METABOLISM OF MIXTURES OF POLYCYLIC AROMATIC HYDROCARBONS (PAHs) BY *CUNNINGHAMELLA ELEGANS*

A Thesis

by

OLUWASEUN ALFRED OLATUBI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Robin Autenrieth
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	Timothy Kramer
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ABSTRACT

Metabolism of Mixtures of Polycyclic Aromatic Hydrocarbons (PAHs) by *Cunninghamella elegans.* (December 2005)

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Chair of Advisory Committee: Dr. Robin L. Autenrieth

Polycyclic aromatic hydrocarbons (PAHs) are environmentally significant compounds due to the toxicity of some members. They are ubiquitous and are persistent bioaccumulative toxins(PBTs). The toxicity of PAHs represents a risk to human health, and there are varied risk assessment approaches to quantifying the risk posed by PAHs based on exposure routes and scenarios. PAHs are not carcinogenic until they are metabolically activated as the body attempts to break them down and forms reactive metabolites that bind to the DNA causing subsequent replication in the cells.

Fundamental to assessing the risk posed by PAHs is understanding the metabolism of PAHs. Since exposure to PAHs is never to single PAHs, understanding what differences may occur in mixtures of PAHs gives accurate assessment of the dangers of PAHs. Understanding the dynamics of complex metabolism vis-à-vis single metabolism of PAHs and possible effects on the toxicity expression of PAHs is a necessary advancement to accurately impact and guide remediation strategies.

Studies were carried out comparing the metabolism of the PAHs Phenanthrene (PHE), Flouranthene (FLA) and Benzo[a]pyrene (BAP) in single, binary and ternary mixtures by monitoring the disappearance of the parent compound. It was observed that

PAH metabolism in the single PAH experiment differed from metabolism in both binary and ternary mixtures. Enzyme competition was evident in the metabolism of mixtures, changing significantly the metabolism patterns of individual PAHs. PAH structure was also seen to affect metabolism in mixtures and the possible creation of toxicity effects during mixture metabolism. PAH concentration changed over time with faster change during single PAH metabolism followed by ternary mixture metabolism and finally binary metabolism. These results affirm that substrate interactions must be considered in the risk assessment approaches to the dangers posed by exposure to PAHs.

DEDICATION

To the Almighty for all His love and the only god He allows me worship – my mother

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Finally I am grateful to my family to my brothers, Oluwole and Sola, to my sister, Funmi, my countless cousins, my uncles and aunts, my grandaunts and grandmothers for all their love and prayers I am eternally grateful. I am especially grateful to Oghogho for helping me follow through with the dream. To my mother, for her prayers and her tears, I say the deepest thank you.

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INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are hydrocarbons with fused benzene rings in linear, angular and cluster arrangements (Cerniglia 1984). They are commonly occurring environmental and health contaminants; many are known to be carcinogenic, mutagenic and persistent in the environment. The United States Environmental Protection Agency (USEPA) has grouped members of this class into the priority pollutant list and are classified as Persistent Bioaccumulative Toxins (PBTs). PAHs are widespread in nature as they are detected in all media in nature on a global scale.

PAHs are formed as by-products of natural and anthropogenic carbon combustion. During combustion, thermal alteration of organic compounds in the organic material being combusted produces PAHs with the type of PAH produced depending on the pyrolysis temperature (Mahaffey et al 1988). The toxicity of PAHs represents a risk to human health, and there are varied risk assessment approaches to quantifying the risk posed by PAHs based on exposure routes and scenarios. PAHs are not carcinogenic until they are metabolically activated as the body attempts to break them down and forms reactive metabolites that bind to the DNA causing subsequent replication in the cells.

Fundamental to assessing risk posed by PAHs is understanding metabolism of PAHs. Since exposure to PAHs is never to single PAHs, understanding what differences may occur in mixtures of PAHs gives accurate assessment of the dangers of PAHs. Understanding the dynamics of complex metabolism vis-à-vis single metabolism of PAHs and possible effects on the toxicity expression of PAHs is a necessary advancement to accurately impact and guide remediation strategies.

This thesis follows the style and format of the *Journal of Environmental Engineering.*

RESEARCH SIGNIFICANCE

While conducting microbial hydroxylation of aromatic substrates it was noticed that there were similarities between microbial metabolites obtained and those from mammalian hydroxylation of aromatic substrates (Smith and Rossaza 1974). Indications were rife that certain microbes exhibited monooxygenase activity similar to those exhibited by humans especially in the liver microsomes. This lead to the discovery of cytochromeP450 monooxygenase enzyme complex in certain filamentous fungi. When parallel studies were carried using filamentous fungi and human liver microsomes to break down PAHs the metabolites were similar and the pathways were also similar (Abourashed et al 1999).

It was then proposed that micro-organisms could be utilized as "microbial models of mammalian metabolism" (Smith and Rossaza 1974), since both fungi and mammals are eukaryotic organisms sharing similar enzyme machinery it comes as no surprise that products and pathways of xenobiotic metabolism would be similar. The exploitation of microbes as models for mammalian metabolism is widespread in pharmaceutical research and industry where filamentous fungi are used in the metabolism of new drugs and in the production of desired metabolites. "It has been astutely recognized that the diverse metabolic abilities and type-reaction specificities exhibited by selected microbial cultures might also be exploited to produce known or suspected mammalian metabolites or drugs and other xenobiotics: perhaps even to predict such metabolites" (Davis 1988).

Pathways of the metabolism of PAHs by various filamentous fungi and their various metabolites have been delineated, particularly the fungus *Cunninghamella elegans* (C. *elegans*). *C.elegans* has been shown to degrade benzo[a]pyrene (Cerniglia et al 1979), flouranthene (Pothuluri et al 1992), phenanthrene (Cerniglia 1980), acenapthene

(Pothuluri et al 1992). The pathways and the metabolites produced in this metabolism where similar to those in human liver metabolism of the same PAHs (Boyland and Wolf 1949).

There is little or no investigation into metabolism of complex mixtures of PAHs by humans or fungi, most if not all studies carried out earlier focused on single PAH metabolism. For bioremediation and study of the breakdown of PAHs lignolytic fungi represent the fungus of interest due to their ability to mineralize xenobiotics. Using filamentous fungi for modeling mammalian metabolism finds most use in pharmaceutical and metabolite research with little attention paid to the other uses of this modeling.

This research utilizes this microbial model of mammalian metabolism for comparative studies of single substrate metabolism and complex mixture metabolism of PAHs. This comparative study would be to offer more information into approaches of human risk assessment to PAHs. Most risk assessment approach need to be furnished with fundamental insights as to how complex substrate metabolism differs from single substrate metabolism and the possible effects this could have on the toxicity expressiono of PAHs.

Though PAH exposure is to varied numbers of PAHs, three PAHs with well characterized microbial and mammalian pathways have been chosen they are BAP, FLA and PHE. This study would repeat the single substrate metabolism and compare it to complex combination mixtures of the three PAHs. Substrate depletion over time would be qualitatively assessed.

LITERATURE REVIEW

Polycyclic Aromatic Hydrocarbons (PAHs) are a ubiquitous large group of organic chemicals some of which are harmful to human and animals and considered environmental contaminants. PAHs have been shown to be persistent in various media in nature at significant levels or at levels to which exposure to human and animal life over a continued period is toxic. A lot of PAHs are recalcitrant and thus their continued presence in the nature is a constant source of risk to human, animal and plant health, where they could be carcinogenic, mutagenic or induce gentotoxic effects in cells of plants and animals. PAH carcinogenicity and toxicity is induced during the metabolism of PAHs by living cells (Cerniglia 1984), for this reason knowledge of PAH metabolism or degradation is very important to help us assess correctly the risk involved in exposure to PAHs. Exposure to PAHs is nature is always to multiple PAHs and rarely to an individual PAH. A better assessment of the risk involved in PAH metabolism would involve understanding the degradation of PAHs in mixtures as it occurs in nature, the complex substrate interactions and its effects on the magnitude of risk assessed.

There are natural and anthropogenic sources of PAHs. Natural PAHs are formed as side-products of natural and anthropogenic carbon combustion. During combustion there is the thermal alteration of organic compounds in the organic material being combusted, this alteration produces PAHs (Mahaffey et al 1988) and the type of PAH produced depends on the pyrolysis temperature. Natural sources of PAHs include volcanoes, the earths' geologic permutations, and crude oil (Juhasz et al. 2000). Other natural sources of PAHs include PAH formation during thermal and geologic production of materials, emissions from trees, burning of vegetation in forest or bush fires and biogenic precursions during early digenesis (Cerniglia 1980). Anthropogenic sources of

PAHs include incomplete combustion of fuels such as wood, petroleum, oil and gas. Other sources include grilling meat, incineration of wastes, and use of industrial power generators. Industrial processes can produce PAHs as can the use of chemical solvents and the various incineration, or high thermal processes. Chemical processes such as the production of coal tar, petroleum, fractional distillation of crude oil, creosote production (Baum 1978) and various industrial processes are significant point sources for PAHs.

Description of PAHs

PAHs are organic chemicals containing two or more fused aromatic (benzene) rings (Kanaly et al. 2000; Juhasz et al. 2000). They range from the simple two (2) fused benzene-ring, naphthalene, to complex hydrocarbons such as the ten (7) fused ring, coronene. Parent compound PAHs are fused benzene rings without any chemical moiety or group added to them(Figure 1). They are the basic skeletal structure of PAHs and serve as the template for modified PAHs. PAHs usually exist in the modified form different from the parent compound through the addition of different chemical groups to the parent compound such as the alkyl, nitro and sulfur groups. These additions can significantly change the character of the parent compound they can confer previously unknown properties to the parent compound such as increased or decreased solubility, generally affecting properties already present in the parent compound such as hydrophobicity.

Addition of these groups considerably modify action and effects due changes in the physical, stearic and chemical properties of the PAH, in effect changing the metabolism and actions of the parent compound.

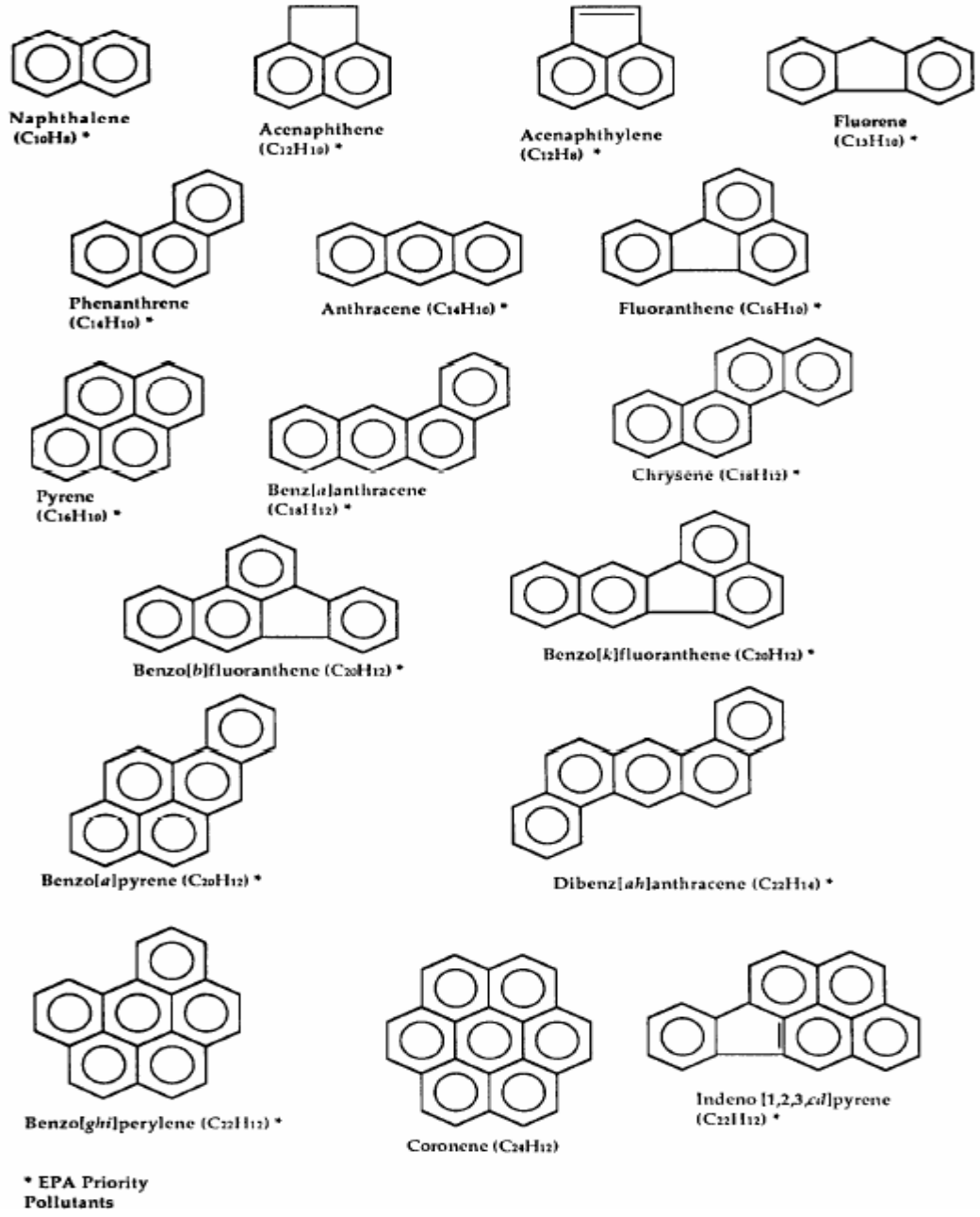

Figure.1. Selected PAH parent compounds. Asterisked are those considered to be EPA priority pollutants (adapted from Dabestani, 1999).

Anthropogenic sources of PAHs account for significant amount of PAHs present in nature today, "concentration of PAH found in soils around urban areas are sometimes up to two orders of magnitude higher that those in less-developed areas (derived from forest fires and air pollution)" (Dabestani 1999). Vehicle emissions alone are believed to be responsible for 35% of the total PAH emission in highly populated and industrialized urban areas of the United States (Bjoerseth et al. 1985). Anthropogenic sources are rapidly increasing the amount of PAHs in the environment as PAH accumulation rate far exceeds the degradation rate of PAHs. Industrialization of developing countries and continued industrial expansion of developed countries is projected to increase the PAH production as projections on PAH production are forecasted to increase in the coming decades (Bjoerseth et al. 1985).

Chemical and Physical Characteristics

The fused benzene rings of PAHs could be in linear, angular, or clustered arrangements (Kanaly et al. 2000). PAHs with less than three benzene rings are termed low molecular weight PAHs (LMW PAHs) while those with more than three benzene rings are called high molecular weight PAHs (HMW PAHs). The chemical properties (Table 1), and hence environmental fate of an individual PAH, is dependent in part upon its molecular size i.e. number of aromatic rings, this determines its solubility, octanol water partitioning co-efficient (KoW), configuration and electrochemical stability (Kanaly et al. 2000; Juhasz et al. 2000; Dabestani and Ivanov 1999).

This general grouping of PAHs as HMW and LMW PAHs becomes useful because each group member share similar characteristics, though with slight variations. The physico-chemical and environmental properties interactions of parent compounds of PAHs in both groups are characteristic of the groups, in the low molecular PAH group behavior within similar range includes, low boiling and melting points, high water solubility which translates into them being easily degradable, similar octanol-water partition co-efficient, similar fugacity values, vapor pressure and other properties. In the high molecular weight PAH group physico-chemical and environmental properties apart from their molecular weights are also shared such as high boiling and melting points, low solubility in water, low octanol-water partition co-efficient, not being easily degradable, higher toxicity to plants and animals, similar fugacity, vapor pressure and other physical properties. It is noticed that this grouping might not hold when parent compounds are substituted as these functional groups greatly alter the properties of the parent compound. This convention is adopted throughout this thesis.

Table 1. Physical properties of PAHs (adapted from Dabestani 1999)

No.	Compound	Vapor pressure (P_a)	Pressure (liquid) (P_s)	Water solubility (g/m^3)	Oct/W† partition constant (log K_{OW})	Henry's law constant (atm M^{-1} $\times 10^{-5}$)	MP‡ (°C)	BP§ (°C)	Fugacity ratio, F (25°C)	Vm‖ (LeBas) (cm^3/mol)	Vi/100¶ (intrinsic) (cm^3/mol)	TSA# ($Å^2$)	TMV* ($Å^3$)
1	Indan	197	197.0	100	3.33	232.8	−51.0	178.0	1.000	143.7	0.00		156.7
2	Naphthalene	10.4	36.81	31.0	3.37	43.01	80.50	218.0	0.283	147.6	0.75	155.8	126.9
3	Biphenyl	1.30	3.706	7.00	3.90	28.64	71	256.0	0.351	185.0	0.92	182.0	155.1
4	Diphenylmethane	0.0885	0.0885	16.0	4.14	0.931	25	264.3	1.000	206.8			171.7
5	Bibenzyl	0.406	0.754	4.37	4.70	16.93	52.2	285.0	0.538				188.0
6	*Trans*-Stilbene	0.065	0.619	0.29	4.81	40.40	124		0.105	221.6		175.0	
7	Acenaphthene	0.300	1.52	3.80	3.92	12.17	96.2	277.5	0.198	173.0	0.92	180.8	148.8
8	Acenaphthylene	0.900	4.14	16.1	4.00	8.400	92	265.0–275.0	0.217	165.7		193.6	
9	Fluorene	0.090	0.715	1.90	4.18	7.870	116	295.0	0.126	188.0	0.96	194.0	160.3
10	Phenanthrene	0.020	0.113	4.57	3.24		101	339.0	0.177	199.0	1.01	198.0	169.4
11	Anthracene	0.0010	0.0778	0.045	4.54	3.960	216.2	340.0	0.0129	197.0	1.01	202.2	170.2
12	Pyrene	0.0006	0.0119	0.132	5.18	0.920	156	360.0	0.0506	214.0	1.15	213.0	186.0
13	Fluoranthene	0.00123	0.00872	0.26	5.22	1.037	111	375.0	0.141	217.0		218.0	187.7
14	Benzo[*a*]fluorene			0.045	5.40		187		0.0249	240.0	1.22	237.4	203.7
15	Benzo[*b*]fluorene			0.020	5.75		209		0.0151	240.0	1.22	239.9	203.7
16	Chrysene	5.7×10^{-7}	1.1×10^{-4}		1.65	5.860	255	407.0	0.0053	251.0	1.27	241.0	212.0
17	Triphenylene	2.3×10^{-6}	1.2×10^{-4}	0.043	5.49	0.012	199	402.0	0.019	251.0	1.27	236.0	211.2
18	*p*-Terphenyl	4.9×10^{-6}		0.018	6.03		213	448.0	0.0138	258.2			
19	Naphthacene	9.3×10^{-9}	1.8×10^{-5}	0.0006	5.76	0.004	357	438.0	0.00052	251.0	1.27	248.0	213.7
20	Benz[*a*]anthracene	2.8×10^{-5}	6.1×10^{-4}	0.011	5.91	0.581	160	435.0	0.0462	248.0	1.27	244.3	212.8
21	Benzo[*a*]pyrene	7.0×10^{-7}	2.1×10^{-5}	0.0038	6.04	0.046	175	495.0	0.0328	263.0	1.41	256.0	228.5
22	Benzo[*e*]pyrene	7.4×10^{-7}	2.4×10^{-5}	0.0040		0.020	178		0.0307			251.4	
23	Perylene	1.4×10^{-8}		0.0004	6.25	0.003	277	495.0	0.00321	263.0	1.41	251.5	227.7
24	Benzo[*b*]fluoranthene			0.0015	5.80		168	481.0	0.0385	268.9			230.3
25	Benzo[*j*]fluoranthene			0.0025	0.0099		166	480.0	0.0403	268.9			230.4
26	Benzo[*k*]fluoranthene	5.2×10^{-8}	4.1×10^{-6}	0.0008	6.00	0.016	217	481.0	0.0126	268.9		266.0	231.1
27	7,12Dimethyl-benz[*a*]Anthracene	3.8×10^{-8}		0.0500	6.00		122		0.110	282.7		267.3	239.4
28	3-Methylchloroanthene	1.0×10^{-6}	3.5×10^{-5}	0.0019	6.42	0.145	178		0.0307	296.0		282.7	250.7
29	Benzo[*ghi*]perylene		2.3×10^{-5}	0.00026	6.50	0.075	277		0.00321	277.0	1.54	266.9	244.3
30	Dibenz[*a,c*]anthracene	1.3×10^{-9}	7.8×10^{-8}	0.0016			205		0.0166	300.0			
31	Dibenz[*a,h*]anthracene	3.7×10^{-10}	9.2×10^{-8}	0.0006			267	524.0	0.0403	300.0	1.53	286.5	255.4
32	Dibenz[*a,j*]anthracene			0.0120			196		0.0195	300.0	1.53	286.4	255.4
33	Pentacene	1.0×10^{-13}	5.3×10^{-11}				>300		0.0019	292.5			
34	Coronene	2.0×10^{-10}		0.00014	6.75		>350	525.0		292.0		282.4	260.8

Generally, PAHs are hydrophobic compounds measured directly by their aqueous solubility in water (Bjoerseth 1985). Generally, PAH hydrophobicity increases with an increase in the number of fused benzene rings. PAH hydrophobicity is a major factor contributing to the persistence of PAHs in the different polar media in the environment and in lipids. The increase in size of PAHs with increasing benzene rings along with increasing angularity confers greater electrochemical stability to PAHs making them be more stable in nature (Kanaly et al. 2000)**.** Hydrophobicity and electrochemical stability are principal factors in the persistence of PAHs in the environment (Kanaly et al. 2000; Juhasz et al 2000; Cerniglia 1985; Dabestani and Ivanov 1999).

The Octanol-water partition constant (Kow) is the ratio of the concentration of a substance in octanol to that in water at equilibrium at a specified temperature (Asante-Duah 2002), Kow is used to predict the fate of PAHs either in the environment or in biological tissues. Kow increases with increasing benzene rings, from LMW PAHs to HMW PAHs. The Kow is used to determine the phase which the PAH shall partition to, this ability is very useful in human exposure analysis to PAHs (Asante-Duah 2002). Measurements of Kow of individual PAHs correlates perfectly with measurements of solubility and hydrophobicity, with increasing hydrophobicity is low solubility and an increasing Octanol-Water partition coefficient.

Distribution of PAHs

Effective transport facilitates PAHs through different media. For example winds can aid in the transference of airborne PAHs from the foci point to regions thousand of miles away from the focal point depending on the wind current and strength, this is a common occurrence as seen in areas with high smoke stacks (Smith and Frankeberg 1979). Also flowing polluted streams might be vectors of pollution as they can transport the pollutants from it source to other water bodies (within medium) or could cause deposition in soil and sediments far off (intermedia) (Chiou et al. 1998), also atmospheric deposition of PAHs is a major source of soil pollution (Chiou et al. 1998; Grimmer 1983), as volatilization of LMW PAHs from the soil could be a source of atmospheric PAH. There are a lot of computational and mathematical models that effectively model transport of PAHs in a medium and through media, Reible et al 2002 have shown Levy-Flight random walk model for bioturbation in benthic environments.

Due to their hydrophobic nature PAHs partition onto surfaces and sediments where they remain attached (Kanaly et al 2000; Juhasz et al. 2000; Geiger and Blumer 1974; Chiou et al. 1998; Grimmer 1983). The more soluble PAHs are found in the water column, but HMW PAHs adhere to sediment and particles or they sink to the bottom or benthic levels of water bodies (Grimmer 1983; Herbes 1977). Table 1 shows us the different water solubilities of PAHs, solubilities range from as high as 1.1mg/l for phenanthrene to as low as 0.0038mg/l for benzo[a]pyrene. Crude oil and petroleum spills are a major source on increasing PAH pollution in waters usually in the ocean bodies at port and harbors or nearby streams to petrochemical refineries, increasing by at least two percent (2%) the PAHs in oceans and water bodies (Lemiere et al 2002). Depending on

the type of crude spilled, the crude may either disperse in emulsions or might sink to the bottom of the water body. Aquatic life in water bodies are themselves not immune from the contamination by oil, filter feeders in water bodies ingest this PAHs into their bodies, they are preyed on by higher aquatic organisms who also ingest this pollutant into their bodies. PAHs in water bodies can be metabolized by micro-organisms (Cerniglia 1980; Cerniglia and Heitkamp 1989).

Sources of PAH contamination to soils are mostly from atmospheric deposition, spills of crude oil and petroleum products, contaminated waste streams (Dabestani 1999). PAHs adhere to soil surfaces and their movement through the soil depends on characteristics of the soil such as porosity, permeability and chemical composition of the soil such as the alkalinity or acidity of the soil (Raber et al. 1997). Some PAHs can be adsorbed onto the surface or leach through the soil and thus enter into the groundwater (McGroddy and Farrignton 1995). Plants grown on contaminated soils can take up water soluble PAHs with plant nutrients as the plant draws nutrients from the contaminated soil. Extensive degradation of this adsorbed PAHs in the presence of PAH degrading organisms such as bacteria and fungi, can reduce them into different metabolites or cause mineralization into carbon-dioxide and water (Kanaly et al 2000; Juhasz et al 2000; Cerniglia 1980; Pointing et al. 2001)

Sources of PAHs in the air is primarily through incomplete combustion of fuels and organic compounds, natural sources such as pollution from pine trees cause air-borne PAH pollution. PAHs in the air exist by adhering to particulate matter in the air where they could be carried to different places or deposited on soil and water bodies (Smith and Frankeberg 1975; Geiger and Blumer 1974).

Effects of PAHs

PAHs have been shown to be harmful primarily because they have been shown to induce tumors. Several studies discuss the mechanism of PAH toxicity in humans and animals (Pinkney et al. 2001;Lemiere et al 2004;Schirmer et al. 2000; Sims 1980; Cavalieri et al. 1995; Melendez-colon et al. 1999; Wogan 1992). The bioaccumulation of PAHs and their bioamgnification along successive trophic levels is another means of exposure to human and animal life. PAHs can cause mutations, malignant and benign tumors, among a host of genotoxic effects (Twiss et al 1999, Geffard et al. 2002; Geffard et al. 2002).

The link between PAHs and harmful effects in humans was made since 1775 by Sir. Percival Pot, surgeon to St. Bartholomew's Hospital in London. He observed the increased incidence of scrotal cancer in chimney sweepers originated from their exposure to soot; (Cerniglia 1980). This observation provided an important link into studying occupational related diseases and their causes. Increasingly, links were made between tumors and occupation that had PAH exposure, in workers such as miners, chemical plant workers, tar-producing industry workers (Gromiec et al. 2002; Cocco et al. 1999; Golden et al. 1995).

Serological investigation of the affected workers in these occupations began to show uniform markers, epidemiology was also helpful as useful links where made in matching possible exposure roots to the different symptoms noticed (Le Moual et al 1995; Wogan 1992). These investigations lead to the identification of common causative agents or groups of causative agents as primary vectors of these symptoms. The identification of these agents or compounds followed and at this stage, researchers where

pretty sure true through repeated animal exposure studies that exposure to certain compounds caused the noticed reactions though they were not sure of the mechanism of induction. Yamagiwa and Ichikawa one hundred and forty years after Pervicial Pott made the link between scrotal cancer and chimney sweeps induced tumors on the ears of rabbits by repeated application of coal tar, thereby giving researchers a confirmation of previous postulates (Cerniglia 1980). PAHs are not carcinogenic but must be metabolically activated to induce cancer. They are activated coincidentally due to metabolic breakdown of the PAH. In the process of breaking down PAHs (metabolism) the body forms reactive metabolites that bind to the DNA causing subsequent replication(Sims 1980)

Mammals metabolize PAHs to trans-dihydrodiols, due to cytochrome P450 monooxygenases (CYP450) and microsomal epoxide hydrolase (Wogan et al. 2004). These epoxides are the further acted upon by mono-oxygenases to form diol-epoxides. “Epoxides where the epoxide group is adjacent to a “bay region”, that is the region between an angular benzo ring and the rest of the molecule are called bay-region epoxides” (Sims 1980; Cavalieri et al. 1995; Wogan et al. 2004). These “bay-region epoxides” are chemically reactive and they bind covalently to exocyclic amino groups of purines in the DNA to form stable adducts (Sims 1980; Cavalieri et al. 1995) thereby inducing tumors in the cells(Wogan et al. 2004). Binding causes disruption to the genetic functioning of the affected cells inducing a gentotoxic effect.

The activated carcinogens can be weak or strong. Carcinogens are speculated to be reactive electrophiles, some of which are weak because they react with small nucleophiles in the cells while the strong carcinogen are believed to be created within the cell and also react with the nucleophilic genetic material (Melendez-Colon et al 1999).

Dibenzo [*a,l*] pyrene has been shown to have the highest carcinogenic potential to date amongst all other PAHs tested, other prominent carcinogens include 7,12-dimethylbenz[a]anthracene and benzo[a]pyrene (Cavalieri et al. 1995).

Another pathway of activation has become prominent, which involves the formation of radical cations that binds on specific elements of purines to "form unstable adducts that depurinate to leave apurinic sites in DNA" (Melendez-colon et al 1999). These apurinic sites are believed to be accountable for mutations in certain critical genes which give rise to cancer initiation and probably mutagenic deformities (Cavalieri et al. 1995).

Overview of Xenobiotic Metabolism by Humans

Most metabolism of xenobiotics in the human body occurs in the liver. Blood from the circulatory system passes through the liver before it is sent to all other parts of the body (Casarett and Doull 1996). The liver purifies blood and removes contaminants to then be excreted of these contaminants. This function, biotransformation of foreign compounds, is not exclusive to the liver, as the enzymes that biotransform foreign compounds are also present in various organs of the body in varying concentrations, though less that that of the liver.

Metabolism of PAHs is believed to be a two stage process with each stage performing a different function and using different enzymes. Phase one reactions are reactions that involve the addition of functional groups to the foreign compound; it may result in an oxidation, reduction or hydrolysis of the PAH. The enzyme of interest in phase I reaction of PAH exposure is the cytochromeP450 enzyme complex. The Phase

two reactions are bio-synthetic reactions where the foreign compounds or phase I derived metabolites are covalently linked to an endogenous molecule to produce a conjugate (Casarett and Doull 1996). The endogenous moieties involved in these conjugations confer upon the lipophilic foreign substance a lessened hydrophobicity, and the ability to undergo significant ionization at physiologic pH so as to facilitate its dissolution in urine and its excretion from the body.

The location of phase I enzymes is believed to be in the microsome of the liver. When the liver is homogenized, the tubular endoplasmic reticulum breaks up and the fragments of the membrane are sealed off to form microvesicles which are referred to as microsomes (Hodgson et al. 2005). They are identified due to their unique spectral ability at certain wavelengths. The endoplasmic reticulum is basically a contiguous membrane made up of proteins and lipids, the presence of the enzymes within this type of matrix is fundamental since the liphophilic foreign substance will partition preferentially into the lipid membrane(Juhasz et al. 2000).

The cytochromeP450 (CYP450) complex containing monooxygenases is the most important enzyme involved in the Phase I reaction. They are different families of the CYP family and they are found in different parts of the body in different organs and they catalyze the biotransformation of different xenobiotic compounds of which they are specific. In the biotransformation of PAH the cytochromeP450 1(CYP1) family is induced by PAHs and would be our cytochromeof interest. The CYP1 gene family contains a single subfamily with two genes in most mammals (Korytko 2000), they are designated according to the agreed cytochromenomenclature as CYP1AI and CYP1A2 and they code for the proteins that are inducible by PAHs.

Phase I reaction of PAHs with CYP1 involves either the hydroxylation of aromatic compounds or either the epoxidation of aromatic compounds, the latter being more common. The mechanism of aromatic hydroxylation involves the addition of oxygen to the carbon hydrogen bond directly while the mechanism of action of the epoxidation of aromatic hydrocarbons involves the addition of oxygen into the carbon-carbon double bond to produce an arene oxide also called epoxides. This represents an overview of phase I as seen in figure 2 below. It should also be noted that Phase I reactions are not only oxidation reactions as Phase I reactions may also involve the reduction (reductive bio-transformation) of PAHs in the absence of oxygen.

Metabolism of PAHs could proceed either by the hydration of the arene oxides formed to give dihydrodiols by the enzyme known as epoxide hydrolase, or the epoxides formed could re-arrange to form phenols and thus are available to take part in Phase II reactions. Phenols arise principally from non-enzymatic re-arrangement of epoxides and several factors may influence phenol formation (Sims 1980). Prroducts of the epoxides (dihydrodiols) could be oxidized by cytochromeP-450 to bay–region epoxides.

Phase II reactions involve the addition of an endogenous molecule covalently to the metabolite of Phase I reactions or to the compound directly in certain cases. Enzymes of different phase II reactions include, glucronosyltransferases, sulfotransferases, methylation, N-acetyl transferases, but of more importance of the covalent bonds and to PAH metabolism is Glutathione trasnferase and its reaction (Hayes et al 2005). Glutathione reaction forms metacapturic products which are soluble in the urine and can thus be excreted from the body. Phase II reactions are of no importance in the activation of carcinogens.

Human BAP Metabolism

Benzo[a]pyrene (BAP) can enter the human body through dermal contact, inhalation, and ingestion. When this xenobiotic enters into the body it is acted upon first by monooxygenase, causing epoxidation of the BAP into simple epoxides such as benzo[a]pyrene-9,10-oxide, benzo[a]pyrene-7,8-oxide, benzo[a]pyrene-4,5-oxide(Baum 1978; Sims 1980;Wogan et al. 2004;Yang et al. 1976; Yang et al. 1975). These simple oxides are hydrated by the enzyme epoxide hydratase (epoxide hydrolase) to give dihyrodiols; this stage also produces the quinones and the phenols which result due to non enzymatic re-arrangement of the simple epoxides. The dihydrodiols are further hydrated by the monoxygenase enzyme to give the diol epoxides. The diol epoxides are the active carcinogen, but only in the bay-region configuration as explained above, in BAP metabolism the two active carcinogens that are known they include the 7,8 and 9,10 oxides(Sims 1980;Yang et al. 1976; Yang et al. 1975). Stereoisomerism of the metabolites is of importance in the comparing the similarities of metabolism of a xenobiotic by different species. Mammalian Metabolism of PAHs always has metabolites predominantly in the Trans configuration; this is due to the action of the CYP450 enzyme complex. On the next page is the graphical representation of the stages in the metabolic activation of BAP adapted from Sims (Sims 1980)

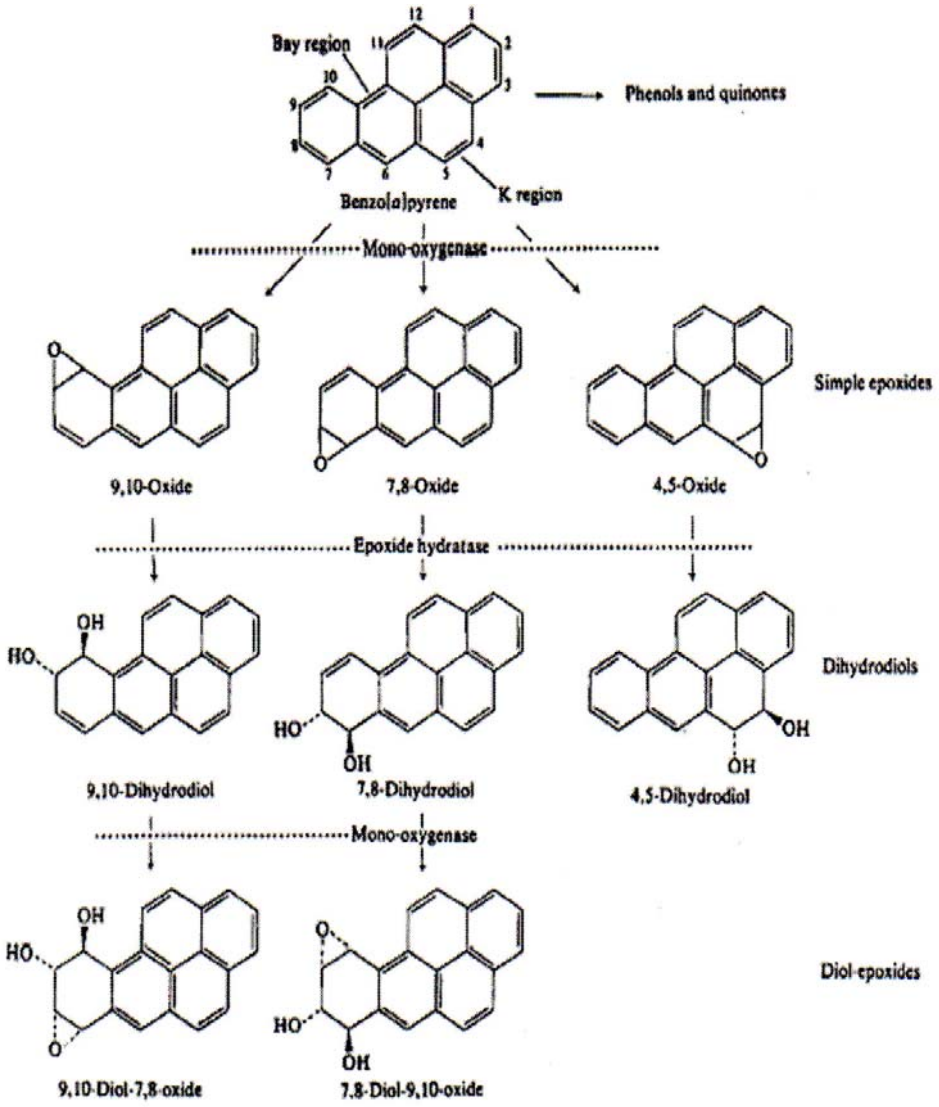

Figure 2. Metabolism of BAP. (adapted from Sims 1980)

Microbial Metabolism of PAHs

Microbial metabolism of PAHs is nature's clean up of PAHs from the environment; due to the versatility of micro-organisms using different substrates, micro-organisms degrade a wide range of environmental contaminants including PAHs.

Microbes are able to degrade parent compound PAHs and substituted PAHs in various environments under different conditions either aerobically and anaerobically (Baek et al. 2004; Yang et al. 1976; Yang et al. 1978). Remediations of contaminated sites are optimized by encouraging the growth of micro-organisms capable of utilizing the contaminant as substrate. The organisms could completely mineralize the contaminant or could break the contaminants to less toxic substrates. Fundamental information about metabolites formed in the metabolism of PAHs were derived from the microbial degradation studies of PAHs (Cerniglia 1984), pathways information, stereochemistry, enzymes and kinetics have been studied using microbial metabolism of PAHs.

Microbial degradation of PAHs has also helped to spur advances in molecular biology where molecular biology tools have been developed from the knowledge gained from detailed metabolism of PAHs (Wogan et al. 1992). It is also hoped that the knowledge derived from a thorough understanding of these metabolic processes would aid in better quantification and understanding of PAH exposure in human risk analysis (Dorne et al. 2005). Studies on microbial metabolism of PAHs has been carried out with bacterial and fungi predominantly, though some work has been done on other microbes such as archea, focus would be on fungi metabolism of PAHs.

Fungi and Bacterial metabolism of PAHs are well characterized and the pathways well elucidated. Different PAHs can be metabolized by both microbes, in some cases as sole carbon source (usually LMW PAHs), and in some cases co-metabolically. In certain cases metabolism is carried out by single strains of either fungi or bacteria or a consortium of bacteria or a consortium of fungi or both, or as in some cases by genetically enhanced bacteria (e.g. plasmid transfer). Though some substituted PAHs

prove to be very difficult at degradation, various strains and species of microbes are very effective at metabolizing these substituted PAHs. Fungi and bacteria metabolism of xenobiotics including PAHs is shown to occur aerobically and also anaerobically, different species living in diverse habitats successfully breakdown PAHs into less harmful products and in some cases to completely mineralize the PAHs to carbon dioxide and water

Bacterial Metabolism of PAHs

Different species of bacteria are able to metabolize PAHs, from naphthalene to benzo[a]pyrene. Examples of bacteria species able to degrade PAHs and have their pathways delineated include certain species of the genus *Pseudomonas, Aeromonas, Moraxella, Beijerincka, Flavobacterium, Achromobacter, Norcadia, Corynebactrium* amongst a few. Particularly of importance in the bacterial metabolism of PAHs are the bacteria *Spinghomonas paucimobilis* and *Spinghomonas yanikuoaye,* these bacteria have been prolific in the metabolism of PAHs especially in the metabolism of HMW PAHs and have contributed immensely to contemporary understanding of PAH metabolism by bacteria (Ye et al. 1996). Table 2 shows different bacteria that are able to metabolize different PAHs.

Table 2. PAHs and the different bacteria species that can metabolize them. (adapted from Juhasz et al 2000)

Organisms
napthalene *Acinetobacter calcoacetius, Alcaligens denitrificans, Mycobactreium sp.,* *Pseudomonas* sp.*, P. putida, P. flourescens,* sp. *paucimobilis,* *Brenvundimonas vesicularis, Burkholderia cepacia,* *Comamonas testosteroni, Rhodococcus sp., Corynebacterium renale,* *Moraxella sp., Streptomyces sp., Neptunnomonas naphthovorans, Cycloclasticus sp.*
Acenapthalene *Beijernickia sp., P. putida, P. flourescens, Bu. cepacia, Pseudomonas sp., Cycloclasticus sp,* *Neptunnomonas naphthovorans, Alcaligens eutrophus, Alcaligens paradoxus*
phenanthrene *Aeromonas sp., A.faecalis, A. dentrificans, Arthrobacter polychromagenes,* *Beijernickia sp., Micrococcus sp., Vibrio sp., Mycobactreium sp., P. putida,* *Sp. paucimobilis, Rhodococcus sp., Norcadia sp., Flavobacterium sp., Streptomyces sp.,* *S. griseus, Acinetobacter sp., P. aeruginosa, P. stutzeri, P. saccarophila, Stenotrophomonas maltophilia,* *Cycloclasticus sp., P. flourescens,* *Acinetobacter calcoaceticus, Acidovorax delafiedii, Gordona sp., Sphingomonas sp.,* *Comamonas testosterone, Cycloclasticus pugetti, Sphingomonas yanoikuaye, Agrobacterium sp., Bacillus sp., Burkholderia sp., Flavobacterium gondwanene,* *Hatomonas meridiana*
Anthracene *Beijernickia sp., Mycobactreium sp., P. putida, Sp. paucimobilis, Bu. cepacia,* *Rhodococcus sp., Pseudomonas sp., Stenotrophomonas maltophilia,* *Acinetobacter calcoaceticus, Acidovorax delafiedii, Gordona sp.,* *Sphingomonas sp., Comamonas testosteroni, Cycloclasticus pugetti*
flouranthene *A. denitrificans, Mycobactreium sp., P. putida, Sp. paucimobilis, Bu. cepacia,* *Rhodococcus sp., Pseudomonas sp., Stenotrophomonas maltophilia,* *Acinetobacter calcoaceticus, Sphingomonas sp., Pasteurella sp.*
Pyrene *A. denitrificans, Mycobactreium sp., Rhodococcus sp., Sp. paucimobilis,* *Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona sp.,* *Sphingomonas sp., P. putida, Bu. cepacia, , P. saccarophila*
Chrysene *Rhodococcus sp., P. marginalis, Sp. paucimobilis, Stenotrophomonas maltophilia,* *Acinetobacter calcoaceticus, Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp.,* *Pseudomonas sp., P. saccarophila*
Benz[a]anthracene *A. denitrificans, Beijernickia sp., P. putida, Sp. paucimobilis, Stenotrophomonas maltophilia,* *Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp., Pseudomonas sp., P. saccarophila* .

Generalized pathways can be made of metabolism of PAHs by bacteria. Exceptions might occur due to carbon sources, the nature of the media and conditions at which the bacteria has to break the PAH, these factors all influence the pathway adopted by the bacteria. A generalized pathway is hereby presented for the metabolism of PAHs without any moiety added i.e. parent compound PAH as depicted in the figure on page 26. The presence of a moiety usually involves stages where cleavage of the moiety has to occur and or before the metabolism of the PAH.

Bacterial metabolism of PAHs generally begins with the oxygenation of the PAHs usually by the enzymatic incorporation of atmospheric oxygen (Kanaly 2000; Juhasz et al. 2000; Cerniglia et al. 1980; Ye et al 1996). Bacterial dioxygenases incorporates two oxygen atoms into the aromatic molecule. The products of this initial oxygenation are cis-dihydrodiols which are then re-aromatized to yield dihydroxylated derivatives (Stingley et al. 2004). This reaction is catalyzed by cis –dihydrodiol dehydrogenases enzymes. These dihydroxylated derivatives are further oxidized leading eventually to the formation of catechols (Stingley et al. 2004). "Catechols can be oxidized via the ortho-pathway which involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield cis, cis-muronic acid or via the meta pathway, which involves cleavage of the bond between the a carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group" (Cerniglia 1980). The metabolites formed, if they are water soluble are easily excreted or they are mineralized completely in some species. The metabolism of naphthalene by *Pseudomonas* is given here as a model for bacteria metabolism of an unsubstituted PAH.

Catabolism of Naphthalene by Pseudomonas

naphthalene catabolism by microorganisms begins with the oxygenation of naphthalene (Figure 3), this reaction is catalyzed by a multi-component enzyme system called naphthalene dioxygenase (NDO) (Gibson et al. 1995). The initial oxygenation is catalyzed by naphthalene1,2-dioxygenase through the incorporation of the two atoms of atmospheric oxygen into the substrate (Naphthalene), this reaction is NADH dependent. The dioxygenase attacks the naphthalene at the carbon I and II and produces the metabolite cis-1,2-dihydroxy-1,2-dihydronapthalene.

This metabolite cis-1,2-dihydroxy-1,2-dihydronaphthalene is then acted upon by a dehydrogenase cis-1,2 dihydroxy-1,2-dihydronapthalene dehydrogenase. This enzyme oxidizes the substrate by removing two hydrogen atoms at the carbon I and II thereby producing 1,2-dihydroxynaphthalene. The pathway till 1,2-hydroxynaphthalene has been mutually agreed on by all researchers, from here there has been divergent views but the view of Eaton and Chapman (Eaton and Chapman 1992) which represents the most recent and most detailed of the divergent views would be adopted.

1,2-dihydroxynapthalene is acted upon by a dehyrogenase; the 1,2-dihydroxynaphthalene dehydrogenase. It cleaves the first ring to an unstable ring cleavage product which spontaneously recyclizes to 2-hydroxychromene-2-carboxylate (HCCA) (Eaton and Chapman 1992). The metabolite HCCA is then acted upon quickly by the enzyme isomerase – 2-hydroxychromene-2-carboxylate isomearse to produce the trans-o-hydroxybenzylidenepyruvate (tHBPA). Eaton and Chapman through recombinant DNA technology argued this to be tHBPA rather than cHBPA that it was believed to be in earlier papers. This stage in the metabolic pathway completes the fission of the first

aromatic ring of naphthalene. The tHBPA formed is then acted upon by a hydratase-aldolase, trans-o-hydroxybenzylidenepyyruvate hyrdatase-aldolase, to produce salicylaldehyde and pyruvate.

Salicylaldehyde is then acted upon by the dehydrogenase salicylaldehyde dehydrogenase in an oxidation reaction to give salicylate. This salicylate is then acted upon by salicylate-5-hydroxylase in an oxidation reaction to give gentisate, which finally goes into the intermediary metabolism. More commonly and of interest, salicylate is hydroxylated by the enzyme salicylate hydroxlase to produce cathecols. These cathecols then go into the nitrobenzene pathway to be acted upon so as to be able to go into the intermediary metabolism to give products which are utilized by the organism for synthesis and maintenance (Eaton and Chapman 1995, University of Minnesota Biocatalysis and Biodegradation database). (Figure 3).

Fungi Metabolism of PAHs

Several fungi species have been found to be able to metabolize PAHs, from naphthalene to benzo[a]pyrene. They include: Mastigomycota – *Phylochytrium, Rhizophlyctis*, *Saprolegnia*, Zygomycota – C*unnighamella, Syncephalastrum, Mucor, Absidia, Rhizophus*, Ascomycota- *Saccharomyces, Neurospora, Candida, Debaryomyces*, Basidiomycota – *Psilocybe, Panaeolus,* Deutromycota – *Aspergillus, Penicilium, Pestalotia*. (Kanaly 2000;Cerniglia 1997; Cerniglia et al. 2000; Pointing 2001). These species have been able to metabolize phenanthrene, flouranthene, acenapthalene, anthracene, fluorene, chrysene, pyrene among a few. Table 4 below shows the different PAHs and their metabolism by different fungi species.

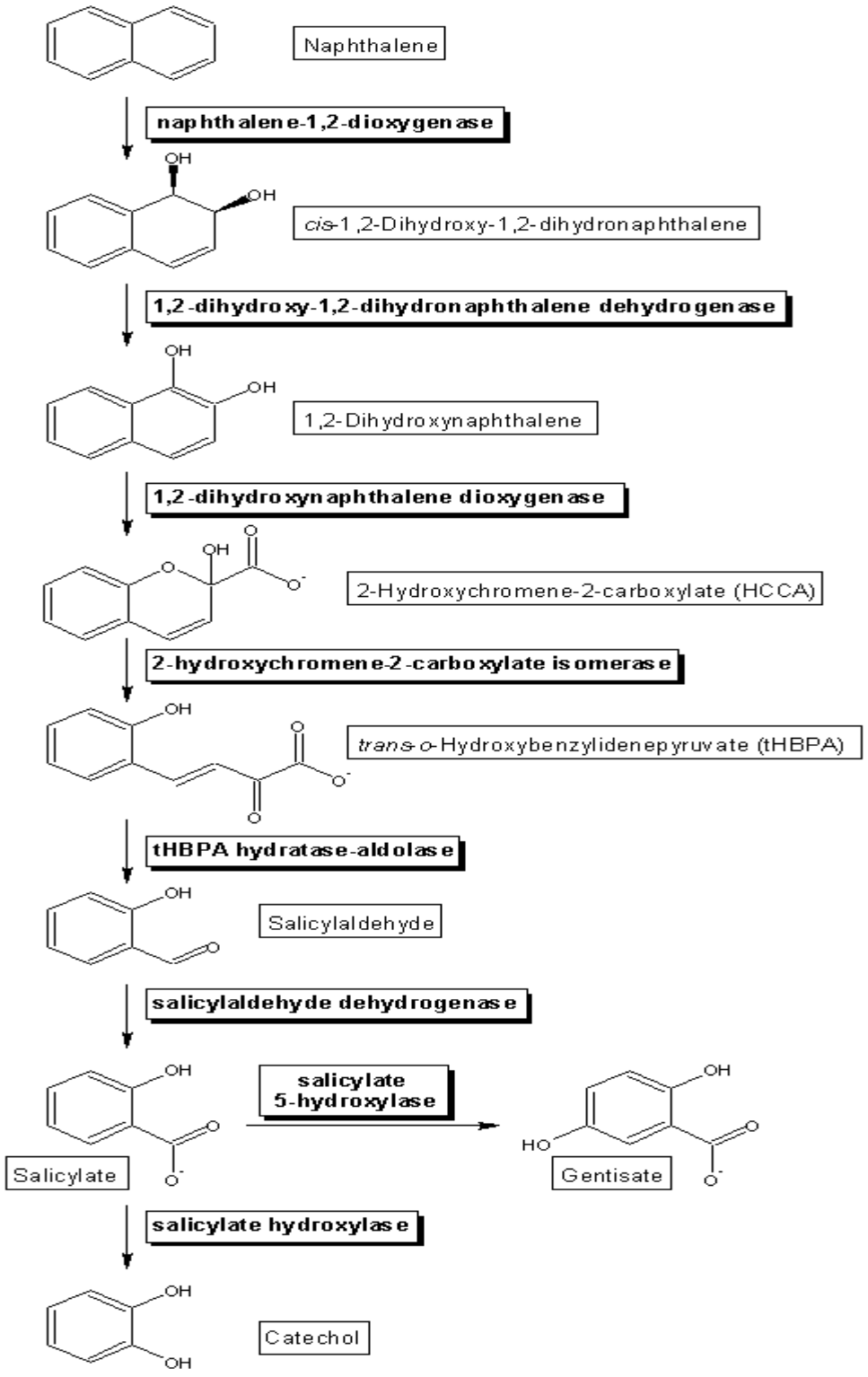

Figure 3. Bacterial metabolism of naphthalene. (adapted from the Universty of Minnesota biocatlysis/biodegradation database, 2005)

Table 3. PAHs and the different fungal species that can metabolize them. (adapted from Kanaly et al 2000)

Compound	Organisms
Acenaphthene	*Cunninghamella elegans*
Anthracene	*Bjerkandera sp, Cunninghamella elegans, Naematoloma frowardii, Phanerochaete chrysosporium, Phanerochaete laevis, Pleurotus ostreatus, Pleurotus sajor-caju, Romaria sp., Rhizoctonia solani, Trametes versicolor*
phenanthrene	*Aspergillus niger, Cunninghamella elegans, Naematoloma frowardii, Phanerochaete chrysosporium, Phanerochaete laevis, Pleurotus ostreatus, Syncephalastrum racemosum, Trametes versicolor*
flouranthene	*Cunninghamella elegans, Naematoloma frowardii, Laetiporus sulphureus, Penicillium sp., Pleurotus ostreatus*
Fluorene	*Cunninghamella elegans, Laetiporus sulphureus, Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor*
Pyrene	*Aspergillus niger, Agrocybe aegerita, Candida parapsilopsis, Crinipellis maxima, Crinipellis perniciosa, Crinipellis stipitaria, Crinipellis zonata, Cunninghamella elegans, Fusarium oxysporium, Kuehneromyces mutabilis, Marasimellus ramealis, Marasimus rotula, Mucor sp., Naematoloma frowardii, Penicillium janczewskii, Penicillum janthinellum, Phanerochaete chrysosporium, Pleurotus ostraeatus, Syncephalastrum racemosun, Trichordema harzarium.*
benzo[a]pyrene	*Aspergillus ochraceus, Bjerkandera adusta, Bjerkandera sp., Candida maltosa, Candida tropicalis, Chrysosporium pannorum, Cunninghamella elegans, Morticella verrucosa, Naematoloma frowardii, Phanerochaete chrysosporium, Pleurotus ostraeatus Phanerochaete laevis, Ramaria sp., Saccharomyces cerevisiae, Syncephalastrum racemosum, Trametes versicolor, Trichoderma sp., Trichoderma viride*
Chrysene	*Cunninghamella elegans, Penicillum janthinellum, Syncephalastrum racemosun*
Benzo[e]pyrene	*Cunninghamella elegans*

Fungal metabolism of PAHs can be the lignolytic or non-lignolytic type of metabolism. Lignolytic Fungi are fungi that produce lignin peroxidase, "a heme containing enzyme with protoporphyrin IX that oxidizes lignin in wood so that the wood carbohydrates can be used as nutrients" (Cerniglia et al 1980), common examples include the white-rot fungi such as *Phanerochaete chrysosporium*. These lignin peroxidases are enzymatic systems utilized in the metabolism of an array of recalcitrant xenobiotics including PAHs. Lignolytic metabolism mineralized PAHs completely to carbon dioxide and water. Lignolytic fungi are finding widespread use in bioremediation and are the fascinating subject of field studies aimed at remediation of contaminated sites (Cerniglia 1997).

Non-lignolytic fungi utilize another enzyme system, cytochromeP450 (CYP450) enzyme complex. These enzyme system have been found in a host of yeast and filamentous fungi (Cerniglia et al 1980). A few of these fungi include Cunninghamella species such as *Cunninghamella elegans, Apspergillus ochraceus, Aspergillus parasiticus, Candida albicans, Candida glabrata*. Non-lignolytic fungi generally do not mineralize PAHs as some lignolytic fungi do, they primarily detoxify by breaking the PAH into less reactive intermediates (Cerniglia 1997; Sutherland 1992) which could then be conjugated with soluble moieties and the excreted from the body, or could result in the creation of activated metabolites.

CYP450 mediated fungal metabolism involves addition of a single oxygen atom to the benzene ring, i.e the ring epoxidation, by the enzyme monooxygenase complex (Sutherland 1992). The unstable products of this epoxidation are hydrated by the epoxide hydrolase to arene oxides or they could undergo non-enzymatic re-arrangement to

phenols. “The monooxygenase enzyme complex that catalyzes the formation of arene oxides generally contains an inducible, membrane bound enzyme, CYP450” (Sutherland 1992). The arene oxide are further hydrated by the epoxide hydrolase to form transdihydrodiols, the monoxygenase can further catalyze the oxidation of the transdihydrodiol resulting in the dihydrodiol epoxide, an example of such possible epoxide is benzo[a]pyrene trans-7,8-dihydrodiol -9,10-oxide, which is the ultimate carcinogen and mutagenic metabolite in mammalian metabolism of BAP as shown in Figure.2. There could also be non-enzymatic rearrangement and action on other enzymes to form quinones, phenols, tetralones similar to phase II reaction of mammalian metabolism of xenobitics explained above. A generic diagram of CYP450 fungi and lignolytic metabolism adopted from (Cerniglia 1997) is shown in Figure 4.

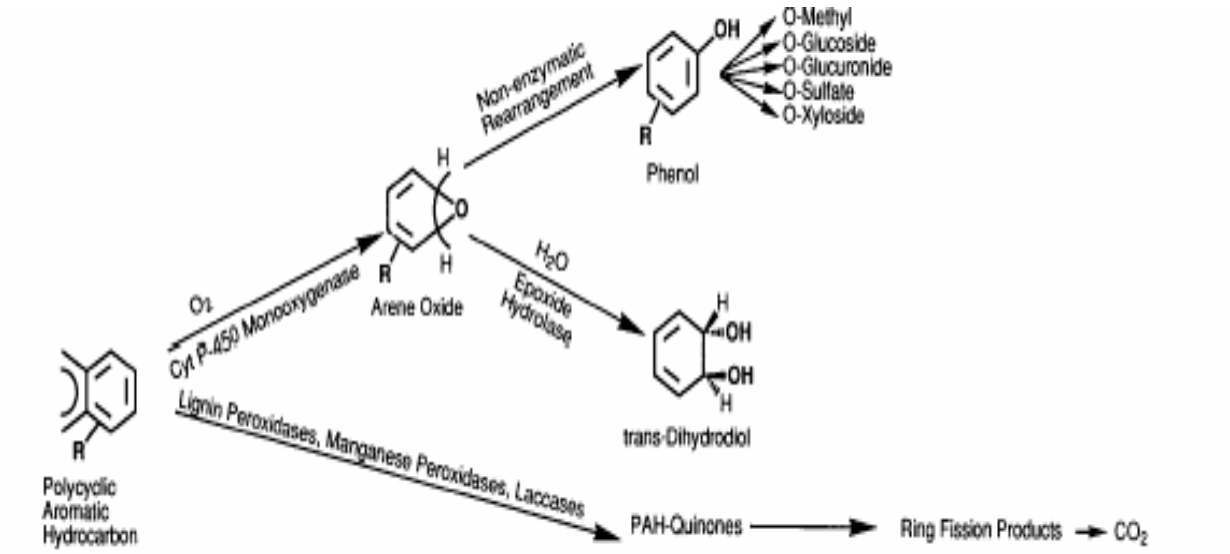

Figure 4. Schematic of PAH CYP450 mediated metabolism and Lignolytic metabolism. (adapted from Cerniglia, 1997)

CYP450 mediated metabolism of phenanthrene shall be used as a model for fungi metabolism of PAHs. *Cunninghamella elegans* (*C. elegans*) through the enzyme mono-oxygenase causes ring epoxidation of phenanthrene through the incorporation of a single atom of oxygen. This can be carried out at two sites giving the metabolites phenanthrene-3,4 oxide and phenanthrene-9,10 oxide, this reaction is catalyzed specifically by phenanthrene-3,4 monooxygenase and phenanthrene-9,10 monooxygenase and a phenol 9-phenanthrol. The oxides which are usually unstable are immediately hydrated to form dihydrodiols of the trans configuration, forming trans- 3,4,-dihydrol phenanthrene and trans-9R,10R- dihydrol phenanthrene, the hydration is catalyzed respectively by the phenanthrene-3,4-epoxide hydrolase and phenanthrene-9,10 epoxide hydrolase respectively.

From this stage non-enzymatic arrangement of the metabolites occurs giving rise to phenols and other conjugated moieties such as sulfate conjugated as shown in the figure 5 which depicts the fungal metabolism of phenanthrene, adopted from the University of Minnesota biocatalysis and biodegradation database. The sulfate conjugates formed include phenanthrene-3,4-dihydrosulfate conjugate, phenanthrene-9,10-dihydrosulfate conjugate, they become active ingredients for various enzymes aimed at detoxification, this is dependent on the species of fungi involved and the existent surrounding which the fungi is living (Cerniglia et al 1985).

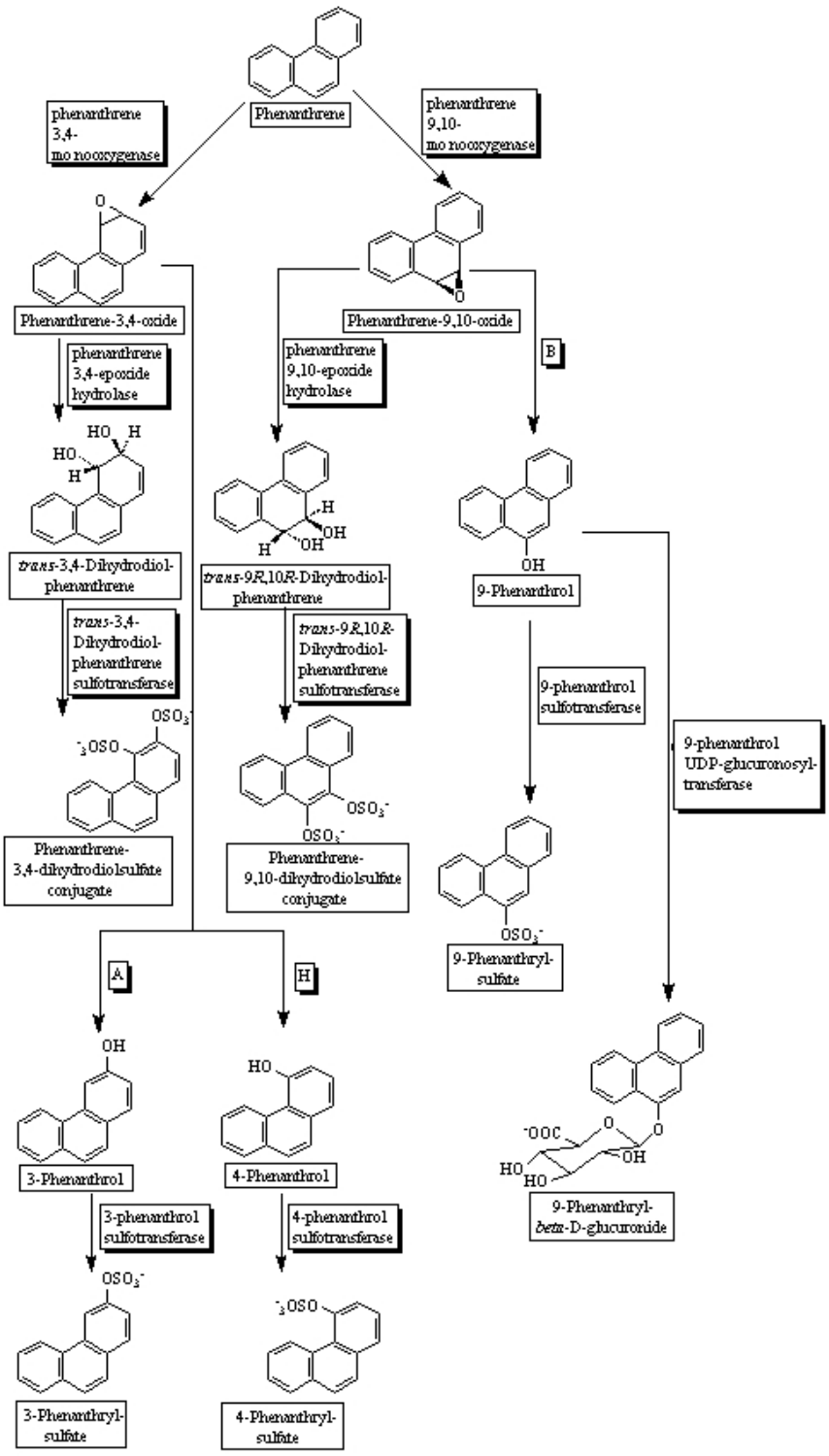

Figure 5. CYP450 mediated fungi metabolism of phenanthrene.(adapted from the University of Minnesota biocatalysis/biodegradation database 2005)

Cytochrome P450

Cytochromes P450 (CYP450s) are a superfamily of enzymes and they are found in virtually all mammals in different organs and parts of the body, there exists databases that have the genetic map of most cytochromes P450 that have been identified. P450s are active in the metabolism of a wide variety of xenobiotics including PAHs, PCBs, organochlorides, pesticides, as well as endogenous compounds (Korykto 2000; Omura et al 1965). CYP 450s carry out the oxidation of a lot of aliphatic and aromatic compounds (Cerniglia et al.1985; Sutherland 1992).They have particular absorption peaks at or near 450nm in the reduced carbon monoxide difference spectrum, ,this is pivotal in their identification and analysis (Omura and Sato 1964; Omura and Sato 1964; Omura et al 1965) .

CYP450s were discovered in mammals in 1964(Omura and Sato 1964), soon after, discoveries were made of CYP450s in yeasts and some filamentous fungi (Ferris et al. 1973; Nelson et al. 1996). The import of the presence of CYP450s in both fungi and mammals would be that the breakdown of the any xenobiotic catalyzed by this enzyme complex would follow the same pathway and bring abut the same intermediates and end products (Cerniglia 1980; Baum 1978). Taking a cursory look at the metabolic pathways in Figure 3 and Figure 6, we would see that the fungal and bacterial pathway of PAHs differ in the intermediates formed and also in their configurations (cis-dihydrodiols for Bacteria and trans dihydrodiols for Fungi), this is due to the different enzyme systems that catalyze the metabolism of PAHs in fungi and bacteria.

In contrast when we compare mammalian metabolism of PAHs and that of fungi (Figure 2 and Figure 6) we see that fungal and mammalian metabolism generate similar

intermediates such as benzo[a]pyrene trans-7,8-dihydrodiol -9,10-oxide. This intermediate is the principal carcinogen and mutagenic metabolite in mammalian metabolism of BAP and it is also produced also by the fungi *C. elegans*. The pathways of both fungi and mammalian metabolism of PAHs produce the same intermediates with the same configuration and stereochemistry because they are catalyzed by the same enzyme system. Fungal metabolism of PAHs can serve as a model system for human metabolism of PAHs based on similar enzyme system for metabolism of PAHs which produce the same intermediates and products. (Cerniglia 1980; University of Minnesota Biocatalysis Biodegradation Database 2005).

Microbial Model of Mammalian Metabolism

Smith and Rossaza while conducting microbial hydroxylation of aromatic substances established firm similarities between microbial metabolites and metabolites from mammalian systems, they also showed that these similarities encompassed both phase I and phase II biotransformation reactions (Smith and Rosaza 1974; Abourashed et al. 1996; Goodhue 1982). Other researchers collaborated these results in the metabolism of xenobitics by microbes that possessed similar enzyme system, this lead to the core of microbial modeling of mammalian modeling(Ferris et al. 1973,Goodhue 1982).“The core assumption behind the microbial model concept, introduced by Smith and Rossaza, is the fact that fungi and mammals both eukaryotic living organisms probably share very similar enzyme machinery for major physiological functions. Therefore, the outcome of xenobiotic metabolism among many other biochemical processes, is expected to be very similar in both fungal and mammalian systems” (Abourashed et al. 1996).

Modeling drug metabolism in the pharmaceutical industry for new and existing drugs has been modeled successfully by using fungi ,and have been successful some of the drugs tested include aminopyrene(Ferris et al. 1973), Cannabanoids (Roberston 1982), Lapachol (Otten and Rosaza 1978), Furosemide (Hezari and Davis 1993), Papaverine(Goodhue 1982).

There are three approaches to utilizing fungi in metabolism modeling(Smith and Rosaza 1975; Abourashed et al. 1996). The retrsopective, prospective, and parallel approaches. In the retrospective approach the microbial modeling follows an already conducted mammalian approach. It (retrospective) primarily aims to validate the microbial model hypothesis and or provide an ample supply of metabolites for evaluation or investigation (Abourashed et al. 1996). The prospective approach is the opposite of the retrospective as its conducted before the mammalian metabolism is evaluated, thereby providing a pathway for investigation and standards for confirming metabolites (Smith and Rosaza 1974; Smith and Rosaza 1975). “The advantage of a higher yield of microbial metabolite can be utilized for further studies” (Abourashed et al. 1996). The parallel approach is a simultaneous study of both mammalian and microbial metabolism; this (parallel) would serve primarily for comparison between both types.

Some immediate gains of using any of these highly popular and reliable models includes procuring results faster especially when using the prospective approach, also this would reduce the use of laboratory animals especially in the light of present concerns and regulations about animal use(Smith and Rosaza 1975). Also metabolites are produced in higher yields in microbial metabolism (Abourashed et al. 1996) and they could be used for studies and evaluation as a map road in drug design, cancer research, human risk

assessment and varied health purposes. Facile manipulation and control also remains a cardinal advantage, as this modeling gives more flexibility to study interactions between varied parameters in the xenobiotic metabolism and also the effects of chemicals either as inhibitors or rate limiting steps in the pathway. This study would be retrospective as mammalian metabolism of individual PAH is firmly established. It would also be prospective as there seems to be little or no studies on mammalian complex mixtures of PAHs.

Conclusion and Main Objective

In human exposure to PAHs, exposure to PAHs in nature is usually to mixtures. One human risk assessment analysis method for PAH exposure is the Relative Potency Factor Approach. This approach is based on assuming additive effects of individual compounds when assessing risk from an exposure scenario (EPA, 1993). "The key assumptions underlying the use of this method is that individual PAH risks are additive and the sum of the risks of the selected PAHs adequately characterizes the risk for the entire PAH component of the mixture" (Versar Inc, 2002). In the additive approach, the total individual parent compound effects is quantitatively added without taking into cognizance the possibility of mixture interactions between the compounds.

This interaction(s) could lessen or increase the risk associated with mixtures. This heightens the possibility that the additive approach could be over-estimating the risk or more worrisome underestimating the risk. A proper understanding of the interactions during metabolism of complex mixtures of PAHs would help in a better assessment of the additive approach to human risk assessment. Understanding the effect of substrate in mixtures during metabolism, and how this changes their individual metabolism, which would indirectly affect the activation of the ultimate carcinogen would be of concern in this study.

This study aims to show the effect(s) of substrate interactions on the metabolism of PAHs. It is expected that mixture metabolism would affect individual metabolism either by inhibition or increasing the substrate metabolism. The objective of this study is to compare the metabolism of the PAHs BAP, PHE and FLA in single substrate metabolism to binary substrate combinations of BAP and FLA and BAP and PHE, and to a ternary substrate metabolism of BAP, PHE and FLA.

MATERIALS AND METHODS

Microbial Methods

The fungi *Cunninghamella echinulata var elegans* with registration number ATCC 36112 lot number 918505 was purchased from American Type Culture Collection (ATCC) Manassas Virginia USA. It was delivered in the dried form. The dried culture was dissolved in sterile distilled water and allowed to soak for about thirty (30) minutes, using autoclaved swab and inoculating loop, the fungi was plated unto prepared potato dextrose agar plates, this was carried out in a sterile laminar flow hood. The fungi were also inoculated into agar slants for storage over long periods in low temperature refrigerators forty eight (48) hours after inoculation. The agar culture was placed into controlled temperature at 24^0C.

After forty eight (48) hours, noticeable mycelia growth had started to occur, the mycelium grows uniformly on the plate surface. It has a white snow white appearance and a cotton-like appearance in general. The plates are then left for between five to seven days at which time the spores would are well developed. The agar is put into 125-Erlenmeyer flasks containing 100ml of distilled water at two plates per flasks. The flasks are then shaken vigorously to dislodge the fungi spores into the water. The fungi spores liquid is then measured in 5ml quantities using sterile pipette tips and put into 30ml of autoclaved sterile sabaround dextrose broth on a gyratory shaker at between 150-180rpm in the controlled room temperature at 24^0C for forty eight (48) hours.

After forty eight (48) hours the fungi grow as small white pellets, though they are not all uniformly sized and shaped they have a general shape and size overall. These pellets are mostly spherical though some are oblong or some of the spheres could join up

forming long chains. It is rationalized that the smaller the pellets the better (Abourashad 1999) as there would be greater diffusion through the sphere, these can be achieved by putting the gyratory at a higher speed. Larger pellets have biofilm problem as the core become anoxic and there is less diffusion of materials and oxygen into the core of the fungi pellets but practical considerations demand that the pellets be not too small. After forty eight (48) hours the fungi broth is changed for fresh broth thereby enriching the medium, this is carried out by decanting the broth from the culture into beakers and immediately adding fresh autoclaved 30ml of sabaround dextrose broth. At this stage the fungi is believed to be in the exponential phase of their growth (Abourashad 1999). The substrate is added after the enrichment. Controls used in the microbial methods include plates which are not inoculated with the fungi and broth not inoculated with the fungi, contamination is inspected in these controls. A dry weight measurement of the fungal biomass was carried out to determine the average biomass weight in the liquid culture.

Chemicals

All chemicals used in the experiments were of high purity. The PAHs used in this experiment include benzo[a]pyrene, Flouraethene and phenanthrene, they were purchased from Alfa Aesar MA, USA and with their purity ranging 99.6-99.9% HPLC grade chemicals. The solvents used include Dimethylsulfoxide, HPLC grade 99.9% purity packed under argon and purchased from Alfa Aesar MA, USA, Methylene Chloride, HPLC grade purchased from EMD Chemicals Inc. New Jersey, USA. Anhydrous sodium sulphate in (granular form) was purchased from EMD Chemicals Inc. All necessary

precautions were observed in the handling of al this toxic chemicals such as wearing of gloves and working in a hood while using the chemicals.

Stock solutions of PAHs are made by dissolving individual PAHs in the solvent dimethysulfoxide (DMSO). DMSO is non-toxic to the fungi and helps in the dissolution of the PAH evenly in the liquid phase (Davis 1988). The PAHs when dissolved in the solvent DMSO are shaken for about twenty minutes to ensure proper dissolution. The PAHs are then added to the fungi using sterile pipettes measuring the different volumes needed. Final concentrations of complex mixture of PAHs is below 0.0038mg/l, the solubility limit of BAP, the less soluble of the three compounds used. The Fungi added PAHs are then put on the gyratory shaker at between 150-180 rpm and samples are taken at various times staring from the zero (0) hour.

Control samples are essential in monitoring results in biotransformation reactions, and we also make use of a control in this section of the experiment. The control used involves autoclaving the fungi and then adding the PAH, this control helps us to monitor if there are other agents of biotransformation or if the PAH breaks down on its own without the aid of the fungi. The control is then treated as a sample and extracted for results.

Extraction

A liquid-liquid extraction (LLE) is carried out on the broth using the solvent Methylene Chloride. The extraction is carried out in 250ml Pyrex extraction flaks. The broth is separated from the fungi, and using three times the volume the solvent is used to extract the broth in three different separate extractions. The fungi are also extracted using

10ml of Methylene Chloride three times. The extractions are carried out manually by shaking vigorously the extraction flasks with the Methylene Chloride and broth or fungi for about ten minutes, in each of the three different extractions.

The extraction results in the Methylene Chloride sinking to the bottom and the broth floating above, in the case of the fungi the pellets also float to the top of the extraction flasks. The Methylene Chloride is then collected in zymax tubes. The extracts are cleaned up using anhydrous sodium sulfate that has been put in the oven at 400^0C for over twenty four (24) hours cooled in a desiccator. Cleanup columns purchased from Corning Incorporated, Corning NY, USA are used for the clean-up. The extract is made to pass through the sodium sulfate three times, the sodium sulfate helps to remove any biological entities and also water from the sample. The extracts are then concentrated using the Zymark Turbo Vap®, set at 24^0C with nitrogen blowing over the extracts to ensure speedy concentration. The samples are then concentrated to about 1ml from 90ml from the extraction and clean up.

Analysis

PAH metabolite determination by – GC/MS-SIM. PAH metabolite identification was carried out using a Hewlett Packard 5890 series II plus gas chromatogram coupled with a Hewlett Packard 5972 Mass spectrum detector. A modified USEPA SW-846 Method 8270C was used for the quantitative determination of polycyclic aromatic hydrocarbons (PAHs). The mobile phase was helium and the capillary column was an Agilent Techologies HP-5MS which is 30m long, 0.25 mm internal diameter and 0.25μL film thickness. It is equipped with an auto sampler and it made 2μL of the sample.

Internal standards were prepared by adding equal volume of commercially purchased and certified 5 alpha androstane purchased from Accu. Standards, New Haven, CT and absolute standard lot # 20016 purchased from Absolute standards Inc. Hamden, CT. A known volume of the internal standard was added to all samples run. Continuing calibration solution at 0.2 μg/ ml where prepared by diluting available solution containing the analyte of interest in Dichloromethane. The continuing calibration standard was run after every five to seven samples run,

The standard blank was dichloromethane and an analytical set consists of standards, samples and quality control samples. All samples where run in duplicates and all experiments performed thrice.

RESULTS

Experiments were conducted to evaluate *C. elegans* performance when exposed to single PAHs and mixtures of PAHs. The results of three single PAH metabolism experiments, two binary PAH metabolism experiments and a ternary PAH experiment are reported along with a comparison of the individual PAH degradation in the tested combination experiments.

Toxicity Experiment

The toxicity of individual PAHs to the fungi, *C. elegan*s, was determined through serial dilutions of the target compounds to determine the toxicity threshold. This series of experiments were to determine the non-toxic concentration for single, binary and ternary evaluation of PAH mixtures to prevent altering the rate of PAH metabolism. Two methods were used. The first method involved the preparation of different concentrations of the individual PAH in Dimethysulfoxide (DMSO) into molten potato dextrose agar at 50^0C. The preparation was then allowed to set and each plate inoculated with an equal amount of the fungi aliquot. Boring cores were used to measure equal amounts of the fungi inoculums which excised sections from the agar cultures. The second method used concentrations of the individual PAHs in Dimethysulfoxide (DMSO) added directly to the surface of the set agar before inoculating with equal amounts of inoculums.

To determine the threshold toxicity concentration PAH doses ranged from 50mg/l – 1000mg/l of the individual PAHs. Growth was monitored over a 96 hour period, the average growth period of fungi in agar without PAHs. The culture seeded with PAH was compared to controls which were not seeded with PAH. The fungal growth diameter in the Petri-dishes was used as a subjective measurement of inhibition when compared to

controls. It was assumed that the threshold toxicity concentration would correlate to the area without growth on the plates.

Both methods yielded comparable result. The control growth diameter was 7cm in 72 hours. The different concentrations, their growth diameter and the percentage area without growth are shown in Tables 4- 7.

Table 4. Different BAP concentrations in agar culture, the diameter of growth and the percent area without growth after 96 hours. Controls have a growth area diameter of 7cm.

Concentration (mg/l)	Diameter of Growth Area (cm)	Percent area without Growth
1500	4.0	67
750	5.2	44
500	6.1	24
250	6.3	19

Table 5. Different FLA concentrations in agar culture, the diameter of growth and the percent area without growth after 96 hours. Controls have a growth area diameter of 7cm.

Concentration (mg/l)	Diameter of fungal Growth(cm)	Percent area without Growth
1000	1.6	94
500	4.5	58
50	6.1	24

Table 6. Different PHE concentrations in agar culture, the diameter of growth and the percent area without growth after 96 hours. Controls have a growth area diameter of 7cm.

Concentration (mg/l)	Diameter of fungal Growth(cm)	Percent area without Growth
100	0	0
50	1.5	95
30	4.2	64

The effect of the aqueous solubility of the PAHs was observed in the results of this test. All treatments to the fungi exceeded the theoretical solubility of all the PAHs due to the doses used. BAP with an aqueous solubility of 0.0038mg/l exhibited growth comparable to the control of 7cm diameter (Table 5). At 250mg/l, well above solubility, the growth area was less than 19% of the control. FLA and PHE with a higher aqueous solubility of 0.24mg/l and 1.1mg/l respectively, showed a reduced growth area at lower concentrations and at 100mg/l PHE showed no fungal growth (Table 6). If the availability of PHE was higher because of its higher aqueous solubility, then higher toxicity is affected at lower concentrations for more soluble PAHs. At concentrations above solubility the PAH would have to be associated with another phase either through complexation or sorption making it less available for fungal exposure. Consequently the less soluble PAHs would have less toxicity due to the lack of availability for fungal exposure.

However, PAH concentrations above the aqueous solubility of the PAHs appear to have the potential to be toxic, especially for the more soluble PAHs. The experiments were repeated with PAH concentrations above the solubility limit of BAP, the least

soluble of the three PAHs. All agar cultures showed 7cm diameter like the control. Although the evidence is not conclusive, at concentrations lower than the aqueous solubility of BAP. Growth was not as restricted. A toxicity threshold at concentrations above 0.0040mg/l (BAP solubility) was assumed.

Table 7. PAHs at 0.0040mg/l concentration in agar culture, the diameter of growth and the percent area without growth after 96 hours. Controls have a growth area diameter of 7cm.

PAH	Concentration (mg/l)	Diameter of fungal Growth(cm)	Percent area without Growth
BAP	0.0040	7	0
PHE	0.0040	7	0
FLA	0.0040	7	0

The liquid culture metabolism experiments were designed to have a total complex mixture PAH concentration of less than 0.0040mg/l. The underlying assumption is that the dynamics of growth in agar culture resembles the dynamics of growth in liquid culture. Thus at concentrations below 0.0040mg/l there would be no toxicity to the fungi.

Single Metabolism Experiment

Single PAH metabolism experiments were performed for PHE, FLA and BAP. The PAHs are cometabolically degraded because this fungus cannot utilize PAHs as a sole substrate (Cerniglia 1990). In liquid cultures, many filamentous micro-organisms tend to aggregate and grow as pellets; the compactness varies considerably. These pellets

are spherical or ellipsoidal masses of hyphae with variable internal structure (Edelstein 1983). Many factors influence pellet formation, including inoculants size, age, type, genetic factors and ability to produce bio-flocculants. “It is often difficult to define a mechanism for pellet formation in filamentous fungi, as often more than one parameter is adjusted by changing only one variable. Even for the most studied, industrially, *Aspergillus* and *Penicillium* species reports are contradictory” (Papagianni 2003).

Experiments to evaluate single PAH degradation were performed in triplicates with sampling performed in duplicate. Fungal dry weight measurements, taken periodically, were constant with an average dry weight of 0.247mg in every liter of liquid culture. Kinetic modeling for filamentous fungi is not well understood due to practical problems with mycelia pellets. Papagianni(2003) reports that “multicelluar structure of the mycelium, the morphological heterogeneity and heterogeneity in physiology and differentiation along the hyphae during fermentation makes it difficult to construct mathematical models of fungi fermentations”. Pothuluri et al (1995) also reported percentage degraded as a means of quantifying the kinetics and this approach has would also be used in discussion of results in this thesis.

PHE Single Metabolism

For single PHE metabolism, the change in concentration was monitored over time. After 2 hours, PHE was completely transformed. The PHE degradation over the two hour incubation period is presented in Figure 6. Curves fits were plotted on the graphs using portions of the data where metabolism could be compared and where active transformation occurred.

The equation of the line fitted to Figure 6 was $y = 0.0011e^{-1.8174x}$, this equation shows that during the time interval compared the transformation of the parent compound was exponential. All single experiments were carried out three times, the values in the graph of all single experiment represent the average values of the last two experiments. Standard deviations were carried out on all experimental values and error bars are shown all readings on the graph. All binary experiments follow the same reporting patterns as in the single experiments. Values of all experiments are reported in the appendix.

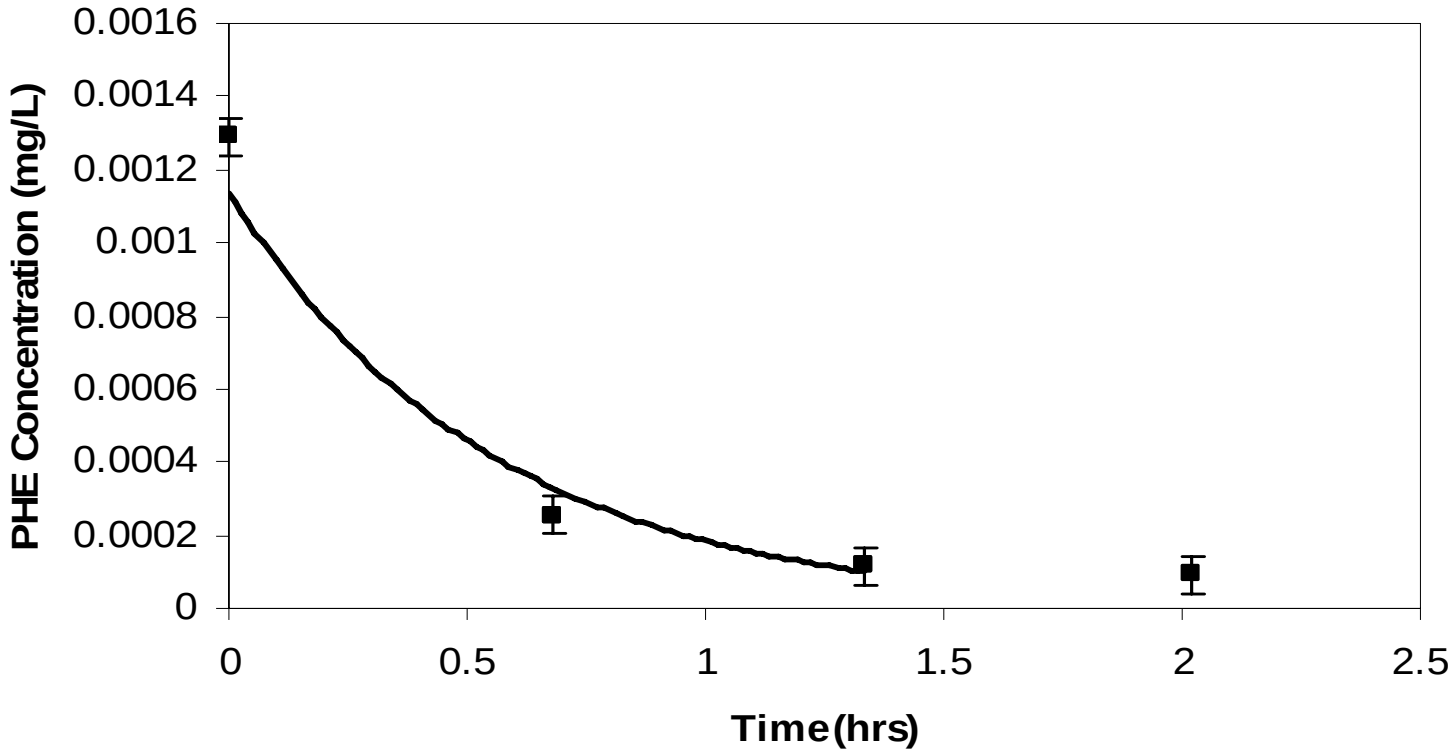

Figure 6. Sole PAH metabolism of phenanthrene. (■). Open symbols represent experimental observation.

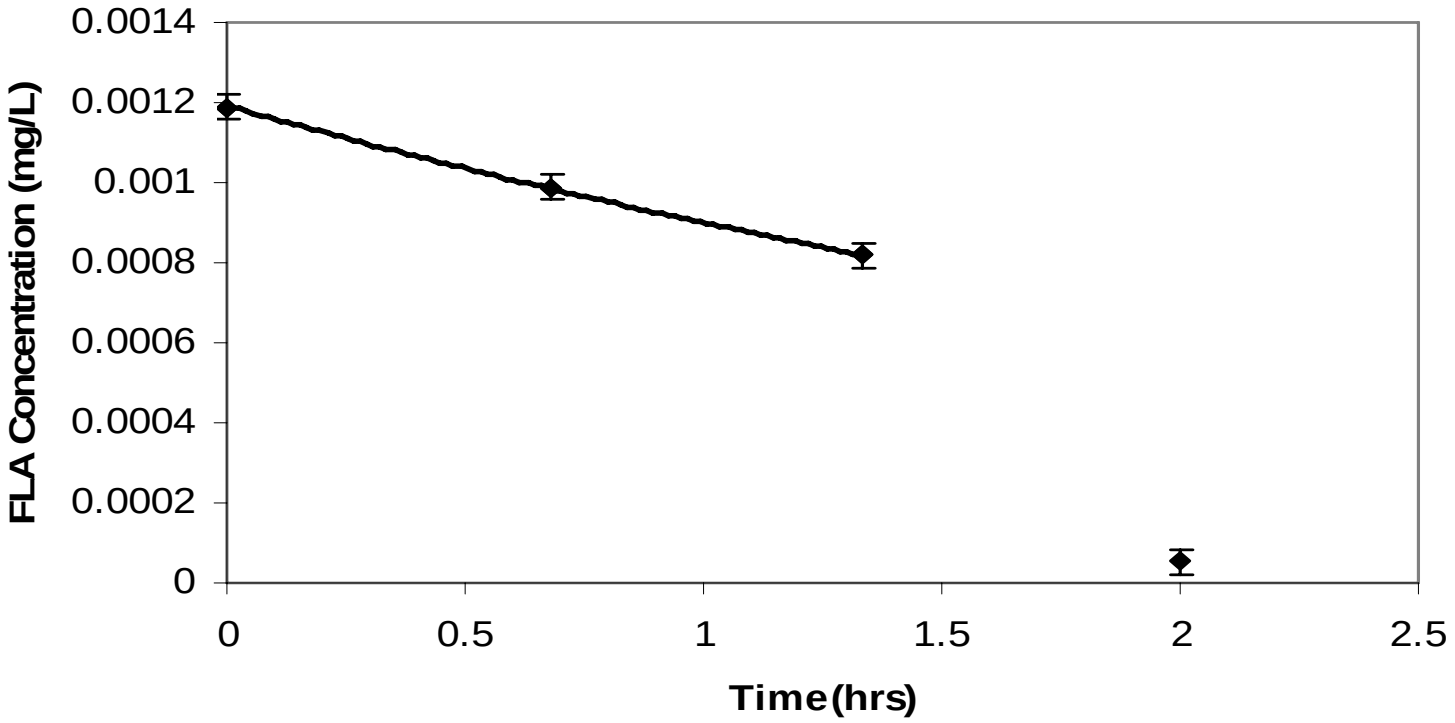

Figure 7. Sole PAH metabolism of flouranthene. (♦). Open symbols represent experimental observation.

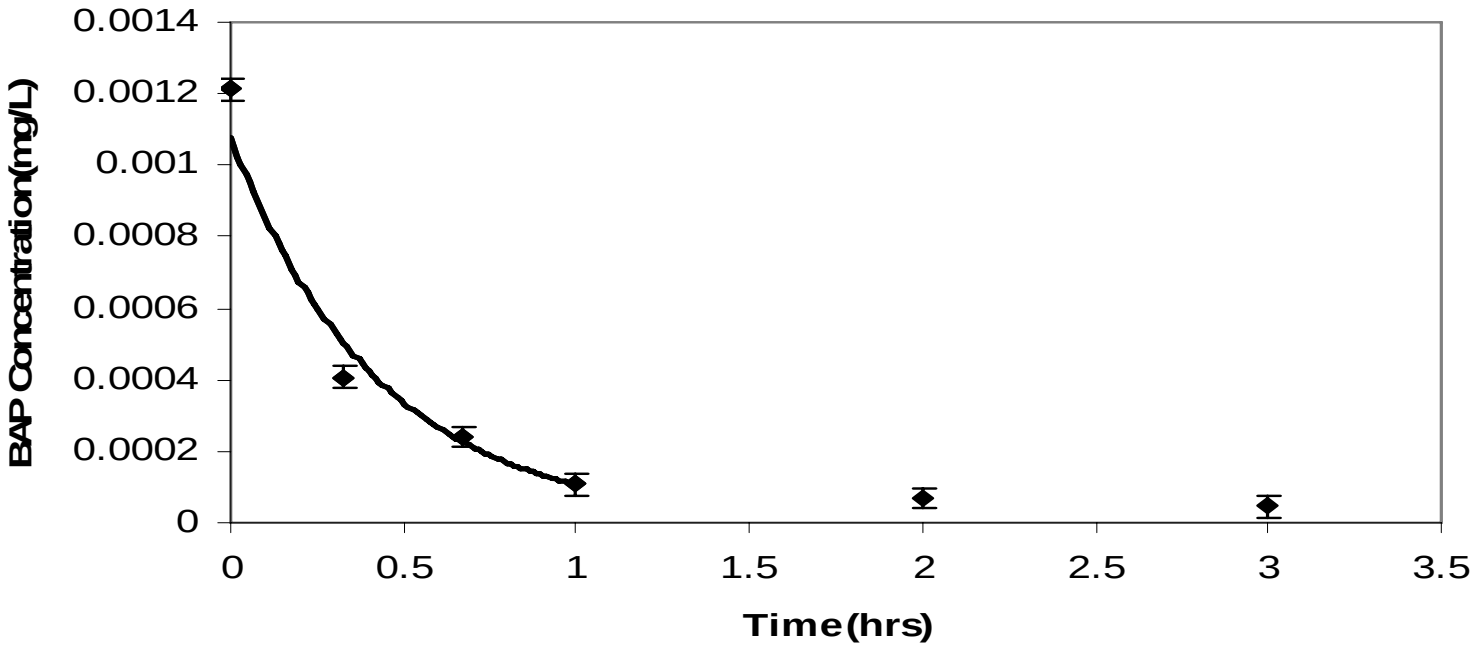

Figure 8. Sole PAH metabolism of benzo[a]pyrene.(♦) Open symbols represent experimental observation.

FLA Single Metabolism

Similarly, FLA was metabolized within 2 hours of incubation (Figure 7). Unlike the exponential decay observed for PHE, FLA was degraded more slowly for the first 1.5 hours and then rapidly dropped to near zero by 2 hours. The equation of the line was $y= 0.0012e^{-0.2818x}$ and the data points chosen was the first 90mins so as to compare with binary and ternary mixtures

BAP Single Metabolism

BAP was metabolized within three hours (Figure 8). The equation of the line fitted was also exponential, it was $y = 0.0011e^{-2.3223x}$, the data points used were for the first hour so as to compare with the binary and ternary mixtures. Unlike the points in phenanthrene and flouranthene single experiments more readings were taken in the experiment.

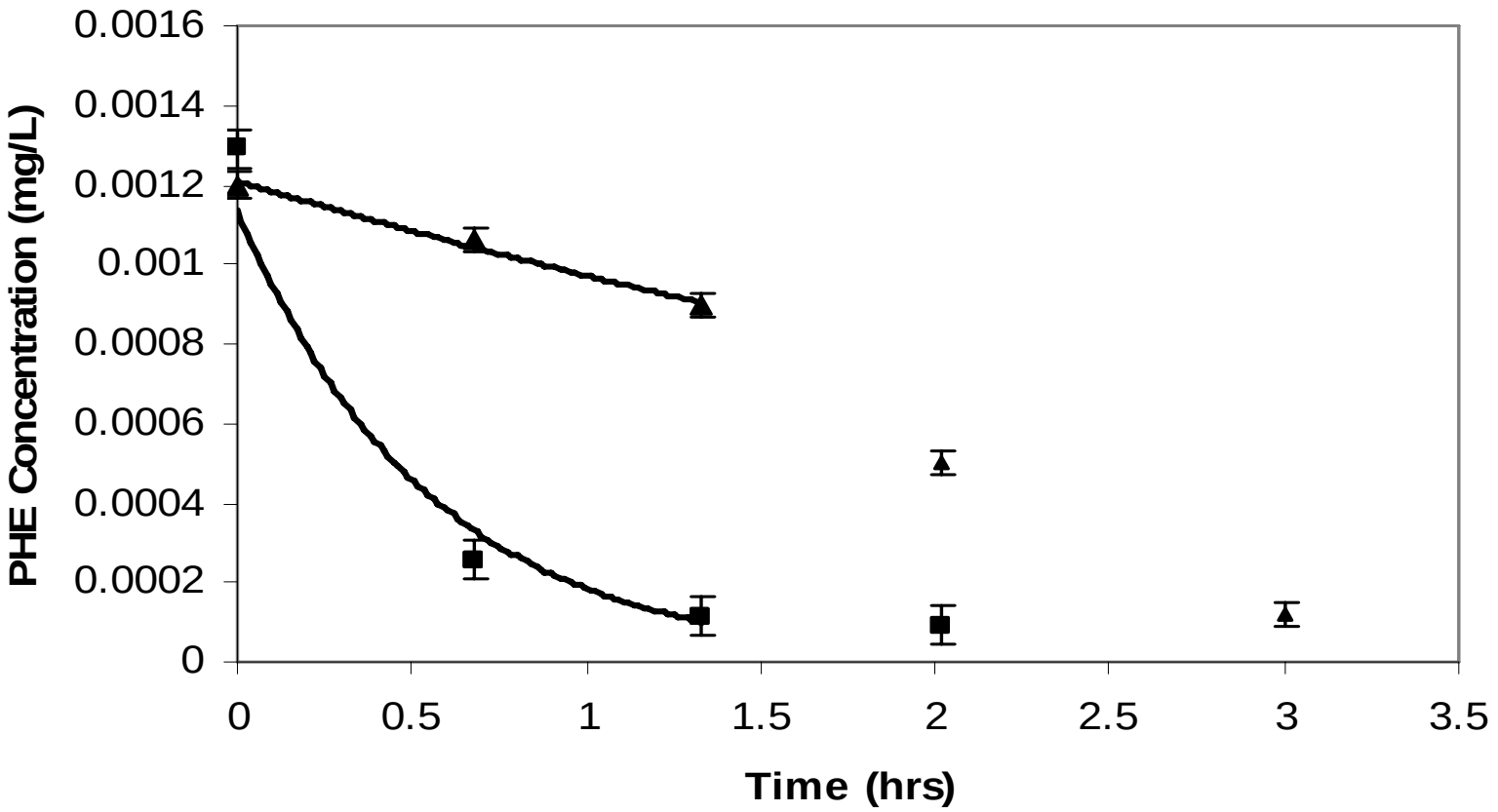

Figure 9. PHE in binary PAH metabolism and single PAH metabolism. (■) represents PHE in single PAH experiment and (▲) represents PHE in binary PAH mixture with BAP.

Binary PAH Metabolism

PHE and BAP

A binary mixture of PHE and BAP was monitored over time at the same concentration of the individual PAH used in single PAH metabolism experiments. In binary experiment there is twice as much PAH as there would be in the single PAH experiments. The experiment was monitored over a three hour period with samples taken at set intervals. The results are shown in Figures 9 and 10, comparing the metabolism of PHE and BAP respectively during single and binary culture conditions.

The points taken to fit the experimental curve were in the first 90minutes as was in the single experiment. The equation of the line for the PHE binary metabolism experiment was $y = 0.0012e^{-0.2818x}$, this also tells us that within this time interval the concentration change was exponential. The equation of the line for BAP in binary mixture with PHE was also exponential and was $y = 0.0012e^{-0.5782x}$.

PHE metabolism in the single experiment was exponential as it decayed rapidly and was transformed approximately 60% in the first hour. Complete transformation was achieved in two hours. In binary mixture with BAP, PHE degraded more slowly, requiring three hours for complete transformation when compared to the 2 hour period in single PAH metabolism. This change in concentration in one hour was approximately 30% in the binary metabolism and this slow transformation was observed until the end of the experiment. A total of three hours was needed in this binary metabolism to reach equivalent concentrations as in the single PAH experiment.

BAP in a binary mixture with PHE demonstrated reduced metabolism when compared to its metabolism in the single experiment. Initial BAP transformation was at a comparable rate to single degradation, but leveled off to a residual concentration of 0.008mg/L. Pothuluri et al (1995) in a quaternary experiment reported that BAP degradation required 8 hours for complete transformation.

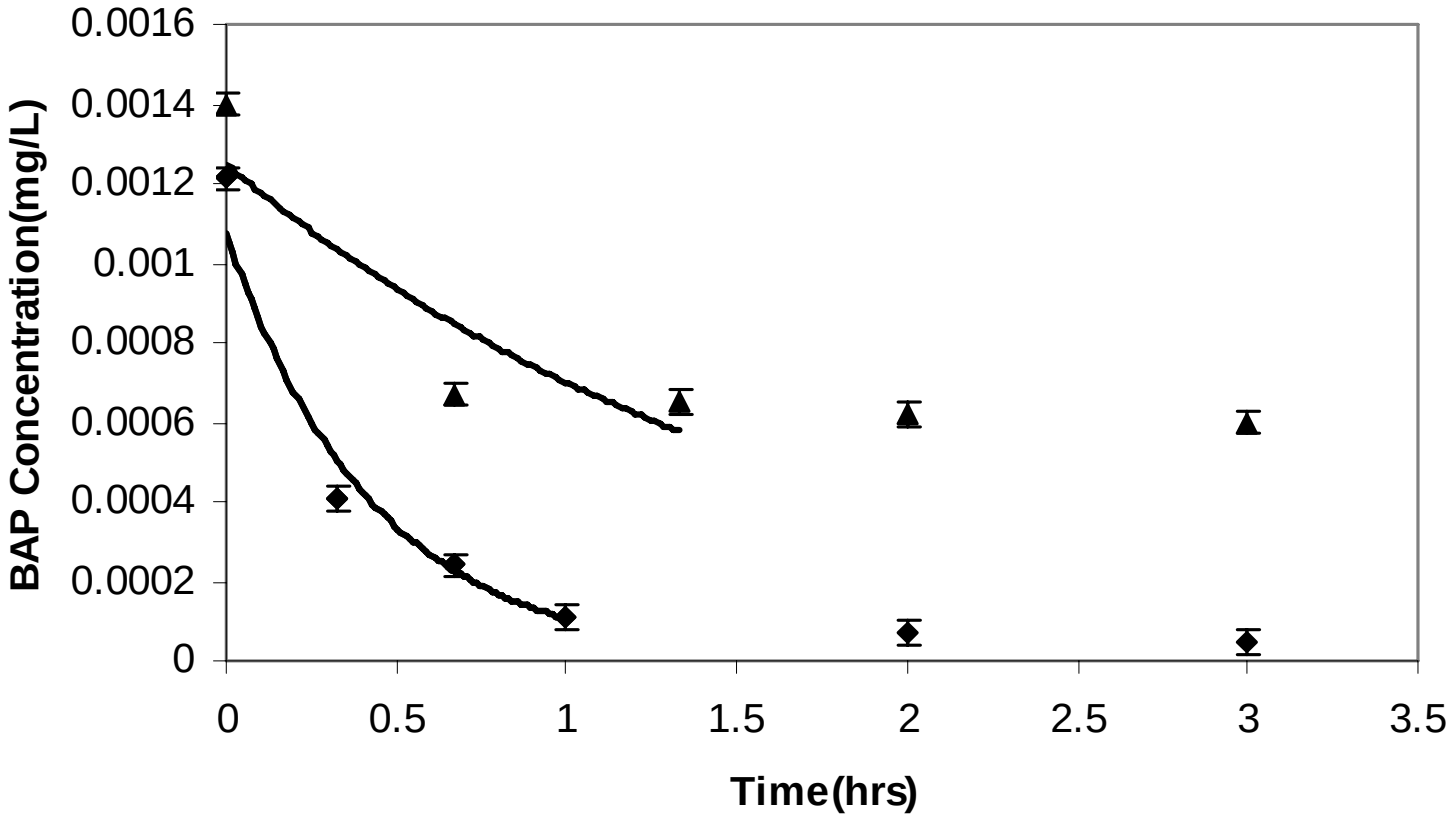

Figure 10. BAP in single PAH metabolism and in binary PAH metabolism with PHE. (▲) represents binary metabolism while (♦) represents single metabolism.

FLA and BAP

FLA was combined with BAP and incubated for 3 hours. Figures 11 and 12 compare the metabolism of FLA in single and in binary mixture with BAP (Figure 11) and BAP during single metabolism and in binary mixture with FLA (Figure 12). The curves fitted to both lines were exponential, the equation of the line for single FLA was $y = 0.0011e^{-0.281x}$ (Figure 7), while the binary was $y = 0.0012e^{-0.376x}$. The slopes look parallel and their values were similar (Table 9)

FLA metabolism in the single and binary mixture was comparable. Both show slow metabolism initially, with the single showing faster metabolism. After two hours FLA metabolism in the binary mixture remains 25% of the substrate. The metabolism as the slopes and equation show are comparable. Though comparable the total time for complete transformation was different and similarities extend only as far as about the initial ninety minutes.

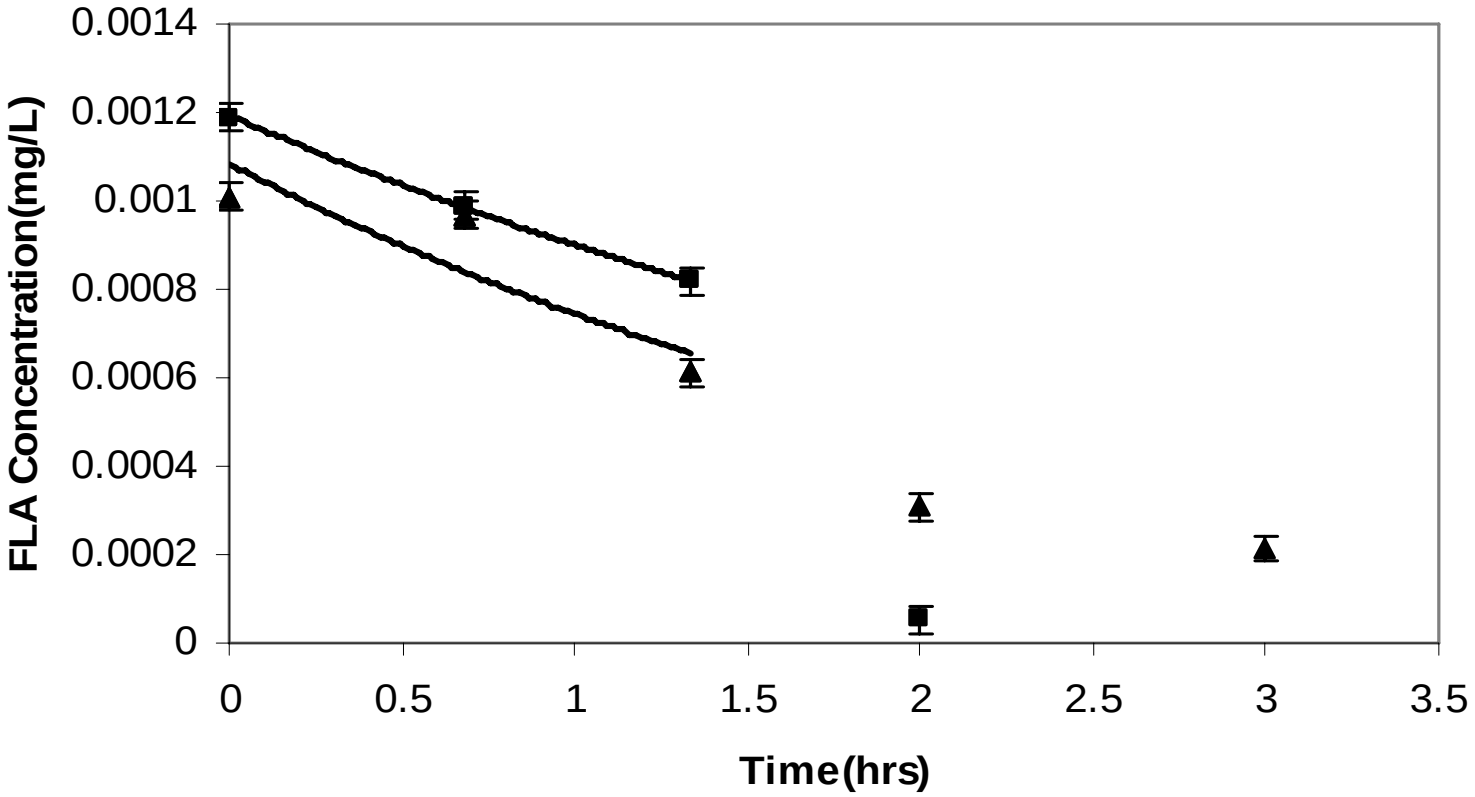

Figure 11. FLA in binary PAH metabolism and single PAH metabolism.(▲) represents binary PAH metabolism and (■) represents single PAH metabolism with BAP.

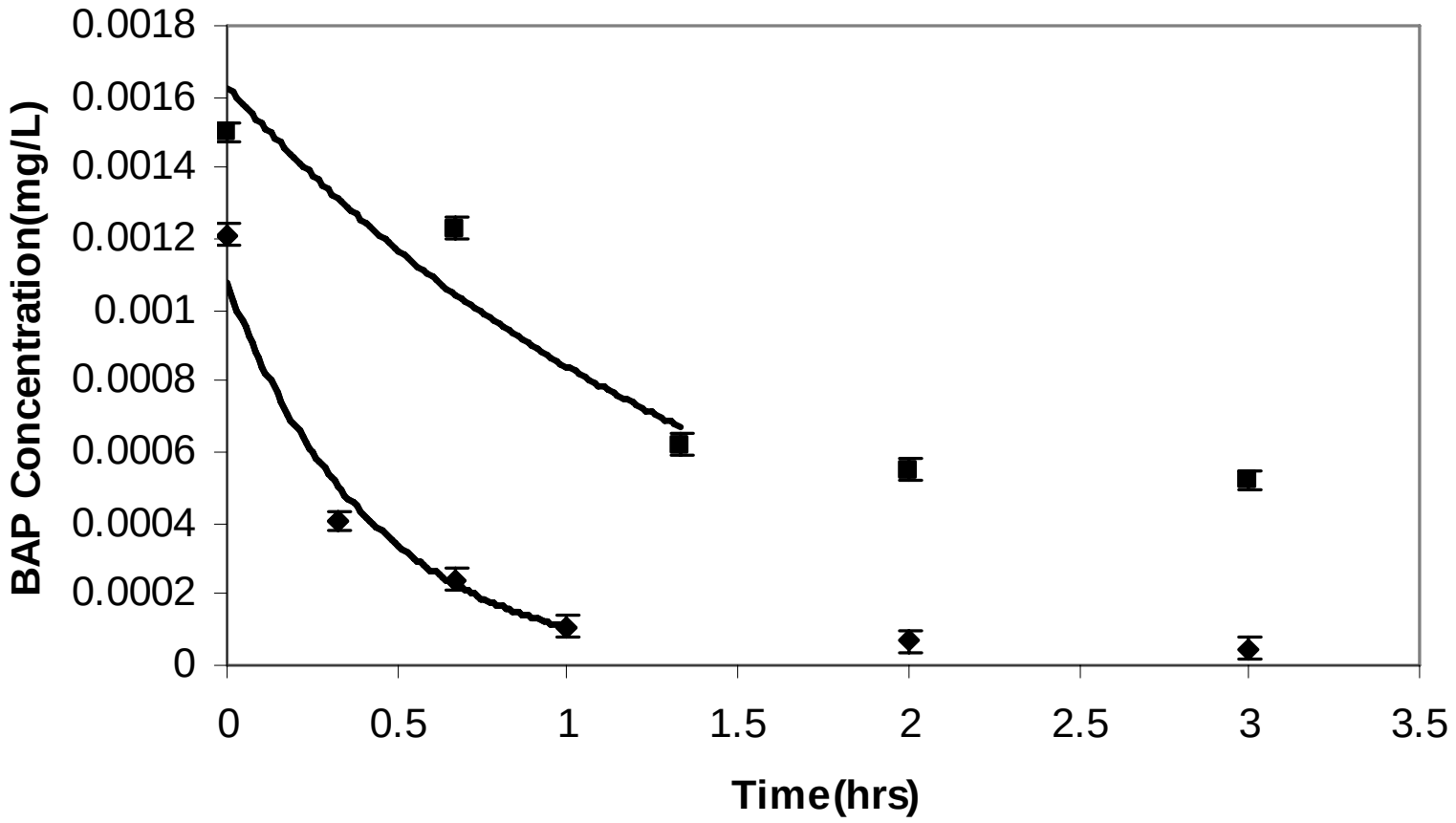

Figure 12. BAP in single PAH metabolism and in binary metabolism with FLA. (♦) represents single BAP metabolism and (■) represents binary metabolism with PHE.

BAP degradation in the presence of PHE demonstrated an initial depletion of 50%, after which there is no further metabolism. BAP degradation with FLA demonstrated a gradual depletion of the substrate with 25% of the BAP remaining after two hours. The presence of PHE reduced the rate of BAP disappearance from -0.100hr^{-1} to 0.005hr^{-1}, while in the presence of FLA the reduction was similar at 0.006hr^{-1}. The residual BAP concentration in binary metabolism with PHE was approximately 0.007mg/L while with FLA it was 0.0003mg/L.

Ternary PAH Metabolism

All three PAHs were combined for the ternary mixture. The treatment concentrations were the same as in the single PAH metabolism experiments. The total PAH concentration was approximately three times that in the single treatments, yet below the solubility limit of BAP. The metabolism pattern of the individual PAHs are compared in single, binary and ternary mixture experiments. (Figures 13-15). The curves fitted for the data for all PAHs in the ternary experiment were exponential and they taken within the time intervals as the binary and single, for the same reason to compare. The equations in the ternary experiments for PHE, FLA and BAP where $y = 0.0012e^{-0.888x}$, $y = 0.0012e^{-0.888x}$, and $y = 0.0014e^{-0.5130x}$ respectively. The equations of the line for both PHE and FLA in ternary experiments are about the same though their slopes differ; their concentrations in the experiments were not the same.

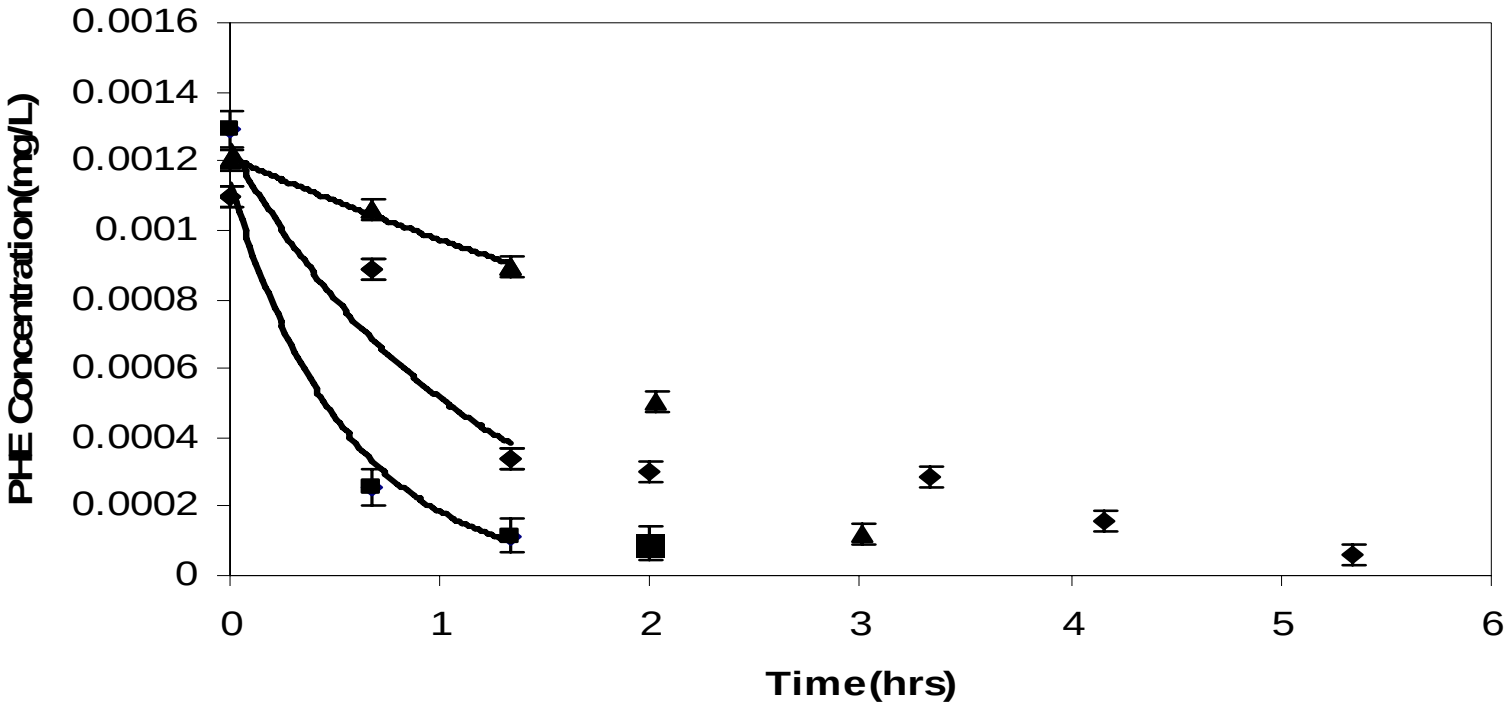

Figure 13. PHE metabolism in ternary, binary and single PAH metabolism. (■) represents PHE in single PAH experiment and (▲) represents PHE in binary PAH mixture with BAP and (♦) represents PAH metabolism in ternary PAH metabolism with FLA and BAP.

In the ternary mixture PHE demonstrates an initial slow metabolism (Figure 13). Between 40 minutes and 90 minutes approximately 25% of the PHE is metabolized; after 90 minutes the metabolism slows and continues to tail for over five hours. Generally in mixtures, degradation requires more time for complete transformation of PHE. Significant metabolism seems to occur within the first 2 hours, thereafter slower.

FLA metabolism in ternary, binary and single PAH metabolism shows similar patterns in the first 2 hours (Figure 14). All conditions are characterized by the initial slow metabolism of FLA to approximately 75% of the initial concentration. After 2 hours the mixtures experience reduced metabolism requiring more time for the ternary culture than the binary with a residual concentration of 0.0002mg/L. When the single and binary were compared previously, no significant difference in this triad comparison was observed.

Binary metabolism of BAP with both PHE and FLA is similar, being characterized by an initial stage of little or no metabolism, though it is more pronounced in the binary mixture with PHE. The binary mixtures follow the initial pattern of the single PAH. In binary with FLA, BAP shows the initial slow degradation characteristic of FLA, and then with PHE it shows an initial exponential-like metabolism before the region where there seems to be inhibition. Ternary and binary metabolism takes a longer time to be completely transformed when compared to single metabolism; this is seen to be characteristic of all mixture metabolism.

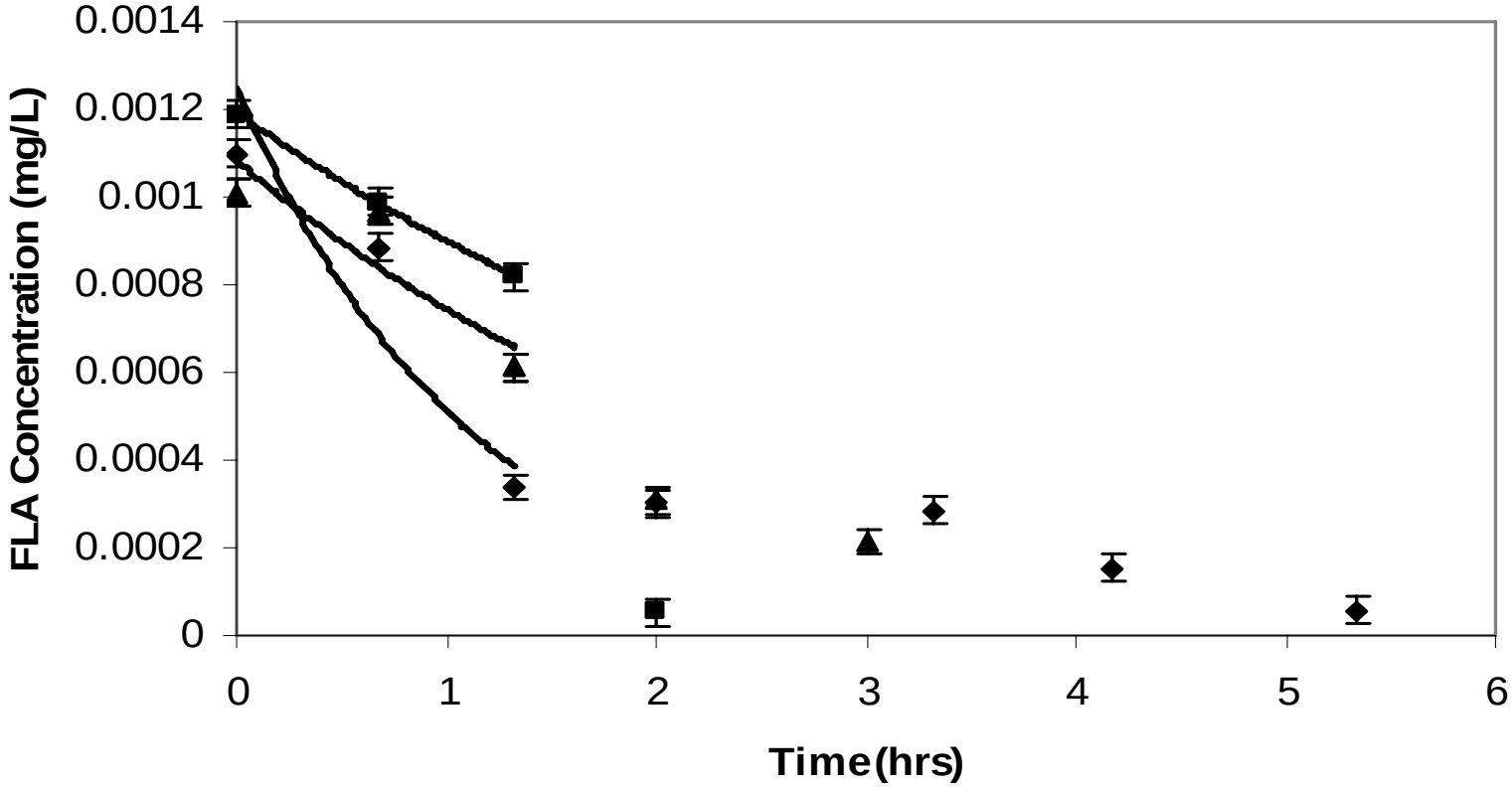

Figure 14. FLA metabolism in ternary, binary and single PAH metabolism. (▲) represents binary FLA metabolism with BAP and (■) represents single FLA metabolism and (♦) represents ternary metabolism with BAP and PHE.

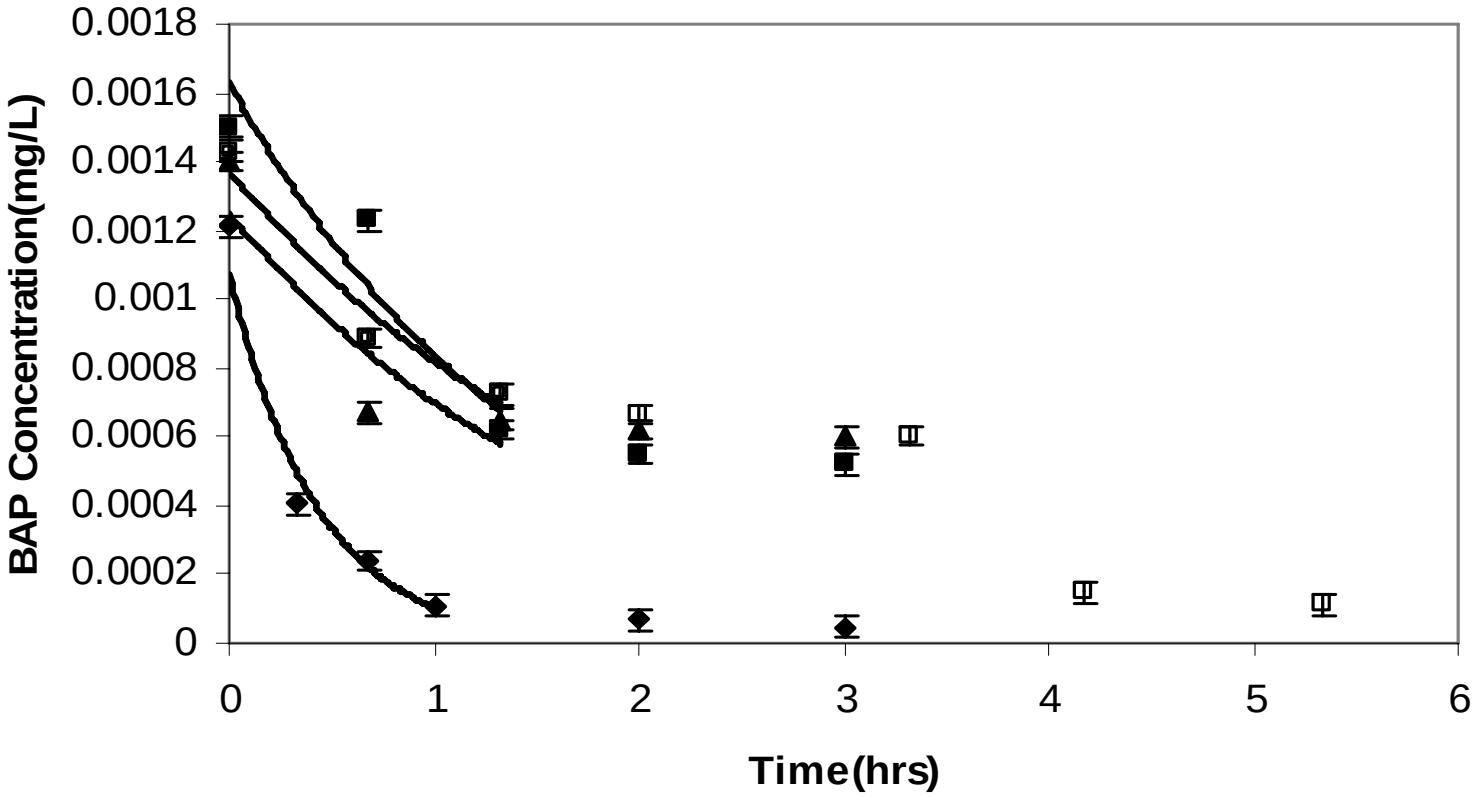

Figure 15. BAP metabolism in ternary, binary and single PAH metabolism. (♦) represents single BAP metabolism and (■) represents binary BAP metabolism with FLA, (▲) represents binary metabolism with PHE and (□) represents ternary metabolism with FLA and PHE.

Binary metabolism of BAP with both PHE and FLA is similar, being characterized by an initial stage of little or no metabolism, though it is more pronounced in the binary mixture with PHE. The binary mixtures follow the initial pattern of the single PAH. In binary with FLA, BAP shows the initial slow degradation characteristic of FLA, and then with PHE it shows an initial exponential-like metabolism before the region where there seems to be inhibition. Ternary and binary metabolism takes a longer time to be completely transformed when compared to single metabolism; this is seen to be characteristic of all mixture metabolism.

DISCUSSION AND CONCLUSION

From the results there is an indication that metabolism of mixtures of PAHs differs from metabolism for single PAHs. There are also differences between mixture experiments depending on the types and number of PAHs in the mixture. Competition for the enzyme is indicated by the PAHs in the mixture experiments, in certain cases there is preferential metabolism of a PAH and the inhibition of the other or there is a reduction of both indicating an equal competition for the enzyme. In mixture metabolism of PAHs there is an indication of no abundance of enzymes because metabolism of individual PAHs differed in the mixtures from the single. If there were an abundance of enzymes, when the total PAH concentration was increased in mixture experiments the rates would remain the same.

The log of the concentration over time, $\log(S/S_o)$, in comparable time intervals was plotted to provide a quantitative estimate of how quickly concentrations changed in the different experiments. The slope (hr^{-1}) for these plots are shown in Tables 8,9 and 10.

Table 8. PHE experiments and slopes.

PHE Combinations	Slope (hr^{-1})
Single	-0.782
Binary	-0.095
Ternary	-0.316

Table 9. FLA experiments and slopes

FLA Combinations	Slope (hr^{-1})
Single	-0.290
Binary	-0.250
Ternary	-0.240

Table 10. BAP experiments and slopes.

BAP Combinations	Slope (hr^{-1})
Single	-0.100
Binary(PHE)	-0.0005
Binary (FLA)	-0.0006
Ternary	-0.0005

The slope for the single BAP combination is approximately ten time that for the binary mixture which is indicative of competitive inhibition. The reduced transformation of PHE is indicative of reduced enzyme availability likely due to PHE and BAP competing for the same sites on the enzyme. In ternary metabolism, the slope is only half that of the single conditions. PHE was chosen because it has bay regions as does BAP. The bay regions are suspected to make metabolism more difficulty for both PAHs in binary mixture. Bouchez et al (1995) reported that there could be decreased degradation rates in the presence of multiple substrates due to toxicity or the formation of toxic metabolites, thus there lies a possibility that toxic effects could play a role in decreased metabolism. In ternary mixtures steeper slope could be due to a preferential metabolism

of PHE and or an inhibition to BAP in the presence of all three PAHs. BAP may also be less available to compete thereby demonstrating less of an effect.

In the metabolism of FLA the slopes were taken in the first hour and a half for single, binary and ternary mixtures were comparable. FLA metabolism rates show no reduction over the time interval calculated for the slopes this might be indicative of no competition for FLA in all combinations carried out. Its metabolism in the ternary structure is about equal and slightly better than in binary mixture, it could be assumed that it does not compete for the same sites as BAP and PHE, but its metabolism is affected by their presence.

BAP metabolism was highly dependent on the presence of another PAH. There is an indication of competitive inhibition for BAP metabolism in mixture experiments. Both PHE and FLA reduced the rate of BAP metabolism similarly. The same was observed for the ternary mixture.

PAHs in mixtures, either ternary or binary, reveal alteration to the individual chemical degradation. Generally, individual PAH degradation is reduced. PAH structure in relation to enzyme binding is speculated to be important in mixture metabolism and there could be toxicity effects on metabolism in mixtures of certain PAHs. Binary metabolism rates were not proven to be different from the ternary for the individual PAHs and in some cases the metabolism was faster in the ternary than the binary.

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APPENDIX

Data values for experiments performed:

Table A-1. PHE Single metabolism.

Time (hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.32E-3	1.37E-3
0.67	2.61E-4	2.98E-4
1.33	1.23E-4	1.67E-4
2	9.25E-5	7.60E-5

Table A-2. PHE binary metabolism with BAP.

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.26E-3	1.31E-3
1.67	1.10E-3	1.17E-3
1.33	8.92E-4	8.10E-4
2	5.20E-4	5.76E-4
3	1.26E-4	1.66E-4

Table A-3. PHE ternary metabolism with FLA and BAP.

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.17E-3	1.21E-3
0.67	8.51E-4	9.20E-4
1.33	3.37E-4	4.16E-4
2.	3.00E-4	2.70E-4
3.33	2.80E-4	1.67E-4
4.16	1.53E-4	2.31E-4
5.33	5.85E-5	9.80E-5

Table A-4. FLA Single metabolism.

Time (hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.19E-3	1.25E-3
0.67	9.80E-4	8.91E-4
1.33	8.22E-4	8.77E-4
2	5.40E-5	9.30E-5

Table A-5. FLA binary metabolism with BAP.

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.10E-3	1.08E-3
1.67	9.72E-4	9.04E-4
1.33	6.11E-4	7.32E-4
2	3.24E-4	4.43E-4
3	2.18E-4	1.63E-4

Table A-6. FLA ternary metabolism withPHE and BAP.

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.00E-3	1.03E-3
0.67	9.80E-4	8.61E-4
1.33	8.72E-4	9.19E-4
2.	2.86E-4	4.01E-4
3.33	2.63E-4	3.90E-4
4.16	1.14E-4	2.21E-4
5.33	8.00E-5	5.90E-5

Table A-7. BAP Single metabolism.

Time (hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.21E-3	1.12E-3
0.33	4.12E-4	5.20E-4
0.67	2.43E-4	2.36E-4
1	1.05E-4	1.99E-4
2	6.90E-5	4.80E-5
3	4.50E-5	4.10E-5

Table A-8. BAP binary metabolism with FLA

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.56E-3	1.49E-3
1.67	1.28E-3	1.19E-3
1.33	6.20E-4	7.10E-4
2	5.51E-4	6.20E-4
3	5.28E-4	4.93E-4

Table A-9. BAP binary metabolism with PHE

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.41E-3	1.34E-3
1.67	6.76E-4	7.70E-4
1.33	6.50E-4	7.00E-4
2	6.27E-4	6.83E-4
3	6.01E-4	5.94E-4

Table A-10. PHE ternary metabolism with FLA and BAP

Time(hrs)	Sample A (mg/L)	Sample B (mg/L)
0	1.48E-3	1.36E-3
0.67	8.81E-4	9.12E-4
1.33	7.29E-4	8.33E-4
2.	6.64E-4	7.20E-4
3.33	6.01E-4	6.84E-4
4.16	1.42E-4	2.01E-4
5.33	1.11E-4	1.54E-4

VITA

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