THE EFFECT OF DISSOLVED HUMIC SUBSTANCES ON THE BINDING AND BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOIL

BY

CHRISTOPHER T. SMYRL

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Soil Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ACKNOWLEDGEMENTS

I would like to express my gratitude to all of the people who were instrumental in making the completion of this thesis possible.

Special thanks to Dr. David Burton for his guidance, advice, and encouragement throughout this project.

I would like to extend my appreciation to the other members of my thesis committee, Dr. Carlos Monreal, Dr. Tee Boon Goh, Dr. Daryl McCartney, and Edwin Yee, for lending their expertise to this project, and for their encouragement and assistance with this research.

Thanks to Mike Nightengale, Rob Ellis, Val Ward, and Aniko Bernatsky for their technical advice, and assistance in the lab.

I would like to thank the staff, faculty, and fellow grad students in the Department of Soil Science for making my stay in the department an enjoyable one.

Finally, I would like to thank my family for their support and encouragement for all these years. Special thanks to my dad for the endless advice and countless rough draft revisions. Yes, Mom, I'm finally done my thesis...

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Smyrl, Christopher Terrance. M. Sc., The University of Manitoba, October 2001. The Effect of Dissolved Humic Substances on the Binding and Biodegradation of Polycyclic Aromatic Hydrocarbons in Soil. Major Professor; Dr. David L. Burton.

Soils are a major environmental sink for polycyclic aromatic hydrocarbons (PAHs) because of their hydrophobic nature and their recalcitrance in the soil. These contaminants are less bioavailable for degradation, since they are bound to the soil particles. For bioremediation of PAH-contaminated soils to be effective, a technique must be found to cause the PAHs to transfer from the soil-sorbed phase to the aqueous phase. This can be achieved by decreasing the interfacial tension between the compound and the water using a surfactant. There is a demand for new techniques and products which may increase the effectiveness of remediation procedures in order to reduce expenses and shorten the time required to restore a contaminated site back to acceptable conditions.

Leonardite is a naturally occurring oxidized form of lignite coal that is rich in humic materials. It is collected during coal-mining procedures, as it is commonly found overlying coal seams. Humic substances have been shown to significantly enhance the aqueous solubilities of PAHs by acting as a surfactant, increasing the water solubility of the PAH and allowing the contaminants to enter the aqueous phase. The use of humic acids on contaminated soils has the potential to greatly enhance the solubility, and

consequently the bioavailability, of PAHs allowing for an easier and more efficient bioremediation procedure.

A fluorescence quenching method was used to determine the association constants (K_b) for various water-soluble humic materials and naphthalene. Results show that partitioning capabilities of these humic material solutions are not influenced by changes in pH, as the differences in association constants at pH 4.0 and pH 7.0 were insignificant. Results indicate that the solution of a 1/1000 dilution of 3.0 g/L Aldrich humic acid was most effective at partitioning naphthalene in aqueous solution, followed by a 1/1000 dilution of L-58, a 1/1000 dilution of L-69, a 1/1000 dilution of L-67D, and lastly, an undiluted L-58. L-58, L-67D, and L-69 are extraction products from leonardite.

The objective of the microcosm study was to determine if a variety of dissolved humic material solution amendments could influence the biodegradation rate of selected PAHs, namely anthracene and benzo[a]pyrene, in a soil with previous exposure to PAHs. Soils were contaminated with ^{14}C-labelled PAHs and, using a microcosm apparatus, total degradation of the PAHs was recorded over a 38 week period. Total respiration and volatilization were also monitored for the duration of the experiment. Significant degradation occurred within the 38 week duration of the experiment, for both anthracene (47.9 - 50.3% of the total initial amount added) and benzo[a]pyrene (24.9 - 31.2% of the total initial amount added), but there were no significant amendment effects, aside from a temporary suppression of benzo[a]pyrene degradation by the glucose amendment. The soils with depressed PAH degradation showed the highest total respiration rates, suggesting that the glucose was preferentially selected as a substrate by the microbiological degrader community.

At the concentrations tested, none of the humic material amendments used had a positive effect on the biodegradation rates of anthracene or benzo[a]pyrene, despite the capability demonstrated by these amendments to bind with PAHs in aqueous solution.

FORWARD

The following thesis was prepared using the manuscript format outlined in "A Guide To Thesis Preparation For Graduate Students In The Department of Soil Science."

CHAPTER 1

Introduction

The contamination of soil and groundwater by highly persistent, hydrophobic substances, such as polycyclic aromatic hydrocarbons (PAHs), is an important environmental problem today. Major sources of PAHs to the aquatic and soil environments include creosote-treated products, spills of petroleum products, metallurgical and coking plants, and deposition of atmospheric PAHs (Government of Canada, 1994). Many PAHs are toxic and carcinogenic, therefore it is important that soils contaminated with these substances be remediated. Many of the remediation techniques presently used are expensive, lengthy, and inefficient. A commonly used *in situ* remediation procedure is bioremediation, which involves the utilization of microorganisms to degrade the contaminants, yielding harmless compounds such as carbon dioxide, microbial biomass, and inorganic forms of nitrogen, phosphorus, and sulfur (Paul and Clark, 1996). The hydrophobic nature of many PAHs make *in situ* bioremediation difficult due to the fact that the contaminants are partitioned onto the soil particles, out of the aqueous phase where microbial degradation occurs. Soils are a major environmental sink for PAHs because of the hydrophobicity factor and the recalcitrance of these compounds (Government of Canada, 1994). For bioremediation techniques to be effective, a way must be found to promote the transfer of the PAHs from the soil-sorbed phase to the aqueous phase. When contaminant availability is reduced due to

partitioning to soil particles, a surfactant may be used to enhance the aqueous solubility of sorbed organic compounds. Chemical surfactants are one answer to the growing demand for new techniques and products which may increase the effectiveness of remediation procedures in order to reduce expenses and shorten the time required to restore a contaminated site back to acceptable conditions. However, there are problems associated with the use of chemical surfactants in bioremediation procedures.

Leonardite is a naturally occurring oxidized form of lignite coal that is rich in humic materials. Leonardite is collected during open-pit coal mining, as it is commonly found overlying coal seams. There has been recent commercial interest in the possibility of using humic extractions of leonardite in environmental remediation procedures in place of synthetic chemical surfactants. Research has shown that water soluble humic and fulvic acids are capable of significantly enhancing the aqueous solubilities of PAHs (Johnson and Amy, 1995) by forming associations with the PAHs. The humic materials act as a surfactant, increasing the water solubility of the PAH and allowing the contaminants to enter the aqueous phase. The use of humic materials on contaminated soils could greatly enhance the bioavailability of PAHs, allowing for a much easier and more efficient bioremediation procedure. Studies have shown an average three-fold increase in PAH concentration in the aqueous phase after the addition of humic acid, and up to a ten-fold increase in solubility for trimethyl naphthalene (Lesage *et al.*, 1996). An advantage of using humic materials extracted from leonardite over a chemical surfactant is that humic materials should not inhibit the activity of the degrader community, as will some chemical surfactants. Toxicity of humic substances to soil microorganisms should not be an issue, as they are a naturally occurring soil component. Synthetic surfactants

have been shown to exhibit aqueous toxicity for soil microorganisms, including those responsible for the metabolism of the contaminants (Kanga *et al.*, 1997). Other reasons for an inhibition to synthetic surfactant-enhanced biodegradation include preferential use of the surfactant as the substrate instead of the contaminant, and a decrease in bacterial adherence to hydrocarbons (Lesage *et al.*, 1996). Humic materials are generally biologically recalcitrant and are therefore not readily metabolized, and would not be expected to act as a preferential substrate (Pyne *et al.*, 1987).

The hypothesis of this thesis is that due to the physical properties of chemically extracted leonardite solutions, these soluble humic material solutions could be utilized to increase the effectiveness of bioremediation procedures on PAH-contaminated soils by binding with the hydrophobic PAHs, resulting in a greater fraction of the PAH in the aqueous phase, consequently making them more accessible to degrading microorganisms.

The objective of the first study was to determine whether the addition of various humic substances to a PAH-water system has any effect on the water solubility of the PAH, and to determine the equilibrium constants for the association of the PAH, naphthalene, with various dissolved humic materials. A fluorescence quenching method was used to determine equilibrium constants for PAHs binding to dissolved humic materials (Gauthier *et al.*, 1986; Kumke *et al.*, 1994).

The second study involved the addition of two ^{14}C-labelled PAHs, anthracene and benzo[a]pyrene, to a previously contaminated soil. A variety of amendments were added to the soils: glucose (C-source only), Tween-80 (a commercial surfactant), Aldrich humic acid (previously reported in the literature to increase PAH solubility), and a number of leonardite-extraction solutions. A microcosm apparatus was used to collect the

radiolabelled carbon molecules which are released as carbon dioxide (CO_2) during the degradation of the PAHs. The amount of ^{14}C-labelled CO_2 released is indicative of the rate and amount of contaminant degradation occurring, which in turn is dependent on factors such as substrate availability. The fate of the PAHs was compared as a function of the differently amended systems to determine if humic material amendments affect PAH water solubility and biodegradation.

Results of the fluorescence quenching experiment were compared to the results of the microcosm degradation study to determine if the partitioning capabilities of these solutions are effective in enhancing the biodegradation of PAHs in soil.

CHAPTER 2

Literature Review

2.1 Polycyclic Aromatic Hydrocarbons

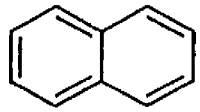
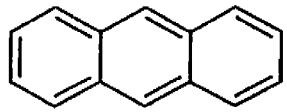
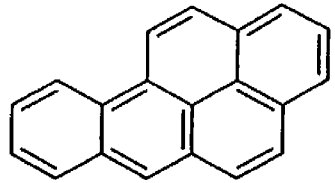
2.1.1 Physical Properties

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemicals comprised of approximately 100 compounds. PAHs are made up of carbon and hydrogen atoms arranged into benzene-like rings of five or six carbon atoms, which are fused together in linear, angular, or cluster arrangements. The physical properties of PAHs are dependent on their structure, mainly the arrangement and number of rings that comprise the compound (Government of Canada, 1994). PAHs are considered to be low molecular weight compounds if they contain less than four rings in their chemical structure. PAHs consisting of four or more rings within their chemical structure are considered to be high molecular weight compounds.

The building block of the PAH structure is the benzene ring. Structurally, benzene is unusually stable (McMurry, 1996), and the influence of this stability is evident when examining the physical properties of various PAHs (Table 2.1). As the number of rings within the chemical structure increases, the stability of the compound increases as well. In general, PAHs have low water solubilities, and high octanol-water partition

coefficients (K_{ow}). These properties play an important role in determining the fate of PAHs in the environment. A high K_{ow} implies the tendency for adsorption to particulates in water and air, and for bioconcentration in organisms (Government of Canada, 1994).

Table 2.1. Physical properties and CCME guidelines for selected PAHs (Government of Canada, 1994; CCME, 1997).

Compound (C.A.S. #)	naphthalene (91-20-3)	anthracene (120-12-7)	benzo[a]pyrene (50-32-8)
Chemical Structure			
Molecular Weight (g/mol)	128.16	178.24	252.32
log K_{ow}	3.5	4.5	6.0
Water Solubility at 25°C (g/L)	3.17×10^{-2}	4.5×10^{-5}	3.8×10^{-6}
Melting Point (°C)	80.5	216	179
Vapour pressure at 25°C (mPa)	11 960	25	0.37×10^{-6}
Recommended Canadian soil quality guidelines (mg/kg)*	0.1 / 0.6 / 22 / 22	none defined	0.1 / 0.7 / 0.7 / 0.7

* Land use: Agricultural / Residential-Parkland / Commercial / Industrial

2.1.2 Sources

In Canada, the occurrence of PAHs is widespread. The presence of PAHs have been recorded in the air, soil, groundwater, fresh and marine surface waters, sediments, and terrestrial and marine biota (Government of Canada, 1994).

Sources of PAHs are both natural and anthropogenic. PAHs are formed by the incomplete combustion of carbonaceous material, such as coal, oil, gas, wood, garbage, tobacco, and even charbroiled meat (U.S. Department of Health and Human Services, 1995). PAHs can also be found in products such as dyes, plastics, pesticides, asphalt, crude oil, coal, coal tar pitch, creosote, and roofing tar. As products of combustion, PAHs are usually produced as complex mixtures of PAHs, not as individual chemicals. Natural sources include volcanoes, forest fires, crude oil, and shale oil (Government of Canada, 1994; U.S. Department of Health and Human Services, 1995).

Deposition from the atmosphere is greatest source of PAHs to the soil (Bjorseth, 1983). PAHs in the atmosphere are usually adsorbed to particulate matter, which can deposit onto the soil by either wet or dry deposition. PAHs adsorbed onto airborn particles are capable of being transported great distances before deposition occurs (Christensen and Zhang, 1993). Table 2.2 lists the atmospheric emissions of PAHs for a given year, 1990, in Canada.

Deposition of PAHs directly into the soil occurs from a variety of sources, including dispersion from creosote-treated material, accidental oil spills, industrial processes, municipal effluents, and burial of wastes containing PAHs. It is estimated that the release of PAHs from creosoted materials into surface waters and soil could be up to 2000 tonnes per year in Canada (Government of Canada, 1994). In Canada, petroleum

hydrocarbon spills release 76 tonnes of PAHs per year, and releases from metal and coking plants result in the deposition of 3.9 tonnes per year into the soil or water environment (Government of Canada, 1994).

Table 2.2 Annual atmospheric emissions of PAHs in Canada during 1990 (Government of Canada, 1994).

Sources	PAH releases	
	tonnes	%
Anthropogenic Sources		
Industrial Processes		
Aluminum plants	925	21
Metallurgical (including ferro-alloy)	19.5	0.4
Coke production	12.8	0.3
Asphalt production	2.5	0.1
Petroleum refineries	0.1	<0.1
Combustion Sources		
Residential Heating		
Wood	474	11.0
Others	29	0.7
Open air fires/agricultural burning	358	8.3
Incineration		
Teepee burners	249	5.8
Municipal (with sludges)	1.3	<0.1
Industrial	1.1	<0.1
Transportation		
Diesel	155	3.6
Gasoline	45	1
Other	1.2	<0.1
Thermal Power Plants	11.3	0.3
Industrial Combustion		
Wood	5.7	<0.1
Other	10.2	0.2
Commercial and Institutional Heating	2.7	0.1
Cigarettes	0.2	<0.1
Natural Sources		
Forest Fires	2010	47
Total	4314	100

2.1.3 Health Effects

The Canadian Environmental Protection Act (Government of Canada, 1994) Priority Substances List Assessment Report on PAHs states, "based on available data, the PAHs benzo[a]pyrene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene and indenol[1,2,3-cd] are entering the environment in a quantity or concentration or under conditions that may constitute a danger to human life or health."

The health effects of exposure to PAHs depends on a number of factors, such as dose, duration of exposure, route of exposure, and individual characteristics (e.g. age and gender). The effects of exposure to PAHs is also dependent on the properties of the specific PAH. Route of exposure for PAHs can include inhalation, oral, and dermal. Very few studies have been conducted on the effects of PAH exposure on humans; however, a large number of experiments have been conducted on laboratory animals. Very rarely is human exposure limited to a single PAH, but is usually a complex mixture of compounds. This makes determining the health effects of individual PAHs difficult (Reeves *et al.*, 2001), as these compounds are known to interact with other chemicals, modifying their mode of action, either additively, antagonistically, or synergistically (U.S. Department of Health and Human Services, 1995).

Most terrestrial invertebrates are not capable of metabolizing PAHs (CCME, 1997). The hydrophobic nature of many PAHs may cause them to be partitioned into fatty tissues in the body, which can lead to bioconcentration of these chemicals to extremely high levels. Bioconcentration factors have been recorded as high as log 6.95 in the amphipod *Pontoporeia hoyi* in Lake Michigan (Landrum *et al.*, 1985).

Bioconcentration factor is the ratio of the chemical concentration in the organism to that in the surrounding water.

One of the most dangerous PAHs to animals, including humans, is benzo[a]pyrene. Benzo[a]pyrene uptake can occur by all routes of exposure: inhalation, oral, and dermal. In laboratory animals, benzo[a]pyrene has demonstrated various carcinogenic properties, regardless of route of exposure (U.S. Department of Health and Human Services, 1995). Absorbed benzo[a]pyrene is distributed rapidly throughout the body through systemic circulation (CCME, 1997). The carcinogenic mechanism of action of benzo[a]pyrene is thought to result from its metabolism to reactive diol-epoxides, which conjugate with glutathione, sulphates and mercapturic or glucuronic acids, followed by elimination from the body. The diol-epoxides are able to interact with DNA, causing mutations, which lead to the carcinogenic activity of benzo[a]pyrene (Figure 2.1)(U.S. Department of Health and Human Services, 1995). Results from reproductive and developmental studies in rodents have shown that *in utero* exposure to benzo[a]pyrene can lead to developmental toxicity, resulting in adverse reproductive effects (U.S. Department of Health and Human Services, 1995). Benzo[a]pyrene crosses the placenta and is readily distributed to the developing foetus (CCME, 1997). Benzo[a]pyrene exposure has also induced immune suppression in mice and their offspring. Acute exposure in mice has also led to decreased longevity and lethality (U.S. Department of Health and Human Services, 1995).

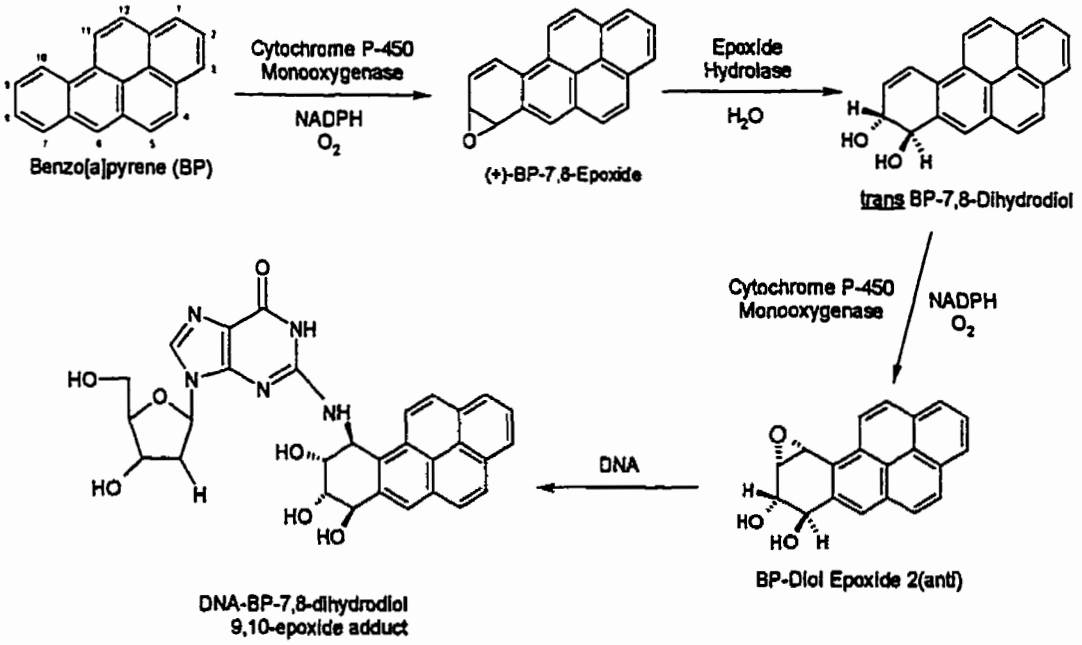


Figure 2.1 The metabolic activation of benzo[a]pyrene in mammalian systems (Pothuluri and Cerniglia, 1994).

2.1.4 Fate of PAHs in Soils

In soils, the most important processes for most high molecular weight PAHs are adsorption and biodegradation (CCME, 1997). Adsorption to soil particles by PAHs is favoured due to their low vapour pressure, low solubility, and tendency to bind to organic matter (U.S. Department of Health and Human Services, 1990).

2.1.4.1 Adsorption

There are a number of factors that influence the extent of adsorption of PAHs to soils, including soil type, soil moisture, temperature, presence of competing molecules and solvents, and pH (CCME, 1997). However, the organic matter content of the soil is the

most important property affecting the adsorption of PAHs to soil (Sims and Overcash, 1983).

A study by Murphy *et al.* (1990) determined that hydrophobic substances are considered to be bound in soils mainly by partitioning to soil organic matter, clay minerals playing only a minor role in systems with low organic matter content. These results are supported by Onken and Traina (1997) who found that the sorption to humic acid-mineral complexes by the PAHs, pyrene and anthracene, was directly related to the fraction of organic carbon in the sorbing complexes. However, Stevenson (1994) states that the individual adsorption effects of organic matter versus clay is difficult to separate, as organic matter and clay are often closely bound. Adsorption of hydrophobic PAHs by soil can be considered to primarily be a matter of partitioning between organic matter and water.

Weissenfels *et al.* (1992) performed a study to determine the soil characteristics that prevent PAH biodegradation and to investigate the correlation of biodegradability with the sorption of PAHs. They determined that inhibition of PAH biodegradation is due to a kind of PAH binding to organic matter within the soil. In one part of their experiment, they found that within seven days of incubation, sand-sorbed PAHs were degraded by the bacteria to below measurable levels. The degradation of soil-sorbed PAHs was significantly slower, and resulted in a PAH fraction of about 23% of the amount initially added, that was not available for biodegradation (Figure 2.2). In addition to the biodegradation data, they found that there were two kinetically distinct processes associated with PAH binding onto the soil material. An initial rapid sorption process was followed by a second sorption process that occurred at an increasingly slower rate over a

long period of time. Sixty percent of the initially applied anthracene oil had become non-extractable within the first few hours of exposure, followed by a slower sorption to approximately 35% non-extractable after 28 days (Figure 2.3). These researchers hypothesized that the initial fast adsorption process is caused by a rapid adsorption of the hydrophobic pollutants onto hydrophobic areas of soil surfaces, whereas the slow adsorption process which occurs later, is due to partitioning into less accessible sites within the soil matrix. Consistent with this decrease in bioavailability, the toxicity of soil washings decreased significantly with increasing amounts of soil organic carbon, determined by measuring the inhibition of bioluminescence of *Photobacterium phophoreum*. They concluded from their findings that the degree of PAH biodegradation in different soils may differ significantly even under the same optimum growth conditions concerning temperature, nutrients, oxygen supply, and occurrence of PAH-degrading bacteria.

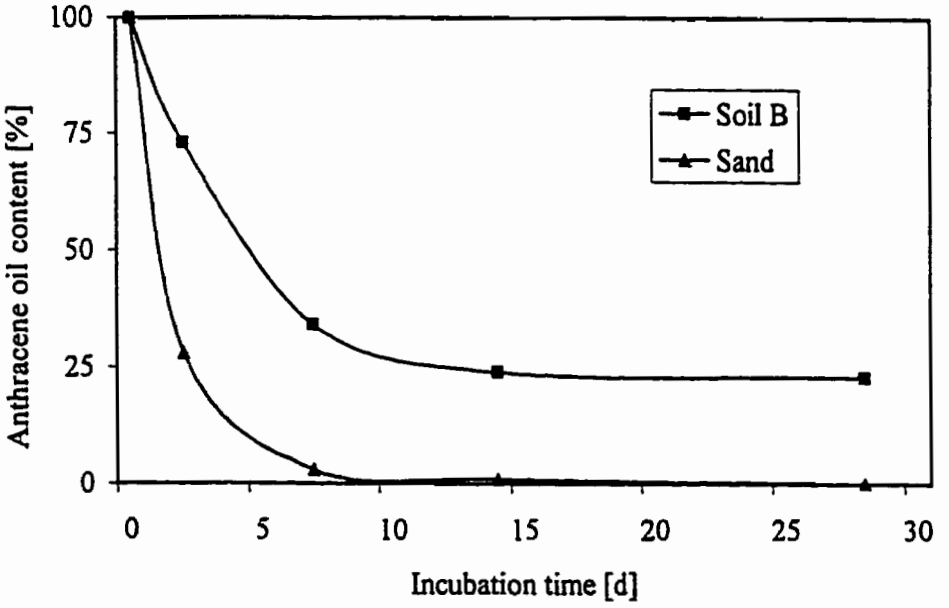


Figure 2.2 Microbial degradation of anthracene oil initially sorbed onto sand (1.0% w/w organic carbon), and soil (13.6% w/w organic carbon) (Weissenfels *et al.*, 1992).

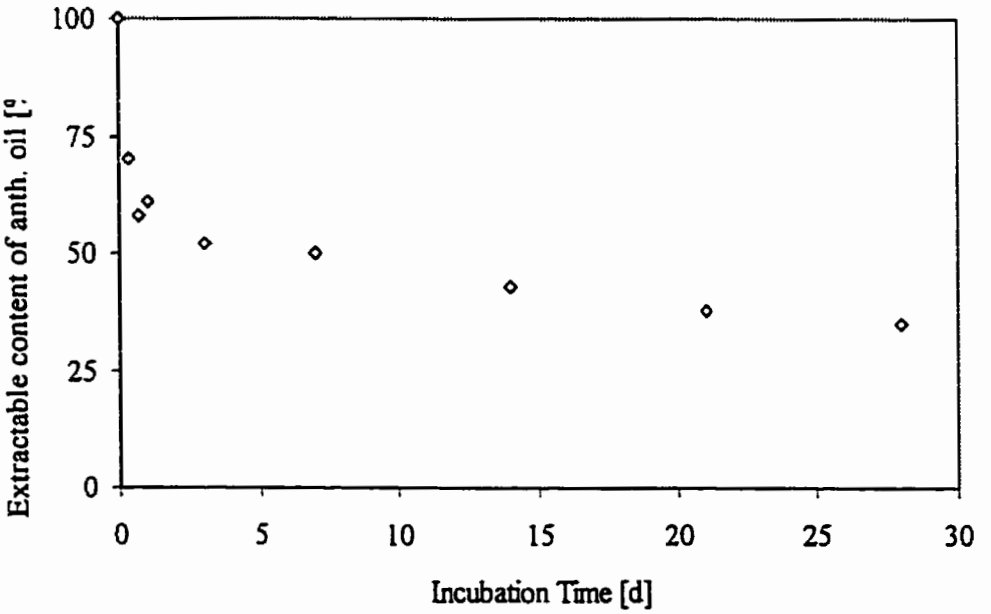


Figure 2.3 Influence of sorption processes on the recovery of anthracene oil added to soil (13.6% w/w organic carbon) (Weissenfels *et al.*, 1992).

Karimi-Lotfabad *et al.* (1996) examined the effect of soil moisture on the interaction of anthracene and soil particles. They found that for a soil with sandy clay loam texture, for their experimental parameters, a soil moisture content below 1% resulted in an enhanced adsorption as measured by a loss of extractable anthracene after four days of loading, as illustrated in Figure 2.4. This suggests that water present in the soil may interfere with the binding of PAHs onto soil particles.

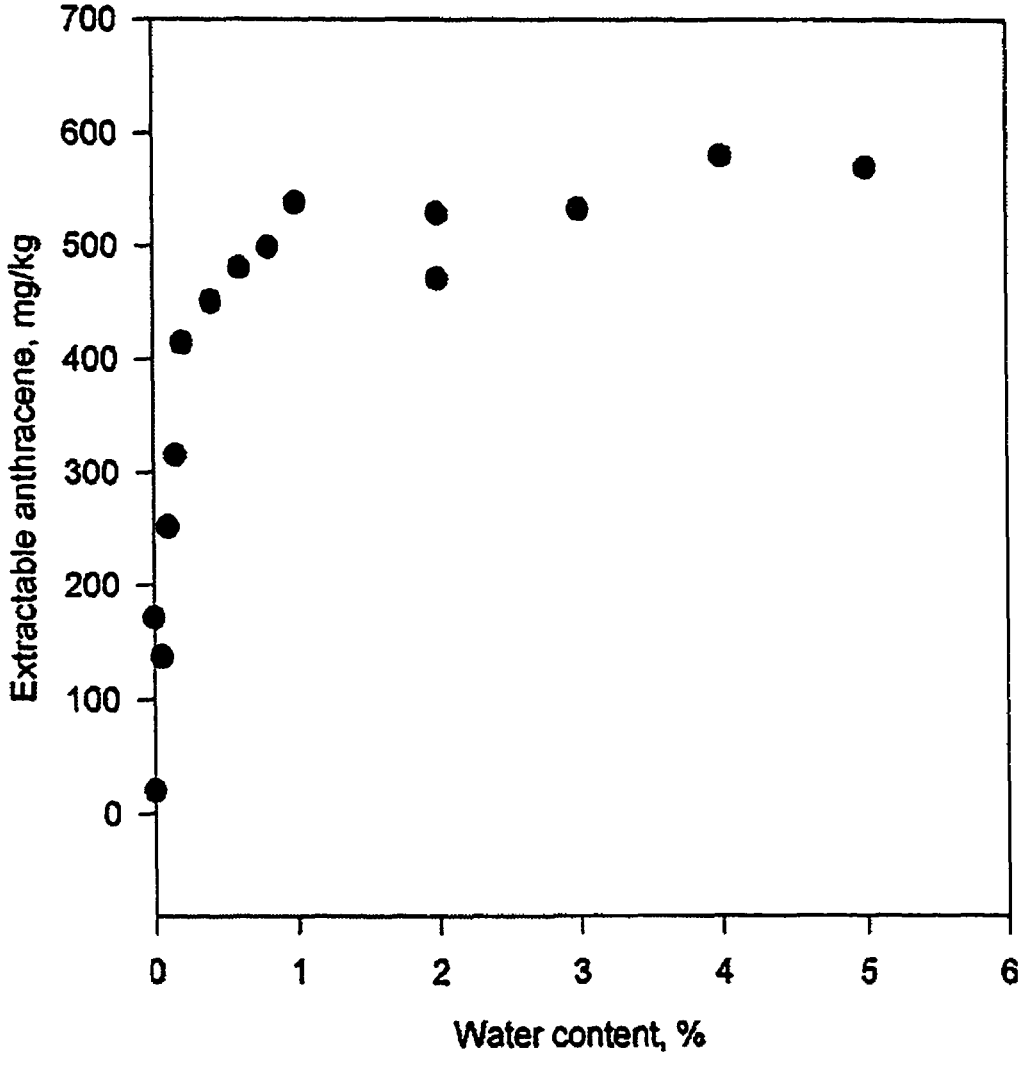


Figure 2.4 Recovery of anthracene as a function of water content (Karimi-Lotfabad *et al.*, 1996).

2.1.4.2 Biodegradation

Most PAHs are highly hydrophobic and tend to partition to a greater extent to soil particles rather than remain in aqueous phase (Lee *et al.*, 1978). Although it has been proven that bound contaminants are often less toxic to the surrounding environment (Bollag, 1992), often the ultimate goal in soil remediation is the complete removal of the harmful compound from the soil. There are many microorganisms capable of degrading various PAHs (Grosser *et al.*, 1991; Morgan *et al.*, 1993; Kastner *et al.*, 1994; Ye *et al.*, 1996), but availability of the hydrophobic contaminant to the soil microorganisms is the key factor for the recalcitrance of many PAHs (CCME, 1997). Contaminants that are bound to soil particles are often less available for degradation (Knaebel *et al.*, 1996) and transport (Liu and Amy, 1993). Studies have demonstrated that most bacteria can use PAHs as a source of carbon and energy from the dissolved state only, and that the rate of biodegradation of PAHs mainly depends on their dissolution rate (Bryniok, 1993).

Environmental factors, microbial flora, and physicochemical properties of the PAH influence degradation rates and the extent of degradation of PAHs in soil systems (Sims and Overcash, 1983). Environmental factors such as temperature, moisture level, aeration, pH, nitrogen, and metabolizable carbon not only influence degradation by affecting the growth conditions of the soil microorganisms (Paul and Clark, 1996), but also by influencing the extent of adsorption between the PAH and soil organic matter (Murphy *et al.*, 1990; Rutherford and Chiou, 1992; Karanfil *et al.*, 1996; Onken and Traina, 1997; Ko *et al.*, 1998). Microbial factors include acclimatization status and

populations present as well as relative proportions of bacteria, fungi, and actinomycetes (Sims and Overcash, 1983). Physicochemical properties of the organic compound of interest that are important in influencing degradation rates include structure, size, concentration, and lipophilicity (Sims and Overcash, 1983).

An obstacle pertaining to the improvement of PAH degradation processes is the uncertainty of the mineralization pathways for many of the higher molecular weight PAHs (Bryniok, 1993). Figure 2.5 and Figure 2.6 illustrate examples of the mineralization pathways for naphthalene and anthracene, respectively. These pathways have been thoroughly studied and are well understood in pure culture studies (Pothuluri and Cerniglia, 1994). However, the bacterial mineralization pathways for the larger, more complex PAHs are extremely complicated, and have yet to be elucidated. More commonly for larger PAHs, such as benzo[a]pyrene, transformation and breakdown are performed by fungi (Pothuluri and Cerniglia, 1994). Figure 2.7 illustrates some of the known fungal transformations of benzo[a]pyrene.

Many high molecular weight PAHs are degraded by cometabolism. Cometabolism is the transformation of the chemical structure of a compound by a microorganism that does not utilize the substrate as a source of energy for growth (Alexander, 1999). Cometabolism of compounds occurs indirectly, as the energy acquired from the breakdown of the cometabolised compound is inadequate to fully sustain growth of the degrading population. As well, no molecular constituents from the cometabolised compound, such as carbon, nitrogen, sulfur, or phosphorus are used by the microorganisms for biosynthetic purposes (Alexander, 1999). Cometabolism occurs

when microorganisms incidentally degrade a compound while in the process of utilizing another compound as substrate (King *et al.*, 1998).

Typically following a contamination event, contaminant concentrations will initially decline rapidly, as the most bioavailable PAH substrates are utilized. Degradation levels will decline over time, as the recalcitrant fraction of the contaminant is left behind (Shuttleworth and Cerniglia, 1995). This fraction is too strongly adsorbed to the soil particles and is inaccessible to the soil microorganisms (Huesemann, 1997).

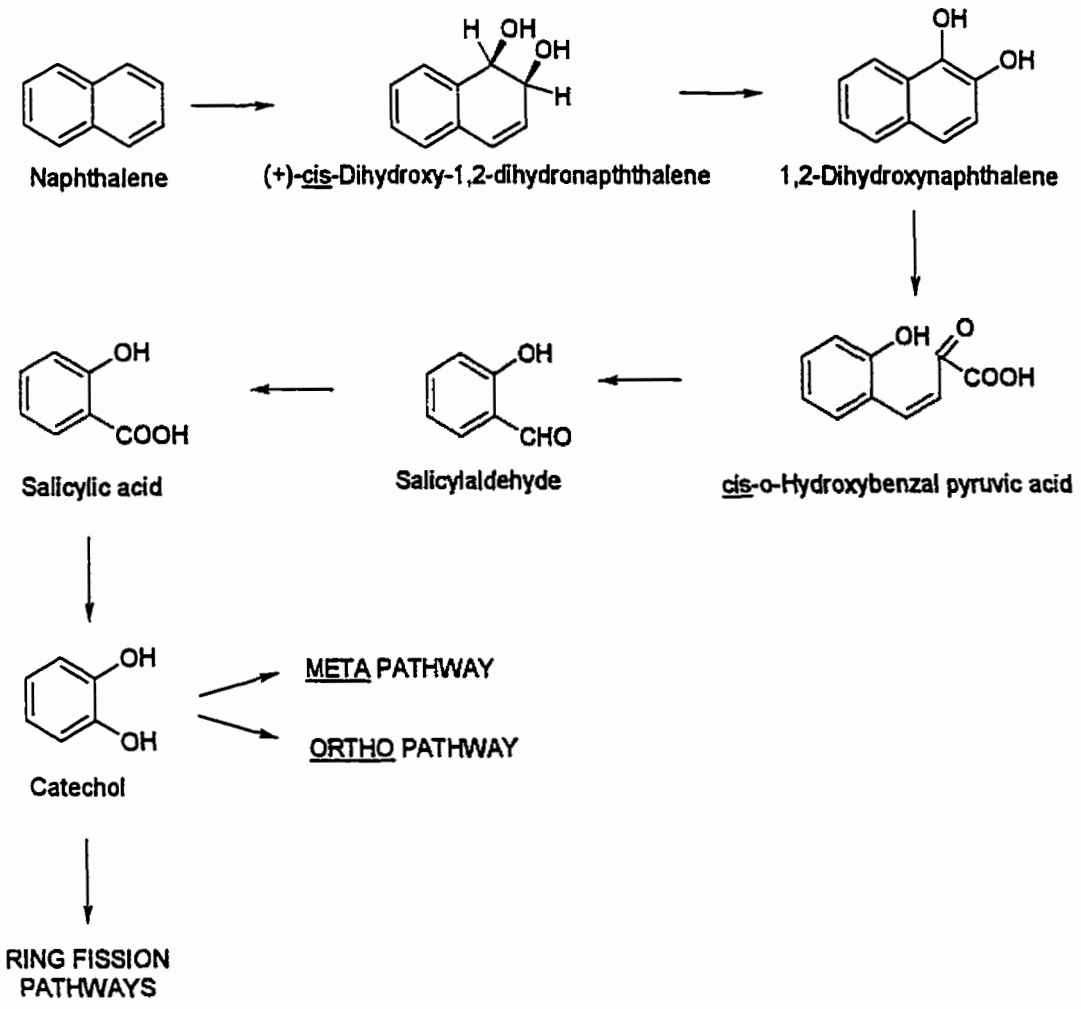


Figure 2.5 Bacterial degradation of naphthalene (Pothuluri and Cerniglia, 1994).

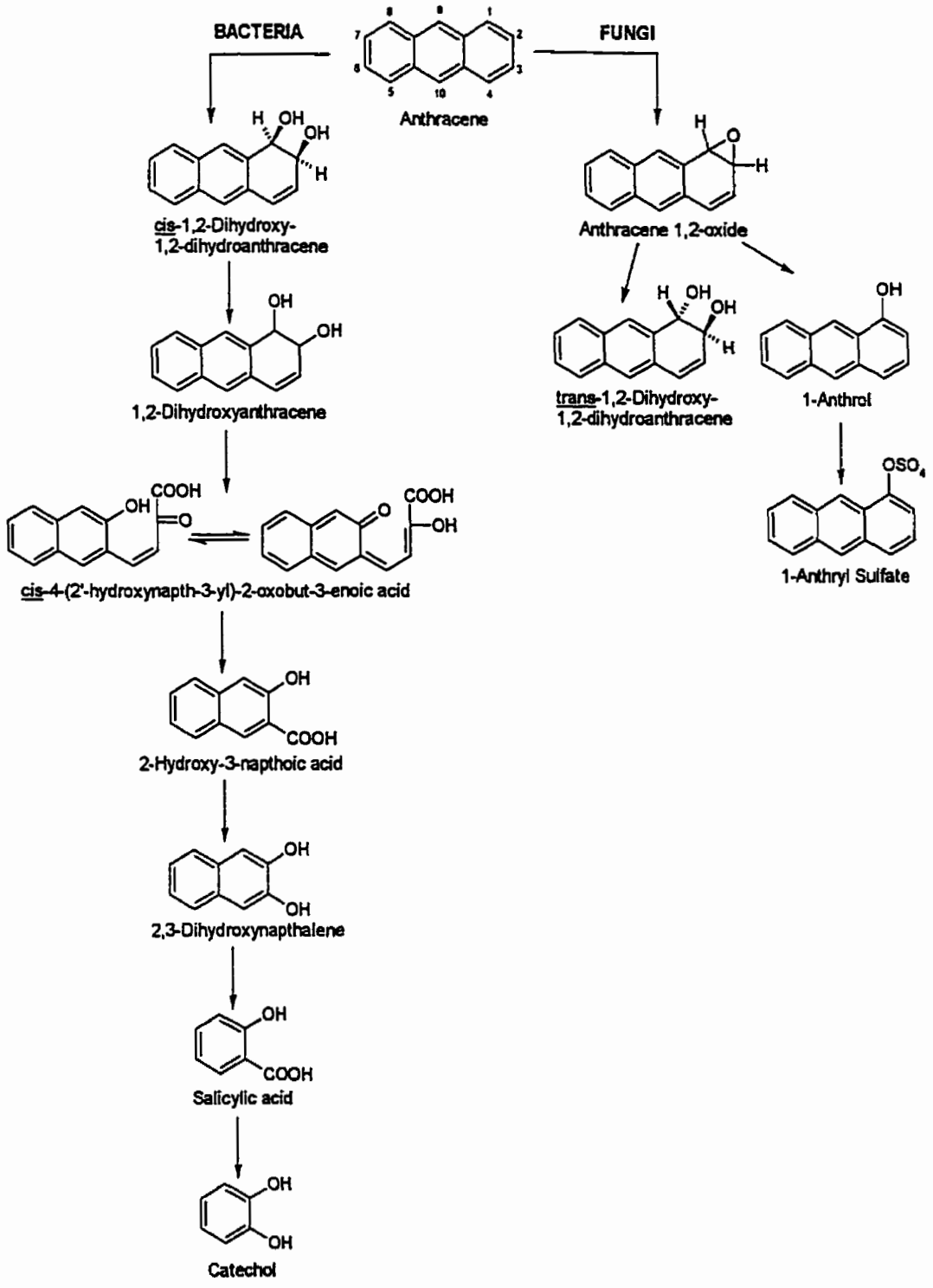


Figure 2.6 Metabolic fate of anthracene (Pothuluri and Cerniglia, 1994).

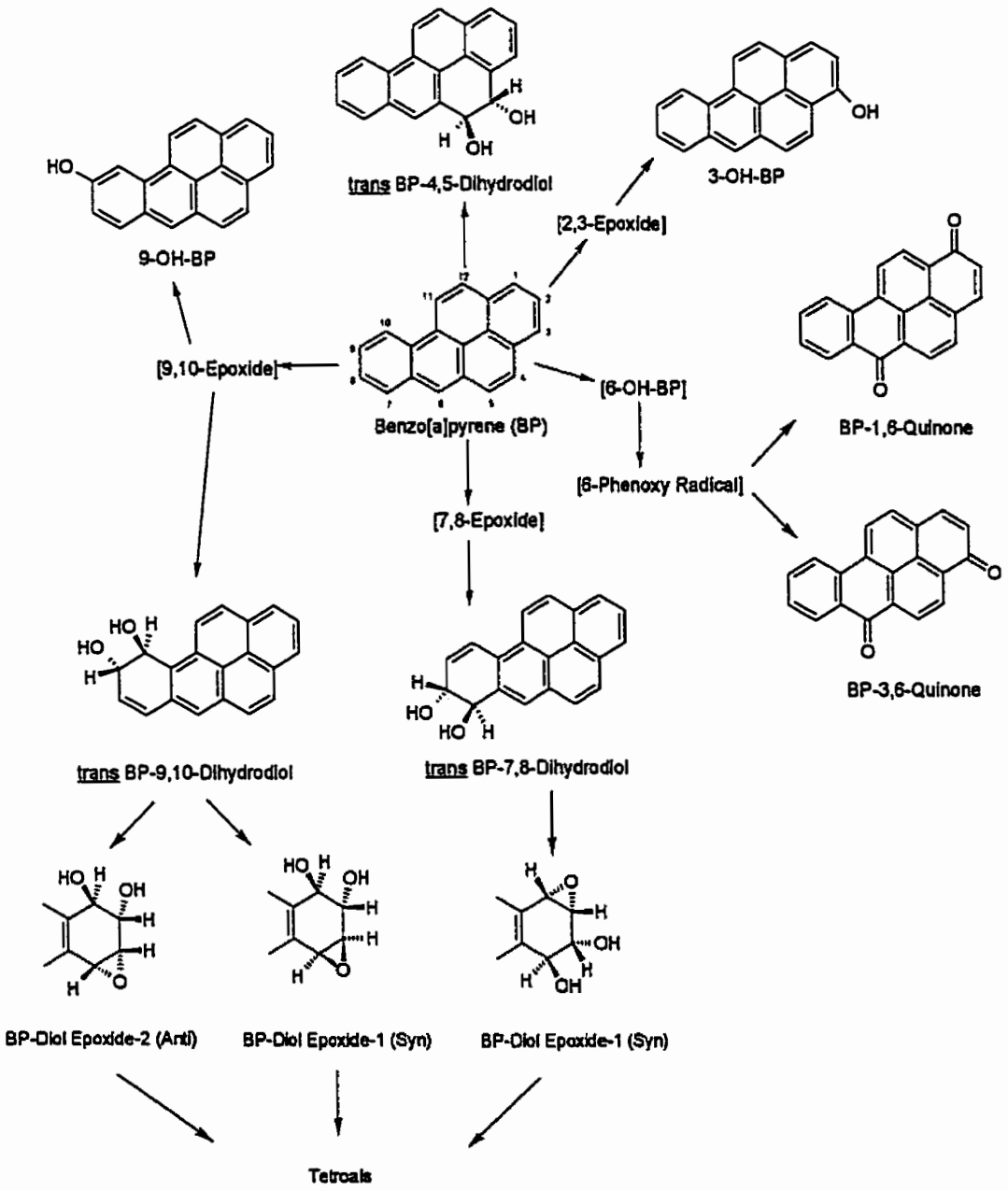


Figure 2.7 Fungal degradation of benzo[a]pyrene (Pothuluri and Cerniglia, 1994).

2.2 Contaminant Solubility / Bioavailability in Soil Remediation

Bioremediation is a process in which microorganisms or microbial processes are used to detoxify and degrade environmental contaminants (Baker and Herson, 1994). The use of microorganisms to remediate a contaminated site can provide an economical method to reduce contaminant toxicity. Bollag and Bollag (1995) state that bioremediation has several advantages over other conventional remediation procedures. As it requires very little equipment and labour to undertake a bioremediation procedure, it is more economical than other methods, and has reduced risk to cleanup personnel and risk of off-site contamination. Because the contaminants can often be completely degraded without requiring the addition of harmful chemicals, the process has minimum environmental impact, and often generates no waste products.

However, a common constraint to efficient bioremediation of PAH-contaminated sites is the low aqueous solubility of many high molecular weight PAHs. Limited contaminant solubility results in reduced degradation rates and lower total amounts of contaminant degraded. When contaminant availability is reduced due to partitioning to soil particles, a surfactant may be used to enhance the aqueous solubility of sorbed organic compounds.

2.2.1 General Information on Chemical Surfactants

The unique feature of surfactant molecules lies in their amphipathic nature; they contain both polar (hydrophilic) and nonpolar (hydrophobic) components. The

hydrophobic component is most commonly a large hydrocarbon chain. The chemical structure of the hydrophilic component is used to classify surfactants into four general groups. The four groups are: 1) anionic, in which the surface active part of the molecule carries a negative charge; 2) nonionic, in which the surface active part of the molecule does not have a charged group; 3) cationic, in which the surface active part of the molecule carries a positive charge; and 4) zwitterionic, in which the surface active part of the molecule can carry a positive or a negative charge or both depending upon the conditions (West and Harwell, 1992). The hydrophobic tail is repelled by water, and the hydrophilic head is easily hydratable and can interact with the structured water molecules. In aqueous solutions surfactant molecules distribute in a way that their concentration at the interfaces of water with gases or solids is higher than in the inner regions of the solution. As a result, there is a lowering of the interfacial tension between water and an adjacent nonaqueous phase, and in a change of wetting properties (Schwarzenbach *et al.*, 1993). Kanga *et al.* (1997) found that the chemical surfactant, Tween-80 (polyoxyethylene sorbitan monooleate), was able to decrease the surface tension of water from 72 to approximately 30 dyne/cm. At low concentration, surfactants are present as individual molecules. However, as the concentration of the surfactant is increased, a concentration is reached where the individual surfactant molecules form aggregates called micelles. The amount of surfactant needed to reach this concentration is called the critical micelle concentration. In a micelle, the hydrophobic ends of the surfactant molecules are clustered in the center of the micelle, and the hydrophilic ends are on the outside toward the water phase. At surfactant concentrations just above the critical micelle concentration, the shape of the micelle is most commonly that of a sphere

(Porter, 1991). Hydrophobic chemicals would be incorporated ito the hydrophobic centre of the micelle and would no longer be bound to the soil matrix (Alexander, 1999). Adsorption at the interfaces and micellar aggregation are the processes by which surfactants may keep otherwise insoluble compounds in the aqueous phase (Schwarzenbach *et al.*, 1993).

2.2.2 Use of Chemical Surfactants In Soil Remediation

The use of chemical surfactants has been proven effective in increasing the amount of hydrophobic PAHs in the aqueous phase during soil remediation procedures (Aronstein *et al.*, 1991; Madsen and Kristensen, 1997; Guha *et al.*, 1998b). When using remediation techniques such as pumping and treatment of groundwater, the use of chemical surfactants is usually acceptable. However, many chemical surfactants can be toxic to both aquatic and terrestrial organisms (Lewis, 1991). For example, Xu *et al.* (1994) state that alkylethoxylates are toxic in the mg/L range to a variety of aquatic organisms. Kanga *et al.* (1997) found that concentrations in the mg/L range of Tween-80, a commonly used chemical surfactant, have detrimental effects on microbial populations due to toxicity. In locations where the environmental impact of a chemical surfactant is unacceptable, other methods of remediating the soil must be investigated.

Another problem arising when chemical surfactants are used in pump and treat remediation procedures is that those surfactants which are biodegradable may cause excessive bacterial growth and clogging in the aquifer (Xu *et al.*, 1994).

The toxicity of chemical surfactants is also of great concern when attempting to utilize bioremediation methods to remediate a contaminated soil. In bioremediation, the

health of the soil microorganisms is of utmost importance, as the soil microorganisms are required to be sufficiently active and present in suitable numbers to degrade the contaminant.

When attempting to bioremediate a site using chemical surfactants, the biodegradability of the surfactant may become an issue. If a surfactant is readily biodegradable by the soil microorganisms, it may become a preferential source of carbon, resulting in decreased degradation rates of the target contaminant. Another effect of the degradation of the surfactant was noted by Tiehm *et al.* (1997), when they found that the rapid degradation of the surfactant, Arkopal N-300, resulted in a lack of oxygen and an inhibition of PAH degradation.

When attempting to improve biodegradation rates using surfactants, another concern is that as PAHs are partitioned by the surfactant, they may remain unavailable to microbial breakdown. However, research to date has been unable to determine whether the use of surfactants causes inhibition or enhancement of biodegradation. Work done by Laha and Luthy (1991) suggests that the bioavailability of PAHs may be decreased upon micellization by nonionic surfactants. This was determined by reduced contaminant degradation, without any signs of toxic effects from the surfactant. Guha *et al.* (1998a) found that above the critical micelle concentration the bioavailability of the micellar-phase PAHs was inversely related to the concentration of the nonionic surfactant, Triton X-100. To counter this data, Zheng and Obbard (2001) found that the use of Tween-80 increased degradation of PAHs containing four to six rings in recently contaminated soils by *Phanerochaete chrysosporium*. Finally, Ye *et al.* (1996) found that the addition of the

surfactants Tween-80 or cyclodextrin to the incubation medium had no effect on the biodegradation of benzo[a]pyrene.

2.2.3 Methods Used To Determine Binding Coefficients of Organic Contaminants to Dissolved Humic Substances

It has become increasingly apparent that dissolved organic matter plays an important role in the fate of hydrophobic organic contaminants, such as PAHs, in the soil. There have been a wide variety of methods developed to measure the binding of these contaminants to dissolved humic substances, including the dialysis method (Carter and Suffet, 1982; McCarthy and Jimenez, 1985), ultrafiltration (Means and Wijayaratne, 1984), reversed phase HPLC (Landrum *et al.*, 1984; Nielsen *et al.*, 1997a; Nielsen *et al.*, 1997b), solubility enhancement (Chiou *et al.*, 1986; Chiou *et al.*, 1987; Kile and Chiou, 1989; Chin *et al.*, 1997), complexation-flocculation (Laor and Rebhun, 1997; Rebhun *et al.*, 1998), and fluorescence quenching (Gauthier *et al.*, 1986; Backhus and Gschwend, 1990; Kumke *et al.*, 1994). The binding values obtained by different techniques are sometimes significantly different from each other, so care must be taken when comparing results obtained by different methods. No single technique has been selected as the best to use, as each technique has its own advantages and disadvantages depending on the parameters of the experiment.

The fluorescence quenching method has become a very popular technique. This technique can only be applied to measure association constant values (K_b) for compounds with high fluorescence efficiencies, such as PAHs. This method is based upon the observation that PAHs fluoresce in aqueous solution, but not when associated with

dissolved organic matter, such as dissolved humic and fulvic acids (Kumke *et al.*, 1994). As a consequence, the fraction of PAH associated with the dissolved organic matter may be determined directly from the fractional decrease in fluorescence upon addition of humic or fulvic substances. Furthermore, this association with water soluble humic materials infers an increase in the amount of PAH in the aqueous phase, as it is no longer bound to solid soil particles. K_b represents the ratio of the total equilibrium concentration of the PAH bound to the humic material to the concentration unbound in solution.

In the fluorescence quenching method it is not necessary to know the initial concentration of pollutant, due to the fact that the data is reported as a fraction of unquenched fluorescence. This is especially useful since most hydrophobic organic pollutants are only very slightly soluble in aqueous solution, making it difficult to prepare solutions in which the hydrophobic organic pollutant concentration is accurately known. The fluorescence quenching method is unique from other methods used to determine association constants, as it allows for constants to be determined directly, without separation of fluorophore and quencher (Puchalski *et al.*, 1992). Eliminating the separation step avoids possible errors due to incomplete separation of free from bound pollutant, which may lead to a number of difficulties and uncertainties in measurements.

2.3 Humic Substances

2.3.1 General Information on Humic Substances

Soil organic matter consists of a mixture of plant and animal products in various stages of decomposition together with substances synthesized biologically and chemically from the breakdown products, as well as microorganisms and small animals and their decomposing remains (Choudhry, 1984). Organic matter is usually divided into two chemical groups: nonhumic substances and humic substances.

Nonhumic substances include a large number of relatively simple, discrete compounds belonging to groups such as polysaccharides and sugars, proteins and amino acids, fats, simple organic acids, and other low-molecular-weight organic substances (MacCarthy *et al.*, 1990). In general, these compounds have a relatively short turnover time in the soil, as they are relatively easily metabolized by microorganisms (Choudhry, 1984). The bulk of the organic matter in most soils and waters consists of humic substances. Humic substances are defined by MacCarthy *et al.* (1990) as being "a category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in colour, of high molecular weight, and refractory...which result from the decay of plant and animal residues, and cannot be classified into any of the discrete categories of compounds such as proteins, polysaccharides, and polynucleotides." Molecular weights of humic substances range from several hundreds to tens of thousands (Choudhry, 1984).

Due to the extremely complex and heterogeneous nature of humic substances, researchers have been unable to separate humic substances into discrete components. Instead, definitions are used that reflect the methods of isolating the various fractions from soil. Based on their solubility in water as a function of pH, three major fractions of humic substances are defined: 1) humic acid, which is not soluble in water under acidic conditions but is soluble at higher pH values; 2) fulvic acid, which is soluble in water under both acidic and basic conditions; and 3) humin, which is not soluble in water at any pH value (Sposito, 1989). An illustration of the scheme for this extraction process is given in Figure 2.8.

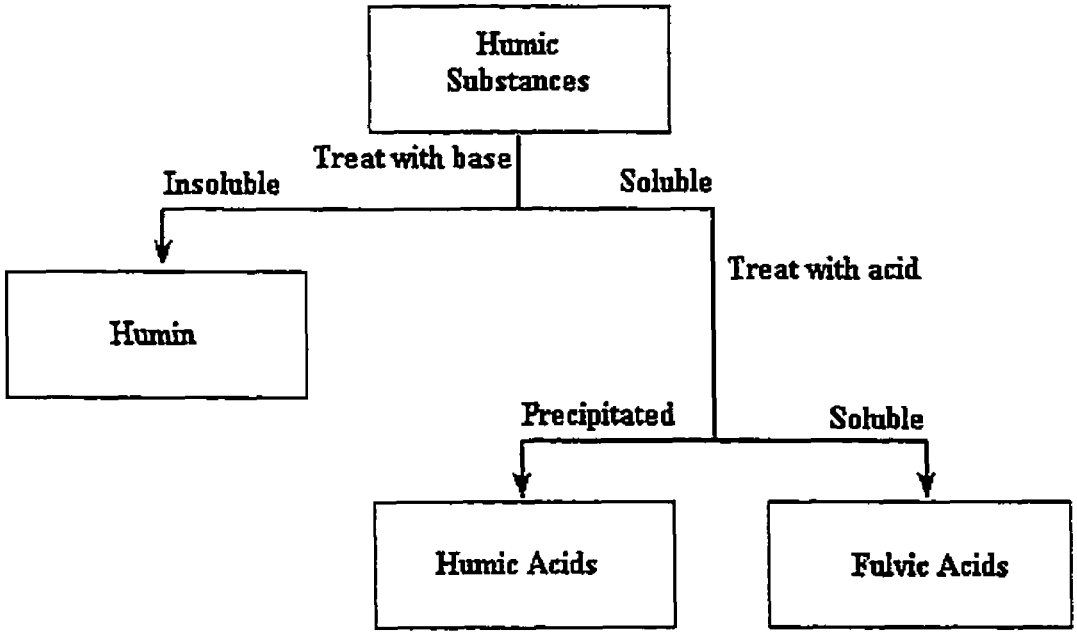


Figure 2.8 Fractionation scheme for humic substances.

Difficulty lies in producing a model for humic substances, mainly due to the fact that they are formed in soils, sediments, and fresh and marine waters, from a variety of

precursors, formation pathways, and environmental influences (Ertel *et al.*, 1988). Stevenson (1994) explains that the structures of humic and fulvic acids are highly variable, dependent on the source. Despite difficulties in the study of humic substances due to their inherent heterogeneity, much information is known about their composition. Elemental analysis and functional group determinations are among the most common methods for characterizing humic substances.

The elements carbon (C), hydrogen (H), nitrogen (N), sulphur (S), phosphorus (P), and oxygen (O) generally account for the entire composition of humic substances on an ash free basis; C and O being the predominant elements (MacCarthy *et al.*, 1990). Table 2.2 gives the average range for the elemental composition of humic substances, as determined by Steelink (1985). Generally, humic acids contain more C and less O than fulvic acids (Choudhry, 1984).

Table 2.3 Usual range for the elemental composition of humic substances (Stevenson, 1994).

Element	Humic Acids (%)	Fulvic Acids (%)
Carbon	53.8-58.7	40.7-50.6
Oxygen	32.8-38.3	39.7-49.8
Hydrogen	3.2-6.2	3.8-7.0
Nitrogen	0.8-4.3	0.9-3.3
Sulfur	0.1-1.5	0.1-3.6

The major functional groups in humic substances are carboxyl, alcohol, phenolic hydroxyl, and carbonyl (MacCarthy *et al.*, 1990). The relative amounts of functional groups are highly variable, depending on the source of the humic substance and the method of functional group analysis (Stevenson, 1994).

There have been a number of theories on the general structure of humic acids. The most recent structural models are based on the premise that humic acids in solution are made up of polymeric macromolecules which possess two kinds of hydrophobic sites; stronger, pH-dependent interior sites, and weaker, pH-independent surface sites.

This theory, advanced by a number of researchers (Engebretson and Wandruszka, 1994; Kumke *et al.*, 1994; Ferreira *et al.*, 2001), suggests that the humic acids have a coiled, flexible macromolecular structure which forms water-protected hydrophobic sites, or pseudomicelles, in the interior of the chemical structure at low pHs. These hydrophobic sites have a very strong affinity for hydrophobic nonpolar compounds, such as PAHs. The structure of the humic acid commonly includes carboxyl and phenolic hydroxyl groups, which become negatively charged at higher pH values. The mutual repulsion of the negatively charged sites causes the humic acid to undergo a conformational change. The humic acid assumes a more linear structure, resulting in a reduction in the number of hydrophobic microenvironments. With fewer pseudomicelles, the humic acid should be less capable of binding to the PAH in solution, resulting in lower K_b values. The hydrophobic sites situated on the surface of the humic acid structure are where hydrophobic moieties such as the poly(methylene) groups are exposed to water but keep their capacity to bind nonpolar chemical compounds. These less protected hydrophobic sites have lower affinity than those interior sites existing at low pH, but still provide some hydrophobic binding capacity at pH values above 5 (Ferreira *et al.*, 2001). Other researchers have proposed that the large molecular sizes may actually be misinterpreted as associations of smaller molecules weakly held together

by hydrophobic forces (Conte and Piccolo, 1999). Figure 2.9 illustrates an example of one of these supramolecular associations.

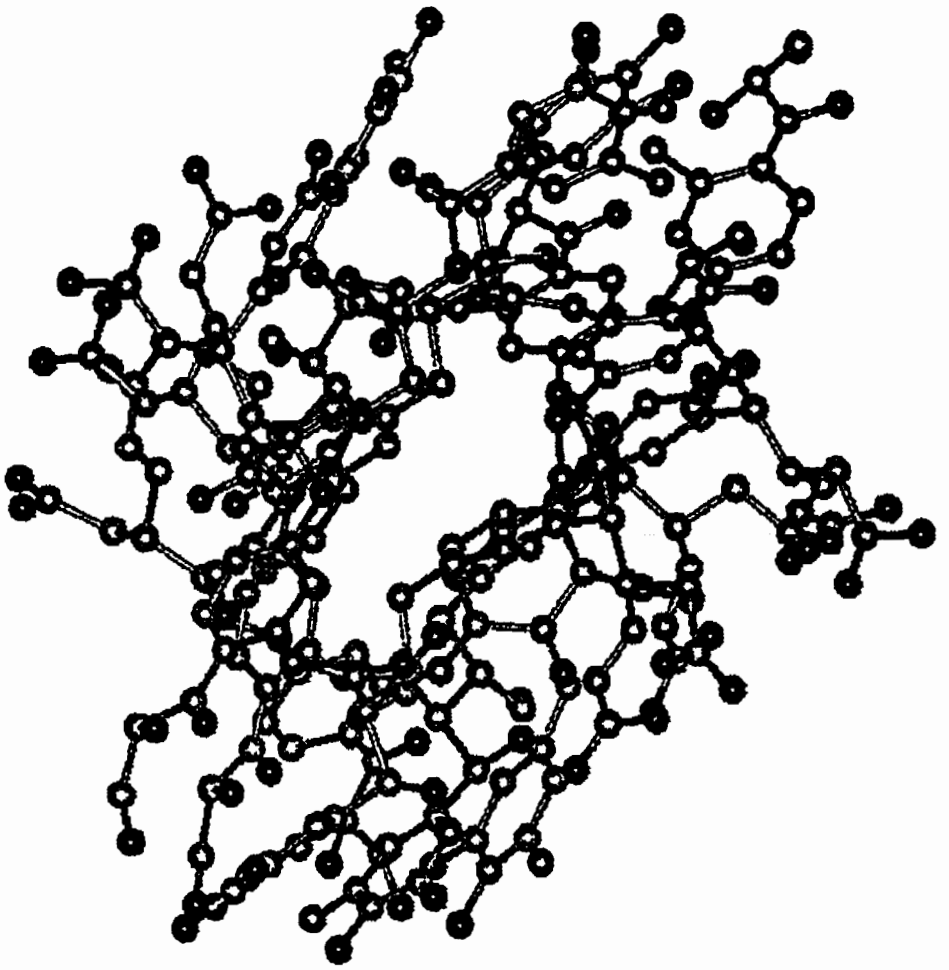


Figure 2.9 A hexamer of amide-linked humic acid building blocks composed of carbon, nitrogen, and oxygen atoms. Hydrogen atoms are not shown for clarity (Sein *et al.*, 1999).

Since the molecular conformation of the humic substances is dependent on parameters such as pH, ionic strength, temperature, concentration of humic substance, and other factors, the occurrence of hydrophobic bonding with humic substances is also

dependent on these properties (Schlautman and Morgan, 1993; Engebretson *et al.*, 1996; Ragle *et al.*, 1997).

Guetzloff and Rice (1994) found that humic acids form micelles only at extremely high concentrations (7.4 g humic acid / L). However, Morra *et al.* (1990) found that despite using humic acid solutions well below the critical micelle concentration, data showed that a close physical association between the fluorophore and quencher occurred, not unlike a partitioning of the former into a hydrophobic micelle interior.

Regardless of what model for humic substances is proposed, a consensus exists on the existence of hydrophobic regions that would be very good sorption sites for nonpolar organic contaminants, such as PAHs (Ferreira *et al.*, 2001). The binding mechanism for neutral PAH molecules to humic substances is referred to as hydrophobic bonding (Gauthier *et al.*, 1987).

2.3.2 Use of Dissolved Humic Substances In Soil Remediation

Research has shown that the presence of water soluble humic materials in soil can significantly enhance the aqueous solubilities of PAHs (Chiou *et al.*, 1987; Johnson and Amy, 1995). Research shows that humic and fulvic acids are both capable of binding with PAHs in aqueous solution; however, humic acids are more effective than fulvic acids, most likely due to differences in their chemical structures (Chiou *et al.*, 1986; Gauthier *et al.*, 1986). Humic acids have surface activity similar to that of chemical surfactants used in the remediation of soil and groundwater (Xu *et al.*, 1994). Humic materials are capable of acting as surfactants, increasing the water solubility of PAHs and allowing the contaminants to enter the aqueous phase. A study using model aquifers has

shown an average three-fold increase in PAH concentration in the aqueous phase after the addition of humic acid, and up to a ten-fold increase in solubility for trimethyl naphthalene (Lesage *et al.*, 1996). The ability of humic materials to bind hydrophobic contaminants is dependent upon the affinities of the humic material with the contaminant and of the contaminant with the soil particles (Johnson and Amy, 1995).

New interest is arising regarding the usefulness of dissolved humic materials in increasing biodegradation rates of hydrophobic PAHs. Dissolved humic materials may have an advantage over chemical surfactants in bioremediation, as they are natural substances found commonly in soil and groundwater, and should pose no threat of toxicity to the soil microorganisms. Strong evidence exists on the capability of humic substances to bind PAHs, increasing the desorption of PAHs from the soil particles where they are biologically unavailable. Presently, there has been little research regarding the effects of humic material partitioning on the bioavailability of PAHs. It must be determined whether the PAHs that have been adsorbed by the soluble humic material are in fact available for degradation by the microbial community. The use of humic materials on contaminated soils could greatly enhance the bioavailability of PAHs, allowing for a much easier and more efficient bioremediation procedure.

Researchers from the National Water Research Institute, Environment Canada performed a study to determine the effect of Aldrich humic acid on the microbial degradation of PAHs (Lesage *et al.*, 1997). Their results indicate that although the presence of the humic acid in the water increased the concentration of PAHs in the aqueous phase, the degradation rate was not increased. They suggest that the humic acid-PAH complex formed is quite stable, and must be broken before degradation can occur.

However, since the nature of the binding between humic acids and PAHs is not yet fully understood (Ferreira *et al.*, 2001), it cannot be stated for certain that this is the case. Further research is required in this area.

2.3.3 Leonardite

Leonardite is a naturally occurring oxidized form of lignite coal that is rich in humic materials. Leonardite is collected during open-pit coal mining, as it is commonly found overlying coal seams. There has been recent commercial interest in the possibility of using humic extractions of leonardite in environmental remediation procedures in place of synthetic chemical surfactants. Leonardite humic acid material has demonstrated the capability to partition organic contaminants from solution in column extraction tests conducted by Yates and Wandruszka (1999), in which they found that the PAH, pyrene, was removed from solution to below detectable limits.

CHAPTER 3

Effect of Humic Substances on the Water Solubility of Naphthalene

3.1 Abstract

A fluorescence quenching method was used to determine the association constants (K_b) for various water-soluble humic substances and naphthalene. Results show that partitioning capabilities of these humic solutions are not influenced by changes in pH, as the differences in association constants at pH 4.0 and pH 7.0 were not significant. Results indicate that the solution of the 1/1000 dilution of 3.0 g/L Aldrich humic acid was most effective at partitioning the naphthalene in solution, followed by the 1/1000 dilution of L-58, the 1/1000 dilution of L-69, the 1/1000 dilution of L-67D, and lastly, the undiluted L-58. L-58, L-67D, and L-69 are extraction products from leonardite. Results of this experiment will be compared to results of the microcosm biodegradation study to determine if the partitioning capabilities of these solutions are effective in influencing the bioavailability of polycyclic aromatic hydrocarbons in soil.

3.2 Introduction

The term fluorescence refers to the absorption of light energy by a molecule at one wavelength, resulting in the emission of light energy at another, usually longer, wavelength (Schenk, 1973):

$$\underset{\text{state}}{\text{ground}} + \text{UV} \xrightarrow{\text{absorption}} \underset{\text{state}}{\text{excited}} \xrightarrow{\text{emission}} \underset{\text{state}}{\text{ground}} + \text{fluorescence} \qquad \text{(Equation 3.1)}$$

A spectrofluorimeter is used to measure fluorescence.

A fluorescence quenching method was used to determine the association constants for various soluble humic substances and naphthalene. The fluorescence quenching method is based upon the observation that polycyclic aromatic hydrocarbons (PAHs) fluoresce in aqueous solution, but not when associated with dissolved organic matter, such as dissolved humic and fulvic acids (Kumke *et al.*, 1994). As a consequence, the fraction of PAH associated with the dissolved organic matter may be determined directly from the fractional decrease in fluorescence upon addition of humic or fulvic substances. Furthermore, this association with water soluble humic and fulvic acids infers an increase in the water solubility of the PAH, as it is partitioned into the aqueous phase and not bound to solid particles. This technique can only be applied to measure association constant values (K_b) for compounds with high fluorescence efficiencies, such as PAHs.

K_b represents the ratio of the PAH's total equilibrium concentration bound to the humic substances to the concentration unbound in solution.

In the fluorescence quenching method it is not necessary to know the initial concentration of pollutant, due to the fact that the data is reported as a fraction of unquenched fluorescence. This is especially useful since most hydrophobic organic pollutants are only very slightly soluble in aqueous solution, making it difficult to prepare solutions in which the hydrophobic organic pollutant concentration is accurately known. The fluorescence quenching method is unique from other methods used to determine association constants, as it allows for constants to be determined directly, without separation of fluorophore and quencher (Puchalski *et al.*, 1992). Eliminating the separation step avoids possible errors due to incomplete separation of free from bound pollutant, which may lead to a number of difficulties and uncertainties in measurements.

Leonardite is a naturally occurring oxidized form of lignite coal that is rich in humic materials. Leonardite is collected during open-pit coal mining, as it is commonly found overlying coal seams. There has been recent commercial interest in the possibility of using humic acid extractions of leonardite in environmental remediation procedures in place of synthetic chemical surfactants. Research has shown that water soluble humic and fulvic acids are capable of significantly enhancing the aqueous solubilities of PAHs (Johnson and Amy, 1995), because of their capability to interact with the PAHs. The humic substance acts as a surfactant, increasing the water solubility of the PAH, thus allowing the contaminants to enter the aqueous phase. The use of humic substances on contaminated soils could greatly enhance the bioavailability of PAHs, allowing for a much easier and more efficient bioremediation procedure. Studies have shown an

average three-fold increase in PAH concentration in the aqueous phase after the addition of humic acid, and up to a ten-fold increase in solubility for trimethyl naphthalene (Lesage *et al.*, 1996).

3.3 Objective of the Study

The objective of this study was to determine the effectiveness of various water-soluble humic substances to bind with PAHs, and to determine the association constants (K_b) for the association of the PAH naphthalene with various dissolved humic substances.

3.4 Materials and Methods

3.4.1 Naphthalene Solution Preparation

The PAH naphthalene was selected for this study because a model chemical was needed that has a similar structure and properties to other more hydrophobic PAHs; however, a PAH with higher water solubility was required to perform this experiment. To prepare the solution of naphthalene saturated in water, 0.1 g of naphthalene (Aldrich) was added to 1.0 L of deionized water in a foil-wrapped volumetric flask. The solution was heated on a heating plate to the melting point of naphthalene (approximately 81°C). The solution was removed from heat and left to equilibrate for 72 hours at room temperature (approximately 20°C). The excess solid naphthalene remaining in the

solution was removed by filtering the solution through a 0.22 μm GVWP filter (Millipore). The filtered solution was stored in the dark at 4°C.

Table 3.1 Selected properties of naphthalene (Government of Canada, 1994).

PAH	MW	log K_{ow}	S_W at 25°C (mg/L)	Melting point (°C)	Vapour pressure at 25°C (mPa)
naphthalene	128.16	3.37	31.7	80.5	11960

3.4.2 Humic Substances Solution Preparation

L-58, L-67D, and L-69 are liquid extracts of leonardite, a mined material rich in humic substances, being developed for proprietary purposes. The objective of this study was to determine if any of these extraction products have the potential to increase the solubility of PAHs in water, through association and binding with the PAH in aqueous solution. Because of factors involving corporate confidentiality, there is little information available on these products. The information available on these products is given in Table 3.2. No functional group data is available for these products. It is assumed that these products contain varying amounts of humic substances. Throughout this thesis, these solutions may be referred to as "humic acids", not because they are entirely comprised of humic acids, but because it is the humic acids within the solution that are believed to be the most active fraction in the partitioning of hydrophobic compounds, such as PAHs. Aldrich humic acid was used to compare the leonardite extractions to a product that has been used in previous studies, and has demonstrated the ability to partition PAHs and other hydrophobic organic compounds (Chiou *et al.*, 1987;

Gauthier *et al.*, 1987; Maxin and Kogel-Knabner, 1995). A blank of distilled water containing no humic acids was also included for reference.

Table 3.2 Elemental analysis data for the leonardite-extraction products.

Product	L-58	L-67D	L-69
pH of solution	2	9	7
Moisture	96.63	90.04	91.42
C (% dry wt.)	0.72	33.65	31.3
H (% dry wt.)	3.04	2.02	2.28
N (% dry wt.)	0.09	0.83	0.78
Ash (% dry wt.)	65.23	49.71	50.83
S (% dry wt.)	0.32	5.6	5.71
O (% dry wt.)	30.60	8.19	9.10

Undiluted L-58, a 1/1000 dilution of L-58, a 1/1000 dilution of L-67D, a 1/1000 dilution of L-69, and a 1/1000 dilution of 3.0 g/L Aldrich humic acid were used. A 1/1000 dilution was necessary for all of the solutions, except L-58, due to the extremely dark colour of the concentrated solutions. Use of full concentration of the solutions resulted in complete absorption of the excitation and/or emission beams, giving zero values for the fluorescence readings. Trials determined that a 1/1000 dilution was necessary to ensure accurate fluorescence readings were obtained from the spectrofluorimeter.

To prepare the 1/1000 dilutions, 1 mL of the humic concentrates were mixed with deionized water in a 1 L volumetric flask. At the concentrations used, all solutions dissolved fully in the water.

3.4.3 Buffer Solutions Preparation

Two aqueous buffer solutions were used in this study, a 0.1 M acetate buffer solution with a pH 4.0, and a 0.1 M phosphate buffer solution with a pH 7.0.

To prepare the 0.1 M acetate buffer solution with pH 4.0, 41.0 mL of 0.2 M acetic acid (glacial) and 9.0 mL of 0.2 M sodium acetate (trihydrate) solutions were combined and the final volume was adjusted to 100 mL with deionized water. The final pH was verified using an Accumet 925 pH Meter with an Accumet Gel-Filled Electrode.

To prepare the 0.1 M phosphate buffer solution with pH 7.0, 39.0 mL of 0.2 M sodium phosphate monobasic (monohydrate) and 61.0 mL of 0.2 M sodium phosphate dibasic (heptahydrate) solutions were combined and the final volume was adjusted to 200 mL with deionized water. The final pH was verified using an Accumet 925 pH Meter with an Accumet Gel-Filled Electrode.

3.4.4 Fluorescence Quenching Method

In a 1 cm x 1 cm x 4 cm quartz fluorescence cuvet, 2.25 mL of the saturated naphthalene solution was combined with 0.250 mL of the appropriate buffer solution. The cuvet was covered, and the contents of the cuvet were mixed for one minute using a vortex mixer, then left to stand for a period of 30 minutes. This equilibration time allows for sorption of the naphthalene to the walls of the cuvet. Gauthier *et al.* (1986) found that after this equilibration period, further sorption processes are negligible. During this 30 minutes, the cuvet was kept in the dark, away from the UV radiation of the

spectrofluorimeter to minimize photodegradation of the naphthalene in solution (Tiller and Jones, 1997). Because the spectrofluorimeter was not equipped with a sample temperature control, all measurements were performed at room temperature (approximately 25°C). Morra *et al.* (1990) have demonstrated that temperature influences fluorescence quenching of naphthalene by soil humic acid solutions, so room temperature was monitored to ensure constancy.

After the 30 minute equilibration period, an initial fluorescence intensity value (unquenched) was recorded using a Perkin-Elmer LS-5 Luminescence Spectrometer. For naphthalene measurements, the spectrofluorimeter was set at an excitation wavelength of 240 nm, slit width 10 nm, and an emission wavelength of 340 nm, slit width 3 nm (Auger *et al.*, 1995).

Once the initial fluorescence intensity value for the solution was recorded, a 10-µL aliquot of a humic solution was transferred to the cuvet using a pipette. Using a vortex mixer, the solution was mixed for one minute, then allowed to stand for one minute. During this time, the cuvet was kept covered, as to not expose it to UV radiation. After the 1 minute equilibration period, a fluorescence intensity value was recorded.

The process of adding 10 µL aliquots of humic solution, mixing, equilibrating and measuring the fluorescence intensity was repeated until 100 µL of humic material solution had been added. It was assumed that any effects on the fluorescence measurements from this dilution was negligible.

After each aliquot of humic material was added, a Pharmacia 3000 spectrophotometer was used to record an absorbance measurement for correction factor calculations (discussed in Section 3.4.6).

A control set containing humic substance only was used to measure the background fluorescence from the humic material (to be subtracted from the total fluorescence intensity measured for naphthalene in the presence of humic substance). The fluorescence of the solution containing humic substance only was measured at both the same concentrations and same instrumental conditions as the measurements with humic substance and PAH together. It was determined by these measurements that the humic substances used in this experiment caused no background fluorescence at the wavelengths used.

Three repetitions were performed for each amendment in this experiment.

3.4.5 Stern-Volmer Equation

Fluorescence intensity of PAH in aqueous solution is proportionately decreased upon the addition of humic or fulvic acids (Gauthier *et al.*, 1986). Equation 3.2 and Equation 3.3 illustrate the association of a PAH with humic or fulvic acids:

$$PAH + Hu \leftrightarrow PAH\text{-}Hu \qquad \text{(Equation 3.2)}$$

$$K_b = [PAH\text{-}Hu] / ([PAH][Hu]) \qquad \text{(Equation 3.3)}$$

where PAH = polycyclic aromatic hydrocarbon;

Hu = humic substance;

PAH-Hu = humic-associated PAH;

K_b = the association constant.

The mass balance on the PAH is described by:

$$C_{PAH} = [PAH] + [PAH\text{-}Hu] \qquad \text{(Equation 3.4)}$$

where C_{PAH} = the total concentration of the PAH.

Combining Equation 3.3 and Equation 3.4 yields:

$$C_{PAH}/[PAH] = 1 + K_b[Hu] \qquad \text{(Equation 3.5)}$$

Assuming that fluorescence intensity is relative to the concentration of unbound PAH in solution:

$$F_0/F = 1 + K_b[Hu] \qquad \text{(Equation 3.6)}$$

where F_0 = fluorescence intensities in the absence of humic material;

F = fluorescence intensities in the presence of humic material.

Equation 3.6 is called the Stern-Volmer equation. The Stern-Volmer equation can be used to determine the association constants for humic substances with fluorescent compounds.

3.4.6 Correction Factor Calculations

The inner filter effect refers to the excessive absorption of the excitation beam, and absorption of emitted radiation by an excess concentration of fluorophore or by the presence of an additional absorbing species in solution (Lloyd, 1981). Corrections can be made by taking into consideration the cell geometry and absorption characteristics of the solution. The higher concentrations of humic substances used in the fluorescence quenching titrations absorbed light at a significant extent at both excitation and emission wavelengths. It was therefore necessary to correct for this effect on the basis of the cell geometry shown in Figure 3.1 and the absorption characteristics of the solution (Gauthier *et al.*, 1986):

$$\frac{F_{cor}}{F_{obsd}} = \frac{2.3dA_{ex}}{1-10^{-dA_{ex}}} 10^{gA_{em}} \frac{2.3sA_{em}}{1-10^{-sA_{em}}} \qquad \text{(Equation 3.7)}$$

where: F_{cor} = corrected fluorescence intensity (no units);

F_{obsd} = observed fluorescence intensity (no units);

d = distance the sample beam must travel through the solution (cm);

A_{ex} = absorbance per centimetre at the excitation wavelength (no units);

g = distance from the sample beam to the edge of the cuvet (cm);

s = thickness of the excitation beam (cm);

A_{em} = absorbance per centimetre at the emission wavelength (no units).

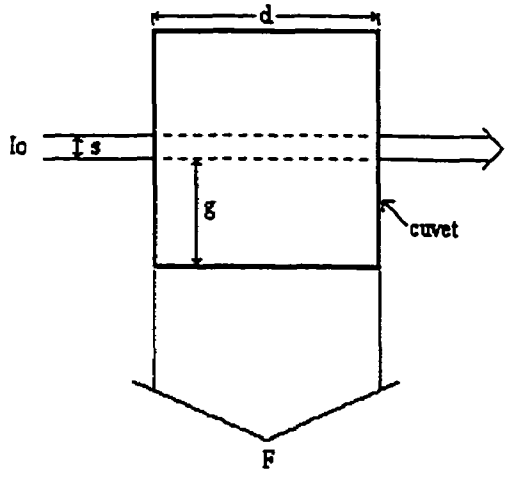


Figure 3.1 Assumed geometry of fluorescence measurement with parameters used to correct for the inner filter effect. Io represents the excitation beam with thickness s = 0.10 cm. The distance the sample beam must travel through the solution d = 1.00 cm. The distance from the edge of the sample beam to the edge of the cuvet g = 0.40 cm. F represents the observed fluorescence intensity with width 1.00 cm (Gauthier *et al.*, 1986).

3.4.7 K_b and K_{oc} Value Calculations

The organic carbon partition coefficient (K_{oc}) values for the solutions in question were determined. The concentration of organic matter in each leonardite extract solution was determined by laboratory analysis conducted by Norwest Labs (Lethbridge, AB), using a combustion furnace method (Canadian Soil Survey Committee, 1978). The results of the quenching experiments were plotted as fluorescence intensity in the absence of humic material over fluorescence intensity in the presence of humic material versus concentration of organic matter in solution. Using the Stern-Volmer equation (Equation 3.6), the value of K_b was determined for each trial. A line was fitted to the data and the equation for the line was determined.

$$F_0/F = 1 + K_b[Hu] \qquad \text{(Equation 3.6)}$$

where F_0 = fluorescence intensities in the absence of humic material (no units);

F = fluorescence intensities in the presence of humic material (no units);

[Hu] = humic acid concentration (mg / L);

K_b = the association constant (mL / g)(units as used by Gauthier *et al.*, 1986).

To calculate the partition coefficient (K_{oc}) value from the K_b, the K_b value was divided by the fraction of organic carbon of the sorbing material:

$$K_{oc} = K_b / f_{oc} \qquad \text{(Equation 3.8)}$$

where K_{oc} = the association constant normalized to organic carbon content (mL / g);

f_{oc} = fraction of organic carbon of the sorbing material;

K_b = the association constant (mL / g).

3.5 Results and Discussion

3.5.1 Fluorescence Quenching by Humic Substances

The Stern-Volmer plots shown in Figures 3.2 through 3.13 display the results obtained from the fluorescence quenching study. These figures illustrate the amount of fluorescence quenching of a naphthalene solution by incremental additions of the humic material solutions. The Y-axis of these graphs represents fluorescence intensity in the

absence of humic material (F_O) over fluorescence intensity in the presence of humic material (F). The X-axis represents the concentration of humic material in solution. Therefore a higher value of F_O/F would result from a greater amount of quenching, caused by a greater association between naphthalene and the dissolved humic material. In a soil or aquatic system, this increased association between PAH and humic material would represent a decrease in the amount of PAH sorbed to soil colloids (Rav-Acha and Rebhun, 1992; Lesage *et al.*, 1996). It should be noted that the scale on the X-axis varies between amendments, due to the fact that the quenching solutions are not all at a standard concentration. To perform comparisons between the solutions, association constants, K_b, should be examined. Table 3.3 and Table 3.4 give the values for the absorbance values and correction factors which have been applied to the data, as described in Section 3.4.5. The maximum value of the correction factor did not exceed 2.2, which is within the recommended acceptable range. Parker (1968) states that as correction values rise above 3, they become increasingly inaccurate. As there was no difference in the absorbance values at pH 4.0 and pH 7.0, the correction values at both pHs are identical.

Inspection of Figures 3.2 through 3.13 shows that a change in pH from 7.0 to 4.0 has little or no effect on the fluorescent quenching capabilities of the tested solutions. This does not coincide with the results of past research (Kumke *et al.*, 1994; Engebretson *et al.*, 1996; Ragle *et al.*, 1997), which suggests that the pH of a solution influences the association index of humic acids. The theory advanced by these workers is that the humic acids have a flexible macromolecular structure, commonly including carboxyl and phenolic hydroxyl groups within the structure of humic acid, which become negatively charged at higher pH values. The mutual repulsion of the negatively charged sites causes

the humic acid to become stretched, resulting in a reduction in the number of hydrophobic microenvironments, or pseudomicelles (Engebretson and Wandruszka, 1994). With fewer pseudomicelles, the humic acid should be less capable of binding to the PAH in solution, resulting in lower K_b values. However, Stevenson (1994) illustrates that the structure of humic acids are highly variable, depending on the source of the humic acid. This theory is applied to soil humic acids, and it is possible that the structure of the altered, non-soil derived humic materials used in this experiment have different molecular structures than natural soil humic acids. Another possible reason for the discrepancies between the results in this experiment and literature results, could be explained by the findings of Guetzloff and Rice (1994) who found that humic acids form micelles only at extremely high concentrations (7.4 g humic acid/L), well above the concentrations used in this experiment. However, Morra *et al.* (1990) found that despite using humic acid solutions well below the critical micelle concentration, a close physical association between the fluorophore and quencher occurred, not unlike a partitioning of the PAH into a hydrophobic micelle interior.

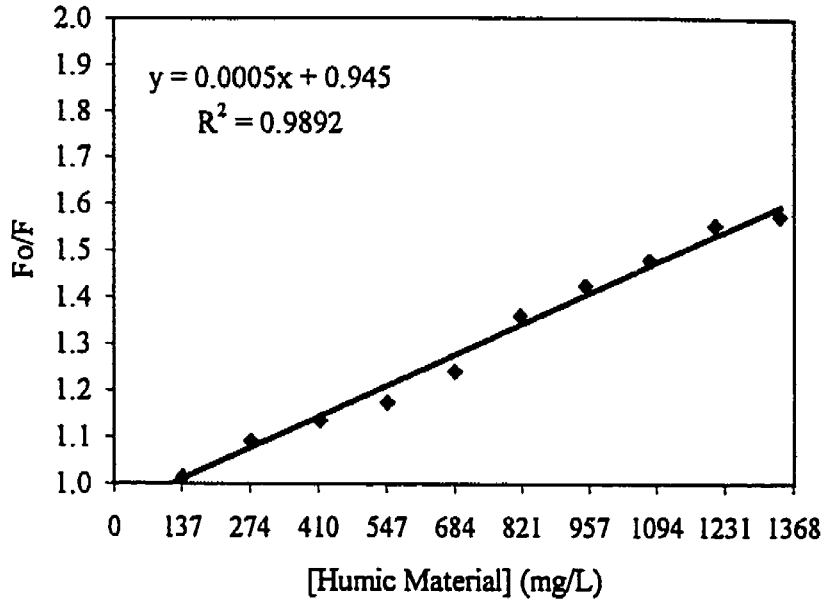


Figure 3.2 Stern-Volmer plot for undiluted L-58, pH 7.0.

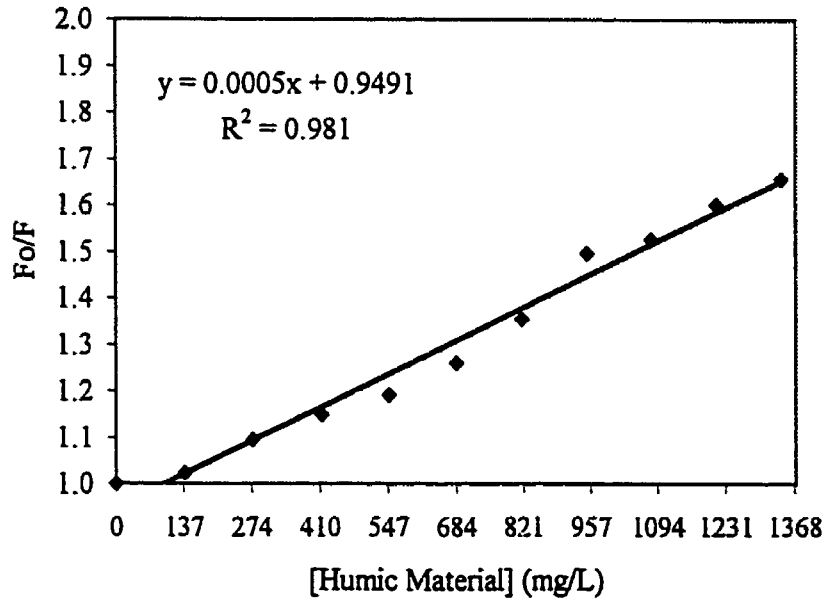


Figure 3.3 Stern-Volmer plot for undiluted L-58, pH 4.0.

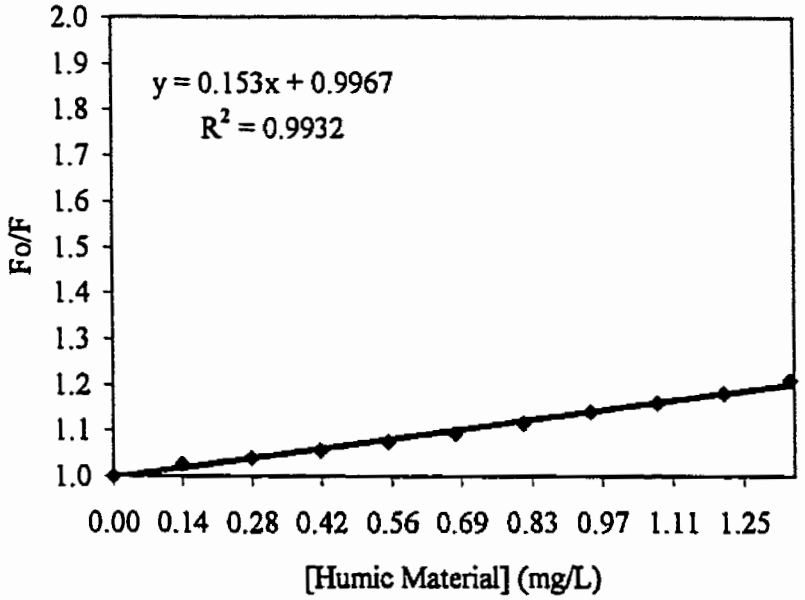


Figure 3.4 Stern-Volmer plot for 1/1000 dilution L-58, pH 7.0.

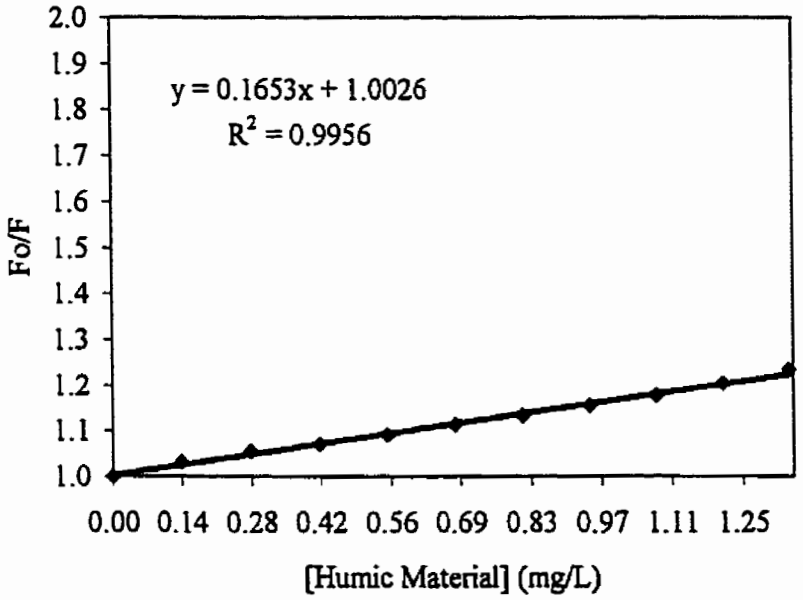


Figure 3.5 Stern-Volmer plot for 1/1000 dilution L-58, pH 4.0.

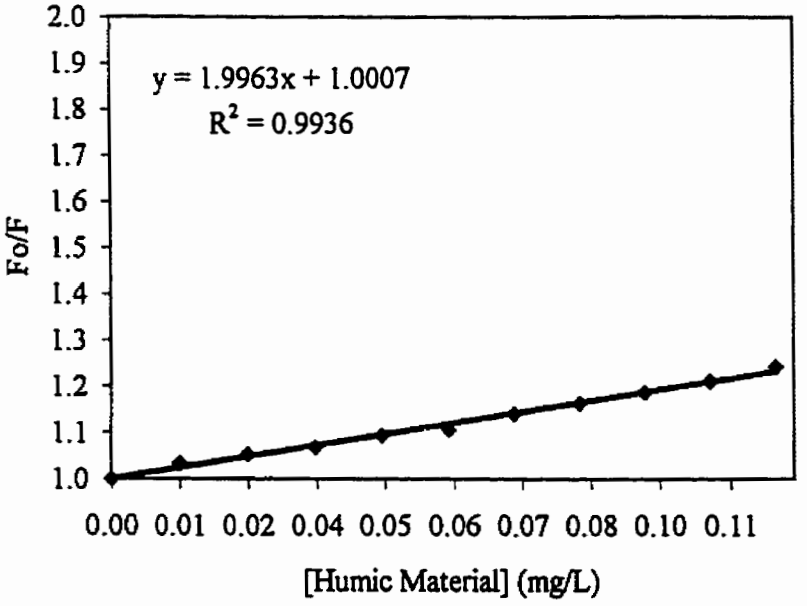


Figure 3.6 Stern-Volmer plot for 1/1000 dilution Aldrich humic acid, pH 7.0.

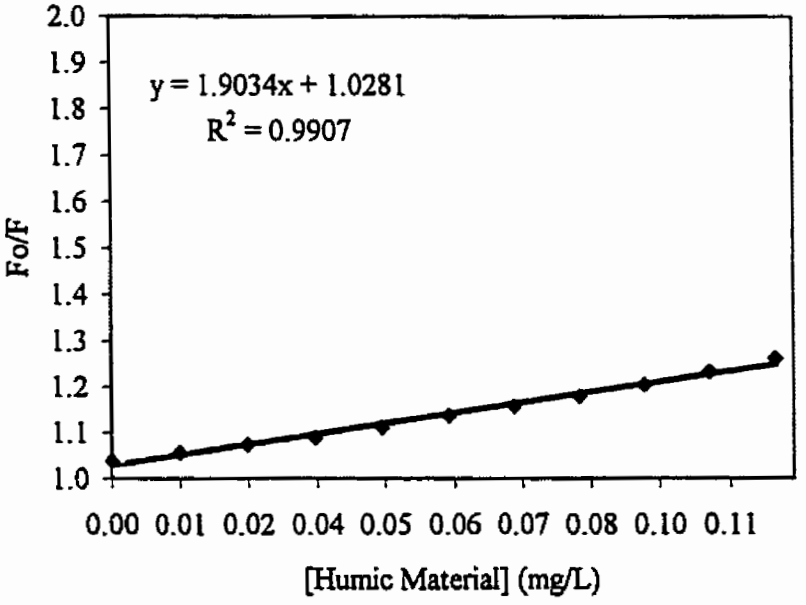


Figure 3.7 Stern-Volmer plot for 1/1000 dilution Aldrich humic acid, pH 4.0.

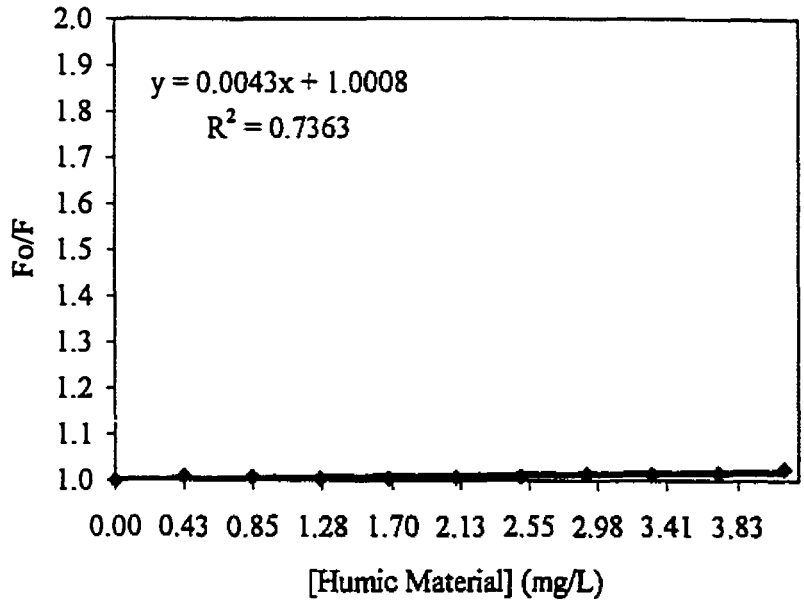


Figure 3.8 Stern-Volmer plot for 1/1000 dilution L-67D, pH 7.0.

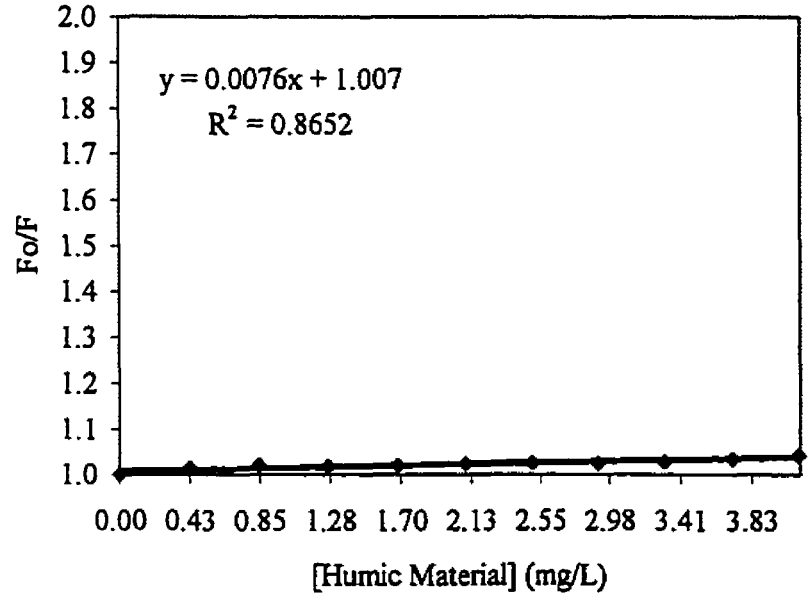


Figure 3.9 Stern-Volmer plot for 1/1000 dilution L-67D, pH 4.0.

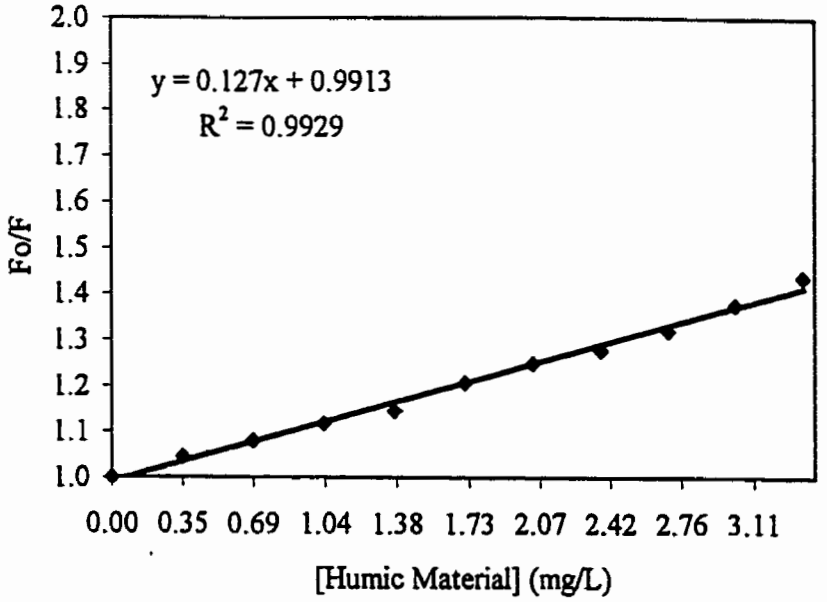


Figure 3.10 Stern-Volmer plot for 1/1000 dilution L-69, pH 7.0.

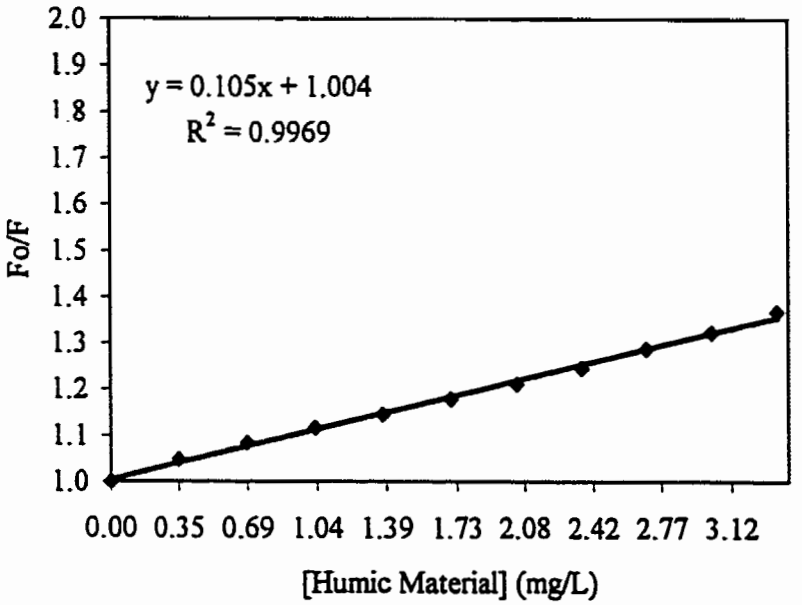


Figure 3.11 Stern-Volmer plot for 1/1000 dilution L-69, pH 4.0.

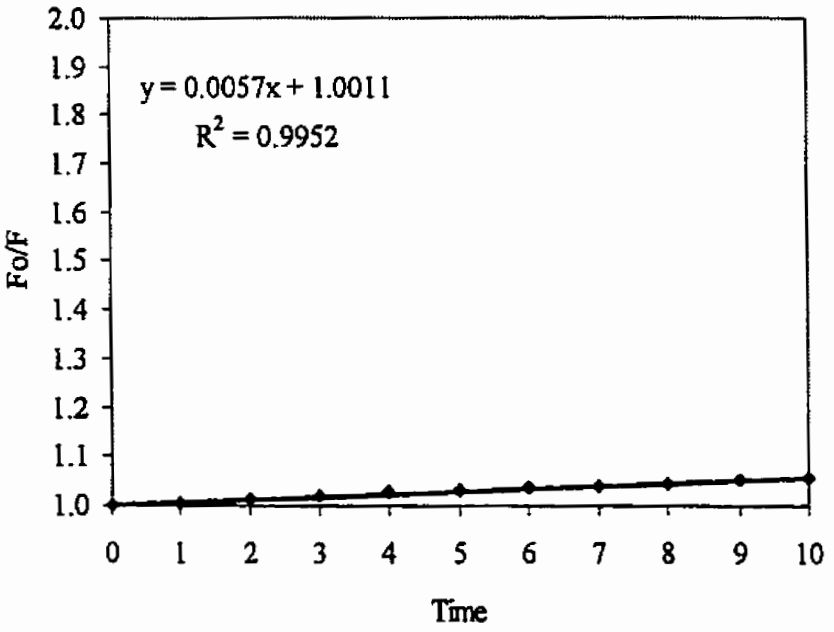


Figure 3.12 Stern-Volmer plot for no amendment (blank), pH 7.0.

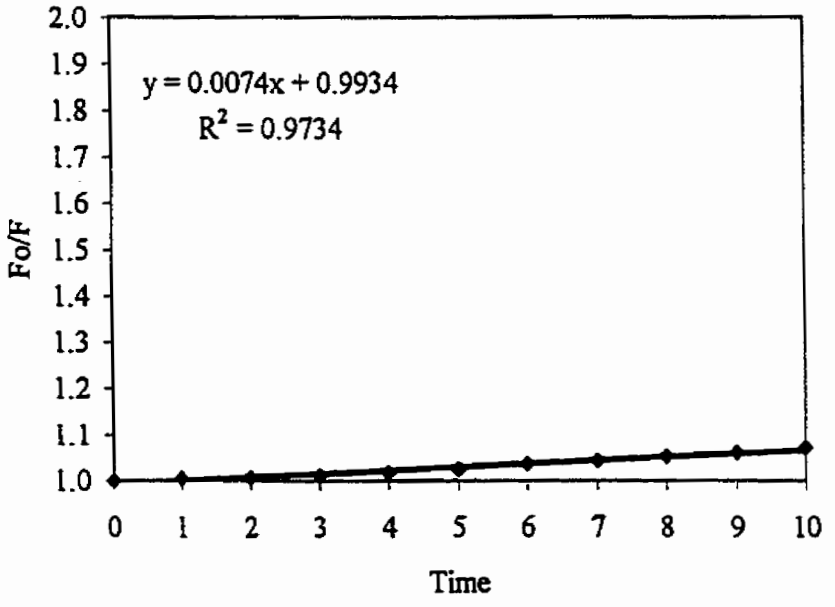


Figure 3.13 Stern-Volmer plot for no amendment (blank), pH 4.0.

Table 3.3 Amendment absorbance at excitation and emission wavelengths. Results are an average of three replications.

µL Added	None		Distilled Water		1/1000 Aldrich HA		L-58		1/1000 L-58		1/1000 L-67D		1/1000 L-69	
	240 nm	340 nm	240 nm	340 nm	240 nm	340 nm	240 nm	340 nm	240 nm	340 nm	240 nm	340 nm	240 nm	340 nm
0	0.223	0.049	0.223	0.049	0.223	0.049	0.223	0.049	0.223	0.049	0.223	0.049	0.223	0.049
10	0.223	0.049	0.223	0.049	0.223	0.049	0.263	0.055	0.223	0.049	0.234	0.054	0.232	0.054
20	0.223	0.049	0.223	0.050	0.224	0.050	0.303	0.062	0.223	0.049	0.245	0.059	0.241	0.058
30	0.223	0.049	0.223	0.050	0.224	0.050	0.343	0.068	0.223	0.049	0.255	0.065	0.250	0.062
40	0.223	0.049	0.223	0.050	0.225	0.050	0.383	0.076	0.223	0.049	0.267	0.071	0.260	0.066
50	0.223	0.049	0.223	0.051	0.225	0.050	0.423	0.083	0.223	0.050	0.277	0.076	0.268	0.070
60	0.223	0.049	0.224	0.051	0.225	0.050	0.463	0.089	0.223	0.050	0.289	0.081	0.277	0.074
70	0.223	0.049	0.224	0.051	0.226	0.050	0.503	0.095	0.223	0.050	0.301	0.087	0.287	0.079
80	0.223	0.049	0.224	0.051	0.226	0.051	0.543	0.101	0.224	0.050	0.312	0.093	0.295	0.083
90	0.223	0.049	0.224	0.052	0.227	0.051	0.583	0.108	0.224	0.051	0.324	0.098	0.305	0.087
100	0.223	0.049	0.224	0.052	0.227	0.051	0.639	0.115	0.224	0.051	0.336	0.102	0.314	0.092

Table 3.4 Correction factors calculated for each amendment solution for both pH 4.0 and pH 7.0.

µL Added	None	Distilled Water	1/1000 Aldrich HA	L-58	1/1000 L-58	1/1000 L-67D	1/1000 L-69
0	1.342	1.342	1.342	1.342	1.342	1.342	1.342
10	1.342	1.342	1.342	1.408	1.342	1.365	1.362
20	1.342	1.344	1.345	1.478	1.342	1.388	1.381
30	1.342	1.344	1.345	1.548	1.342	1.411	1.399
40	1.342	1.344	1.346	1.625	1.342	1.438	1.420
50	1.342	1.345	1.346	1.702	1.344	1.460	1.438
60	1.342	1.346	1.346	1.779	1.344	1.486	1.457
70	1.342	1.346	1.348	1.859	1.344	1.514	1.480
80	1.342	1.346	1.349	1.941	1.345	1.540	1.498
90	1.342	1.348	1.351	2.028	1.346	1.567	1.520
100	1.342	1.348	1.351	2.147	1.346	1.593	1.542

3.5.2 Association Constant Values for Humic Substances

Table 3.5 and Table 3.6 give K_b and K_{oc} values for naphthalene with the five humic material solutions at pH 4.0 and pH 7.0, respectively. Some of the log K_b values obtained in this experiment are higher than literature values for naphthalene with humic acids. Literature values for log K_b range from approximately 2.1 to 4.7 (Mackay *et al.*, 1992). It should be noted that the previous studies used to determine these K_b values were obtained using different methodologies, and most experiments were performed using soil humic acids. The materials tested in this experiment are synthetic solutions, and may be more active than humic materials of natural origins, which were used in the previous work. It should also be noted that most experiments of this nature are conducted with humic acids that have been isolated from soils. As mentioned in Section 3.4.2, the solutions used in this experiment are not strictly humic acids, and it is possible that some quenching may have been caused by materials in the solution other than the humic acids. Because of factors involving corporate confidentiality, there is little information available on these products, making further inspection into this matter very difficult. In this experiment, it is assumed that any fluorescence quenching caused by substances within the leonardite extract solutions other than the humic acids is negligible. However, if this assumption were to be incorrect and quenching is occurring due to substances within the solution other than the humic acid, the calculations for quenching caused solely by the humic acid would determine it to be greater than its true value. For the purposes of this thesis, however, the relative values of the solutions within this experiment are more

important than the absolute values obtained, as the values are compared against each other.

Results indicate that the solution of the 1/1000 dilution of 3.0 g/L Aldrich humic acid was most effective at quenching naphthalene fluorescence, which implies an increase in the amount of PAH in the aqueous phase (Gauthier *et al.*, 1986; Schlautman and Morgan, 1993; Auger *et al.*, 1995). This was followed by the 1/1000 dilution of L-58, the 1/1000 dilution of L-69, the 1/1000 dilution of L-67D, and lastly, the undiluted L-58.

Most K_{oc} values obtained in this experiment are also higher than literature values, which range between log 2.4 and log 5.0 (Mackay *et al.*, 1992). The K_{oc} values were calculated for these solutions, but it is suspected that these values do not accurately represent the true partitioning capabilities of the solutions. K_{oc} values will not be considered in the discussion, due to the relatively high quenching capabilities of certain solutions despite a very low organic carbon content in the solution, particularly L-58. It is possible that any of the leonardite-extracted humic type solutions, which are not strictly humic or fulvic acids, may contain other reactive constituents which are not organic.

Table 3.5 Interaction of naphthalene with five humic material solutions at pH 4.0.

Solution	Undiluted L-58	1/1000 L-58	1/1000 AHA	1/1000 L-67D	1/1000 L-69
slope (L/mg)	0.0005	0.1653	1.9034	0.0076	0.1050
intercept	0.949	1.028	1.028	1.007	1.004
R^2	0.9810	0.9956	0.9907	0.8652	0.9969
log K_b (mL/g)	2.6	5.2	6.4	4.0	5.0
f_{oc}	0.0072	0.0072	0.3903	0.3365	0.3130
log K_{oc} (mL/g)	4.8	7.4	6.8	4.5	5.5

Table 3.6 Interaction of naphthalene with five humic material solutions at pH 7.0.

Solution	Undiluted L-58	1/1000 L-58	1/1000 AHA	1/1000 L-67D	1/1000 L-69
slope (L/mg)	0.0005	0.153	1.9963	0.0043	0.1270
intercept	0.945	1.001	1.001	1.001	0.991
R^2	0.9892	0.9932	0.9936	0.7363	0.9969
log K_b (mL/g)	2.6	5.2	6.3	3.7	5.1
f_{oc}	0.0072	0.0072	0.3903	0.3365	0.3130
log K_{oc} (mL/g)	4.8	7.3	6.7	4.1	5.6

3.6 Summary and Conclusions

The fluorescence quenching experiment gives insight into how well the solutions in question are able to partition hydrophobic contaminants in aqueous solution, such as PAHs. Results show that partitioning capabilities of these humic material solutions are not influenced by changes in pH, as the differences in association constants at pH 4.0 and pH 7.0 are insignificant. Results indicate that the solution of the 1/1000 dilution of 3.0 g/L Aldrich humic acid was most effective at quenching naphthalene fluorescence. This was followed by the 1/1000 dilution of L-58, the 1/1000 dilution of L-69, the 1/1000 dilution of L-67D, and lastly, the undiluted L-58.

Results from this experiment indicate that the leonardite-extract solutions tested have a greater binding capacity than values reported in the literature for soil or sediment-derived humic and fulvic acids. This should not be unexpected, as these solutions have undergone extraction processes, which may have caused the solutions to become more reactive than humic and fulvic acids from natural sources. A greater binding capacity for these extracted humic substances would allow for a greater amount of PAH to be partitioned away from the soil particles, into the aqueous phase, compared to unaltered soil humic and fulvic acids.

It is difficult to state with certainty what the effect of using any of these products would have on the water solubility of PAHs in the soil, as the ability of humic materials to bind hydrophobic contaminants is dependent upon the affinities of the humic material with the contaminant and of the contaminant with the soil particles (Johnson and Amy, 1995). However, from the high binding coefficients measured in this experiment, it seems that there is potential for the products tested to be useful in enhancing the solubilization of PAHs into the aqueous phase in certain soil systems.

CHAPTER 4

Effect of Humic Substances on the Biodegradation of Anthracene and Benzo[a]Pyrene in Soil

4.1 Abstract

The objective of this study was to determine if a variety of dissolved humic material amendments would influence the biodegradation rate of selected polycyclic aromatic hydrocarbons (PAHs), namely anthracene and benzo[a]pyrene, in a soil with previous exposure to PAHs. Soil samples were treated with ^{14}C-labelled PAHs and, using a microcosm apparatus, total degradation of the PAHs was recorded over a 38 week incubation period. Total respiration and PAH volatilization were also monitored for the duration of the experiment.

Significant degradation occurred within the 38 week duration of the experiment, for both anthracene (47.9 - 50.3% of the total initial amount added) and benzo[a]pyrene (24.9 - 31.2% of the total initial amount added), but there were no significant amendment effects, aside from a temporary suppression of PAH degradation by the glucose amendment. The soils with depressed degradation showed the highest total respiration rates, suggesting that the glucose was preferentially selected as a substrate by the

degrader community. It was determined that volatilization is not a major source for losses of anthracene or benzo[a]pyrene from the soil.

These results suggest that the dissolved humic material amendments would not enhance the microbial decomposition of PAHs from soil, when utilized in the manner tested.

4.2 Introduction

The contamination of soil and groundwater by highly persistent, hydrophobic substances, such as PAHs, is an important environmental problem today. Major sources of PAHs to the aquatic and soil environments include creosote-treated products (railway ties, transmission and telephone poles), spills of petroleum products (gasoline, oil, and diesel fuel), metallurgical and coking plants, and deposition of atmospheric PAHs (Government of Canada, 1994). Many PAHs are toxic and carcinogenic, therefore it is important that soils contaminated with these substances be remediated. Many of the remediation techniques presently used are expensive, lengthy, and inefficient. Bioremediation involves the utilization of microorganisms to degrade contaminants, yielding harmless compounds such as carbon dioxide, microbial biomass, and inorganic forms of nitrogen, phosphorus, and sulfur (Paul and Clark, 1996). The hydrophobic nature of many PAHs makes *in situ* remediation difficult due to the fact that the contaminants are partitioned onto the soil particles, out of the aqueous phase. Because most bacteria can use PAHs as a carbon source from the dissolved state only, these sorbed contaminants are less available for biodegradation. For bioremediation techniques

to be effective, a way must be found to promote the transfer of the PAHs from the soil-sorbed phase to the aqueous phase. When contaminant availability is reduced due to partitioning to soil particles, a surfactant may be used to enhance the aqueous solubility of sorbed organic compounds. Chemical surfactants are one answer to the growing demand for new techniques and products which may increase the effectiveness of remediation procedures in order to reduce expenses and shorten the time required to restore a contaminated site back to acceptable conditions. However, there are problems associated with the use of chemical surfactants in bioremediation procedures.

Leonardite is a naturally occurring oxidized form of lignite coal that is rich in humic materials. Leonardite is collected during open-pit coal mining, as it is commonly found overlying coal seams. There has been recent commercial interest in the possibility of using humic extractions of leonardite in environmental remediation procedures in place of synthetic chemical surfactants. Research has shown that water soluble humic and fulvic acids are capable of significantly enhancing the aqueous solubilities of PAHs (Johnson and Amy, 1995) by forming associations with the PAHs. The humic materials act as a surfactant, increasing the water solubility of the PAH and allowing the contaminants to enter the aqueous phase. The use of humic materials on contaminated soils could greatly enhance the bioavailability of PAHs, allowing for a much easier and more efficient bioremediation procedure. Studies have shown an average three-fold increase in PAH concentration in the aqueous phase after the addition of humic acid, and up to a ten-fold increase in solubility for trimethyl naphthalene (Lesage *et al.*, 1996). An advantage of using leonardite over a chemical surfactant is that humic materials should not inhibit the activity of the degrader community, as will some other chemical

surfactants. Toxicity of humic and fulvic acids to soil microorganisms should not be an issue, as they are a naturally occurring, common soil component. Synthetic surfactants have been shown to exhibit aqueous toxicity for soil microorganisms, including those responsible for the metabolism of the contaminants (Kanga *et al.*, 1997). Other reasons for an inhibition to synthetic surfactant-enhanced biodegradation include preferential use of the surfactant as the substrate instead of the contaminant, and a decrease in bacterial adherence to hydrocarbons (Lesage *et al.*, 1996). Humic materials are generally biologically recalcitrant and are therefore not readily metabolized, and would not be expected to act as a preferential substrate (Pyne *et al.*, 1987).

4.3 Objective of the Study

The objective of this study was to assess whether a number of humic and fulvic acid solutions could be utilized to increase the effectiveness of bioremediation procedures on PAH-contaminated soils. The rate of degradation for two PAHs, anthracene and benzo[a]pyrene, was examined to determine the effect of the dissolved humic material amendments on the bioavailability of PAHs in soil.

4.4 Materials and Methods

4.4.1 Soil Properties

The soil used in this study was obtained from the site of a crude oil pipeline rupture in 1994. At the time of the spill, the soil was exposed to high contaminant levels, but has since been remediated. In previous laboratory experiments, the soil from this location has shown a limited ability to degrade PAHs (Heaman, 1998).

Immediately after collection in the spring of 2000, the soil sample was mixed thoroughly and air dried. The soil was ground and passed through a 2-mm sieve. Soil characteristics were determined, including particle size, pH, electrical conductivity, field capacity, and bulk density. Characteristics of the soil are summarized in Tables 4.1 and 4.2.

Table 4.1 Selected properties of the soil used in this study.

pH	Electrical Conductivity	Field Capacity	Bulk Density
	(dS/m)	(%)	(g/cm^3)
7.6	0.77	39.8	1.08

Table 4.2 Particle size distribution of the soil used in this study.

Total Sand	Total Silt	Total Clay	Sand Fractions					Textural Class
(%)	(%)	(%)	% VCS	% CS	% MS	% FS	% VFS	
19	47	34	01	02	03	05	08	Clay Loam

4.4.1.1 Bulk Density and Field Capacity

The bulk density and field capacity of the soil were determined using acrylic cylinders 15 cm long by 5 cm in diameter with a cloth secured over the bottom end of the cylinder. Using air dried, sieved soil, the cylinders were filled to 13 cm height, and water was slowly added to each sample until the wetting front had moved one-third the way down the sample. The open end of the cylinder was covered with Parafilm to prevent evaporation, and left undisturbed for 48 hours (Veihmeyer and Hendrickson, 1949). After 48 hours, the wetted portion of the soil was removed from the cylinder, weighed, and placed in a drying oven at 110°C for 48 hours, after which time the oven dry weight of the soil was determined. The average bulk density of the soil was calculated using the computed volume of soil, weight of air dry soil, and air dry water content. The volumetric field capacity was calculated using gravimetric water content and bulk density. The results are an average of six replicates. See the Appendix for calculations. Table 4.1 shows the results of the bulk density and field capacity measurements.

4.4.1.2 Soil pH

Soil pH was determined using the pH in water method of Thomas (1996). An Accumet 925 pH Meter with an Accumet Gel-Filled Electrode was used. The results are an average of five replicates. Table 4.1 shows the results of the soil pH measurements.

4.4.1.3 Electrical Conductivity

Soil electrical conductivity was determined using an Orion Model 160 Conductivity Meter with an Orion 016010 Conductivity Cell. A 1:1 soil-water suspension was prepared in a 250 mL beaker, using 50 g of air dried, sieved soil and 50 mL of distilled water. The solution was stirred well, allowed to stand for 10 minutes, and stirred again. The electrical conductivity of the soil was measured after allowing the soil and water mixture to stand another 5 minutes. The results are an average of five replicates. Table 4.1 shows the results of the electrical conductivity measurements, reported in units of decisiemens per meter (dS/m).

4.4.1.4 Particle Size Distribution

The soil particle size analysis was performed by the Manitoba Soil Survey according to the method of Haluschak (1986). The soil was determined to be a clay loam soil. Table 4.2 shows the results of the particle size analysis.

4.4.2 Soil Preparation for Degradation Study

The equivalent of 10.0 g oven dried soil was weighed into 30 mL pyrex beakers. All soil samples were brought to field capacity and pre-incubated at 20°C for approximately four weeks. This was done to allow the soil organisms to recover from the sampling and air-drying procedures, and to adapt to more stable, optimal environmental conditions for the experiment. Soils were weighed and rewet to field capacity on a weekly basis. Five replicates of each set were prepared.

4.4.3 Microcosm Apparatus

Glass jars (500 mL) with air-tight metal lids (Richards Packaging, Winnipeg, Manitoba) were used for the microcosm container. Within each microcosm jar, three 20 mL borosilicate scintillation vials were placed alongside the 30 mL pyrex beaker containing the soil (see Figure 4.1). The first scintillation vial contained 0.5 g Ambersorb 563 (Aldrich), which was present to trap any volatile hydrocarbons released from the soil. The second scintillation vial contained 5 mL acidified water (pH 3.0), which was used to maintain humidity within the jar without sorbing carbon dioxide. The third scintillation vial contained 15 mL of 0.1 M sodium hydroxide (NaOH), which was used to trap carbon dioxide released from the soil. The NaOH traps collected carbon dioxide as sodium carbonate (Na_2CO_3). All components within the microcosm were made of glass to minimize partitioning of the PAHs onto the walls of the vessels.

At approximate three-week intervals, at the time of the trap change, the beaker containing the soil was removed from the microcosm and weighed to determine if the soil had lost water during incubation. If necessary the soil was re-wet to field capacity levels.

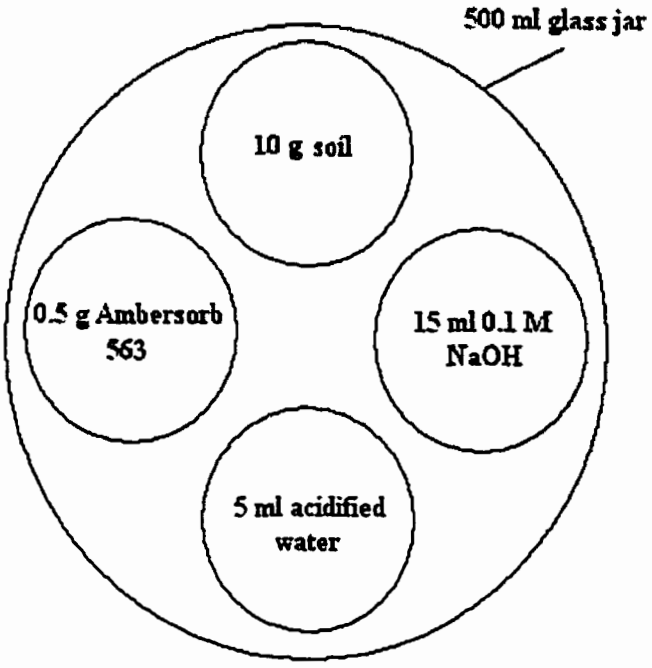
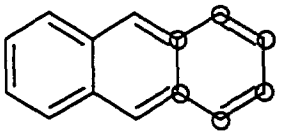


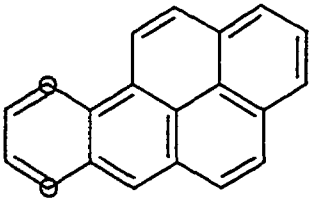
Figure 4.1 Illustration of the components of the microcosm used in this study.

4.4.4 ^{14}C-Labelled PAHs

Stock solutions were prepared using diesel fuel #2, hexane, and either ^{14}C-1,2,3,4,4A,9A-anthracene or ^{14}C-7,10-benzo[a]pyrene (illustrated in Figure 4.2). Table 4.3 lists selected properties of anthracene and benzo[a]pyrene. A 1.0 mL aliquot of stock solution was added to each soil plug. Each aliquot was prepared to contain the carrier solvent hexane, enough diesel fuel to produce contamination levels of 5000 μg/g soil, and either enough anthracene to produce contamination levels of 100 μg/g soil, or enough benzo[a]pyrene to produce contamination levels of 7 μg/g soil. After the stock solutions were added to the soils, the hexane was allowed to evaporate, and the soils were rewet to field capacity.



^{14}C-1,2,3,4,4A,9A-anthracene



^{14}C-7,10-benzo[a]pyrene

Figure 4.2 Locations of the ^{14}C-labels (o) on the PAH structures.

After running four 1.0 mL stock solution samples through the scintillation counter (1.0 mL stock solution and 5.0 mL ScintiSafe 30%), the amount of radioactivity in microcuries (μCi) added to each sample was calculated: anthracene 0.485 μCi/sample and benzo[a]pyrene 0.569 μCi/sample.

Table 4.3 Selected properties of anthracene and benzo[a]pyrene (Government of Canada, 1994).

	Anthracene	Benzo[a]pyrene
Molecular Weight (g/mol)	178.24	252.32
log K_{ow}	4.5	6.0
Water Solubility at 25°C (mg/L)	0.045	0.0038

4.4.5 Addition of Amendments

One week (168 hours) after the soils were contaminated, amendments were added at a concentration of 1500 μg/g. Amendments were dissolved in 1.0 mL of reverse osmosis-purified water per sample. 1.0 mL of distilled water was added to the blanks to

keep the soil moisture levels relatively uniform. The amendments used for this study were as follows:

- Product L-58
- Product L-67D
- Product L-69
- Blank (distilled water)
- Aldrich humic acid
- Tween-80
- Glucose

Products L-58, L-67D, and L-69 are extractions of leonardite, a mined humic substance high in humic materials, being developed for proprietary purposes. The objective of this study was to determine if any of these products have the potential of enhancing or improving PAH degradation rates in soil. Because of factors involving corporate confidentiality, there is little information available on these products. The information available on these products is given in Table 3.2. No functional group data is available for these products. The assumptions are that these products contain humic materials, and it is also known that L-67D contains glucose. The Aldrich humic acid was used to compare these leonardite extracts to a product that has demonstrated the ability to partition PAHs and other hydrophobic organic compounds (Chiou *et al.*, 1987; Gauthier *et al.*, 1987). The glucose amendment was used to compare a strict glucose amendment to the leonardite extract that contains glucose (L-67D). Tween-80 (polyoxyethylene sorbitan monooleate) is a product commonly used as a surfactant in soil remediation. It has been shown to enhance the amount of PAH solubilized in a soil-water system (Yeom *et al.*, 1996; Zheng and Obbard, 2001). A set of blanks, with no amendment, was also included for reference. Distilled water was added to the blank set to keep a uniform soil

moisture level across all soils.

4.4.6 Monitoring $^{14}CO_2$ Evolution and Total Respiration

Alkali (15 mL of 0.1 M NaOH) traps were changed at 24, 48, 72, 120, and 168 hours (one week) after the start of incubation. Following the addition of the amendments (at 168 hours), traps were changed at 6, 12, 24, 48, 72, 120, 168 hours, and then weekly for the following 26 weeks, after which time trap changes were performed bi-weekly until the conclusion of the experiment after 38 weeks.

4.4.6.1 Determining $^{14}CO_2$ Evolution

NaOH traps were subsampled in order to determine the amount of radioactive carbon dioxide that had evolved. To do this 1.0 mL was removed from the NaOH trap with a pipette and placed in a 7 mL scintillation vial. To this 5.0 mL of ScintiSafe 30% was added and mixed, and the samples were then stored in the dark for 48 hours. The radioactivity in disintegrations per minute was measured using a Beckman LS 7500 scintillation counter. Final results were corrected for background radiation and adjusted for the amount of radioactivity that was present in the entire trap.

4.4.6.2 Determining Total Respiration

Total respiration was measured using a Technicon AutoAnalyzer II. To determine total carbon dioxide (CO_2) production, a modified version of the Total Organic Carbon / Dissolved Organic Carbon method was used (industrial method no. 455-

76W/A). The NaOH traps collected CO_2 as sodium carbonate (Na_2CO_3). The AutoAnalyzer method used converts the Na_2CO_3 to CO_2 by mixing the sample with acid. The CO_2 is dialyzed through a silicone rubber membrane and reacts with a phenolphthalein indicator. The colour change in the indicator is proportional to the concentration of the original carbon concentration. Corrections were made for baseline drift and sensitivity drift. Final values were adjusted for background respiration, then converted to units of mg of carbon per trap.

Quality control measures were put in place to monitor system performance and aid in troubleshooting if necessary. Duplicate samples were run every fourteen samples to monitor system stability and reproducibility. Duplicates were compared to determine if the system was producing results within acceptable ranges of variability. Increased variability was usually an indicator of required system maintenance, such as line changes. Although there was some variability in the duplicate measurements, no values were considered to be outside of the acceptable range. QA/QC data for the respiration measurements may be found in the Appendix.

4.4.7 Volatilization

To test for any volatile organic carbon that may evolve during incubation, vials containing 0.5 g Ambersorb 563 were placed in the microcosms. The volatile organic carbon traps were replaced by fresh traps three hours after the initial contamination of the soil, and at the conclusion of the experiment. The traps were sampled at three hours to determine if any ^{14}C-PAHs were lost during the time period in which the hexane was evaporating from the soil, and again at the end of the 38 week experiment. At the

conclusion of the study, 0.2 g was removed from the Ambersorb traps to be oxidized in a Packard Biological Oxidizer. To ensure complete and uniform combustion, a few milligrams of non-radiolabelled cellulose and 0.3 mL of Combustaid (Canberra-Packard) were mixed with all samples before being placed in the oxidation furnace. The carbon in the samples is oxidized by heating the sample in a stream of oxygen. The resultant $^{14}CO_2$ is bubbled into ScintiSafe 30% scintillation cocktail (Fisher Scientific) where it is trapped. The collected sample in scintillation cocktail was stored in the dark for 48 hours, then was counted using a Beckman LS 7500 scintillation counter, and the total ^{14}C in the combusted sample was determined.

4.4.8 Mathematical and Statistical Analysis of CO_2 Evolution

Degradation results used were the average of five sets, plotted as cumulative percent $^{14}CO_2$ collected over time. Degradation results for anthracene and benzo[a]pyrene were fitted to a linear mineralization model, and a first-order mineralization model, as performed by Knaebel *et al.* (1994). For the first-order model, the Marquardt method in the NLIN module of SAS (SAS Institute Inc., Cary, NC) was used to fit the data to the following equation:

$$P = P_0 [1-e^{(-kt)}] \qquad \text{(Equation 4.1)}$$

where: P = the percentage of compound mineralized at time t (%);

P_0 = the asymptotic percentage of compound converted to $^{14}CO_2$ (%);

k = the first-order rate constant (day^{-1});

t = time (days).

Degradation results were fitted to a linear mineralization model using JMP IN statistical software (SAS Institute Inc., Cary, NC). Equation 4.2 was used:

$$P = k_L t + b_L \qquad \text{(Equation 4.2)}$$

where: P = the percentage of compound mineralized at t (%);

b_L = the y-intercept of the linear curve;

t = time (days);

k_L = the linear rate constant (day^{-1}).

To determine which mineralization model, the linear or the first-order, was most appropriate for each treatment, an F-test was performed to determine if there was a significant reduction in the residual sum of squares with increasing complexity of the model. The F-statistic is calculated by subtracting the larger from the smaller of the residual sum of squares of the two models and then dividing this difference by the residual mean square of the first-order model. This calculated F value is compared to the suitable value in an F table. The degrees of freedom at $P \leq 0.05$ level can be derived by using the value 1 and the number of data points minus the number of parameters in the equation (Robinson, 1985). If the calculated F value is greater than the F value found in the table, the increased model complexity resulted in a better fit for the data, and the more complex model should be used.

The first-order rate constant was used to calculate a mineralization half-life for the treatments that fit the first-order mineralization model, as affected by the various amendments. The half-life calculation assumes that all of the chemical that was available

for degradation has been degraded. The following equation was used to calculate the mineralization half-life values:

$$t_{1/2} = \ln 2 / k \qquad \text{(Equation 4.3)}$$

where: $t_{1/2}$ = the mineralization half-life (days);

k = the first-order rate constant (day^{-1}).

The specific activity for each set was calculated for each sampling date, as well as for the cumulative values at 38 weeks. The specific activity of respired CO_2 reflects the degradation of the labelled compound in relation to total metabolism. An increasing specific activity indicates that a greater percentage of total metabolism is accounted for by the metabolism of the labelled compound. Specific activity of a substance is the fraction of carbon dioxide released due to contaminant degradation over total respiration, as represented by the following equation:

$$\text{Specific Activity} = \frac{\text{g } CO_2 \text{ released by PAH degradation}}{\text{g } CO_2 \text{ total respiration}} \qquad \text{(Equation 4.4)}$$

SAS was used to perform one way ANOVAs and Duncan's Multiple Range tests at the 5% level on the final cumulative degradation values, as well as the total respiration data and specific activity values, for anthracene and benzo[a]pyrene. Results determine whether the amendments used had a statistically significant effect on PAH degradation and respiration.

4.5 Results and Discussion

4.5.1 PAH Degradation

Figure 4.3 and Figure 4.4 illustrate the degradation results for ^{14}C-labelled anthracene and benzo[a]pyrene, respectively. Results are plotted as cumulative $^{14}CO_2$ over time, expressed as a percent of the total radioactivity initially added and represent the mean of five replicates. The standard deviation of the samples was calculated, but will not be presented in this thesis, as it does not affect the interpretation of the data. A sample graph with the standard deviations present is given in the Appendix. Over the 38 week duration of the experiment, the soils contaminated with anthracene degraded between 47.9% and 50.3% of the total initial radiolabelled PAH added. Benzo[a]pyrene contaminated soils degraded between 24.9% and 31.2% of the total initial radiolabelled PAH added. In general, the anthracene was degraded more quickly and to a greater extent in the time given than the benzo[a]pyrene. This is to be expected for a number of reasons. Due to the greater number of rings in its molecular structure, benzo[a]pyrene is comparatively more hydrophobic, and has a higher octanol-water partition coefficient (K_{ow}) than anthracene. These factors would result in a greater fraction of benzo[a]pyrene adsorbing to soil particles, reducing its availability to degrader microorganisms (Sims and Overcash, 1983). This would result in lower degradation rates and a lower degree of degradation overall, as compared to anthracene. The influence of the greater structural complexity of benzo[a]pyrene is also illustrated by the three week lag period before the start of any significant degradation. By the end of the time allowed for this experiment, the degradation curves for anthracene were beginning to plateau, indicating that any

radiolabelled anthracene remaining in the soil had become unavailable for biological degradation. At the end of 38 weeks, the degradation curve for benzo[a]pyrene was still nearly linear, due to the slow rate of degradation of this highly hydrophobic compound.

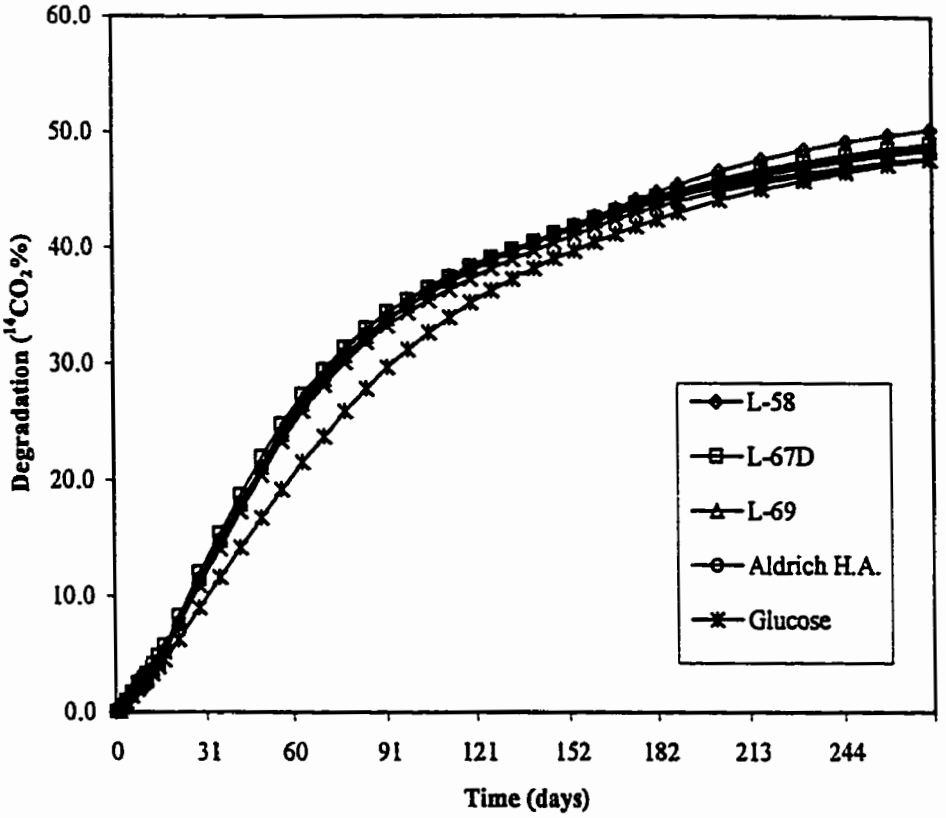


Figure 4.3 Cumulative degradation of anthracene with various amendments. Points represent the mean of five replicates.

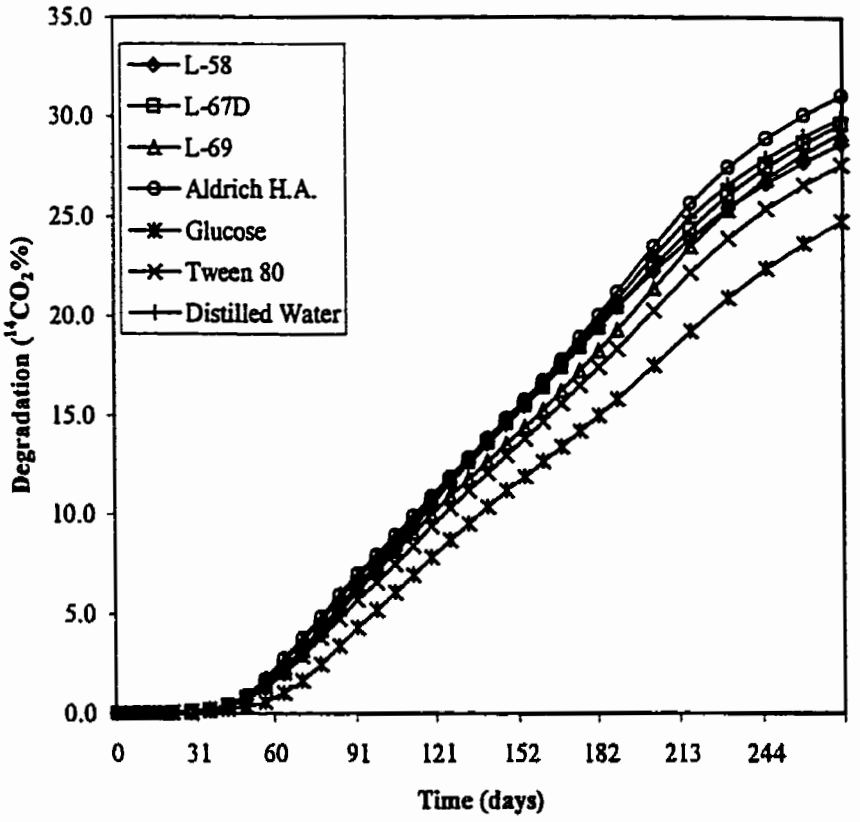


Figure 4.4 Cumulative degradation of benzo[a]pyrene with various amendments. Points represent the mean of five replicates.

None of the amendments tested increased the biodegradation rate of either anthracene or benzo[a]pyrene (Table 4.4 and Table 4.5 respectively). The only amendment that produced a cumulative degradation value that was statistically different than the blank was glucose. The soils amended with glucose had statistically significant lower rates of degradation than the rest. Microorganisms would preferentially degrade glucose over anthracene and benzo[a]pyrene because it is much easier to degrade than the complex PAHs. The depression of PAH degradation rates was not matched by L-67D, the leonardite-extract product containing glucose. It is unknown why the glucose within the solution did not have a similar effect on degradation rates as strict glucose. It is suspected that the amount of glucose within the amendment was insufficient to have a significant effect on anthracene or benzo[a]pyrene degradation.

Table 4.4 Calculations of cumulative 38 week anthracene total degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative PAH Degradation (mg C) [a]	
Anthracene	L-58	0.001634	A
Anthracene	L-67D	0.001595	A
Anthracene	L-69	0.001587	A
Anthracene	Aldrich H.A.	0.001577	A
Anthracene	Water	0.001556	A
Anthracene	Glucose	0.001552	A
Anthracene	Tween-80	0.001550	A

[a] Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

Table 4.5 Calculations of cumulative 38 week benzo[a]pyrene total degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative PAH Degradation (mg C) [a]	
Benzo[a]Pyrene	Aldrich H.A.	0.001681	A
Benzo[a]Pyrene	Water	0.001619	B A
Benzo[a]Pyrene	L-67D	0.001603	B A
Benzo[a]Pyrene	L-69	0.001579	B A
Benzo[a]Pyrene	L-58	0.001550	B A
Benzo[a]Pyrene	Tween-80	0.001494	B
Benzo[a]Pyrene	Glucose	0.001341	C

[a] Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

When the supply of glucose has been depleted, the degrader community would then begin to degrade other less-available carbon sources, such as PAHs. This is evident in Figure 4.3, where the cumulative degradation values of anthracene with the glucose substrate eventually catch up to the level of the other amendments after a lag period. No plateau was observed for the benzo[a]pyrene degradation curve, because the slow rate of degradation of the chemical resulted in there still being bioavailable benzo[a]pyrene left in the soil after 38 weeks. It is hypothesized that if this experiment had been conducted over a longer period of time, eventually the soil microorganisms would degrade all of the bioavailable radiolabelled chemical, at which point any benzo[a]pyrene remaining in the soil would be partitioned onto soil particles.

The PAH degradation curves were analyzed, and it was determined that the anthracene degradation is best characterized by a first order model, while the benzo[a]pyrene is best characterized by a linear model during the 38 week incubation. Mineralization half-lives were calculated for the contaminants with first order

degradation curves (Table 4.6). The mineralization half-life values for anthracene ranged from 53.7 to 77.0 days.

Table 4.6 Curve fitting analysis of the PAH degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Best Fit Model	k	P_0 or b_L	Mineralization Half-Life (days)
Anthracene	L-58	First Order	0.011	52.262	63.0
Anthracene	L-67D	First Order	0.012	50.098	56.4
Anthracene	L-69	First Order	0.012	50.218	57.8
Anthracene	Aldrich H.A.	First Order	0.012	49.906	57.3
Anthracene	Glucose	First Order	0.009	52.756	77.0
Anthracene	Tween-80	First Order	0.012	49.289	57.8
Anthracene	Distilled Water	First Order	0.013	48.952	53.7
B[a]P	L-58	Linear	0.148	-7.226	
B[a]P	L-67D	Linear	0.144	-6.662	
B[a]P	L-69	Linear	0.134	-5.999	
B[a]P	Aldrich H.A.	Linear	0.145	-6.338	
B[a]P	Glucose	Linear	0.119	-6.476	
B[a]P	Tween-80	Linear	0.130	-6.163	
B[a]P	Distilled Water	Linear	0.144	-6.396	

Results of the 38 week cumulative specific activity calculations for anthracene and benzo[a]pyrene are given in Table 4.7 and Table 4.8, respectively. The specific activity values show that for both anthracene and benzo[a]pyrene, the glucose amendment caused the greatest reduction in specific activity. The second lowest specific activity values, for both anthracene and benzo[a]pyrene, were for the soils amended with Tween-80. For both contaminants, these were the only amendments that were statistically different than the blank treatments (i.e. the distilled water amendment) at $\alpha = 0.05$. These results suggest that the glucose and Tween-80 amendments were used as a

substrate by the soil microorganisms, rather than the desired targets of bioremediation efforts, the contaminants.

Figure 4.5 and Figure 4.6 show the specific activity values for anthracene and benzo[a]pyrene, respectively, at each sampling period. These figures illustrate that over the 38-week period, the degradation of the labelled compound relative to total respiration is quite different for the anthracene degraded soils, as compared to the benzo[a]pyrene degraded soils. The anthracene contaminated soils show a rapid peak in specific activity at approximately 30 days, after which it gradually declines. In comparison, the specific activity values for benzo[a]pyrene peak at approximately 180 days, before beginning to decline. It is believed that the lag phase for benzo[a]pyrene is due to the greater complexity of the chemical compared to other substrates available in the soil system. Catabolite repression would occur as long as more easily degraded substrates are available in the soil.

Table 4.7 Specific activity calculations of cumulative 38 week ^{14}C-anthracene degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative Respiration (mg)	PAH Degradation (mg)	Specific Activity (*10^{-3} %)[a]			
Anthracene	L-69	30.28	0.001587	5.24		A	
Anthracene	Water	30.00	0.001556	5.19	B	A	
Anthracene	L-58	31.72	0.001634	5.15	B	A	
Anthracene	Aldrich H.A.	31.05	0.001577	5.08	B	A	C
Anthracene	L-67D	31.88	0.001595	5.00	B		C
Anthracene	Tween-80	31.67	0.001550	4.90			C
Anthracene	Glucose	33.25	0.001552	4.67	D		

[a] Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

Table 4.8 Specific activity calculations of cumulative 38 week ^{14}C-benzo[a]pyrene degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative Respiration (mg)	PAH Degradation (mg)	Specific Activity (*10^{-3} %)[a]	
B[a]P	Water	30.29	0.001619	5.35	A
B[a]P	Aldrich H.A.	32.18	0.001681	5.22	A
B[a]P	L-69	30.51	0.001579	5.17	A
B[a]P	L-58	30.12	0.001550	5.14	A
B[a]P	L-67D	31.45	0.001603	5.10	A
B[a]P	Tween-80	32.80	0.001494	4.55	B
B[a]P	Glucose	34.24	0.001341	3.92	C

[a] Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

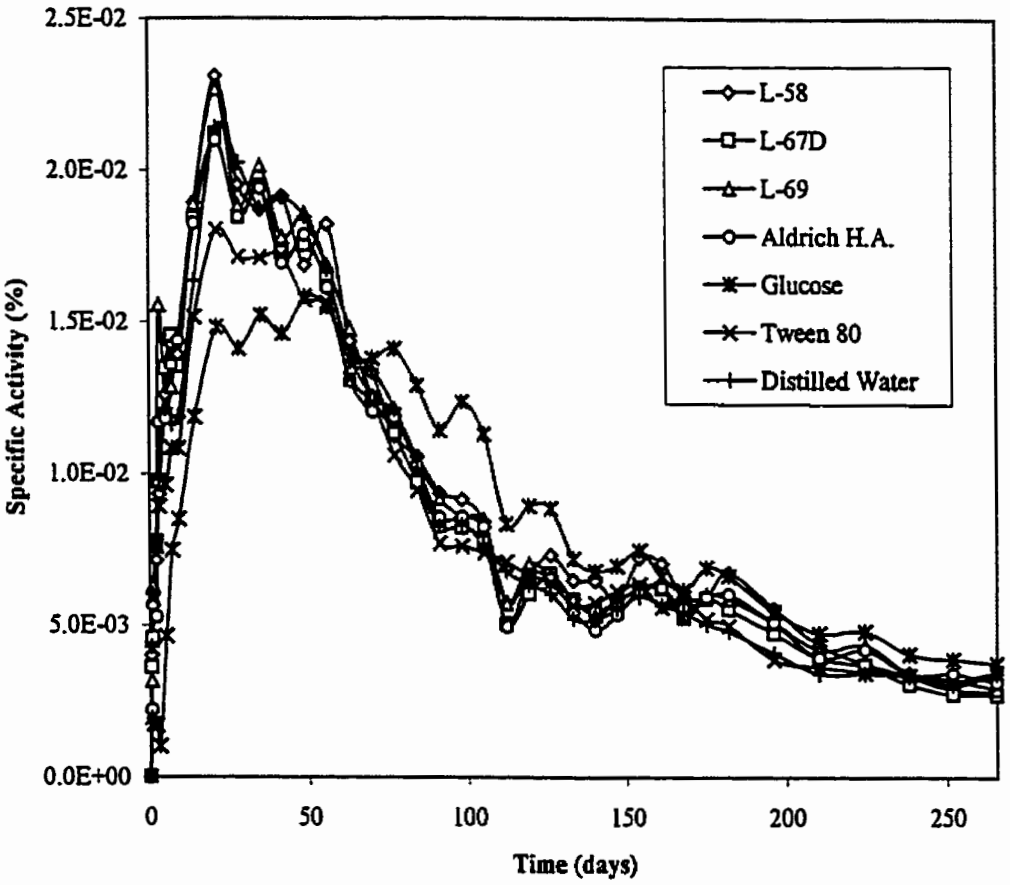


Figure 4.5 Specific activity over time plotted for anthracene with various amendments. Points represent the mean of five replicates.

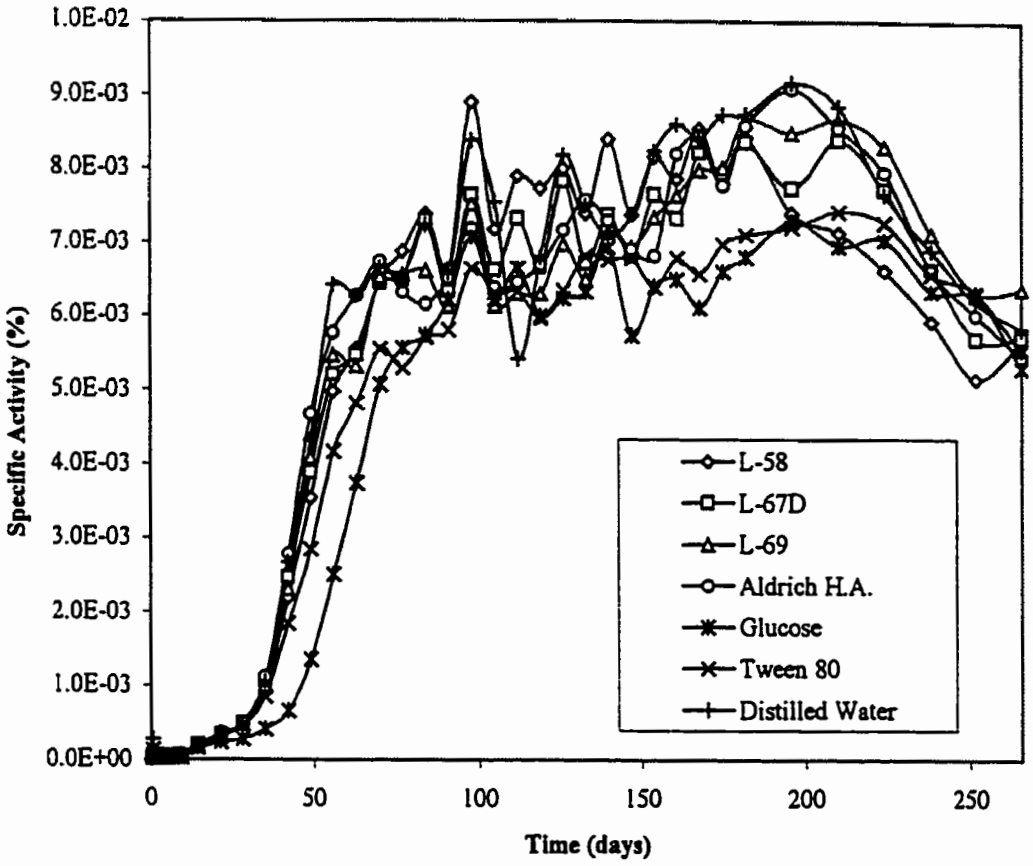


Figure 4.6 Specific activity over time plotted for benzo[a]pyrene with various amendments. Points represent the mean of five replicates.

4.5.2 Respiration

Figure 4.7 and Figure 4.8 show the cumulative CO_2 evolved for the anthracene and benzo[a]pyrene-contaminated soils, as influenced by the various amendments. The standard deviation of the samples was calculated, but will not be presented in this thesis, as it does not affect the interpretation of the data. A sample graph with the standard deviations present is given in the Appendix. For the anthracene-contaminated soils, the only amendment that had a significant effect on respiration at $\alpha = 0.05$ was the glucose amendment (Table 4.9). For the benzo[a]pyrene-contaminated soils, L-67D, Aldrich humic acid, Tween-80, and glucose all had statistically significant effects on soil respiration at $\alpha = 0.05$ (Table 4.10). The soils with the glucose amendment showed the highest rate of respiration of all the amendments, most likely due to the readily-available nature of glucose as a substrate. Glucose was utilized quickly by the soil microorganisms, as in the first week after the addition of the glucose, 4.20 mg of carbon was trapped from the benzo[a]pyrene-contaminated soils and 4.33 mg of carbon was trapped from the anthracene-contaminated soils. Assuming a 60% utilization efficiency for sugars (Paul and Clark, 1996), over two-thirds of the glucose was metabolized within the first seven days. The amendment L-67D, which contains glucose, did not have the same effect on respiration rates. The type of contaminant did not seem to influence total respiration rates, with anthracene-contaminated soils ranging from 30.0 - 33.2 mg of carbon per trap cumulative over 38 weeks, and benzo[a]pyrene-contaminated soils ranging from 30.1 - 34.2 mg of carbon per trap cumulative over 38 weeks.

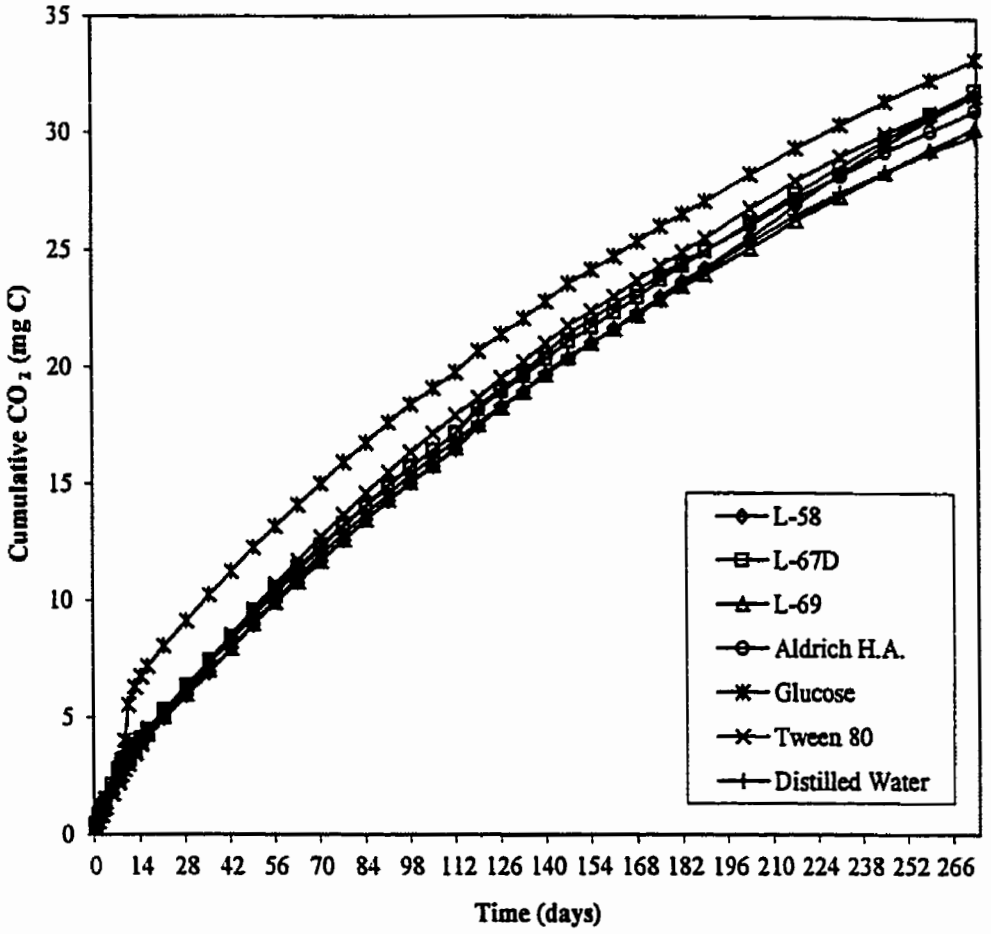


Figure 4.7 Cumulative soil respiration of anthracene with various amendments. Points represent the mean of five replicates.

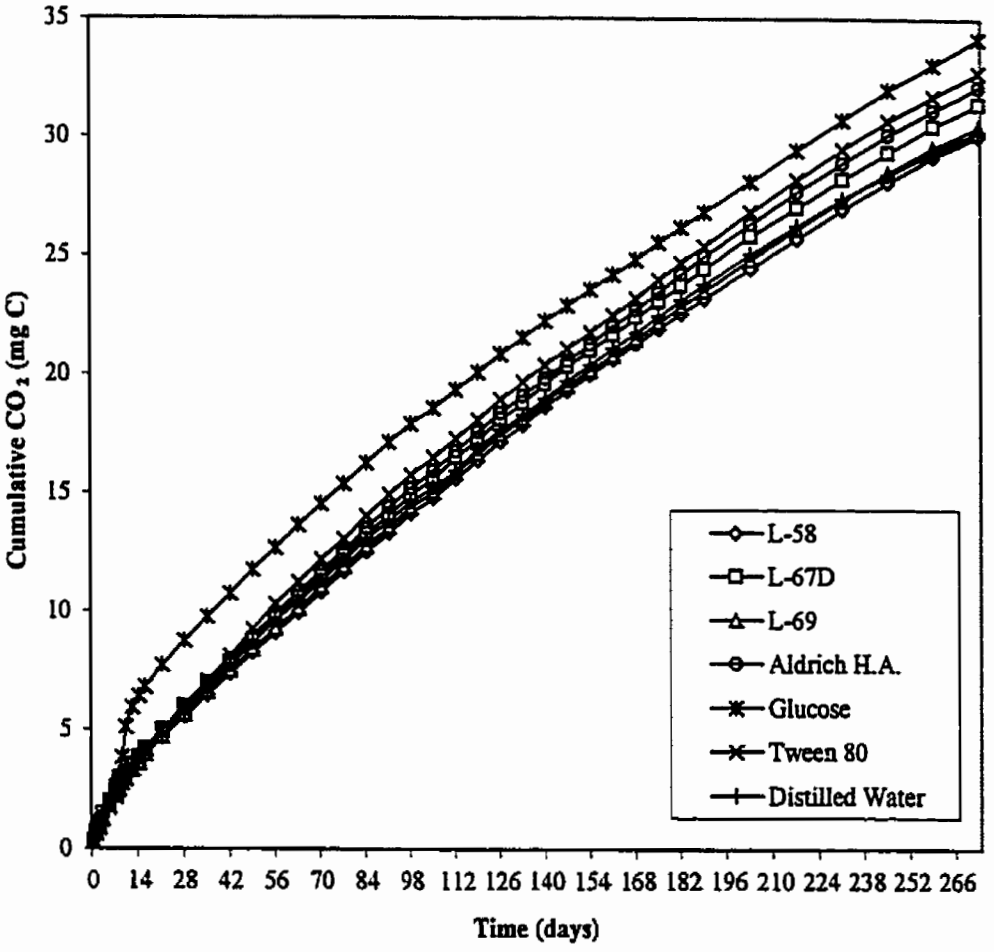


Figure 4.8 Cumulative soil respiration of benzo[a]pyrene with various amendments. Points represent the mean of five replicates.

Table 4.9 Total respiration calculations of cumulative 38 week anthracene degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative Respiration (mg C)[a]	
Anthracene	Glucose	33.2	A
Anthracene	L-67D	31.8	B A
Anthracene	L-58	31.7	B A
Anthracene	Tween-80	31.6	B A
Anthracene	Aldrich H.A.	31.1	B
Anthracene	L-69	30.3	B
Anthracene	Water	30.0	B

[a]Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

Table 4.10 Total respiration calculations of cumulative 38 week benzo[a]pyrene degradation results. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	Cumulative Respiration (mg C)[a]		
Benzo[a]Pyrene	Glucose	34.2	A	
Benzo[a]Pyrene	Tween-80	32.7	B	
Benzo[a]Pyrene	Aldrich H.A.	32.1	B	
Benzo[a]Pyrene	L-67D	31.4	B	C
Benzo[a]Pyrene	L-69	30.4		C
Benzo[a]Pyrene	Water	30.3		C
Benzo[a]Pyrene	L-58	30.1		C

[a]Means with the same letter are not significantly different at α=0.05 using Duncan's New Multiple-Range Test.

4.5.3 Volatilization

Oxidation of the Ambersorb 563 traps, sampled at 3 hours and 38 weeks, indicated that there was no significant loss of ^{14}C-labelled compounds due to volatilization. Table 4.11 shows the amounts of radioactivity collected by the volatile organic carbon traps. As suggested by their relative vapour pressures (anthracene: 25 mPa at 25°C, and benzo[a]pyrene: $0.37*10^{-6}$ mPa at 25°C), the amount of anthracene lost due to volatilization was up to ten times higher than benzo[a]pyrene. The greatest loss due to volatilization over the 38 week period represented only 0.1% of the total radioactivity initially added. Volatilization does not seem to be a significant mechanism for loss of anthracene or benzo[a]pyrene from the soil.

Table 4.11 Percent of radioactivity lost due to volatilization. Values shown in the table represent the mean of five replicates.

Contaminant	Amendment	% of Total Radioactivity	
		3 hours	38 weeks
Anthracene	L-58	0.005	0.083
Anthracene	L-67D	0.002	0.080
Anthracene	L-69	0.005	0.094
Anthracene	Aldrich H.A.	0.005	0.081
Anthracene	Glucose	0.005	0.079
Anthracene	Tween-80	0.007	0.100
Anthracene	Distilled Water	0.007	0.070
B[a]P	L-58	0.003	0.006
B[a]P	L-67D	0.006	0.006
B[a]P	L-69	0.001	0.006
B[a]P	Aldrich H.A.	0.001	0.008
B[a]P	Glucose	0.001	0.007
B[a]P	Tween-80	0.003	0.007
B[a]P	Distilled Water	0.002	0.006

4.6 Summary and Conclusions

Although significant levels of anthracene and benzo[a]pyrene degradation were observed in this experiment, none of the amendments were able to significantly increase the degradation of PAHs by the microbial community within the soil. As expected by their chemical structures, the amount of anthracene metabolized in the 38 week period was greater than the amount of the more structurally complex benzo[a]pyrene.

Glucose temporarily depressed PAH degradation by providing a preferential alternate substrate, but this carbon source was quickly utilized and the cumulative PAH degradation curve for anthracene eventually reached similar levels to those shown by the other substrates. The duration of the experiment was not long enough to determine if the degradation curve for benzo[a]pyrene would eventually show similar characteristics.

The leonardite extract L-67D, which contained glucose in the solution, did not have the same effect as the strict glucose amendment. There was no significant difference in metabolism or respiration for the soils amended with the L-67D product than the blank. Most likely, the product did not contain sufficient amounts of glucose to influence the metabolism of the contaminant.

Tween-80 was used in this experiment to represent a chemical surfactant. Although the literature confirms the effectiveness of Tween-80 to enhance the solubility of certain hydrophobic contaminants (Yeom *et al.*, 1996; Zheng and Obbard, 2001), it had significantly lower specific activity values than the blank. This suggests that the Tween-80 was metabolized by the soil microorganisms, instead of increasing the solubility of the contaminants. As the Tween-80 is degraded, any solubility

enhancements caused by the surfactant would be lost. This data suggests that the effects of Tween-80 must be carefully considered before using it as a tool in bioremediation, as it may serve as a substrate for the microbial community in the soil, lowering the amount of contaminant metabolized. Research has shown that using higher concentrations of Tween-80 in the soil could cause other problems for bioremediation procedures, as many synthetic chemical surfactants are toxic, and have detrimental effects on microbial populations when used at high concentrations (Kanga *et al.*, 1997). Also, work done by Laha and Luthy (1991) suggests that the bioavailability of PAHs may be decreased upon micellization by nonionic surfactants.

The products L-58, L-69, and Aldrich humic acid had no significant effect on the specific activity values in this experiment. Previous research with Aldrich humic acid has demonstrated its ability to sorb hydrophobic organic contaminants (Chiou *et al.*, 1987; Gauthier *et al.*, 1987), but had no significant effect on the specific activity values in this experiment. It does not appear that the L-58, L-69, or Aldrich humic acid was used as a substrate by the microbial community, as specific activity levels were not significantly different from the blank. It is possible that the conditions of this experiment did not allow for these amendments to partition the PAHs into the aqueous phase. Other experiments involving PAH-partitioning in soil systems use a soil material with a very high sand content (Lesage *et al.*, 1996). The soil used in this experiment had quite a high clay content (34%), which may have bound the hydrophobic contaminants too strongly to be influenced by these amendments.

The respiration measurement data coincided with the degradation results. For a short time period after the amendment addition, increased respiration was observed in the

glucose amended soils. When the glucose had been consumed by the soil organisms, the respiration rate returned to similar levels as those of the other amendments.

Anthracene and benzo[a]pyrene volatilization were both very low (below 0.1% of total radioactivity), and not considered a major loss pathway from the soil. Anthracene losses were higher than those for benzo[a]pyrene, as would be expected from the higher vapour pressure of anthracene.

Although some of the amendments used in this study have demonstrated the ability to sorb hydrophobic compounds such as anthracene and benzo[a]pyrene (Chiou *et al.*, 1986; Zheng and Obbard, 2001), this capability did not increase the rate of degradation or the total amount of ^{14}C-labelled PAHs degraded. A potential explanation for this outcome could be that solubility was not a major limitation for the biodegradation of the PAHs in this soil, as the degradation rates observed in this experiment were quite fast compared to values listed in the literature (Mackay *et al.*, 1992). If the soil used in this experiment had been exposed for longer periods with contaminants, a greater fraction of the PAHs would be strongly sorbed to the soil particles. With a greater fraction of PAH strongly sorbed to the soil particles, solubility would become a more important limitation to degradation. It is possible that in a situation where contaminant solubility was more of an concern, the addition of these amendments may have an enhanced effect on rates of contaminant biodegradation.

It is notable that none of the amendments used caused a reduction in contaminant degradation, suggesting that the amendments used have no adverse effects on soil microorganisms at the concentrations tested.

CHAPTER 5

General Discussion

The objective of this study was to determine the effectiveness of various test humic material solutions at binding polycyclic aromatic hydrocarbons (PAHs) in aqueous solution, and the effect of this binding on the biodegradation rates of these contaminants. Naphthalene was used in the fluorescence quenching study to determine to what extent each test solution could bind to PAHs in solution. Anthracene and benzo[a]pyrene were used in a microcosm study to determine the influence that the addition of these humic material amendments on PAH bioavailability and biodegradation. The PAH naphthalene was selected for the fluorescence quenching study because a model compound was needed that has a similar structure and properties to other more hydrophobic PAHs; however, a PAH with higher water solubility was required to perform this experiment. Although naphthalene has a much higher aqueous solubility than anthracene and benzo[a]pyrene, it was assumed that since they all belong to the same class of compounds and have similar chemical structures, the binding of anthracene and benzo[a]pyrene by dissolved humic substances will follow similar trends to those observed for the binding of naphthalene.

5.1 Binding Capabilities of Humic Solutions

A fluorescence quenching method was used to determine the association constants (K_b) for various water-soluble humic materials and naphthalene. Results show that partitioning capabilities of these humic material solutions are not influenced by changes in pH, as the differences in association constants at pH 4.0 and pH 7.0 were insignificant. This contradicts past research (Kumke *et al.*, 1994; Engebretson *et al.*, 1996), which suggests that the pH of a solution influences the association index of humic acids. The theory advanced by these workers was that the humic acids have a flexible macromolecular structure, commonly including carboxyl and phenolic hydroxyl groups within the structure of humic acid, which become negatively charged at higher pH values. The mutual repulsion of the negatively charged sites causes the humic acid to assume a more linear structure, resulting in a reduction in the number of hydrophobic microenvironments, or pseudomicelles. With fewer pseudomicelles, the humic acid should be less capable of binding to the PAH in solution, resulting in lower K_b values. This theory (Engebretson and Wandruszka, 1994) is applied to soil-derived humic acids, and it is possible that the structure of the altered, non-soil derived humic materials used in this experiment have different molecular configurations than natural soil humic acid. Stevenson (1994) illustrated that the structure of humic acids is highly variable, dependent on the source. Another possible reason for the discrepancies between the results in this experiment and previous studies, could be explained by the findings of Guetzloff and Rice (1994) who found that humic acids form micelles only at extremely high concentrations (7.4 g humic acid / L), well above the concentrations used in this experiment. However, Morra *et al.* (1990) found that despite using humic acid solutions

well below the critical micelle concentration, data showed that a close physical association between the fluorophore and quencher occurred, not unlike a partitioning of the former into a hydrophobic micelle interior.

Results show that the solution of the 1/1000 dilution of 3.0 g/L Aldrich humic acid was most effective at quenching naphthalene fluorescence, followed by the 1/1000 dilution of L-58, the 1/1000 dilution of L-69, the 1/1000 dilution of L-67D, and lastly, the undiluted L-58. This experiment has shown that these solutions possess the potential to bind to PAHs in aqueous solution. The microcosm biodegradation study will determine if these binding capabilities are effective in influencing the bioavailability of PAHs in soil.

5.2 Biodegradation of PAHs

The objective of this study was to determine if a variety of humic material solution amendments would influence the biodegradation rate of selected PAHs, namely anthracene and benzo[a]pyrene, in a soil with previous exposure to PAHs. Soils were contaminated with ^{14}C-labelled PAHs and, using a microcosm apparatus, total degradation of the PAHs was recorded over a 38 week period. Total respiration and volatilization were also monitored for the duration of the experiment.

Significant degradation occurred within the 38 week duration of the experiment, for both anthracene (47.9 - 50.3% of the total initial amount added was degraded) and benzo[a]pyrene (24.9 - 31.2% of the total initial amount added was degraded), but there were no significant amendment effects, aside from a temporary suppression of PAH degradation by the soils with the glucose amendment. The soils with depressed

degradation showed the highest total respiration rates, suggesting that the glucose was preferentially selected as a substrate by the degrader community.

5.3 Influence of Binding on Biodegradation Rates

Since the hydrophobic nature of PAHs is the limiting factor in biodegradation of these compounds in soils (CCME, 1997), it was hypothesized that by increasing the solubility of PAHs, bioavailability of the contaminants would increase as well. These experiments were conducted to determine if the addition of dissolved humic substances to a contaminated soil would increase the amount of PAHs in solution, thereby enhancing biodegradation. However, when partitioning data is compared to the biodegradation rates of the PAHs, no amendment effect is observed. Despite a variety of amendments used, many of which showed binding capabilities in the fluorescence quenching experiment, none were able to cause a significant increase in the rate of metabolism of anthracene or benzo[a]pyrene in the soil. There are a number of possible explanations for this. It is possible that the conditions of this experiment did not allow for these amendments to partition the PAHs. Most other experiments involving PAH-partitioning in soil systems use a soil material with a very high sand content and containing little organic matter (Lesage *et al.*, 1996). The soil used in this experiment had quite a high clay content (34%), which may have bound the hydrophobic contaminants too strongly to be influenced by these amendments. It is also possible that the solutions used in this experiment did in fact partition the PAHs into solution, but did not cause an increase in the amount of free contaminant in solution. This agrees with results obtained by Lesage

et al. (1997), who found that although the addition of humic acid to PAH-contaminated soils increased the concentration of PAHs in the aqueous phase, the degradation rate was not increased. If the surfactant is bound too tightly, or if the bound contaminant is isolated from the aqueous phase due to the complex structure and large size of the humic molecule, it may also restrict the soil microorganisms from accessing the contaminant for degradation. Research has shown that while surfactants increase the amount of hydrophobic contaminant in solution, it may be unavailable to microorganisms, thereby inhibiting biodegradation (Laha and Luthy, 1991). Another potential explanation for this outcome could be that solubility was not a major limitation for the biodegradation of the PAHs in this soil, as the degradation rates observed in this experiment were quite fast compared to values listed in the literature (Mackay *et al.*, 1992). If the soils used in this experiment had been aged with contaminants, a greater fraction of the PAHs would be strongly sorbed to the soil particles. With a greater fraction of PAH strongly sorbed to the soil particles, solubility would become a more important limitation to degradation. It is possible that in a situation where contaminant solubility was more of an concern, the addition of these amendments may have an enhanced effect on rates of contaminant biodegradation.

The results obtained by these experiments do not provide sufficient evidence to dismiss the use of dissolved humic materials; however, it does highlight the fact that consideration may be required as to the environmental conditions in which these amendments are to be used. The effectiveness of these amendments would vary with a number of environmental conditions, including changes in soil type, contaminant type, and microorganism type. However, these issues are the same for all surfactants, synthetic

or natural, and can be overcome with understanding of the interactions between all of the components of the system.

If the binding of the PAHs by the dissolved humic materials is too strong to be broken by the soil microorganisms, rendering the partitioned PAH unavailable for biodegradation, another possible use for these amendments in the remediation of contaminated soils could be in a pump-and-treat procedure, in which a surfactant is used to increase the proportion of the contaminant in the aqueous phase, allowing for increased efficiency of contaminant removal when the water is pumped from the ground for *ex situ* treatment. The use of these materials may be advantageous over synthetic chemical surfactants, as they are of natural origin and non-toxic. Unlike some synthetic chemical surfactants (Lewis, 1991; Xu *et al.*, 1994; Kanga *et al.*, 1997), they should have no negative impacts on the soil or aquifer.

CHAPTER 6

Summary and Conclusions

The effect of humic substances on the binding and biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil was assessed. A fluorescent quenching study indicated that the humic substances were all capable of binding with the PAH naphthalene in aqueous solution to some extent. This binding implies an increase in the amount of PAH in the aqueous phase of the system. However, as observed in the microcosm biodegradation study using the PAHs, anthracene and benzo[a]pyrene, a binding to water-soluble humic material does not increase contaminant bioavailability, as expressed by the contaminant degradation rates. None of the amendments tested: leonardite extracts L-58, L-67D, and L-69, Aldrich humic acid, glucose, or Tween-80, demonstrated a significant positive amendment effect on the respiration or degradation rates of the contaminants. The soils amended with glucose and Tween-80 both had significantly lower specific activity values, as both amendments were preferentially utilized by the soil microorganisms as substrate.

Recommendations for further study of the effects of humic material amendments in contaminant remediation include: 1) conducting a microcosm biodegradation study similar to the one conducted for this experiment, but using soils with a variety of textures.

and therefore a variety of binding capacities for PAHs (e.g. sand, silt, clay); 2) allowing the microcosm biodegradation study to run for a longer duration to determine if the amendments have an effect after all readily available contaminants have been metabolized; and 3) testing the effects of amendment additions on aged contaminated soils. Another area for potential to conduct research is using these solutions in pump-and-treat soil remediation programs, which may overcome any issues involving bioavailability when the contaminant is associated with a surfactant.

CHAPTER 7

Contribution to Knowledge

This study found that amendments with various dissolved humic material solutions to a PAH-contaminated soil had no effect on the degradation rates of the contaminant. Despite the fact that an amendment may act to increase the amount of hydrophobic contaminant in the aqueous phase of a soil system, it may not lead to increases in the degradation rate of the compound. Utilizing glucose and the chemical surfactant Tween-80 as amendments actually depressed PAH biodegradation, as they served as a more readily available carbon source for mineralization for the soil microorganisms.

Although the leonardite-extracted humic material solutions (L-58, L-67D, L-69) did not increase biodegradation rates at the concentrations tested, they had no negative effects on the ability of the soil microorganisms to mineralize PAHs, and had no negative impact on the rate of respiration of the soil microbial community.

Despite the fact that in this study these amendments did not enhance the degradation of PAHs in soils, under different circumstances they may be more successful at increasing the amount of contaminant available for biodegradation. The lack of evidence of toxicity, along with the high binding coefficients, suggests that these

dissolved humic materials may be used successfully as surfactants in remediation procedures in which contaminant solubility, but not contaminant bioavailability, is an issue, such as pump-and-treat of groundwater or active barrier systems.

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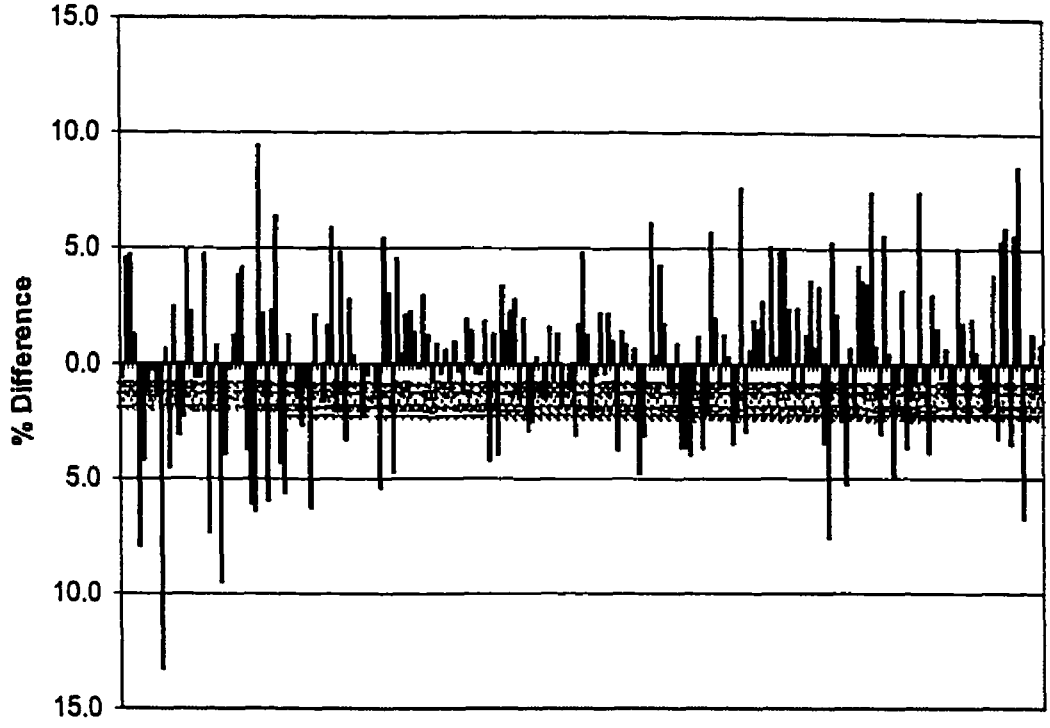
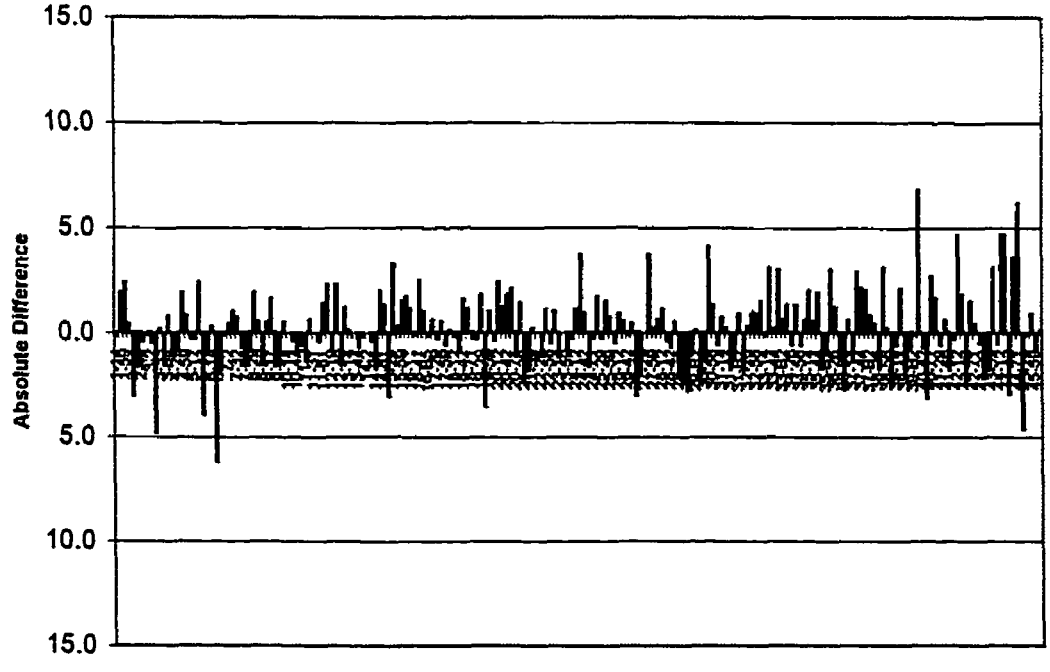
APPENDICES

I. Soil Bulk Density and Field Capacity Calculations

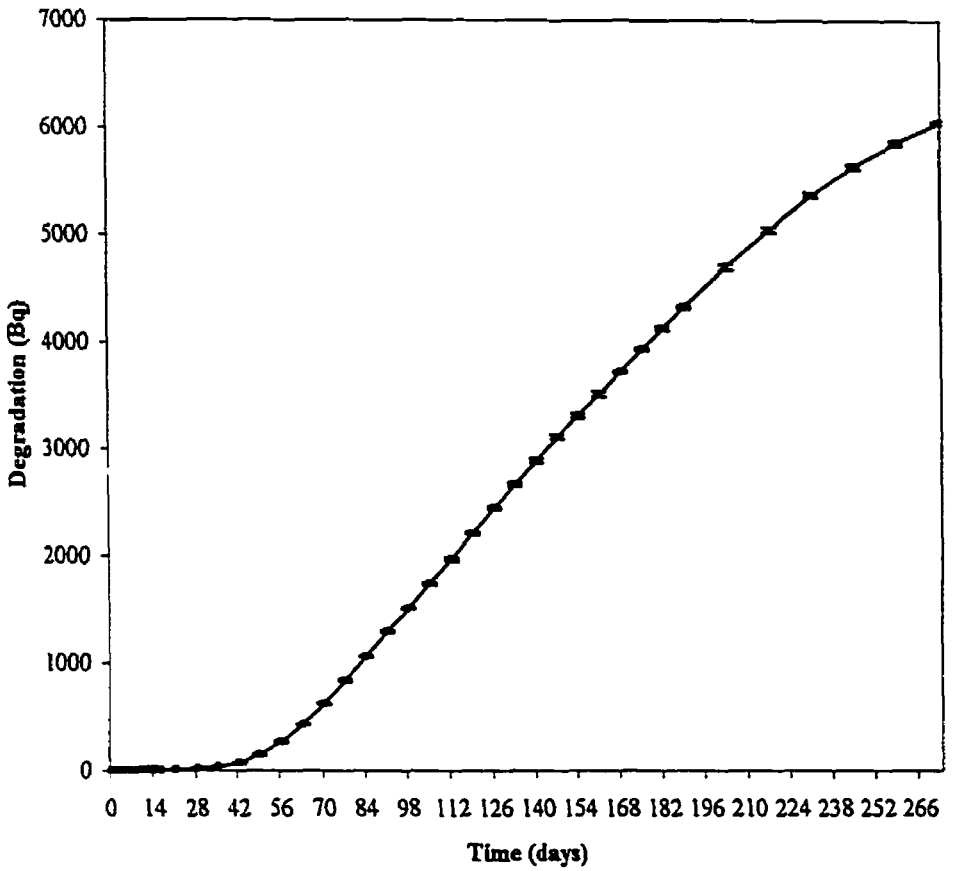
Measurement	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
Time	48 hours	48 hours	48 hours	48 hours	48 hours	48 hours
Determination of Bulk Density						
Soil Height (cm)	13.00	13.00	13.00	13.00	13.00	13.00
Mass Cylinder (g)	79.38	78.78	77.32	77.48	79.37	80.25
Volume Soil (cm3)	147.44	147.44	147.44	147.44	147.44	147.44
Mass Cylinder+AD Soil (g)	251.50	252.84	249.11	245.83	246.14	242.51
Mass AD Soil (g)	172.12	174.06	171.79	168.35	166.77	162.26
Mass OD Soil (g)	161.23	163.05	160.92	157.70	156.22	152.00
Bulk Density (g/cm3)	1.09	1.11	1.09	1.07	1.06	1.03
Mean Bulk Density (g/cm3)						1.08
Determination of Volumetric Field Capacity						
Mass Beaker (g)	59.10	63.51	74.79	109.75	95.69	108.31
Mass Beaker+AD Soil (g)	148.10	129.47	140.17	169.34	164.72	178.84
Mass AD Soil (g)	89.00	65.96	65.38	59.59	69.03	70.53
Mass Beaker+OD Soil (g)	124.30	111.76	122.64	153.02	146.30	159.45
Mass OD Soil (g)	65.20	48.25	47.85	43.27	50.61	51.14
Mass Water (g)	23.80	17.71	17.53	16.32	18.42	19.39
Grav. Water Content (%)	36.50	36.70	36.64	37.72	36.40	37.92
Mean Grav. Water Content (%)						36.98
Volumetric Field Capacity (%)						39.76
Determination of AD Water Content						
Mass Beaker (g)	27.98	27.94	28.55			
Mass Beaker+AD Soil (g)	38.00	39.00	38.43			
Mass AD Soil (g)	10.02	11.06	9.88			
Mass Beaker+OD Soil (g)	37.40	38.28	37.79			
Mass OD Soil (g)	9.42	10.34	9.24			
AD Water Content (%)	6.37	6.96	6.93			
Mean AD Water Content (%)			6.75			

II. Variability in Duplicate Measurements for AutoAnalyzer QA/QC





III. Sample of the standard deviation plots for degradation data in Chapter 4. Error bars show standard deviation of five repetitions for benzo[a]pyrene contaminated soils with L-58 amendment. Size of data points has been reduced to show error bars.



IV. Sample of the standard deviation plots for total respiration data in Chapter 4. Error bars show standard deviation of five repetitions for benzo[a]pyrene contaminated soils with L-67D amendment.