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ABSTRACT

VITAMIN EFFECT ON BIOREMEDIATION OF PHENANTHRENE, ANTRACENE, FLUORANTHENE, AND PYRENE IN A CONTAMINATED SOIL FROM AN INDUSTRIAL SITE

by
Ilkay Cam

Bioremediation of organic wastes is often a cost-effective technology for the *in situ* treatment of contaminated soils. In this study, the possibility of increasing the rate and extent of biodegradation for phenathrene, anthracene, fluoranthene, and pyrene in an industrial PAH contaminated soil via vitamin mixture supplementation was explored.

Soil contained with PAHs was obtained from the Bayway refinery site formerly owned by Exxon (Linden, NJ). Experiments were conducted in which the contaminated soil was amended with a mixtures of four vitamins (niacin, biotin, folic acid, and panthothenic acid). Peat moss, a common soil amendment, was also added in some experiments.

The results showed that different vitamin mixture concentrations had a significant effect on the biodegradation rate and extents of phenanthrene, anthracene, fluoranthene, and pyrene, provided that peat moss was absent. The presence of peat moss resulted in an increase in the rate and extent of PAH biodegradation, independently of the presence of vitamins.

VITAMIN EFFECT ON THE AEROBIC BIODEGRADATION OF PHENANTHRENE, ANTRACENE, FLUORANTHENE, AND PYRENE IN A CONTAMINATED SOIL FROM AN INDUSTRIAL SITE

by
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VITAMIN EFFECT ON THE AEROBIC BIODEGRADATION OF PHENANTHRENE, ANTRACENE, FLUORANTHENE, AND PYRENE IN A CONTAMINATED SOIL FROM AN INDUSTRIAL SITE

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To my family

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CHAPTER 1

INTRODUCTION

1.1 Objective

The purpose of this study was to determine the effect of different vitamin mixture concentrations on the aerobic biodegradation of phenanthrene, anthracene, fluoranthene, and pyrene in soil.

Vitamin effect on the degradation of aliphatic hydrocarbons in soil was studied by Susmita GuptaPal in 1996 (Guptapal, 1996). She determined the optimum vitamin mixture to maximize the rate of degradation. In the present study, the same vitamin mixture concentration level was used for polycyclic aromatic hydrocarbons (PAHs) degradation in soil.

The effect of different vitamins was studied by other researchers also. For example, in 1993, a group of researchers from the University of Groningen, The Netherlands, studied the effect of biotin on *Xanthobacter* autotrophic GJ10 for optimal growth on 1,2-dichloroethane (Wijngaard 1993). Effect of B12 on reductive dechlorination of a polychlorinated biphenyl congener and hexachlorobenzene in 1992 by Assaf-Anid in University of Michigan (Assaf-Anid, 1992). Vitamin and nutrient mixture effect on pentachlorophenol was another study that was done in 1996 (Cole 1996).

1.2 Background Information

1.2.1 Bioremediation

Bioremediation is a widely used remediation technique, which utilizes the degradative abilities of microorganisms to eliminate organic contaminants. Microorganisms metabolize organic substances by frequently using them as the carbon and energy sources. Such microorganisms can metabolize organic substances into carbon dioxide and water. Bioremediation may rely on either indigenous microorganisms (those that are native to the site) or exogenous microorganisms (those that are imported from other locations). In either case, bioremediation technologies optimize the environmental conditions, so the appropriate microorganisms will flourish and mineralize the maximum amount of contaminants (USEPA, 1999).

Bioremediation can take place under aerobic and anaerobic conditions. In aerobic condition, the carbon and energy source is broken down by a series of enzyme-mediated reactions in which oxygen serves as an external terminal electron acceptor (Cookson, 1995). Under anaerobic conditions the chemical compounds are broken down by series of enzyme mediated reactions in which nitrates, sulfates, carbon dioxide, and other oxidized compounds (excluding oxygen), serve as terminal electron acceptors (Cookson, 1995).

1.2.2 Bioremediation Methods

Bioremediation can be used as a cleanup method for contaminated soil and water. Bioremediation applications can be *in situ* or *ex situ*. *In situ* bioremediation process

treats the contaminated soil and ground water at the location in which it was found. *Ex situ* bioremediation process require excavation of contaminated soil or pumping of ground water before they can be treated (USEPA, 1995).

In situ bioremediation involves the enhancement of the environment inhabited by indigenous microorganisms through the addition of oxygen, electron acceptors, nutrients, and/or additional microorganisms to stimulate microbial degradative activity (USEPA, 1995).

Two specific examples of *in situ* bioremediation that can be applied in contaminated aquifers and when the contaminant is found in the vadose zone are bioventing and air sparging. Bioventing involves supplying oxygen at low flow rates to microorganisms in order to stimulate aerobic biodegradation. Air sparging involves displacing water from the soil matrix by injecting air directly into the saturated formation below the water table (USEPA, 1993)

Ex situ bioremediation techniques require excavation of the contaminated soil. Slurry phase bioremediation and solid phase bioremediation are the examples for *ex situ* techniques. Slurry phase bioremediation involves placing the contaminated soil combined with water and other additives in a large tank called a "bioreactor" and mixing to keep the microorganisms in contact with the contaminated soil (USEPA, 1995).

Solid phase bioremediation treats soil in above ground treatment areas equipped with collection systems to prevent any contaminant from escaping the treatment. Moisture, heat, nutrients, and oxygen are controlled to enhance biodegradation (USEPA, 1995).

1.2.3 Land Farming

Land farming is a bioremediation technology. Contaminated soils are mixed with soil amendments such as soil bulking agents and nutrients, and then tilled into the earth. The material is periodically tilled for aeration. Contaminants in soil are degraded, transformed, and immobilized by microbiological processes and by chemical oxidation. Soil conditions are controlled to optimize the rate of degradation of the contaminants. Moisture content, frequency of aeration, and pH are all conditions that may be controlled (Land farming, 1998).

CHAPTER 2

LITERATURE REVIEW

2.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants, some which are on the United States Environmental Protection Agency priority pollutant list (EPA 1995). The lower molecular weight PAHs are amenable to bioremediation; however, high molecular weight PAHs seem to be recalcitrant to microbial degradation. The rates of biodegradation of PAHs are highly variable and are dependent on PAH structure, physicochemical parameters of the site, and number and types of microorganisms present (Crawford, 1996).

PAHs generally exist as colorless, pale yellow or white solids. Because they do not dissolve easily in water, and they can persist in the environment for months to years (EPA 1999). Microbial degradation represents one of the major routes by which PAH can be removed from the environment. Many different microorganisms such as, *Pseudomonas, Flavobacterium, Alcaligenes, Arthrobacter, Micrococcus, Bacillus, Beijerinckia, Nocardia, Cynobacteria,* and *Mycobacteria* have the ability to degrade PAHs (Crafword, 1996).

The degradation of PAHs depend on the complexity of the PAH chemical structure and the extent of enzymatic adaptation. In general, PAHs containing 2 or 3 aromatic rings are readily degradable. PAHs containing four or more aromatic rings are significantly more difficult. PAHs can be readily degraded or extremely resistant or can yield intermediate products that are highly carcinogenic (Cookson, 1995).

Under optimized metabolic conditions, the degradation rates of some PAHs are dependent on the soil to water ratio and the partition coefficient. PAHs are strongly hydrophobic. Therefore, a significant portion of the PAHs is adsorbed on soil particles possibly being entrapped in intraparticle micropores (Lotfabad *et.al.*, 1996).

2.2 General Pathways of Microbial Polycyclic Aromatic Hydrocarbon Metabolism

Bacteria, fungi, and algae play an important role in the aerobic metabolism of PAHs in terrestrial and aquatic environments.

First, bacteria initially oxidize aromatic hydrocarbons that range in size from benzene to benzo*[a]*pyrene to *cis*-dihydrodiols. The *cis*-dihydrodiols are formed by the incorporation of both atoms of molecular oxygen into the aromatic nucleus. The dioxygenase that catalyzes these initial reactions is generally a multicomponent enzyme system; the terminal oxygenase is an iron-sulfur protein (Cerniglia, 1984).

Second, *cis*-dihydrodiols are rearomatized through a *cis*-dihydrodiol dehydrogenase to yield a dihydroxylated aromatic derivatives (Cerniglia, 1984).

Third, further oxidation of *cis*-dihydrodiols leads to the formation of catechols that are typically substrates for other dioxygenases which can metabolize the enzymatic cleavage of the aromatic rings. Catechol can be oxidized via the ortho pathway, which involves cleavage of the bond between carbon atoms of the two hydroxyl groups, to yield *cis*, *cis*-muconic acid, or via the meta pathway, which involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group (Cerniglia, 1984).

The same biodegradative mechanism is then extended to the other aromatic rings of the PAH molecule. The products of the aromatic ring cleavage are further degraded into Krebs cycle where they are used as a source of carbon and then converted into carbon dioxide and water.

2.3 Soil Reactions with Hazardous Materials

Soil governs the concentrations of hazardous materials in water found in both unsaturated and saturated zone soils. There are several important facts regarding soil reactions with hazardous materials (Dragun, 198).

First, soil is a heterogeneous, reactive mass of solids and water. These solids possess distinct physical and chemical properties which exert a profound effect on the concentration of hazardous materials in water.

Second, soil possesses solids with significant surface area that mediate various physical and chemical surface reactions. The primary physical and chemical surface reactions occurring at soil surfaces, which govern the concentration of hazardous materials in water, are hydrolysis, oxidation, reduction, bound residue formation, and various fixation reactions. Hazardous materials dissolved in water are not isolated from these surfaces but are in intimate contact with them (Dragun, 1988).

Third, soil contains a significant amount of water in which chemical reactions may occur. Water may comprise from 33 to 50 percent of the total volume of a saturated soil or from 107,000 to 163,000 gal/acre-foot of soil. Water is usually the solvent in a soil system that is responsible for moving dilute concentrations of hazardous materials.

Fourth, although solids occupy 50 to 67 percent of the soil bulk volume, 33 to 50 percent is comprised of an interconnected network of pores. In the unsaturated zone, soil water and the soil atmosphere occupy these pores (Dragun, 1988).

2.4 How Microorganisms Degrade Organic Chemicals

The biodegradation of an organic chemical is the modification or decomposition of the chemical by soil microorganisms to produce ultimately microbial cells, carbon dioxide, and water. It is most important to recognize that microorganisms possess numerous enzymes within these cells, which are responsible for the biodegradation of organic chemicals (Dragun, 1988).

After an organic chemical has set up into the microbial cell, it will interact with enzymes. Whether or not the chemical transforms as a result of this interaction depend upon the chemical binding to the enzyme and the conformational changes at the enzyme's active site. Recalcitrant or non-reacting chemicals are not bound at all or fail to produce an alignment that leads to reaction (Dragun, 1988).

2.5 Peat Moss

Peat moss is a natural, organic soil conditioner that regulates moisture and air around plant roots for ideal growing conditions. It will help to:

Save water:

Peat retains up to 20 times its weights in moisture, and releases water slowly as plants need it.

Aerate heavy, clay soil:

Peat moss allows for proper root growth by loosening and aerating soils.

Bind sandy soil:

Canadian peat helps it retain moisture and nutrients.

Reduce leaching:

Peat moss reduces leaching of nutrients in or added to the soil, releasing them over time. This will save on fertilizer.

Protect soil:

Peat moss protects soil from hardening and adds organic material (Online, 1998).

2.6 Studies on Biodegradation

The following information was obtained by using ProQuest Direct and Applied Science Technology Index database, and also keyword search on online and electronic journals.

Hydrocarbons are water-insoluble compounds. Their uptake is a difficult process for microbes, but there are organisms that can utilize hydrocarbons. The general pathways of microbial PAH metabolism was described in previous pages.

The effect of bioremediation on PAH residues in soil was studied by Wang et. al (Wang et. al, 1990). They studied spills of diesel oil having a high content of PAH and total aromatics.

A study was conducted by Boldrin et. al on degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. (Boldrin et. al, 1990). They identified several metabolites of phenanthrene and fluorene metabolism. They also found

that *Mycobacterium* sp. was able to utilize phenanthrene, pyrene, and fluoranthene as a sole carbon and energy source.

Thibault et. al studied the effect of four different surfactants on pyrene degradation in soil. In the presence of Witconol SN70, pyrene mineralization was 86% under unsaturated conditions (Thibault et. al, 1996).

The effects of phosphorus, nitrogen, and temperature were studied for the bioremediation of a petroleum contaminated cryic soil by Walworth and Reynolds (Walworth and Reynolds, 1995). They studied four different concentration levels of nitrogen and phosphorus at two temperatures.

Nitrogen and phosphorus play a very important role in biodegradation rates of soil. Soil microorganisms also need vitamin for their growth. Most of the dominant species either get required vitamins from soil or they are able to synthesize them (GuptaPal, 1996).

The effect of vitamins during aerobic and anaerobic biodegradation has been studied by some researchers. Kafkewitz et. al studied the effect of vitamins used in the present work on aerobic degradation of 2-chlorophenol, 4-chlorophenol, and 4-chlorobiphenyl (Kafkewitz, et. al, 1996).

The effect of vitamin B12 was studied in many aerobic and anaerobic degradation of hydrocarbons. Reductive chlorination of polychlorinated compounds was studied in the presence of B12 by Assaf-Anid et. al (Assaf-Anid et. al, 1992).

B12 was also studied as a factor in biological dehalogenation of polychlorinated hydrocarbons by *Dehalospirillum multivorans* (Lee et. al, 1998).

The effect of niacin, biotin, folic acid, thiamin, and panthothenic acid on the biodegradation of aliphatic hydrocarbons and diesel fuel was studied by GuptaPal (GuptaPal, 1996). She studied the effect of individual vitamins as well as a mixture, and she found that the vitamin mixture was more effective than individual vitamins. Also, the addition of vitamin mixtures stimulated the initial growth of the microorganisms. The optimum vitamin mixture concentration, V_b, that she was reported in her study was used as the starting point for the present study.

Another important study was done by the Composting Technology Center at Olds College. Many oil companies joined to conduct for a remedial project. Chevron Corp. began to use peat moss to reclaim an industrial site contaminated with hydrocarbons (Biocycle, 1997).

The above studies show that vitamins and minerals are important for microbial growth. In the present study, the effects of vitamin mixture on the biodegradation of soil PAHs were studied in the presence and absence of peat moss.

CHAPTER 3

MATERIALS AND EXPERIMENTAL METHODS

3.1 Reagents and Chemicals

The following chemicals were obtained from Fisher Scientific (Fair Lawn, NJ): HPLC grade acetone, hexane, methylene chloride, cyclohexane, methanol, acetonitrile, and pentane. Also, anhydrous sodium sulfate (12-60 mesh), silica gel (200 mesh), ammonium chloride, potassium phosphate (monobasic), and ferrous sulfate. Sodium chloride, magnesium sulfate (heptahydrate) and calcium chloride (dihydrate) were obtained from Sigma (Bellefonte, PA). PAHs standards, 99% pure phenanthrene, 99.9% anthracene, 98% fluoranthene, 99% pyrene, and 99.3%pure 2-fluorobiphenyl (2-fbp) were obtained from Chem Service Inc. (West Chester, PA). Nitrogen gas was obtained from Matheson Co. (Rutherford, NJ).

Folic acid and biotin were obtained from Roche Scientific (Nutley, NJ). Panthothenic acid was purchased from Sigma (St. Louis, MD), and niacin was purchased from National Biochemical Corporation (Cleveland, OH)

The Canadian sphagnum peat moss that was used for the study was bought from Home Depot (Woodbridge, NJ).

3.2 Glassware

The following materials were used for Soxhlet extraction, (EPA standard method 3540C), drying, concentration, and silica gel cleanup (EPA method 3630C) procedures: 40mm ID Soxhlet extractors, 500ml round bottom flask, 20mm and 10mm ID chromatographic

columns, 10ml and 25ml concentrator tubes, 500ml evaporation flasks, three-ball Synder columns, and various sizes of beakers and graduated cylinders.

3.3 Analytical Instruments

The HPLC system was manufactured by Waters Corp. (Milford, MA), and consisted of an auto sampler (Model 717), pump (Model 660E), and photodiode array detector (PDA, Model 996). Mobile phase reservoirs for acetonitrile, tetrohydrofuran, and water, were put under a degassing module, which was manufactured by Alltech (Deerfield, IL).

The column was made by Supelco (Bellefonte, PA), and the specifications were: 250mmx3.0mm ID Supelcosil LC-PAH 5μm column. The column was placed in a water jacket, to keep the temperature constant. The software that was used for the HPLC analysis, Millenium32, was also developed by Waters Corporations.

3.4 Experimental Soil

The soil that was used in experiments was collected from Exxon at Bayway location in Linden, NJ. Soil was collected from near the sludge Lagoon #2, and 6 feet across from the roadway and at an approximate depth of 100+ cm.

The soil was put in 3 and 5 gallons pails. The soil had dark brown color, a heavy gasoline smell, and sandy clay loam texture. The pH of the soil was 6.5 in distilled water. After bringing it to the lab, the soil was stored at 4 °C in a cold room.

3.5 Standard Solutions

3.5.1 Internal Standard

2-fluorobiphenyl (2-fbp) was used as an internal standard to monitor the recovery rate of extraction. 2-fbp is also a PAH, but it was not expected to be present in the sample.

100 μg/ml (100ppm) stock solution was prepared by dissolving 2.5 mg of 2-fbp in 25 ml of acetonitrile. Then, 1ml of this solution was added to 10 g of soil samples, which were used for extraction. Most of the extractions showed 60-75% recovery rate for 2-fbp. For each sample, the concentration of each PAH was calculated as follows:

$$\text{Residual PAH Concentration in Soil} = \text{PAH Concentration in Soil Sample} \times 1/\text{ Recovery Rate of Internal Standard (2-fbp)}$$

3.5.2. Calibration Standards

EPA method 8310 provides high performance liquid chromatographic condition for the detection of PAHs. Six concentration levels were prepared through the dilution of stock standards in acetonitrile. The preparations of calibration levels are given in Table B1.

The retention times of the standards from the HPLC analysis and the correlation coefficients, R^2, of the regression between concentration and area under the chromatogram are given in Table B2.

3.6 Soxhlet Extraction

EPA method 3540C is a procedure for extracting nonvolatile and semi-volatile organic compounds in solids. The Soxhlet extraction ensures intimate contact of the solid

samples with the extraction solvent. This method is applicable to recover water insoluble and slightly water-soluble organics from solids and semi-solids mixtures.

The procedure consists of six steps: extraction, drying, concentration, solvent extraction, solvent exchange, cleanup, and solvent exchange.

Extraction: For each extraction, approximately 40 g of sampled soil, which was taken randomly from of each tray, and placed in a desiccator to allow dry. When the soil was completely dried, aggregations of particles were broken down in a mortar, with a rubber pestle. Then, the soil was passed through a 1mm sieve to prepare for the test. 10g of soil sample was blended with 10g of anhydrous sodium sulfate in a cellulose extraction thimble, and spiked with 1ml of the 100ppm internal standard solution. A piece of glass wool was placed above the sample, and then the thimbles were placed in the extractors. 300ml of extraction solvent which was prepared by mixing 150ml of acetone and 150ml of hexane for each sample in a 500ml round bottom flask. Two boiling teflon chips were added to the flasks, and the flasks were attached to the extractors. Then, samples were extracted for twenty hours at 4-6 cycles per hour by adjusting the temperature control unit. After the extraction was complete, extracts were allowed to cool to room temperature.

Drying: Kuderna-Danish (K-D) concentrators (i.e., 10ml concentrator tubes) were attached to 500ml evaporation flasks and two or three of boiling teflon chips were put into the tubes. 50g of anhydrous sodium sulfate was put into the 20mm ID drying columns. The bottoms of the columns were plugged with glass wool. The columns were

pre eluted with 50ml extraction solvent, which was collected in a beaker and discarded. Then, extracts were passed through the column and collected in to the prepared K-D apparatus. The empty round bottom flasks were rinsed with 50ml of extraction solvent, which was passed through the column and collected in the K-D apparatus also.

Concentration: Three-ball Synder columns were attached to the evaporation flasks after prewetting the columns with 1ml of methylene chloride. The apparatus assembled (concentrator tube, evaporation flask, and Synder column) was placed a boiling water bath by partially immersing the concentrator tube in hot water, and allowing the lower surface of the flask to be bathed with stream. Extracts were concentrated to around 5-6ml by raising the temperature of the water bath. Then, the K-D apparatus was removed from the water bath and allowed to drain and cool to room temperature. After removing the Synder columns, the flasks and the joints were rinsed into the concentrator tube with 1-2ml of cyclohexane.

Solvent Exchange: The tubes with concentrated extracts were placed in a warm water bath at approximately 35°C, and then the solvent solution in the extract was partially evaporated with a gentle stream of dry nitrogen. When the solvent in the tube was down to 2ml, 2ml of cyclohexane was added to the tube and then evaporated again to 2ml. That procedure was repeated a total of five times. After the fifth addition, the sample volume was brought down to 1ml.

Sample Cleanup with Silica Gel: EPA method 3630C, silica gel cleanup, was followed after the solvent extraction step. Silica gel is a regenerative adsorbent of silica with weakly acidic properties. It is produced from sodium silica and sulfuric acid. Silica gel can be used in column chromatography for the separation of analytes (such as PAHs) from interfering compounds of a different chemical polarity (USEPA, 1996).

Silica gel was preliminary activated by heating it in oven at 130°C for 16 hours before use. It was placed in a glass tray and loosely covered with foil.

10mm ID columns with stopcocks at the were plugged with glass wool. A slurry of 10g of silica gel in 10 ml methylene chloride was poured into the 10mm ID columns. Methylene chloride was removed by opening the stopcock after the silica gel was settled. Then, 3g of sodium sulfate was added to the top of the silica gel. Each column was eluted with 40ml of pentane first, and the eluate was discarded (the Na_2SO_4 was always covered by pentane so as to prevent exposure to the air). Sample extracts were from the concentrator tubes transferred to the columns and the PAHs were adsorbed on the silica gel. Then, each concentration tube was rinsed with 2ml of cyclohexane, which was then transferred to the column. Columns were eluted with 25ml of pentane that was discarded also. Next, the column was eluted with 25ml of methylene chloride and pentane (2:3, v/v) to eluate the PAH from the silica gel. Final eluate was collected into 25ml concentrator tubes. Then, the concentrator tubes were placed in a water bath for solvent exchange. Solvent was partially evaporated to 2ml with nitrogen.

Solvent Exchange: Then 2ml of acetonitrile added and evaporated to 2ml again for a total of five times. The final volume was brought down to 1ml, and allowed to cool for 10

minutes. Then, the extracts were passed through 0.22 μm sterile filters and stored in vials for HPLC analysis.

3.7 HPLC Analysis

3.7.1 Setting up the Method

Millenium32 software was used to do the analyses. The method set contained three methods: instrument method, processing method, and report method.

3.7.2 Instrument Method

Instrument method controlled all HPLC units. The software allowed us to control everything from the computer. Several working conditions have been tested in order to get the best separations and to save time and solvents. The following conditions were achieved. The column was placed in water jacket to keep it at constant temperature. Solvents' reservoirs were attached to the degassing system. Before each set of runs, the lamp was turned on for an hour and allowed to warm up. The mobile phase condition, which was 80% acetonitrile and 20% water, was set to get the pressure stabilized. Also, the needle was washed with a methanol and water (50:50) solution before each set of run.

After preparing them, the sample vials were placed in the autosampler tray, and then run was started. Each run time was 10 minutes, and the injection volume was 10μl. The pressure was 2100psi at 1.0ml/min flow rate.

3.7.3 Processing Method

A Photodiode Array Detector (PDA) was used for detection (996PDA, Waters Corp). PDA measures light intensity in a specific wavelength range. Some of the specifications are given below:

Wavelength range : 190-800nm

Light source : Prealigned, Deuterium lamp (2000 hr life)

Spectral resolution : 1.2 nm per photodiode

Wavelength accuracy : ∓ 1 nm

Three different processing methods were developed for the method set. For antracene, phenanthrene, and 2-fluorobiphenyl, peaks were measured at 250nm after they were scanned between 215nm and 350nm. For fluoranthene, the peak was measured at 285nm, and the pyrene peak was at 334nm.

3.7.4 Report Method

In the report page generated by the PDA analyzer, PDA component results appear in the library match table. When performing a peak match, Millenium 32 software chooses the components from the processed component table that closely match the targeted unknown peaks. To accomplish this, the software uses the time period defined in the processing method.

The most appropriate peak for a component is determined by calculating how close the retention time of the peak to the retention time of the component. Retention times of the components are given in Table B.2. Figure 3.2, 3.3, 3.4, 3.5, and 3.6 show the adsorption spectra of library peak, sample peak, and the difference between the peaks for 2-fbp, phenanthrene, anthracene, fluoranthene, and pyrene respectively. Match angle indicates the difference in spectral shapes between and acquired spectrum and library spectrum. Small values indicate that that spectra are similar.

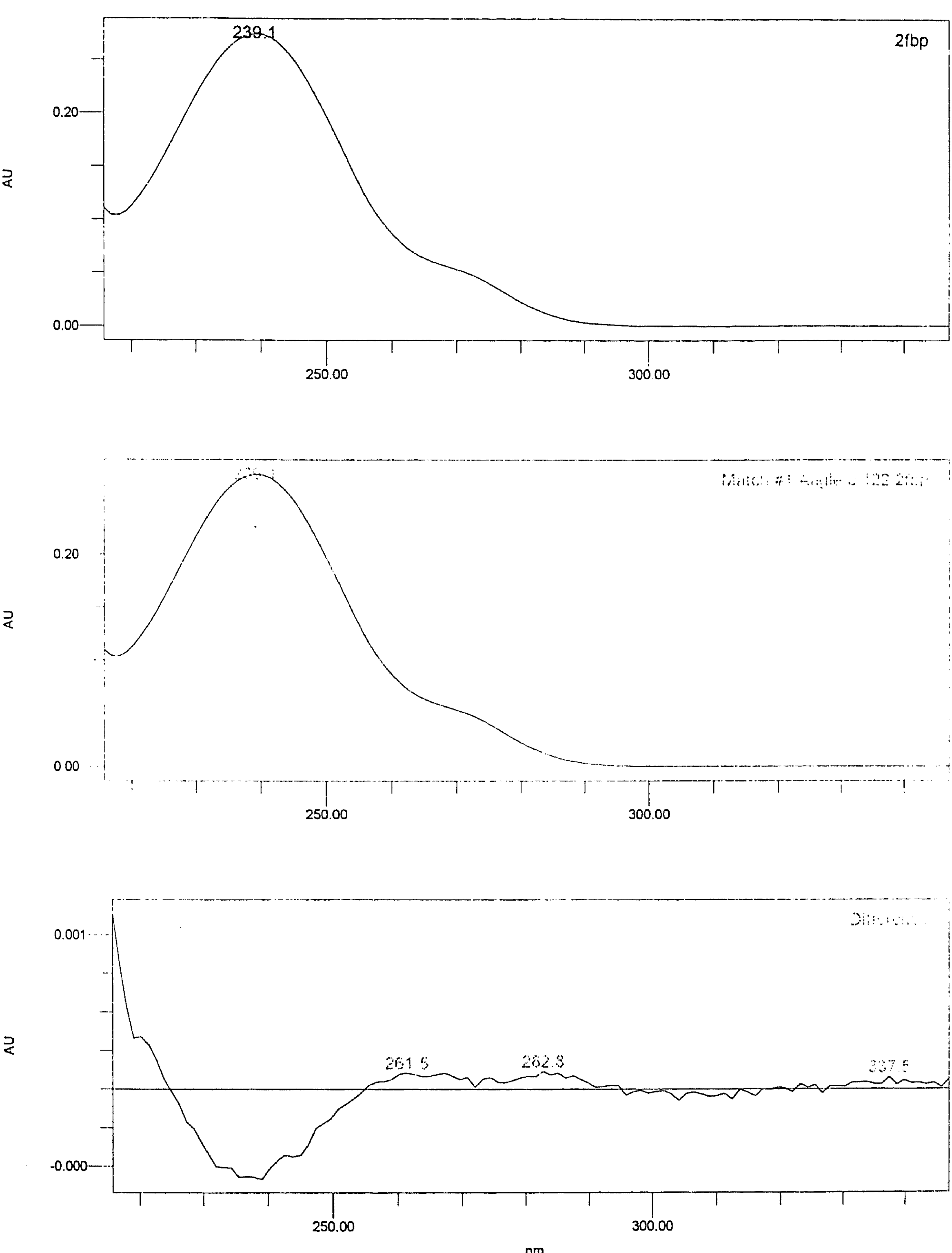

Figure 3.1 Library Spectra of 2-fluorobiphenyl

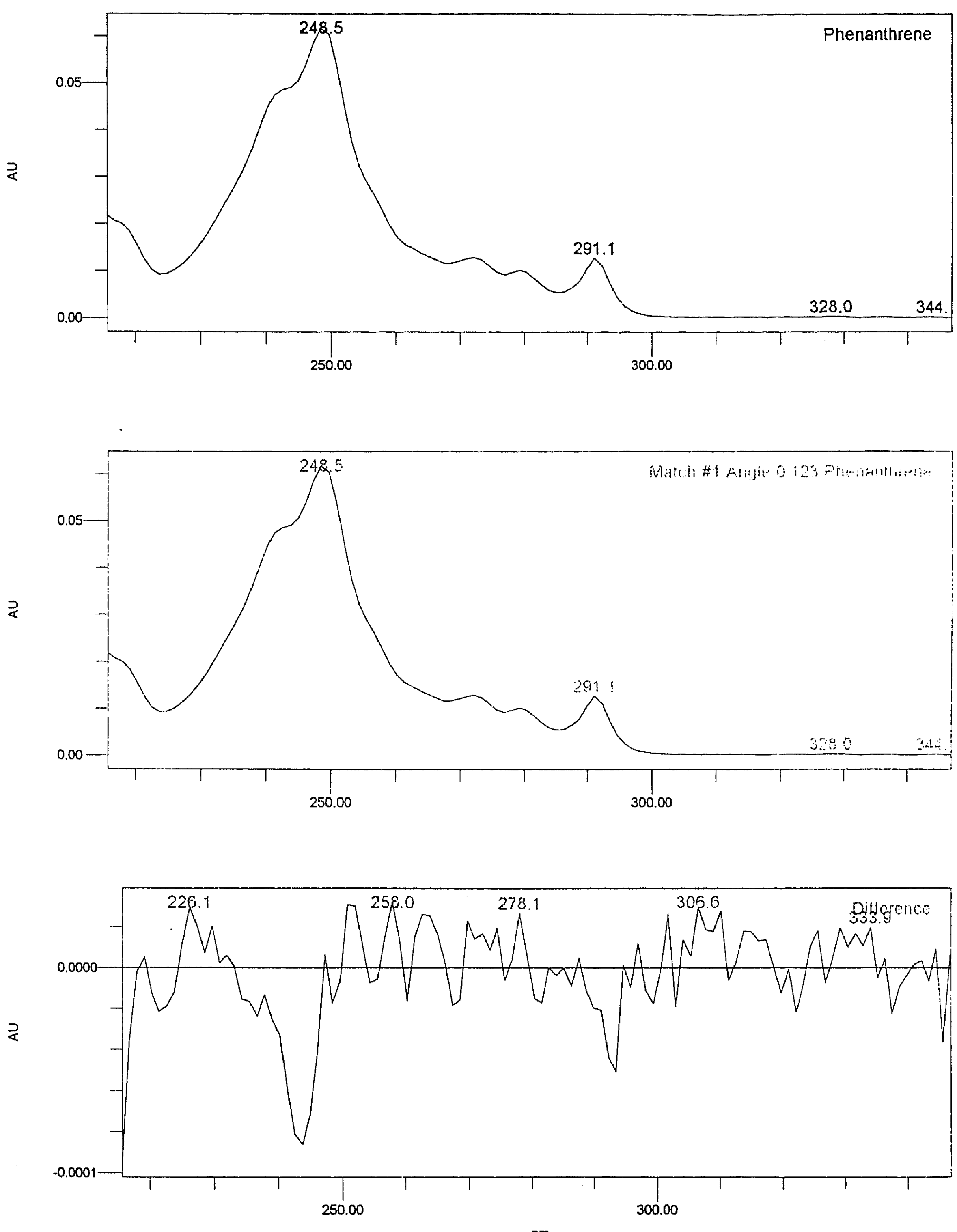

Figure 3.2 Library Spectra of Phenanthrene

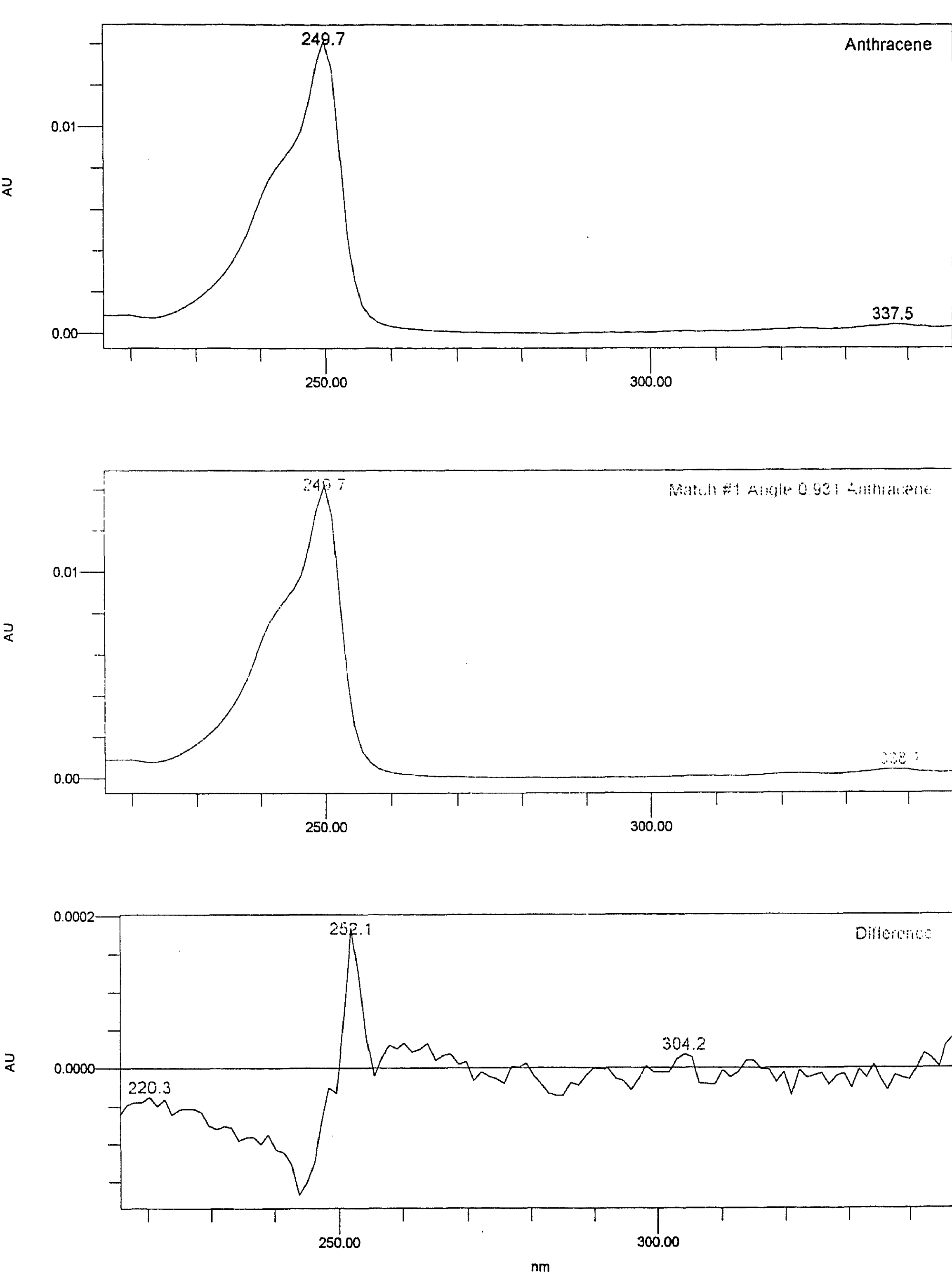

Figure 3.3 Library Spectra of Antracene

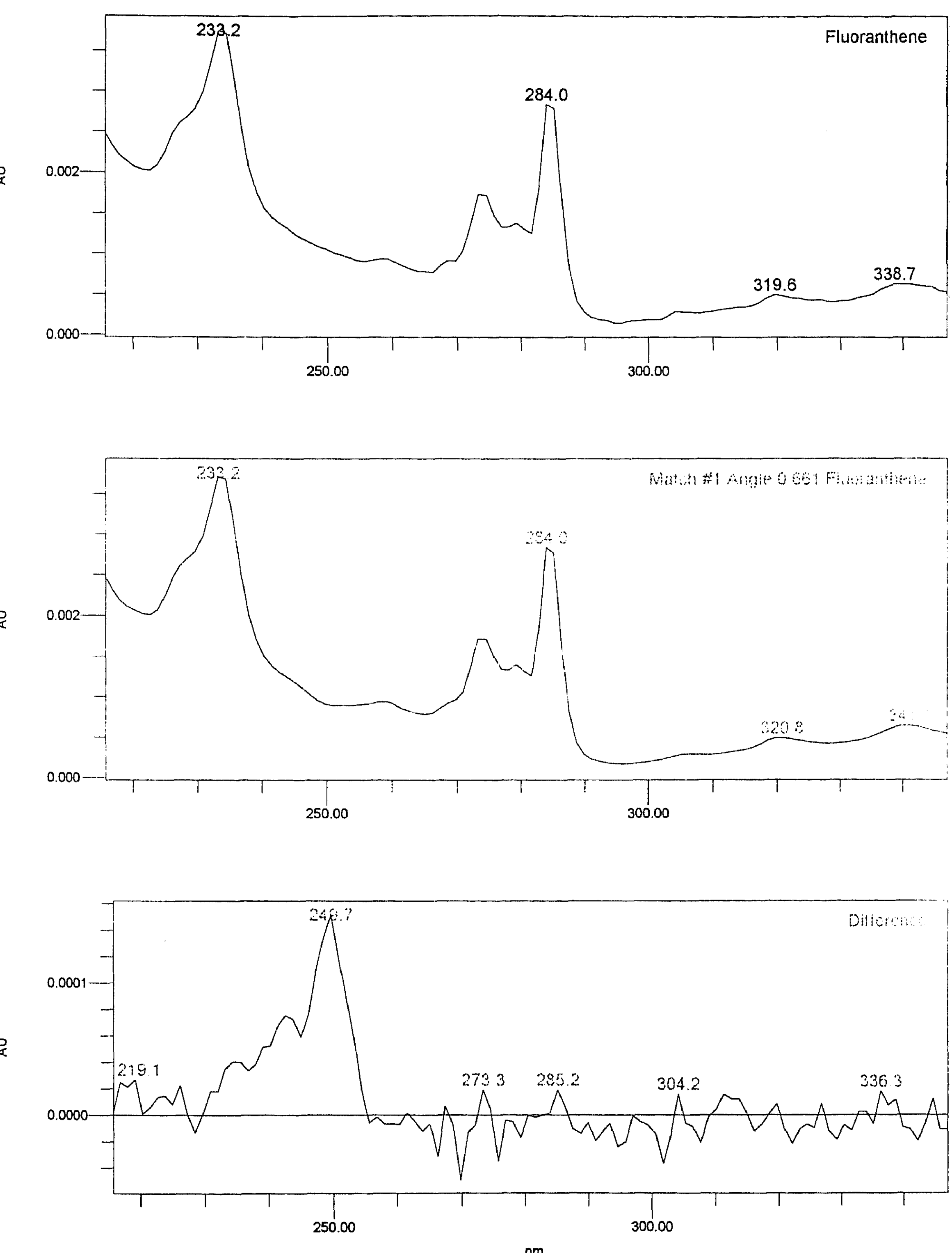

Figure 3.4 Library Spectra of Fluoranthene

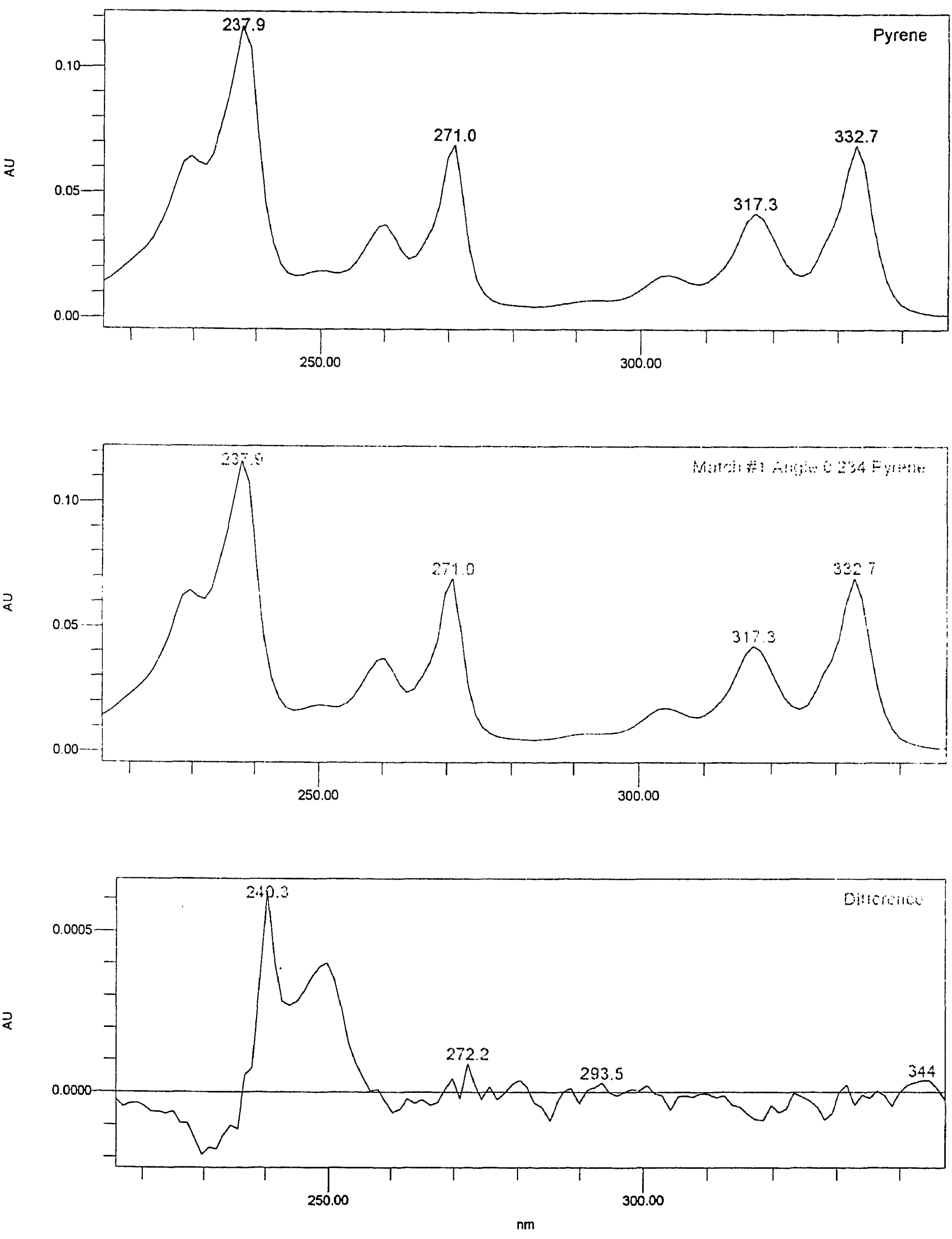

Figure 3.5 Library Spectra of Pyrene

3.8 Preparation of Mineral Salt Medium

First a mineral salt stock solution was prepared. It contained 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.2 g of $MgSO_4.7H_2O$, 0.1g of $CaCl_2.2H_2O$, 0.1 g of NaCl, and 0.01 g of $FeSO_4.7H_2O$ in 1 liter of Milli-Q water. Then, the mineral salt medium was prepared by diluting 1:10 the mineral salt stock solution.

3.9 Preparation of Vitamin Solutions

Based on a study already done on the use of vitamins (GuptaPal, 1996), a specific vitamin solution was prepared. To prepare a vitamin stock solution, 20.0mg of niacin, 20.0mg of biotin, 40.0mg of folic acid, and 40.0mg of pantothenic acid were dissolved in a one liter flask with Milli-Q water. Then three different vitamin solutions having different vitamin concentrations were selected to add to soil microcosms. These vitamin solutions (V_a, V_b, and V_c) were prepared as follows:

V_a was prepared by diluting 3.0ml of the vitamin stock solution into 1 liter of mineral salt medium. V_a contained 20ppb of niacin, 20ppb of biotin, 40ppb of folic acid, and 40ppb of panthothenic acid in dry soil.

V_b was prepared by diluting 7.5ml of the vitamin stock solution into 1 liter of mineral salt medium. V_b contained 50ppb of niacin, 50ppb of biotin, 100ppb of folic acid, and 100ppb of panthothenic in dry soil acid.

V_c was prepared by diluting 15.0ml of the vitamin stock solution into 1 liter of diluted mineral salt medium. V_c contained 100ppb of niacin, 100ppb of biotin, 200ppb of folic acid, and 200ppb of panthothenic acid in dry soil.

3.10 Preparation of Experimental Soil

The experimental soil from Exxon (Linden, NJ) was stored at 4°C in the cold room of Biological Science Department of Rutgers University Newark Campus. The soil was in dark brown color, and had heavy gasoline smell. Before setting up sample microcosms, coarse gravels and other large size materials; such as, rocks, glass particles, and woods were removed by hand, and also by using 3.5mm sieve.

3.11 Determination of Natural Moisture Content of Experimental Soil

The moisture content of the soil is an important parameter, which not only affects the transportation of contaminants, but may also affect the biodegradative activity of the microorganisms.

The water content of the soil may influence aeration, nutrient transport, and the motility and survival of microorganisms. The pH, redox potential, type of existing microorganisms and available nutrients within the soil likewise help in deciding which type of remediation efforts must be made (Cerniglia, 1989).

Natural Moisture Content Determination Test:

To determine the original moisture content of the experimental soil, three standard drying containers were taken. The weight of the containers and their lids were recorded. Approximately 50g of sieved soils were placed in the containers and weights were recorded again. Then, the containers were placed in oven at 105°C for 24 hours. After 24 hours, containers were removed from the oven and placed in desiccator for 10-15

minutes to allow the samples to cool down to the room temperature. When the samples were cool enough, the weights were recorded again to calculate the percent moisture content. The calculation was as follows:

$$\text{Moisture content (w)} = \frac{\text{loss of moisture}}{\text{dry soil mass}} \times 100\%$$

$$w = \frac{\text{mass of wet soil and container} - \text{mass of dry soil and container}}{\text{mass of dry soil and container} - \text{mass of container}} \times 100\%$$

The value of *w* for each individual moisture content specimen was calculated, and then the average value was calculated (Head, 1986).

The values for the determination of natural moisture content are given in Table 3.1, and the average natural moisture content was taken 20%.

3.12 Determination of Plastic and Liquid Limit of Experimental Soil

The plastic limit is the moisture content at which a soil passes from the plastic state to the solid state. And, the liquid limit is the moisture content at which soil passes from the plastic to the liquid state (Head, 1986).

For the liquid and plastic limit tests, 250g of soil was first dried. The aggregations of particles were broken down in a mortar with a rubber pestle. Then, the soil was sieved through a 425 μm sieve on a receiving pan.

Plastic Limit Test:

About 20g of sieved soil was put in a mixing glass, and distilled water was added. Soil and water were mixed with a spatula. Then, the soil was rolled into threads between the palms of hand by applying a steady pressure until the threads' diameter was down to 3mm. Soil was broken up again to dry it more, and then it was rolled again to 3mm diameter until the tread crumbled. The first crumbling point was taken as the plastic limit. The crumbled threads were gathered and placed in a container immediately and closed up with the lid. By following the same procedure, two other pieces of soil were prepared and placed in two other containers. Then, the containers were placed in the even at 105°C for overnight, and the moisture content was calculated.

The results of the experiment are given in Table 3.2., and the average value for the plastic limit was taken 22%.

Liquid Limit Test:

The liquid limit was determined with a Casagrande apparatus (Head, 1986). 50g of soil was well mixed with some distilled water. A portion of well mixed soil was placed in Casagrande liquid limit apparatus, and the surface of the soil was pressed with spatula in order to make the level of soil level parallel to the base. Then, the soil was cut by grooving tool by dividing it half. After that, the handle of the apparatus was turned in a rate of two revolutions per second, until the groove was closed down to around 13mm, and the number of blows was recorded. The test must restart if the number of blow exceeds 50 blows by mixing in a little more water. The experiment was repeated two

more times. A small quantity of soil from the middle of the bowl was placed in a container and put in the oven at 105 °C for over night. To calculate the moisture content for each blow count, as in the moisture content test, moisture content as ordinate was plotted against the corresponding number of blows as abscissa (logarithmic scale). Drawn straight line is called "flow curve" (Head, 1996). By using the semi logarithmic curve the moisture content is the value that corresponds to 25 blows (Head, 1996). Flow curve in Figure 3.6, showed that the moisture content was 35% for the present study.

By using a semi-logarithmic chart, the moisture content is the value that corresponds to 25 blows. In our experiment, the moisture content value for liquid limit of the experimental soil was 35%.

The results of the experiment are given in Table 3.3.

3.13 Determination of pH

To determine the pH value of the soil, 50 g of dry soil was placed in a beaker, and 50 ml of distilled water was added to it. The beaker was shaken vigorously until the soil was in suspension. Then, the aliquot of the suspension was transferred into another beaker, and the pH was measured by ATI Orion pH meter. The pH value of the experimental soil was 6.52 in distilled water. When the microcosms were sampled for the moisture content determination, the pH was measured also. The pH value of the soil microcosms were in a range of 6.0 to 6.9 with peat moss, and 6.7 to 7.4 without peat moss.

3.14 Preparation of Experiment Trays

The purpose of the work was to determine the effect of different vitamin mixture concentrations on the aerobic biodegradation of PAHs. For this purpose, nine sample microcosms were set up by treating them differently. Table 3.4 shows the microcosm preparation. 4000g of soil was put in 33cmx38cmx9cm size nesting trays, and 2000g of soil was placed in 20cmx30cmx6cm size nesting trays which were purchased from Ward's Natural Science Establishment Inc. (Rochester, NY). Therefore, at time zero the vitamin concentration in the trays were as follows (in mg of vitamin / kg of dry soil):

Tray #2 and #6: 20 μg of biotin, 20 μg of niacin, 40 μg of panthothenic acid, and 40 μg of folic acid per kg of dry soil.

Tray #3, #7, and #9: 50 μg of biotin, 50 μg of niacin, 100 μg of panthothenic acid, and 100 μg of folic acid per kg of dry soil.

Tray #4 and #8: 100 μg of biotin, 100 μg of niacin, 200 μg of panthothenic acid, and 200 μg of folic acid per kg of dry soil.

According to the calculated values of natural moisture content and liquid limit of the soil, 30% initial moisture content level ((30g of water / 100g of dry soil), in no peat moss case, and (30g of water / 100g of dry soil with peat moss), in peat moss case) was chosen appropriate to keep the soil samples to have efficient biodegradation.

Soil microcosms were maintained in an incubator at 24 °C. The soil in each tray was manually mixed with a spatula 2-3 times a week to homogenize the moisture content. At the same time to determine the moisture content, soil was sampled. When the

measurement was below 22%, MSM (contain no vitamin) bring the moisture content up to 30%. In some cases it was visually observed that the soil appeared dry even after the calculated MSM addition was made. Therefore, extra MSM was added to them. A summary of moisture content analysis data and MSM additions is reported in Table 3.5.

After the preparation of sample trays, approximately 50g of soil was sampled from each tray for Soxhlet extraction and pH measurement at time zero. The trays were than loosely covered with aluminum foil and placed in incubator at 24°C. Tray # 9 was placed in anaerobic chamber in order to determine the effect of vitamin mixture and peat moss on depletion as a result of microbial activity.

Table 3.1 Natural Moisture Content Determination Test

Container	Container and wet soil	Container and dry soil	%Moisture Content
1. 1.935g	53.661g	44.368g	21.900
2. 1.912g	46.607g	38.997g	20.520
3. 1.937g	39.545g	32.634g	22.514

Table 3.2 Plastic Limit Determination Test

Container	Container and wet soil	Container and dry soil	%Moisture Content
1. 4.31g	7.14g	6.63g	21.79
2. 4.30g	8.34g	7.55g	24.44
3. 4.30g	9.91g	8.80g	24.58

Table 3.3 Liquid Limit Determination Test

Container	Container and wet soil	Container and dry soil	# of blows	%Moisture content
1. 4.300g	17.012g	13.623g	15	36.40
2. 4.309g	14.434g	11.797g	26	36.20
3. 4.327g	13.635g	11.230g	30	34.83

Table 3.4 Preparation of Microcosms

Tray #	Soil Amount (g)	Peat Moss Amount (g)	Vitamin Mixture	Initial MSM Amount (ml)
1	4000	0	0	400
2	2000	0	Va	200
3	2000	0	Vb	200
4	4000	0	Vc	400
5	4000	400	0	400
6	4000	400	Va	400
7	4000	400	Vb	400
8	4000	400	Vc	400
9	2000	200	Vb	200

Table 3.5 MSM Addition (in ml) and Percent Moisture Content

Tray#	Time (day) 4	8	12	14	17	20	24	28	32	35	38	42	48	52
1	0-(25%)	320-(22%)	0-(25%)	320-(22%)	0-(26%)	0	400-(20%)	0	0-(25%)	320-(22%)	0	400-(20%)	0	0-(25%)
2	0-(28%)	0	0-(26%)	160-(22%)	0-(25%)	0	180-(21%)	0	0-(24%)	200-(20%)	0	200-(20%)	100-(22%)	0-(25%)
3	0-(25%)	160-(22%)	0-(25%)	0	0-(22%)	180-(20)	0	150-(22%)	0-(28%)	0	0	200-(20%)	150-(22%)	0-(25%)
4	0-(27%)	0	0-(25%)	360-(21%)	0-(26%)	0	320-(22%)	0	0-(23%)	420-(20%)	0	360-(21%)	0	0-(25%)
5	0-(27%)	0	0-(25%)	230-(22%)	0-(26%)	0	320-(22%)	0	0-(25%)	230-(22%)	0	350-(22%)	0	0-(25%)
6	0-(27%)	0	0-(25%)	230-(22%)	0-(26%)	0	320-(22%)	0	0-(25%)	230-(22%)	0	230-(22%)	0	0-(25%)
7	0-(27%)	0	0-(25%)	230-(22%)	0-(26%)	0	320-(22%)	0	0-(25%)	230-(22%)	0	230-(22%)	0	0-(25%)
8	0-(27%)	0	0-(25%)	230-(22%)	0-(26%)	0	0	320-(20%)	0-(26%)	0	260-(20%)	0	260-(20%)	0-(25%)
9	0-(27%)	0	0-(25%)	110-(22%)	0-(22%)	0	160-(20%)	0	0-(25%)	110-(22%)	0	110-(22%)	0	0-(25%)

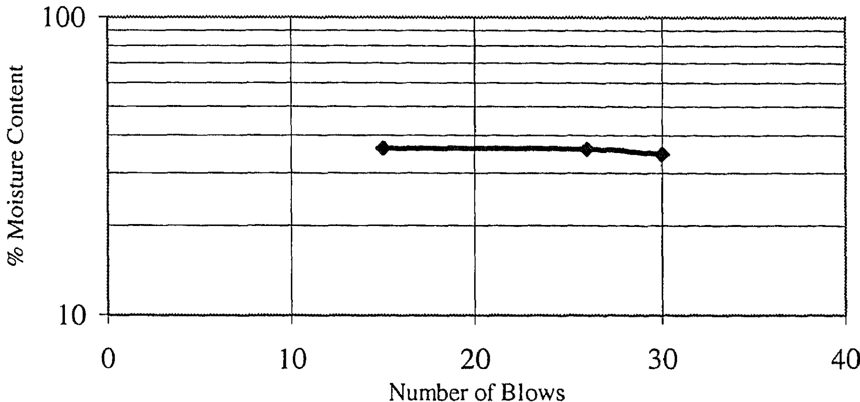

Figure 3.6 Flow curve of Experimental Soil

CHAPTER 4

RESULTS AND DISCUSSIONS

All the results of the HPLC analysis after Soxhlet extraction are given in Table 4.1. The values in parenthesis show the actual values for the duplicate samples.

After the microcosms were set up, the concentration of the target compounds at time zero was determined (in duplicates) for untreated soil, tray #1, #3, #6, and #9.

The PAH concentration in the original soil in tray #1, and #3 were very similar. Therefore, an average value of these initial three concentrations was taken as the initial concentration for the other trays that did not have peat moss. Tray #5, #6, #7, #8 and #9 had 10% peat moss by weight. The results for trays #6 and #9 were calculated by considering the peat moss amount. These results were also used for tray #7 and #8.

The biodegradation percentages of phenanthrene, anthracene, fluoranthene, and pyrene as a function of vitamin concentration at the end of experiments are given in Table 4.2. The initial and overall biodegradation rates are given in Table 4.3 and 4.4. For the calculation of initial degradation rates 14-day data, and for the calculation of overall degradation 56-day data was used.

On the basis of data presented in Table 4.2, it can be concluded that peat moss (no vitamin) played a significant role in the aerobic degradation of PAHs in soil. The overall average degradation was 90% for PAHs in the presence of peat moss (no vitamin) while it was 75% in the absence of peat moss (no vitamin).

On the basis of data presented in Table 4.3 and 4.4, initial and overall biodegradation rates of PAHs were higher in the presence of peat moss and V_a and V_b

vitamin mixture concentrations. In the absence of peat moss, V_c vitamin concentration was more effective in the initial biodegradation rates of higher molecular weight PAHs.

Figures 4.1, 4.2, 4.3, and 4.4 show the effects of vitamins on the aerobic biodegradation of phenanthrene, anthracene, fluoranthene, and pyrene, respectively, in the contaminated soil supplemented with peat moss (10% by weight).

As shown in Figure 4.1, the soil phenanthrene was strongly biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was 75%, except in the anaerobic microcosm, used as control, in which only a slight depletion of the original phenanthrene concentration was observed (10%). Vitamins were found to significantly enhance the phenanthrene biodegradation rate, especially when they were used at the V_a and V_b concentrations. However, they did not influence the final phenanthrene removal from the soil. V_c concentration of vitamins apparently did not show any effect on the phenanthrene biodegradation rate and extent in the soil.

As shown in Figure 4.2, the soil antracene was strongly biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was 98% except in the anaerobic microcosm, used as control, in which a slight depletion of the original anthracene concentration was observed (4%). Vitamins were found to significantly enhance the antracene biodegradation rate, especially when they were used at the V_a and V_b concentrations. However, V_c vitamin concentration did not influence the final antracene removal from the soil as much as other concentration levels.

As shown in Figure 4.3, the soil fluoranthene was almost completely biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was almost 100% except in the anaerobic microcosm, in which a slight depletion of the original anthracene concentration was observed (12%). Vitamins significantly enhanced the fluoranthene biodegradation rate, at all concentrations. In addition, V_b vitamin concentration apparently also enhanced the final removal of fluoranthene.

As shown in Figure 4.4, the soil pyrene was biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was 70% except in the anaerobic microcosm, in which a slight depletion of the original anthracene concentration was observed (12%). Vitamins enhanced the pyrene biodegradation rate, especially when they were used at the V_c concentration, which, however, did not influence the final pyrene removal from the soil. V_a concentration of vitamins apparently did not have effects on the pyrene biodegradation in the soil. The lower degradation rate and extent observed for pyrene was probably due to its chemical structure make this compound more recalcitrant with respect on the other PAHs.

On the basis of the data presented above, it can be concluded that typically vitamins play a minor role in the aerobic biodegradation of PAHs in the microcosms with the contaminated soil supplemented with peat moss. Vitamin additions were found to enhance the initial biodegradation rate of phenanthrene, anthracene, fluoranthene, and pyrene, especially when they were used at the V_b concentration, without significantly modifying the final removal of the xenobiotics from the soil. Considering the

insignificant PAH disappearance in the anaerobic microcosms, the PAH depletion described can be reasonably ascribed to the biological biodegradative activity of the soil aerobic indigenous bacteria.

Figures 4.5, 4.6, 4.7, and 4.8 show the effects of vitamins on the aerobic biodegradation of phenanthrene, anthracene, fluoranthene, and pyrene, respectively, in the contaminated soil without peat moss.

As shown in Figure 4.5, the soil phenanthrene was strongly biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was 76%. Vitamins were found to significantly enhance the phenanthrene biodegradation rate and extent especially when they were used at the V_b and V_c concentrations. They also influenced the final phenanthrene removal from the soil (76%).

As shown in Figure 4.6, the soil antracene was biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was 90%. Vitamins were found significantly enhance the antracene biodegradation rate and extent, especially when they were used at the V_b and V_c concentrations. V_a vitamin concentration did not influence the biodegradation rate and extent of anthracene in the soil.

As shown in Figure 4.7, the soil fluoranthene was significantly biodegraded by the indigenous microorganisms under all treatment conditions. The average value of the depletion observed in the different aerobic microcosms at the end of the treatment was

almost 85%. Vitamins were found significantly enhance the fluoranthene biodegradation rate and extent in the soil at all concentrations.

As shown in Figure 4.8, the soil pyrene was biodegraded by the indigenous microorganisms by an average 65%. Even though the higher vitamin concentration has had higher biodegradation rate of pyrene, vitamins did not enhance the pyrene biodegradation in soil.

On the basis of the data presented above, it can be concluded that vitamins played a significant role in the aerobic biodegradation of PAHs in the microcosms with contaminated soil when peat moss was not present. They were found to enhance the biodegradation rate and extent of phenanthrene, anthracene, and fluoranthene especially when used at the V_b and V_c concentrations. However, they did not display the same positive effect on the biodegradation of pyrene; this result could be ascribed to the higher recalcitrance of this PAH.

Higher and more interesting vitamin effects were observed in the absence of peat moss, which probably masked the effect of vitamins. In fact, peat moss probably released additional carbon sources and nutrients for the indigenous specialized microorganisms which, under these conditions, did not get any benefits from vitamins. Because of the release of these additional nutrients by the peat moss, a faster and larger PAH removal was observed; however, it is likely that peat moss favored the bacterial activity by increasing the water and oxygen availability in the soil.

On the basis of the data presented, it can be concluded that vitamins applied at the V_b concentration gave the larger effect on the PAHs removal from the soil.

The effect of vitamins was also studied by other researches for the degradation of different compounds both in aerobic and anaerobic conditions were mentioned in Chapter 2. Biotin was found to be a necessary supplement for optimal growth in the biodegradation of 1,2-dichloroethane (Wijngaard *et al.* 1993). They had studied the effects of organic nutrients and cocultures on the competitive behavior of 1,2-dichloroethane-degrading bacteria. They found the strain AD25 not dominant in the lack of vitamins, which are necessary for optimal growth of this strain.

The effect of vitamin B 12 was studied for the degradation of 1,2-dichloroethane in groundwater (Lasage, 1997). B12 was also studied on reductive dechlorination of biphenyls and benzenes by Assaf-Anid *et al.* in 1992. Finally, Kafkewitz et al. (1996) found that the same vitamin mixture used in this work was capable of enhancing the biodegradation rate and extent of monochlorophenols, mono chlorobenzoic acids and 4-chlorobiphenyl.

It is possible to speculate that vitamins enhanced the indigenous specialized bacterial activity by favoring the activity of the deoxygenates responsible for the PAH aromatic ring attack and cleavage. In fact, these enzymes require niacin as cofactor to express their activity.

Boldrin et. al (Boldrin et.al, 1993) found Mycobacterium sp. strain able to utilize phenanthrene, pyrene, and fluoranthene as sole sources of carbon and energy and to degrade fluorene cometabolically. The growth rates for phenanthrene, pyrene, and fluoranthene were 0.069, 0.056, and 0.040 mg/kg. h^{-1} respectively.

In one of the recent studies, Thiabult et. al found 63% mineralization for pyrene upon inoculation with K-12 and B-24 bacterial isolates (Thiabult et. al, 1996). In present

study, pyrene was degraded to 81% in the presence of peat moss (no vitamin), and 75% in the absence of peat moss (V_c) without any inoculation with bacterial isolates.

In present study, phenanthrene was degraded 57% by indigenous soil microorganisms in two weeks. Fluoranthene was degraded 75% in two weeks, but it was degraded almost 100% in six weeks by indigenous microorganisms. However, Heitkamp and Cerniglia found phenanthrene degradation 51% in two weeks by gram positive rod bacterium, and in the same study, fluoranthene was degraded 89% (Heitkamp and Cerniglia, 1988).

Field et. al found one of the new isolates, *Bjerkandera* sp. strain Bos 55, was the best degrader of both anthracene and benzo(*a*)pyrene, removing 99.2 and 83.1% of these compounds after four weeks, respectively (Field et. al, 1992). At the end of present study, anthracene degradation was 97% in the presence of V_b .

The present study showed that the indigenous microorganisms were able to degrade the specific PAHs in the presence of peat moss or vitamin. Vitamin mixture had significant effect on indigenous microorganisms to enhance the biodegradation rates. They are comparable to the results in other studies that were done with specific microorganisms.

Table 4.1 The results of HPLC analyses

	Time (days)	Residual Phenanthrene Concentration (mg/kg) in soil	Residual Anthracene Concentration (mg/kg) in soil	Residual Fluoranthene Concentration (mg/kg) in soil	Residual Pyrene Concentration (mg/kg) in soil	2-fbp Recovery Rate (%)
Tray#1	0	6.9 (7.3-6.5)	6.38 (6.78-5.98)	6.57 (6.84-6.30)	8.88 (9.93-7.83)	65
	14	5.2	5.48	5.26	6.04	62
	28	3.53	1.51	2.23	4.41	64
	42	3.13	0.94	1.96	3.12	57
	56	2.57	0.65	0.98	2.57	60
Tray#2	0	6.9	6.38	6.57	8.88	64
	14	3.94 (4.56-3.32)	5.43 (6.31-4.55)	2.09 (2.44-1.74)	4.87 (5.53-4.21)	58
	28	3.96	3.25	1.05	5.24	59
	42	2.52	2.35	ND*	ND	68
	56	1.87	1.85	ND	ND	51
Tray#3	0	6.9 (6.9-ND)	6.38 (8.23-4.53)	6.57 (7.82-5.32)	8.88 (11.42-6.34)	68
	14	3.92	4.08	2.45	4.96	52
	28	2.66	1.48	1.67	6.04	57
	42	2.31	0.28	1.13	4.03	69
	56	1.77	0.19	0.87	3.75	52

* ND: not detected

Table4.1 (continued)

	Time (days)	Residual Phenanthrene Concentration (mg/kg) in soil	Residual Anthracene Concentration (mg/kg) in soil	Residual Fluoranthene Concentration (mg/kg) in soil	Residual Pyrene Concentration (mg/kg) in soil	2-fbp Recovery Rate (%)
Tray#4	0	6.9	6.38	6.57	8.88	60
	14	3.78	2.97	2.58	4.98	46
	28	2.66	0.37	1.24	4.76	47
	42	1.97 (2.49-1.45)	0.39 (0.53-0.25)	1.44 (2.04-0.84)	3.44 (4.33-2.55)	48
	56	1.12	0.26	1.12	2.58	52
Tray#5	0	7.67	7.09	7.32	9.87	63
	14	4.81	5.04	2.46	5.17	69
	28	1.96 (2.51-1.41)	0.42 (0.6-0.24)	1.11 (1.85-0.37)	3.3 (4.75-1.85)	71
	42	1.92	0.2	0.99	3.27	73
	56	1.12	0.1	0.12	1.85	62
Tray#6	0	7.67 (10.22-5.12)	7.09 (9.44-4.74)	7.32 (9.79-4.85)	9.87 (15.32-4.42)	63
	14	4.14	3.46	3.18	4.86	57
	28	3.92	0.33	1.63	5.58	77
	42	2.42	0.57	1.93	4.72	72
	56	2.06	0.41	0.13	3.46	75

Table 4.1 (continued)

	Time (days)	Residual Phenanthrene Concentration (mg/kg) in soil	Residual Anthracene Concentration (mg/kg) in soil	Residual Fluoranthene Concentration (mg/kg) in soil	Residual Pyrene Concentration (mg/kg) in soil	2-fbp Recovery Rate (%)
Tray#7	0	7.67	7.09	7.32	9.87	56
	14	3.39	2.82	1.84	5.87	68
	28	2.81	0.21	0.48	5.49	58
	42	2.77	0.45	0.07	5.09	74
	56	1.37	0.19	0.07	ND	64
Tray#8	0	7.67	7.09	7.3	9.87	52
	14	5.71	2.81	3.26	6.02	58
	28	3.61	2.29	1.55	3.95	64
	42	3.76	1.13	0.12	4.38	62
	56	2.58	0.84	0.11	2.39	72
Tray#9	0	7.67 (10.92-4.42)	7.09 (10.07-4.11)	7.3 (9.97-4.63)	9.87 (16.02-3.72)	72
	14	6.96	6.8	7	9.5	54
	28	6.51	6.42	6.56	9.28	62
	42	6.63	5.61	5.82	8.88	68
	56	6.65 (8.79-4.51)	5.84 (7.71-3.97)	6.1 (8.11-4.09)	8.78 (11.99-5.57)	66

Table 4.2 Percentage Degradation of PAHs at the end of the Experiments (t=56 d)

	With Peat Moss (10% w/w)					Without Peat Moss			
Vitamin Concentration	**V0**	**Va**	**Vb**	**Vc**	**Anaerobic (Vb)**	**V0**	**Va**	**Vb**	**Vc**
Phenanthrene	85	50	82	66	13	63	73	74	84
Anthracene	98	88	97	88	13	90	73	97	96
Fluoranthene	98	95	99	98	13	85	NA	87	83
Pyrene	81	29	NA	75	7	71	NA	57	70

Table 4.3 Initial Degradation Rates (mg/(kg.day)) of PAHs

	With Peat Moss (10% w/w)					Without Peat Moss			
Vitamin Concentration	**V0**	**Va**	**Vb**	**Vc**	**Anaerobic (Vb)**	**V0**	**Va**	**Vb**	**Vc**
Phenanthrene	2.16	3.29	3.99	1.83	0.66	1.76	3.06	3.08	3.23
Anthracene	1.50	3.66	4.30	4.31	0.29	1.01	1.06	2.58	3.82
Fluoranthene	4.47	4.04	5.35	3.95	0.29	1.42	4.87	4.48	4.34
Pyrene	2.98	3.63	2.89	2.79	0.27	2.28	3.23	3.15	3.14

Table 4.4 Overall Degradation Rates (mg/(kg.day)) of PAHs

	With Peat Moss (10% w/w)					Without Peat Moss			
Vitamin Concentration	**V0**	**Va**	**Vb**	**Vc**	**Anaerobic (Vb)**	**V0**	**Va**	**Vb**	**Vc**
Phenanthrene	1.50	1.31	1.47	1.19	0.24	1.12	1.30	1.33	1.54
Anthracene	1.76	1.68	1.74	1.57	0.31	1.60	1.27	1.73	1.71
Fluoranthene	1.75	1.75	1.77	1.76	0.29	1.52	NA	1.55	1.48
Pyrene	1.41	1.16	NA	1.35	0.20	1.27	NA	1.03	1.27

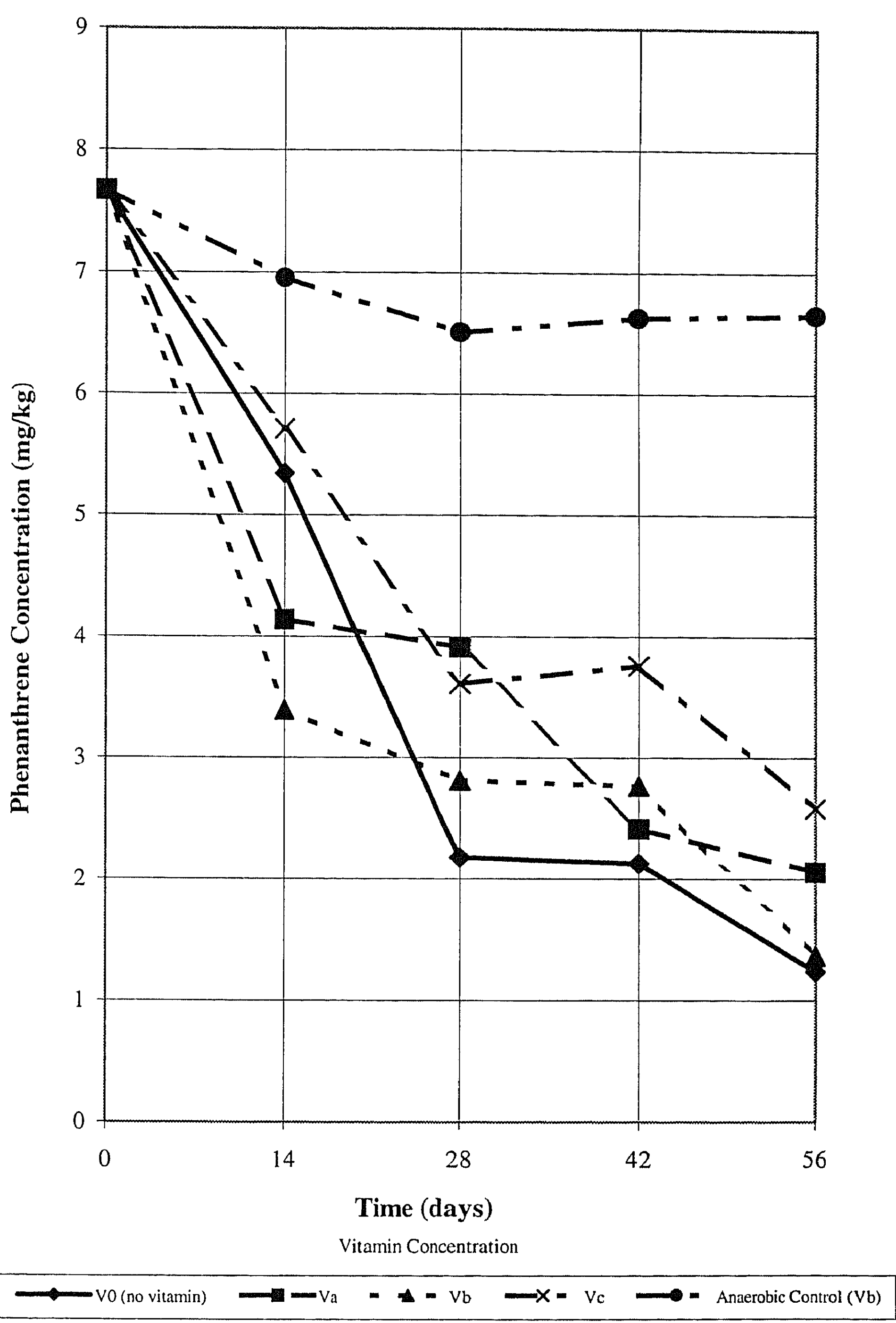

Figure 4.1 Phenanthrene Degradation in the Presence of Peat Moss

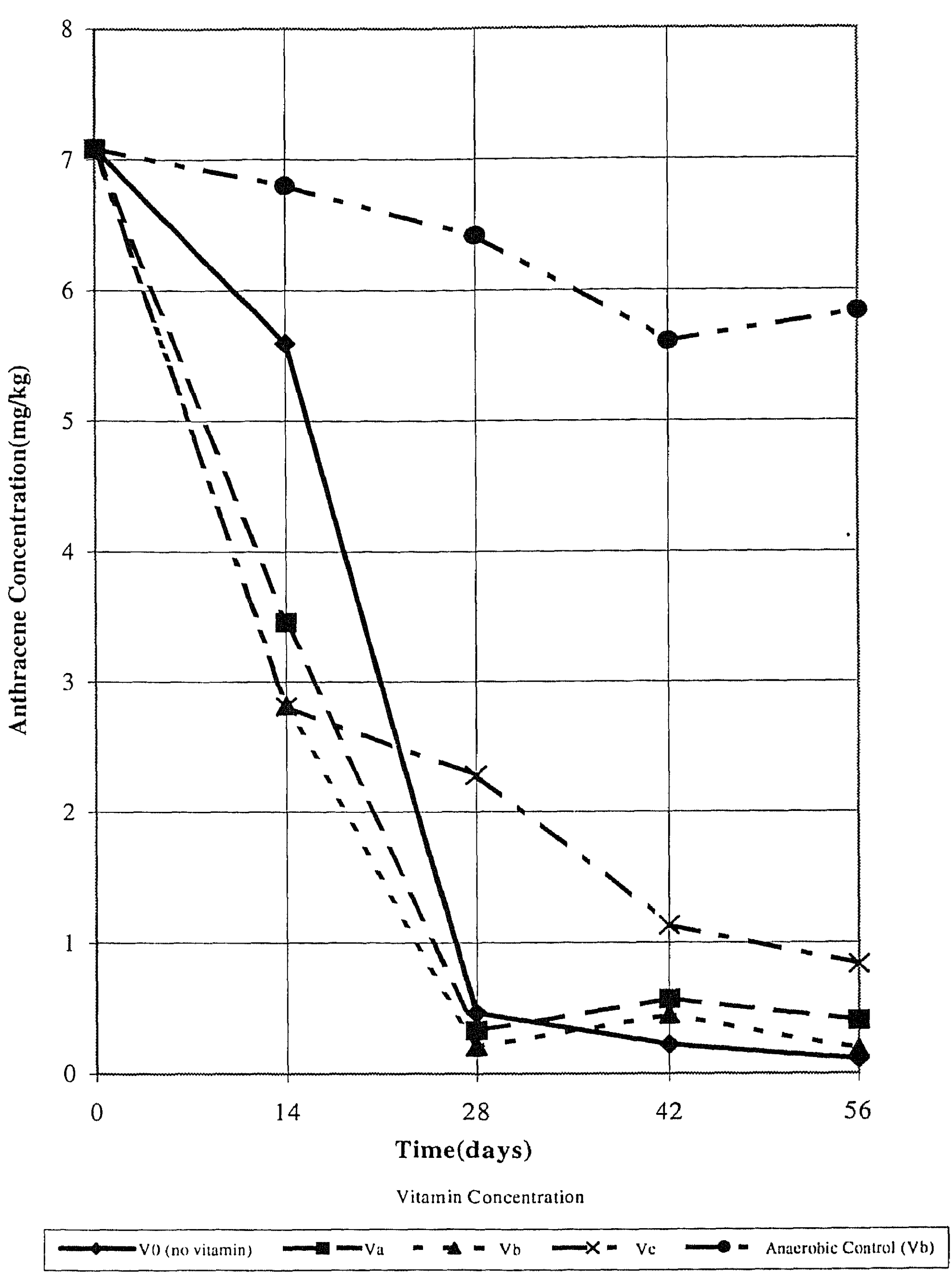

Figure 4.2 Anthracene Degradation in the Presence of Peat Moss

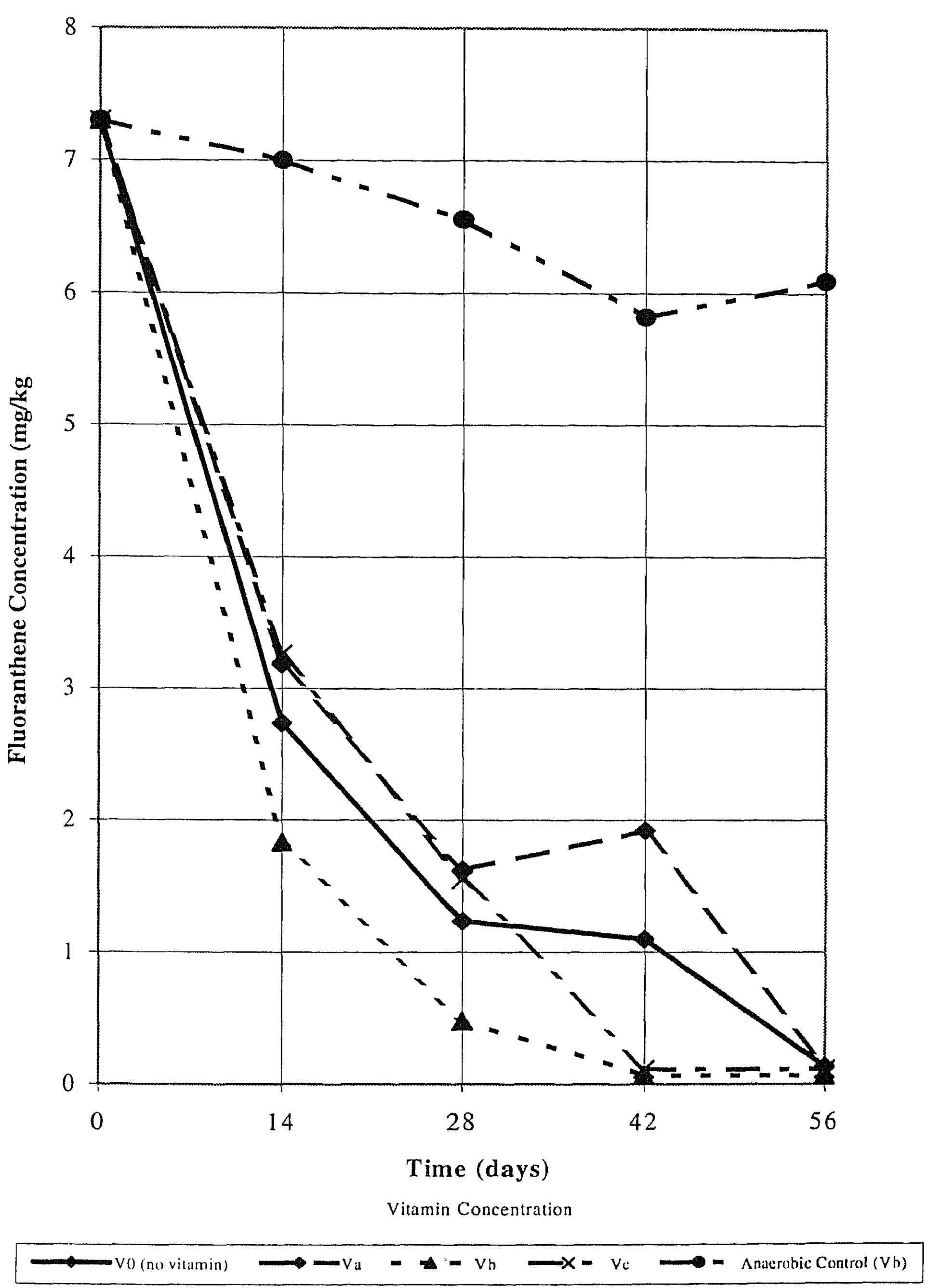

Figure 4.3 Fluoranthene Degradation in the Presence of Peat Moss

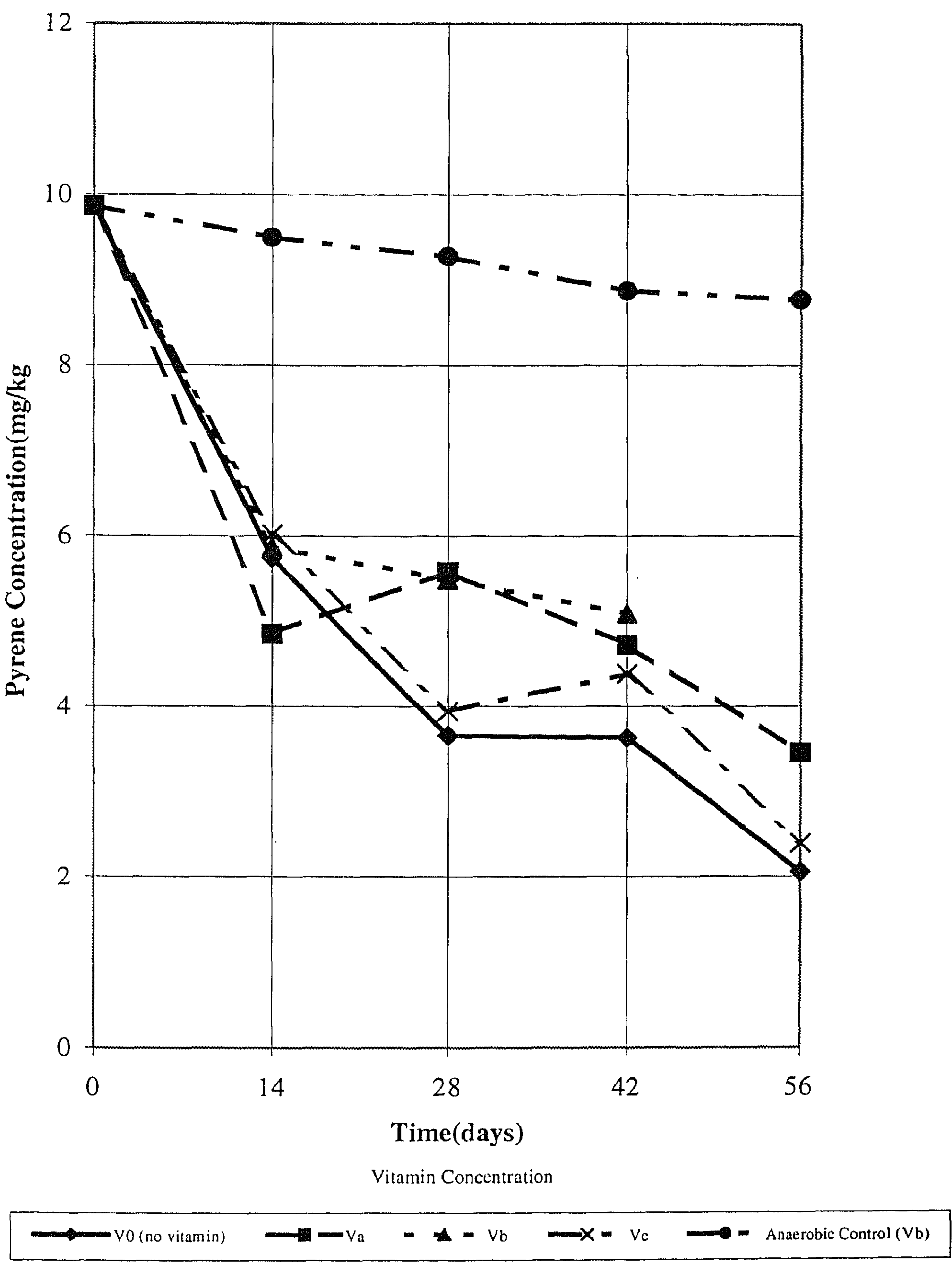

Figure 4.4 Pyrene Degradation in the Presence of Peat Moss

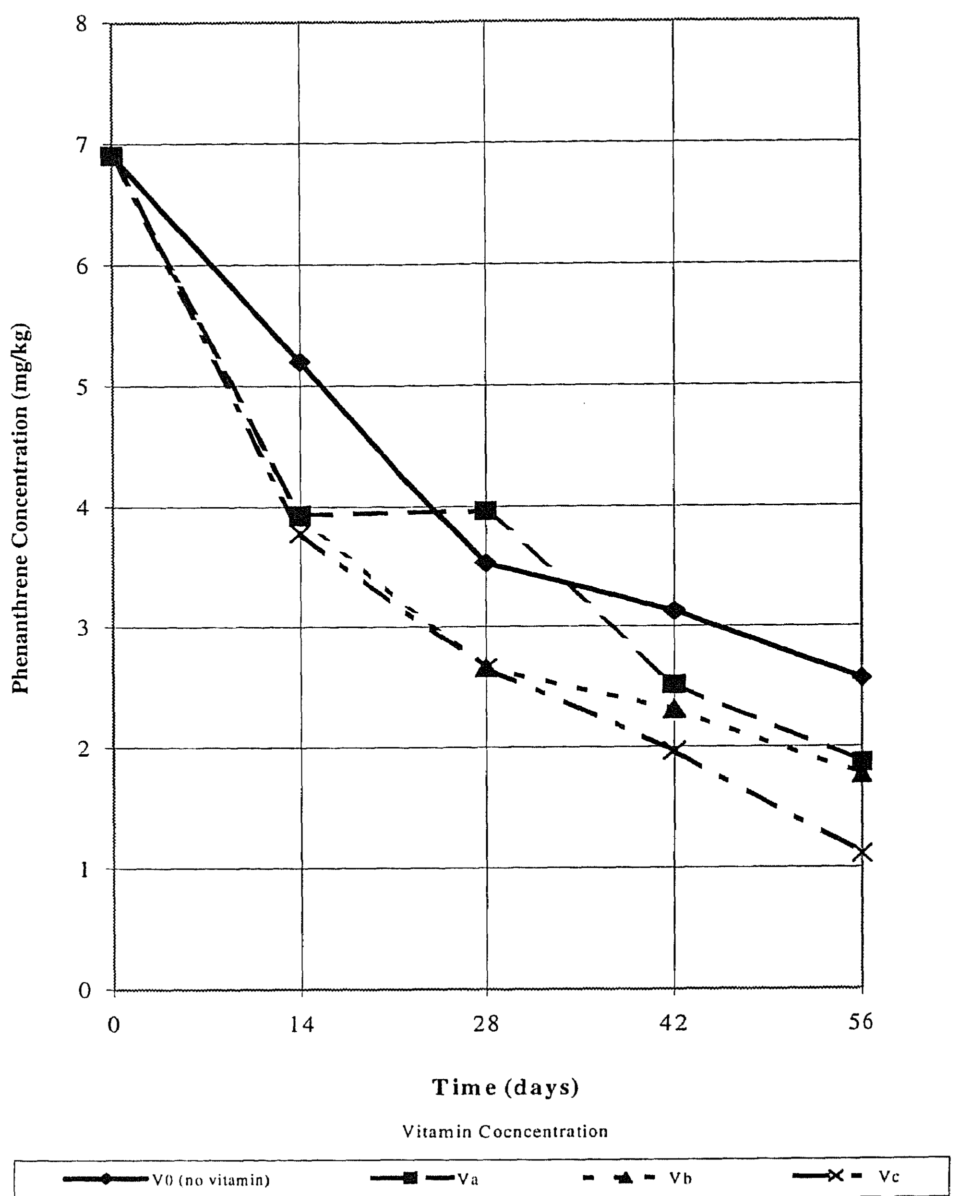

Figure 4.5 Phenanthrene Degradation in the Absence of Peat Moss

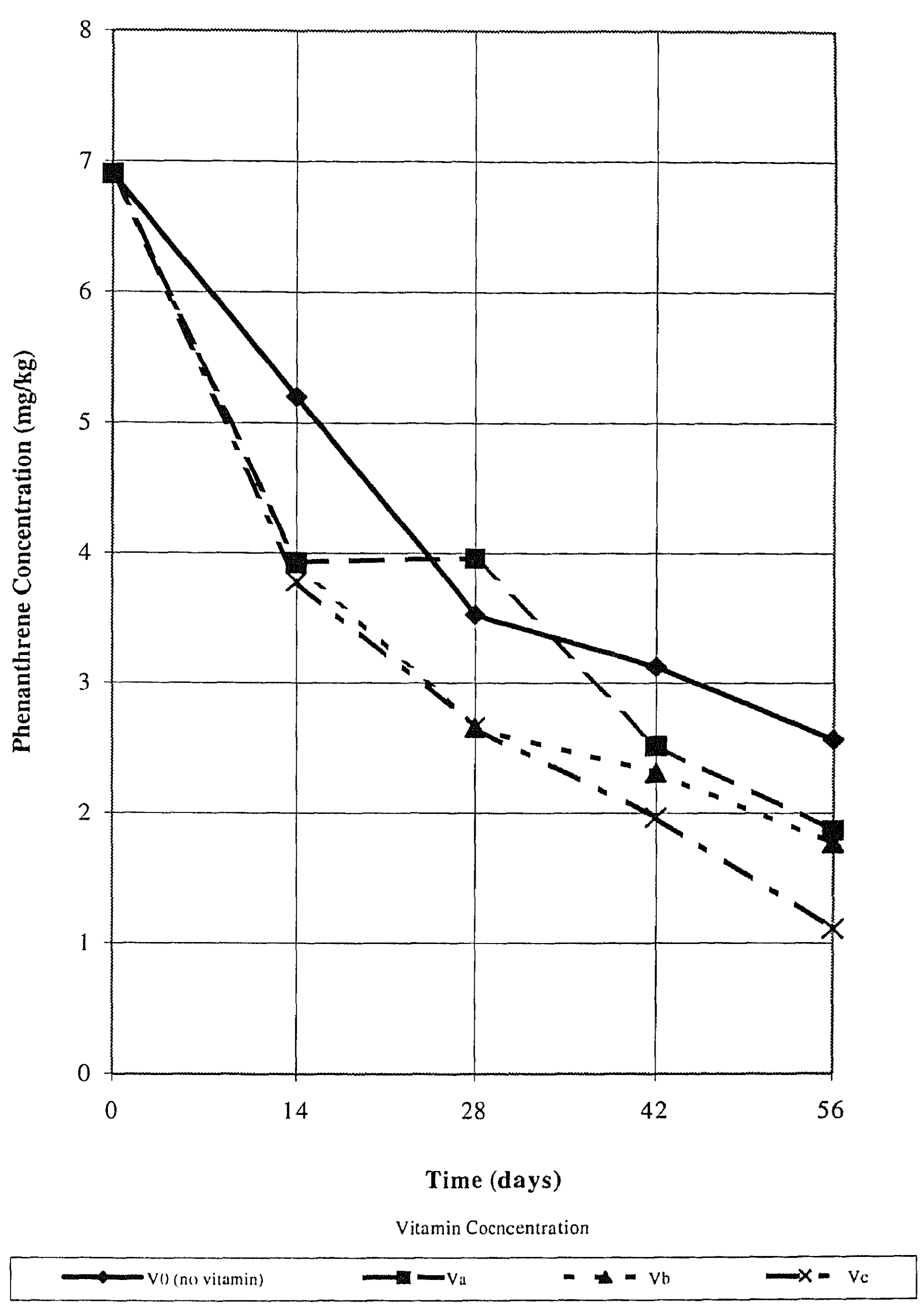

Figure 4.6 Antracene Degradation in the Absence of Peat Moss

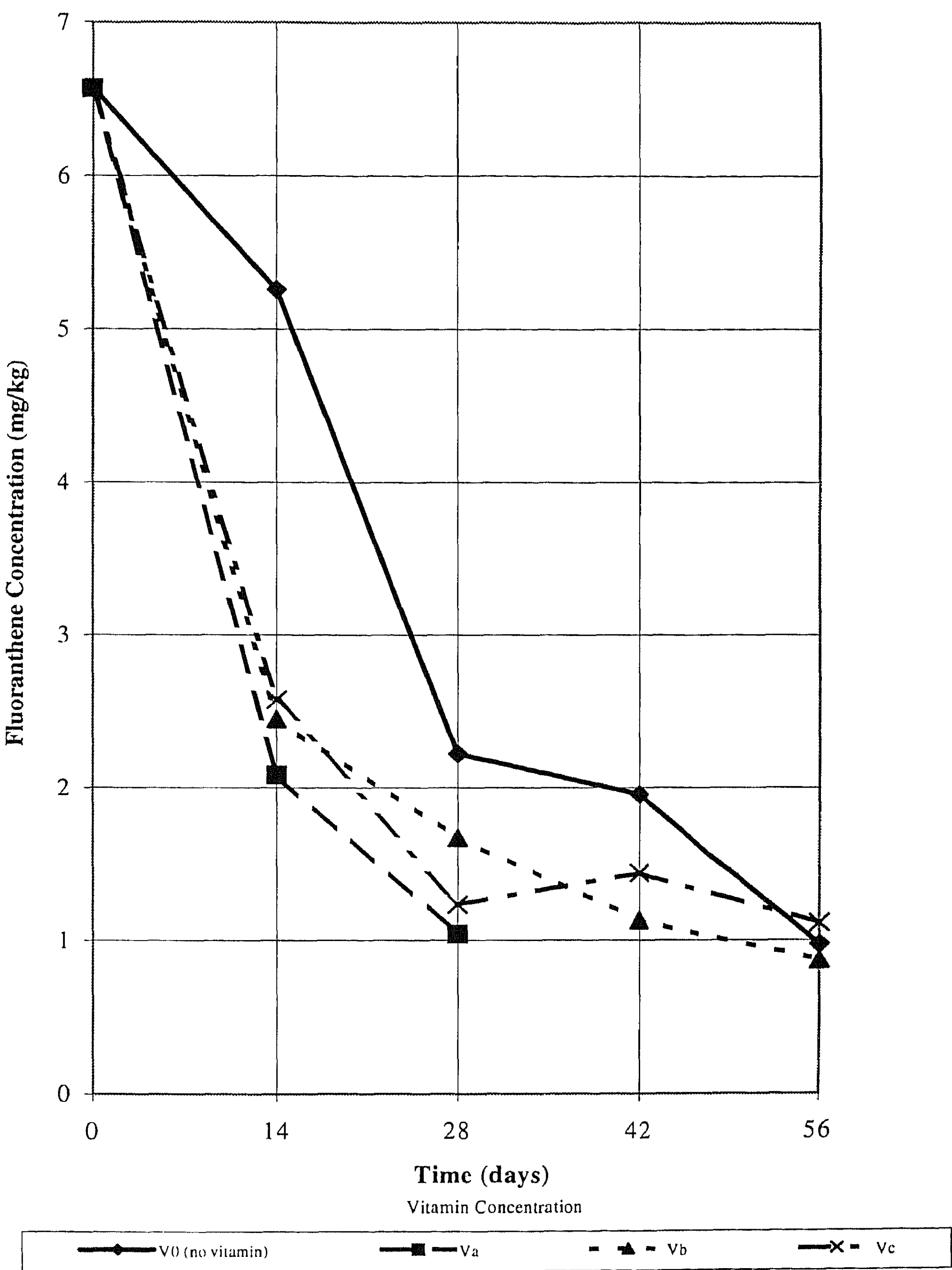

Figure 4.7 Fluoranthene Degradation in the Absence of Peat Moss

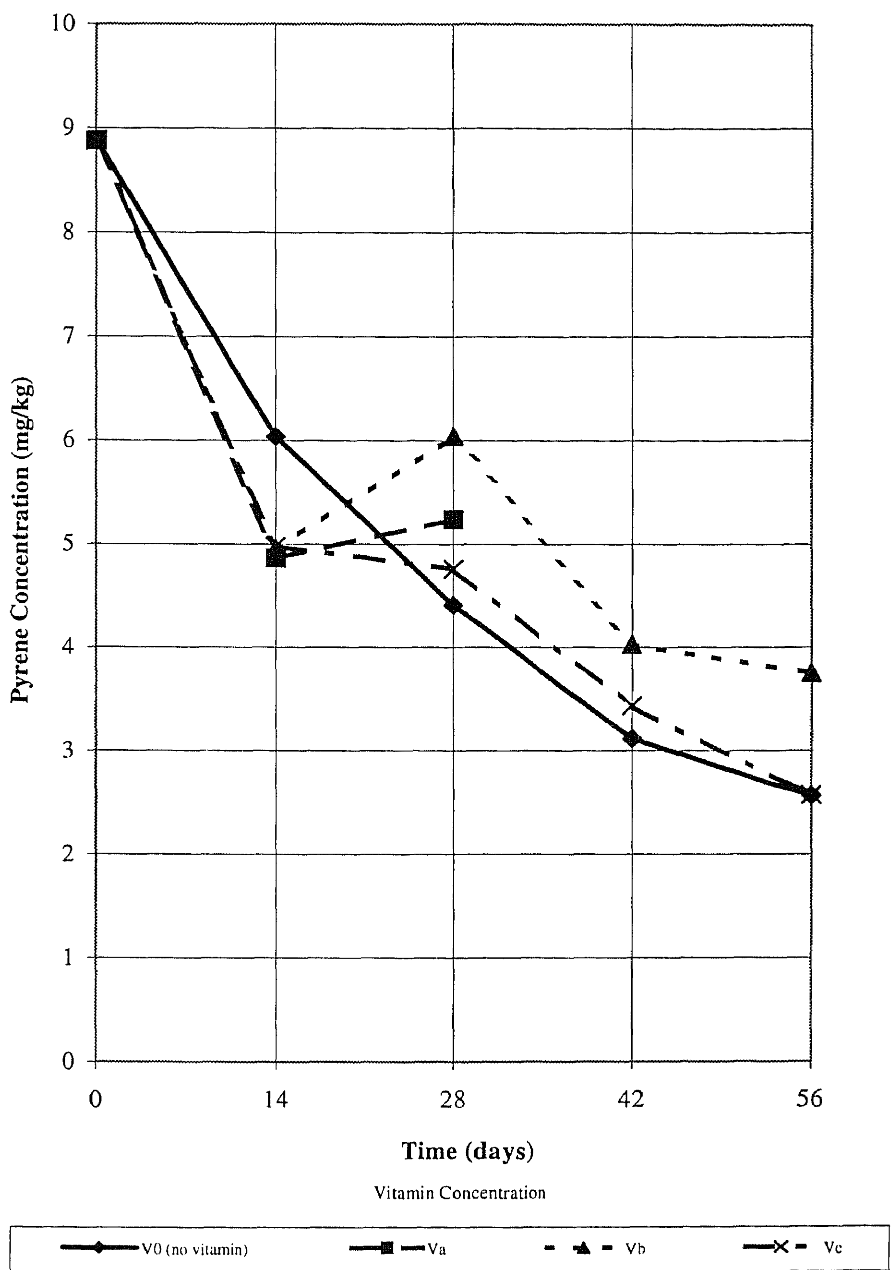

Figure 4.8 Pyrene Degradation in the Absence of Peat Moss

CHAPTER 5

CONCLUSION

The study showed that vitamin mixture had an effect on biodegradation of aromatic hydrocarbons.

The addition of vitamin mixtures containing different initial vitamin concentrations was found to enhance significantly the biodegradation rate of the phenanthrene, anthracene, and fluoranthene in the contaminated soil in the absence of peat moss. The study also showed that the same vitamin mixtures enhanced the final removal of four-ring PAH, fluoranthene, in the absence of peat moss. Peat moss, which can release easily metabolized carbon sources, masked the vitamin effect on the PAHs biodegradation. Peat moss significantly enhanced PAH biodegradation probably by increasing the availability of organic compounds, oxygen, water, and nutrients to the soil indigenous microorganism.

Thus, this study was the first in which the effect of vitamin on aerobic biodegradation of aromatic hydrocarbons has been demonstrated.

On the basis of experimental data presented in this thesis and considering the findings already described in the literature (Kafkewitz, *et al.* 1996, GuptaPal 1996) we can conclude that the vitamin mixture which has been used in this work could be successfully applied to the larger scale bioremediation of organic xenobiotics contaminated soil. However, further studies on this topic are necessary in order to assess if this approach could be cost effective at the industrial scale.

APPENDIX A

PAH DEGRADING MICROORGANISMS

The following table shows the PAHs that were used on our study and organisms capable of degrading them (1).

Table A1. Hydrocarbons and Microorganisms Capable of Biodegrading Them.

Hydrocarbon	Microorganisms
Antracene	Stream bacteria, (*Flavobacterium, Beijerinckia sp., Cunnighamella*) (*Pseudomonas /Acinetobacter* sp., *Arthrobacter* sp.)
Fluoranthene	Sewage sludge *Pseudomonas* spp.
Phenanthrene	*Beijerinckia*, (*Pseudomonas putida*, *Cunnighamella elegans*), *Pseudomonas* spp., *Flavobacterium*.
Pyrene	Stabilization pond organisms, (*Pseudomonas /Alcaligenes* sp., *Acinetobacter* sp., *Arthrobacter* sp.)

APPENDIX B

CALIBRATION LEVELS

Table B.1 shows the preparation of calibration standards. Table B.2 shows the retention times of the compounds and correlation coefficients,R^2. All of the standards were obtained from Chem. Service, West Chester, PA. 2-fluorobiphenyl was 99.3%, phenantherene was 99%, antracene was 99.9%, fluoranthene was 98%, and the pyrene was 99% pure.

Table B1. Preparation of Calibration Solutions

Source	Stock Solution		Working Soluiton							
	Amount used (mg)	Concentration (mg/ml)	Volume used (ml)	Final Concentration (mg/l)	Std.1 (mg/l)	Std.2 (mg/l)	Std.3 (mg/l)	Std.4 (mg/l)	Std.5 (mg/l)	Std.6 (mg/l)
Anthracene	20.02	2.002	1.00	20.02	10.01	5.005	3.003	1.001	0.05	0.05
Fluoranthene	20.04	2.004	1.00	20.04	10.02	5.01	3.006	1.002	0.501	0.05
Phenanthrene	20.03	2.003	1.00	20.03	10.015	5.008	3.005	1.002	0.501	0.05
Pyrene	20.03	2.003	1.00	20.03	10.015	5.008	3.005	1.002	0.501	0.05
2-fluorobiphenyl	20.07	2.007	8.00	160.56	80.28	40.14	24.084	8.028	4.014	0.401

Standards were dissolved in 10ml acetonitrile. Then 1ml from each standard and 8ml from 2-fluorobiphenyl were combined and diluted to 100ml with acetonitrile.

Table B.2 Retention Times and Correlation Coefficients

Standards	Retention Times (min)	Correlation Coefficients
Anthracene	4.653	0.999787
Fluoranthene	5.475	0.999833
Phenanthrene	3.861	0.999812
Pyrene	6.43	0.999838
2-fluorobiphenyl	2.618	0.999829

APPENDIC C

POLYCYCLIC AROMATIC HYDROCARBONS

In recent years, the concern about the presence of polycyclic aromatic hydrocarbons (PAH) in air, soil, and water systems has increased, since this important class of chemicals is carcinogenic in experimental animals and a potential health risk to man. A summary of the structure, toxicity, and genotoxicty of PAH commonly found in soils and aquatic ecosystems is given in Table C1. (5)

Table C1. Chemical Structures, Physical and Toxicological Characteristics of Specific PAHs[1].

PAH	Molecular Weight	Solubility (mg/l)	Genotoxicity
Antracene	178.2	0.070	—
Phenanthrene	178.2	1.300	—
Fluoranthene	202.3	0.260	Weak Carcinogen
Pyrene	202.3	0.140	Ames +UDS +SCE

[1] The Symbols are: (Ames) Salmonella Typhimurium Reversion Assey, (UDS) Unscheduled DNA Synthesis, (SCE) Sister Cromatid Exchange. (4)

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