air inflow tube, an outflow tube with carbon dioxide trapping filter lodged in it and a carbon dioxide trapping chamber. The inflow tube had sterile moist air flowing into the bioreactors which were regulated individually by one-way air valves. There were six replicate bioreactors for each sampling day from day zero to day 50 for each treatment group and control. The CO_2 trapping chamber contained 50 ml 1.5 N KOH, and the trapping filters were sterile cotton plugs saturated with 1.5 KOH solution. Mineralization of anthracene in each bioreactor up to day 50 was determined by analyzing the trapping solution in the chambers and the filters in the outflow tubes. These bioreactors were then detached from the remediation set up and discarded. After day 50, instead of discarding individual bioreactors, mineralization of anthracene was analyzed by replacing fresh filters in the outflow tube on sampling days.

Soil (50 g) collected from the contaminated site was placed into all bioreactors and autoclaved for 45 minutes in liquid cycle at 121° C under 25 pounds per square inch (psi) for three consecutive days with 24 hours between each autoclaving cycle.

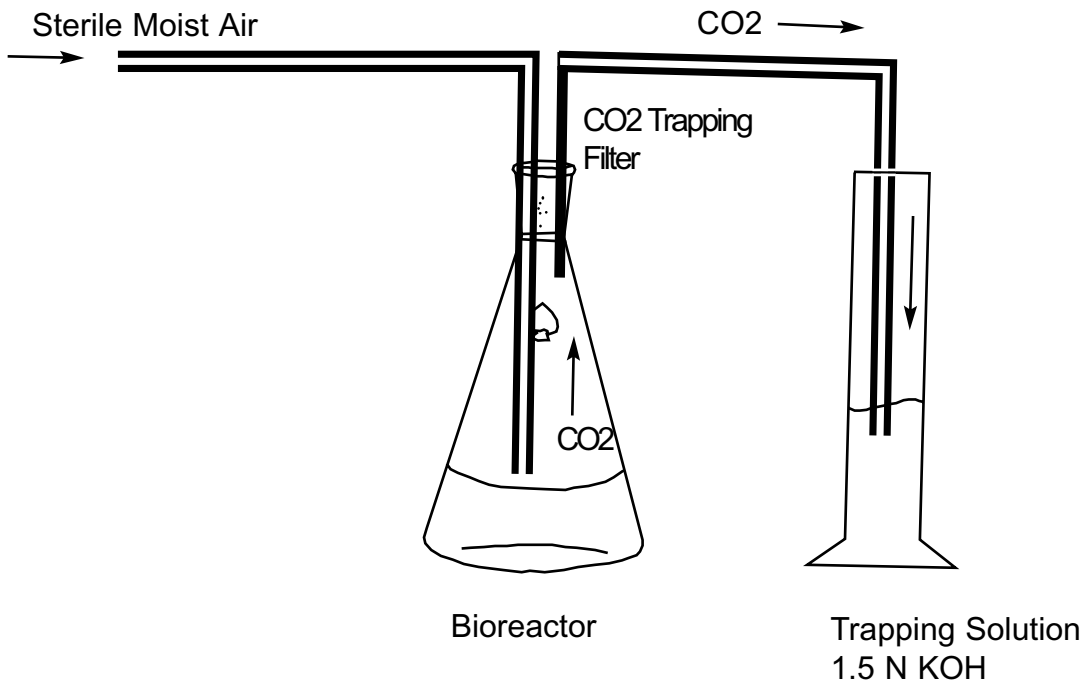

Figure 4. Individual Bioreactor Setup for Both Remediation Studies (BAS and BAA).

After the final liquid cycle, soil samples were autoclaved for 15 minutes in a dry cycle at 121° C under 25 psi.

Control Group. Radiolabelled anthracene (2 ml) and 1ml unlabelled anthracene (60 mg/ml of methylene chloride) was placed in a 2L sterile beaker and methylene chloride was allowed to evaporate in a sterile laminar air flow hood. Sterile distilled water (1850 ml) was added into the beaker and stirred with a magnetic stirrer. The amount of unlabeled anthracene was calculated to be 0.0324 mg/ml (or 3.24×10^4) ng/ml).

In each of the control bioreactors (already containing sterile soil) was added 70 ml control media which was calculated to contain 1.578×10^4 ng or 89 nmoles of radiolabelled anthracene and, 2.27×10^6 ng or 12751 nmoles of unlabeled anthracene, making a total of 2.285×10^6 ng or 12841 nmoles in each control bioreactor. Addition of unlabeled anthracene was for quantification purposes and did not affect mineralization of labeled anthracene.

Non-F-500 Treatment Group. Radiolabelled anthracene (2 ml) and 1 ml unlabelled anthracene (60 mg/ml of methylene chloride) was placed in a 2L sterile beaker and methylene chloride was allowed to evaporate in a sterile laminar air flow hood. Sterile salts media (1050 ml) and sterile distilled water (800 ml) was added into the beaker and stirred.

In each of non F-500 treatment chambers, 65 ml of media was added which contained 1.465×10^4 ng or 82 nmoles of radiolabeled anthracene and 2.106×10^6 ng or 11830 nmoles of unlabeled anthracene making a total of 2.121×10^6 ng or 11914 nmoles in each bioreactor. Enriched microbial culture (5 ml), corresponding to approximately 8.75×10^7 cfu previously harvested, was also added into each bioreactor, bringing the microbial concentration to 1.25×10^6 cfu/ml.

F-500 Treatment Group. Radiolabelled anthracene (2 mL) and 1 mL unlabelled anthracene (60 mg/ml of methylene chloride) was placed in a 2L sterile beaker and methylene chloride was allowed to evaporate in a sterile laminar air flow hood. Sterile salts media (1050 ml) and sterile distilled water (800 ml) was added into the beaker and stirred. F-500 corresponding to a final surfactant concentration of 9.47×10^{-4} M was added into the beaker and stirred.

Media (65 ml) was added into each of the F-500 treatment bioreactors. The quantity of radiolabeled and unlabeled anthracene were the same as in non F-500 treatment bioreactors. Enriched microbial culture (5 ml), corresponding to approximately 8.75×10^{7} cfu, was also added into each bioreactor, bringing the microbial concentration to 1.25×10^{6} cfu/ml.

All the bioreactors were carefully connected with all their respective tubing and aerated by passing sterile moist air through them. On day 50 F-500 treatment bioreactors were reinjected with F-500 (to make final concentration of 9.47×10^{-4} M).

Analytical Methods

The radioactivity of standard concentrations of [9-^{14}C] anthracene prepared in 2 ml scintillation fluid (Ecoscint: *Fisher Chemicals*) was determined using a liquid scintillation counter (Beckman 2000) counting for 2 minutes. The signal obtained in disintegration per minute (DPM) was plotted as a standard curve (Figure 4) against [9-^{14}C] anthracene concentration. The amount of radiolabeled anthracene in control, non F-500 and F-500 treatment bioreactors was quantified from the standard plot after analyzing by liquid scintillation. Soil control media (1 ml) was pipetted into a 4 ml scintillation vial to which 2 ml scintillation fluid was added, shaken and analyzed by counting for 2 minutes.

Remediation was monitored by quantifying $^{14}CO_2$, the end product of [9-^{14}C] anthracene degradation, trapped in the filters and trapping solution of the remediation system. The filters and 1 ml trapping solution were transferred into 4 ml scintillation vials which were filled with 2 ml scintillation fluid, shaken and analyzed by liquid scintillation counting for 2 minutes.

The radioisotope ^{14}C of trapped $^{14}CO_2$ emits β^- particles (Neame and Homewood, 1974), and the passage of this ionizing radiation through the solvent-solute system causes excitation of the solvent molecules by promotion of electrons from their ground state to higher energy levels known as singlet states. These excited singlet states undergo internal conversion to the lowest excited singlet state (S_{1x}), and when they are in close proximity to the solute particles of scintillation fluid, energy transfer (Beckmen Operating Manual, 1993) takes place as the lowest excited singlet state (S_{1y}) of the scintillation fluid solute is lower than S_{1x}. This scintillation emission is detected and recorded by photomultiplier tubes (Dyer, 1974). Liquid scintillation methods have short resolution times, and high rates of disintegration can be measured especially for penetrating radiations like β^- particles (Neame and Homewood, 1974). $^{14}CO_2$ trapped was quantified by sacrificing individual bioreactors on specific days - 0, 1, 2, 4 and 16. From day 50 onwards i.e. days 64, 78, and 91, bioreactors were not discarded, instead the trapping filters were replaced by fresh filters. There were six replicates for each treatment and control group for the specific days.

The amount of [9-^{14}C] anthracene mineralized over the remediation period was quantified from the standard plot (Figure 4), because for every mole of $^{14}CO_2$ evolved, one mole of [9-^{14}C] anthracene was mineralized as stated earlier that the purity of radiolabeled anthracene used was 99.7%. The quantification in nmoles was then converted into ng of anthracene using its molecular weight for calculations.

Bioremediation of Anthracene in Aqueous Media (BAA)

The second part of this bioremediation study was conducted in order to determine the effect of soil on anthracene bioavailability for microbial degradation. The experiment was engineered the same way as the latter half of BAS. Filters were replaced instead of discarding bioreactors. The contents in each flask was similar for the treatment and control groups except soil was not added to any reaction chamber.

Cell Harvest: In order to maintain similar conditions as the previous study, microbial cells were grown to a population of 5.0×10^7 cfu/ml which was determined by dilution and plate count. Enriched microbial culture was harvested by centrifuging in batches of 5 ml culture at 2500 rpm for 5 minutes at 25°C. The microbial pellets were washed three times with sterile salts media at 2500 rpm and 25°C and resuspended finally in 5 ml salts media.

Control Group. Radiolabelled anthracene (250 µl) and 1 ml unlabelled anthracene (60 mg/ml of methylene chloride) was placed in a 2 L beaker and methylene chloride was allowed to evaporate in a sterile laminar air flow hood. Sterile distilled water (1400 ml) was added into the beaker and stirred, counted, and the amount of radiolabeled anthracene was quantified from the standard plot. This media (200 ml) was then palced into individual bioreactors, and the amount of anthracene was quantified as 3.926×10^3 ng or 22 nmoles radiolabeled and 8.57×10^6 ng or 48146 nmoles unlabeled.

Non-F-500 Treatment Group. Unlabeled and labeled anthracene were used in the same quantities as those used in control of BAA. Sterile salts media (794.6 ml) and sterile distilled water (604.8 mL) was then added into the beaker to make a total volume of 1400 ml. The amount of radiolabeled anthracene in the media was quantified after counting 1 ml of the solution. Enriched microorganisms corresponding to 2.5×10^8 cfu

were added into the beaker and stirred with a magnetic stirrer. This media (200 mL) was placed into non-F-500 treatment bioreactors which then had a microbial population of 1.25×10^6 cfu/ml. The amount of radiolabeled and unlabeled anthracene in each bioreactor were the same as those in control bioreactors of BAA.

F-500 Treatment Group. This mixture contained the same components as non-F-500 treatment group including the quantity of microbial population. F-500 corresponding to a final surfactant concentration of 9.47×10^{-4}M was also added into the beaker and stirred with a magnetic stirrer. This solution (200 ml) was placed into F-500 treatment bioreactors which then had a microbial population of 1.25×10^6 cfu/ml. Radiolabeled and unlabeled anthracene were in the same quantities as those in non-F-500 treatment bioreactors of BAA.

Analytical Methods

On days 0, 1, 2, 4, 19 and 41 trapping filters and trapping solution (1 ml) were analyzed by liquid scintillation counting similar to BAS. The amount of anthracene mineralized over the BAA study period was quantified in the same manner as BAS.

Toxicity Tests

In order to determine the toxic range of F-500, a respiration inhibition test on activated sludge was performed in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines method 209 (1984) (OECD, 1984, Kleèka et al, 1985, Volskay and Grady, 1988). Activated sludge was fed with standard amount of modified synthetic sewage feed (16 g peptone, 11 g yeast extract, 3 g urea, 0.7 g NaCl, 0.4 g $CaCl_2.2H_2O$, 0.2 g $MgSO_4.7H_2O$ and 2.8 g K_2HPO_4 in 1L distilled water with pH 7). The respiration rate of activated sludge was measured after 30 minutes of

contact time as a control parameter. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance (F-500 in this case) under identical conditions was also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls from which an effective concentration (EC) range to cause 50% inhibition in respiration rates was calculated.

The formula for percent inhibition of microorganisms is (OECD, 1984, Kleèka et al, 1985, Hemond and Fechner, 1994):

$$1 - 2Rs/ Rc_1 + Rc_2 * 100$$

where Rs = oxygen consumption rate at tested concentration of test substance (F-500 in this case); Rc_1 = oxygen consumption rate, control 1 and Rc_2 = oxygen consumption rate, control 2.

Microbial Innoculum. Activated sludge was collected in glass jars (2L) from Clemson University waste treatment plant and transported to the laboratory. The sludge was allowed to settle for 30 minutes and the supernatant was discarded. The suspended solids were washed three times with distilled water, and 1ml of the washed sludge was weighed and dried. From this, the amount of wet sludge to be used was calculated and suspended in distilled water in order to obtain an activated sludge with a mixed liquor suspended solids concentration of 4000 ± 400 mg/L. This solution produced a concentration of 1600 mg of activated sludge per liter in the test medium.

Control Test. At time zero, synthetic sewage feed (16 ml) was diluted up to 300 ml with distilled water. Microbial innoculum (200 ml) was then added. The total mixture (500 ml) was poured into a 1 L beaker (control C1) and aerated for 30 minutes using a Pasteur-pipette aeration device. The second control (C2) test was performed at the end of the experiment, following the exact procedure as C1.

Treatment Test. After aerating C1 30 minutes, the first treatment test (F-500 1) was performed. Synthetic sewage feed (16 ml) was made up to 200 ml with distilled water and 100 ml of test substance was added. Microbial innoculum (200 ml) was added to this mixture and the total content (500 ml) was poured into a 1L beaker (F-500 1) and aerated for 30 minutes. This procedure was repeated for all the concentrations of F-500 (F-500 2, F-500 3, F-500 4, F-500 5 and F-500 6) as listed in Table IV.

Table IV.

Concentrations of F-500 Used to Determine its EC_{50} Range on Activated Sludge

Treatment	Concentration of F-500 (mg/L)	Concentration of F-500 (M)	Volume of F-500 (ml in 500 ml)
F-500 1	100	0.02	0.05
F-500 2	300	0.05	0.15
F-500 3	550	0.95	0.28
F-500 4	1000	1.72	0.50
F-500 5	3000	5.15	1.50
F-500 6	10000	17.18	5.00

After incubation (contact) the media was transferred to a 250 ml flat-bottomed biological oxygen demand (BOD) bottle to a level that allowed no head space. A dissolved oxygen (DO) electrode (Orion 97-08) connected to a DO meter (Orion 230A) is fiited into the BOD bottle and DO levels in ppm were measured over a 10 minute period recorded at intervals of 15 seconds.

A reference substance 3, 5-dichlorophenol (3,5-DCP) was also tested in the same way as the F-500 treatments. The stock solution of 3,5 DCP was prepared fresh at the start of the study by dissolving 0.5 g of 3, 5 DCP in 10 ml of 1 N NaOH diluted to 30 ml with distilled water. Approximately 8 ml of 1N H_2SO_4 was then added under stirring conditions to the point of incipient precipitation, and finally this mixture was diluted to 1 L

with distilled water (pH was 7.5). Three different concentrations were tested from this stock solution (0.5, 0.25 and 0.125 g/L).

The rate of respiration of microbial culture for the different treatment groups was determined by plotting time dependant oxygen consumption graphs and by calculating rates from the linear range of the plots.

Percent respiration inhibition of microorganisms were determined from these rates and plotted log-normally against concentration. The EC_{50} range of F-500 on activated sludge was extrapolated from the log-normal plot.

Statistical Analysis

The $^{14}CO_2$ evolution data collected for all the specific days were analyzed by SAS. For BAS, $^{14}CO_2$ evolution data were analyzed in two parts. The first part included data obtained on days 0, 1, 2, 4, 16 and 50 analyzed for ANOVA and normality of distribution. As the CO_2 evolution data (in the replicate bioreactors) was not distributed normally (results and discussion section), the data was log transformed and analyzed non-parametrically using the Kruskal-Wallis test with rejection p-value > 0.05. The latter part of this study (days 64 and 91) was analyzed using a nested design. The data was not normally distributed and thus was log transformed and non-parametrically analyzed using the Kruskal-Wallis test. Data obtained in BAA was analyzed similar to the second half of BAS as the experimental procedure was similar. The rejection areas were also p-value > 0.05.

CHAPTER V

RESULTS AND DISCUSSION

Soil Characteristics

The soil used was mostly sandy with a relatively small amount of clay, low in organic matter (2.6%) as noted in Table V, and low in most of the inorganic elements as noted in Table VI. Though microorganisms are known to degrade PAHs, inorganic nutrients at concentrations that are less than optimum for microbial growth (Weissenfels et al., 1992) may hinder microbial attack on aromatic compounds. In order to minimize this limitation, soil in all the treatment and control groups were supplemented with mineral salts media. However, it has been suggested that the organic carbon content of soil is the single most important factor determining the sorption of hydrophobic molecules (Weissenfels et al; 1992). Means et al. (1980) demonstrated that adsorption of PAHs to soil under water-wet conditions was correlated to organic content, not to clays or other inorganic soil components, but according to Chiou et al. (1985) clay in dry soils could strongly adsorb aromatic hydrocarbons. The sorbed quantity of anthracene in this case would be unavailable for microbial degradation. In a study performed by Weissnfels et al. (1992), PAH degradation was reduced due to high organic content (13.6%) in soil. However, over 2 days 70% removal of anthracene was observed from a soil slurry which contained only 0.62% organic matter (Gray et al., 1994). Autoclaving may alter the soil characteristics (Skipper and Westermann, 1973), but this effect was taken into account due to the comparative nature of this study (Meininger, 1993).

Table V

Soil Characteristics

% Sand						% Silt	% Clay	% Soil Moisture
Very Coarse	Coarse	Medium	Fine	Very Fine	Total			
6.9	20.1	21.8	16.2	7.7	72.7	21.9	5.4	7.4

Note: Determined by the soil laboratory, Department of Agriculture, Clemson University

Table VI

Soil Test Results

pH	P Kg/Ha	K Kg/Ha	Mg Kg/Ha	Ca Kg/Ha	Zn Kg/Ha	Mn Kg/Ha	NO_3-N (ppm)	Organic Matter %
4.1	22.42 L	33.63 L	30.27 L	297.0 7 L	3.03 S	2.24 L	2	2.6

Note: Phosphorous (P), potassium (K), magnesium (Mg), Calcium (Ca) and Manganese were low (L) and zinc (Zn) was found in satisfactory (S) amounts.

Critical Micellar Concentration Determinations

In Figure 5, the CMC of F-500 was calculated to be 3.98×10^{-3} M, which is relatively higher than other commercial nonionic surfactants (See Table III). This can be due to a difference in the analyzing instruments or, F-500 may contain a high CMC surfactant like CHAPS (3-[(3-cholamidopropyl)-dimethylamino]-1-propane sulfonate).

Once the CMC of F-500 was calculated, the surfactant concentration to be used in the bioremediation study was determined. This concentration was determined to be 9.47×10^{-4} M and was used in the F-500 treatment groups. Very little information was available on the properties F-500, including the effect of F-500 on microorganisms and solubilization of PAHs in F-500. Therefore, a low concentration of F-500 was chosen to test the effect of F-500 on remediation. The concentration of F-500 used in remediation was less than its CMC which enhances microbial degradation (Aronstein and Alexander, 1992, Aronstein et al., 1991). According to Comeau et al. (1993) the recommended microbial population from an engineering perspective, should be between 10^6-10^8 cfu/ml for decontamination. The microbial population used in this study was 1.25×10^6 cfu/ml, which was on the lower end of the recommended scale. Thus, a very high surfactant concentration was not used in order to minimize the inhibition of anthracene degraders. Using F-500 at low concentrations may have minimized the possibility of competition between the surfactant and the contaminant as a nutrient source by microorganisms (the concentrations of anthracene added in the F-500 trt bioreactors were approximately 1.19×10^{-2} M in soil and 4.82×10^{-2} M in aqueous media). This effect was observed in Tiehm's study (1994), where PAH degradation was inhibited because anionic surfactant sodium dodecyl sulfate (SDS) was preferred as a growth substrate. However, the F-500 concentration used was less than the CMC which would also minimize micelle formation in the system.

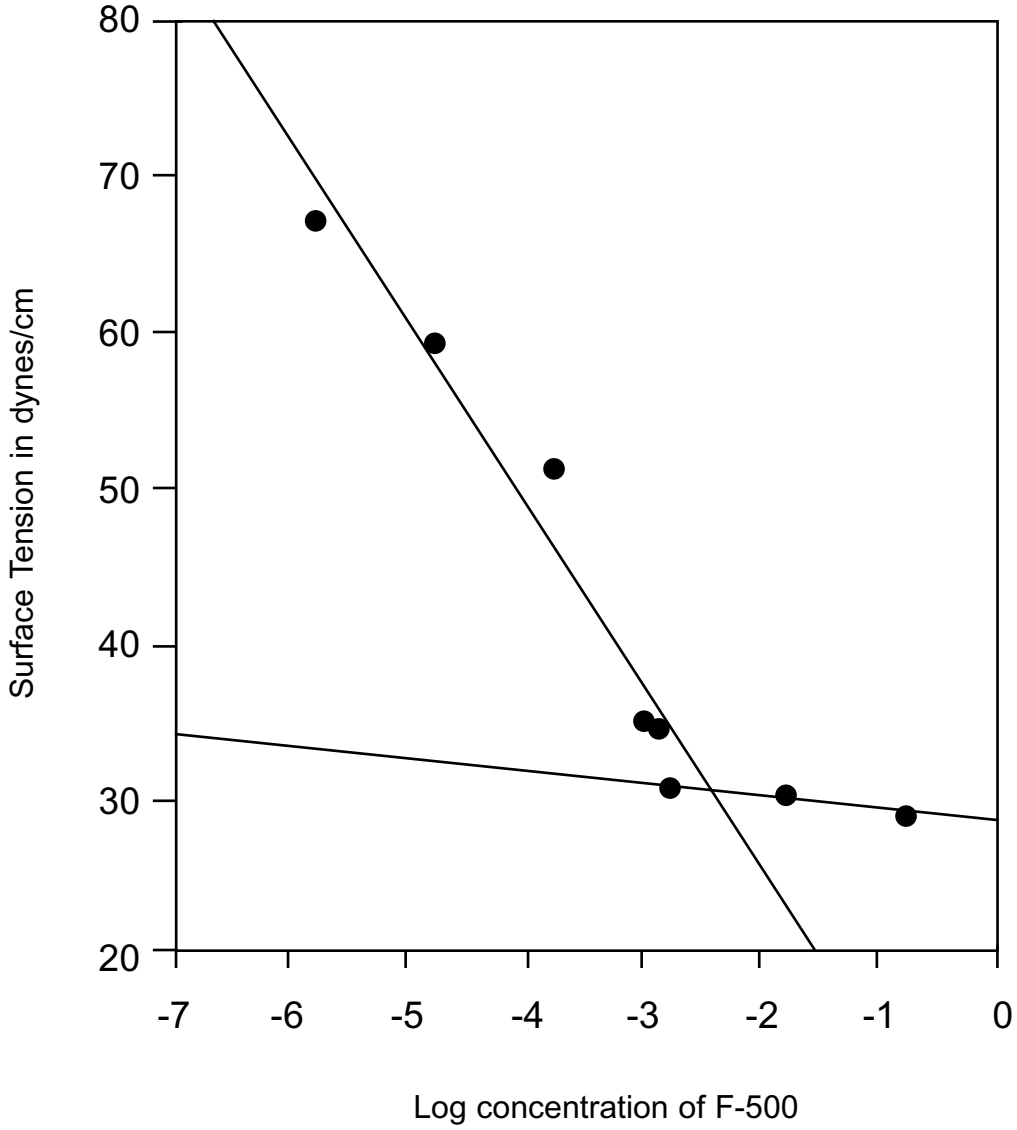

Figure 5. Critical Micellar Concentration (CMC) Determination of F-500. A double linear regression plot intersected at 3.98 x 10-3 M which was determined to be the CMC of F-500.

Toxic Range of F-500 on Microorganisms

In Figure 6, the respiration inhibition tests on activated sludge microorganisms using F-500 revealed the effective concentration range of F-500 to inhibit 50% (EC_{50}) of the rate of respiration when compared to control (no F-500) was between 2000 and 3000 mg/L. This concentration corresponds to 0.012 and 0.017 M.

The concentration of F-500 used in this study was significantly below the EC_{50} range for microorganisms. Microorganisms used in this study were a mixed population from soil and the toxicity test was performed using activated sludge which was essentially comprised of a mixed microbial population. Enriched soil microorganism biomass could not be brought up to levels comparable to activated sludge in order to determine toxicity of F-500. Therefore, the EC_{50} range of F-500 for activated sludge microorganisms was presumed to be the EC_{50} range for the soil microorganisms. Toxicity tests on microorganisms are also performed by plate count or spectrophotometric or turbidometric methods, but it has been suggested that these methods may not be adequate in determining toxicity. Remediation is mostly concerned with oxidation capacity of microorganisms rather than viability because degradation and respiration both involve the utilization of oxygen to produce energy. The inhibition of degrading potential can be determined by measuring inhibition of respiration rates. Aerobic degradation of carbon compounds is after all, the utilization of oxygen to produce CO_2, a respiration end product.

F-500 concentration used in both bioremediation studies (BAS and BAA) caused respiration inhibition of approximately 7% of the microbial population. Some growth or damage to cellular components may also have occurred. However, the inhibitory effect of surfactants on microorganisms does depend on specific environmental conditions (Talmage, 1994), the type of acclimation history (Talmage, 1994) of bacteria, and the

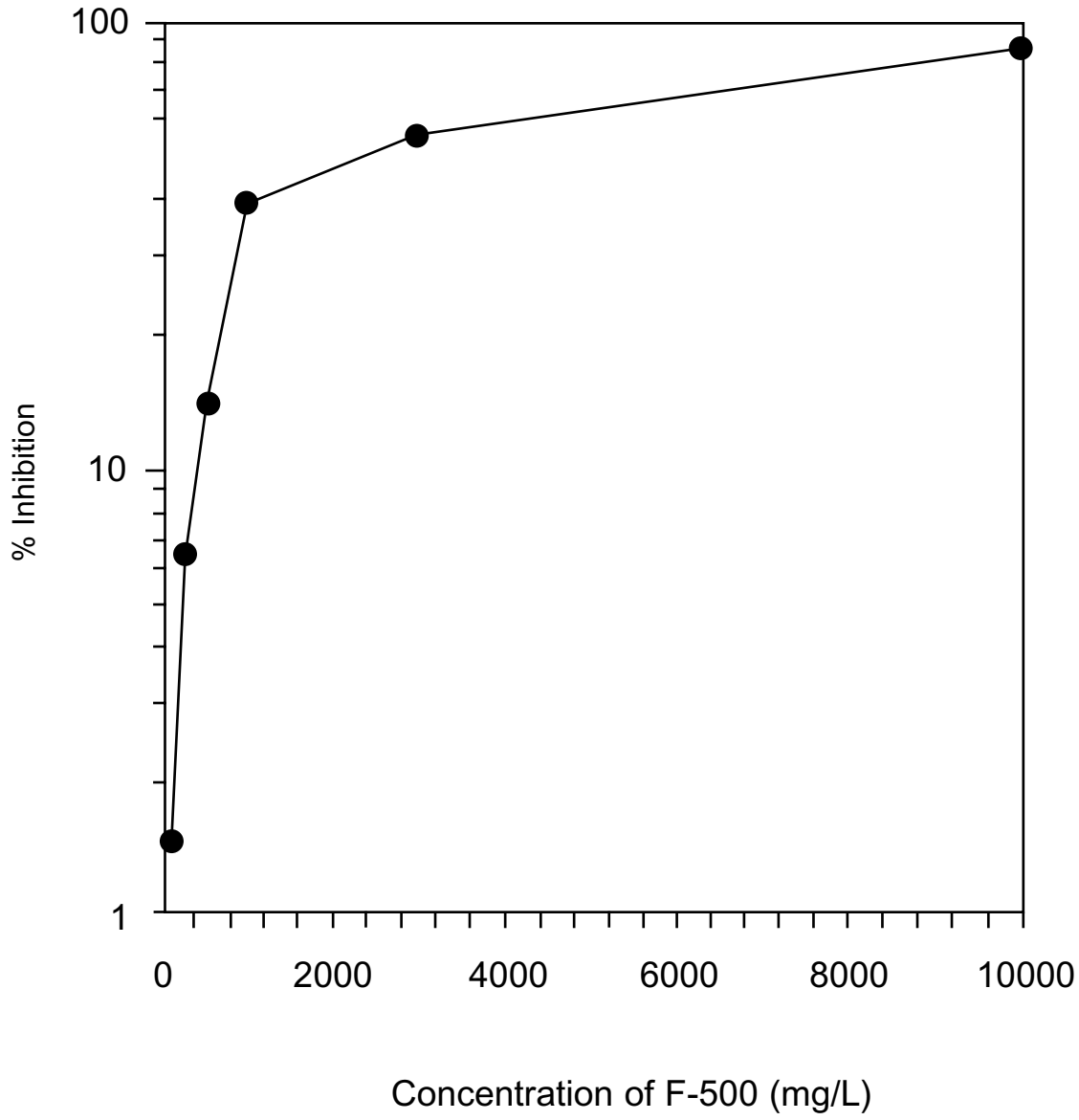

Figure 6. EC_{50} Range Determination of F-500 (F-500) on Activated Sludge Microorganisms. The log-normal plot determined the EC_{50} range to be between 2000 and 3000 mg/L.

presence of other nutrient factors. If F-500 is used for remediation purposes in the field, then other external factors also come into play. Rain, sunlight and weathering actions can also alter the effect of surfactant toxicity. The fate of surfactants in soil are of major concern because of their diverse domestic and industrial application, but presently there is no information describing environmental levels of nonionic surfactants or their degradation products in soil. However, F-500 being nonionic, is nontoxic to terrestrial vertebrates (Hunt et al., 1995). F-500, if compared to general nonionic surfactants, would most likely undergo rapid primary and ultimate biodegradation under aerobic conditions. According to Swisher (1987), surfactant biodegradation under anaerobic conditions is relatively slow. Therefore, use of nonionic surfactants in biodegradation under aerobic conditions should be an agent of choice in future remediation studies, where nonionic surfactants would serve the purpose of cleaning up contaminated sites, without causing any degenerative effect themselves.

Bioremediation of Anthracene in Soil (BAS)

The degradation of [9-^{14}C] anthracene in soil was monitored over a period of 91 (aging of anthracene in soil was not conducted before remediation). Radiolabeled anthracene degradation product $^{14}CO_2$ was counted in disintegration per minute (DPM) and the data collected were analyzed in two parts. Data for the first 50 days showed significant difference between control and treatment groups ($p = 0.0001$). However, the distribution was not normal when determined by box, stem and leaf histogram and normal probability plot of residuals (W:Normal = 0.75; $p = 0.0001$) over remediation period and for replicate samples. The data was then log transformed and analyzed non-parametrically by Kruskall-Wallis (KW) test. The results showed significant difference between the treatment groups (KW test statistic = 35.502; $p = 0.0001$). The amount of

anthracene degraded within each control and treatment group on respective sampling days were also significantly different (KW test statistic = 15.098; p-value = 0.01).

The second part of the BAS (i.e., after day 50) was analyzed using nested variance design because the experimental measurements were altered i.e. individual reactors were not discarded, but filters in reactors from day 50 were analyzed and replaced with fresh filters on specific collection days. The distribution of data was not normal (W:Normal = 0.922; p = 0.0026). The data was then log transformed, and analyzed by KW test. The results showed significant difference between treatment groups (KW test statistic = 36.20; p = 0.0001). However, within each treatment and control group there was no significant difference between individual sampling days (KW test statistic = 1.5539; p = 0.4598).

The total amount of anthracene mineralized in soil was plotted in Figure 7, which shows the antilogarithm of the geometrical mean values (distribution of data was not normal) of the total amount of anthracene degraded in soil against time.

Anthracene mineralization was quantified by considering that for every mole of [9-^{14}C] anthracene mineralized, one mole of $^{14}CO_2$ was liberated. For every ng of labeled anthracene mineralized, 144 ng of unlabeled anthracene was assumed to be mineralized because of the ratio of labeled to unlabeled anthracene in the system. Table VII lists the antilog of the geometric mean quantities of anthracene mineralized in soil in ng and parts per billion (ppb).

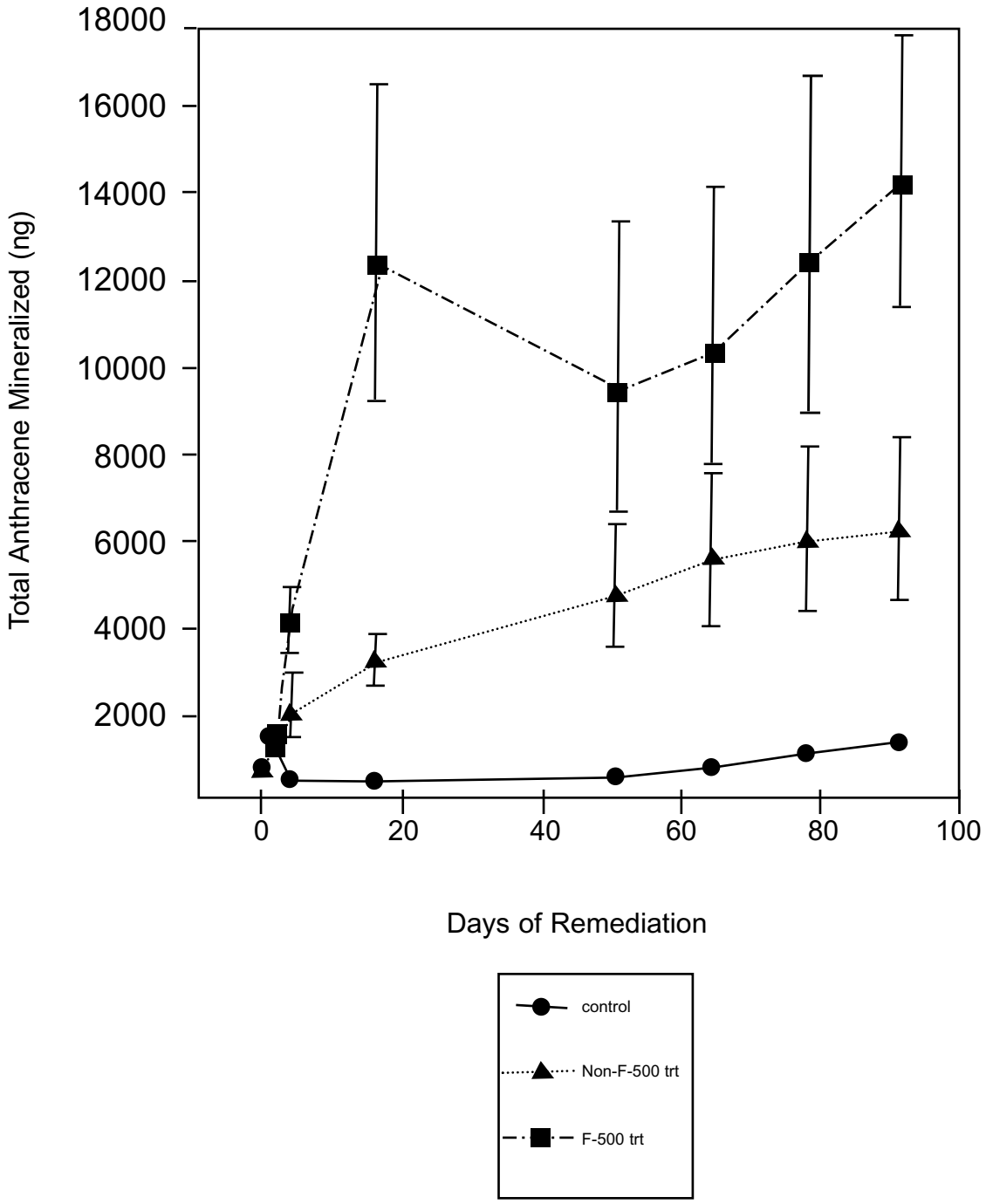

Figure 7. Effect of Nonionic Surfactant F-500 on the Mineralization of Anthracene in Soil. This effect was observed in control, non-F-500 treatment (non-F-500 trt) and F-500 treatment (F-500 trt) bioreactors plotted against time.

Table VII

Quantity of Total Anthracene Mineralized (ng and ppb) in Soil Enriched with Anthracene Degrading Microorganisms

Days	Control (ng)	Control (ppb)	Non-F-500 trt (ng)	Non-F-500 trt (ppb)	F-500 trt (ng)	F-500 trt (ppb)
0	817.62	16	720.94	14	910.91	18
1	1488.74	30	1314.61	26	1300.99	26
2	1289.76	26	1165.41	23	1520.96	30
4	488.75	10	2053.14	41	4144.51	83
16	421.20	8	3228.54	65	12487.44	250
50	436.38	9	4819.54	97	9687.99	194
64	649.05	13	5635.33	112	10556.56	211
78	976.65	20	6009.85	120	12624.56	252
91	1228.79	25	6348.70	127	14227.96	285

Note: Geometric mean values listed above.

The control group accounted for any mineralization other than biological, such as photo- and chemical- degradation because the environment in the control reactors was sterile. Non-F-500 treatment bioreactors accounted for bacterial degradation without any enhancement. Degradation of anthracene in soil was observed to be most efficient in F-500 treatment bioreactors where surfactant was used. This indicates that F-500, as a surfactant, was successful in making the nonpolar contaminant, anthracene, more available to the degrading microorganisms by mass transfer of anthracene from the hydrophobic phase to the hydrophilic phase and thus, increasing its solubility. From Figure 8, the total amount of anthracene mineralized in soil using surfactant was observed to be approximately 0.7%, which can be considered as a low amount, but was 96% greater than control mineralization and 50% greater than non F-500 mineralization of anthracene in soil. This low quantity of total anthracene mineralization may have occurred due to low F-500 concentrations used. According to a study conducted by

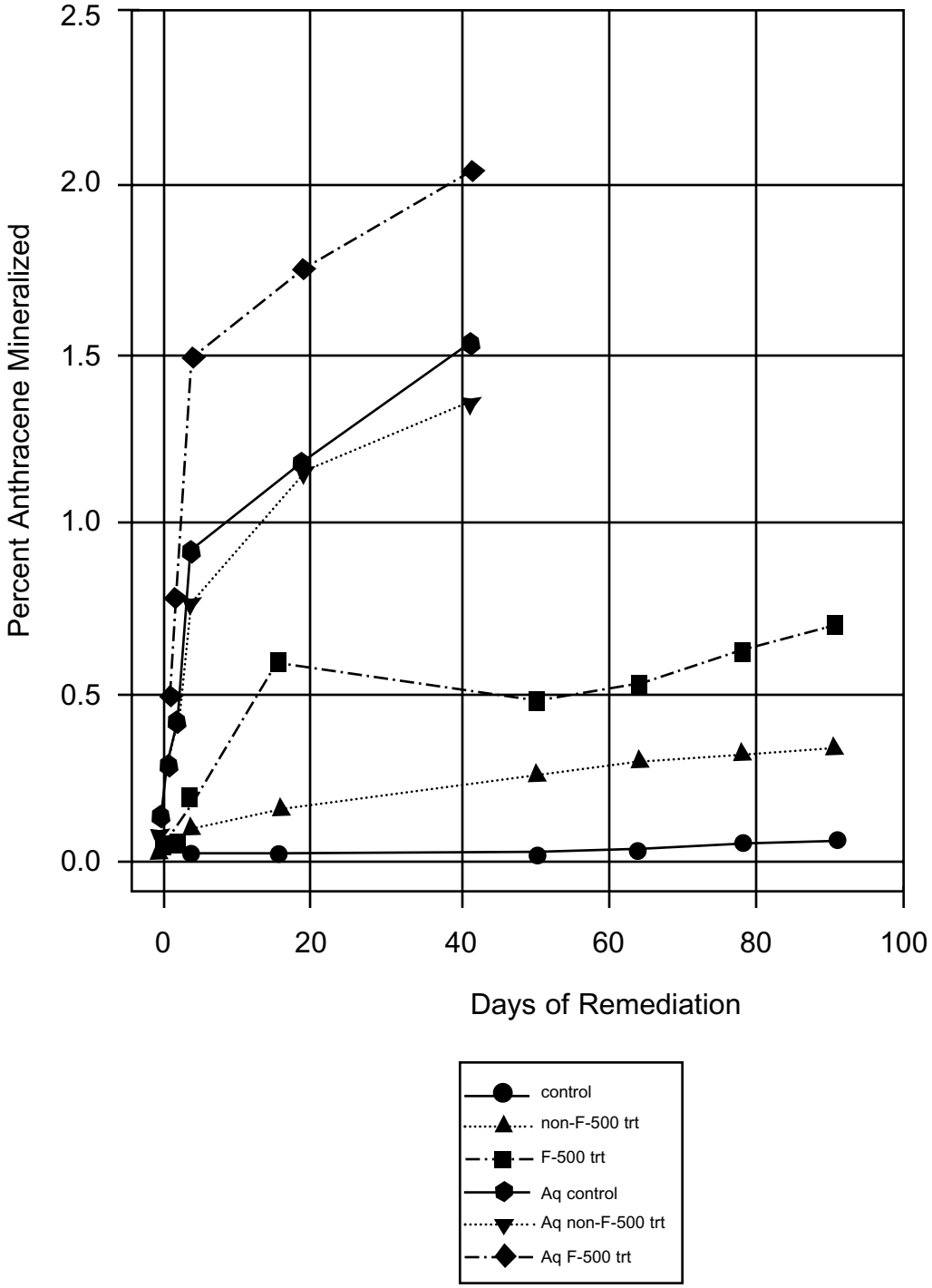

Figure 8. Comparison of Percent Anthracene Mineralized in Soil and Aqueous Media. Observed in soil control, soil non-F-500 treatment (non-F-500 trt), soil F-500 treatment (F-500 trt), aqueous control (Aq control), aqueous non-F-500 treatment (Aq non-F-500 trt) and aqueous F-500 treatment (Aq F-500 trt).

Laha and Luthy (1992), phenanthrene biomineralization was inhibited at surfactant levels that solubilize phenanthrene, and at lower concentrations of nonionic surfactants, phenanthrene mineralization was not significantly different from control.

In Figure 8, it can be seen that only 0.05% of the total amount of anthracene was mineralized in the control bioreactors, and 0.3% in the non-F-500 treatment bioreactors, at the end of the study period. Such low quantities may have even resulted due to mineralization of radiolabeled carbon compounds other than anthracene that were present as impurities with the [9-^{14}C] anthracene used in this study. The purity of [9-^{14}C] anthracene used in this study, as stated earlier in Chapter IV, was 99.7%, indicating the presence of 0.3% impurities. Therefore, the control and the non-F-500 treatment may indicate mineralization of the impurities. In the case of F-500 treatment bioreactors, the total amount of compound mineralized was 0.7% which indicates that anthracene must have been mineralized in F-500 treatment system.

Since mineralization was monitored and not substrate disappearance, minor oxidation or assimilation was not detected by the method used. Therefore, as seen in Figures 1 and 2, before CO_2 could be liberated from [9-^{14}C] anthracene, two of the three rings had to be cleaved (Fedorak et al., 1982). Anthracene may have been degraded, but the final aerobic degradation product detected was a conservative estimation of mineralization, as only one carbon atom of anthracene was labeled and only the evolved end product, $^{14}CO_2$ would be trapped and detected. Most of the labeled intermediates (Figures 1 and 2) of anthracene degradation may be ionized and retained in the water as anions. As a result, these intermediates would not volatilize as readily as CO_2. As seen in Table VII, the maximum amount of anthracene was mineralized by day 16 (250 ppb or 12487.44 ng) in soil with F-500 treatment. After day 16, degradation occurred, but the process was relatively slow and not significantly different from day 16. This

phenomenon may have taken place because anthracene was mixed in the media with surfactant and then added to the bioreactors. This may have solubilized some of the anthracene and thus be made potentially degradable. This hypothesis is supported by the observation using non-F-500 treatment where, approximately 65 ppb or 3228.54 ng of anthracene was mineralized by day 16, which was significantly less than the enhanced degradation with F-500 treatment, indicating a poorly bioavailable amount. In the control, only 8 ppb or 421.20 ng was mineralized by day 16. After day 50, although mineralization with F-500 treatment was still significantly greater than with non-F-500 treatment, the mineralization pattern indicates that a degradation saturation in the system may have eventuated. The maximum amount of anthracene mineralized at the end of the study was 25 ppb, 127 ppb and 285 ppb for control, non-F-500 treatment and F-500 treatment respectively. With non-F-500 treatment, the limiting factor could be due to sorption of anthracene onto soil. Prolonged incubation of PAHs in soil can lead to their increased migration into the organic matter of soil until equilibrium is achieved thus reducing the amount available for degradation. Similarly in F-500 treatment, anthracene may have bound to soil making transfer by F-500 more difficult.

According to Laha and Luthy (1992), bulk addition of surfactant to water does not represent the actual aqueous phase surfactant concentration because some amount of surfactant adsorbs onto soil particles thereby reducing the aqueous phase surfactant concentration. Surfactants which have high CMC require higher concentrations of surfactant to form micelles in solutions that are soils (Swisher, 1987). F-500 has a relatively high CMC, and since a low concentration was used in the study, micelle formation was likely to be minimized, and hence the amount of anthracene solubilized was less than optimal. This could also explain for low mineralization quantities because although each bioreactor had sufficient quantities of anthracene as energy source, only

the soluble amounts were available to the organisms for degradation. Higher concentrations of F-500 may have resulted in greater quantities of anthracene mineralization, but due to lack of information on F-500 only one concentration was tested in this study which was a relatively low concentration.

Bioremediation of Anthracene in Aqueous Media (BAA)

Mineralization of [9-^{14}C] anthracene in the absence of soil showed no significant difference between control and treatment groups when analyzed by ANOVA. However, the distribution of data was not normal (W:Normal 0.804; p = 0.0001) over remediation period and for replicates. Data were log transformed and analyzed nonparametrically using KW test. The results showed no significant difference between treatment groups (KW test statistic = 0.4299; p = 0.4982). However, there was significant difference within control and treatment groups between sampling days (KW test statistic = 75.263; p = 0.0001).

Similarly, in this study, for every mole of $^{14}CO_2$ trapped, one mole of anthracene was mineralized which was quantified and listed in Table VIII. The total amounts of anthracene mineralized in the control and the treatment groups were plotted in Figure 9. Figure 9 was obtained by plotting the antilogarithm of the geometrical mean values (as data was not normally distributed) of the total amount of anthracene mineralized in aqueous media against time.

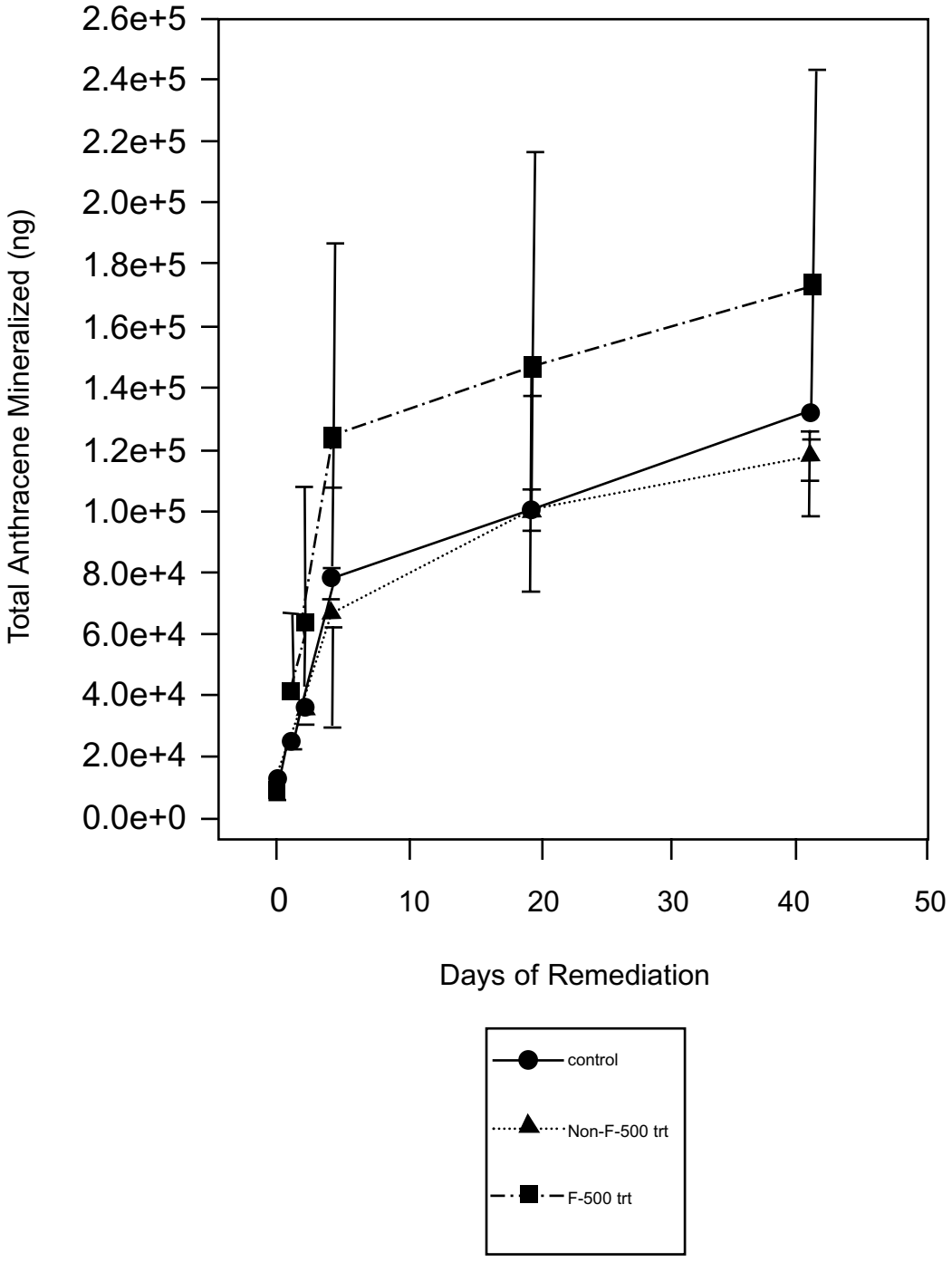

Figure 9. Effect of Nonionic Surfactant F-500 (F-500) on Anthracene Mineralization in Aqueous Media. Effect was observed in control, non-F-500 treatment (non-F-500 trt) and in F-500 treatment (F-500 trt) bioreactors against time.

Table VIII

Quantity of Anthracene Mineralized in Aqueous Media in ng

Day	Control (ng)	Non-F-500 trt (ng)	F-500 trt (ng)
0	11996.58	8535.17	7701.18
1	24558.87	24037.51	42484.01
2	35874.89	34941.28	66736.02
4	78541.47	65397.87	127654.67
19	100520.34	98465.53	149522.63
41	129601.87	114700.62	173298.43

Note: Geometric mean values used above.

From Figure 8, it was observed that the percent of total anthracene mineralized was significantly greater than ($p<0.05$) the total percent of anthracene mineralized from BAS indicating that soil did bind anthracene rendering it less available to the microorganisms for degradation. However, mineralization with F-500 treatment was not significantly different from non-F-500 treatment or control in aqueous media, which suggests that enhanced mineralization did not occur with F-500 in aqueous media.

Degradation half-life of a chemical is defined as the time for half of the reactant initially present to decompose (Voet and Voet, 1994). As the parent compound (anthracene) concentration in both the remediation studies was not monitored over the study period, degradation half hife could not be determined. However, having quantified mineralization in soil and aqueous media, mineralization rate constants and half-lives of anthracene were determined and listed in Table IX. It was assumed that anthracene was degraded by first-order reactions in both BAS and BAA. Once the quantity of anthracene mineralized on each sampling day was determined, concentration of parent compound [A] on those specific days were calculated by subtracting the amount of anthracene mineralized from the initial quantity of anthracene [Ao] on day zero. The total [Ao] in each

BAS bioreactor was 228500 ng (or 2.285 x 10^6 ng), and the [Ao] in each BAA bioreactor was 8573926 ng (or 8.574 x 10^6 ng).

Half-life of a chemical is calculated using the formula: $t_{1/2} = 0.693/k$ (Voet and Voet, 1994), where $t_{1/2}$ is the half-life, *k* is the degradation rate constant or in this case, mineralization rate constant. The factor 0.693 is obtained from first-order kinetics, [A]/[Ao] = c¤ which equals to 0.5 for half-life calculations (Voet and Voet, 1994).

In Figures 10 and 11, the natural log of [A] was plotted against time, and a linear curve was fitted to determine the regression equation for each control and treatment group in BAS and BAA. The slope of each curve was determined to be *-k* from which $t_{1/2}$ was calculated for all controls and treatment groups in BAS and BAA as listed in Table IX.

Table IX

Mineralization Rate Constants and Half-lives of Anthracene in Soil and Aqueous Systems

Treatment	Mineralization Rate Constant *k* (per day)	Mineralization Half-life $t_{1/2}$ (hours)
Soil Control*	-	-
Soil non-F-500	3.3 x 10-5	504000
Soil F-500	7.9 x 10-5	210531
Aqueous Control	3.0 x 10-4	55440
Aqueous non-F-500	2.7 x 10-4	61600
Aqueous F-500	3.9 x 10-4	42646

*****Mineralization rate for soil control was not significant.

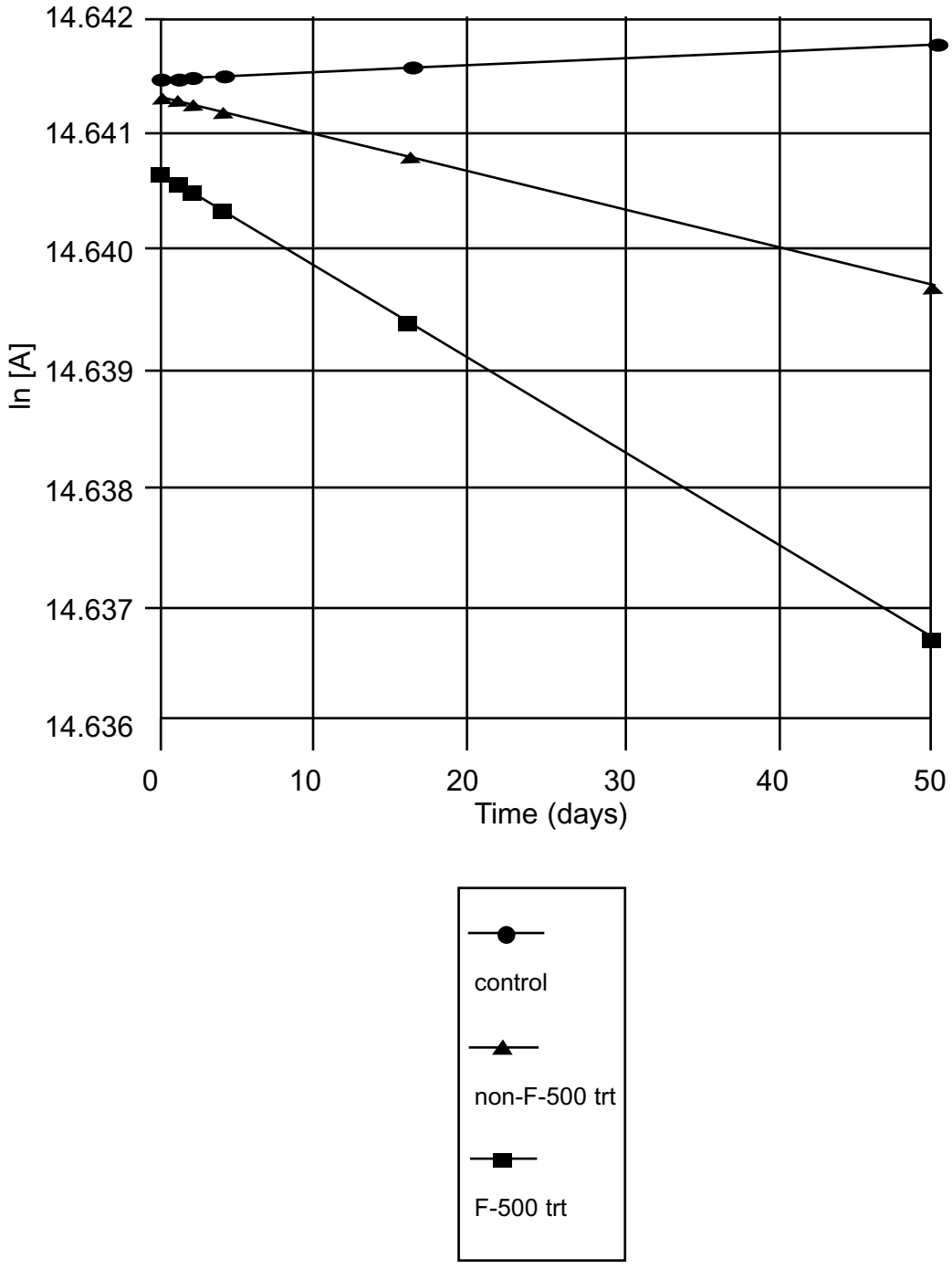

Figure 10. Determination of Mineralization Rate Constant (K) of Anthracene in Soil. K (per day) was determined for control, non-F-500 treatment (non-F-500 trt) and F-500 treatment (F-500 trt) bioreactors by plotting the natural log (ln) of concentration of anthracene [A] over time.

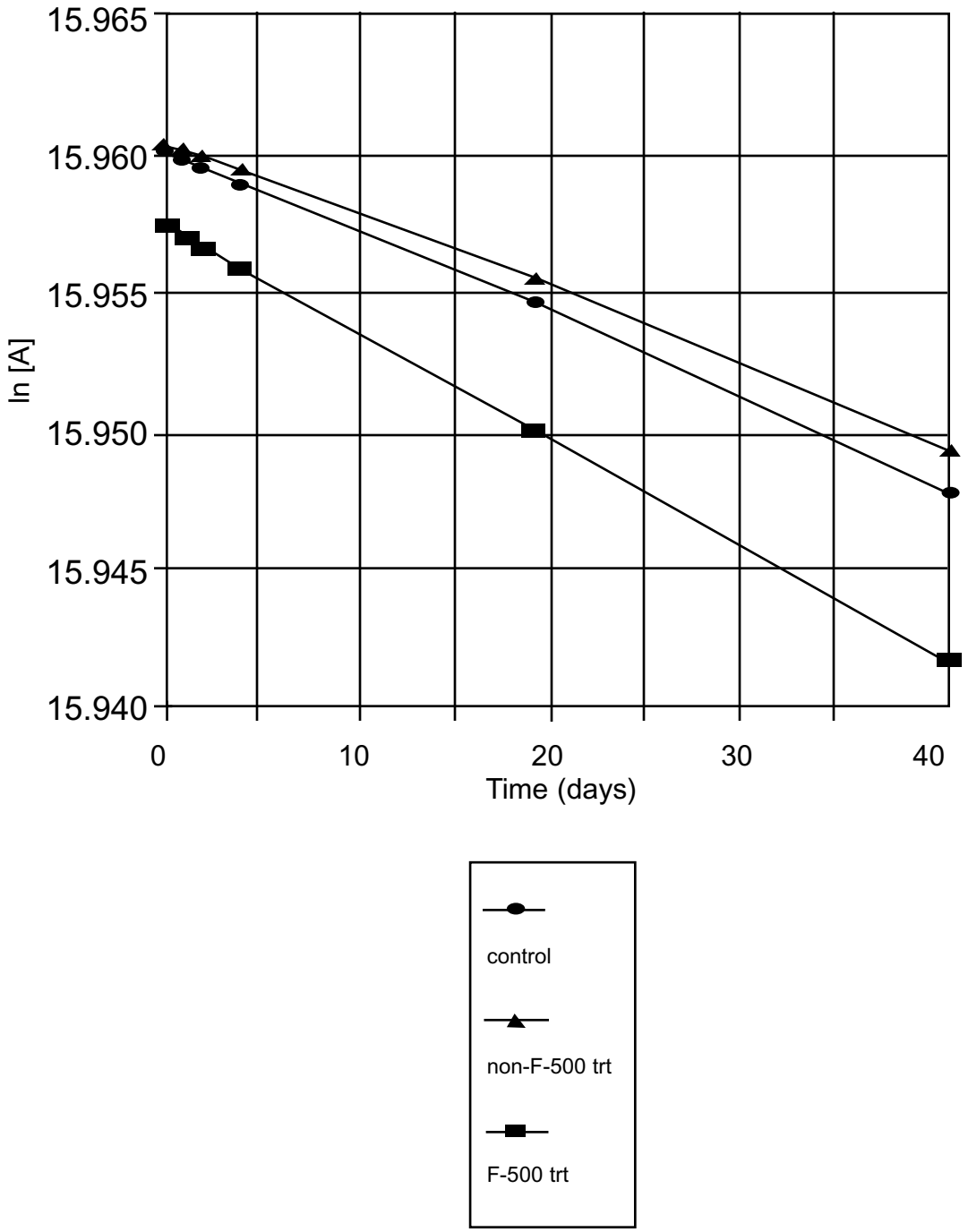

Figure 11. Determination of Mineralization Rate Constant (K) of Anthracene in Aqueous Media. K (per day) was determined for control, non-F-500 treatment (non-F-500 trt) and F-500 treatment (F-500 trt) bioreactors by plotting the natural log (ln) of concentration of anthracene [A] over time.

In the BAS control, the mineralization rate constant could not be determined because the linear regression for the soil control data was not significant ($p = 0.262$). Aqueous half-lives for the control, the non-F-500 and the F-500 treatments in BAA study were not significantly different from each other (p-value = 0.5). However, the mineralization half-life of anthracene for both remediation studies indicated a longer mineralization half-life of anthracene in soil than in aqueous media. This supports the fact that the soil in the BAS study did bind anthracene and interfered with its degradation. Compared to the literature values of anthracene degradation half-life (in soil 1200 to 11040 hours, and in aqueous media 0.58 to 1.7 hours), the mineralization half-life of anthracene is greater in both soil and aqueous media.

In aqueous media, F-500 did not enhance the ability of microorganisms to degrade anthracene. However, the BAS study did show enhanced mineralization of anthracene using F-500 in a soil system, which indicated that anthracene did bind to soil and F-500 was able to desorb the bound anthracene from soil making a greater amount bioavailable for degradation. This could explain the significant difference observed between treatment groups in BAS and the lack of it in BAA compounded by the fact that the half-life of anthracene in soil-water systems is much higher than water systems alone.

CHAPTER VI

CONCLUSIONS

An experimental investigation was conducted to determine the toxic range of anonionic surfactant (F-500) to microorganisms. The EC_{50} range was observed to be between 2000 and 3000 mg/L. The efficacy of using F-500 to enhance mineralization of a polycyclic aromatic hydrocarbon, anthracene, from soil, in a bioremediation process was also assessed. To achieve this, F-500 concentrations to be used in this study were determined by measuring its CMC, which was 3.98×10^{-3} M. Using F-500 at a concentration below its CMC for remediating anthracene in soil showed enhanced anthracene mineralization by 96% compared to the control, and 50% compared to the non-F-500 treatment by enhancing its partitioning into the aqueous phase from soil. However, the total amount of anthracene remediated was not significant as it was less than 1% in soil, and approximately 2% in aqueous media.

This study was further investigated to determine the effect of soil on contaminant bioavailability. Soil did bind anthracene because firstly, it was observed that the percent of anthracene mineralized in aqueous media was between 78% and 98% greater than in the presence of soil and secondly, the mineralization rates and half-lives of anthracene in aqueous media was significantly less than in soil.

Therefore, soil can bind hydrophobic anthracene and increase its half-life in the soil compartment of the environment. However, F-500 is capable of desorbing bound anthracene and rendering it more bioavailable for microbial degradation when used at specific concentrations non-inhibitory to microorganisms.

This study monitored mineralization of anthracene and not substrate disappearance. Complete removal of anthracene from soil and aqueous systems was

the most conservative estimate possible. Very little information exists describing the effect of surfactant on the biodegradation of individual aromatic hydrocarbons (Aronstein et al., 1991) in soil, and on the characteristics of F-500 as a surfactant. Studies conducted by other researchers indicate that surfactants show a range of effects on PAH solubilization and degradation depending on the concentration used which may be characteristic for specific surfactants.

Use of nonionic surfactants also shows a good potential for bioremediation since this class of surfactants is nontoxic to terrestrial and aquatic organisms as well as plants. These surfactants are easily degradable and can be environmentally safe to use, without worrying about surfactants causing a hazardous problem. There are, however, many factors to determine before an ideal process can be developed and this may even vary from site to site depending on the contaminant, microbial population, soil quality, aging of the contaminant and climate conditions because the degrader (i.e. the microorganisms) and the enhancer (i.e., the surfactant), are dependent on these factors. F-500 indicated a potential to enhance biodegradation of aromatic hydrocarbons, but further studies need to be conducted in order to optimize remediation of contaminants at laboratory scale and also under natural environmental conditions.

APPENDICES

Appendix A

Soil Bioremediation Data (BAS)

Table A-I

Mineralization of Anthracene in Soil on Day 0

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	171.65	0.04108	5.90976916	5.950849164	1059.25	21.19	0.04634	3.024999	1.326029
2	133.83	0.03193285	4.59385979	4.625792642	823.391	16.47	0.03602	2.915606	1.216636
3	112.15	0.02668932	3.83952571	3.866215026	688.186	13.76	0.03011	2.837706	1.138736
4	126.01	0.0300415	4.32177065	4.351812155	774.623	15.49	0.03389	2.88909	1.19012
5	135.86	0.03242383	4.66449163	4.696915453	836.051	16.72	0.03658	2.922233	1.223263
6	125.04	0.0298069	4.28802046	4.317827363	768.573	15.37	0.03362	2.885685	1.186715
mean	134.09	0.03199573	4.60290623	4.634901967	825.013	16.5	0.03609	2.912553	1.213583
geo mean	132.92	0.03170916	4.56167968	4.59338884	817.623	16.35	0.03577	2.911998	1.212269
std err	8.25	0.00199566	0.28709496	0.289090611	51.4581	1.029	0.00225	0.025574	0.025574

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	99.24	0.02356691	3.38750704	3.411073943	607.171	12.14	0.02863	2.783311	1.084341
2	104.45	0.024827	3.56863261	3.593459604	639.636	12.79	0.03016	2.805933	1.106963
3	114.11	0.02716337	3.90446236	3.931625723	699.829	14	0.033	2.844992	1.146022
4	136.84	0.03266085	4.69467045	4.727331302	841.465	16.83	0.03968	2.925036	1.226066
5	140.65	0.03358234	4.82712504	4.86070738	865.206	17.3	0.0408	2.937119	1.238149
6	115.68	0.02754309	3.95904338	3.986586469	709.612	14.19	0.03346	2.851021	1.152051
mean	118.495	0.02822392	4.05690681	4.085130737	727.153	14.54	0.03429	2.857902	1.158932
geo mean	117.5137	0.02798295	4.02226983	4.050252782	720.945	14.42	0.034	2.857342	1.157555
std err	6.886103	0.00166547	0.23939527	0.241060748	42.9088	0.858	0.00202	0.025338	0.025338

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	86.31	0.02043965	2.93799579	2.958435442	526.602	10.53	0.02483	2.721482	1.022512
2	163.98	0.03922493	5.63819213	5.677417066	1010.58	20.21	0.04765	3.004571	1.305601
3	196.49	0.04708781	6.76840178	6.815489585	1213.16	24.26	0.05721	3.083917	1.384947
4	126.59	0.03018178	4.33832936	4.368511144	777.595	15.55	0.03667	2.890753	1.191783
5	132.3	0.0315628	4.53683742	4.568400227	813.175	16.26	0.03835	2.910184	1.211214
6	226.38	0.05431701	7.80752716	7.861844171	1399.41	27.99	0.06599	3.145944	1.446974
mean	155.3417	0.03713567	5.33788061	5.375016273	956.753	19.14	0.04512	2.959475	1.260505
geo mean	148.0806	0.0353563	5.08211399	5.117470286	910.91	18.22	0.04295	2.956166	1.25256
std err	20.77	0.00502255	0.72194072	0.726963262	129.399	2.588	0.0061	0.062205	0.062205

Table A-II

Mineralization of Anthracene in Soil on Day 1

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	202.72	0.0485946	6.99081898	7.039413579	1253.02	25.06031	0.054818	3.098	1.398986
2	353.64	0.0850961	12.2419306	12.32702676	2194.21	43.88422	0.095994	3.3413	1.642308
3	213.98	0.0513179	7.38259951	7.433917451	1323.24	26.46475	0.05789	3.1216	1.422668
4	202.73	0.048597	6.99116692	7.039763938	1253.08	25.06156	0.054821	3.098	1.399008
5	179.56	0.0429931	6.18498976	6.227982879	1108.58	22.17162	0.048499	3.0448	1.345797
6	347.23	0.0835458	12.018901	12.10244684	2154.24	43.08471	0.094245	3.3333	1.634323
mean	249.9767	0.0600241	8.6350678	8.695091908	1547.73	30.95453	0.067711	3.1728	1.473849
geo mean	240.5817	0.0577364	8.30596537	8.363701814	1488.74	29.77478	0.06513	3.1706	1.469214
std err	32.11	0.0077652	1.11709907	1.124864256	200.226	4.004517	0.00876	0.053	0.053026

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	412.33	0.0992909	14.272076	14.37136693	2558.1	51.16207	0.120628	3.4079	1.708948
2	210.19	0.0504013	7.24468206	7.295083357	1298.52	25.9705	0.061232	3.1135	1.41448
3	207.97	0.0498644	7.1675038	7.217368162	1284.69	25.69383	0.06058	3.1088	1.409829
4	205.04	0.0491557	7.06564239	7.114798107	1266.43	25.32868	0.059719	3.1026	1.403613
5	191.9	0.0459777	6.6088305	6.654808168	1184.56	23.69112	0.055858	3.0736	1.374586
6	131.19	0.0312943	4.49824829	4.52954263	806.259	16.12517	0.038019	2.9065	1.207504
mean	226.4367	0.0543307	7.80949718	7.863827893	1399.76	27.99523	0.066006	3.1188	1.419827
geo mean	212.8688	0.0510256	7.33441844	7.385444036	1314.61	26.29218	0.061991	3.1154	1.412429
std err	39.11	0.0094587	1.35960027	1.369059013	243.693	4.87385	0.011491	0.0661	0.066109

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	272.89	0.0655659	9.42444659	9.490012516	1689.22	33.78444	0.079656	3.2277	1.528717
2	146.6	0.0350214	5.0339767	5.068998106	902.282	18.04563	0.042547	2.9553	1.256372
3	182.53	0.0437114	6.28308259	6.326794034	1126.17	22.52339	0.053105	3.0516	1.352634
4	174.48	0.0417645	6.00322447	6.044988934	1076.01	21.52016	0.05074	3.0318	1.332846
5	202.19	0.0484664	6.96656219	7.015028599	1248.68	24.9735	0.058882	3.0964	1.397479
6	339.22	0.0816085	11.730408	11.81201652	2102.54	42.05078	0.099146	3.3227	1.623774
mean	219.6517	0.0526897	7.57361676	7.626306452	1357.48	27.14965	0.064013	3.1143	1.415304
geo mean	210.656	0.0504969	7.25842969	7.308926626	1300.99	26.01978	0.061349	3.1118	1.409979
std err	29.55	0.0071463	1.02720303	1.034349285	184.114	3.682283	0.008682	0.0556	0.055566

Table A-III

Mineralization of Anthracene in Soil on Day 2

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	1039.77	0.2510437	36.1151431	36.36618678	6473.181	129.4636	0.2831936	3.81112	2.11215
2	152.79	0.0365185	5.25355418	5.290072696	941.6329	18.83266	0.0411953	2.97388	1.27491
3	120.21	0.0286387	4.11996541	4.148604121	738.4515	14.76903	0.0323063	2.86832	1.16935
4	105.32	0.0250374	3.60188263	3.626920049	645.5918	12.91184	0.0282438	2.80996	1.11099
5	119.19	0.028392	4.08447552	4.112867536	732.0904	14.64181	0.032028	2.86456	1.16559
6	348.75	0.0839134	12.0717879	12.15570136	2163.715	43.2743	0.0946598	3.3352	1.63623
mean	314.3383	0.0755906	10.8744681	10.95005876	1949.11	38.98221	0.0852711	3.11051	1.41154
geo mean	209.1091	0.0500194	7.19579744	7.245816884	1289.755	25.79511	0.0564252	3.09134	1.37207
std err	149.78	0.036225	5.21132654	5.247551522	934.0642	18.68128	0.0408641	0.16015	0.16015

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	212.25	0.0508995	7.31629793	7.367197457	1311.361	26.22722	0.0618377	3.11772	1.41875
2	197.46	0.0473224	6.80212381	6.849446224	1219.201	24.38403	0.0574918	3.08608	1.38711
3	179.68	0.0430221	6.18400239	6.227024527	1108.41	22.16821	0.0522675	3.0447	1.34573
4	184.5	0.0441879	6.35156961	6.395757518	1138.445	22.7689	0.0536837	3.05631	1.35734
5	234.93	0.0563849	8.10476778	8.161152693	1452.685	29.0537	0.0685018	3.16217	1.4632
6	138.99	0.0331808	4.76941517	4.802596018	854.8621	17.09724	0.0403113	2.9319	1.23293
mean	191.3017	0.045833	6.58802945	6.633862406	1180.828	23.61655	0.0556823	3.06648	1.36751
geo mean	188.8531	0.0452346	6.5020263	6.547260931	1165.412	23.30825	0.0549554	3.06563	1.36558
std err	13.29	0.0032141	0.46199623	0.465210342	82.80744	1.656149	0.0039048	0.03207	0.03207

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	216.09	0.0518283	7.44979547	7.50162374	1335.289	26.70578	0.062966	3.12558	1.42661
2	175.09	0.041912	6.02443111	6.06634311	1079.809	21.59618	0.0509188	3.03335	1.33438
3	246.1	0.0590865	8.49309265	8.552179148	1522.288	30.44576	0.071784	3.1825	1.48353
4	194.48	0.0466017	6.69852416	6.745125827	1200.632	24.01265	0.0566162	3.07941	1.38044
5	332.15	0.0798986	11.4846196	11.56451813	2058.484	41.16968	0.0970685	3.31355	1.61458
6	368.03	0.0885765	12.7319872	12.82056372	2282.06	45.64121	0.1076113	3.35833	1.65936
mean	255.3233	0.0613173	8.81374169	8.875058945	1579.76	31.59521	0.0744941	3.18212	1.48315
geo mean	245.9518	0.0590349	8.48567442	8.544709309	1520.958	30.41917	0.0717213	3.17993	1.47848
std err	31.82	0.0076952	1.10610127	1.11379642	198.2558	3.965115	0.0093488	0.05298	0.05298

Table A-IV

Mineralization of Anthracene in Soil on Day 4

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	38.34	0.0088376	1.27138	1.280217619	227.8787	4.557575	0.009969	2.357704	0.65873
2	60.94	0.0143037	2.05772457	2.072028233	368.821	7.376421	0.016135	2.566816	086785
3	59.05	0.0138465	1.9919639	2.005810443	357.0343	7.140685	0.01562	2.55271	0.85374
4	65.68	0.0154452	2.22195229	2.237397529	398.2568	7.965135	0.017423	2.600163	0.90119
5	115.97	0.0276132	3.97243881	4.000052042	712.0093	14.24019	0.03115	2.852486	1.15352
6	258.67	0.0621267	8.93754371	8.999670386	1601.941	32.03883	0.070083	3.204647	1.50568
mean	99.77167	0.0236955	3.40883388	3.432529375	610.9902	12.2196	0.02673	2.689087	0.99012
geo mean	80.48121	0.0189548	2.7268361	2.745790887	488.7508	9.775016	0.021382	2.575972	0.95611
std err	33.47	0.008096	1.16468446	1.172780419	208.7549	4.175098	0.009133	0.121624	0.12162

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	529.58	0.127649	18.3482704	18.47591947	3288.714	65.77427	0.15508	3.517026	1.81806
2	274.52	0.0659602	9.48111351	9.547073673	1699.379	33.98758	0.080135	3.23029	1.53132
3	594.73	0.1434062	20.6132092	20.7566154	3694.678	73.89355	0.174224	3.567577	1.86861
4	61.96	0.0145504	2.09146842	2.106018775	374.8713	7.497427	0.017677	2.573882	0.87491
5	307.43	0.0739198	10.6252291	10.69914893	1904.449	38.08897	0.089805	3.279769	1.5808
6	817.24	0.1972225	28.3487659	28.5459884	5081.186	101.6237	0.239605	3.705965	2.007
mean	430.91	0.1037847	14.9180094	15.02179411	2673.879	53.47759	0.126088	3.312418	1.61345
geo mean	332.281	0.0725251	10.4247523	11.53448579	1053.138	41.06277	0.096816	3.289935	1.5605
std err	109.80	0.0292061	4.19809051	3.843601029	684.161	13.68322	0.032262	0.164833	0.16483

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	977.51	0.2359855	33.9205499	34.15653539	6079.863	121.5973	0.286698	3.783894	2.08492
2	1119.3	0.2702788	38.8498771	39.12015589	6963.388	139.2678	0.328361	3.842821	2.14385
3	710.86	0.1714934	24.6504668	24.82196026	4418.309	88.36618	0.208347	3.645256	1.94629
4	328.46	0.0790061	11.3563368	11.43534287	2035.491	40.70982	0.095984	3.308669	1.6097
5	552.94	0.1332989	19.1603805	19.29367936	3434.275	68.6855	0.161944	3.535835	1.83687
6	623.8	0.1504371	21.6238273	21.77426437	3875.819	77.51638	0.182766	3.588363	1.88939
mean	718.8117	0.1734166	24.9269064	25.10032302	4467.857	89.35715	0.210683	3.617473	1.9185
geo mean	667.0775	0.1608661	23.1228896	23.28375571	4144.509	82.89017	0.195436	3.61321	1.91032
std err	117.80	0.0284907	4.09526014	4.123750886	734.0277	14.68055	0.034613	0.077915	0.07791

Table A-V

Mineralization of Anthracene in Soil on Day 16

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	101.26	0.0240555	3.46061896	3.484674425	620.272	12.40544	0.02714	2.792582	1.09361
2	122.6	0.0292168	4.20312309	4.232339845	753.3565	15.06713	0.03296	2.877001	1.17803
3	56.81	0.0133048	1.91402532	1.927330099	343.0648	6.861295	0.01501	2.535376	0.83641
4	56.87	0.0133193	1.91611296	1.929432251	343.4389	6.868779	0.01503	2.53585	0.83688
5	45.54	0.010579	1.52189685	1.532475868	272.7807	5.455614	0.01193	2.435814	0.73684
6	61.42	0.0144196	2.0744257	2.088845449	371.8145	7.43629	0.01627	2.570326	0.87136
mean	74.083333	0.0174825	2.51503381	2.532516323	450.7879	9.015758	0.01972	2.624491	0.92552
geo mean	69.447488	0.0163351	2.34997349	2.366308635	421.2029	8.424059	0.01843	2.619936	0.91295
std err	12.47	0.0030151	0.43374918	0.436764261	77.74404	1.554881	0.0034	0.069844	0.06984

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	275.4	0.066173	9.5117067	9.577879696	1704.863	34.09725	0.08039	3.231689	1.53272
2	544.67	0.1312967	18.8728741	19.00417275	3382.743	67.65486	0.15951	3.529269	1.8303
3	1090.53	0.2633205	37.8496885	38.11300897	6784.116	135.6823	0.31991	3.831493	2.13252
4	405.9	0.0977358	14.0485372	14.14627292	2518.037	50.36073	0.11874	3.401062	1.70209
5	557.2	0.1343292	19.3084793	19.44280852	3460.82	69.2164	0.1632	3.539179	1.84021
6	534.85	0.1289236	18.5314819	18.66040554	3321.552	66.43104	0.15663	3.521341	1.82237
mean	626.63	0.1511216	21.7222122	19.8240914	3528.688	70.57377	0.1664	3.509006	1.81004
geo mean	590.59076	0.1423837	20.466234	18.13784205	3228.536	64.57072	0.15224	3.504434	1.8012
std err	108.75938	0.0263046	3.78101823	3.974413126	707.4455	14.14891	0.03336	0.080421	0.08042

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	2896.88	0.7002047	100.647418	101.3476228	18039.88	360.7975	0.85068	4.256234	2.55726
2	3269.19	0.7902516	113.590769	114.3810211	20359.82	407.1964	0.96007	4.308774	2.6098
3	1248.77	0.3015925	43.3508996	43.65249207	7770.144	155.4029	0.3664	3.890429	2.19146
4	3179.95	0.768668	110.488342	111.2570103	19803.75	396.075	0.93385	4.296747	2.59778
5	2992.75	0.7233918	103.980337	104.703729	18637.26	372.7453	0.87885	4.270382	2.57141
6	579.5	0.1397227	20.0837385	20.22346115	3599.776	71.99552	0.16975	3.556275	1.85731
mean	2361.1733	0.5706385	82.0235842	82.59422274	14701.77	294.0354	0.69327	4.096474	2..3975
geo mean	2006.317	0.4846911	69.6694965	70.15418757	12487.45	249.7489	0.58885	4.086296	2.37937
std err	468.77718	0.1133785	16.2970313	16.41040982	2921.053	58.42106	0.13774	0.125859	0.12586

Table A-VI

Mineralization of Anthracene in Soil on Day 50

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	156.46	0.0374061	5.38124819	5.418654331	964.5205	19.2904	0.0422	2.984311	1.285341
2	57.42	0.0134523	1.93524967	1.948701978	346.869	6.93738	0.01518	2.540165	0.841195
3	70.09	0.0165167	2.37608974	2.392606424	425.8839	8.51768	0.01863	2.626291	0.930321
4	64.37	0.0151332	2.17706802	2.19220126	390.2118	7.80424	0.01707	2.5913	0.89233
5	47.81	0.011128	1.60087925	1.612007288	286.9373	5.73875	0.01255	2.457787	0.758817
6	71.21	0.0167876	2.41505903	2.431846596	432.8687	8.65737	0.01894	2.636356	0.937386
mean	77.89333	0.018404	2.64759898	2.666002979	474.5485	9.49097	0.02076	2.639869	0.940899
geo mean	71.886	0.0169239	2.43467052	2.451594412	436.3838	8.72768	0.01909	2.634926	0.927944
std err	16.10934	0.0038962	0.5605085	0.564404703	100.464	2.00928	0.0044	0.073986	0.073986

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	413.10	0.0994771	14.2988451	14.3983222	2562.901	51.258	0.12085	3.408732	1.709762
2	505.58	0.1218444	17.5139108	17.6357552	3139.164	62.7833	0.14803	3.496814	1.797844
3	587.79	0.1417277	20.3719402	20.51366789	3651.433	73.0287	0.17218	3.562463	1.863493
4	733.38	0.1769401	25.4333743	25.61031441	4558.636	91.1727	0.21496	3.658835	1.959865
5	777.33	0.1875699	26.9612953	27.14886523	4832.498	96.65	0.22788	3.684172	1.985202
6	3109.54	0.7516387	108.04054	108.7921784	19365.01	387.3	0.91316	4.287018	2.588048
mean	1021.12	0.246533	35.4366509	35.68318388	6351.607	127.032	0.29951	3.683006	1.984036
geo mean	775.5616	0.1870669	26.8890006	27.07606756	4819.54	96.3908	0.22727	3.672617	1.965636
std err	421.3854	0.1019164	14.6494583	14.75137463	2625.745	52.5149	0.12382	0.127781	0.127781

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	274.96	0.0660668	9.4964101	9.562476684	1702.121	34.0424	0.08026	3.23099	1.53202
2	2189.05	0.5290088	76.0397192	76.56872795	13629.23	272.585	0.64269	4.134471	2.435501
3	2106.89	0.5091375	73.1834281	73.69256559	13117.28	262.346	0.61855	4.117844	2.418874
4	2025.14	0.4893655	70.3413906	70.83075604	12607.87	252.157	0.59453	4.100642	2.401672
5	2224.31	0.5375368	77.2655325	77.80306929	13848.95	276.979	0.65305	4.141417	2.442447
6	2499.06	0.6039878	86.817212	87.42119986	15560.97	311.219	0.73378	4.192037	2.493067
mean	1886.568	0.4558505	65.5239488	65.97979924	11744.4	234.888	0.55381	3.986233	2.287263
geo mean	1557.515	0.3760321	54.0508528	54.42688486	9687.986	193.76	0.45684	3.970165	2.256437
std err	328.9243	0.0795537	11.4350481	11.51460177	2049.599	40.992	0.09665	0.151571	0.151571

Table A-VII

Mineralization of Anthracene in Soil on Day 64

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	204.05	0.04891627	7.03709501	7.086011283	1261.31	25.2262	0.055181	3.10082	1.401852
2	94.68	0.02246402	3.23167439	3.254138415	579.2366	11.58473	0.025341	2.76286	1.063886
3	93.85	0.02226326	3.20279536	3.225058644	574.0604	11.48121	0.025114	2.75896	1.059988
4	105.25	0.02502049	3.59944705	3.624467538	645.1552	12.9031	0.028225	2.80966	1.110694
5	72.57	0.01711649	2.46237888	2.479495376	441.3502	8.827004	0.019309	2.64478	0.945813
6	102.18	0.02427797	3.49262945	3.516907424	626.0095	12.52019	0.027387	2.79658	1.097611
mean	112.0967	0.02667642	3.83767002	3.864346447	687.8537	13.75707	0.030093	2.81228	1.113307
geo mean	105.9569	0.02517149	3.62116986	3.646341345	649.0488	12.98098	0.028395	2.80894	1.105243
std err	18.97303	0.00458862	0.6601476	0.664736422	118.3231	2.366462	0.005176	0.06241	0.06241

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1*									
2	445.58	0.10733277	15.4280118	15.53534452	2765.291	55.30583	0.130396	3.44174	1.742771
3	639.45	0.1542222	22.1678993	22.32212149	3973.338	79.46675	0.187364	3.59916	1.900185
4	820.61	0.19803759	28.4659239	28.66396146	5102.185	102.0437	0.240595	3.70776	2.008786
5	824.43	0.1989615	28.5987261	28.79768761	5215.988	102.5198	0.241718	3.70978	2.010808
6	3175.71	0.76764254	110.340939	111.1085813	19777.33	395.5465	0.932606	4.29617	2.597198
mean	1181.156	0.28523932	41.0003	41.28553927	7348.826	146.9765	0.346536	3.75092	2.05195
geo mean	906.5103	0.21873133	31.440441	31.65917236	5635.333	112.7067	0.265736	3.74028	2.033149
std err	503.4883	0.12177379	17.5037639	17.62553773	3137.346	62.74691	0.147943	0.14482	0.144823

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	354.72	0.08535735	12.2692653	12.35462261	2199.123	43.98246	0.1037	3.34225	1.643279
2	2343.01	0.56624556	81.3921362	81.95838175	14588.59	291.7718	0.68793	4.16401	2.456043
3	2183.72	0.52771965	75.8544218	76.38214147	13596.02	271.9204	0.641125	4.13341	2.434442
4	2120.88	0.51252115	73.6697902	74.18231135	13204.45	264.089	0.62266	4.12072	2.42175
5	2353.36	0.56874881	81.7519538	82.32070259	14653.09	293.0617	0.690971	4.16593	2.466959
6	2633.51	0.63650594	91.4913642	92.12787012	16398.76	327.9752	0.773289	4.21481	2.515841
mean	1998.2	0.48284974	69.4048219	69.88767165	12440.01	248.8001	0.586613	4.02352	2.324553
geo mean	1696.678	0.40974528	58.8967861	59.30653143	10556.56	211.1313	0.497796	4.0107	2.300479
std err	336.6079	0.08141205	11.7021677	11.78357972	2097.477	41.94954	0.098907	0.1369	0.136902

*Bioreactors were damaged therefore, discarded.

Table A-VIII

Mineralization of Anthracene in Soil on Day 78

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	253.89	0.0609706	8.77122835	8.832198938	1572.1314	31.44263	0.06878	3.19649	1.49752
2	143.49	0.0342692	4.92996991	4.964239125	883.63456	17.67269	0.03866	2.94627	1.2473
3	136.21	0.0342692	4.92996991	4.964239125	883.63456	17.67269	0.03866	2.94627	1.2473
4	160.26	0.0325085	4.67666953	4.709178007	838.23369	16.76467	0.03667	2.92337	1.2244
5	127.47	0.0383252	5.51346541	5.551790629	988.21873	19.76437	0.04323	2.99485	1.29588
6	156.1	0.0303946	4.3725699	4.402964522	783.72768	15.67455	0.03429	2.89417	1.1952
mean	164.264	0.0384562	5.53231217	5.570768391	991.59677	19.83194	0.04338	2.98357	1..2846
geo mean	158.921	0.0373423	5.37206397	5.409406272	962.87432	19.25749	0.04212	2.98195	1.28097
std err	21.04	0.0046272	0.6656749	0.670302139	119.31378	2.386276	0.00522	0.04467	0.04467

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1*									
2	516.47	0.1244782	17.8925015	18.01697973	3207.0224	64.14045	0.15123	3.5061	1.80713
3	697.82	0.1683396	24.1971314	24.36547101	4337.0538	86.74108	0.20452	3.63719	1.93822
4	857.82	0.2070372	29.7595289	29.96656815	5334.0488	106.681	0.25153	3.72706	2.02809
5	845.75	0.204118	29.3399156	29.54403354	5258.838	105.1768	0.24798	3.72089	2.02192
6	3229.8	0.7807248	112.221377	113.0021015	20114.374	402.2875	0.9485	4.30351	2.60454
mean	1229.53	0.2969395	42.6820908	42.97903039	7650.2674	153.0053	0.36075	3.77895	2.07998
geo mean	968.764	0.2333143	33.5366046	33.76991892	6011.0458	120.2209	0.28345	3.76951	2.06345
std err	503.87	0.1218662	17.5170431	17.63890929	3139.7259	62.79452	0.14805	0.13707	0.13707

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	574.84	0.1385956	19.9217336	20.06032926	3570.7386	71.41477	0.16838	3.55276	1.85379
2	2568.79	0.6208528	89.2413744	89.86222713	15995.476	319.9095	0.75427	4.204	2.50503
3	2266.04	0.5476296	78.7162753	79.26390492	14108.975	282.1795	0.66531	4.1495	2.45053
4	2271.94	0.5490566	78.9213887	79.4704453	14145.739	282.9148	0.66705	4.15063	2.45166
5	3384.09	0.8180414	117.585266	118.4033076	21075.789	421.5158	0.99384	4.32378	2.62481
6	2706.16	0.6540771	94.0170403	94.67111738	16851.459	337.0292	0.79464	4.2264	2.52767
mean	2295.31	0.5547088	79.7338464	80.28855526	14291.363	285.8273	0.67391	4.10122	2.40225
geo mean	2028.26	0.4900131	70.4344865	70.92449962	12624.561	252.4912	0.59532	4.09291	2.38727
std err	382.555	0.0925248	13.2995201	13.39204496	2383.784	47.67568	0.11241	0.11275	0.11275

*Bioreactors were damaged therefore, discarded.

Table A-IX

Mineralization of Anthracene in Soil on Day 91

Control	14-C DPM	Anthracene hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	290.62	0.0698541	10.0492123	10.11906636	1801.1938	36.02388	0.0788	3.25556	1.55659
2	182.41	0.0436824	6.28415268	6.327835103	1126.3546	22.52709	0.049277	3.051675	1.35271
3	169.31	0.0436824	6.28415268	6.327835103	1126.3546	22.52709	0.049277	3.051675	1.35271
4	201.32	0.040514	5.82835118	5.868865234	1044.658	20.89316	0.045702	3.018974	1.32
5	175.92	0.048256	6.94210737	6.990363364	1244.2847	24.88569	0.054436	3.09492	1.39595
6	194.56	0.0421127	6.05833957	6.10045232	1085.8805	21.71761	0.047506	3.035782	1.33681
mean	202.3567	0.048017	6.90771929	6.955736248	1238.1211	24.76242	0.054166	3.084764	1.38579
geo mean	198.8621	0.0471407	6.78166013	6.828800827	1215.5265	24.31053	0.053178	3.083758	1.38362
std err	18.29492	0.0044931	0.64638257	0.650875701	115.85587	2.317117	0.005069	0.03568	0.03568

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1*									
2	558.92	0.1347452	19.3682751	19.50302029	3471.5376	69.43075	0.163702	3.540522	1.84155
3	736.02	0.1775786	25.5251538	25.70273248	4575.0864	91.50173	0.21574	3.660399	1.96143
4	927.04	0.2237788	32.1659612	32.38973994	5765.3737	115.3075	0.271868	3.760827	2.06186
5	886.19	0.2138988	30.7458115	30.95971034	5510.8284	110.2166	0.259865	3.741217	2.04225
6	3281.9	0.7933257	114.032632	114.8259581	20439.021	408.7804	0.963809	4.31046	2.61149
mean	1278.014	0.3086654	44.3675668	44.67623223	7952.3693	159.0474	0.374997	3.802685	2.10372
geo mean	1020.935	0.2464203	35.4204595	35.66687989	6348.7046	126.9741	0.299375	3.793876	2.08835
std err	505.13	0.1221708	17.5608284	17.68299918	3147.5739	62.95148	0.148425	0.132729	0.13273

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	ppb	% mineral	log ng	log ppb
1	781.45	0.1885663	27.1045271	27.29309343	4858.1706	97.16341	0.229089	3.686473	1.9875
2	2921.05	0.7060504	101.487688	102.1937382	18190.485	363.8097	0.857778	4.259844	2.56087
3	2459.28	0.5943666	85.4342609	86.02862758	15313.096	306.2619	0.722094	4.185063	2.48609
4	2438.74	0.5893988	84.7201882	85.30958699	15185.106	303.7021	0.716059	4.181418	2.48245
5	3599.32	0.8700969	125.067734	125.9378307	22416.934	448.3387	1.057078	4.350576	2.65161
6	2891.29	0.6988527	100.453082	101.1519345	18005.044	360.1009	0.849034	4.255394	2.55642
mean	2515.188	0.6078886	87.3779133	87.98580192	15661.473	313.2295	0.738522	4.153128	2.45416
geo mean	2285.358	0.5522293	79.3774457	79.92967502	14227.482	284.5496	0.670902	4.147179	2.44365
std err	387.1406	0.0936339	13.4589384	13.55257235	2412.3579	48.24716	0.113756	0.096682	0.09668

*Bioreactors were damaged therefore, discarded.

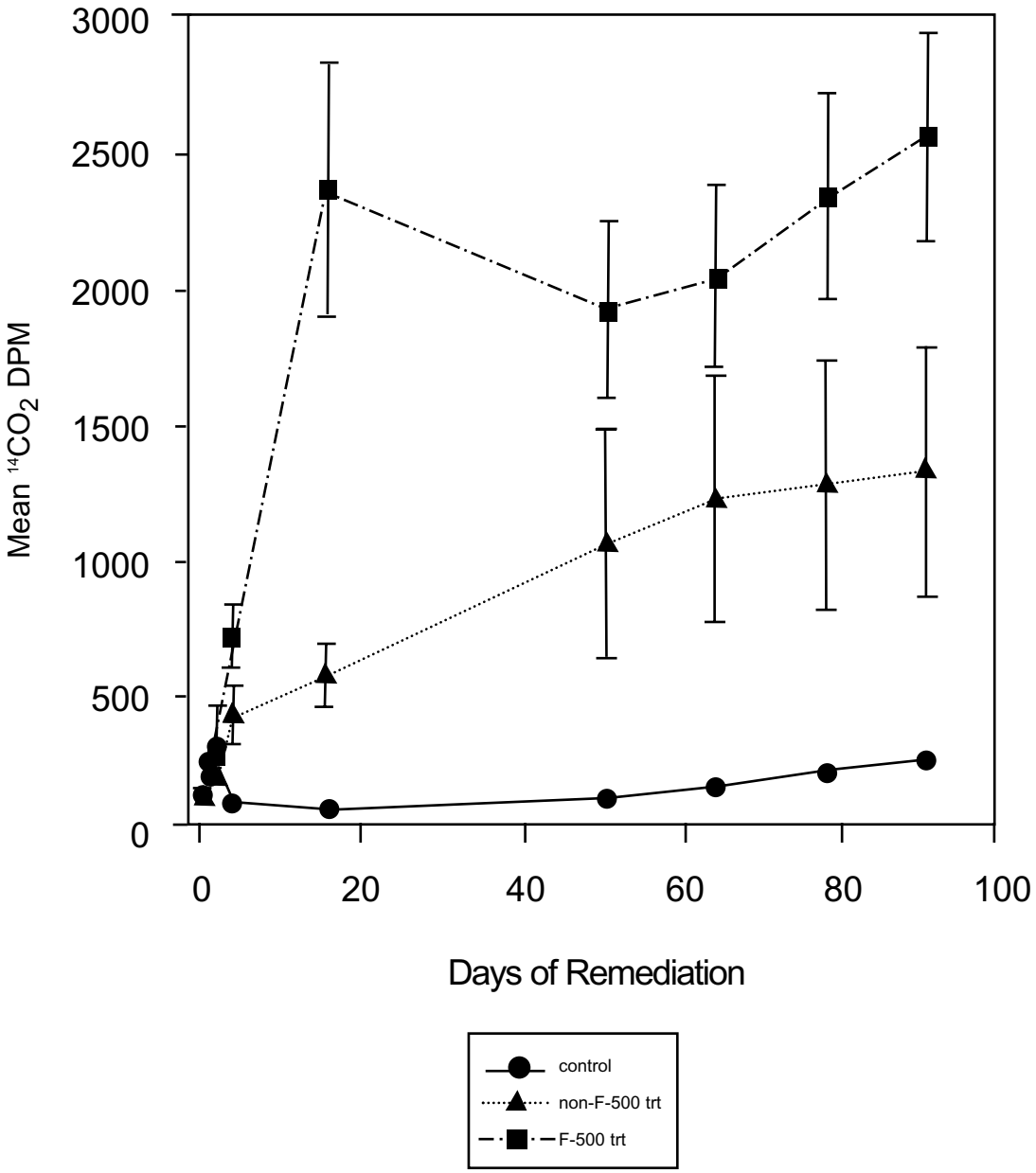

Figure A-1. Mineralization of [9-^{14}C] Anthracene in Soil Mean $^{14}CO_2$ DPM Vs Time. Observed in control, non-F-500 treatment (non-F-500 trt) and F-500 treatment bioreactors.

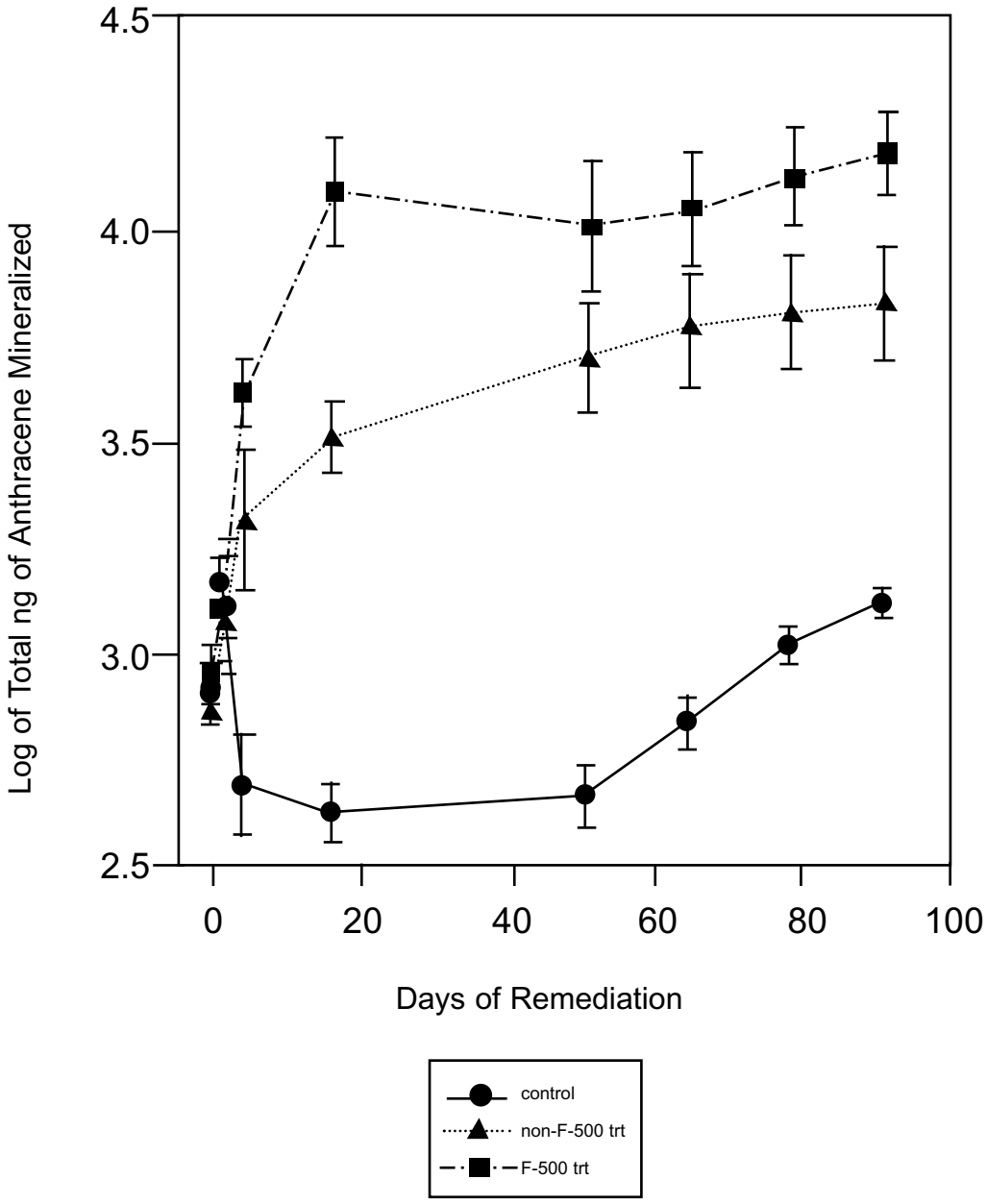

Figure A-2. Mineralization of [9-^{14}C] Anthracene in Soil Log Total ng of Anthracene Vs Time. Observed in control, non-F-500 treatment (non-F-500 trt) and F-500 treatment bioreactors.

Appendix B

Aqueous Media Bioremediation Data (BAA)

Table B-I

Mineralization of Anthracene in Aqueous Media on Day 0

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	185.51	0.044432141	96.97314835	97.017580496	17269.1293	0.20141446	4.23727044
2	185.65	0.044466002	97.04704874	97.091514739	17282.2896	0.20156796	4.23760128
3	117.65	0.028019508	61.15257718	61.180596686	10890.1462	0.12701468	4.03703371
4	124.44	0.029661739	64.73674573	64.766407474	11528.4205	0.13445904	4.06176981
5	102.97	0.024469001	53.4035942	53.4280632	9510.19525	0.11091994	3.97818943
6	90.79	0.021523144	46.97426091	46.995784055	8365.24956	0.09756614	3.9224789
mean	134.501667	0.032095256	70.04789585	70.079991108	12474.2384	0.14549037	4.07905726
geo mean	129.487295	0.030866264	67.36562085	67.396487109	11996.5747	0.13991925	4.07729066
std err	16.8405994	0.004073071	8.889476729	8.8935497999	1583.05186	0.01846358	0.05382798

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	89.07	0.021107144	46.06634193	46.087449069	8203.56593	0.09568038	3.91400267
2	94.94	0.022526863	49.1648794	49.18740626	8755.35831	0.10211608	3.94227392
3	96.14	0.022817096	49.79831125	49.821128343	8868.16085	0.10343173	3.94783356
4	77.2	0.018236264	39.8006452	39.818881462	7087.7609	0.08266645	3.85050906
5	106.22	0.025255046	55.1191388	55.144393843	9815.7021	0.11448315	3.99192137
6	94.61	0.02244705	48.99068564	49.013132687	8724.33762	0.10175428	3.94073246
mean	93.03	0.02206491	48.15666703	48.178731944	8575.81429	0.10002201	3.93121218
geo mean	92.6063796	0.021960336	47.92843286	47.9503932	8535.16999	0.09954797	3.93097823
std err	3.8989879	0.00094301	2.05811927	2.0590622804	366.513086	0.00427474	0.01913464

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	79.56	0.018807054	41.04639451	41.065201559	7309.60588	0.08525388	3.86389396
2	78.13	0.018461194	40.29155488	40.310016076	7175.18286	0.08368607	3.85583297
3	91.88	0.021786771	47.54962818	47.571414947	8467.71186	0.09876118	3.92776607
4	86.04	0.020374308	44.4669265	44.487300809	7918.73954	0.09235837	3.89865606
5	90.55	0.021465097	46.84757454	46.869039638	8342.68906	0.09730301	3.92130606
6	77.44	0.01829431	39.92733157	39.945625879	7110.32141	0.08292958	3.85188923
mean	83.9333333	0.019864789	43.3549017	43.374766485	7720.70843	0.09004868	3.88655739
geo mean	83.7300398	0.019814549	43.2452534	43.265067949	7701.18209	0.08982094	3.88643504
std err	2.62256829	0.000634295	1.384348573	1.3849828678	246.52695	0.00287531	0.01379795

Table B-II

Mineralization of Anthracene in Aqueous Media on Day 1

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	248.43	0.05964998	130.1860917	130.2457173	23183.742	0.27039817	4.36518354
2	267.17	0.06418244	140.0781858	140.14236826	24945.3416	0.29094417	4.39698945
3	519.59	0.1252328	273.3205757	273.44580848	48673.3539	0.56769031	4.68729127
4	216.14	0.05184032	113.1414964	113.19333667	20148.4139	0.23499633	4.30424086
5	244.09	0.05860031	127.8951799	127.9537802	22775.7729	0.26563992	4.35747312
6	182.49	0.04370172	95.37901153	95.422713253	16985.243	0.19810342	4.23007176
mean	279.65166667	0.06720126	146.6667568	146.7339581	26118.6445	0.30462872	4.39020834
geo mean	263.1456044	0.06318808	137.9079776	137.97116564	24558.8675	0.28643662	4.38793126
std err	49.477180492	0.01196656	26.11701835	26.128984914	4650.95931	0.05424538	0.06404067

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	289.61	0.06960979	151.9233614	151.99297122	27054.7489	0.31554676	4.43224351
2	408.02	0.09824845	214.4272493	214.52549779	38185.5386	0.44536813	4.58189892
3	246.6	0.05920738	129.2201082	129.27931555	23011.7182	0.26839181	4.36194905
4	290.48	0.06982021	152.3825995	152.45241973	27136.5307	0.3165006	4.43355432
5	182.12	0.04361224	95.18370338	95.227315611	16950.4622	0.19769776	4.22918154
6	189.46	0.04538749	99.05819486	99.103582353	17640.4377	0.20574513	4.24650936
mean	267.715	0.06431426	140.3658695	140.43018371	24996.5727	0.2915417	4.38088945
geo mean	257.58065782	0.06184666	134.9803356	135.04218226	24037.5084	0.28035587	4.37923609
std err	33.950611948	0.0082113	17.92116581	17.929377111	3191.42913	0.03722249	0.05392825

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	166.77	0.03989968	87.08105428	87.120953962	15507.5298	0.18086846	4.19054262
2	2189.05	0.52900872	1154.561524	1155.0905328	205606.115	2.39803898	5.31303603
3	229.31	0.05502562	120.0934109	120.14843654	21386.4217	0.24943554	4.33013813
4	1904.62	0.46021642	1004.42234	1004.882556	178869.095	2.08619798	5.25253531
5	202.13	0.04845186	105.7461795	105.79463135	18831.4444	0.21963616	4.27488363
6	274.12	0.06586337	143.746812	143.81267533	25598.6562	0.29856396	4.40821717
mean	827.6666667	0.19974428	435.9418867	436.14163099	77633.2103	0.90545685	4.62822548
geo mean	454.66976386	0.10930808	238.5648937	238.67420178	42484.0079	0.49550232	4.60551823
std err	387.54432867	0.09373155	204.5691012	204.66283278	36429.9842	0.42489263	0.20915741

Table B-III

Mineralization of Anthracene in Aqueous Media on Day 2

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	309.73	0.07447601	162.543902	162.61837815	28946.0713	0.33760575	4.46158963
2	336.9	0.08104736	176.885855	176.96690232	31500.1086	0.36739417	4.49831205
3	964	0.23271788	507.906783	508.13950101	90448.8312	1.05492885	4.95640296
4	302.18	0.07264997	158.55856	158.63121004	28236.3554	0.32932815	4.45080864
5	378.4	0.09108455	198.79204	198.88312437	35401.1961	0.4128936	4.54901794
6	276.89	0.06653333	145.208984	145.27551714	25859.0421	0.30160091	4.41261243
mean	428.0166667	0.10308485	224.982687	225.08577217	40065.2674	0.4672919	4.55479061
geo mean	383.5725963	0.09230334	201.452036	201.54433958	35874.8924	0.41841844	4.55120461
std err	108.1215467	0.0261503	57.0730262	57.099176512	10163.6534	0.1185414	0.08250837

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	383.54	0.09232772	201.50524	201.59756729	35884.367	0.41852895	4.55490529
2	592.52	0.14287166	311.817396	311.96026809	55528.9277	0.6476487	4.74451929
3	362.87	0.08732847	190.594376	190.68170441	33941.3434	0.39586694	4.53072903
4	478.3	0.11524639	251.525241	251.6404878	44792.0068	0.52242113	4.65120052
5	255.47	0.06135268	133.902225	133.96357795	23845.5169	0.27811663	4.37740674
6	269.8	0.06481854	141.466457	141.53127583	25192.5671	0.29382763	4.40127242
mean	390.4166667	0.09399091	205.135158	205.2291469	36530.7881	0.42606833	4.54333888
geo mean	373.5864903	0.08990124	196.209457	196.29935801	34941.2857	0.40752954	4.5415069
std err	52.32409519	0.01265512	27.6197904	27.63244551	4918.5753	0.05736668	0.05776874

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	241.25	0.05791343	126.396058	126.45397126	22508.8069	0.26252622	4.35235248
2	4070.66	0.98409528	2147.78795	2148.7720404	382481.423	4.4609829	5.58261035
3	332.83	0.08006299	174.737465	174.81752825	31117.52	0.36293194	4.49300498
4	3673.52	0.88804292	1938.15367	1939.0417169	345149.426	4.02556985	5.53800715
5	297.02	0.07140197	155.834803	155.90620509	27751.3045	0.32367087	4.4432834
6	368.04	0.08857888	193.323411	193.41199038	34427.3343	0.40153519	4.5369034
mean	1497.22	0.36168258	789.372226	789.73390872	140572.636	1.63953616	4.82436029
geo mean	712.885241	0.17170664	374.749745	374.92145175	66736.0184	0.77835999	4.79717862
std err	752.9423915	0.18210679	397.448077	397.63018413	70778.1728	0.82550471	0.23414196

Table B-IV

Mineralization of Anthracene in Aqueous Media on Day 4

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	707.29	0.17062995	372.3998744	372.57050434	66317.5498	0.773479	4.821628
2	576.02	0.13888097	303.1077085	303.24658945	53977.8929	0.629559	4.732216
3	3983.16	0.96293251	2101.600206	2102.5631385	374256.239	4.36505	5.573169
4	496.89	0.11974257	261.3381565	261.45789907	46539.506	0.542803	4.667822
5	909.4	0.21951232	479.0856339	479.30514622	85316.316	0.995067	4.931032
6	471.25	0.11354127	247.8038296	247.91737056	44129.292	0.514692	4.644727
mean	1190.6683	0.28753993	627.5559014	627.84344136	111756.133	1.303442	4.895099
geo mean	837.69946	0.20208117	441.0421524	441.24423362	78541.4736	0.91605	4.885348
std err	562.35991	0.13601248	296.8472294	296.98324216	52863.0171	0.616555	0.142284

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	694.09	0.1674374	365.432124	365.59956143	65076.7219	0.759007	4.813426
2	843.49	0.20357131	444.2943895	444.4979608	79120.637	0.922805	4.89829
3	697.71	0.16831293	367.3429768	367.51128971	65417.0096	0.762976	4.815691
4	804.78	0.1942089	423.8609337	424.05514259	75481.8154	0.880365	4.877842
5	697.19	0.16818717	367.0684896	367.23667681	65368.1285	0.762406	4.815366
6	502.56	0.12111392	264.331122	264.45223592	47072.498	0.549019	4.672767
mean	706.83667	0.17047194	372.0550059	372.22547788	66256.1351	0.772763	4.815564
geo mean	697.53256	0.1682637	367.2355204	367.4037841	65397.8736	0.762753	4.81502
std err	48.419892	0.01171084	25.5589182	25.570629043	4551.57197	0.053086	0.032206

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	562.1	0.13551427	295.759899	295.89541328	52669.3836	0.614297	4.721558
2	4294.42	1.03821392	2265.901871	2266.9400849	403515.335	4.706307	5.60586
3	465.59	0.11217234	244.8161424	244.92831474	43597.24	0.508486	4.639459
4	4793.87	1.15901099	2529.541486	2530.7004969	450464.688	5.25389	5.653661
5	776.4	0.18734491	408.8802704	409.06761532	72814.0355	0.84925	4.862215
6	1516.51	0.36634806	799.5546431	799.92099121	142385.936	1.660685	5.153467
mean	2068.1483	0.49976742	1090.742385	1091.2421527	194241.103	2.265486	5.106037
geo mean	1360.577	0.32844564	716.832617	717.16106262	127654.669	1.48887	5.092096
std err	799.89869	0.19346365	422.2344244	422.4278881	75192.1641	0.876986	0.180456

Table B-V

Mineralization of Anthracene in Aqueous Media on Day 19

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	870.27	0.21004833	458.430477	458.64052529	81638.0135	0.9521659	4.91189243
2	747.8	0.18042771	393.783478	393.96390567	70125.5752	0.8178933	4.84587644
3	5035.52	1.21745651	2657.09882	2658.3162815	473180.298	5.5188281	5.67502665
4	703.21	0.16964316	370.246203	370.41584926	65934.0212	0.7690061	4.81910956
5	1949.31	0.47102515	1028.0124	1028.4834226	183070.049	2.1351948	5.2626173
6	626.32	0.15104653	329.65906	329.81010677	58706.199	0.6847061	4.76868396
mean	1655.405	0.39994123	872.871741	873.27168184	155442.359	1.8129657	5.04720106
geo mean	1188.15839	0.28683217	626.011211	626.29804318	111481.052	1.3002333	5.03724069
std err	705.157964	0.17054964	372.224595	372.39514494	66286.3358	0.7731152	0.14479948

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	1058.28	0.25552046	557.673412	557.92893269	99311.35	1.1582948	4.99699889
2	1341.25	0.32395964	707.041921	707.36588094	125911.127	1.4685351	5.10006411
3	1116	0.26948063	588.141484	588.41096489	104737.152	1.2215773	5.02010076
4	1092.41	0.26377515	575.68927	575.95304494	102519.642	1.1957139	5.01080708
5	1001.44	0.24177313	527.669857	527.91163001	93968.2701	1.095977	4.97298123
6	770.25	0.18585747	405.633932	405.81978965	72235.9226	0.8425068	4.85875322
mean	1063.27167	0.25672775	560.308313	560.56504052	99780.5772	1.1637675	4.99328422
geo mean	1049.30757	0.25334423	552.923781	553.17712529	98465.5283	1.1484297	4.99276484
std err	76.412431	0.01823927	39.8071964	39.82543571	7088.92756	0.0826801	0.03212098

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	707.69	0.1707267	372.611018	372.78174504	66355.1506	0.7739178	4.82187464
2	4416.16	1.06765798	2330.16353	2331.2311902	414959.152	4.8397793	5.61800535
3	667.67	0.16104745	351.486066	351.64711356	62593.1862	0.730041	4.79652706
4	5559.07	1.34408241	2933.45986	2934.8039454	522395.102	6.0928335	5.7179991
5	80.07	0.0189304	41.315603	41.334533444	7357.54695	0.085813	3.86673304
6	1746.09	0.42187432	920.740713	921.16258778	163966.941	1.9123902	5.21475629
mean	2196.125	0.53071988	1158.29613	1158.8268526	206271.18	2.4057958	5.00598258
geo mean	1083.92205	0.26086114	569.329429	569.59029034	101387.072	1.1825045	4.96506047
std err	921.408128	0.22285195	486.37438	486.59723226	86614.3073	1.0102058	0.27693457

Table B-VI

Mineralization of Anthracene in Aqueous Media on Day 41

Control	14-C DPM	hot (nmoles)	Anthracene cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	1025.23	0.247527	540.2276433	540.4751703	96204.58	1.12205969	4.98319575
2	917.79	0.2215415	483.5143783	483.7359198	86104.994	1.00426552	4.93502834
3	5749.5	1.3901398	3033.980219	3035.370359	540295.92	6.30161553	5.73263169
4	1269.95	0.306715	669.4055122	669.7122272	119208.78	1.39036377	5.07630823
5	1243.47	0.3003106	655.4277826	655.7280932	116719.6	1.36133185	5.06714379
6	811.31	0.1957883	427.3078587	427.5036469	76095.649	0.88752386	4.88135983
mean	1836.20833	0.4436704	968.3105657	968.7542361	172438.25	2.01119337	5.11261127
geo mean	1380.80996	0.3334556	727.7669437	728.1003993	129601.87	1.5115812	5.1050572
std err	786.075348	0.1901203	414.9376357	415.127756	73892.741	0.86183075	0.12774608

Non F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	1221.46	0.2949872	643.8095868	644.104574	114650.61	1.33720071	5.05937639
2	1547.29	0.3737925	815.8021701	816.1759626	145279.32	1.69443149	5.1622038
3	1278.67	0.308824	674.0084503	674.3172743	120028.47	1.39992413	5.07928429
4	1307.71	0.3158477	689.3375011	689.6533487	122758.3	1.43176276	5.08905085
5	1109.76	0.2679714	584.8476386	585.1156101	104150.58	1.21473599	5.01766169
6	949.52	0.2292157	500.2633721	500.4925879	89087.681	1.03905339	4.94981765
mean	1235.735	0.2984398	651.3447865	651.6432263	115992.49	1.35285141	5.05956578
geo mean	1222.01259	0.2951159	644.090408	644.3855239	114700.62	1.33778397	5.05914357
std err	82.0737139	0.0198504	43.32342043	43.34327079	7715.1022	0.0899833	0.0292081

F-500 trt	14-C DPM	hot (nmoles)	cold (nmoles)	Total (nmoles)	ng	% mineral	log ng
1	829.93	0.2002917	437.1366096	437.3369013	77845.968	0.9079383	4.89123613
2	4589.09	1.1094829	2421.446341	2422.555823	431214.94	5.02937487	5.6346938
3	804.66	0.1941799	423.7975905	423.9917704	75470.535	0.88023299	4.87777743
4	5888.35	1.4237221	3107.273563	3108.697285	553348.12	6.45384673	5.74299844
5	1180.69	0.2851266	622.2887396	622.5738662	110818.15	1.29250163	5.04461089
6	1856.67	0.4486192	979.1114585	979.5600778	174361.69	2.03362696	5.24145108
mean	2524.89833	0.6102371	1331.842384	1332.452621	237176.57	2.76625358	5.23879463
geo mean	1845.89692	0.4458835	973.1407965	973.58668	173298.43	2.02122582	5.22783019
std err	888.055124	0.2147852	468.7686673	468.9834525	83479.055	0.97363849	0.15273096

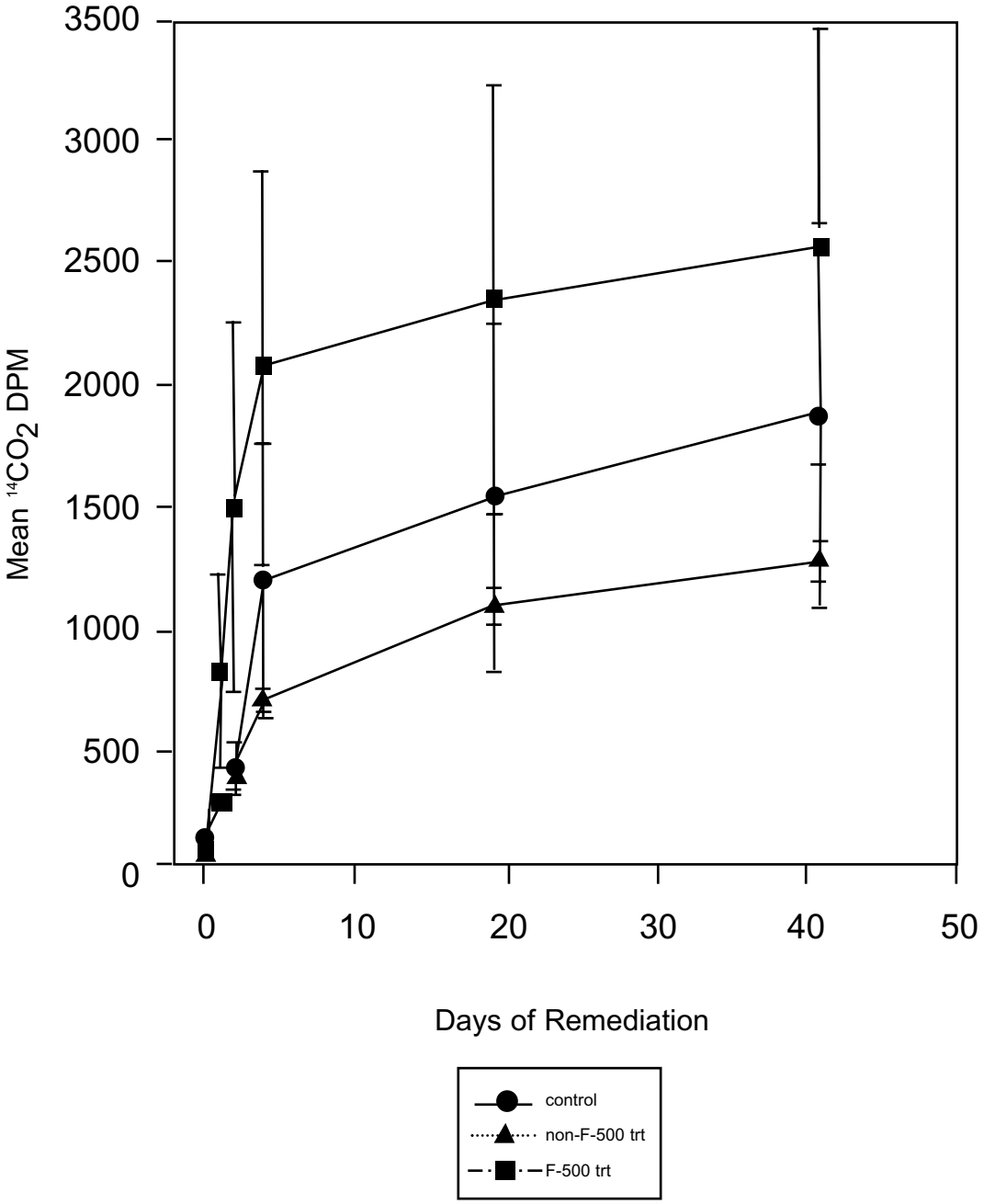

Figure B-1. Mineralization of [9-^{14}C] Anthracene in Aqueous Media Mean $^{14}CO_2$ DPM Vs Time. Observed in control, non-F-500 treatment (non-F-500 trt) and F-500 treatment bioreactors.

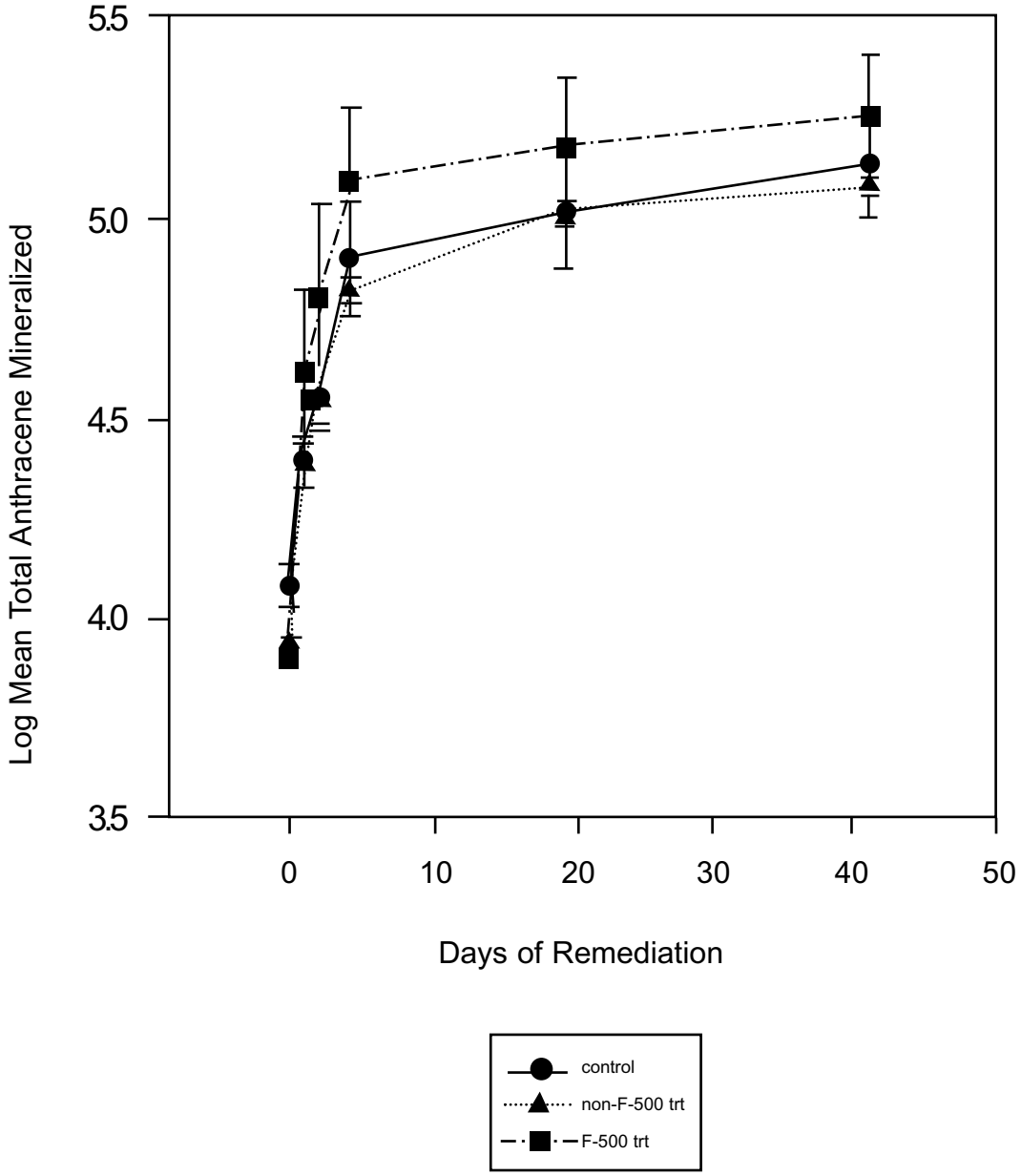

Figure B-2. Mineralization of [9-^{14}C] Anthracene in Aqueous Media Log Total ng of Anthracene Vs Time. Observed in control, non-F-500 treatment (non-F-500 trt) and F-500 treatment bioreactors.

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APPENDIX B

A Research Report

Entitled:

ACUTE ORAL TOXICITY LIMIT TEST IN RATS

For:

REDUCED HAZARDS OF HYDROPHOBIC CHEMICALS IN SOILS AND GROUND WATERS

TIWET Study Number 09440.5

Sponsor:

Hazard Control Technologies, Inc.
150 Walter Way
Fayetteville, Georgia 30214

Submitted by:

The Institute of Wildlife and Environmental Toxicology
P.O. Box 709, 1 TIWET Drive
Pendleton, South Carolina 29670

December 22, 1994

DOCUMENT CONTROL STATEMENT

QUALITY ASSURANCE PRACTICES

This study will be conducted under the auspices of the Institute of Wildlife and Environmental Toxicology Quality Assurance Program. This study will be conducted under Tier II QA Requirements as outlined in The Institute of Wildlife and Environmental Toxicology and The Department of Environmental Toxicology Quality Assurance Manual, Clemson University, August 1993. The assigned Study Director will be Katherine Hunt.

The Quality Assurance Unit verbally notified the Study Director immediately of any problems encountered during audits. Written audit reports were also submitted to the Study Director and Management. Audits were preformed on and written reports were generated for the following phases of the project:

Auditable Research Activity:	Dosing
Audit Dates:	11/29/94
Dates Submitted to Study Director	12/12/94
Dates Submitted to Management	12/12/94

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1.0 INTRODUCTION

1.1 Study Scope and Design

This study was performed to determine whether a single oral dose of compound administered at the limit rate of 5 g/kg (as defined by 40 CFR Part 798, July 1993) was lethal to laboratory rats (*Rattus norvegicus*), the preferred rodent species.

2.0 OBJECTIVES

To determine whether a single acute limit dose (5 g/kg) of compound is lethal to a single group of male and female rats. If no lethality is demonstrated, no further testing for acute oral toxicity is necessary.

3.0 EXPERIMENTAL DESIGN AND PROCEDURES

3.1 Test System

a.	Species:	Rat (*Rattus norvegicus*), Sprague-Dawley
b.	Source:	Harlan Sprague-Dawley, Dublin, VA
c.	Age:	Young adult, at least 6 weeks old
d.	Body Weight:	100-250 grams
e.	Sex:	Equal numbers of each sex
f.	Number:	20 (10 each dosed and control; 5 of each sex)

g.	Housing:	Singly, in polypropylene boxes (16¾ x 10½ x 7½, in.) with Aspen Shavings (Northeastern Products Corp., Warrensburg, NY). Light Schedule: 12 L:12 D
h.	Identification:	By each individual's cage number

3.2 Animal Care

Study rooms were maintained at 22C ± 2C and 45% - 75% relative humidity with approximately 15 air changes per hour. Animal rooms, cages and records were maintained according to TIWET SOP 901-01-01. Feed and water were provided *ad libitum* and were tested, along with aspen shavings, for the presence of chemical contaminants on a per lot (feed and bedding) and monthly (water) schedule. Rats were housed singly and acclimated to test cages for a period of at least seven days before testing. Rats were observed for 14 days following dosing.

3.3 Diet

Wayne Rodent Blox were used for feed. See Appendix A for feed component list.

3.4 Assignment to Test Groups

Doses and sexes were assigned to cage positions by random draw. Individuals were placed sequentially into male or female designated cages as they were removed from their shipping crate until all rats had been distributed. One alternate of each sex was acclimated along with the test group. One of these alternates, male rat number 21, was

substituted for rat number 3 which was supposed to be a male but turned out, instead, to be a female.

3.5 Treatment Levels, Route, Method and Frequency of Administration

A single dose of 5 g/kg of compound was administered by oral gavage at a rate of 5 mls/kg. Since the density of the compound was 1 g/ml, the compound was administered neat according to the weight of the animal. The vehicle control was water. Rats were fasted overnight (>16 hours) prior to test substance administration.

3.6 Body Weights

Each animal was weighed upon receipt (11/15/94), during the acclimation phase at one week pre-dose (11/22/94), at the beginning of the testing phase (11/29/94), one week post-dose (12/06/94), and at the completion of the test (12/13/94). The differences between the mean body weights of treatment and control groups for each sex were tested using a student's t-test (Zar, 1974).

3.7 General Health, Behavior, and Survival

Rats were monitored closely the first two hours post-dose, and then at least two more times the day of dose. Thereafter, each animal was observed at least once daily to determine general health and survival. Cage-side observations following dosing included an evaluation of the animal's skin, fur, eyes, fecal droppings, activity, and general behavior at that point in time. Particular attention was directed to the observation of

possible symptoms of exposure including tremors, convulsions, lethargy, salivation, and diarrhea. When necessary, rats were handled to appraise their physical condition.

3.8 Gross Necropsy

A necropsy to determine gross pathology of major organ systems and muscle tissue was performed on all animals euthanized at the completion of the study (TIWET SOP 101-01-04).

4.0 STUDY ARCHIVE

At the end of the study, all original data, correspondence, reports, and magnetic media were archived at TIWET. Six months after the final report is issued, the Sponsor will be notified of archiving procedures and will be asked to determine the fate of any remaining samples.

5.0 RECORDS

Completed data forms were collected on a daily basis, reviewed for errors and placed in project-specific red three-ring binders for temporary storage at TIWET research facilities in Pendleton, SC, until permanently bound and archived. Project computer files were backed up using PCTools Deluxe Version 5.5 (Central Point Software, Inc., 1989); full back-ups were performed once a week.

6.0 RESULTS

6.1 Survival

No mortality was observed during the two week period of the test.

6.2 Body Weights

Body weights increased in all rats during the acclimation phase and in all but one female (control number 13) during the testing phase of the study (Table 1). This rat lost about 1.5 g during the last week of the test, a decrease of 0.8% from that recorded the previous week. Males gained more weight than females, as was expected for this species. Individual body weight data collected at each phase of the test are shown in Table 1. Mean body weights separated by treatment and control groups within each sex are shown in Table 2. Mean body weights were not significantly different between treatment and control groups as measured at 7 and 14 days following administration of the test substance (Student's t-test, $p > 0.05$).

6.3 General Health and Behavior

Rats were observed prior to dose to establish normal behavior patterns. Rats constructed shallow nests in the shavings and generally slept and/or rested most of the day. Activity was greatest in response to external stimuli such as noise, room doors opening, and food and water changes. Rats were especially active in the fresh shavings which were available immediately following weekly cage changes. All normal maintenance behaviors, such as grooming, eating, drinking, resting, and stretching, were

similar to those observed previously in our laboratory for captive deer mice. Vocalizations occurred only when rats were handled, i.e. during cage changes, dosing, and occasionally during weighing.

Immediately following gavage, several rats expelled a small amount of blood through their nostrils. This appeared to be a normal response to the physical abrasion of the gavage needle against the sensitive skin of the upper palate, since it was observed in both control and treated animals. It appeared to cause no additional distress, though, and all symptoms disappeared by 24 hours post-dose in the affected animals.

Additional symptoms observed included congested or raspy breathing which was first noted 45 minutes post-dose in a single, dosed male rate (number 14). This symptom became more severe, progressing to a coughing/sneezing reaction and increased difficulty breathing, but subsided later within the same hour. Less severe, raspy sounding respirations continued to be heard intermittently up to one week post-dose, and after that only sporadically. This symptom disappeared completely by 9 days post-dose.

Three other rats were heard making low, squeaky or raspy sounds, but these were not heard again after the day of dose. Compound dosed rats number 10 (male) and 15 (female) also exhibited slight tremors approximately an hour post-dose.

Five out of the 10 compound dosed rats, (number 1,7, 9, 10, and 17) exhibited diarrhea/loose fecal droppings at 24 hours post-dose. This symptom disappeared by 48 hours post-dose and all fecal droppings of previously affected rats thereafter appeared normal.

Though not measured quantitatively, no gross changes in food and or water consumption were noted over the two week testing period.

6.4 Gross Necropsy

No mortality was recorded during the two week period of the test following dosing, therefore all rats were necropsied upon euthanasia at the completion of the test. Rat number 14, the one which had difficulty breathing, exhibited subtle, lightly colored areas along the edges of the lungs which suggested some aspiration of test substance had occurred. This may have explained the raspy breathing sounds noticed in this rat. No signs of severe aspiration pneumonia, however, were observed. No other gross pathologic changes in the lungs or any other organs were noted in that or any other animal. Nor was any apparent reason found to describe the small weight loss of control female rat number 13.

7.0 CONCLUSIONS

Administration of the test substance was found to cause no compound-related mortality, no abnormal behavioral manifestations, no differences in mean weights between treated and control groups, and no gross pathologic changes upon necropsy. Symptoms of compound associated diarrhea disappeared by 48 hours post-dose in all affected animals, and observed respiratory symptoms were most likely a result of aspiration of a small amount of the test substance into the lungs rather than a toxic response. Based

on the results of survival, cage-side observations, and gross necropsies, the limit test was met and no further LD50 testing is necessary.

Tables

Table 1. Body weights of individual rats.

Treatment	Sex	ID	Receipt 11/15/94	Acclimation 11/22/94	Dose 11/29/94	Post-dose 12/06/94	Euthanasia 12/13/94
Control	F	8	103.90	142.30	157.15	183.98	199.19
	F	11	105.87	149.03	162.36	196.88	214.92
	F	12	106.16	151.33	169.74	194.59	214.21
	F	13	104.99	136.47	148.35	175.71	174.28
	F	18	107.85	148.98	163.73	188.27	200.74
Dose	F	1	105.13	136.16	152.91	176.50	188.45
	F	5	105.51	148.78	160.66	189.61	200.25
	F	15	103.99	138.30	150.39	169.92	182.29
	F	19	107.58	156.93	165.59	194.91	200.87
	F	20	105.69	150.48	168.63	196.70	212.10
Control	M	2	104.88	154.47	189.79	228.32	256.09
	M	4	104.39	161.60	193.75	245.05	270.63
	M	6	102.65	168.17	211.47	273.43	309.54
	M	16	107.31	162.56	194.55	242.49	262.19
	M	21	101.00	158.87	193.73	245.07	277.17
Dose	M	7	102.35	159.43	193.13	220.99	252.02
	M	9	100.02	153.79	188.86	217.79	254.99
	M	10	101.09	167.22	208.68	247.06	286.28
	M	14	100.14	164.84	202.16	229.71	269.83
	M	17	101.83	159.58	195.13	234.98	274.12

Table 2. Comparison of mean rat weight data (N=5 for each value).

	Receipt 11/15/94	Acclimation 11/22/94	Dose	Post-dose 11/29/94	Euthanasia 12/06/94	12/12/94
Females						
Control	105.75±1.47	145.62±6.13		160.27±8.03	187.89±8.51	200.67±16.47
Dose	105.58±1.30	146.13±8.71		159.64±7.88	185.53±11.78	196.73±11.65
Males						
Control	104.05±2.38	161.13±5.03		196.66±8.49	246.87±16.40	275.12±20.85
Dose	101.9±1.02*	160.97±5.24		197.59±7.84	230.11±11.68	267.45±14.12

*Significantly different from same sex controls, student's t-test, $t_{0.05}(2),8=2.306$, $P<0.05$

Appendices

Appendix A: Feed Component List of Wayne Rodent Blox

Appendix B: Time and Event Schedule

Acclimation of Rats to Test Facilities	15 November 1994
Initiation of Test/Dose Administration	29 November 1994
Euthanization and Gross Necropsies	13 December 1994

Appendix C: Standard Operating Procedures

SOP 101-01-04	Postmortem examination
SOP 202-06-02	Dosing procedure for avian and mammalian test systems
SOP 901-01-01	Small rodent colony maintenance and care
SOP 901-05-01	Pre-test preparation and maintenance of animals
SOP 902-01-01	Euthanasia of small mammals and birds
SOP 902-02-01	Animal procurement