CHANGES IN THE BIOAVAILABILITY OF PHENANTHRENE IN MONTMORILLONITE CLAY WITH AGING

BY

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

MASTER OF SCIENCE

Department of Soil Science
University of Manitoba
Winnipeg, Manitoba

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ABSTRACT

Lanoie, Joanne Marie. M.Sc., The University of Manitoba, February, 2004.
Changes in the Bioavailability of Phenanthrene in Montmorillonite Clay with Aging.
Major Professor; Barbara L. Sherriff.

Changes in the bioavailability of aged phenanthrene in montmorillonite clay were investigated by measuring $^{14}CO_2$ respiration in microcosms and percentage of compound extracted. The biodegradation of phenanthrene in montmorillonite clay was extremely slow with less than 5 % degraded in most microcosms. This indicated that montmorillonite was a poor environment for microbial degradation to occur. Aging decreased the bioavailability of ^{14}C labelled phenanthrene in all treatments (average of 0.1% biodegraded). No significant extraction differences were apparent between aged and non-aged samples when using a dichloromethane shaker extraction or an ASE extraction. Extraction efficiencies were ineffective and ranged from 0 to 12.2 % recovery.

Changes in the structure of phenanthrene in montmorillonite that occurred with time were monitored using NMR and XRD. The ^{13}C CP MAS NMR spectrum of phenanthrene amended clay was better resolved than pure phenanthrene and improved with age. This indicated that changes were occurring to the chemical environment of the carbon and hydrogen with time. A 1ppm change in the ^{29}Si chemical shift indicates that aging of phenanthrene in montmorillonite caused a reduction in shielding of the Si tetrahedra. No changes were observed in the ^{27}Al MAS NMR spectra. XRD indicated that montmorillonite with 0.25 g/g phenanthrene had d-spacings that remained 0.31Å

wider than the control after drying for 3 months. This indicates that the phenanthrene

was preventing the closure of the d-spacing.

Montmorillonite clay strongly sorbs phenanthrene and decrease its bioavailability

and extractability. This phenomenon may be linked to the changes that occur with time

as carbon and hydrogen relaxation rates change, Si becomes less shielded and interlayer

spacings remain larger.

ACKNOWLEDGEMENTS

FOREWORD

The following thesis was prepared using the manuscript format outlined in the

Guide to Thesis Preparation for Graduate Students in the Department of Soil Science.

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CHAPTER 1. INTRODUCTION

Soil remediation decisions are often based on the volume of extractable contaminants detected from a sample, assuming that all of the contaminants are biologically available. Total contaminant concentration or amount extracted is, however, not necessarily an accurate predictor of potential risks. Biological availability (bioavailability) of some organic contaminants change substantially as compounds reside in soil over long periods of time.

Approximately 60% of Canada's polluted sites are contaminated with petroleum hydrocarbons (CCME, 2001). Each year, Canada experiences about 100 oil spills offshore and about 1000 inland. Although most of these spills are small, the majority of spills are caused by human error (Government of Canada, 1991).

Petroleum includes carbon chains, known as the aliphatic fraction, and six membered carbon rings, known as the aromatic fraction. Aliphatic and simple aromatic fractions degrade relatively easily in the environment while polycyclic aromatic hydrocarbons (PAHs) are often much more difficult to degrade. PAHs are produced by the incomplete combustion of organic-carbon during petroleum refining, coal gasification and coal liquefaction (Miller *et al.*, 1998). Many PAHs are carcinogenic and persistent once they have entered the soil/sediment environment and thus are considered to be priority pollutants. When petroleum products enter the environment they may volatilize, be degraded by biota or may sorb to soil and sediments. A portion of these petroleum products may become less available to microbial degradation with time through the

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process of aging. Riley *et al.*, (2001) defined aging as:

> "the process by which lipophilic organic chemicals are incorporated into natural solids (e.g., soils and sediments). The magnitude of the process is influenced by the length of the contact time of the organic compound with the natural solid. Extensive aging results in the accumulation of a resistant fraction of chemical that exhibits decreased bioavailability and slow release to the environment."

The data available in the literature on aging and clay are confounded by the numerous factors involved when comparing whole soils with varying amounts of organic matter, sand, silt, clay and mineral types (Chung and Alexander, 1998; Johnson *et al.*, 2001). The role of clay minerals in bioavailability has not been elucidated conclusively (Chung and Alexander, 1998; Alexander and Alexander, 2000). Some consider organic matter to be the most important factor controlling bioavailability with age (Alexander, 1994), but that clays are important in dry conditions and in soils with low organic content (Hwang and Cutright, 2002).

To test if the presence of clay results in an aging effect with PAHs, experiments were used to examine changes that occurred with time with regards to the degradability (as measured by respiration), extractability, and the structural changes that occur in the clay. To reduce the number of variables, phenanthrene and benzene were sorbed separately onto pure montmorillonite clay.

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CHAPTER 2. LITERATURE REVIEW

2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Petroleum is composed of both aliphatic and aromatic fractions. The solubility of the aromatic fraction of petroleum decreases and stability increases as the number of rings increase in the aromatic molecule (Wilson and Jones, 1993). The octanol-water partitioning coefficient (K_{ow}) and the melting point of aromatic hydrocarbons increase with the number of rings while the vapour pressure and the Henry's law constant decrease (Government of Canada, 1994). Many polyaromatic hydrocarbons (PAHs) are carcinogenic and persistent once they have entered the soil/sediment environment and thus are listed as "priority substances" in Canada (Government of Canada, 1994) and as "priority pollutants" in the United States (Wilson and Jones, 1993).

Phenanthrene, a three ring polyaromatic hydrocarbon (Figure 1.1), and benzene, a one ring aromatic hydrocarbon (Figure 1.2), are two components of petroleum. The difference in the number of rings influences the physical properties of these compounds (Table 2.1) which, in turn, affect how the contaminant moves within the environment and how it is removed by abiotic and biotic processes.

Bay-region

3

4

2

5

13 12

1

6

11

7

10

14

8

9

K-region
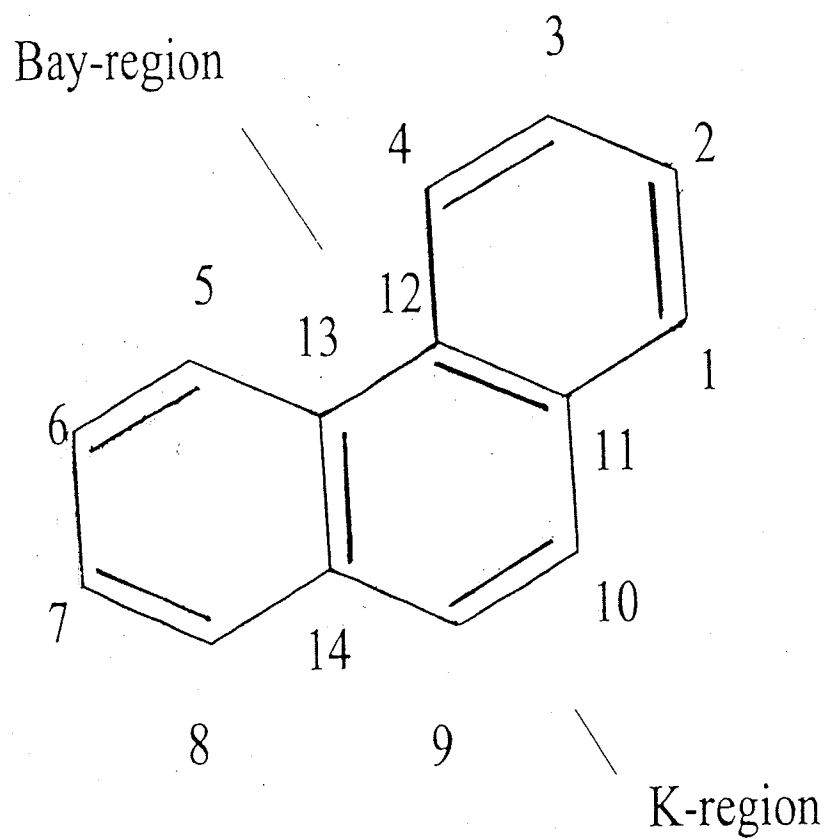
Figure 1.1 Phenanthrene molecule

1

6

2

5

3

4
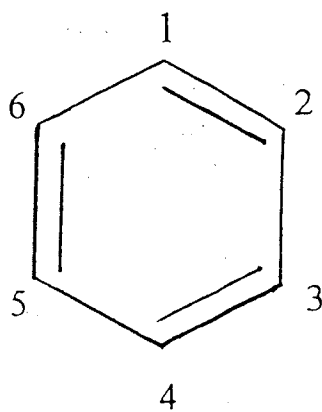
Figure 1.2 Benzene molecule

Table 2.1 Physical properties of phenanthrene and benzene

Physical Property	Phenanthrene	Benzene
Chemical formula	$C_{14}H_{10}$ [a]	C_6H_6 [c]
Molecular weight	178.24 [a]	78.11 [c]
Melting point	95°C [a]	5.5°C [c]
Water solubility at 25°C	1.1 mg/L [b] 3.42 mg/L [a]	180 mg/L [c]
Vapour pressure at 25°C	0.586 atm [a]	0.13 atm [c]
Log K_{OW}	4.5 [a]	2.36 [d]
Width	8.031 Å	6.740 Å
Length	11.752 Å [e]	7.406 Å [e]
Thickness	3.888 Å [e]	3.883 Å [e]
Molecular Volume	169.5 Å^3 [f]	-----
Molecular Surface Area	198 Å^2 [f]	-----

[a]Government of Canada, 1994; [b]Yalkowsky *et al.*, 1998; [c]EPA, 1988; [d]Crittenden and Hand, 1997; [e] Sander and Wise, 2003; [f] Clemson University, 1998.

2.1.1 Source

PAH's, such as phenanthrene, are most likely to be released into the environment from the incomplete combustion of organic compounds including wood and fossil fuels. Industrial sources of phenanthrene include the incomplete combustion of organic-carbon during petroleum refining, coal gasification and coal liquefaction (Miller *et al.*, 1998). Petroleum hydrocarbon spills occur via production plants and facilities, underground storage tanks and tankers. Phenanthrene is a major component of the total PAH content in the environment and is more commonly found in refined oil products than crude oil

(Irwin *et al.*, 1997). Human exposure results primarily from ingestion of food contaminated with phenanthrene (Irwin *et al.*, 1997).

Benzene is a common industrial chemical with 98% being produced in the manufacturing of styrenes, phenols, cyclohexanes and other organic chemicals. It is also used for making detergents, pesticides, solvents and paint removers. The US coast guard emergency response notification system reported that in 1993 benzene was one of the most frequently reported non-petroleum spill (Irwin *et al.*, 1997). In gasoline, benzene represents less than 2% of the total volume yet ambient air in heavy traffic and filling stations is a major source of human exposure to benzene (Irwin *et al.*, 1997) and leaking underground storage tanks contaminate many aquifers with benzene. Cigarette smoke, vehicle emissions and natural gas dehydrators are the major anthropogenic sources of benzene. The average Canadian ambient levels of benzene in 1998 were 0.3-1.0 $\mu g/m^2$ in rural areas and 1.0-3.2 $\mu g/m^2$ in urban areas (CCME, 2000). Although benzene is very volatile, it is one of the most soluble components of petroleum and can be a significant hazard in ground water (Irwin *et al.*, 1997).

2.1.2 Health Concerns

Acute toxicity is rarely reported as a result of exposure to low levels of a single PAH compound and thus is more frequently associated with chronic risks such as cancer (Irwin *et al.*, 1997). Health effects normally result from exposure to complex mixtures of chronic-risk aromatics such as PAHs and benzenes. The toxicity of petroleum appears to

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be a function of its di-aromatic and tri-aromatic hydrocarbons (Irwin *et al.*, 1997). Phenanthrene has been found to be toxic to marine diatoms, gastropods, mussels, crustaceans, and fish (Ellis *et al.*, 2001). It was once thought to be carcinogenic to humans but now it is believed to be neither mutagenic nor carcinogenic to people (Ellis *et al.*, 2001). Phenanthrene has been declared a carcinogen for modelling purposes by the Environmental Protection Agency (EPA) in the United States (Irwin *et al.*, 1997) since it is the smallest aromatic hydrocarbon to have a "bay-region" and a "K-region"(Ellis *et al.*, 2001) (Figure 1.1) and since the structure is found in carcinogenic PAHs such as benzo[a]pyrene, benz[a]anthracene, and 3-methylcholanthrene.. Epoxide intermediates formed in the bay region are considered the carcinogenic form of PAHs that pose a health risk (Kenneth and Hollenstein 1998; Government of Canada, 1994).

Benzene is a carcinogen and a non-threshold toxicant which means that there is some probability of harm for critical effects at any level of exposure (CCME, 2000). It can be inhaled, ingested or absorbed through the skin. Occupational exposures have been shown to cause an increase in nonlymphocytic leukemia and can have a 2-50 year latent period (Irwin *et al.*, 1997). Benzene causes acute irritation of the mucous membranes, producing restlessness and convulsions which may cause respiratory failure that can lead to death. Benzene may also cause central nervous system disorders and chromosome aberrations (Irwin *et al.*, 1997). The adult lethal oral dose of benzene has been estimated at 15 ml to 30 ml (Irwin *et al.*, 1997). Its carcinogenic properties are thought to be caused by the metabolite 1,2,4-benzenetriol which forms a semiquinone radical and active oxygen while its mutagenic properties come from the primary metabolite, benzene oxide

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(Irwin *et al.*, 1997). Benzene is not persistent in the environment nor is it bioaccumulative (CCME, 2000).

2.2 Hazards, Exposure and Risk

Petroleum hydrocarbon spills onto soils are common throughout much of the world. The risk associated with these spills is a combination of exposure and hazard. A compound, such a phenanthrene, has an intrinsic toxicity or hazard, yet only if exposure occurs will there be a risk to people or the environment. To reduce or eliminate risk, the hazard is often removed to eliminate exposure. This normally occurs by physically removing the soil from the site and/ or remediating it. Remediation can be costly and take a long time when soils have a high clay content, have been contaminated for long periods of time and are contaminated with high molecular weight polyaromatic hydrocarbons. At equal concentrations, the exposure and risk of a sequestered toxicant is less than if it were fully available (Chung and Alexander 1998). Therefore, it is important to understand the process by which sequestering occurs and the resultant changes in bioavailability.

2.3 Remediation Criteria

The Canadian Council of Ministers of the Environment (CCME) has produced a three-tier approach to the remediation of contaminated land. Tier-1 is based on numerical criteria (Table 2.2). If the contaminant is over the specified concentration, then the site requires remediation. Tier-2 allows for site-specific adjustments to Tier-1 levels based on environmental characteristics found on-site. Tier-3 requires a site-specific risk assessment to examine the specific pathways and interactions the contaminants will have within a particular environment. According to the CCME, site-specific remediation objectives should be developed using risk assessment if "site conditions, receptors and/ or exposure pathways differ significantly from those assumed in the derivation of guidelines" (CCME, 1999). Manitoba still prefers to use the criteria based Tier 1 system for contaminated sites (pers. comm., Lemoine, 2002). If contaminants have reduced bioavailability then a Tier 3, site-specific risk assessment may be advisable. It is necessary to understand and predict the movement and disappearance of contaminants when performing a risk assessment.

Table 2.2 Canadian environmental quality guidelines

Land Use	Phenanthrene	Benzene
	mg/kg	
Agricultural	0.1	0.05
Residential-parkland	5	0.5
Commercial	50	5
Industrial	50	5

CCME 1999.

2.4 Petro-Canada's Soil Recycling Facility

The Petro-Canada Soil Recycling Facility (Figure 2.1 and 2.2) commenced operation in the fall of 1993 and was operated until its decommissioning in the fall of 2001. The facility consisted of a contaminated stockpile, a treatment cell, a remediated stockpile, and a sump connected to a retention pond. Contaminated soil underwent aeration by spreading and mixing with a backhoe to facilitate volatilization and aerobic biodegradation. Nutrients were added as required to stimulate the microbial population and remediated soil was used as backfill or cover material. As part of the decommissioning process, the soil was remediated to agricultural land use guidelines. The clay rich soil under the facility may have had extended contact with contaminants and some issues were raised about the effectiveness of remediation during the decommissioning and remediation of the site.

Figure 2.1 Petro-Canada Soil Recycling Facility. The backhoe is aerating the soil to promote aerobic biodegradation and volatilization.

Figure 2.2 Petro-Canada Soil Recycling Facility. Remediated soil is stored until it is used as a backfill material on an industrial site.

2.5 Environmental Fate of Phenanthrene and Benzene in the Environment

2.5.1 Non-Biological Transformations

Volatilization is the most common fate of low molecular weight compounds, such as benzene, but is expected to be insignificant for higher molecular weight compounds such as phenanthrene (Irwin *et al.*, 1997). Conversly, research has indicated that long range transport of phenanthrene may be appreciable and occurs though volatilization dependent on temperature (Dimashki *et al.*, 2001). Volatile phenanthrene is expected to adsorb rapidly to particulate matter and may photolyze or be subject to wet and dry deposition. The half-life of sorbed phenanthrene on ash undergoing photolyzation is 49 hours (Irwin *et al.*, 1997). The vapour phase of phenanthrene will react with photochemically generated, atmospheric hydroxyl radicals with a half-life of 1.67 days (Irwin *et al.*, 1997). In an aqueous solution, phenanthrene will adsorb to suspended sediments and will be removed from solution. Photolysis occurs at the surface of water and biodegradation within the water column. Phenanthrene binds strongly to soil and is not expected to leach into groundwater. Oxidation, bioconcentration (Irwin *et al.*, 1997) and hydrolysis (Sims and Overcash, 1983) is insignificant. Once phenanthrene enters the environment, microbial biodegradation is its most probable fate.

Benzene is not expected to be persistent in the environment due to its tendency to volatilize and biodegrade. In water, benzene volatilizes with a half life of 5.23 hours in a wind-wave tank with an artificial wind speed of 7.09 m/s and has a biodegradation half

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life of 16 days in an aerobic river test (Irwin *et al.*, 1997). Once in the atmosphere, benzene undergoes photo-oxidation whereby it reacts with hydroxyl radicals with a half life of 13.4 days or it can be washed out by rain. In cold or nutrient deficient soils photodegration is important and has a half life of 17 days (Irwin *et al.*, 1997) while the portion remaining in soil is expected to leach into groundwater where it could be more persistent.

2.5.2 Biodegradation

Biodegradation can be defined as the biologically catalysed reduction in complexity of chemical compounds. Organic compounds can be converted into less complex organic chemicals but are more often converted into inorganic products such as CO_2. In the biological sciences this is known as mineralization. However, since the geological reference for mineralization is the formation of solid inorganic minerals, which would not include CO_2 formation, the term ultimate or complete biodegradation will be used in this thesis.

The initial oxidation of PAHs may be the rate limiting step for degradation which may explain the absence of detectable PAH intermediate degradation products in soil and sediments (Carmichael *et al.*, 1997). Once the initial oxidation has occurred, the degradation products become available to a larger portion of the microbial community.

2.5.2.1 Phenanthrene Biodegradation

Phenanthrene biodegradation can occur through both bacterial (Figure 2.3 and 2.4) and fungal (Figure 2.5) pathways. In an aerobic environment, bacteria metabolization of phenanthrene most often occurs with initial dioxygenation at the 3,4 position and then metabolized to 1-hydroxy-2-naphthoic acid where it can be degraded by oxidizing to 1,2-dihydroxynaphthalene which is then metabolized via salicylic acid (Pinyakong *et al.*, 2000) through the naphthalene pathway (Ellis *et al.*, 2001). In the phthalate pathway, the 1-hydroxy-2-naphthoic acid undergoes ring-cleavage and then is metabolized by o-phthalic acid and protocatechuic acid (Pinyakong *et al.*, 2000 and Ellis *et al.*, 2001). Dioxygenation can occur at the 1,2 or 3,4 positions of phenanthrene (Pinyakong *et al.*, 2000). The fungal metabolism of phenanthrene uses monooxygenases and epoxide hydrolases and involves oxidative attack at the K-region (Figure 1.1). Anaerobic degradation, once thought not possible, has now been shown to occur (Rockne and Strand 1998), although the degradation is 1-2 orders of magnitude slower than aerobic conditions (Rockne *et al.*, 1998; McNally *et al.*, 1998).

2.5.2.2 Benzene Biodegradation

Benzene tends to be persistent under anaerobic conditions although anoxic benzene biodegradation has been demonstrated using multiple species (Harwood and Gibson, 1997). Aerobically, benzene degradation first involves the oxidation of benzene to cis-dihydrobenzene-diol (Figure 2.6) which is then turned into a catechol.
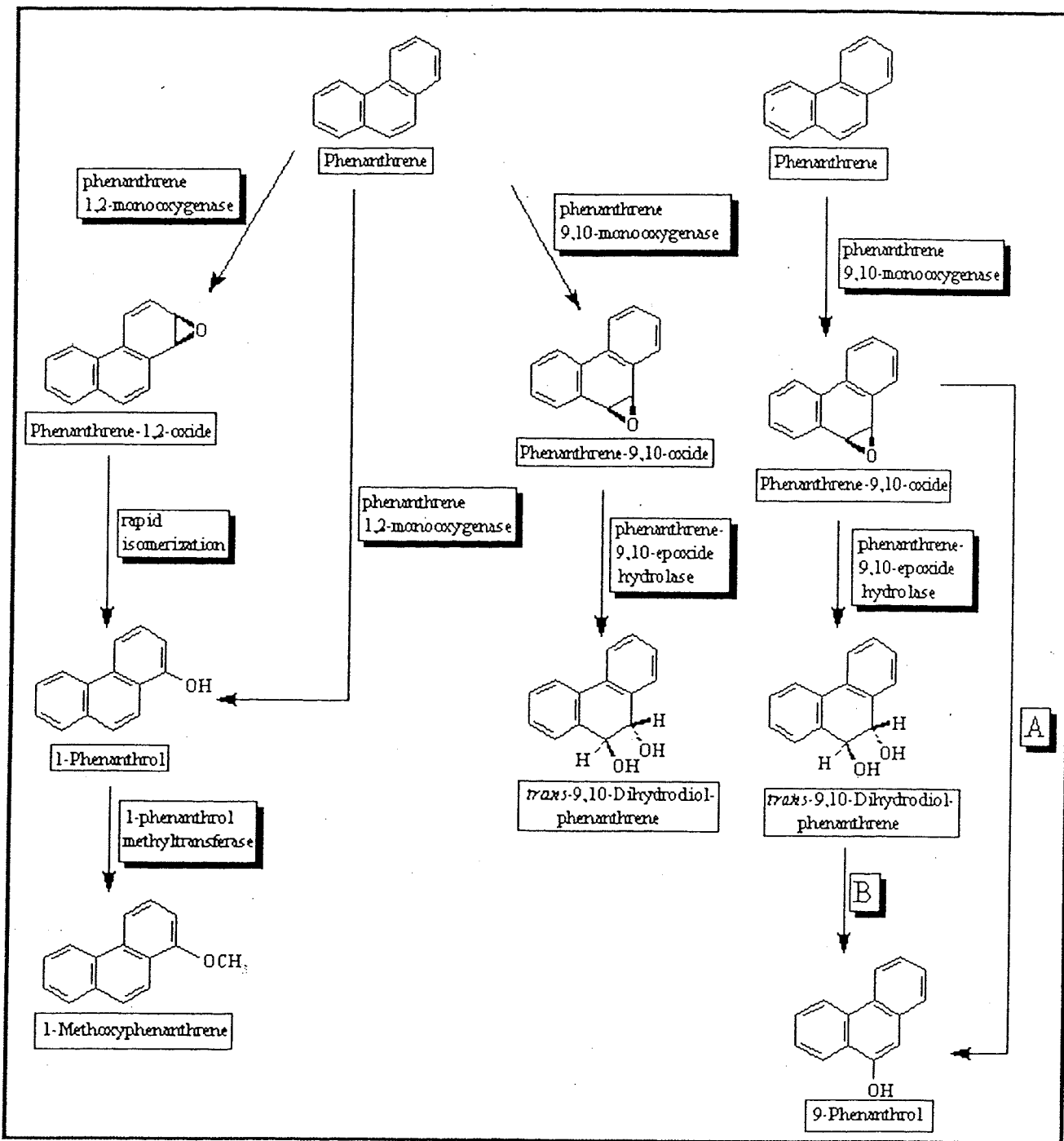
Figure 2.3 Possible bacterial pathways for phenanthrene biodegradation. With permission from Ellis et al 2001, The University of Minnesota Biocatalysis/Biodegradation Database.
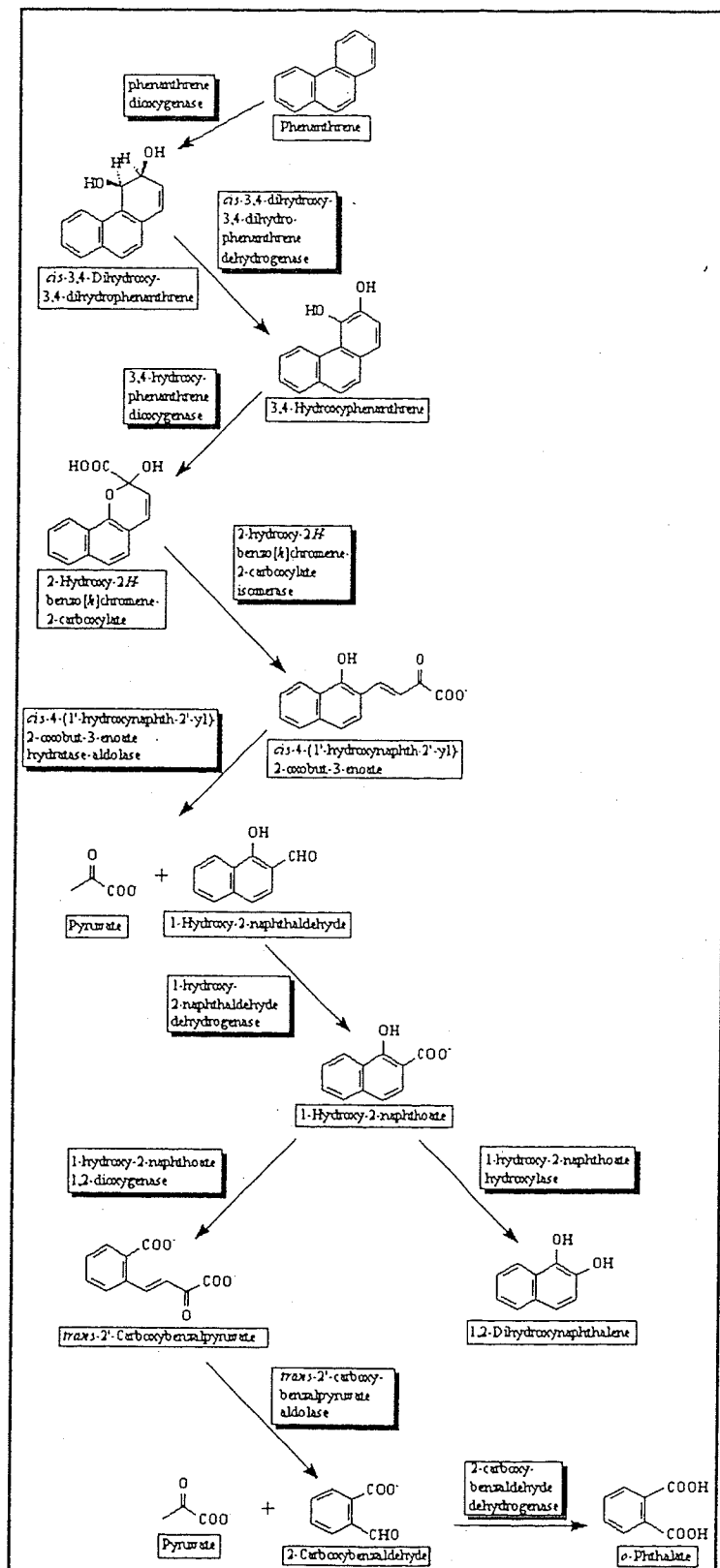
Figure 2.4 Possible bacterial pathways for phenanthrene biodegradation. With permission from Ellis et al 2001, The University of Minnesota Biocatalysis/Biodegradation Database.
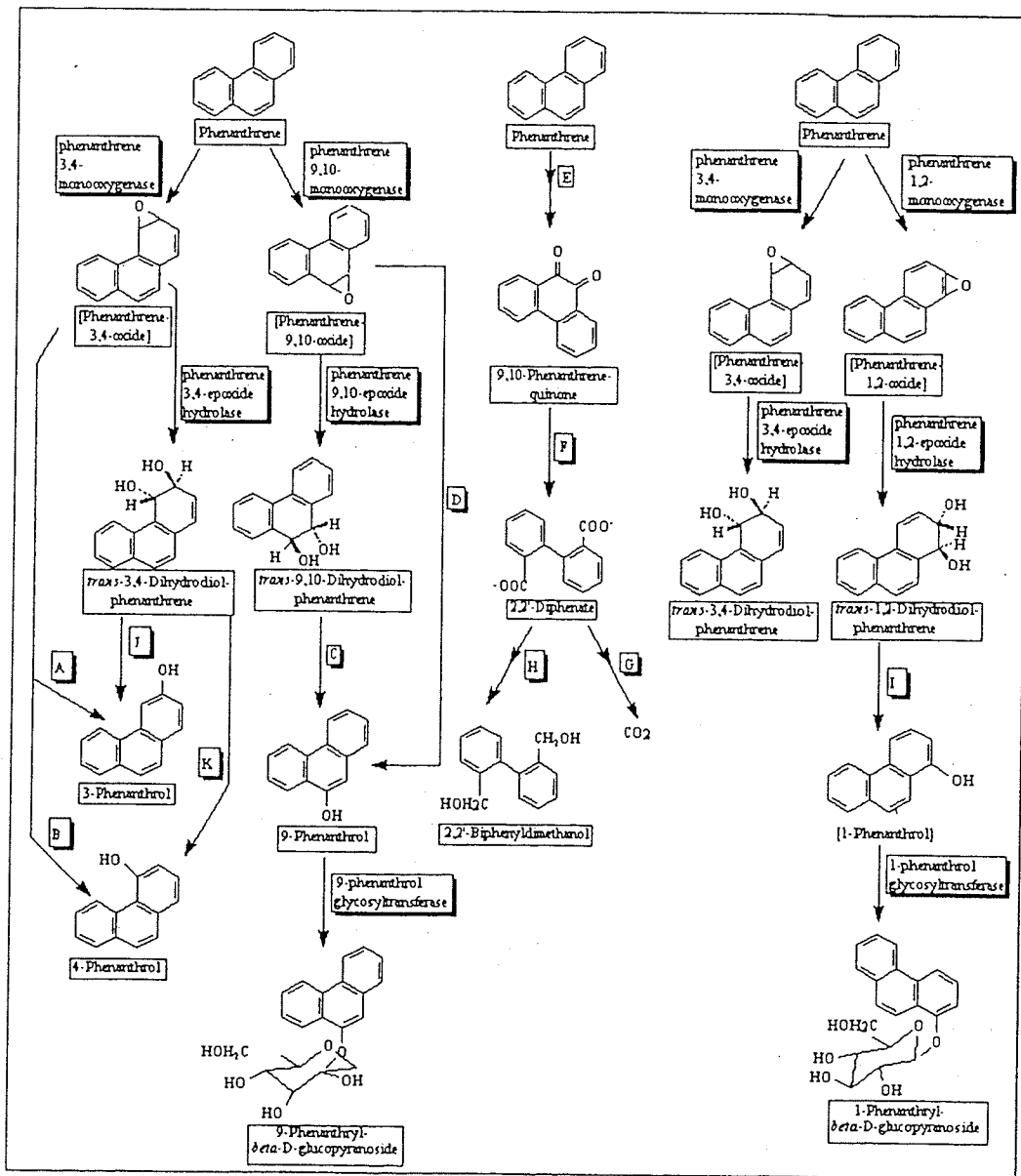
Figure 2.5 Possible fungal pathways for phenanthrene biodegradation. With permission from Ellis et al 2001, The University of Minnesota Biocatalysis/Biodegradation Database.
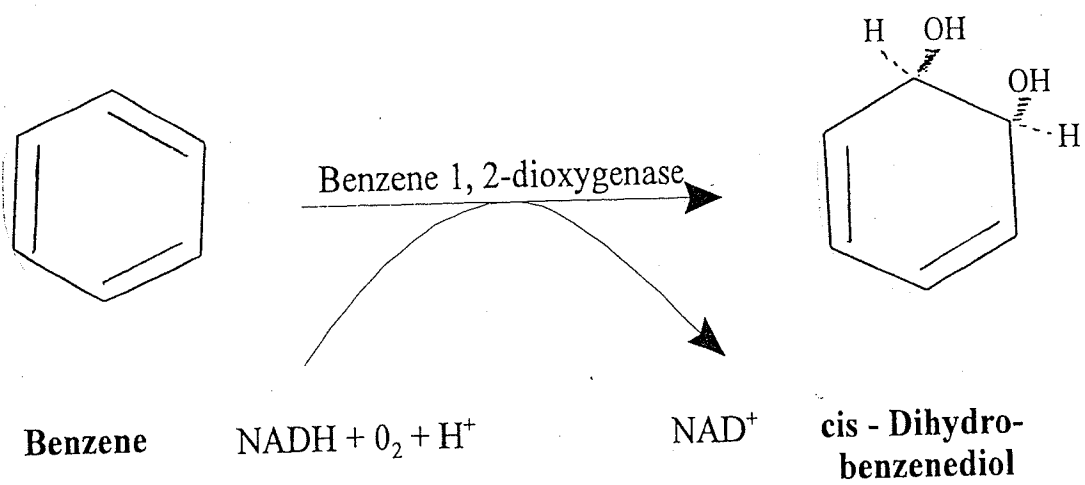
Figure 2.6 Oxidation pathway of benzene to cis-dihydrobenzene-diol

The catechol can then degrade via numerous pathways depending on the microbial population (Ellis *et al.* 2001). Four possible products are pyruvate, acetaldehyde, formate or cis,cis-muconate as shown in Figure 2.7.

In the human body, benzene is primary oxidized in the liver to phenol (hydroxybenzene), catechol (1,2-dihydroxybenzene) or quinol (1,4-dihydroxybenzene). These are then conjugated and excreted in urine (Irwin *et al.*, 1997).

2.5.3 Humification

Complete degradation of a contaminant to CO_2 may not be possible due to organic matter (OM) formation. Ressler *et al.* (1999) found a dark-coloured residual fraction of dissolved organic matter (DOM) and particulate organic matter (POM) that comprised about 10% of the carbon from individual PAHs or 20% from a mixture of seven PAHs. They found that the OM produced was highly resistant to bacterial degradation and had a molecular size distribution similar to natural humic acids. Excretions of metabolites during degradation were identified as known precursors for humification (Ressler *et al.*, 1999). Phenolic compounds and their degradative intermediates, which resemble naturally occurring humic acids, have decreased toxicity and bioavailability through OM production (Bollag, 1992).

```
Benzene
   |
   |
   |
cis-Dihydro-benzene-diol
   |
   |
   | cis-1,2-dihydrobenzene-1,2-diol dehydrogenase
   |
   |
   Catechol-------------------------------------------------+
   |                                                         |
   |                                                         |
   | catechol 2,3-di-oxygenase             | catechol 1,2-dioxygenase
   |                                                         |
   |                                                         |
2-Hydroxymuconic semialdehyde            cis,cis-Muconate
   |
   | 2-hydroxy-muconate semialdehyde hydrolase
   |-----------------------------------------------+
   |                                               |
   |                                               |
cis-2-Hydroxy-                               Formate
penta-2,4-dienoate
   |
   |
   | 2-oxopent-| 4-enoate hydratase
   |
   |
   |
4-Hydroxy-2-oxovalerate
   |
   |
   | 4-hydroxy- 2-oxo-valerate aldolase
   |----------------------+
   |                      |
   |                      |
Pyruvate              Acetaldehyde
```

Figure 2.7 Four possible biodegradation pathways of benzene. Modified with permission
from Ellis *et al*. 2001, The University of Minnesota Biocatalysis/Biodegradation
Database.

2.6 Factors Affecting the Rate of Hydrocarbon Degradation

Factors that affect the rate of hydrocarbon degradation include the amount of oxygen, water, and nutrients available as well as pH, temperature, cometabolism or competitive metabolism, initial PAH concentration and the prior exposure of the microbial community to the PAH. Under anaerobic conditions, unsubstituted PAHs are extremely resistant to microbial degradation (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Since soil pores are either filled with air or water, the optimal soil moisture content for degradation is 30-90 % of water holding capacity (Wilson and Jones, 1993) resulting in an oxygen rich, aerobic, environment. Aerating the soil increases phenanthrene degradation, particularly when phosphorous is also added (Manilal and Alexander, 1991). The addition of phosphorus does not increase the total amount of phenanthrene degraded, but instead reduces the time required to reach maximum biodegradation (Manilal and Alexander, 1991). Petroleum spills create high carbon to phosphorus and nitrogen ratios, which can be compensated for by adding nutrients.

The pH of a soil will greatly influence the composition of the microbial community. Fungi are more tolerant of acidic conditions than bacteria. At low pH, fungi have a competitive advantage for growth whereas a pH value between 7 and 8 is favourable for bacteria (Wilson and Jones, 1993).

Optimal degradation occurs at a temperature range of 20 to 30°C (Wilson and Jones, 1993). As temperatures increase, the vapour pressure and solubility of PAHs increase and adsorption to soil particles decrease. The rate of enzymatic activity is also

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dependent on temperature. The effect that temperature has on enzymatic activity is called the Q_{10} effect (Sylvia, 1998). According to Q_{10}, for every 10°C change in temperature there is a multiple factor by which a reaction rate will change. Thus a Q_{10} of 2 means that a reaction rate will double with an increase of 10°C. There is a limit to the effectiveness of increasing temperature beyond the optimal as enzymes denature and become ineffective.

Cometabolism and competitive metabolism can be important influences on biodegradation. Cometabolism is the metabolic transformation of a substance while a second substance serves as primary energy or carbon source (BioTech Resources, 1998). Competitive metabolism occurs if microbes prefer to degrade one compound rather than another. In this case, one compound may have to be completely degraded before the degradation of the other begins.

The rate of degradation is usually directly proportional to the initial concentration of the contaminant in the soil exhibiting first-order degradation kinetics. Thus degradation may be very slow at low concentrations (Aust, 1990). The onset of contaminant biodegradation normally follows a period of adaptation or acclimation of indigenous microbes, the length of which depends on the contaminant structure. Prior exposure to a contaminant through repeated spills will create an environment where the biodegradation pathway is maintained within an adapted community (Maier *et al.*, 2000). Adaptation most commonly occurs by the induction of enzymes required for biodegradation followed by an increase in the population of organisms capable of degrading that compound (Maier *et al.*, 2000). Where previous exposure may not have

occurred and analogous materials do not exist, genetic mutation or gene transfer may be involved in adaptation to develop new metabolic abilities. These abilities may take weeks or years to develop once exposure has occurred (Maier *et al.*, 2000).

2.7 Bioavailability

The American Heritage Dictionary of the English Language (1992) defines bioavailability as "The degree to which a drug or other substance becomes available at the physiological site of activity after administration." Bioavailability can be visualised as an organism having access to a substance in the environment and can change depending on the form and location of the substance. Factors affecting bioavailability of a substance to an organism include the biochemical activity of the organism and the mass transfer of the chemical to the microorganism (Bosma *et al.*, 1997). Adding nutrients or bioaugmentation can increase the intrinsic activity of the microbial cells but cannot increase the mass transfer and thus cannot increase bioavailability (Bosma *et al.*, 1997). Bioavailability is controlled by physical and chemical processes such as sorption, desorption, diffusion, and dissolution (Bosma *et al.*, 1997). Adsorption-desorption kinetics are major factors in determining the bioavailability of a compound for degradation (Wilson and Jones, 1993).

In most studies of bioavailability, the solution is analysed, not the solid phase. Bioavailability can be underestimated when sampling the solution (Hwang and Cutright,

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2002) as organisms in contact with a sorbed contaminant may have a larger exposure of extended duration and increased frequency than in a single aqueous or solvent extract (Alexander and Alexander, 2000). Thus, studying spiked soil samples is required to understand bioavailability changes that occur within the soil environment.

2.7.1 Sorption to Soil Particles

Sorption is a general term for either adsorption or absorption. Adsorption refers to the temporary retention of solutes by the surfaces of a solid material while absorption refers to the longer term retention within the mass.

The sorption of a volatile aromatic hydrocarbon has been shown to occur onto clay minerals in the following three phases; a gas-like labile phase, a liquid-like sorption phase and a resistant to desorption phase. These phases have sorption/desorption time scales of minutes, hours and weeks, respectively, for toluene (Chang *et al.*, 2003). The initial phase of sorption is rapid as the hydrophobic adsorbate associates with hydrophobic mineral surface areas of the soil. The second phase is much slower as the organic compound is transported to less accessible hydrophobic sites within the soil matrix. Sorption will increase over time until an equilibrium is reached and all the sites are occupied.

Inhibition of biodegradation, when compounds are sorbed hydrophobically, is demonstrated by the inability of microorganisms to metabolize certain sorbed PAHs (Weissenfels *et al.*, 1992). Sorption can alter the availability of organics, change the

levels of organic and inorganic nutrients, modify pH, render inhibitors less toxic, retain microorganisms, bind extracellular enzymes, and make the micro-environment on the surface less favourable for microbes (Alexander 1994). Conversely, sorption may concentrate nutrients at the surface so that the growth of organisms may be enhanced and biodegradation stimulated, especially if the bulk solution has a low concentration of nutrients (Alexander 1994). Active surfaces include clay minerals, organic or humic substances, complex carbonaceous matter, amorphous iron, and aluminum oxides and hydroxides (Alexander, 1994). Sorption is influenced by pH, temperature, major cations, exchange capacity, surface area, and the type and quantity of clay minerals, organic matter and solutes in solution (Alexander 1994).

Cationic organic molecules may be sorbed to clays and humic surfaces. Anionic compounds are poorly sorbed by clay minerals but may be retained by organic surfaces. The complex organic matter of soil is often the chief sorbent for non-ionic organic compounds (Alexander, 1994). Cumulative sorption can occur when dissolved soil organic matter (SOM) first interacts with the soil, thus creating new sorption sites onto which the PAH sorbs (Abdul *et al.*, 1990).

Sorption to SOM is responsible for increasing the time required for biodegradation (Manilal and Alexander, 1991), yet Carmo *et al.* (2000) found that Freundlich coefficient isotherms deviated from linearity, indicating another sorption mechanism in addition to partitioning into soil organic matter. They also found that there was not a systematic trend with specific surface area (Carmo *et al.*, 2000). Nonlinear sorption isotherms may imply that adsorption rather than absorption is occurring (Murphy

et al., 1990).

It has been proposed that sorption includes both partitioning into organic matter and adsorption onto mineral surfaces (Karickhoff, 1997; McCarty *et al.*, 1981). At low organic carbon fractions (F_{oc} = 0.0002), mineral effects become important for hydrophobic organic compounds with log octonal/water partition coefficients (K_{ow}) < 4 (Curtis *et al.*,1986). Both soil organic matter and clay minerals can have significant effects on sorption when the organic matter content of the soil is less than 6% (Murphy *et al.*, 1990; Hwang and Cutright, 2002). Clay minerals sorbed 12.2% more phenanthrene than pure organic matter, based on equivalent solid mass (Hwang and Cutright, 2002). As well, sorption of hydrophobic compounds to clay minerals has been found to dominate in dry conditions whereas the sorption to soil organic matter is the main interaction for saturated soils (Murphy *et al.*, 1990; Hwang and Cutright. 2002).

The sorption of a humic acid for hydrophobic organic compounds varies for kaolinite and hematite. This may indicate that the orientation and structure of the humic substance on the mineral may affect the surface area and the accessibility of hydrophobic sites that control sorptivity (Murphy *et al.*, 1990). It has been suggested that organics can fit between the silicate sheets of the clay mineral and thus become protected (Morrill *et al.*, 1982).

The properties of the soil and compound will determine if the compound is bound by ionic or covalent bonds, or adsorbed by Van der Waals attractions, hydrogen bonding, charge transfer or hydrophobic bonding (Bollag and Loll, 1983; Morrill *et al.*, 1982). Adsorption is considered easily reversible unless covalent bonds have been formed

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(Bollag, 1992). If the formation of stable covalent linkages occurs, the result is a new chemical species (Alexander, 1994). Hydrogen bonding on clay surfaces is important for large molecules but ion exchange is more important for low molecular weight organic compounds (Morrill *et al.*, 1982; Chung and Alexander, 1998).

2.7.2 Desorption

Clay minerals and SOM effect the desorption of PAH's. Clay minerals have been shown to decrease the desorption of pyrene by 65% compared to pure SOM, yet phenanthrene desorption was inhibited by adding dissolved SOM (Hwang and Cutright, 2002). In an experiment comparing pure clay to pure SOM, the desorption-resistant fraction was thought to be a result of PAHs binding to clay minerals since the fraction bound to SOM was easily desorbed (Hwang and Cutright, 2002).

Desorption is concentration and time dependent. First-order desorption rates decrease with decreasing concentrations of applied phenanthrene (Johnson *et al.*, 2001) by an order of magnitude (Hwang and Cutright, 2002). Aging decreased desorption by a factor of 2 but was unaffected beyond 3 months. Desorption from subsoil was 1-2 orders of magnitude lower than from contaminated shales (Johnson *et al.*, 2001).

The more reduced and condensed organic matter is, the greater the sorption-desorption hysteresis. This reduces the desorption rate and decreases the bioavailability of the contaminant sorbed to the organic matter (Lueking *et al.*, 2000).

2.7.3 Mechanisms of Utilization of Sorbed Compounds by Organisms

There are two hypotheses to explain the mechanism of utilization of sorbed compounds by organisms. The first is that organisms use the compound in solution and subsequent degradation is governed by the rate of spontaneous desorption into the aqueous phase. The second hypothesis is that microorganisms excrete metabolites that facilitate desorption so that the rate of biodegradation is greater than the rate of spontaneous desorption in sterile conditions. This may involve the use of surfactants that release the compound from the surface, cations that displace charged compounds, extracellular enzymes that utilize sorbed compounds or microbiologically induced changes in the surface pH (Liu *et al.*, 1991; Alexander, 1994). High concentrations of most synthetic surfactants are needed to bring hydrophobic compounds into aqueous solution, but low concentrations of two non-ionic alcohol ethoxylate surfactants have been shown to enhance the rate and/or extent of phenanthrene degradation significantly (Aronstein *et al.*, 1991; Aronstein and Alexander, 1992).

Thomas and Alexander (1987) speculated that microbes may directly utilize compounds which adhere to the surface, bringing them into the cell without dissolution. In this way, molecules may be fully available, partially available or completely resistant to degradation. Sorption often reduces the rate and extent of biodegradation but does not necessarily prevent it (Alexander, 1994).

Extracellular enzymes may slowly metabolize compounds sorbed within the lattice of clays or may sorb onto reactive surfaces and lose catalytic activity as is the case for the enzyme pronase in montmorillonite (Alexander, 1994). The availability of a

substrate acted on by extracellular enzymes, may be diminished or become negligible because of reduced accessibility if shielded from the catalyst. Steric effects may also prevent the formation of the substrate-enzyme complex necessary for catalysis. While extracellular enzymes are important for large molecular weight compounds, low molecular weight compounds are believed to be metabolised via intracellular enzymes.

2.8 Aging of Contaminants in Soils

Biodegradation of ^{14}C phenathrene in heavy fuel oil on kaolinite has been shown to drop from 20% to 6% after 2 months of aging (Dictor *et al.*, 2003). Chung and Alexander (1998) found a statistically significant reduction in bioavailability after 20 days of aging phenanthrene in a variety of soils. Sequestration was not significantly increased from 120 to 200 days and was considered to have ceased at this time or was occurring extremely slowly. The reduction in biodegradation was attributed to diminished bioavailability, although conclusions were not reached about causality (Chung and Alexander, 1998). Reduced pyrene biodegradation has been observed after 120 days of aging (Guthrie-Nichols et al, 2003) and decreases in biphenyl bioavailability have been shown to be most pronounced within the first 80 days of aging (Feng *et al.* 2000). Soil column experiments show that the highest contaminant retardation corresponds to the longest aging times (Thomsen *et al.*, 1999).

Sequestration occurs at both low and high concentrations but the percentage of

both aged and unaged compounds susceptible to microbial degradation increases with

contaminant concentration (Chung and Alexander, 1999).

2.9 Sorption Capacity of Clays

Non expanding 1:1 clays such as kaolinite have smaller surface areas and a lower

capacity for sorption than expandable 2:1 clays. Expandable 2:1 clays such as

montmorillonite have structures that can expand, allowing organic molecules, inorganic

ions and water to penetrate between the layers (Figure 2.8). Therefore, while adsorption

occurs only on the surface and on broken and frayed edges of 1:1 clays it may also occur

on internal surfaces of expandable 2:1 clays. Interlayer ions also influence sorption.

Weakly hydrated cations (K^+ and Cs^+) result in greater sorption of dinitrophenol

herbicides than more strongly hydrated cations (Na^+ and Ca_2^+) (Sheng et al., 2002).

The total amount of expandable clay, smectite and vermiculite, correlate well with

the rate and extent of phenanthrene and pyrene degraded and may govern substrate

bioavailability (Hwang and Cutright, 2003). Absorption of cesium occurs in two stages

in expandable clays with an initial rapid uptake being followed by a slower penetration

into the interlayer (Ejeckam, 2003). The adsorbed cations are separated from the clay

surfaces by water molecules, moving freely throughout the interlayer, and exchanging

rapidly between surface-bonded and hydrated species (Sullivan et al., 1998).

Si tetrahedral

Al octahedral

Interlayer space
filled with water
and cations
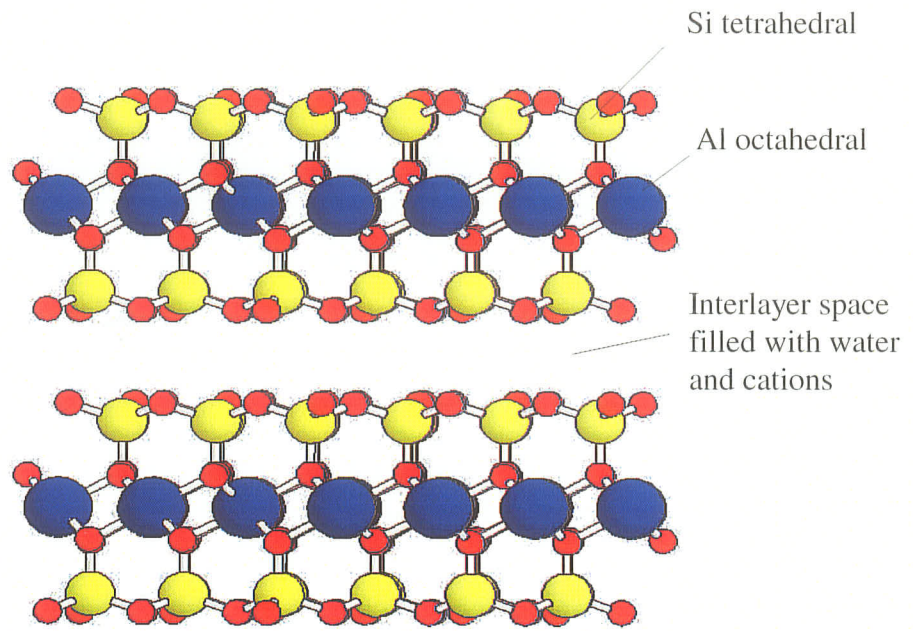
Figure 2.8 Montmorillonite structure

A clay mineral with metal cations in the interlayer exchange sites, tends to be hydrophilic and not a good adsorbent for poorly water-soluble organic species, which cannot compete with highly polar water molecules. When the interlayer cations are replaced by alkylammonium cations, the surfaces become strongly organophilic (Ogawa *et al.* 1995). Being hydrophobic, alkylammonium-exchanged clays have a greater propensity for sorbing non-ionic organic compounds such as PAHs, than the unmodified materials (Jaynes and Boyd, 1990). Alkylammonium-smectites, such as montmorillonite saturated with tetradecyltrimethylammonium (TDTMA), sorb 20-30 wt. % of phenanthrene. These alkylammonium-smectite clays have the potential to contain and entrap PAHs in contaminated sites. It is believed that intercalated phenanthrene is no longer biodegradable (Theng *et al.*, 2001) due to hydrophobic interactions (Ogawa *et al.*, 1995).

2.10 Extractions

Mild extractions have been used to estimate bioavailability while other extractions are used to remove all of the remaining PAH. Numerous different extractions have been used including water, $Na_2P_2O_7$ solution (Tsao *et al.*, 1998), dichloromethane (Guthrie and Pfaender, 1998; Northcott and Jones 2001), methanol (Northcott and Jones, 2001), acetone-pentane mixture (Jonge *et al.*, 1999), ethanol (Chung and Alexander, 1998), and n-butanal (Alexander and Alexander, 2000; Northcott and Jones, 2001). Different solvents have different efficiencies of extraction depending on the substance being extracted, the matrix, agitation, temperature, and duration. Sequential soil extractions

have also been used (Kelsey *et al.*, 1997; Guthrie and Pfaender, 1998; Northcott and Jones, 2001).

Harsh or complete extraction techniques remove nearly all of the compounds from a sample. Soxhlet is a widely used extraction technique (Banerjee and Gray, 1997; Tsao *et al.*, 1998; Tang *et al.*, 1998; Chung and Alexander, 1998; Smith *et al.*, 1999; Reid *et al.*, 2000; Alexander and Alexander, 2000; Käcker *et al.*, 2002) that meets EPA standards. Soxhlet is time consuming and requires large volumes of solvent. A newer method of extraction, Accelerated Solvent Extraction (ASE™) with Dionex Application Note 313 meets the requirements of U.S. EPA Method 3545. Using this method, Dionex (date unknown) was able to extract 19.2 % more phenanthrene from soil than the Soxhlet method. ASE is faster and uses less solvent than Soxhlet and has similar or greater efficiencies.

Research has shown that the amount of a compound extracted with a mild extraction decreases in aged soils (Feng *et al.*, 2000; Smith *et al.*, 1999; Chung and Alexander, 1998) and is correlated with bioavailability (Kelsey *et al.*, 1997; Chung and Alexander, 1998). Chung and Alexander (1998), however, did not observe a strong correlation between compound extractability and bioavailability among 16 soils tested, suggesting that the rates and extent of declined availability to a particular solvent and to bacteria occur at different rates making it difficult to find just one surrogate assay (Chung and Alexander, 1998). Research in finding surrogate assays is increasing. If residues are non-extractable with organic solvents, they can be considered a long term sink in the environment (Richnow *et al.*, 1995). The stability of these complexes and the possibility

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of future release of these pollutants depends on the type of pollutant, soil conditions, microbial activity, and binding mechanisms. The release of bound residues is gradual and is thought to occur via microbial activity (Bollag, 1992). After 168 and 300 days, extractable phenanthrene and fluoranthene were still detected by ^{13}C NMR, yet no metabolites could be identified. It was thought that the polarity of the degradation products enabled them bind to organic matter quickly and thus they could not be extracted (Käcker *et al.*, 2002).

2.11 Digestions

Complete digestion of a soil, sediment or sludge is often used to determine the total volume of an element in a sample. Aqua regia digestion (HCl and HNO_3) ISO standard 11466, is used extensively in Europe (Quevauviller *et al.*, 1993;Vercoutere *et al.*, 1995) to estimate the maximum element availability to plants (Vercoutere *et al.*, 1995). Aqua regia, however, leaves residual elements bound to silicate minerals. Nitric acid-perchloric acid digestion is recommended for P, K, Ca, Mg, S, Al, Se, and the trace elements, with the exception of B, and extracts all trace elements from SiO_2 (Carter, 1993). Hydrofluoric digestion (HF) is used for the decomposition of silicate rocks, usually in combination with nitric, perchloric or sulphuric acid. This dissolves all of the fluorine and silica leaving a residue of alkali metals, alkaline earths, iron, aluminium, titanium, manganese, and phosphorus to be dissolved in dilute acid. Acid resistant

minerals such as zircon, topaz, corundum, sillimanite, tourmaline, chromite, rutile and barium sulphate may remain as residues (Jeffery and Hutchison, 1981). Nitric acid is often added prior to HF digestion to decompose carbonate minerals, oxidise sulphides and organic matter and to convert iron and other elements into their higher valence states (Jeffery and Hutchison, 1981).

It is important to note that measuring the recoverable compounds from a sample, rather than completely digesting the sample, is becoming common in environmental studies (Kane, 1995, Chen and Ma, 1998). Sample preparation techniques for total analysis that use acid digestion and thermal decomposition can result in losses, incomplete recoveries and sample contamination (Smith, 1994; Smith and Arsenault, 1996). Open system digestions can introduce atmospheric contamination and volatilization losses during the oxidation of organic substances (Kubrakova, 1997; Quevailler et al., 1993).

2.12 CO_2 Capture in Microcosms

$^{14}CO_2$ evolution has been used to monitor the degradation of many PAHs including ^{14}C-phenanthrene (Reid et al., 2000; Smith et al., 1997) and ^{14}C-benzene (Tao et al., 1998). Degradation experiments are often studied in microcosms that consist of a glass jar containing an atmosphere, a matrix (such as soil), a community of microorganisms, and the substrate that is being monitored. Radiolabelled compounds are

biodegraded into $^{14}CO_2$, which can be measured by injecting a portion of the gas in the head space directly into a gas chromatograph (Smith *et al.*, 1997) or by trapping the $^{14}CO_2$ within an alkali trap such as sodium hydroxide (NaOH). $^{14}CO_2$ gas dissolves in an aqueous NaOH solution to form bicarbonate (HCO_3^-) (Equation 2.1). Adding acid causes the reaction to reverse and releases CO_2 from the water (Equation 2.2). Thus, a container of acidified water can be added to the microcosm to humidify the atmosphere without absorbing CO_2 from the atmosphere.

$$CO_2 + Na^+ + OH^- \rightarrow HCO_3^- + Na^+$$ **Equation 2.1**

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O$$ **Equation 2.2**
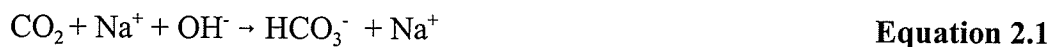
Some species of bacteria or fungi may not produce CO_2 directly from the substrate in question; however, the organic products excreted by one species will likely be converted to CO_2 by another species. These species are then consumed by protozoa. Therefore, in the presence of O_2, the net effect is still of complete biodegradation to CO_2 (Alexander, 1994).

2.13 Nuclear Magnetic Resonance Spectrometry

Nuclear magnetic resonance spectrometry (NMR) measures the energy of a nucleus associated with a magnetic moment interacting with an applied magnetic field. An electromagnetic pulse generates a small magnetic field which excites the sample. When the pulse is removed, the nucleus releases energy as it relaxes to its original ground state. More frequent pulses selectively receive information from nuclei that relax within a short time frame, excluding the slower relaxing nuclei. Iron, protons, and water can speed the relaxation of ^{13}C nuclei allowing slower relaxing nuclei to relax faster.

Magic Angle Spinning (MAS) involves the rotation of a powdered sample at an angle of 54.7° to the magnetic field. This removes many of the nuclear interactions in solids which would produce broad spectra which are difficult to interpret. Solid state, MAS NMR has been used extensively to study cations in phyllosilicates (Weiss et al., 1990; Kirkpartick, 1998; Sullivan, 1998; Liang and Sherriff, 1993; Ejeckam, 2003). ^{29}Si and ^{27}Al MAS NMR spectra of Pb sorption in vermiculite have indicated more shielding after Pb exchange than before, indicating that Pb affected the local chemical environments of the SiO_4 and AlO_4 tetrahedra. There were no observable peak changes for montmorillonite, hectorite or kaolinite with Pb exchange and thus the Pb exchange was remote from the SiO_4 and AlO_4 tetrahedra (Liang and Sherriff, 1993). ^{29}Si MAS NMR also indicated clay minerals becoming more shielded with Cs absorption (Ejeckam, 2003). ^{133}Cs MAS NMR indicated that Cs adsorbs rapidly onto the oxygens in an outer sphere of water and then slowly bonds to the oxygen atoms of the SiO_4 tetrahedral layer

(Ejeckam, 2003).

Cross polarization (CP) links ^{13}C and ^{1}H nuclear spins allowing ^{13}C to relax at the rate of ^{1}H thus enhancing the signal strength of the slow relaxing less abundant ^{13}C. ^{13}C CP MAS NMR has been used to study the interactions of PAHs and soil organic matter revealing non-covalent associations (Guthrie et al., 1999). There has also been evidence of covalent bonding with organic matter. Of the phenanthrene metabolites produced from ^{13}C labelled phenanthrene, 50-70% were ester-bound via their carboxyl groups to organic matter (Käcker et al., 2002). The higher polarity of PAH metabolites makes them much more able to form covalent bonds to soil organic matter (Käcker et al., 2002). ^{13}C CP NMR has also been used to monitor aromatic carbon degradation (Selifonov et al., 1998). If the ^{13}C label remains in the aromatic structure, whether it is in its original state or as a metabolite, it will give an NMR signal at about 125 ppm. If the aromatic structure is broken, the NMR signal will change. For example, carboxylic carbon will give an NMR signal between 160-190 ppm (Käcker et al., 2002). ^{13}C CP MAS NMR has also been used to monitor the sorption of 2-phenethylamine onto clays (Bank et al., 1993) and to determine the structure of organic carbon in peat (Johnson et al., 2001), shales and soils (Lueking et al., 2000).

2.14 X-ray Diffraction

Crystal structure refinements of clay minerals and monitoring of ion exchange processes with X-ray diffraction (XRD) are difficult due to fine grain size, structural disorder, platy nature, and highly mobile exchangeable cations. In fact, there are no crystal structure refinements for many clay minerals such as members of the smectite family (Güven, 1998). XRD can, however, be used to determine changes in the thickness of the interlayer of smectites. An interlayer spacing of 12 to 12.5 Å was maintained with 2 dinitrophenal herbicide amended smectite clays independent of the presence of water. The amendments were interacting with the opposing clay layers, minimizing contact with water (Sheng *et al.*, 2002).

The thickness of the interlayer of tetradecyltrimethylammonium (TDTMA) montmorillonite was compared to that of phenanthrene and TDTMA montmorillonite in a solid-solid reaction with XRD using air-dried aqueous suspensions of clay on glass slides. Intercalation of phenanthrene altered the orientation of TDTMA chains in the interlayer space as was indicated by the change from 1.8 to 3.4 nm in the basal spacing (Theng *et al.*, 1998). The thickness of an individual silicate layer is 9.6 Å (Theng, 1975).

CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

All chemicals were purchased from Fisher Scientific, Fairlawn, New Jersey and were HPLC or GC purity unless otherwise stated.   Phenanthrene-9- [14]C (13.1 mCi/mmol, 1.4 mg, 100 μCi) and benzene-UL-[14]C (6.9 mCi/mmol, 0.874 mg, 100 μCi) were purchased from Sigma Chemical Company.

In order to add solid radioactive phenanthrene to the clay, 998.6 mg analytical grade phenanthrene and 1.4 mg ^{14}C-phenanthrene were initially mixed with 200 ml dichloromethane to give a final concentration of 5.0 mg phenanthrene /ml dichloromethane with a radioactivity of 1 x 10^6 disintegrations per minute (DPM)/ml. Liquid scintillation counting (LSC) indicated that 924,630 DPM/ml was achieved. The 0.874 mg/μl of ^{14}C-benzene was mixed with 3 ml analytical grade benzene to give a final radioactivity of 7.4 x 10^7 DPM/ml based on LSC.

Calcium montmorillonite clay (Texas Montmorillonite Stx-1) was purchased from the Source Clay Minerals Repository, University of Missouri-Columbia, MO, USA. The 2:1 clay (Figure 2.8) was selected due to its expandable nature, high ion exchange capacity and relevance to expandable clays found in Manitoba. The sample locality is the Manning Formation, Jackson Group (Eocene), Gonzales County, Texas, USA. It is a dioctahedral clay with Mg^{2+} in the octahedral layer. The tetrahedral layer of Si^{4+} is

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partially substituted by Al^{3+} creating an excess charge of -0.68. The total surface area is 83.79 m^2/g and it has a cation exchange capacity (CEC) of 84.4 meq/100g. The gravimetric water content of the air dried montmorillonite at 20°C is 0.154 (+/-0.00082). Montmorillonite is formed by alteration of volcanic ash or basic igneous rocks (Deer et al., 1998). The chemical composition is given in Table 3.1 and the structural formula, as given by the Clay Society Repository, is:

$$(Ca_{0.27} Na0_{.04} K_{0.01})[Al_{2.41} Fe^{3+}_{0.09} Mn_{trace} Mg_{0.71} Ti_{0.03}][Si_{8.00}]O_{20}(OH)_4$$
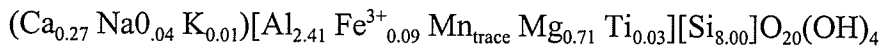
Table 3.1 Chemical composition of montmorillonite clay

Source	SiO_2	Al_2O_3	TiO_2	Fe_2O_3	FeO	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5
a	70.1	16	0.2	0.65	0.2	Trace	3.69	1.6	0.27	0.1	0
b	66.1	14.2	0.2		0.6	0	3.35	1.5	0.29	0.1	0

a. Clay Society Repository information b. Analysis performed using ICP-OES at the U of M.

3.2 Inoculum

Industrial soils taken from the Petro-Canada Soil Recycling Facility (south perimeter, Winnipeg, Manitoba) and the MidCanada Soil Remediation Facility (Ile des Chenes, Manitoba) were used to prepare the inoculum. While sampling, preference was given to fuel contaminated soils visually identified as having a high clay content (type, quantity and age of clay and fuel were not analysed). Composite mixing of the samples

allowed the greatest microbial variation. To ensure that the contaminated soil could be used to produce an active inoculum for future experiments, water was added periodically to prevent desiccation and to provide a favourable microbial environment.

A basic nutrient solution was prepared (Table 3.2), which could be stored for up to one year in a sealed container at room temperature without precipitation. This was used to prepare the inoculum (Table 3.3). The inoculum was produced by mixing 100 g industrially contaminated soil, 10 ml basic nutrient solution, and 0.15 g analytical phenanthrene and bringing the volume to 1 L with double distilled water. The inocula were placed on a platform shaker for 7 days to produce an active microbial consortium.

Table 3.2 Basic nutrient solution

Mineral	Amount g/L
NaCl	80
NH_4Cl	100
KCL	10
KH_2PO_4	10
$MgSO_4 \cdot 7H_2O$	20
$CaCl_2 \cdot 2H_2O$	4
double distilled water	make up to 1 L

Table 3.3 Inoculum

Component	Amount per L
basic nutrient solution	10 ml
phenanthrene	0.15g
industrially contaminated soil	100g
double distilled water	make up to 1 L

3.3 Most Probable Numbers

Most probable number (MPN) determinations of contaminant degrading bacteria were conducted in the Department of Soil Science. The MPN procedure is a statistical method of estimating the number of culturable organisms in the soil that are able to use a certain energy source. The organisms are diluted to extinction and evaluated as to whether a positive sign is present. Published tables are used to interpret the results (Pepper *et* al,. 1995). The MPN indicates the highest soil dilution that will still provide growth in a suitable medium (Paul and Clark, 1996).

Three samples of industrially contaminated soils were used in which no nutrients were added prior to the MPN testing. For each soil sample, 10 g of soil were transferred into a 90 ml dilution blank, shaken for 30s, rested for 30s, and shaken for another 30s. Dilutions were made by transferring 1 ml portions of 10^{-3} through to 10^{-8} dilutions, in

replicates of five, into MPN tubes containing 9 ml of Bushnell-Haas Broth with resazurin, which was then incubated for 7 days in the dark. A positive result was indicated by a change in colour representing the reduction of resazurin by microbial oxygen consumption.

3.4 Microcosms

All microcosm treatments were conducted in sets of four in the Department of Soil Science. Montmorillonite clay (5 g) was measured into 20 ml glass beakers and brought up to approximately field capacity by adding 1.55 ml deionized water (31 wt %). The beakers were then autoclaved on the liquid cycle to minimize water losses, with a sterilization time of 40 min at 121°C and an exhaust time of 11 min. The clay was then allowed to sit for 24 hours to allow for any spore activity to occur before a second autoclaving. Just prior to the second autoclaving, the phenanthrene dichloromethane solution was added and the dichloromethane allowed to volatilize under a flowhood to reduce microbial contamination.

The phenanthrene amended clay was placed into a 50 ml jar and placed within a 2 L glass mason jar. An alkali trap, consisting of 5 ml 1 M NaOH, was added to a 15 ml scintillation vial, placed within a 20 ml beaker to prevent spillage and added to the mason jar. 5 ml acidified water (pH 2) was added to a 20 ml beaker and placed within the mason jar to keep the microcosm environment humid. The acidified water was covered with aluminum foil to prevent the acidic water vapour, produced during autoclaving, from

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condensing onto the mason jar, alkali trap or clay containing beaker. The mason jars were then autoclaved as one unit. Note that the clay has been autoclaved twice, 24 hours apart, to increase the chances of eliminating spore forming microbes. Ambersorb™ (0.5 g) was added to the sterilized microcosms under the flowhood in pre-sterilised 20 ml beakers. The ambersorb had been previously pasteurised at 50°C for 24 hours to remove any volatile components that may have been present and to reduce the chances of microbial contamination. Autoclaving the ambersorb would have destroyed its ability to trap volatile hydrocarbons. The complete microcosm is shown in Figure 3.1. Four control (sterile) microcosms contained only amended clay, acidified water and an alkali trap, sterilized as one unit and not opened until the end of the first experiment. The sterile samples were then used as the aged samples for the next experiment.

The benzene microcosms followed the same procedure except that the ^{14}C benzene was added under a flowhood following the second autoclaving and was sterilized by a syringe filter, pore size 1 μm. While adding the benzene to the surface of the montmorillonite clay, the liquid became contaminated. The ^{14}C benzene was accidentally sucked into the rubber bulb used to pipette the liquid and contaminated the remaining benzene. A separation of phases occurred, indicating at least one other liquid has been introduced into the system. The contaminated benzene was used despite this unknown contaminant which could have been radioactive. Samples were taken from the bottom to avoid the upper layer which was visibly different in colour and density than the original liquid. The final sample taken had a radioactivity of 3,984,927 DPM/ml. The addition of the benzene solution resulted in approximately 797,000 DPM/g in the clay.

Ambersorb to trap volatile hydrocarbons

5ml of 1M NaOH in scintillation bottle to capture CO_2

5 ml acidified water to humidify the environment while not capturing CO_2

^{14}C radio-labelled phenanthrene contaminated montmorillonite clay
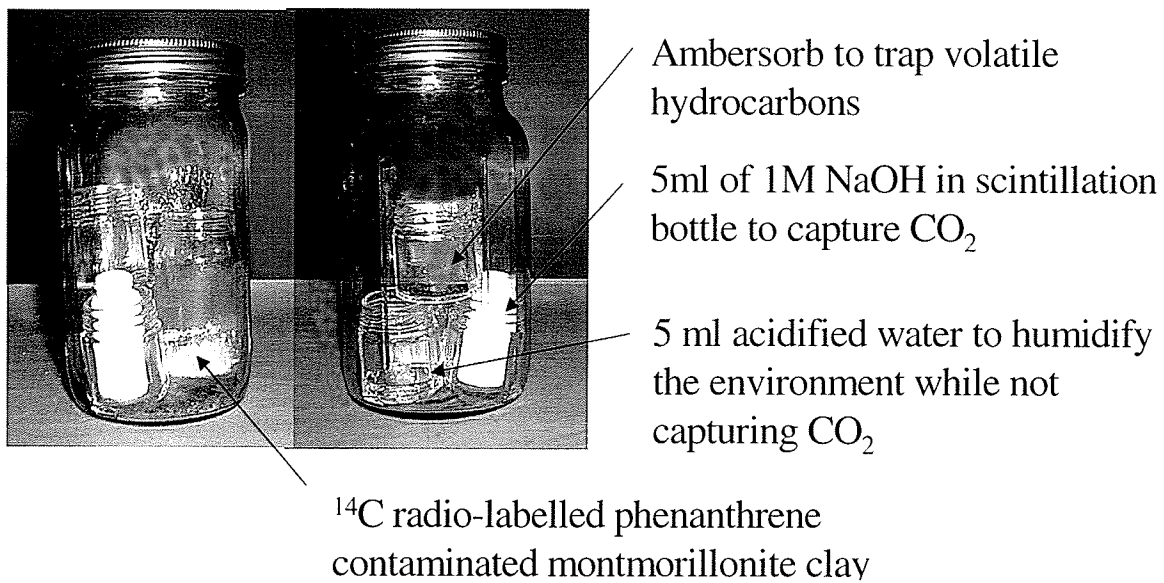
Figure 3.1 Microcosm apparatus

To start Experiment A, either 1 ml of the inoculum (A_{PIN}, A_{BIN}) or 1 ml of the basic nutrient solution (A_{PNN}, A_{BNN}) was added as different treatments to the amended clay to determine if inoculation was required for biodegradation to occur (Table 3.4). It was thought at the time that these two additions would have the same volume of water and concentration of nutrients (See Appendix I). The microcosms were incubated in the dark at 21°C. Degradation was measured as the radioactive respiration of $^{14}CO_2$ in the alkali traps which were periodically removed and replaced with fresh traps. For each sample, the sealed jars created an enclosed environment that allowed the labelled compound to be confined within the system. After the traps were removed from the microcosms, 10 ml of 30% Ecolite Scintillation cocktail (ICN Biochemical Inc. Aurora, OH) was added and the vials were placed in the dark for approximately 24 hours to dissipate chemoluminescence. The level of radioactivity was determined as DPM using a Beckman LS 7500 scintillation counter, and used to calculate the percentage of phenanthrene or benzene that had degraded to $^{14}CO_2$ during the incubation period.

After 70 days of the experiment, there was no significant degradation of phenanthrene. High amendment concentrations may have been inhibiting microbial degradation or temperatures may not have been optimal since a malfunction in the incubator caused a spike in both the temperature and the $^{14}CO_2$ trapped. Experiment B was started using the same procedure as Experiment A but with only 0.5 ml phenanthrene solution and incubated at 31°C. To try to increase the degradation rate, a new treatment of occasional mixing was included and thus treatments consisted of inoculated (B_{PIN}), non-inoculated (B_{PNN}) and inoculated but mixed occasionally (B_{PINM}).

Table 3.4 Microcosm treatments (in replicates of four)

Experiment	Temp (°C)	Matrix	Amendment	Inoculated/ non-inoculated	New/aged/ sterile	Duration (days)	Code*
Objective: To age microcosms and to determine the rate and/or extent of degradation in non-aged microcosms. Inoculated and non-inoculated treatment of phenanthrene and benzene were started to determine if inoculation was required for degradation.							
A	21	Montmorillonite	Phenanthrene	Inoculated	New	361	A_{PIN}
A	21	Montmorillonite	Phenanthrene	Non-inoculated	New	361	A_{PNN}
A	21	Montmorillonite	Phenanthrene	Non-inoculated	Sterile	204	A_{PNS}
A	21	Montmorillonite	Benzene	Inoculated	New	361	A_{BIN}
A	21	Montmorillonite	Benzene	Non-inoculated	New	361	A_{BNN}
A	21	Montmorillonite	Benzene	Non-inoculated	Sterile	204	A_{BNS}
Objective: To determine if the rate of degradation could be increased by increasing the temperature and decreasing the initial concentration of phenanthrene. Benzene experiments were not included (no more ^{14}C labelled benzene). An additional treatment was included where the clay was periodically mixed to promote biodegradation.							
B	31	Montmorillonite	Phenanthrene	Inoculated	New	289	B_{PIN}
B	31	Montmorillonite	Phenanthrene	Non-inoculated	New	289	B_{PNN}
B	31	Montmorillonite	Phenanthrene	Inoculated- mixed	New	289	B_{PINM}
B	31	Montmorillonite	Phenanthrene	Non-inoculated	Sterile	132	B_{PNS}

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Objective: To determine if the slow rate of biodegradation in experiments A and B were occurring due to a problem inherent in the method or if it was caused by the microbial environment within the clay.

| C | 21 | Industrial soils | Phenanthrene | Inoculated | New | 142 | C_{PIN} |

Objective: To determine if aged microcosms had lower biodegradation than newly amended microcosms under the same conditions as listed in experiment A. An additional treatment was included where the clay was not autoclaved prior to the experiment to determine if autoclaving the phenathrene amended montmorillonite clay hindered biodegradation.

D	21	Montmorillonite	Phenanthrene	Inoculated	New	157	D_{PINX}
D	21	Montmorillonite	Phenanthrene	Inoculated	Aged	157	D_{PIA}
D	21	Montmorillonite	Phenanthrene	Inoculated	New	157	D_{PIN}
D	21	Montmorillonite	Phenanthrene	Non-inoculated	New	157	D_{PNN}
D	21	Montmorillonite	Benzene	Inoculated	Aged	157	D_{BIA}

Objective: To determine if aged microcosms had lower biodegradation than newly amended microcosms under the same conditions as listed in experiment B.

E	31	Montmorillonite	Phenanthrene	Inoculated	New	157	E_{PIN}
E	31	Montmorillonite	Phenanthrene	Inoculated	Aged	157	E_{PIA}
E	31	Montmorillonite	Phenanthrene	Non-inoculated	Aged	157	E_{PNA}
E	31	Montmorillonite	Phenanthrene	Non-inoculated	New	157	E_{PNN}

* Code is comprised of up to five parts: 1. Experiment letter (A, B, C, D or E); 2. Amendment - phenanthrene (p) or benzene (b); 3. Inoculated (i) or non-inoculated (n); 4. New (n), aged (a) or sterile (s); and 5. Optional - occasionally mixed (m) or not sterilized prior to experiment (x).

At 204 days for experiment A and 130 days for experiment B, the sterile controls (A_{PNS}, A_{BNS}, B_{PNS}) were tested for $^{14}CO_2$ and restarted as "aged" samples within Experiments D and E by adding 1 ml of freshly prepared inoculum (D_{PIA}, D_{PIA}, E_{PIA}) or 1 ml of the basic nutrient solution (E_{PNA}) with the same experimental procedure as above. Newly amended treatments of inoculated (D_{PIN}, D_{PINX}, E_{PIN}) and non-inoculated (D_{PNN} E_{PNN}) microcosms were prepared and the original microcosms (A_{PIN}, A_{PNN}, A_{BIN}, A_{BNN}, B_{PIN}, B_{PNN}, B_{PINM}) were continued (Table 3.4).

Experiment C was started to determine if degradation rates improved with the use of clayey soils with previous exposure to hydrocarbons as compared to pure montmorillonite clay. The ^{14}C-phenanthrene microcosm was started using the same procedures as above but containing a composite sample of Petro-Canada and Mid-Canada soils instead of the montmorillonite clay (C_{PIN}) (Table 3.4).

Degradation rates continued to be low for experiments A and B. A plausible explanation was that there was insufficient nutrients for microbial degradation to occur. The inoculant added to the microcosms may have been leached of nutrients during the process of mixing the solution with the nutrient deficient industrially contaminated soils. Dr. Racz, an expert in the field of nutrients, suggested that the non-inoculated microcosms (A_{PNN}, A_{BNN}, B_{PNN}, D_{PNN}, E_{PNA}, E_{PNN}) required twice the amount of phosphorous than originally added and that the inoculated microcosms (A_{PIN}, A_{BIN}, B_{PIN}, B_{PINM}, D_{PINX}, D_{PIA}, D_{PIN}, D_{BIA}, E_{PIN}, E_{PIA}) required both nitrogen and phosphorous. Therefore 1 g KH_2PO_4 was added to 100 ml double distilled water to make 10 g/l stock solution. Of this, 20 ml was then diluted to 1000 ml with double distilled water and 0.5

ml aliquots were carefully administered to each of the non-inoculated microcosms, distributing the solution evenly over the surface of the amended clay. This doubled the amount of phosphorus in the system. Then 0.2 g of KH_2PO_4 was added to 20 ml of the original basic nutrient solution and added to the inoculated microcosms in 0.5 ml aliquots evenly over the surface of the previously inoculated clays. These additional nutrients were added at day 220 for experiments A, day 148 for experiments B, and day 18 for experiments D and E.

3.5 Aqueous Sorption

Hwang and Cutright (2002) found that thermodynamic equilibrium of aqueous sorption of phenanthrene took 144 hours. Therefore batch equilibrium sorption experiments were performed in replicates of four, over periods of 7 days, to quantify the sorption of ^{14}C labelled phenanthrene from water to montmorillonite clay.

Several attempts were made to produce an aqueous solution with adequate radioactivity. The final solution was produced by transferring 1ml of phenanthrene stock solution to a small glass vial which was placed under a fume hood to allow the dichloromethane to volatilize. Once the dichloromethane volatilized, the glass vial and its contents were added to 1L deionized water in a 1L flask, placed on a magnetic stirrer and heated for 12 hours. Part of the solution was then transferred to 50 ml Teflon tubes and centrifuged for 10 minutes at 10,000 RPM to remove flocculent phenanthrene from

the solution. 5 ml of this phenanthrene solution was added to 1g montmorillonite clay in 50 ml Teflon centrifuge tubes resulting in 67.4 DPM per tube. The tubes were placed in a rotary shaker for 7 days to reach equilibrium. The clay slurries were then centrifuged at 10,000 RPM for 10 minutes. A 1 ml sub-sample was taken from each tube and placed in a 15 ml scintillation vial with 10 ml of 30% Scintisafe solution (Fisher Scientific, Fairlawn, New Jersey) to measure the phenanthrene concentration remaining in solution. The samples were stored in the dark for 24 hours to reduce chemoluminescence and the radioactivity was quantified with a Beckman LS 7500 scintillation counter (LSC). The radioactivity detected was subtracted from the radioactivity initially applied to give the total ^{14}C phenanthrene sorbed by the soil constituents, calculated as a percentage of the original radioactivity applied. The sorption distribution coefficient, Kd [ml g^{-1}] was calculated assuming linear partitioning (1/n = 1) according to the following equation:

Kd = [sorbed]/[solution]   **Equation 3.1**

where: [sorbed] = the concentration of phenanthrene sorbed by montmorillonite at equilibrium (mg ml^{-1}) and [solution] = the concentration of phenanthrene in solution at equilibrium (mg ml^{-1}).

3.6 Extractions

Phenanthrene and benzene were extracted from the microcosm clay using a dichloromethane shaker extraction in the Department of Soil Science to mimic the bioavailable fraction of the amendment remaining in the clay. After the microcosm studies were complete, 50 ml dichloromethane was added to the clay which was shaken on a platform shaker for 2 hours. Surrogate spikes were also included. A 5 ml subsample was then pipetted into a 15 ml scintillation vial with 10 ml of 30% Scintisafe solution. The samples were stored in the dark for 24 hours to reduce chemoluminescence and radioactivity was quantified with a LSC.

After the mild extraction was complete, phenanthrene and benzene was further extracted using an Accelerated Solvent Extraction (ASE™) method at the Department of Microbiology to remove the remaining amendments from the clay. The amendments removed by ASE, but not by the mild dichloromethane extraction, would be considered to be less bioavailable and thus more difficult to degrade. A Dionex ASE 200 Accelerated Solvent Extractor was used with 11-ml stainless steel extraction cells. The ASE procedure used was that described in Dionex Application Note 313, meeting U.S. EPA Method 3545. Samples were mixed in equal proportions with anhydrous sodium sulfate (Fisher Scientific). A cellulose disk was placed at the outlet end of each extraction cell. Samples were then added to the cells and filled with Ottawa sand (Fisher Scientific) leaving approximately 1 cm headroom at the top of the cell. The collection vials were capped with septa and run with the parameters given in Table 3.5. Surrogate spikes and

matrix blanks were also included. The volumes extracted were recorded and 5 ml subsamples were pipetted into 15 ml scintillation vials with 10 ml of 30% Scintisafe solution. The samples were stored in the dark for 24 hours before the radioactivity was quantified with a LSC. The results were then multiplied according to the volume of the original extract.

Table 3.5 ASE settings

Property	Setting
System pressure	14 MPa (2000 psi)
Oven temperature	100°C
Sample size	1-5 g
Oven Heat-up Time	5 minutes
Static Time	5 minutes
Solvent	dichloromethane/acetone (1:1)
Flush Volume	60% of extraction cell volume
Nitrogen Purge 1 MPa	1 MPa (150 psi) for 60 seconds

3.7 Digestions

Digestions were used to determine if any additional phenanthrene or benzene could be recovered from the amended clay. The procedure for the hydrofluoric (HF)

digestion consisted of passing the clay, that had previously undergone biodegradation and extraction, through a brass sieve, mesh number 35, aperture 500 MIC (Endecotts Ltd. London England) to remove the larger sand fraction. A small hole was drilled in the lid of a 50 ml digestion tube to fit a flexible Teflon tubing. Grease was added to the joint of the lid and tubing to ensure no gas escaped from the digestion tube other than through the tubing. The other end of the tubing was then placed into a 15 ml scintillation vial containing 15 ml 1M NaOH solution to capture any CO_2 evolved during the digestion procedure. In the 50 ml digestion tube, 8.5 ml trace metal grade nitric acid was added to a 0.5 g clay sample and shaken. Then 2 ml hydrofluoric acid was added, the tube was sealed, shaken and placed in a DigiPrep Jr. digestion block (SCP Science) at 90°C for 1 hour. The acid portion of the samples was diluted to 50 ml with deionized water and a 2 ml subsample was taken. The diluted acid and NaOH trap was then mixed with 10 ml 30% Scintisafe solution and stored in the dark for 24 hours. The radioactivity was then quantified with a LSC. Preliminary tests indicated that radioactivity did not escape as gases into the NaOH and therefore, subsequent digestions used the 50 ml digestion tube without alteration to the cap.

The sample preparation required for the digestion introduced many errors into the experiment (appendix A) and thus was only performed for a few samples.

3.8 Nuclear Magnetic Resonance Spectrometry

NMR was used to determine changes in the chemical environment of ^{27}Al, ^{29}Si and ^{13}C within the phenanthrene amended montmorillonite clay with aging.

Solid state NMR spectra were obtained on 25 mg phenanthrene/g montmorillonite clay and on pure montmorillonite clay. High concentrations of phenanthrene were required since ^{13}C MAS NMR is not a highly sensitive technique. ^{27}Al and ^{29}Si MAS spectra were obtained on a Bruker AMX-500 spectrometer with a Doty Scientific MAS probe at a magnetic field of 11.7 Tesla at the Department of Chemistry. Air-dried samples were packed into zirconia rotor sealed with Kel-F caps and spun between 6 and 10 kHz at an angle of 54.7° to the magnetic field. The Larmor frequency was 130.3 MHz for ^{27}Al and 99.36 MHz for ^{29}Si. Relative peak positions were measured in ppm with reference to $Al(H_2O)_6$ for ^{27}Al and tetramethylsilane, $Si(CH_3)_4$, (TMS) for ^{29}Si.

Solid-state ^{13}C CP/MAS NMR data were obtained at the Department of Chemistry at the University of Queens, Ontario, courtesy of Dr. Gang Wu, on a Bruker ASX-200 NMR spectrometer operating at 50.36 MHz. The samples were spun at 14 kHz. The RF field strength at the 1H frequency was approximately 60 kHz. The contact time during the Hartmann-Hahn match was 2 ms and the recycle time was 5 s. An average of 17900 transients were collected and ^{13}C chemical shifts were referenced to TMS.

An NMR spectrum of phenanthrene in a $CDCl_3$ solution was obtained on a Bruker Avance 300 MHz System in the Department of Chemistry, University of Manitoba. The

^{13}C chemical shifts were referenced to TMS.

The program SpinWorks, courtesy of Dr. K. Marat, was used for all data processing.

3.9 X-Ray Diffraction

XRD data were obtained in the Department of Geological Sciences to determine if changes occurred as phenanthrene amended montmorillonite clay dried, compared to a pure montmorillonite control. Samples were prepared by adding either 0.025 g or 0.25 g of solid phenanthrene to 1.00 g montmorillonite clay and grinding with a pestle and mortar. Pure phenanthrene and pure montmorillonite samples were also used. The samples were mounted on glass slides by spreading a small amount of powder evenly on the glass and adding a few drops of double distilled water. The samples were then "chopped" with a blade at right angles, spreading the material uniformly over the slide. The XRD trace was acquired after air drying the samples for 24 hours.

Continuous scan XRD data were collected on a Philips automated diffractometer system PW1710 Bragg-Brentano goniometer equipped with incident- and diffracted-beam Soller slits, 1.0 divergence and anti-scatter slits, a 0.2 mm receiving slit and a curved graphite diffracted-beam monochromator. A normal focus Cu X-ray tube was operated at 40kV and 40 mA. The samples were scanned from 2.0 to 45.0 degrees with 0.02 step per second. The wavelength to compute d-spacing was 1.54056A (Cu/K-alpha1). The

program JADE 6.0 was used to identify diffraction peaks.

3.10 Mathematical and Statistical Analysis

[14]C-phenanthrene degradation was calculated from the ^{14}C that evolved as carbon dioxide and reported as a percentage of the radioactivity that was initially added to the clay samples. A one-way analysis of variance (ANOVA) (significance level of 0.05), the Tukey-Kramer HSSD for multiple means comparisons and multiple regression was performed using JMPin[TM] Statistical Discovery Software (SAS Institute).

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Inoculum

Most Probable Numbers (MPN) of diesel fuel degraders indicated that 1.17×10^6 bacteria/g of soil were present with an upper confidence level of 3.86×10^6 and a lower confidence level of 3.55×10^5 (Table 4.1). Previous exposure to a contaminant through repeated spills would create an environment in which a biodegradation pathway was maintained within an adapted community (Maier *et al.*, 2000). The Petro-Canada soil produced an active inoculum supplied with an adequate number of metabolically capable bacteria required for these experiments.

4.2 Microcosms

4.2.1 Volatilization

The ambersorb that was placed in the microcosms to absorb volatile hydrocarbons could not be analysed due to technical problems with the oxidising equipment. This procedure had previously shown phenanthrene volatilization, under similar conditions, to be insignificant after three months (Nicolas, 2001). The volatile component of the benzene was expected to be significant but could not be measured.

Table 4.1 Most probable number of diesel fuel degraders (industrially contaminated soils)

Soil	Replication	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Sample 1	1	●	●	●	●		
Sample 1	2	●	●		●		
Sample 1	3	●	●	●	●	●	
Sample 1	4	●	●	●			
Sample 1	5	●	●	●			
Sample 2	1	●	●	●	●	●	
Sample 2	2	●	●	●	●		
Sample 2	3	●	●			●	
Sample 2	4	●	●	●	●		●
Sample 2	5	●	●	●	●		
Sample 3	1	●	●	●	●		
Sample 3	2	●	●	●	●		
Sample 3	3	●	●	●			
Sample 3	4	●	●	●	●		
Sample 3	5	●	●	●	●		

Note: Positive response indicated by a ●

4.2.1 Phenanthrene Respiration

Phenanthrene degradation in pure montmorillonite clay at 21°C (Table 4.2, Figure 4.1) was extremely slow. After 204 days the inoculated treatment A_{PIN} had only degraded 2.0 +/- 0.7 % and the non-inoculated treatment A_{PNN} degraded 1.3 +/- 0.1 %. The four sterile microcosms A_{PNS}, after 204 days averaged 0.01 +/- 0.01% $^{14}CO_2$ in the alkaline trap, showing that they provided a suitable environment to "age" the phenanthrene for use in D_{PIA}. The A_{PNS} experiment was also used to determine if inoculating the clay was necessary to provide the required microorganisms for degradation. The inoculated microcosm A_{PIN} did out perform the non-inoculated microcosm A_{PNN} but after 361 days, the inoculated microcosm A_{PIN} had only degraded 2.4 +/- 0.9 % and the non-inoculated microcosm A_{PNN} degraded only 1.5 +/- 0.1 % of the ^{14}C-phenanthrene into CO_2. The inoculated treatment degraded more phenanthrene than the non-inoculated soil but the difference was not statistically significant (P = 0.09).

Due to the slow degradation of phenanthrene at 21°C, experiment B was kept at 31°C to determine if degradation rates would increase with a 10°C increase in temperature as predicted by the Q_{10} effect. The microcosms also contained less phenanthrene within the clay (82,500 DPM vs 185,000DPM) and an additional treatment was included in which the clay was mixed occasionally throughout the experiment to reduce the chance that the amendment was distant from the microorganisms capable of degrading it. Degradation rates increased (Figure 4.2). The inoculated microcosm (B_{PIN}) experienced the most degradation (4.5 +/- 0.2% degraded after 132 days) followed by the

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Table 4.2 Total % recovered (averages of four replicates for all procedures and treatments except digestion)

Treatment	% Sterile $^{14}CO_2$ captured	% $^{14}CO_2$ captured	% Shaker extracted ***	% ASE extracted ***	% digestion recovered	Total % recovered
			Phenanthrene			
			---- 361 Days ----			
A_{PIN}		2.4 +/- 0.9	0.062a +/- 0.010	0.060a +/- 0.006	7.2/7.2	9.72
A_{PNN}		1.5 +/- 0.1	0.066a +/- 0.004	0.066a +/- 0.004	2.3	3.94
			---- 289 Days ----			
B_{PIN}		4.5 +/- 0.2	0.19 +/- 0.014	0.073ab +/- 0.008		4.69
B_{PNN}		1.2 +/- 0.1	0.124a +/- 0.008	0.075ab +/- 0.010		1.4
B_{PINM}		2.7 +/- 0.3	0.198a +/- 0.010	0.075ab +/- 0.014		2.98
			---- 142 Days ----			
C_{PIN}		35.1 +/- 2.0	0.123 +/- 0.059	0.101 +/- 0.059	44.7/47.5	81.42
			---- 157 Days ----			
D_{PINX}		2.0 +/- 0.1	12.205b +/- 0.081	0.908b +/- 0.081		15.11
D_{PIA}	0.01* +/- 0.003	0.17 +/- 0.03	0.122a +/- 0.005	0.060a +/- 0.005	3.2	3.56
D_{PIN}		25.7 +/- 2.4	0.093 +/- 0.012	0.125a +/- 0.012		25.92
D_{PNN}		1.8 +/- 0.1	12.214b +/- 0.299	1.161b +/- 0.299		15.17
			---- 157 Days ----			

E_{PIN}		3.9 +/- 2.0	0.166a +/- 0.007	0.062ab +/- 0.007		4.13
E_{PIA}	0.12 +/- 0.1**	0.1 +/- 0.04	0.131a +/- 0.014	0.062ab +/- 0.018		0.41
E_{PNA}	0.12 +/- 0.1**	0.1 +/- 0.2	0.146a +/- 0.006	0.090b +/- 0.006		0.45
E_{PNN}		2.8 +/- 0.3	0.194a +/- 0.014	0.052a +/- 0.014		3.04

Benzene

---- 361 Days ----

A_{BIN}		12.2 +/- 6.0	0.004a +/- 0.001	0.004ab +/- 0.001	2.5/4.8	16.26
A_{BNN}		37.8 +/- 9.3	0.004a +/- 0.001	0.001a +/- 0.001	4.8/2.1	43.69

---- 158 Days ----

D_{BIA}	0.01 +/- 0.006*	0.1 +/- 0.1	0.036a +/- 0.004	0.006b +/- 0.004		0.15

Controls

---- Within 30 Minutes ----

Shaker surrogate spikes ****		3.283 +/- 0.822			94	97.3
ASE surrogate spikes ****				6.190 +/- 0.522		6.2
Digestion control					98.3	98.3

* Measured after 204 days of aging prior to the start of the experiment.

** Measured after 132 days of aging prior to the start of the experiment.

*** Means followed by the same letter within an experiment are not significantly different (Tukey-Kramer's all pairs comparisons).

**** Matrix blanks where below detection limits.

Figure 4.1 Degradation of phenanthrene in montmorillonite clay at 21°C.

Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.

Figure 4.2 Degradation of phenanthrene in montmorillonite clay at $31^{\circ}C$.

Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.

mixed inoculated (B_{PINM}) microcosm (2.7 +/- 0.3 %) and the non-inoculated (B_{PNN}) microcosm (1.2 +/- 0.1%). Periodic mixing may have allowed the $^{14}CO_2$ to escape without capture while the jar was open. The NaOH solutions in the eight sterile microcosms B_{PNS} were found to have captured an average of 0.12 +/- 0.1% ^{14}C and were suitable to use as aged samples for the treatments D_{BIA} and D_{BNA}.

The very slow degradation rate in the montmorillonite clay raised questions as to whether the montmorillonite clay was causing the extremely slow degradation rates or if there was a problem inherent in the method. To test this, a ^{14}C-phenanthrene microcosm was started using the same procedures as for montmorillonite clay but with a composite sample of Petro-Canada and Mid-Canada soils (C_{PIN}). Degradation was 30.5 +/- 1.8 % after 34 days (Figure 4.3) indicating the slow degradation of experiments A and B was due to the characteristics of the pure montmorillonite clay such as poor structure, lack of organic matter and low nutrients. At the end of experiment C (142 days), 35.1 +/- 2.0 % of the phenanthrene had been degraded to CO_2. Since there was not a problem inherent in the method being used, experiments D and E were started.

Microcosms were then prepared to compare the degradation of ^{14}C phenanthrene aged in clay with newly amended clay. Aged inoculated (D_{PIA}, previously A_{PIS}) microcosms had statistically lower degradation (0.17 +/- 0.03%) than newly inoculated (D_{PIN}) microcosms (25.7 +/- 2.4 %) at 21°C (Figure 4.4). The aged microcosm had been inoculated with the same inoculum, at the same time with the same nutrient and moisture levels, thus indicating a clear decrease in the bioavailability of phenanthrene when aged in montmorillonite clay. The new inoculated (D_{PIN}) microcosms also exhibited

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Figure 4.3 Degradation of phenanthrene in industrial contaminated clayey soil at 21^0C. Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.
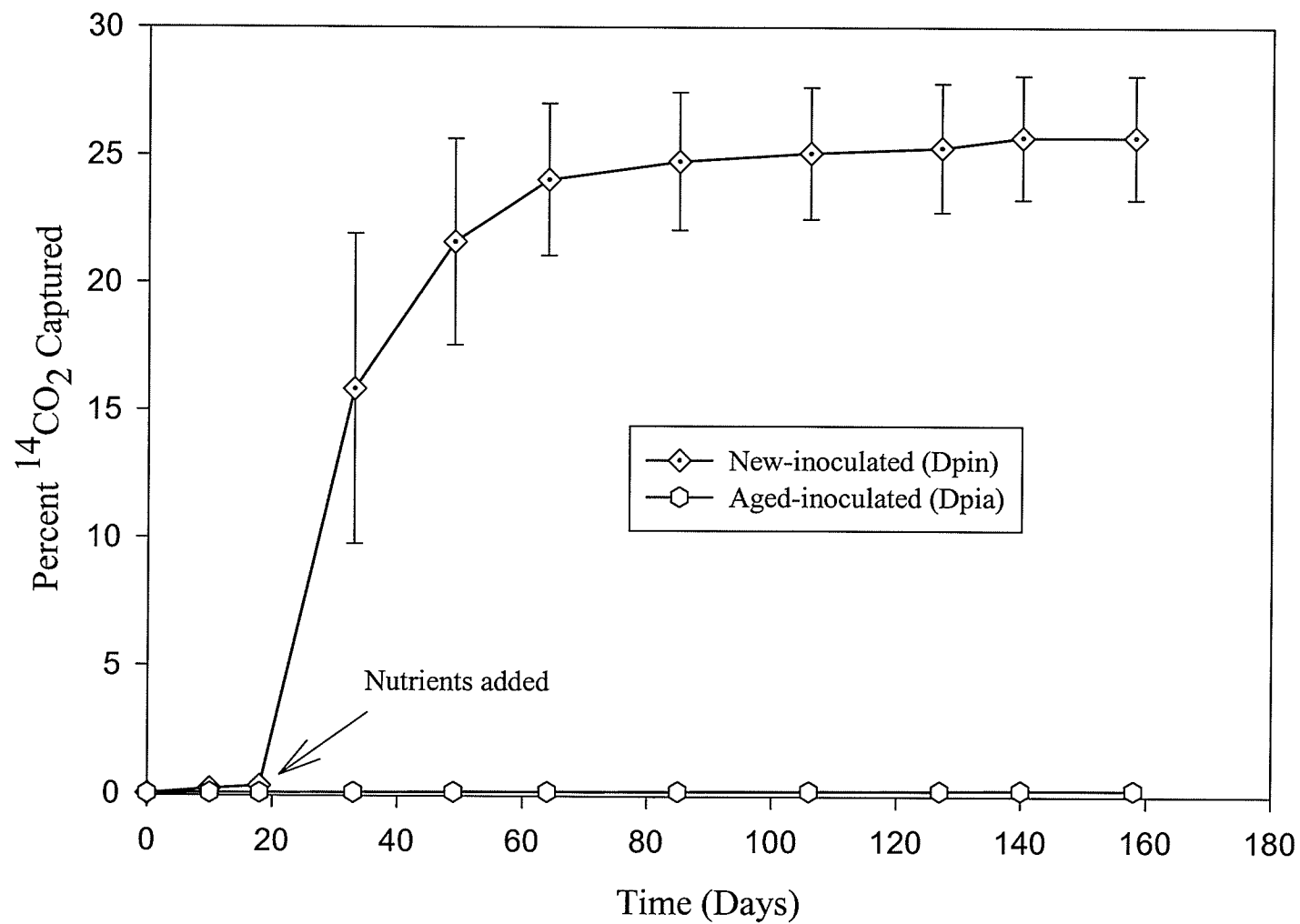
Figure 4.4 Degradation of new and aged, inoculated phenanthrene in montmorillonite clay at 21°C. Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.

degradation that was significantly higher than both the new non-inoculated (D_{PNN}) (1.8 +/- 0.1 %) and the non-sterilized (D_{PINX}) (2.0 +/- 0.1 %) treatments. The additional 25% degradation caused by adding a microbial consortium to the microcoms provided statistically significant increases in degradation. The results also indicated that degradation of phenanthrene within the non-sterilized microcosms (D_{PINX}) was greater than if sterilized and then inoculated (D_{PIN}).

The aged inoculated microcosm E_{PIA} (0.1 +/- 0.04 %) and the aged non inoculated microcosm E_{PNA} (0.1 +/- 0.2 %) had statistically lower degradation rates for phenanthrene than the newly inoculated E_{PIN} (3.9 +/-2.0 %) and newly non-inoculated E_{PNN} (2.8 +/- 0.3 %) microcosm at 31°C (Figure 4.5 and 4.6). This indicated that the aged samples had lower bioavailability at 31°C with lower phenanthrene concentrations than at 21°C at higher concentration similar to that of experiment A. The amount of CO_2 captured by NaOH solution may have been slightly depressed for all 31°C treatments due to a pressure build up that occurred in these microcosms.

4.2.2 Benzene Respiration

The benzene microcosms showed much higher degradation rates than phenanthrene microcosms (Figure 4.7). After 204 days the inoculated microcosm A_{BIN} degraded 12.2 +/- 6.0 % of the ^{14}C-benzene and the non-inoculated microcosm A_{BNN} degraded 37.8 +/- 9.3 %. Although A_{BNN} was not inoculated, it did not remain sterile due to the periodic opening of the microcosm to change the NaOH trap.
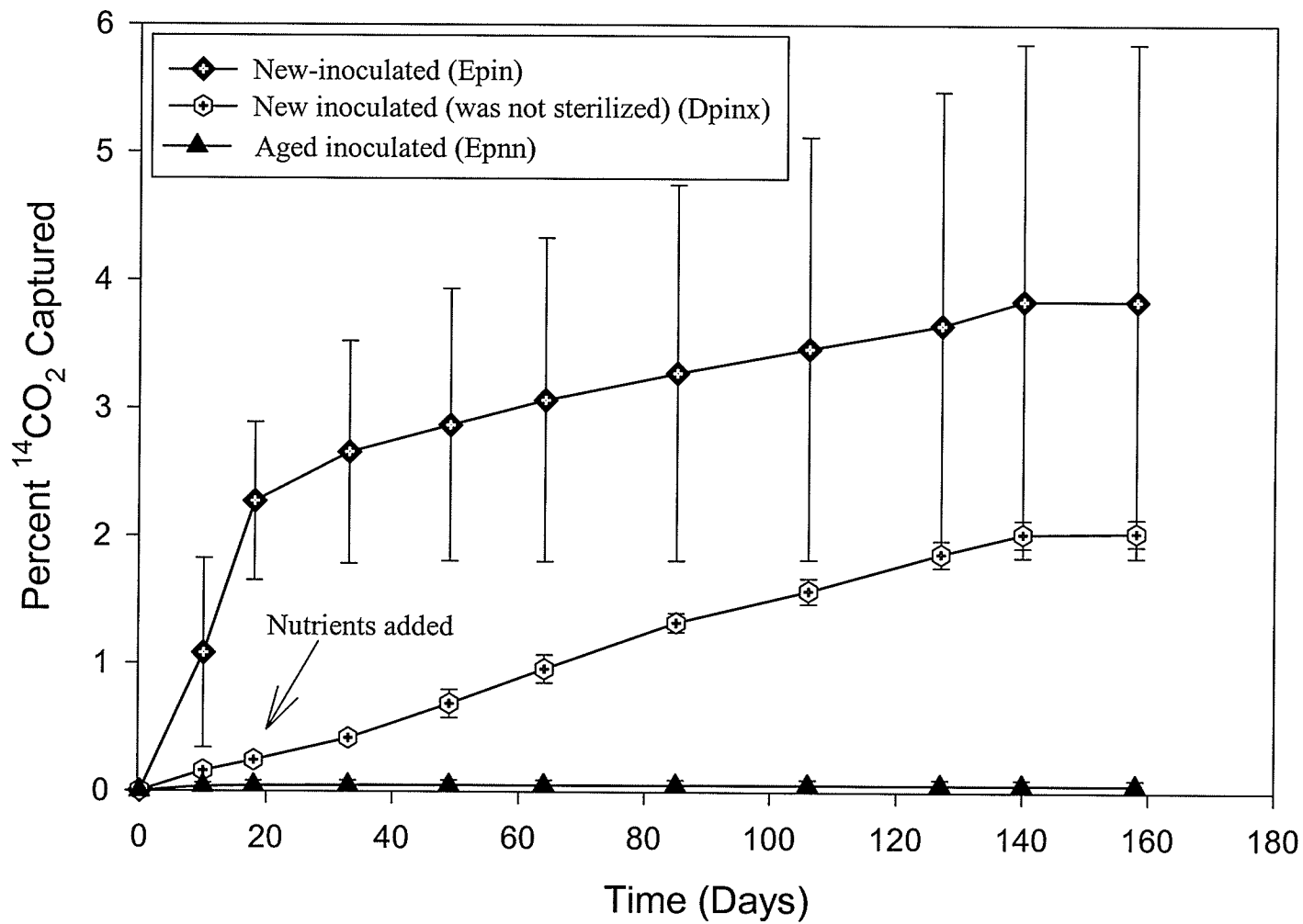
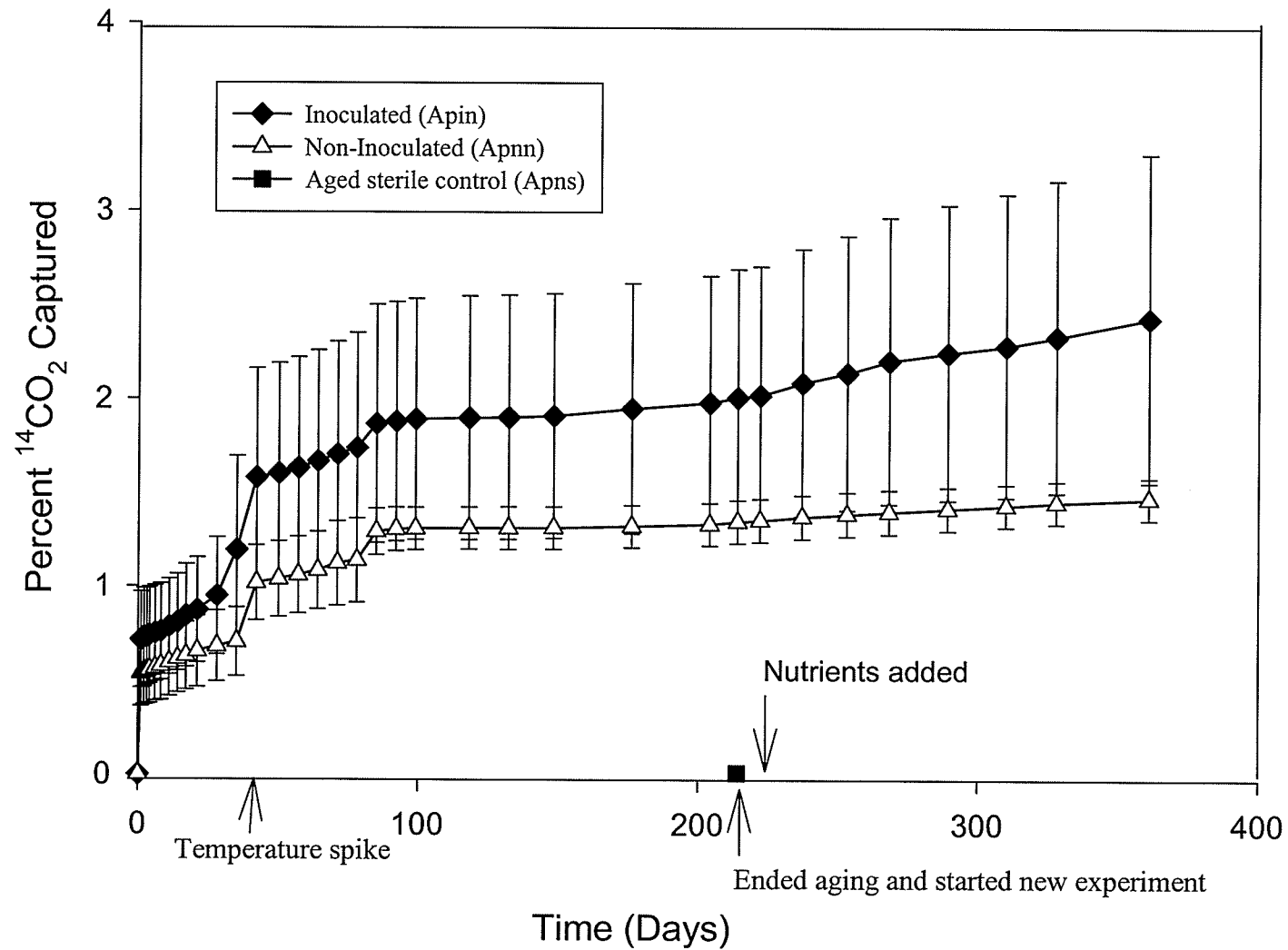**Figure 4.5 Degradation of new and aged (inoculated) phenanthrene in montmorillonite clay at 31°C. Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.**
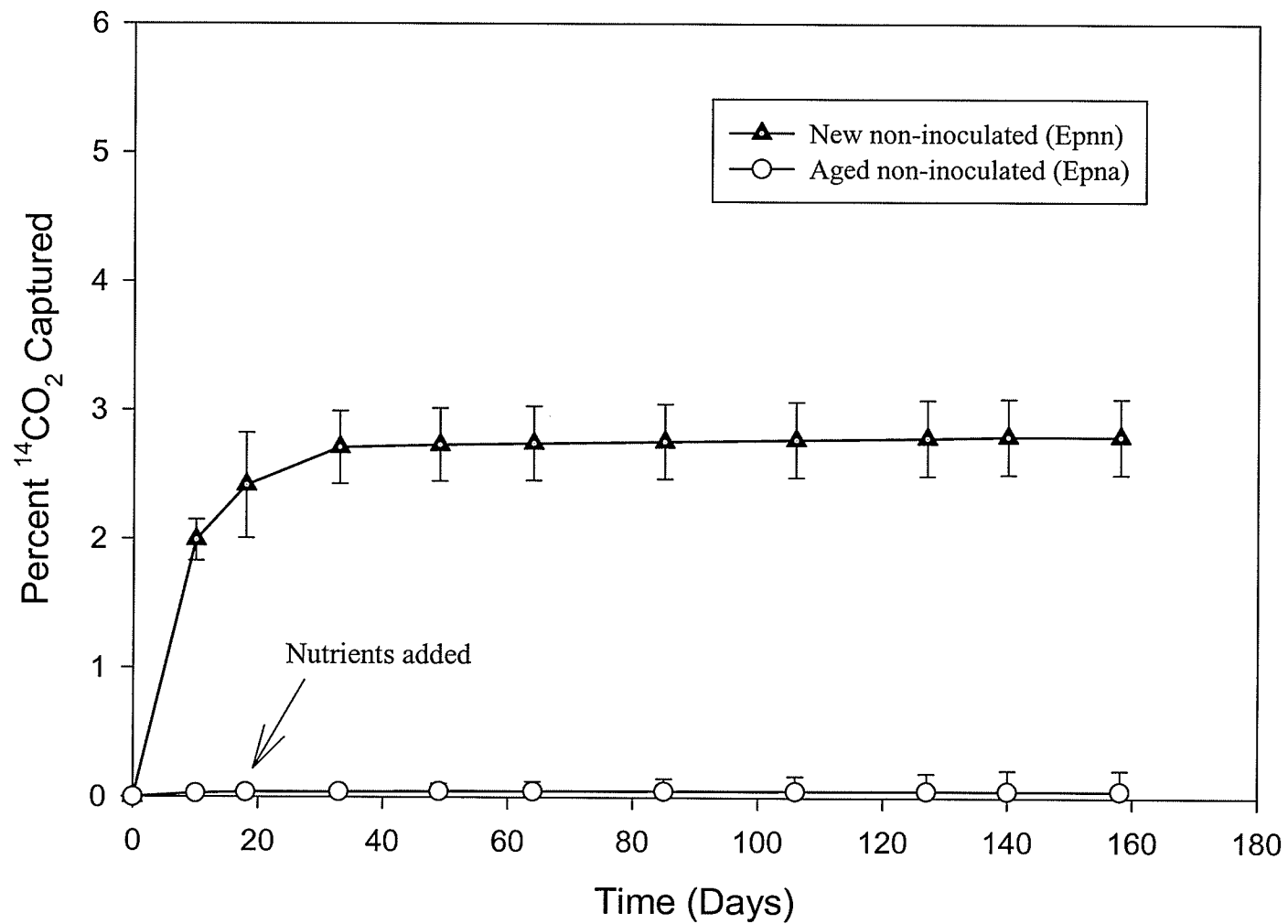
Figure 4.6 Degradation of new and aged (non-inoculated) phenanthrene in montmorillonite clay at 31^0C. Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.
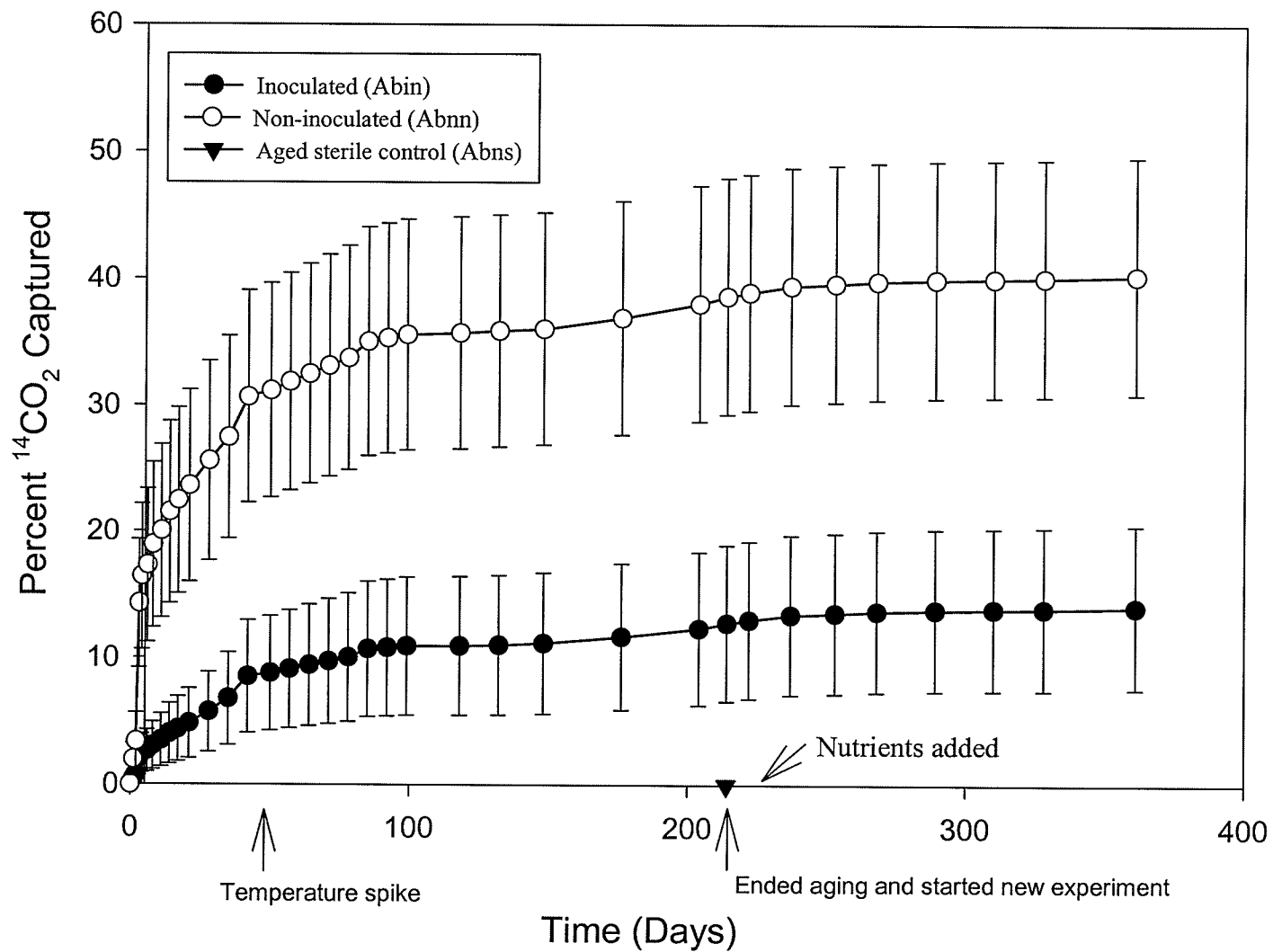
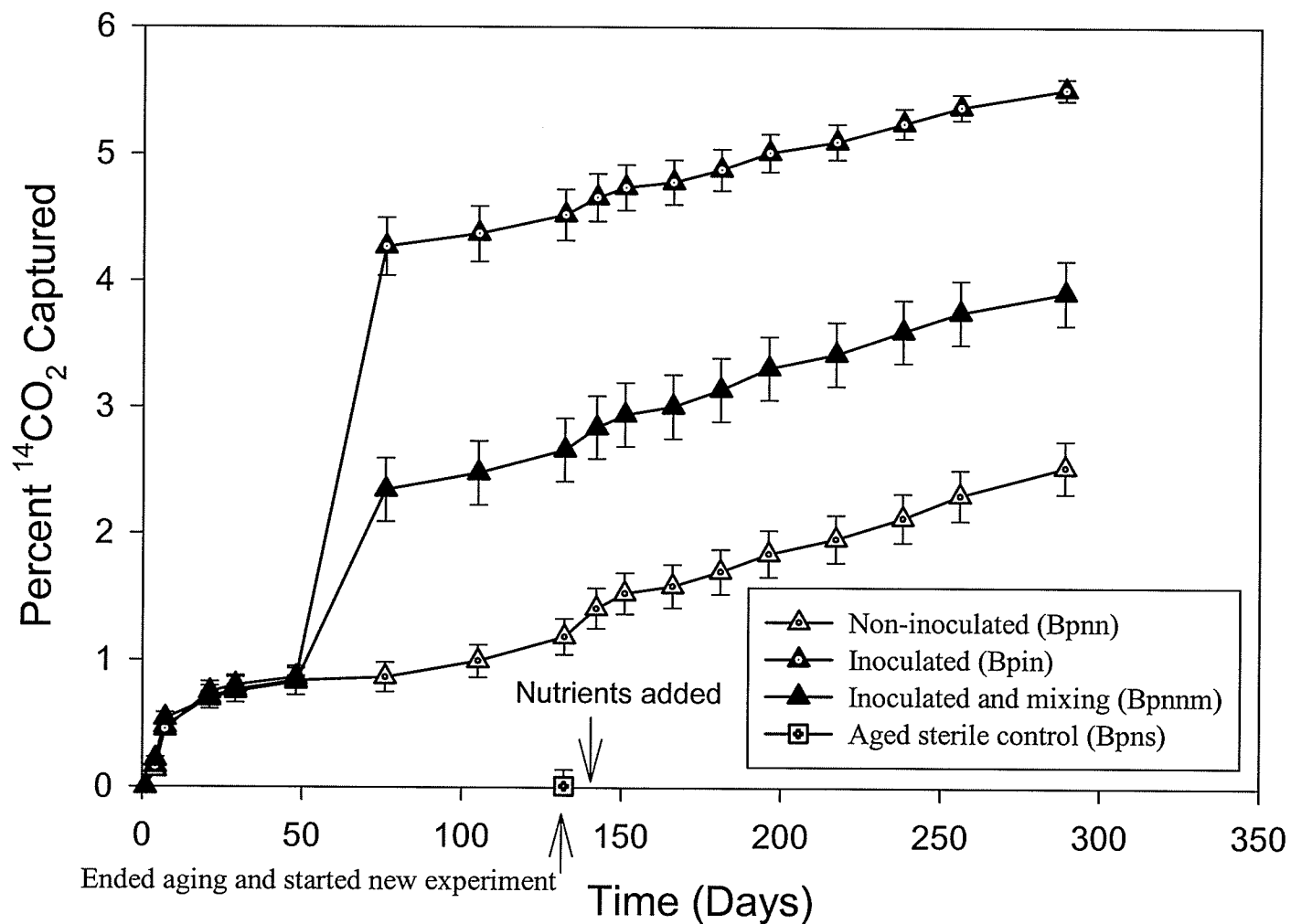**Figure 4.7 Degradation of benzene in montmorillonite clay at $21^{\circ}C$.**
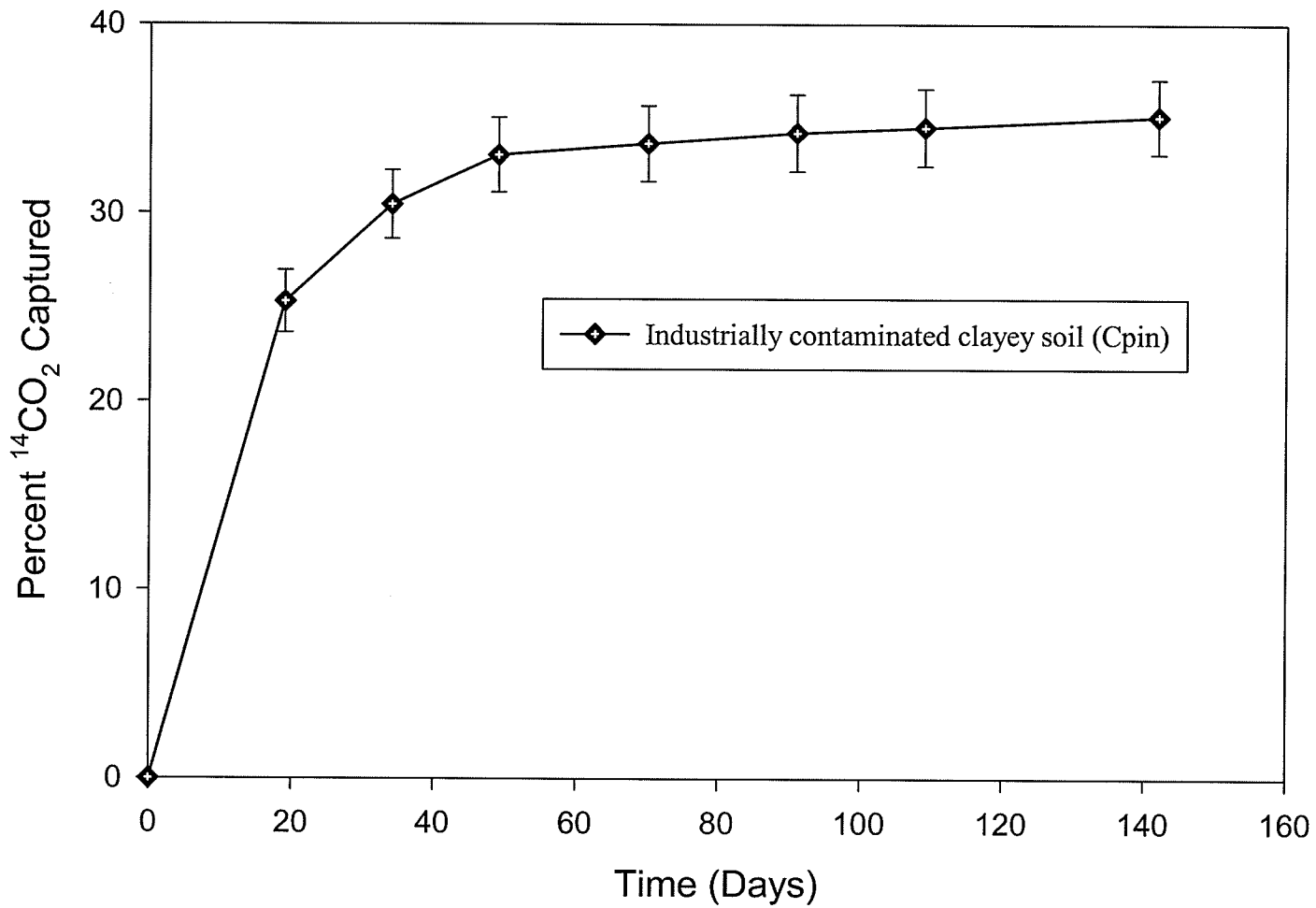**Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.**

The higher degradation rate of the non-inoculated microcosm could be explained by naturally occurring microbes using the higher nutrient values found in that microcosm. An error was made in assuming that non-inoculated and inoculated treatments received the same concentration of nutrients. The sorption capacities of soils vary according to the type and amount of minerals, soil organic matter and nutrients they contain. The industrial soils were very low in nutrients and consequently when the growth medium was added, the nutrients may have sorbed to the soil and thus were taken out of solution during the growth of the inoculum. When the 5 ml microbial inoculum was subsequently taken, the nutrients might have been sorbed from the solution to the soil, thus producing a nutrient deficient inoculum. The non-inoculated microcosms received the basic growth medium without alteration and thus presumably received more nutrients.

The four sterile benzene microcosms A_{BNS} were found to have 0.01 +/- 0.01 % $^{14}CO_2$ captured in the alkaline trap after 204 days. Compared to the 12 and 38 % captured for the inoculated and non-inoculated treatments, the degradation of the sterile microcosms were not significant and thus provided a suitable environment to "age" the benzene in the clay. The sterile A_{BNS} treatments were then inoculated and treated as the aged samples D_{BIA}. The aged benzene samples (D_{BIA}) could not be compared to the inoculated and non-inoculated benzene samples (A_{BIN}, A_{BNN}) since experimental conditions were different for each of the experiments, including starting concentrations, inoculum, and duration. At 21°C, the aged benzene (D_{BIA}) microcosm only degraded 0.1 +/-0.1 % after 158 days (Figure 4.8) which is much less than A_{BIN} (11%) and A_{BNN} (35%) had degraded at day 158. Although these experiments cannot be directly
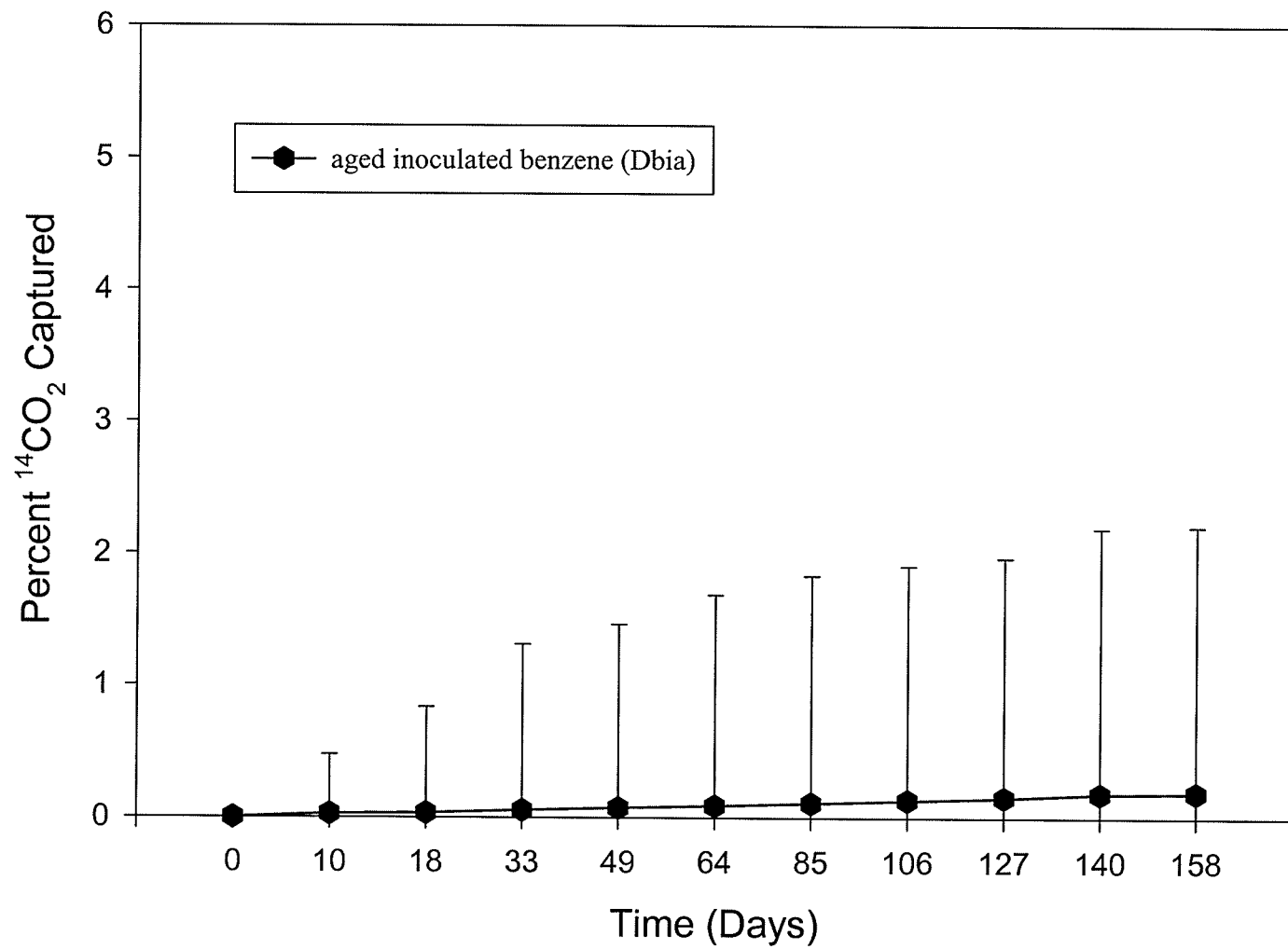
Figure 4.8 Degradation of aged benzene in montmorillonite clay at 21°C.
Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$.

compared, the large difference between the experiments is important. Since the benzene was not degraded, it may have been volatilized into the ambersorb. Another possibility would be that the benzene had an aging effect in montmorillonite clay. This would indicate that the benzene became sorbed to the clay and subsequently became biologically unavailable. However, a low weight single aromatic ring would be expected to degrade relatively quickly and not to have an aging effect.

4.3 Aqueous Sorption

A seven day sorption experiment indicated that 75% of the aqueous phenanthrene was sorbed onto montmorillonite clay. The sorption distribution coefficient, Kd, was found to be 3.1ml g^{-1} . Sorption to soil organic matter has been shown to be dominant in saturated soils whereas sorption to clay minerals dominates in dry conditions (Murphy *et al.*, 1990; Karickhoff *et al.*, 1997; Hwang and Cutright. 2002). Thus clays are not expected to be good sorbents in wet conditions, such as the batch aqueous sorption experiments. However, the aqueous phase in the experiments was deionized water which increases sorption compared to 0.01 M $CaCl_2$ solution, used in many published aqueous sorption studies (Thomsen *et al.*, 1999) because it mimics more closely the natural soil solution.

4.4 Extractions

The dichloromethane shaker extraction recovery of phenanthrene ranged from 0.0 to 12.2 +/- 0.3 % for experimental samples and 3.3 +/- 0.8 % for spiked controls (Table 4.2) which were extracted within 30 minutes. If these values are indicative of the bioavailable fraction of the amendment, then it would appear that phenanthrene has become less available for biodegradation within 30 minutes of being added to the montmorillonite clay. ASE was used as the second extractant and the results ranged from 0.0 to 1.2 +/-0.3 % for experimental samples and 6.2 +/- 0.5 % for spiked controls (Table 4.2). ASE did not prove to be an efficient extraction method even with the addition of anhydrous sodium sulfate to dry the clay and Ottawa sand to improve fluid flow through the sample.   Most treatments within each trial had similar extraction rates, however, there are a few exceptions. In the 21°C trial, the inoculated, non-sterilized treatment (D_{PINX}) and the non-inoculated treatment (D_{PNN}) had significantly higher recoveries than the other treatments for both the mild and ASE extractions. The mild extraction had about 12 % more extractable phenanthrene than the other extractions while the ASE extraction was 1% higher. The non-inoculated phenanthrene (B_{PNN}) sample had statistically higher recoveries with the ASE than the aged non inoculated (Bpna) in the 31°C trials. However, since all of the recovered amounts were low, it is unlikely that these differences are meaningful. Previous studies, which have used a variety of soil types, found sandy soils with low organic matter content had the highest extraction efficiencies while soils with a high clay and high organic matter content had lower

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extraction efficiencies. Sorbent pore size and soil organic matter were assumed to be causing the differences (Reid *et al.*, 2000).

Neither the mild dichloromethane extraction nor the ASE were apparently able to remove benzene from the montmorillonite clay. This could have been due to the ineffectiveness of the methods or it may be that there was no benzene to extract if it had already volatilized.

4.5 Digestions

HF digestion of spiked samples had a recovery of 98.3 +/- 2.0 % and therefore twelve experimental samples were selected for testing to determine whether the amendments remained in the clay (Table 4.2). The highest HF recovery was for the spiked dichloromethane shaker extraction control (#4), with a digestion recovery of 94%. The industrially contaminated samples also had relatively good recoveries ranging from 44.7 to 47.5%. The experimental samples that had previously been extracted with both the shaker and ASE had recoveries ranging from 2.3 to 16.8 with a mean of 5.7 +/- 5.2 % for phenanthrene while the benzene amended clay had a mean recovery of 3.5 +/- 1.9%. This indicates that the digestion had poor recoveries for the samples that had undergone ASE, or in the case of benzene, may have volatilized. A correction factor was applied to ASE samples to account for the anhydrous sodium sulfate but the volume of sand was unknown and variable, making it difficult to apply a correction factor (Appendix 1).

4.6 Total Recovery

The total recoveries of the ^{14}C added to the microcosms were very low and ranged from 1 to 43.7 % for phenanthrene amended montmorillonite clay. The recovery for the phenanthrene amended industrial soil averaged 81.4%. The clay sample that had been spiked as the dichloromethane shaker extraction control (#4) had a total recovery of 97.3% and the digestion control had a recovery of 98.3 % (Table 4.2).

The higher the percentage of phenanthrene recovered through CO_2 capture and extractions, the more that was recoverable through HF digestion (Figure 4.9). It is important to note that the total error in the digestion of the treatment samples is large and thus only provided for general comparisons.

4.7 Nuclear Magnetic Resonance Spectrometry

The ^{13}C NMR spectrum of phenanthrene dissolved in $CDCl_3$ has seven peaks (Figure 4.10). The small peaks at 132.1 and 130.3 ppm each represent two non-protonated carbon atoms in the phenanthrene molecule corresponding to positions $C_{11, 14}$ and $C_{12, 13}$ respectively. The peaks at 128.5, 126.9, and 122.7 each represent two protonated carbon atoms at positions $C_{9,10}$, $C_{8,1}$ and $C_{5,4}$ respectively and the peak at 126.6 ppm represents the four protonated carbon atoms at $C_{6,7, 2,3}$.
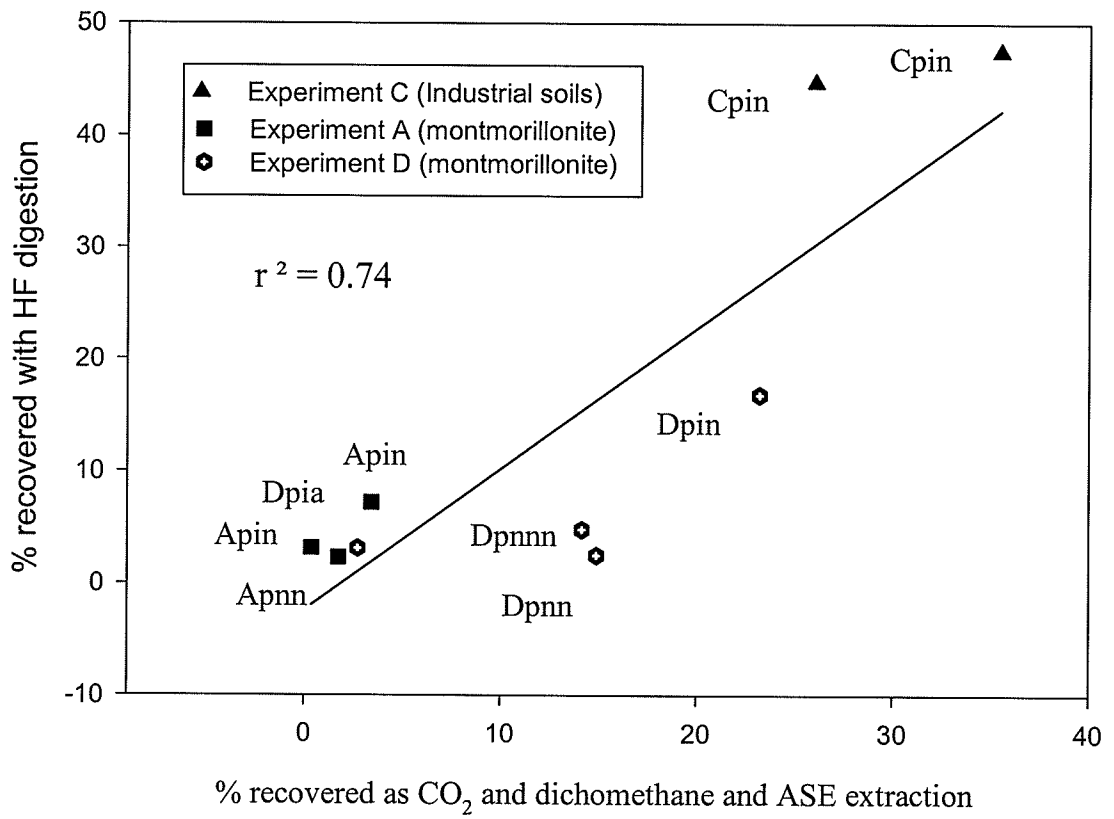
Figure 4.9 Phenanthrene recovered with HF verses total phenanthrene recovered through CO_2 capture and extractions.
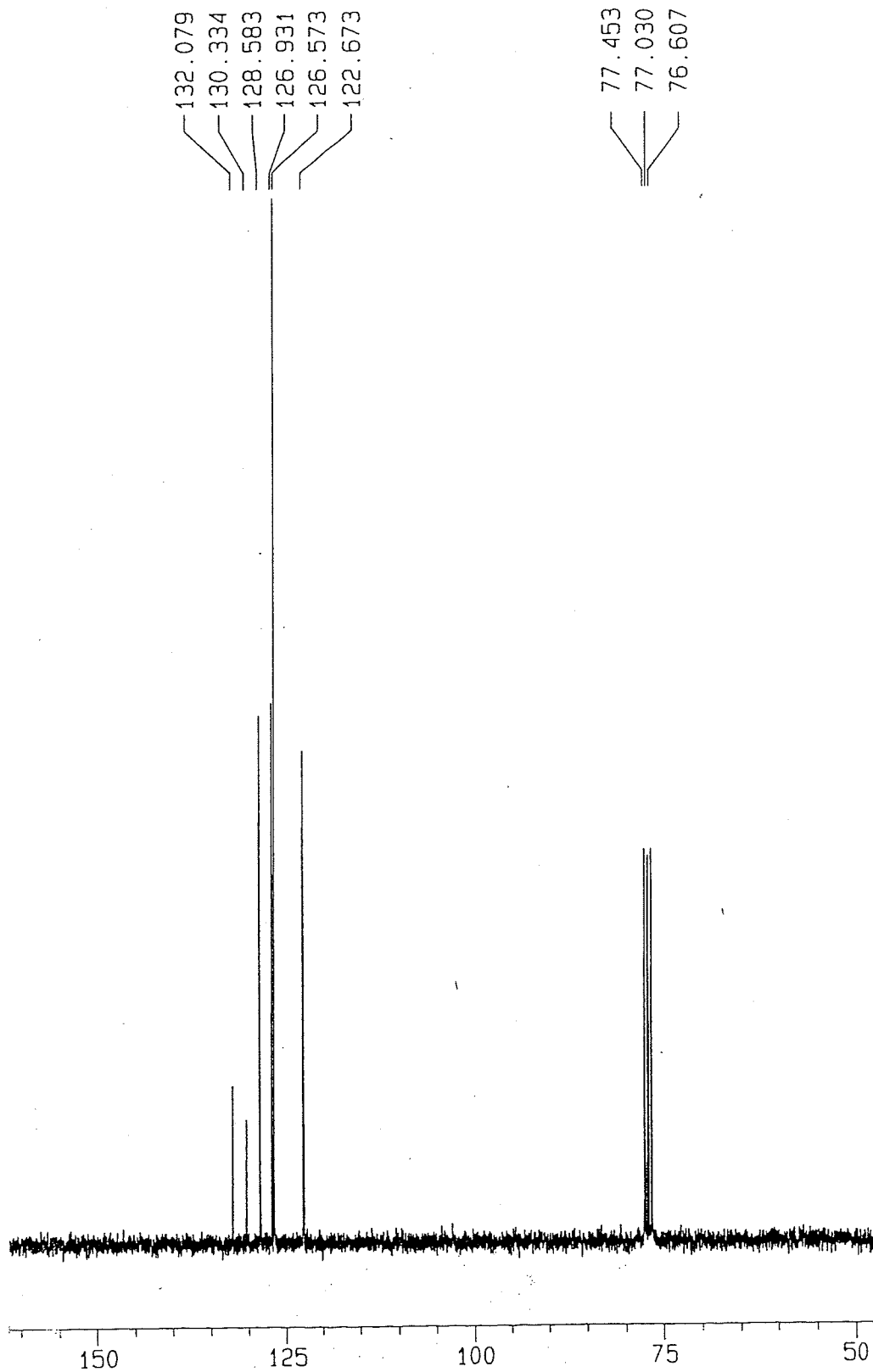
Figure 4.10 ^{13}C NMR spectrum of phenanthrene (122.67 - 132.08 ppm) in CDCl3 (76.61 - 77.45)

The ^{13}C CP MAS NMR spectrum consists of envelopes of resonances which can be related to the relative intensities and chemical shifts of the solution peaks (Figure 4.11). The pure phenanthrene spectrum is one broad envelope without separately resolved peaks centred around 127 ppm. The freshly amended 25 mg phenanthrene / g montmorillonite clay gives three distinguishable ^{13}C NMR peaks, one around 127 ppm and two smaller peaks at approximately 123 and 129 ppm. The aged 25 mg phenanthrene / g montmorillonite clay has five visible peaks at approximately 123, 127,129, 130, and 132 ppm.

The relative intensity of the phenanthrene peaks in clay are slightly different from the solution spectrum (Figure 4.11). This is inherent in the CP MAS process since protonated carbons relax faster and thus are selectively enhanced over non-protonated carbons.

A relaxation delay (RD) of 600 s (10 min) was required over three days of data collection to obtain the solid state spectrum of pure phenanthrene, while the samples of phenanthrene mixed with montmorillonite were obtained with a 1 s RD. Relaxation rates were not measured due to the length of time that would be required. The increased resolution of phenanthrene when sorbed to clay may be caused by the proximity to mobile protons of the clay structure. The increased resolution of the aged phenanthrene in montmorillonite compared to the freshly amended montmorillonite indicates that changes have occurred as phenanthrene became sorbed to the montmorillonite clay.
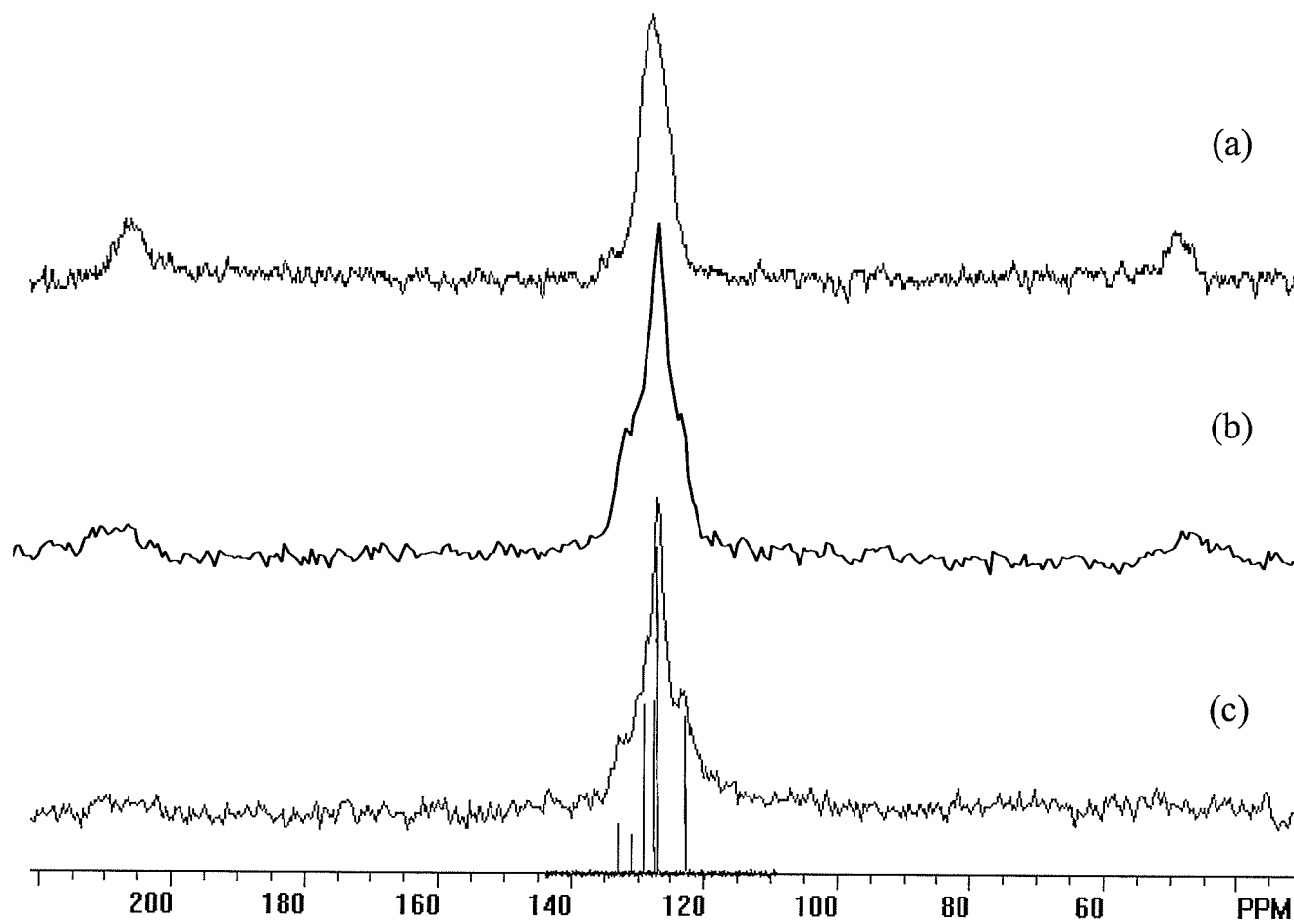
Figure 4.11 ^{13}C CP MAS NMR of (a) pure phenanthrene, (b) freshly mixed 25 mg/g phenanthrene in montmorillonite clay and (c) aged 25 mg/g phenanthrene in montmorillonite clay with the phenanthrene/CDCl$_3$ solution spectrum for reference.

The ^{29}Si MAS NMR spectra of pure montmorillonite (Figure 4.12) and newly amended phenanthrene in montmorillonite have tetrahedral SiO$_4$ peaks at -94.8 ppm relative to TMS (Table 4.3). The spectra of aged samples have tetrahedral SiO$_4$ peaks at -94.7 ppm relative to TMS. It appears that the Si is becoming less shielded as phenanthrene is aged in montmorillonite clay.

The small peak at -111 ppm is due to amorphous SiO$_2$. The ASE extracted sample had a peak at -108 ppm originating from the sand added as part of the ASE procedure. Previously, cations added to clays have been shown influenced the ^{29}Si and ^{27}Al chemical shifts, helping to determine sorption sites and binding mechanisms. (Kirkpartick, 1998; Sullivan, 1998; Ejeckam, 2003).

Table 3.3 ^{29}**Si MAS NMR parameters comparing aged and non-aged phenanthrene amended montmorillonite**

Treatment	Chemical shift (ppm)	Full width at half height (ppm)
pure montmorillonite	-94.9	3.5
montmorillonite with dichloromethane added, then volatilized	-94.8	4.0
New PHN amended MONT.	-94.8	3.8
New PHN amended MONT.	-94.8	4.0
Aged PHN amended MONT.	-94.6	4.0
(2 samples)	-94.7	4.0
ASE extracted aged PHN amended MONT.	-94.7	4.0
Shaker extracted aged PHN amended MONT.	-94.7	3.5
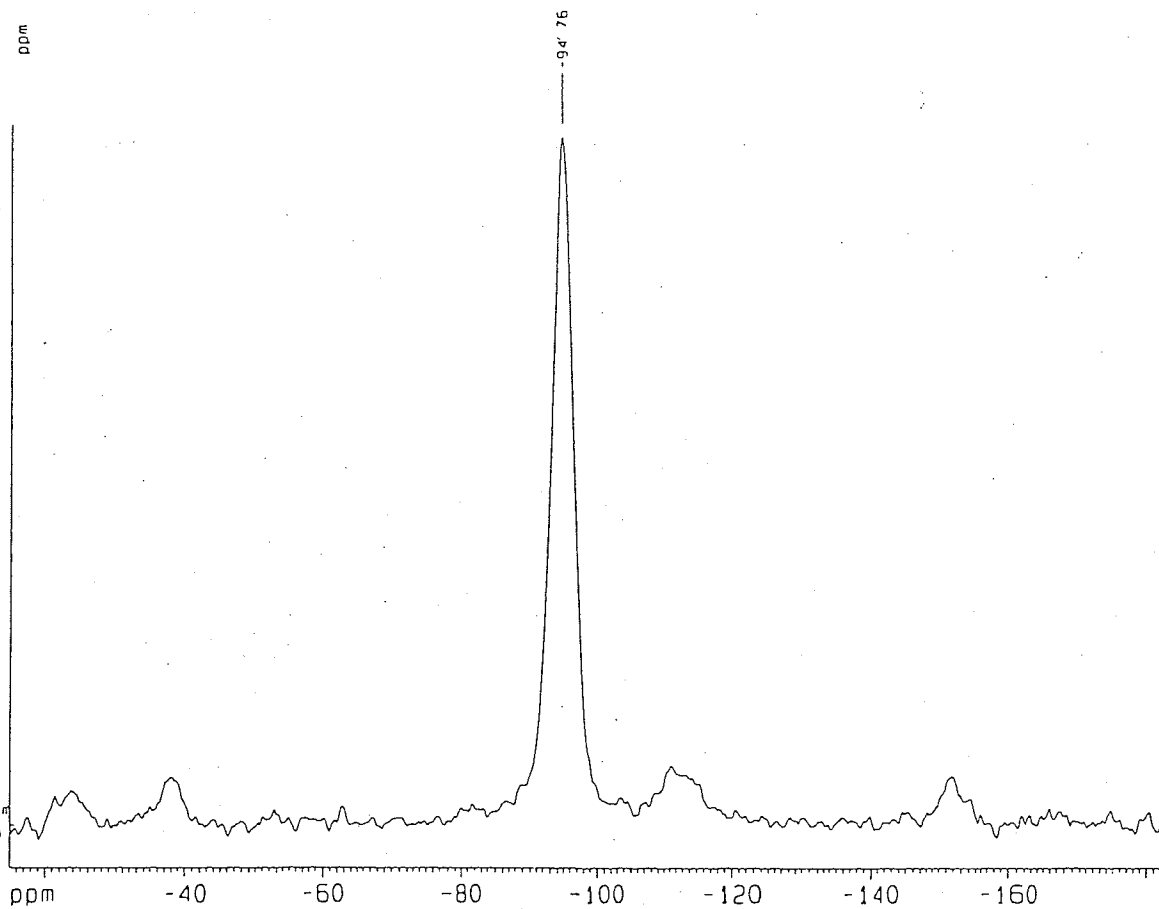
Figure 4.12 ^{29}Si MAS NMR of phenanthrene in montmorillonite clay.

The ^{27}Al MAS NMR spectra show the octahedral AlO_6 peak at approximately 2.4 ppm, with reference to $Al(H_2O)_6$ (Figure 4.13). No significant trend was seen in the chemical shift of the Al regardless of concentration, age or if it had been extracted. Thus ^{27}Al MAS NMR could not detect any changes to the chemical environment of the Al octahedral within the montmorillonite.

4.8 X-Ray Diffraction

XRD data were collected for pure phenanthrene (Figure 4.14), pure montmorillonite (Figure 4.15), and samples containing 0.025 g/g and 0.25 g/g phenanthrene in montmorillonite (Figure 4.16). The d-spacing for the [001] peak of the montmorillonite control was 15.33 Å at day one and decreased by 0.36 Å to 14.97 Å after two months (Table 4.4) (Figure 4.17). The largest decrease occurred during the first month of aging. The [001] d-spacing for the 0.025 g/g phenanthrene in montmorillonite sample decreased 0.31 Å from 15.23 to 14.92 Å (Figure 4.18) while the [001] peak of the 0.25 g/g phenanthrene in montmorillonite sample decreased only 0.06 Å from 15.44 to 15.39 Å (Figure 4.19). The sample containing 0.25 g/g phenanthrene in montmorillonite initially had a [001] d- spacing of 15.44 Å which after a month decreased to 15.12 Å and then increased to 15.38 Å after three months. This trend was not seen in either the montmorillonite control or the lower concentration of phenanthrene amended clay. In general, all of the montmorillonite peaks shifted to smaller d-spacings and the intensities
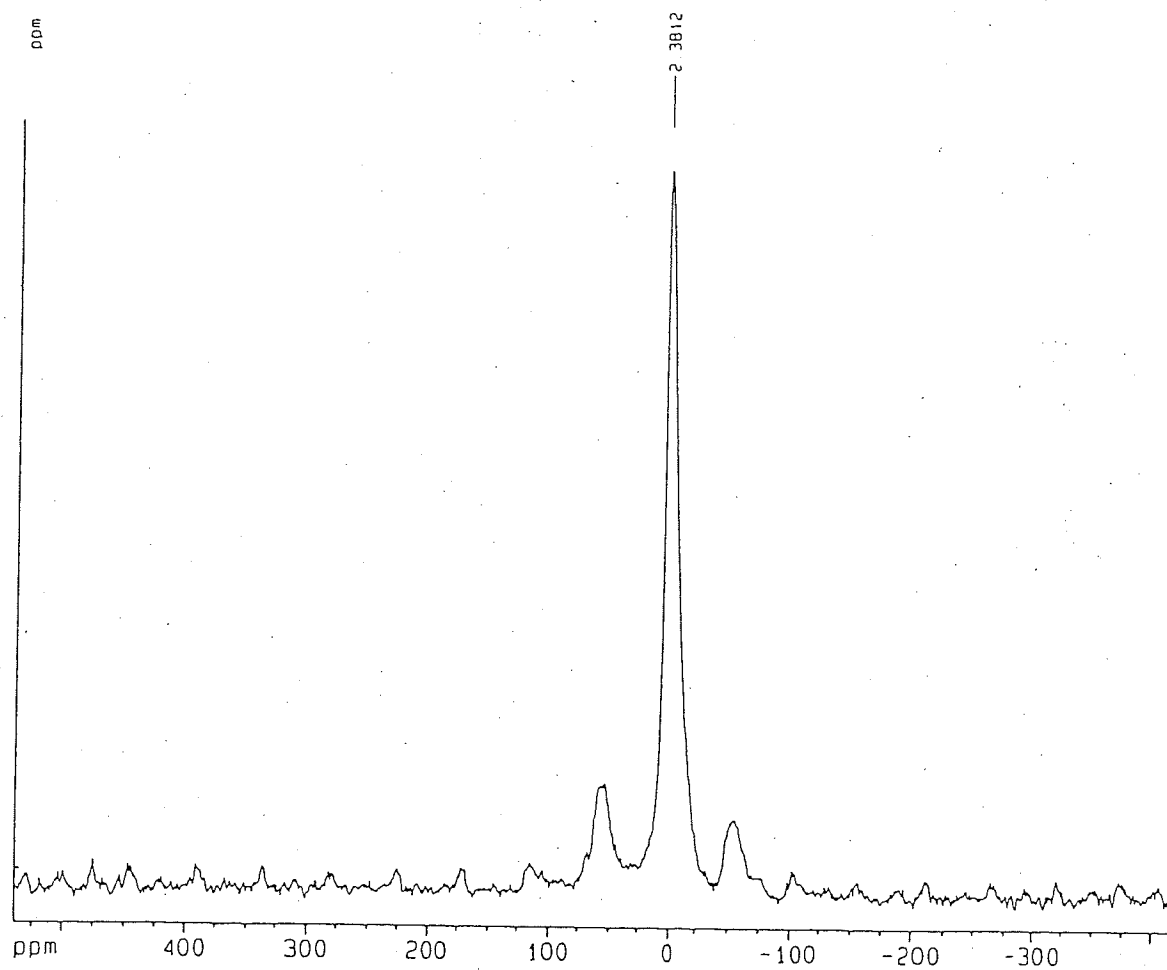
Figure 4.13 ^{27}Al MAS NMR of phenanthrene in montmorillonite clay.
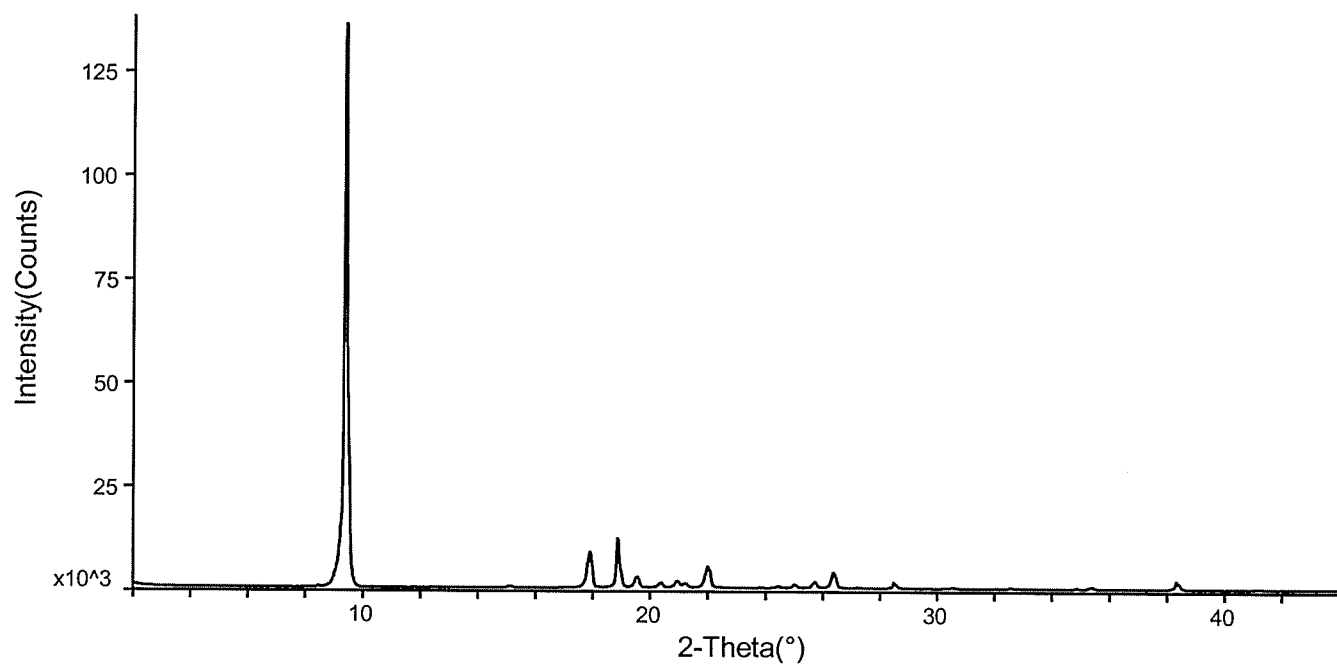
Figure 4.14 XRD pattern of pure phenanthrene.
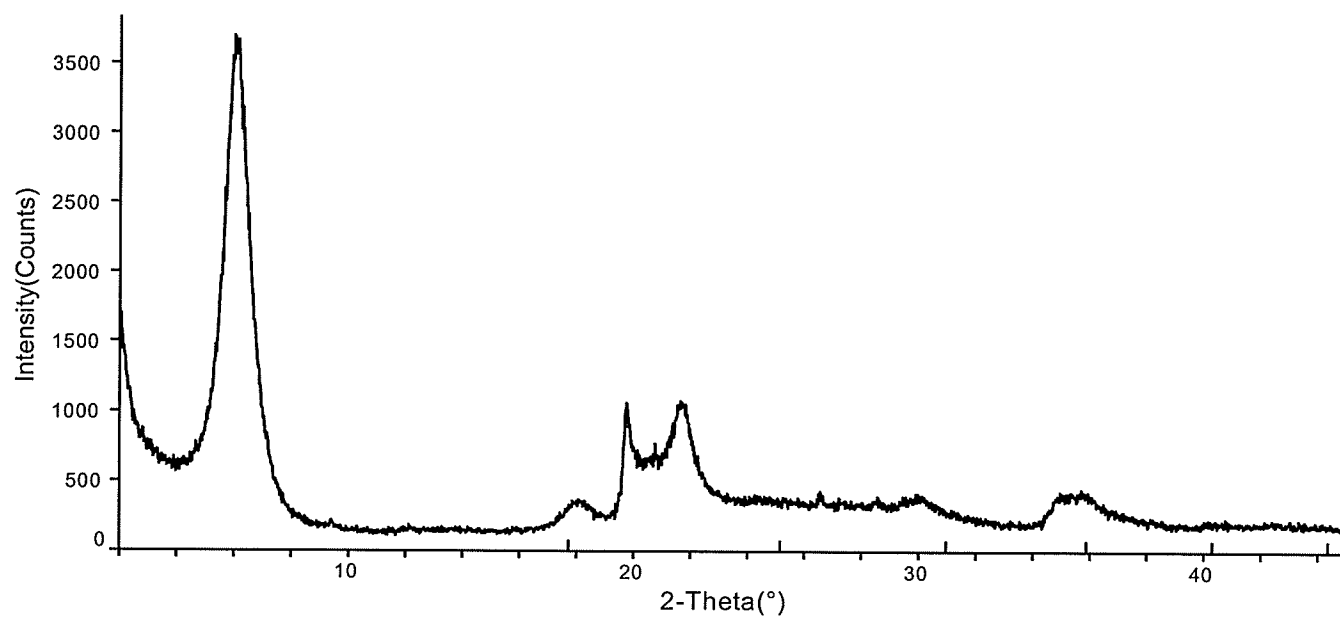
Figure 4.15 XRD line tracing of pure montmorillonite.
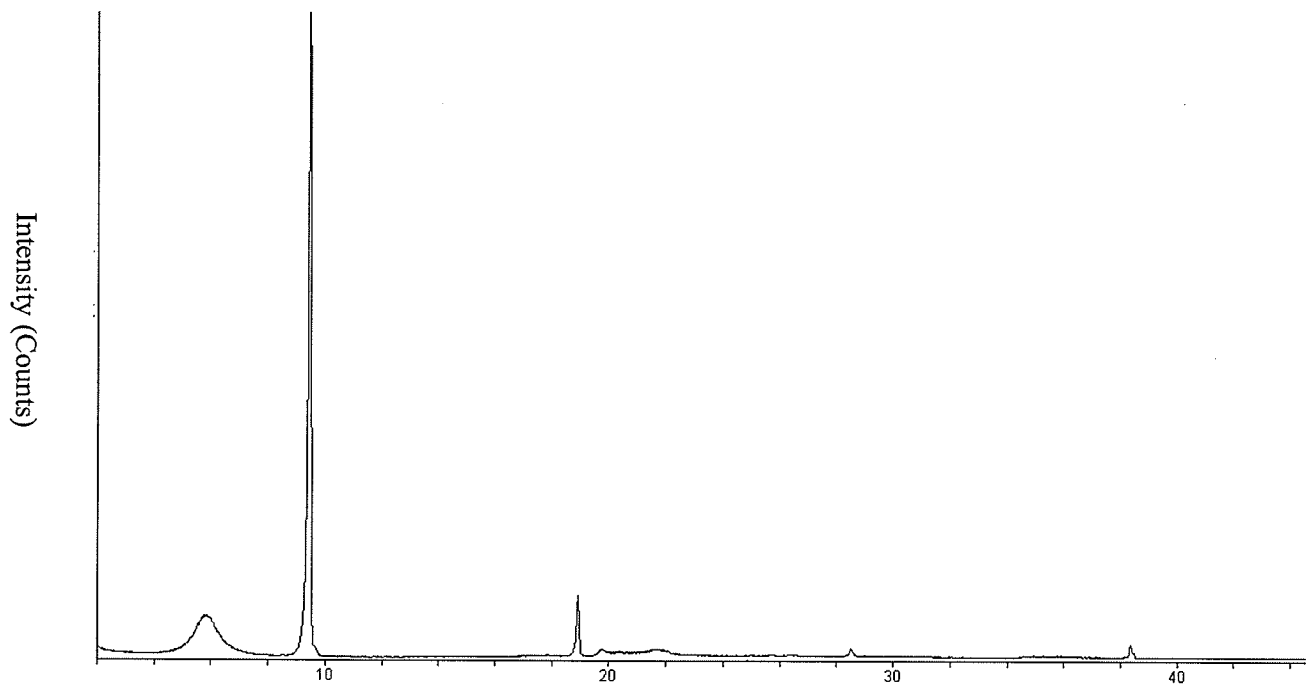
Figure 4.16 XRD line tracing of 0.25 g/g phenanthrene in montmorillonite.

Table 4.5 Interlayer d-spacings of the major phenanthrene and montmorillonite peaks (Å)

Time/ Peak	Phenanthrene		Montmorillonite			0.025 g Phn/g mont.			0.25 g Phn/g mont.		
	1 day	3 months	1 day	1 month	3 months	1 day	1 month	3 months	1 day	1 month	3 months
MONT			15.33	15.02	14.97	15.23	15.02	14.92	15.44	15.12	15.38
PHN	9.40	9.40				9.42			9.40	9.40	9.38

*The data for all peaks have been included in Appendix IV.
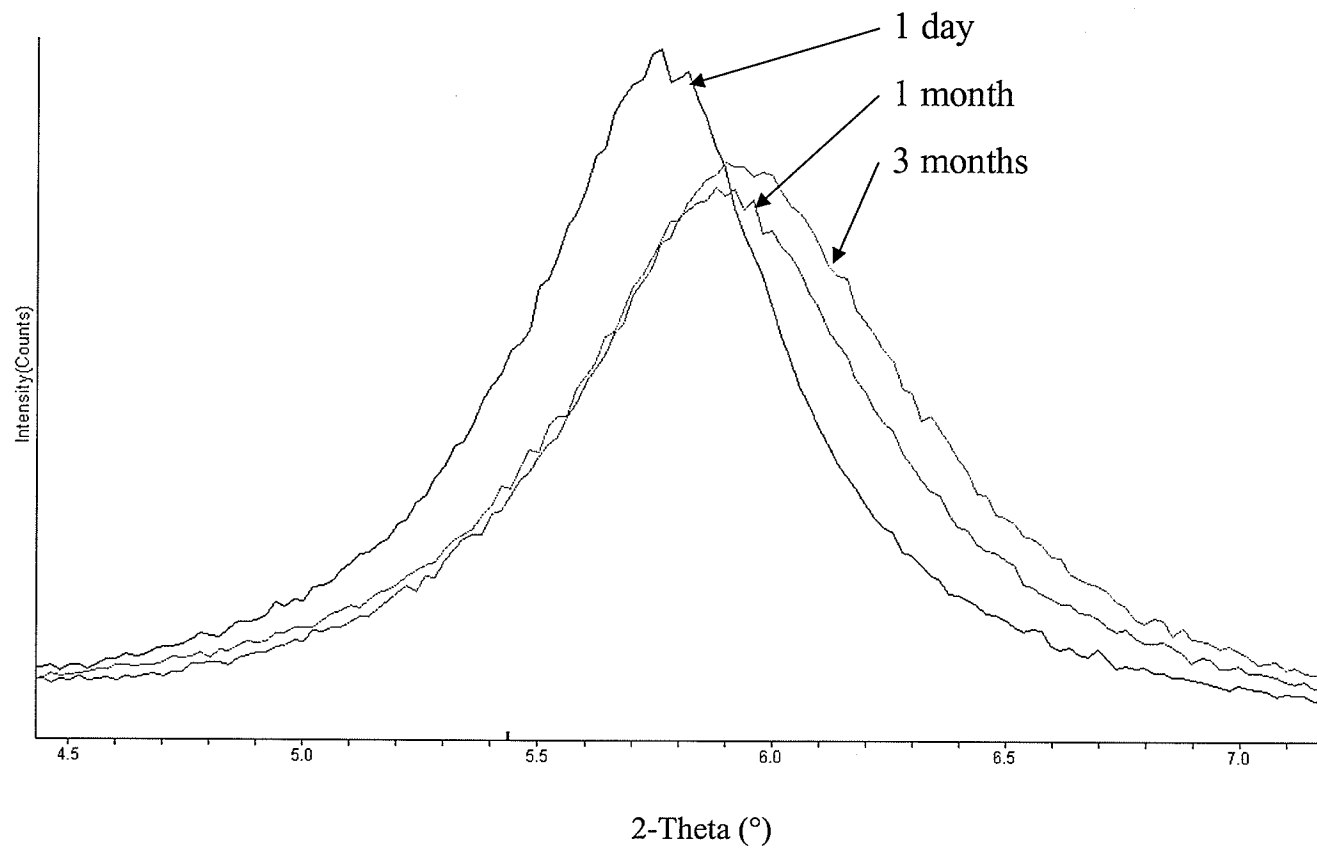
Figure 4.17 XRD line tracing of montmorillonite 001 peak in montmorillonite control.
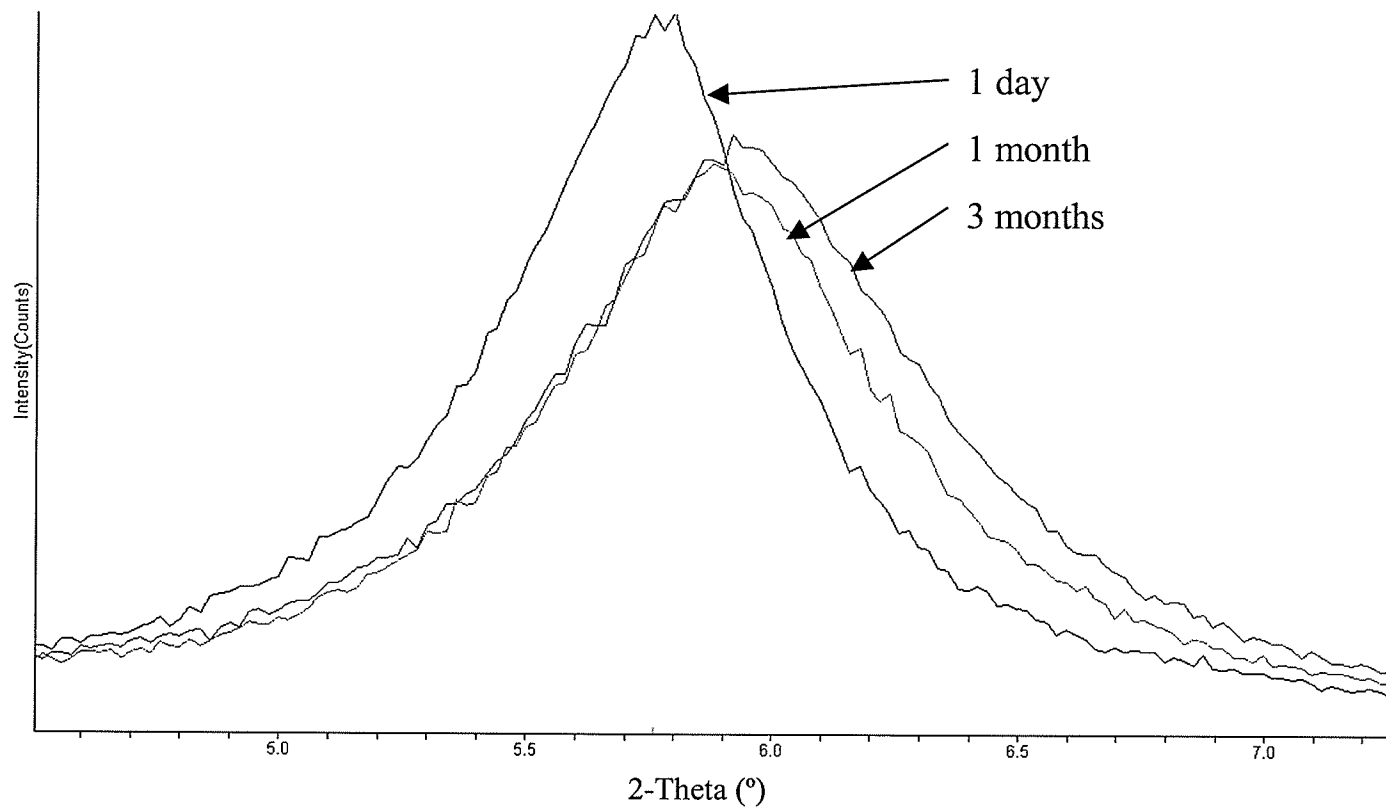
Figure 4.18 XRD line tracing of montmorillonite 001 peak in 0.025g/g phenanthrene
in montmorillonite
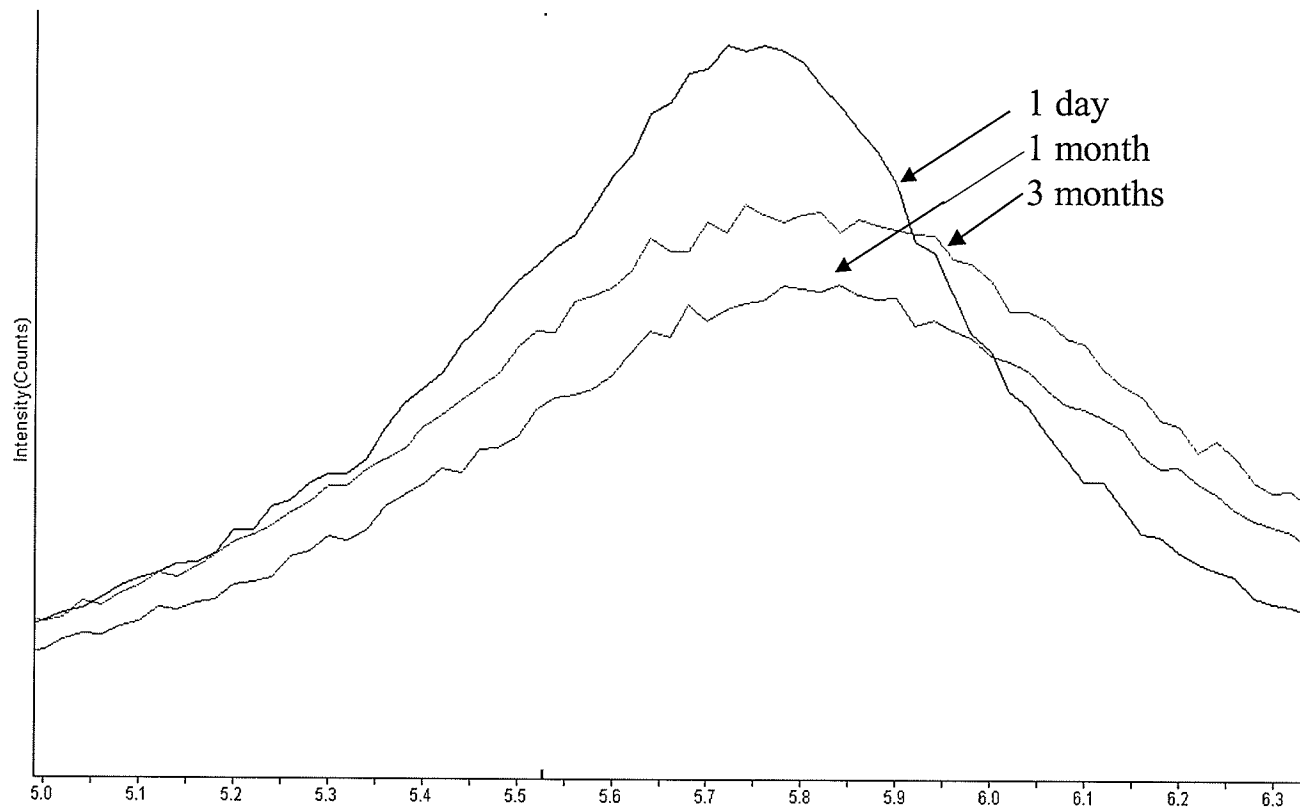
Figure 4.19 XRD line tracing of montmorillonite 001 peak in 0.25g/g phenanthrene in montmorillonite.

of the peaks decreased after a month or more of aging. The d-spacing of the 0.25 g phenanthrene /g montmorillonite clay was 0.11 Å larger than the control after day 1, 0.10 Å larger after 1 month and 0.41 Å larger after 3 months. This indicates that the phenanthrene may have be preventing the closure of the interlayer space as water evaporated and the samples dried. The thickness of the alumino silicate layer in montmorillonite is 9.6 Å (Theng, 1975) and the [001] d-space can be reduced to 9 Å if heated to 300°C for an hour to expel the adsorbed water (Faure, 1998). Ethylene glycol can be used to expand the interlayer spacing to swell during glycolation and is used to identify the expandable clay. The d-spacing of the 0.25 g/g sample was only 0.41 Å wider than the control sample, which is much smaller than the dimensions of the phenanthrene molecule which has a width of 8.031Å, a length of 11.752Å and a thickness of 3.888Å (Sander and Wise, 2003). As the interlayer spacing is about 5 to 6 Å, it is possible that the phenanthrene molecules could lie within the interlayer, displacing cations and water molecules, preventing closure.

The largest phenanthrene peak within the montmorillonite did not vary with time or concentration and had an average value of 9.4 +/- 0.02 2θ.

During the XRD procedure, the 0.25 g/g phenanthrene in montmorillonite sample turned a pastel blue corresponding to the hex code RGB colour #66CCCC. Colour changes have been reported for other experiments. A blue solution was formed during the protonation of phenanthrene in the attempts to generate a phenanthrenium cation (Laali *et al.*, 1997). Colour generating reactions have also been reported when mixing clays with organic compounds (Hasegawa, 1963; Cozens, 1993; Tao and Maciel, 1998)

including a yellow and greenish yellow colour when carbon tetrachloride and benzene were mixed with montmorillonite (Tao and Maciel, 1998). The causes of these changes have not yet been determined.

CHAPTER 5. SUMMARY AND CONCLUSIONS

When petroleum contaminants enter the environment and sorb to soil and sediments, they may become less available for microbial degradation with time. This aging results in a resistant fraction that is less bioavailable and has a slow release to the environment (Riley *et al.*, 2001). The major factors affecting bioavailability are sorption and sequestration. Organic matter has been shown to be the main sorbent for non-ionic organic compounds (Alexander, 1994) but sorption onto clay minerals is important when the organic matter content of the soil is less than 6% (Murphy *et al.*, 1990; Hwang and Cutright, 2002). This research study has shown that clay strongly influences sorption as well as bioavailability. Pure montmorillonite clay strongly sorbed the petroleum component phenanthrene causing a number of changes to occur. The sorption of phenanthrene with time reduces the bioavailability of phenanthrene to microbes, reduces the closure of the montmorillonite d-spacing as it dries and changes the chemical environment of C and Si in montmorillonite.

Microcosm experiments revealed that the degradation rate of phenanthrene was much greater in freshly amended than aged montmorillonite. Since overall degradation was extremely slow, numerous experimental conditions were attempted to enhance biodegradation. The microcosms at 21°C had much higher degradation then at 31°C and inoculated trials degraded more extensively than non-inoculated trials. In all cases, the phenanthrene that had been recently added to the montmorillonite clay degraded more than the aged samples. The experimental conditions enabling the phenanthrene to

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degrade faster in the 21°C experiment could have been the lower temperature, higher phenanthrene concentration, or a more active inoculum. Studies have indicated that higher rates of degradation occur with higher concentrations until the point where the chemical becomes toxic to the degrading organisms (Aust, 1990). Overall, degradation was extremely slow in the montmorillonite clay. The most extensive phenanthrene degradation was 26% over 160 days, while industrials soils degraded 35% after only 142 days. The poor degradation rates could be related to an unfavourable microbial environment. The montmorillonite clay used in the experiments was nutrient deficient, lacked organic matter and the platy expandable nature may have hindered water and nutrient movement.

Up to 38% of benzene in montmorillonite clay degraded in non-aged microcosms and degradation was much lower within aged samples. Benzene, a light weight aromatic hydrocarbon, was not expected to experience an aging effect since it is highly volatile and relatively easy for microbes to biodegrade. The lower degradation in the aged samples may have been caused by benzene volatilizing during the aging process, which was not measured due to technical problems. Strangely, the non-inoculated benzene treatments outperformed the inoculated benzene treatments. This may have been because the solution was contaminated with other chemical compounds during the preparation and thus the treatments were not comparable due to these impurities. Additionally, the inoculated treatment received less nutrients than the non-inoculated treatment. This could have effected the benzene experiments more than the phenanthrene experiments due to the higher degradation rates in the benzene microcosms.

The sorption capacity for soils varies according to the type and volume of mineral, soil organic matter and nutrients. The industrial soils were very low in nutrients and consequently when the growth medium was added, the nutrients sorbed to the industrial soil and thus were taken out of solution during the growth of the inoculum. When the microbial inoculum was subsequently taken, the nutrients had been leached from the solution and sorbed to the soil, thus producing a nutrient deficient inoculum. The non-inoculated microcosms received the basic growth medium without alteration and thus were given more nutrients. The subsequent addition of nutrients increased biodegradation rates, but only slightly. It could be that nutrients were not the limiting factor or that not enough nutrients were added.

Research has shown that the amount of a contaminant extracted with a mild extraction decreases in aged soils (Feng *et al.*, 2000; Smith *et al.*, 1999; Chung and Alexander, 1998) and is correlated with bioavailability (Kelsey *et al.*, 1997; Chung and Alexander, 1998). Dichloromethane shaker extractions and acetone/ dichloromethane ASE extractions were ineffective at extracting phenanthrene from montmorillonite clay with efficiencies ranging from 0.0 to 12.2%. Phenanthrene and benzene extractions have been successful previously using soils of various textures and OM (Dionex, date unknown). It is believed that the physical properties of the clay inhibited the extraction of phenanthrene from the matrix and that the benzene may have volatilized. After the microcosm and extraction experiments were completed, HF digestion was used to account for the remaining phenanthrene and benzene. The total recovery of the shaker and ASE controls was 70 to 94 % but microcosm treatments that were digested had much

lower recoveries. Due to the methods followed during the extraction process, large errors are associated with the digestion and thus the results are to be used qualitatively, not quantitatively. Total recoveries (% CO_2 captured, extracted and digested) were less than 40% for phenanthrene and benzene. It has been suggested that the polarity of petroleum degradation products enable them bind to organic matter quickly and prevent extraction (Käcker *et al.*, 2002). Similarly, it may be possible that the experimental amendments were unable to be extracted in their parent form or that the degradation products were binding to the clay.

The ^{13}C CP MAS NMR spectra showed the resolution of the amended montmorillonite to be much better than pure phenanthrene immediately after mixing and increased further after aging. The increased resolution after mixing was probably due to protons dissipating energy within the sample and thus allowing nuclei to relax faster. The increase in resolution from non-aged to aged samples may be from changes that occur as phenanthrene sorbs and becomes less bioavailable with time. The ^{29}Si spectra showed a 0.1 ppm shift from non-aged to aged phenanthrene amended montmorillonite samples indicating that the Si tetrahedra is becoming less shielded with time. There were no observable changes in the peak position for the ^{27}Al. This may indicate that the phenanthrene is distant from the Al octahedra (Liang and Sherriff, 1993) or that the technique was not sensitive enough to detect these changes.

The XRD data showed the 0.25 g/g phenanthrene in montmorillonite to have a d-spacing 0.41 Å greater than the control montmorillonite after 3 months of air drying. Theng et al., (2001) indicated that a change in basal spacing from 0.18 to 0.44 Å meant

that phenanthrene migrated into the interlayer and rearranged the orientation of previously sorbed tetradecyltrimethylammonium that had been added to increase the sorption capability of the montmorillonite. Thus phenanthrene may have displaced cations or water within the interlayer and prevented the interlayer from closing as much as the pure montmorillonite clay. Although this provides evidence of phenanthrene entering the interlayer, it does not disprove other theories stating that large hydrophobic molecules become physically entrapped within micro-pores or become sorbed to exterior and/or broken edges. It is likely that a combination of locations are possible since binding methods have not been determined.

No agreement in the literature has been reached about the significance clay plays in the bioavailability of aged hydrophobic compounds in the environment. Studying interactions in an environmental context becomes complicated and will not reflect the pure clay testing performed for several reasons. The sorption of phenanthrene and benzene to montmorillonite clay was favoured in several ways. Sorption of hydrophobic compounds to clay minerals dominate in dry conditions (Murphy *et al.*, 1990; Karickhoff *et al.*, 1997; Hwang and Cutright. 2002). Although the experiments started at 60% water holding capacity and contained acidified water to humidify the environment, the clay was dry and powdery at the end of the 361 days. As well, all treatments were autoclaved (except D_{PINX}) to sterilize the clay and other contents of the microcosm so that aged and non-aged treatments would be prepared similarly. Sorption has been found to increase with soil autoclaving (Thomsen *et al.*, 1999). Although the NMR spectra of phenanthrene amended montmorillonite clay did not indicate any visible changes with

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respect autoclaved verses non-autoclaved samples, any changes to the clay structure may impact the sorption process and thus effect the bioavailability of a compound with age.

The data gathered from microcosm degradation studies, extractions, XRD and NMR indicate that changes occur as phenanthrene is aged in montmorillonite clay. It may be that the processes causing the increased resolution in the CP NMR, reduced shielding of the Si and the wider d-spacing in the XRD are the same that are causing the reduced bioavailabity in the microcosms. Understanding bioavailability and the structural changes that occur with time may help explain why the remediation of older contaminated sites is often more difficult than sites that have been recently contaminated with PAHs such as phenanthrene.

CHAPTER 6.   CONTRIBUTION TO KNOWLEDGE

This research studied changes in the bioavailability of phenanthrene and benzene in pure montmorillonite clay and to tried to correlate these changes with structural and chemical changes occurring in the clay with time. The research gathered data from microcosm degradation studies, extractions, XRD and NMR to form a multidisciplinary view of the changes that occur with aging. Microcosms studies showed that aged phenanthrene amended montmorillonite clay had lower degradation rates than newly amended montmorillonite in all trials. Reduced degradation rates are thought to be caused by reduced phenanthrene bioavailability in the clay.

Acetone/ dichloromethane ASE and dichloromethane shaker extractions were ineffective at extracting phenanthrene from montmorillonite clay. These procedures are routinely used and are effective with a wide variety of soil types. The ineffectiveness of the extractions may have been caused by the low permeability of the clay to the solvents.

The resolution of the ^{13}C CP MAS NMR of phenanthrene amended clay increased after mixing, compared to pure phenanthrene and increased further with aging. This indicates that changes occurred within the chemical environment of the carbon and hydrogen. The 1ppm change in the ^{29}Si chemical shift indicates that aging of phenanthrene in montmorillonite caused a reduction in shielding of the Si tetrahedra. ^{27}Al MAS NMR did not show any evidence of changes to the chemical environment of the Al octahedral.

The d-spacing of the interlayer of montmorillonite clay was 0.41Å wider than the control montmorillonite clay after 3 months of drying. This indicates that the phenanthrene was preventing the closure of the interlayer, most likely by rearranging the interlayer contents.

The processes indicated by the increased resolution in the ^{13}C CP MAS NMR, the slight shift in the ^{29}Si MAS NMR and the wider d-spacing in the XRD may be causing reduced microbial bioavailability. More studies are required to determine the specific binding locations and methods involved in reducing the effectiveness of remediation. Understanding binding locations and methods will help determine how contaminants are degraded or leached from the soil. Only after these processes are fully understood can the information help to assess the risk of a contaminant within the environment. Understanding this could help improve soil remediation procedures, saving money while ensuring the safety of people and the environment.

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CHAPTER 8. APPENDICES

I. General Problems Encountered

Preliminary analyses with Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) in Soil Science determined that HPLC would be the most successful method of measuring phenanthrene and benzene as the peak of benzene was very close to that of the carrier liquid with the GC. The initial recoveries were in the 90% range for phenanthrene and benzene solutions.

In early tests of ASE with spiked samples, a bi-layer was formed as the water removed from the clay floated on top of the dichloromethane/acetone solution. Phenanthrene and benzene were not detected in the water phase but measurements within the solvent phase were within the expected range.

Early experiments, in the Department of Geological Sciences, consisted of montmorillonite clay contaminated with non-radioactive phenanthrene and benzene. These samples were to be tested at 0, 3, 6 and 12 month intervals with a dichloromethane shaker extraction in Geological Sciences and ASE in Microbiology to determine if aging reduced the extractable portion of the compound. Experimental ASE extractions encountered "vapour pressure exceeded" where samples had been brought up to 100°C and 2000 psi but were not held for the 5 minute static time and only produced approximately half of the solvent required. These samples were extracted a second time and the resultant solvents combined. Extractions were able to recover 37.1% of phenanthrene with the shaker extraction and 33.3% with ASE while for benzene 42.4 % was recovered with the shaker extraction and 48.6% for ASE. Since the ASE extractions were lower than the dichloromethane extractions, it was decided that it would be repeated

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at the end of the experiment.

During the extractions of samples which had been aged for 3 months, more technical problems were encountered with ASE including "vapour pressure exceeded", "solvent does not reach collection vial or rinse vile" and leaks in the hoses. Each time a problem was encountered, the appropriate measures were taken, which included cleaning, flushing solvents through the system and sometimes replacing parts. At the six month sampling period, the ASE in Microbiology was not functional and so the extraction was done at the Freshwater Institute, Department of Fisheries and Oceans, Government of Canada, accredited laboratory no. 452. HPLC analysis was done at the Department of Soil Science where the HPLC was incorrectly calibrated by the technician who left samples to evaporate overnight and used incorrect settings. At this point the experiment was discontinued and not restarted since this would take another 6 months and the testing problems remained. For MPN determination using HPLC, 0.5 g montmorillonite clay was placed in 10 ml test tubes and 1 ml of 10 ppm phenanthrene in dichloromethane was added to each tube, including three controls. The dichloromethane was then evaporated and 8.9 ml basic nutrient solution added. The tubes were capped with MPN lids and sterilized using an autoclave set on the liquid cycle. 1 ml aliquots of the inoculum used for the experiments were then diluted to extinction, using 10^{-1} to 10^{-9} dilutions, in triplicate. This technique was used for the initial extraction experiments as well as for experiments A, B, C, D and E. The MPN tubes were incubated in the dark, to prevent photo-degradation, until the end of each experiment. A positive result indicated by the depletion of the phenanthrene in the diluted MPN tubes when compared to the controls,

as measured by HPLC after being extracted with ASE. All MPN extraction results were below detection limit. Phenanthrene may not have been measured due to its strong sorption to the clay and benzene may have volatilised.

It was then decided to change the experimental technique to measure biodegradation by trapping respired $^{14}CO_2$ from radio-labelled phenanthrene and benzene in microcosms. The clay was extracted using a shaker and ASE extraction method following biodegradation experiments. Extraction efficiencies of the shaker and ASE extractions were very low and a large portion of the radioactive carbon could not be recovered. The microcosm jars at 31°C were often under pressure and when opened small explosions of hot moist air were emitted from the microcosm allowing $^{14}CO_2$ to escape. Water condensed on lids and, although every effort was made to have the condensation drip into the clay containing jar, some spillage did occur. These losses would cause biodegradation to be underestimated.

Mass balance of the microcosms could not be calculated due to technical problems with the oxidizer preventing the measurement of volatile hydrocarbons sorbed to the ambersorb. The oxidizer had not been used for some time and required substantial maintenance, unable to be completed at that time. It was decided that a new oxidizer was required but would not be available for this study. As 4 to 6 months had passed after microcosm experiments were complete, and volatile components might have been lost from the ambersorb, it was decided that the ambersorb would not be analysed. The amount of volatile phenanthrene was expected to be minor but the amount of volatile benzene was expected to be significant.

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The technical problems of the ASE and HPLC overshadowed the real issue, which was that the mild and ASE extractions had very poor recovery rates for compounds added to pure montmorillonite clay. Two digestion methods, nitric-perchloric acid and hydrofluoric acid digestion, were attempted to determine if the phenanthrene and benzene remained in the clay. The nitric-perchloric digestion of spiked samples had recovery rates of only 45.8 +/- 4.5% and so a hydrofluoric (HF) digestion was used.

The sample preparation required for the HF digestion introduced many errors into the experiment and thus was only done for a few samples as a qualitative assurance that the phenanthrene and benzene remained in the montmorillonite clay. Since samples that were digested had been used for both the mild and ASE extractions, transfer losses were possible. More importantly, Ottawa sand and anhydrous sodium sulphate had been added to the clay during the ASE extractions. The sand had been added in an unknown amount as a filler material. The digestion samples had to be small, 0.5 g or less, and the silica sand was unlikely to have any of the contaminant sorbed to it, yet could be a significant portion of the mass. The difference in weight and form of the clay and sand made it difficult to take a homogeneous sample. Therefore the larger sand fraction was removed by sieving the samples. The sample then consisted of the clay, an equal weight of anhydrous sodium sulfate and an unknown amount of fine silica sand that had been able to pass through the sieve. 0.5 g of this mixture was used for the digestions. Experimental samples had low digestion recovery rates but dilution factors were uncertain and sample volumes were very small.

II. ^{14}C Raw Counts for Microcosms and Extractions

Experiment A Raw Counts

Day	1	2	3	4	6	8	11	14	17	21	28	35
Background	26.2		24.2	27.7	31.9	31.9	24.2	24.2	26.3	26.3	27.3	28
ABIN1	17836.2	15438.2	10708.1	8919.3	4465.6	8150.4	7425.5	7873.3	5867.5	9093.3	15316.3	13014.2
ABIN2	5197	3769.8	3132.4	2876.7	2406.4	3720.6	6440.1	9785.5	6734.3	5101.4	16890.6	21883.4
ABIN3	5879.5	3317.9	2787	2407.3	1562.1	2809.1	2158.1	4140	3621.7	5123.2	10915.6	17458
ABIN4	36420	31622	21282.5	15613.7	6725.2	12977.7	14575.6	14061.7	6650.8	8206.4	11921.2	11697.4
ABNN1	118105.9	83410.8	48742.1	33908.7	9978	22277.6	2037.9	13163.3	9604.7	11165	13159.7	6246.2
ABNN2	1042.2	242.4	166.5	123.7	103.9	121.4	88.5	81.3	86.3	121.3	101.1	90.3
ABNN3	96164.5	78070.1	44792.8	29551.3	10917.7	20101.4	17238.1	12162.9	4411.8	10648.5	13323.9	4710.6
ABNN4	96687.1	65939	38269.2	26602.9	15892.2	17135.6	9938	17301.5	9141.7	9465.9	12645	3834.6
APIN1	7280.3	89.4	99.1	107.4	180.6	149	476.3	391.9	398.9	582.3	1480.9	4817.3
APIN2	7955.4	139.7	101.9	87.9	122.7	116.8	226.8	246.1	706.8	236	561.5	2559.1
APIN3	8142.5	71.1	71.7	73.8	105.3	83.7	103.4	102.2	178.8	163.7	505.7	1529.4
APIN4	3129.8	74.8	85.7	95.2	134.7	103.1	290.9	183	159.7	212.1	363.6	229.9
APNN1	6329.8	91	81.3	73.4	103.8	303.9	162.5	235.3	206.5	524.1	185.2	187.2
APNN2	3309.6	62.3	70.5	66.4	107.7	100.1	246.7	182.2	184.7	124.1	189.7	255.6
APNN3	3894	55.5	68.9	73.3	168.9	80	193	208.7	168.2	249.4	416.3	305.7
APNN4	6298.8	91.4	121.5	74.3	109.6	71.7	186.3	213.2	162.2	83.2	332.3	211.7

42	50	57	64	71	78	85	92	99	148	176	204
28	27.7	27.7	26	25.7	26.6	25.4	24	27.1	24.1	24.3	27.1
28422.6	6217.4	6764.9	5514.4	5223.7	5394.8	12957.2	2063.6	2264.9	4585.2	10303.6	12147.3
32387.7	2453.8	4609.4	5666.2	5212.6	5370.5	9299.9	1447.1	1727.1	3191.4	6914.9	8965.4
23957.3	2862.1	3144.9	3689.1	3315.1	3502.5	7535.5	1251.9	1392.8	3104	6853.6	8727.5
20363.9	1941	4602.3	4166.6	4702.3	4257.6	9072.6	2008.2	2232.4	3264.6	6147.3	8629.3
11467.5	3487.7	4578	3470	3642.7	3021.7	6774	2016.9	2012	2048.1	2418.2	3384.3
216.6	175.5	124.4	65.2	79.5	74	195.9	75.3	45.2	57.7	64.6	87.1
16574.7	3354.2	5006.3	3654.6	4212	3254.3	7939.6	1908.7	1862.5	2105.3	2407.7	3338.3
13170.7	2645.2	3415.7	3415.7	3746.9	3869.8	5701.5	1943.8	1910.3	2145.3	2487.8	3393.5
3897.4	269.7	402.4	535.1	577.2	580.3	1807.7	157.7	210.5	322.9	595.7	409.6
5393.4	301.9	264.75	227.6	205.7	202.8	555	94.5	69.4	56.1	100.5	127.9
2049.6	161	193.8	226.6	477.1	425.9	1586.5	195.8	272.3	287.1	770.7	615.9
3075.2	172.4	323.95	475.5	201.1	163.9	903.3	86.2	42.2	43.8	93.1	127.9
2918.4	183.8	175	247.5	155.1	157.5	653.8	85.9	37.7	46.8	70.2	98.2
2398.6	183.5	162.9	232.7	215.5	154.3	2535.1	233.4	107.4	46.7	108	121.9
3456.5	266.4	257	269.9	197.3	190.6	2044.3	182.4	63.5	70	153.9	162
2959.1	227.2	323.2	280.7	914.1	163.1	550.6	84.1	36.4	47.6	93.4	131.5

214	222	237	253	268	289	310	328	361	Acidified Water	Shaker Extraction	ASE
23.2	27.3	27	25.4	25.6	24.2	27.6	25.5	28	28	28	28
11363.6	7607.7	10859.2	3392.2	4566.3	2869.4	1439.3	1103.8	4264.9	227.9	213.2	219.9
4594.6	1954.8	4281.3	1375.4	1185.6	738	658.8	411.9	850.8	75.5	163	190.6
2755.1	1132.4	2574.5	1012	755.9	687.1	574.8	421.2	953.3	102.9	180.4	142.1
3505.9	1039.8	2186.3	979.8	786	464.8	341.1	266.3	647.2	137	194.8	145.3
489.4	36.7	66.6	64.7	55.4	75.9	62.3	59	93	106.2	227.9	36.8
36.5	22.7	26.7	24.6	25.2	24.9	25.6	27.8	24.3	30	59.1	27.6
759	27.2	56.1	49.6	43.7	54.2	48.9	36.6	69.5	145.8	243.8	63
687.5	28.8	45.8	39	47.1	43.4	69.8	48.8	77.3	103.2	263.4	81.1
282.2	140.9	720.3	543.5	880.6	618.7	561	620	1134.2	82.4	566.4	566.9
203.1	112.4	519.5	430.9	559.5	419.7	395.4	400	1054.3	83.4	731.7	657
529.3	215.2	1170.9	863.7	905.5	527.2	421.9	709.5	1311.6	89.1	523.5	524.1
108	58.2	165.7	168	177.1	112.4	100.7	140.7	258.9	63.7	601.2	570.9
186.3	85.5	207.8	141	126.1	159.3	162.2	174.9	191.8	63.4	1354.2	661.3
124.3	87.7	166.6	136	126.4	204.2	213	193.6	219.5	58.6	1382.5	653
152	94	246.9	217.6	175.7	218.3	225.2	239.7	258.5	49.2	1066.8	592.3
135.8	107.3	165.9	128.2	102.8	143.6	134	138.9	182.9	130.1	1404.6	661

Experiment B Raw Counts

Day	1	4	7	21	29	48	76	105	132	142	151	166
Background	28.3	25.2	25.7	29.3	23	24.1	24.3	27.1	25.3	25.7	27.3	27
BPNN1	120.1	410.4	167.2	60.9	59.3	46.8	143.3	225.5	243.2	160.2	86	172.7
BPNN2	105.8	242.7	136.4	70.3	69.6	46	107.1	194	193.7	101.9	67.2	115
BPNN3	247.5	516.3	580.5	156.2	179.2	70	230.5	302.6	365.8	155.6	137.1	181.9
BPNN4	188.3	343.8	165.9	75.1	88.1	47.6	146.5	181.2	216.9	175	63.3	109
BPIN1	194.6	474.8	612.4	36	40.2	4585.2	62.6	75.5	71	59.8	40.6	54.7
BPIN2	239.5	421.3	302.8	117.4	100.1	3191.4	136.6	220.7	208.5	121.2	106.6	126.2
BPIN3	145.7	216.5	241.2	107.5	125.7	3104	163.3	236.2	215.9	123.2	49.5	166.8
BPIN4	197.6	199.9	154.3	68.8	88.4	3264.6	153.5	193.3	174.2	126.7	86.4	166.2
BPINM1	193.6	324.3	156.6	62.8	71.1	2048.1	145.6	212	204.7	104.5	91.1	174.1
BPINM2	287.1	367	162.7	94	85.3	57.7	148.2	239.3	228.9	126.9	84.9	179.4
BPINM3	173.3	225.6	171.3	136.4	164.4	2105.3	215.4	190.6	167.5	110.8	90.7	125.7
BPINM4	344.5	514.1	251.9	63.5	85.2	2145.3	141.7	230.4	235.1	173.3	109.3	179.6

181	196	217	238	256	289	No Water	Shaker Extraction	ASE
25.4	25.6	24.2	27.6	25.5	28		28	28
168.8	155.9	229.5	215.4	261.9	208.9		829.6	351.8
132.6	104.7	156.9	173.4	220.8	146.1		1176.3	390.6
215.4	152.9	213.7	257.9	295.6	155.2		767.9	290.7
169	170	180.8	201.1	244.8	172.7		603.2	322.8
57.6	55.4	48.3	59.2	63.6	49.3		531.2	284.6
247.4	132.1	164	141	228.3	167.7		613.4	338.2
185	148	202.4	197.3	218.6	170.8		543.6	368.3
166.1	125.7	278.1	243	158.6	143		469.7	322.5
154.5	124.6	234	196.7	207.5	129.9		562.1	344.2
230.1	138.3	238.6	162.8	173.8	190.2		556.4	372.7
177.8	126.2	160.3	154	150.9	108.7		555.9	252.7
243.6	171.5	208.2	210.6	214.4	234.3		591.3	384.9

Experiment C Raw Counts

Days	19	34	49	70	91	109	142	Acidified Water	Shaker	ASE
Background	27	25.4	25.6	24.2	27.6	25.5	28	28	28	28
CPIN1	17994.9	39146.8	26466.4	7687.6	5431	4453.9	4343.1	50.9	1169.6	805.9
CPIN2	108302.9	18235.2	10451.7	2627.3	2480.2	1333.5	2612.5	36.6	180.9	321.5
CPIN3	96505.9	21210.9	10370.7	2369.5	2214	1171.3	2539.7	37.1	310.8	337.9
CPIN4	108268.5	24392.6	11735	2559	2481.5	1163.2	1981.9	39.8	483.9	305.2

Experiment D Raw Counts

	10	18	33	49	64	85	106	124	157	Acidified water	shaker	ASE
Days												
Background	23.2	27.3	27	25.4	25.6	24.2	27.6	25.5	28	28	28	28
DPIN1	1595.6	1325.9	170435.6	41355.5	11530.3	2581.8	203.5	1324.6	2359.8	59.4	888.7	1030.1
DPIN2	1584.8	1162	67705.5	79048.7	32854.5	8708.8	4682.2	2592	4978.3	56.9	810.2	1146.5
DPIN3	1642	1017.6	123263.9	47234.6	27130.4	9364.9	3447.4	2303.4	3968.8	53.8	825.6	1078.2
DPIN4	1702.9	1020.8	187319.8	35989.6	15200.3	4808.4	3024.7	1527.1	3139.5	70	857.2	1267
DPNN1	1416.5	584.1	1316.4	2837.6	1932.9	2632.4	2296.5	1423.3	1225.9	137.1	106082.6	10210.7
DPNN2	1127.3	694.1	1474.3	2138.6	1745.2	3117.9	2894.1	2539.8	1243.7	107	97736.1	14032.3
DPNN3	1199.5	495.6	1239.9	1663.2	1484.9	2693.1	2432.5	2382.7	1502.2	127.2	108406.7	8465
DPNN4	952.9	599.4	1383.1	2320.9	1850.3	2766.8	2431.4	2007.2	1450.5	104	119344.3	8409.6
DPINX1	1199.2	830.2	1467.9	1839	2094.6	3559.2	3075.2	2448.9	1469.9	156	109623.9	8296.1
DPINX2	1574.9	813.7	1434.2	3764.1	2154.3	2858.6	271.1	2636.6	1417.4	125.2	99488.9	8896.8
DPINX3	997.5	776.3	1725.7	2447.9	2407	3617.4	2957.5	2818.7	1468.2	114.7	105541.5	7229.5
DPINX4	1902.6	656.4	1657.2	1631.6	3120.8	2856	2659.1	2487	1392.3	119.5	116610.7	7751.7
DPIA1	297.2	112.6	224.5	216.3	145.1	159.9	170.5	189.9	280.6	75	1307.6	602.2
DPIA2	214.4	96.3	220	180	166.7	238.5	210.8	194.4	254	72.6	1117.3	488.6
DPIA3	227.5	96.6	173.6	140	133.8	112.4	100.2	107.3	152.1	164.4	901.7	572.7
DPIA4	261.3	87.3	210.8	183.8	176	201.4	212.4	206.1	296.2	98.5	1109.9	552.1
DBIA1	103.6	65.1	163.2	95	93.4	52	63.1	57.5	85.7	462.9	216.4	60.7
DBIA2	612.9	144.8	329.5	160.4	141.5	81.9	74.7	64.5	136.8	660.8	727	130.1
DBIA3	84.7	45.1	124	75.1	77.3	47.6	54.4	43.7	79.3	255.7	224.7	68.8
DBIA4	85.4	67	128.7	72.1	64.8	48.1	52.7	48.7	75.5	302.6	216.6	80.2
EPNA1	104.5	91.1	174.1	154.5	124.6	234	196.7	207.5	129.9		562.1	344.2
EPNA2	126.9	84.9	179.4	230.1	138.3	238.6	162.8	173.8	190.2		556.4	372.7
EPNA3	110.8	90.7	125.7	177.8	126.2	160.3	154	150.9	108.7		555.9	252.7
EPNA4	173.3	109.3	179.6	243.6	171.5	208.2	210.6	214.4	234.3		591.3	384.9
EPIA1	206.1	76.7	83	70.6	45.2	47.2	49.7	30.3	29.2		645.2	395.7
EPIA2	99.3	51	31.3	31	30.1	31.5	30.1	26.9	28		629.8	434.3
EPIA3	70.5	29.9	26.8	28.9	24.3	26.3	28.6	31	29.8		607.5	380.9
EPIA4	215	79.6	41.2	31	29.6	29.9	30.8	30.4	26.2		631.1	388.2
EPIN1	87.5	32	33.2	25.5	27.9	26.4	29	25.9	24.2		984.5	261.1

EPIN2	338.1	112.9	56.2	34.9	33.6	32.6	34.2	34.9	34.9		738.6	395.4
EPIN3	170.6	55.7	42.6	40.6	34.2	31.2	32.4	31.9	29.2		834.6	252.1
EPIN4	101.9	32.3	33	38.5	32.7	27	28	25.2	28.2		637.2	233.6
EPNN1	5143.8	3882.2	2275.4	1308.3	1291.2	1407	1387.4	1346.3	1558.5		855.5	297.7
EPNN2	6918.7	5420.5	3182.2	1986.2	1868.9	1898.2	1648	1531.8	1489.4		745.4	306.9
EPNN3	5851.6	4268.4	442.7	139.9	90.5	135.6	132.4	127.5	104		783.8	244.3
EPNN4	28.7	6176.2	550.8	188.1	97.5	145.8	126.9	116.5	124.2		464.8	281.6
EPINM1	8234.6	21.6	3139.3	102.9	80.4	101.5	94	91	90.7		488.3	299.9
EPINM2	7316.8	2144.7	612.6	97.2	59.6	73.1	70.3	72.2	72.8		571.1	166
EPINM3	8541.2	1508.2	720	112.6	76.6	77.1	84.6	96	81.6		541.8	267
EPINM4	8835.8	3454.3	521.2	116.1	94.4	100.5	98.2	102.1	60.2		1703.9	238

Spiked Controls for Extractions

Shaker C1	31491.2
Shaker C2	30518
Shaker C3	38971.5
Shaker C4	20460
ASE C1	55780
ASE C2	57608
ASE C3	51983.8
ASE C4	63560.9

III. XRD Data

0.25g/g phenanthrene contaminated montmorillonite (Time 1)

JADE: Peak Search Report (12 Peaks, Max P/N = 134.6)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=72818, 09/11/02 14:20

PEAK: 25-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.720	15.4365	647	4888	6.7	168821	32.0	0.587
9.401	9.4000	185	72633	100.0	527382	100.0	0.123
17.580	5.0407	223	235	0.3	8245	1.6	0.596
18.918	4.6871	258	6333	8.7	44222	8.4	0.119
19.780	4.4848	260	619	0.9	12481	2.4	0.343
20.399	4.3501	273	426	0.6	16131	3.1	0.644
21.942	4.0475	514	348	0.5	11572	2.2	0.565
25.777	3.4533	306	80	0.1	889	0.2	0.189
26.400	3.3732	306	183	0.3	1778	0.3	0.165
28.540	3.1250	338	788	1.1	5587	1.1	0.121
29.541	3.0214	323	159	0.2	5011	1.0	0.536
38.382	2.3433	189	1211	1.7	10335	2.0	0.145

0.25 g/g phenanthrene contaminated montmorillonite (Time 2)

JADE: Peak Search Report (16 Peaks, Max P/N = 115.6) SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=53817, 10/18/02 11:46

PEAK: 23-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.840	15.1215	769	2962	5.5	126399	33.2	0.725
9.400	9.4009	192	53625	100.0	381119	100.0	0.121
17.847	4.9659	253	98	0.2	2286	0.6	0.397
18.882	4.6958	268	5242	9.8	35878	9.4	0.116
19.741	4.4935	266	599	1.1	17882	4.7	0.508
20.340	4.3624	265	434	0.8	17313	4.5	0.678
21.581	4.1144	495	365	0.7	14090	3.7	0.656

25.057	3.5509	313	61	0.1	424	0.1	0.118
25.718	3.4612	305	95	0.2	973	0.3	0.174
26.398	3.3735	299	175	0.3	1891	0.5	0.184
27.617	3.2273	292	77	0.1	580	0.2	0.128
28.520	3.1271	298	650	1.2	5564	1.5	0.146
34.881	2.5701	251	70	0.1	1283	0.3	0.312
35.878	2.5009	284	69	0.1	1011	0.3	0.249
38.364	2.3444	177	911	1.7	8184	2.1	0.153
41.162	2.1912	155	53	0.1	671	0.2	0.215

0.25 g/g phenanthrene contaminated montmorillonite (Time 3)

JADE: Peak Search Report (11 Peaks, Max P/N = 128.9)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=66894, 12/18/02 10:17

PEAK: 27-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.740	15.3837	888	3444	5.2	150582	36.2	0.743
9.419	9.3821	197	66697	100.0	415980	100.0	0.106
17.820	4.9732	281	118	0.2	2869	0.7	0.413
18.918	4.6870	294	6160	9.2	42892	10.3	0.118
19.723	4.4976	293	698	1.0	22127	5.3	0.539
20.380	4.3540	309	507	0.8	19498	4.7	0.654
21.741	4.0845	547	466	0.7	17020	4.1	0.621
25.019	3.5563	355	64	0.1	1414	0.3	0.376
26.377	3.3761	348	100	0.1	1787	0.4	0.304
28.522	3.1269	322	797	1.2	8018	1.9	0.171
38.398	2.3423	204	1286	1.9	10170	2.4	0.134

0.025g/g phenanthrene contaminated montmorillonite (Time 1)

JADE: Peak Search Report (8 Peaks, Max P/N = 33.6)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=5981, 09/11/02 13:37

PEAK: 33-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.799	15.2270	777	5204	100.0	176519	100.0	0.577
9.377	9.4240	134	1937	37.2	13513	7.7	0.119
17.559	5.0466	162	261	5.0	9022	5.1	0.588
19.780	4.4847	168	832	16.0	22746	12.9	0.465
21.759	4.0811	506	435	8.4	16160	9.2	0.632
26.600	3.3483	279	119	2.3	1304	0.7	0.186
29.620	3.0135	284	250	4.8	7234	4.1	0.492
34.822	2.5743	165	210	4.0	3263	1.8	0.264

0.025 g/g phenanthrene contaminated montmorillonite (Time 2)

JADE: Peak Search Report (8 Peaks, Max P/N = 29.0) SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=4727, 10/18/02 12:29

PEAK: 31-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.880	15.0177	735	3992	100.0	156324	100.0	0.666
9.403	9.3981	128	51	1.3	297	0.2	0.099
17.760	4.9901	169	131	3.3	4910	3.1	0.637
19.760	4.4892	181	765	19.2	21560	13.8	0.479
21.640	4.1033	490	474	11.9	16315	10.4	0.585
26.600	3.3483	277	155	3.9	928	0.6	0.102
30.263	2.9509	246	68	1.7	2607	1.7	0.652
34.861	2.5715	157	158	4.0	5485	3.5	0.590

0.025 g/g phenanthrene contaminated montmorillonite (Time 3)

JADE: Peak Search Report (8 Peaks, Max P/N = 29.0)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=4968, 12/18/02 11:00

PEAK: 33-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.920	14.9158	880	4088	100.0	167470	100.0	0.696
9.343	9.4584	143	47	1.1	392	0.2	0.142
18.042	4.9127	208	138	3.4	4431	2.6	0.546
19.779	4.4849	218	895	21.9	22066	13.2	0.419
21.700	4.0920	552	524	12.8	18505	11.0	0.600
30.220	2.9550	285	64	1.6	2290	1.4	0.608
35.023	2.5600	189	213	5.2	7348	4.4	0.586
35.767	2.5084	347	78	1.9	1607	1.0	0.350

Montmorillonite (Time 1)

JADE: Peak Search Report (8 Peaks, Max P/N = 40.6)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=8514, 09/11/02 12:52

PEAK: 31-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.760	15.3311	1026	7488	100.0	258637	100.0	0.587
9.377	9.4233	135	84	1.1	1147	0.4	0.232
17.538	5.0527	175	377	5.0	11879	4.6	0.536
19.800	4.4802	170	745	9.9	17019	6.6	0.388
21.759	4.0811	490	530	7.1	18614	7.2	0.597
26.642	3.3431	283	92	1.2	575	0.2	0.106
29.520	3.0234	311	212	2.8	8086	3.1	0.648
35.916	2.4983	287	93	1.2	3563	1.4	0.651

Montmorillonite (Time 2)

JADE: Peak Search Report (6 Peaks, Max P/N = 35.2) SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=6813, 10/18/02 13:29

PEAK: 41-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.880	15.0174	1001	5812	100.0	225817	100.0	0.661
17.857	4.9630	183	168	2.9	6476	2.9	0.655
19.779	4.4849	198	666	11.5	17697	7.8	0.452
21.699	4.0922	483	458	7.9	16649	7.4	0.618
30.277	2.9495	256	96	1.7	3513	1.6	0.622
35.059	2.5574	158	203	3.5	11693	5.2	0.979

Montmorillonite (Time 3)

JADE: Peak Search Report (11 Peaks, Max P/N = 35.1)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=7121, 12/18/02 11:44

PEAK: 31-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
5.900	14.9665	1202	5919	100.0	243059	100.0	0.698
9.379	9.4215	153	95	1.6	834	0.3	0.149
17.920	4.9457	209	194	3.3	7860	3.2	0.689
19.778	4.4851	232	795	13.4	17877	7.4	0.382
20.564	4.3154	604	83	1.4	5401	2.2	1.106
21.661	4.0994	541	576	9.7	21842	9.0	0.645
26.568	3.3523	318	91	1.5	433	0.2	0.081
28.635	3.1148	317	62	1.0	487	0.2	0.134
29.925	2.9835	298	98	1.7	3731	1.5	0.647
34.941	2.5658	174	204	3.4	5914	2.4	0.493
35.869	2.5015	335	74	1.3	2219	0.9	0.510

Phenanthrene (Time 1)

JADE: Peak Search Report (23 Peaks, Max P/N = 184.6) SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=136894, 05/08/02 16:00

PEAK: 17-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
8.481	10.4175	212	487	0.4	4113	0.4	0.144
9.397	9.4038	280	136614	100.0	1114056	100.0	0.139
15.122	5.8539	269	423	0.3	4581	0.4	0.184
17.901	4.9511	433	8501	6.2	96206	8.6	0.192
18.863	4.7006	470	11873	8.7	96160	8.6	0.138
19.560	4.5346	565	2396	1.8	23618	2.1	0.168
20.380	4.3540	514	992	0.7	9623	0.9	0.165
20.942	4.2385	485	1568	1.1	23281	2.1	0.252
21.239	4.1798	510	897	0.7	14597	1.3	0.277
22.001	4.0368	470	4993	3.7	59331	5.3	0.202
24.441	3.6389	451	339	0.2	2944	0.3	0.148
25.022	3.5558	457	767	0.6	7621	0.7	0.169
25.740	3.4583	468	1408	1.0	15473	1.4	0.187
26.399	3.3734	434	3714	2.7	42128	3.8	0.193
28.485	3.1309	353	1370	1.0	13661	1.2	0.170
29.041	3.0722	353	94	0.1	1175	0.1	0.213
30.598	2.9193	322	204	0.1	2590	0.2	0.216
32.616	2.7431	281	214	0.2	4117	0.4	0.327
34.877	2.5703	261	206	0.2	2960	0.3	0.244
35.399	2.5336	263	503	0.4	6752	0.6	0.228
38.325	2.3466	222	1966	1.4	21282	1.9	0.184
41.238	2.1873	221	242	0.2	3831	0.3	0.269
44.079	2.0527	225	246	0.2	3629	0.3	0.251

Phenanthrene (Time 3)

JADE: Peak Search Report (24 Peaks, Max P/N = 214.8)

SCAN: 2.0/45.0/0.02/1(sec), Cu, I(max)=184965, 12/18/02 12:33

PEAK: 17-pts/Parabolic Filter, Threshold=3.0, Cutoff=0.1%, BG=3/1.0, Peak-Top=Summit
NOTE: Intensity = Counts, 2T(0)=0.0(°), Wavelength to Compute d-Spacing = 1.54056A (Cu/K-alpha1)

2-Theta	d(A)	BG	Height	I%	Area	I%	FWHM
8.498	10.3965	174	607	0.3	3634	0.3	0.102
9.402	9.3982	223	184742	100.0	1314614	100.0	0.121
15.120	5.8548	216	462	0.3	5531	0.4	0.204
17.920	4.9458	394	7799	4.2	89683	6.8	0.195
18.898	4.6919	425	16940	9.2	116750	8.9	0.117
19.541	4.5389	434	2269	1.2	26057	2.0	0.195
20.381	4.3537	446	974	0.5	8817	0.7	0.154
20.960	4.2347	435	1590	0.9	21293	1.6	0.228
21.222	4.1832	457	815	0.4	14199	1.1	0.296
22.020	4.0333	438	5127	2.8	53256	4.1	0.177
24.461	3.6361	432	370	0.2	3547	0.3	0.163
25.022	3.5558	443	823	0.4	7665	0.6	0.158
25.740	3.4582	455	1272	0.7	13393	1.0	0.179
26.418	3.3709	420	3959	2.1	43251	3.3	0.186
28.503	3.1290	358	1694	0.9	14952	1.1	0.150
29.076	3.0686	350	136	0.1	1747	0.1	0.218
30.540	2.9247	318	215	0.1	3549	0.3	0.281
32.601	2.7444	290	183	0.1	3495	0.3	0.325
34.920	2.5673	264	207	0.1	2895	0.2	0.238
35.381	2.5348	251	460	0.2	6602	0.5	0.244
36.420	2.4649	239	112	0.1	1325	0.1	0.201
38.363	2.3444	229	2426	1.3	31356	2.4	0.220
41.201	2.1893	220	240	0.1	3566	0.3	0.253
44.043	2.0543	221	258	0.1	3712	0.3	0.245