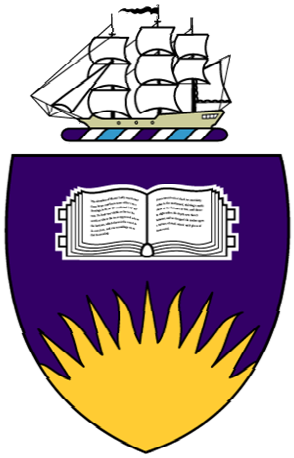
Biodegradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons in Soils by Defined Bacterial and Fungal Cocultures

A Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Christopher WM Lease B.App.Sc (Env Health) B.Sc (Hons)



School of Biological Sciences
Flinders University of South Australia
Adelaide, South Australia

AUSTRALIA

Declaration

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Christopher WM Lease

School of Biological Sciences

Flinders University of South Australia

Adelaide Australia

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"Everything that lives, lives not alone, nor for itself"
William Blake

Publications

Conference Abstracts

Lease C, Bentham R and McClure N (2002) *Degradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons by Defined Fungal-Bacterial Co-Cultures in* Proceedings of the Environmental Engineering Research Event Blackheath NSW (Poster)

Stewart R, Juhasz AL, Lease C, Dandie C, Waller N and Bentham R (2004) *An Emerging Technology for High Molecular Weight PAH Bioremediation – Bacterial – Fungal Co-Cultures in* Proceedings of Enviro 04 Conference and Exhibition, Sydney, Australia (Poster)

Lease C, Bentham R, Juhasz AL and Stanley G (2004) *Breaking Benzo[a]pyrene: The Case for Cocultures in* Proceedings of the 4th International Conference on the Remediation of Chlorinated and Recalcitrant Compounds, Monterey, United States of America (Platform Presentation – Winner of Student Paper Prize)

Manuscripts in Preparation

Juhasz AL, Waller N, Lease C, Bentham R and Stewart R (*submitted*) *Pilot Scale Bioremediation of Creosote-Contaminated Soil – Efficacy of Enhanced Natural Attenuation and Bioaugmentation Strategies*

Summary

Despite microbial degradation being the primary route of degradation of PAHs in soils, high molecular weight polycyclic aromatic hydrocarbons (such as benzo[*a*]pyrene) have consistently proven to be resistant to microbial attack. However, recent research has demonstrated the potential for bacterial-fungal co-cultures to achieve biodegradation of high molecular weight PAHs. The aim of this research was to determine the efficacy of co-culture bioaugmentation for the remediation of high molecular weight PAH-contaminated soils.

PAH degrading bacteria were enriched on multiple PAHs and isolated on pyrene from both contaminated (soil from a former manufactured gas plant) and uncontaminated (agricultural soil, termite mound matrix and kangaroo faeces) sources. The bacterial isolates were identified using 16SrRNA analysis as *Mycobacterium* sp. Strain BS5, *Mycobacterium* sp. Strain KA5 and *Mycobacterium* sp. Strain KF4 or fatty acid methyl ester (FAME) analysis as *Ralstonia pickettii* and *Stenotrophomonas maltophilia*.

The initial phase of assessment of PAH degradation by fungal and bacterial coculture components was undertaken using liquid media. Two fungal isolates from a previous investigation into the coculture process (*Penicillium janthinellum*) and the American Type Culture Collection (*Phanerochaete chrysosporium*) were assessed for their ability to degrade benzo[*a*]pyrene in minimal media and MYPD. The fungal isolates were found to be able to degrade benzo[a]pyrene cometabolically in MYPD. The bacterial isolates and two others from previous investigations were assessed for their ability to degrade single PAHs (fluorene, phenanthrene, fluoranthene, pyrene and benzo[a]pyrene) in liquid culture. This process was used as an initial screen to select the best bacterial isolates for further investigation of PAH degradation by axenic cultures and cocultures with the fungal isolates using a PAH mixture. Based on the results of these experiments four bacterial isolates (VUN 10,010, *Mycobacterium* 1B,

Mycobacterium sp. Strain BS5 and *Mycobacterium* sp. Strain KA5) and the two fungal isolates were selected to investigate further using a PAH mixture composed of the previously mentioned PAHs. It was found that the use of a fungal bacterial coculture increased the degradation of the PAH mixture beyond that of axenic bacterial cultures. Based on these experiments, the coculture composed of *P. janthinellum* and VUN 10,010 was selected for assessment of its ability to degrade the same PAH mixture in spiked soil microcosm experiments.

Natural attenuation, axenic *P. janthinellum*, axenic VUN 10,010 and a coculture of these two organisms were assessed for PAH degradation in soil microcosms over a 100 day period. Inoculation of microcosms with the coculture resulted in the removal of benzo[a]pyrene by 11 mg/kg (± 1.21 mg/kg) (30%) over the 100 day incubation period. Substantial PAH degradation was also observed in the microcosms assesing natural attenuation

Using an alternative sequential inoculation method, initially inoculating with *P. janthinellum* then 50 days later with VUN 10,010 significantly enhanced the removal of benzo[*a*]pyrene. After 100 days incubation, benzo[*a*]pyrene was degraded below detection limits in two of three microcosms, compared to a 4.95 mg/kg (± 4.64 mg/kg) (14.7 %) reduction in soil microcosms inoculated using an alternative inoculation process of VUN 10,010 followed by *P. janthinellum*.

Attempts were made to optimise the process using sequential inoculation and soil amendments intended to enhance the performance of the fungal component using distilled water and 1% glucose. The addition of distilled water was not observed to substantially influence the ability of the coculture to degrade PAHs, whereas the addition of 1% glucose was found to inhibit PAH degradation.

Symbols and Abbreviations

%	Percent
BaP	Benzo[*a*]pyrene
BSM	Basal Salts Medium
BSMY	Basal Salts Medium with Yeast Extract
BSMY3	Basal Salts Medium with Yeast Extract (3%)
^{o}C	Degree Celsius
cfu	Colony Forming Unit
DCM	Dichloromethane
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic Acid
EPA	Environment Protection Authority (Australia)
FID	Flame Ionisation Detector
g	Gram
GC	Gas Chromatography
$HgCl_2$	Mercuric Chloride
K_{ow}	Octanol/Water partition coefficient
kg	Kilogram
l	Litre
LB	Luria-Bertani
LiP	Lignin Peroxidase
LOI	Loss on ignition
MnP	Manganese Peroxidase
MGP	Manufactured Gas Plant
MW	Molecular Weight
µm	Micrometre
µmols/mL	Micromoles per milliliter
µg	Microgram
mg	Milligram
mM	Millimolar
ml	Millilitre
MPN	Most Probable Number
MYPD	Malt Yeast Peptone Dextrose Broth
NA	Nutrient Agar
NB	Nutrient Broth
nm	Nanometre
NSWEPA	New South Wales Environment Protection Agency
PAHs	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA or PDB	Potato Dextrose Agar or Potato Dextrose Broth
pH	Hydrogen Ion Concentration (minus log of)
rpm	Revolutions per Minute

rDNA or rRNA	ribosomal Deoxynucleic Acid or ribosomal Ribonucleic Acid
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) aminoethane
USEPA	United States Environment Protection Authority
UV	Ultraviolet
VUN	Victoria University Strain Number (Gram negative bacterium)
v/v	volume per volume
w/v	weight per volume
WHC	Water Holding Capacity
x g	times gravity

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Since the Industrial Revolution, numerous sites throughout the world have been subjected to a variety of activities that have resulted in soil and groundwater being contaminated with persistent organic pollutants. Often these pollutants present serious risks to human health and the environment. Urban consolidation has necessitated the development of former industrial sites for residential use, requiring the effective and economical removal of contaminants in soil.

The method of removal of contaminants from soil is often determined by the nature of the contaminant, the nature of the site and various legal and environmental limitations. The conventional methods used for the remediation of contaminated soils in Australia are (i) the excavation and removal of contaminated soil to landfill or (ii) excavation and incineration of contaminated soil or (iii) isolation of the pollutant via on-site containment (Natusch, 1997). These methods are expensive and containment either on or off site simply confines or transfers the contaminant without its destruction, necessitating long-term management (Mueller *et al.*, 1996).

Bioremediation is an emergent technology intended to achieve the remediation of soil via biologically mediated transformation of pollutants. It includes augentation with various pollutant degrading organisms (bioaugmentation) or the provision of amendments to exploit the existing natural degradative capacity of the soil's indigenous microflora (biostimulation). Allowing a pollutant to degrade via intrinsic bioremediation (where the target pollutant is biodegraded without intervention) is usually a slow process. Consequently, enhanced

bioremediation, involving either biostimulation or bioaugmentation is increasingly being investigated (Mueller *et al.*, 1996).

Although extensively investigated, the routine use of bioremediation for the remediation of contaminated soils has been limited. The reasons for this are two-fold. Firstly, the commercial development and application of bioremediation is still in its infancy. Secondly, the highly refractory nature of some common pollutants, for example, high molecular weight Polycyclic Aromatic Hydrocarbons (PAHs), limits the perceived potential for treatment via bioremediation (Harayama, 1997). Biodegradation of lower molecular weight PAHs (≤ 3 fused benzene rings) has been repeatedly achieved (e.g.: Cheung and Kinkle, 2001; Kastner and Mahro, 1998 and Smith *et al.*, 1999), whilst degradation of high molecular weight PAHs (≥ 4 fused benzene rings) is yet to be demonstrated consistently. This failure to demonstrate consistent degradation has made identifying the mechanisms of high molecular weight PAH (e.g. benzo[*a*]pyrene) degradation an immediate research priority for the development of appropriate bioremediation strategies (Juhasz and Naidu, 2000a).

The recalcitrance of high molecular weight PAHs to bioremediation has prompted numerous attempts to achieve microbially mediated degradation for the treatment of soils contaminated with these compounds. To date, few studies have demonstrated high molecular weight PAH biodegradation and those that have had a degree of success, have failed to demonstrate it consistently. Many of these studies have assessed single organisms to degrade high molecular weight PAHs, often resulting in partial degradation of the compounds with a concomitant increase in soil / soil-solution toxicity (Boonchan *et al*., 2000).

The failure of single organisms to achieve complete degradation of high molecular weight PAHs has prompted investigations using microbial cocultures or consortia in an attempt to achieve consistent degradation. The work presented in this thesis was intented to demonstrate the feasibility of bioaugmentation,

using a defined bacterial and fungal co-culture, for the remediation of soils contaminated with high molecular weight PAHs. The introduction is a review of the literature relevant to this investigation detailing the various organisms and conditions involved in the degradation of PAHs in soils.

1.2 POLYCYCLIC AROMATIC HYDROCARBONS

1.2.1 Chemical Character

PAHs are a group of over 100 chemically similar compounds that share general properties whilst each possessing unique characteristics dependent largely on structure (Figure 1.1). Generally, PAHs are compounds containing two or more fused benzene rings. The diversity of structures represented by the PAH group of compounds can be broadly categorised into alternant and non-alternant classes. Alternant PAHs contain only fused benzenoid rings (e.g. anthracene, phenanthrene and pyrene) whereas non-alternant PAHs contain four, five or six membered rings (e.g. fluorene and fluoranthene) (Harvey, 1997). PAHs have melting points above room temperature and boiling points above 100^{o}C and have relatively low aqueous solubility (International Programme on Chemical Safety, 1998).

The benzenoid rings that comprise PAHs possess delocalised electrons, often depicted in structure diagrams as a circle within the benzenoid ring, considered to more accurately reflect the nature of electron sharing than a structure of alternating single and double bonds. The delocalised electrons confer a greater chemical stability than molecules possessing localised electrons as the polarity of the bonds is reduced, making the breaking of bonds difficult (Harvey, 1997). The reactions of particular PAHs can be predicted on this basis (Dabestani and Ivanov, 1999).

The effect of electron sharing on the reactivity of the PAH molecule results in its structure being important in determining its environmental stability and potential toxicity. The impact of the PAH structure on its chemical behaviour can be generally categorised as follows:

- PAHs with a linear structure are most unstable and their angular counterparts most stable; and
- Increased size and angularity of the PAH structure increases hydrophobicity and electrochemical stability.

These structural aspects impact on the PAHs chemical and photochemical reactivity, as well as its ionisation potential, vapour pressure, solubility and adsorption characteristics (Karcher, 1992).

The reduction in aqueous solubility tends to follow an increase in molecular weight corresponding with an increase in the number of rings contained in the PAH molecule (Table 1.1). This reduction in aqueous solubility can be countered by ring hydroxylation, ring fission or the addition of substituents (e.g. methyl substitution) on the PAH molecule (Harvey, 1997). Hence, many of the reactions resulting in the removal of PAHs from the environment or detoxification in higher organisms are reliant upon these reactions enhancing aqueous solubility.

Volatility also decreases with an increasing number of fused benzene rings. Theoretical and experimental evidence has shown that the association of a contaminant with organic matter in soil (expressed as the organic normalised partition coefficient K_{oc}) is a function of the hydrophobicity of the compound (expressed as the octanol-water partitioning coefficient K_{ow}). As PAHs are characterised by high K_{ow} and low vapour pressures, naturally occurring organic material is an excellent sorbent for these compounds (Accardi-Dey and Geschwend, 2002)

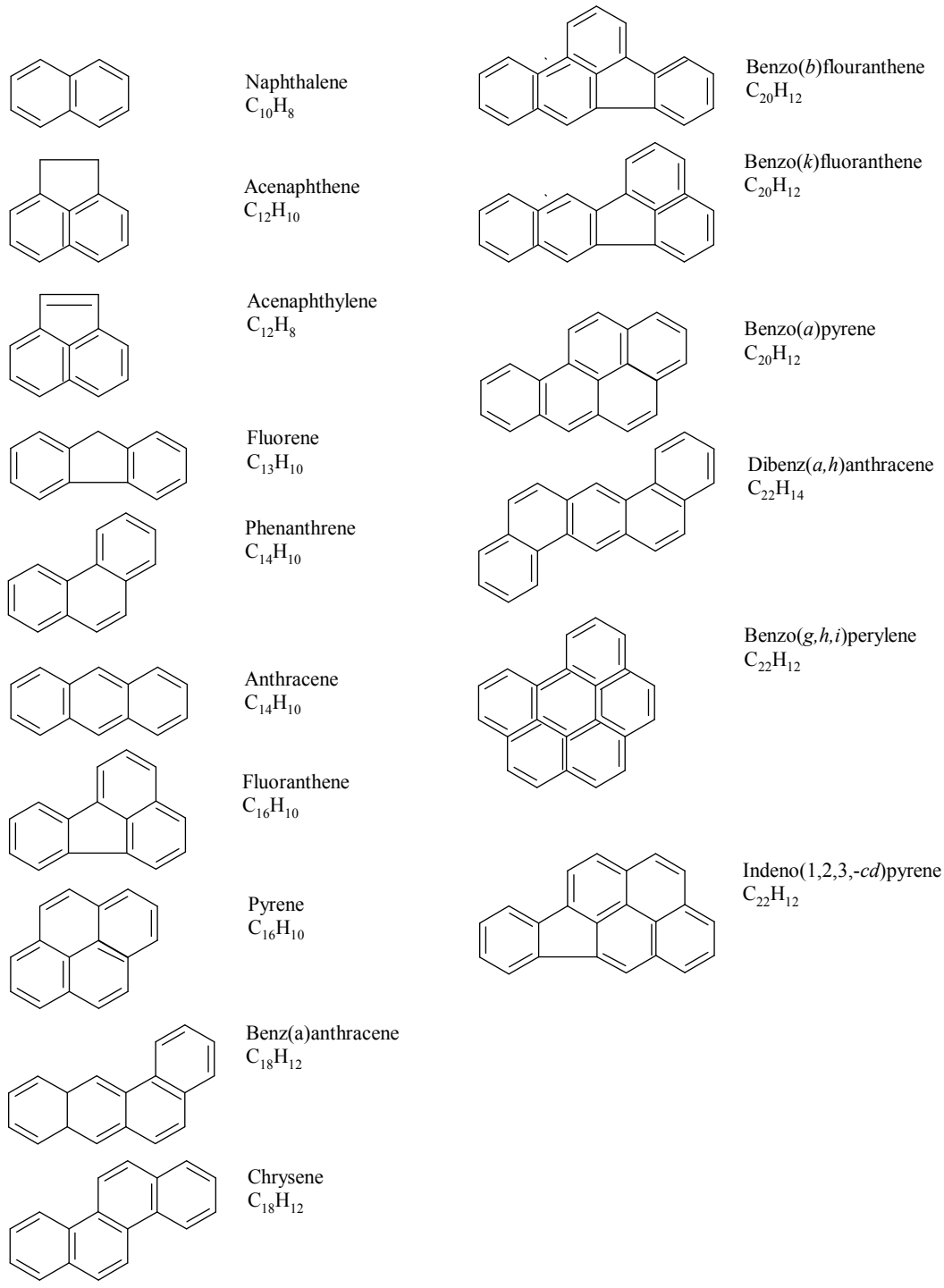


Figure 1.1: Structure and Molecular Formula of 16 Polcyclic Aromatic Hydrocarbons Designated Priority Pollutants by the USEPA (*adapted from Harvey, 1997*)

Table 1.1: Physical and Chemical Properties of USEPA 16 Priority Polycyclic Aromatic Hydrocarbons
(*Adapted from IPCS, 1998, with information from Harvey, 1991 and Debestani and Ivanov, 1999*)

Compound	**Number of Rings 6 member (5 member)**	**Melting Point (^{o}C)**	**Boiling Point (^{o}C)**	**Vapour Pressue (Pa at 25^{o}C)**	**Density**	**n-Octanol: water Partition Coefficient (log K_{ow})**	**Solubility in Water at 25^{o}C (µg/litre)**	**Henry's Law Constant at 25^{o}C (kPa)**	**Ionisation Potential (eV)**
Naphthalene	2	81	217.9	10.4	1.154	3.4	$3.17x10^{4}$	$4.89x10^{-2}$	8.12
Acenaphthylene	2 (1)	92-93		$8.9x10^{-1}$	0.899	4.07		$1.14x10^{-3}$	8.22
Acenaphthene	2 (1)	95	279	$2.9x10^{-1}$	1.024	3.92	$3.93x10^{3}$	$1.48x10^{-2}$	7.68
Fluorene	2 (1)	115-116	295	$8.0x10^{-2}$	1.203	4.18	$1.98x10^{3}$	$1.01x10^{-2}$	7.88
Anthracene	3	216.4	342	$8.0x10^{-4}$	1.283	4.5	73	$7.3x10^{-2}$	7.439
Phenanthrene	3	100.5	340	$1.6x10^{-2}$	0.98	4.6	$1.29x10^{3}$	$3.98x10^{-3}$	8.19
Fluoranthene	3 (1)	108.8	375	$1.2x10^{-3}$	1.252	5.22	260	$6.5x10^{-4}$	7.9
Pyrene	4	150.4	393	$6x10^{-4}$	1.271	5.18	135	$1.1x10^{-3}$	7.5
Benz[*a*]anthracene	4	160.7	400	$2.8x10^{-5}$	1.226	5.61	14		7.54
Chrysene	4	253.8	448	$8.4x10^{-5}$ (20^{o}C)	1.274	5.91	2.0		7.8
Benzo[*b*]fluoranthene	4 (1)	168.3	481	$6.7x10^{-5}$ (20^{o}C)		6.12	1.2 (20^{o}C)	$5.1x10^{-5}$	*na*
Benzo[*k*]fluoranthene	4 (1)	215.7	480	$1.3x10^{-8}$		6.84	0.76	$4.4x10^{-5}$	*na*
Benzo[*a*]pyrene	5	178.1	496	$7.3x10^{-7}$	1.351	6.5	3.8	$3.54x10^{-5}$	7.23
Benzo[*g,h,i*]perylene	6	278.3	545	$1.4x10^{-8}$	1.329	7.1	0.26	$2.7x10^{-5}$	*na*
Indeno[1,2,3-*cd*]pyrene	5 (1)	163.6	536	$1.3x10^{-8}$		6.58	62	$2.9x10^{-5}$ (20^{o}C)	*na*
Dibenzo[*a,h*]anthracene	5	266.6	524	$1.3x10^{-8}$	1.282	6.5	0.5 (27^{o}C)	$7x10^{-6}$	7.57

na = not available

1.2.2 Toxicology

The differences conferred by chemical structure also contribute to the differing toxic, carcinogenic and mutagenic properties of PAHs. Many PAHs and their transformation products exhibit acute toxic, mutagenic, teratogenic or carcinogenic properties. In many cases, metabolic activation is required for the initiation of carcinogenesis - for example mammalian systems oxidise PAHs to reactive electrophilic intermediates (Pothuluri and Cerniglia, 2000). As a general rule, lower molecular weight PAHs exhibit acute toxicity whereas many of the high molecular weight PAHs exhibit chronic effects such as genotoxicity (Juhasz and Naidu, 2000).

In humans, there is potential for PAHs to be absorbed through the pulmonary and gastrointestinal tract and the skin. Absorption of benzo[*a*]pyrene following ingestion is thought to be low, and absorption of PAHs following dermal or inhalation exposure is thought to be mediated by the vehicle of administration (e.g. the presence of carrier materials such as particulates or solvents). PAHs can be widely distributed throughout the body and following distribution can be found in almost all organs, particularly those high in lipids due to their low aqueous solubility. The fate of PAHs in the body is dependent of structure and the detoxification process. Metabolism of PAHs may result in the excretion of PAHs in the urine and faeces, although some PAHs are activated through metabolism creating metabolites with the potential to bind to DNA (particularly diol epoxides) that can intiate tumor formation (IPCS, 1998).

Most of the non-substituted PAHs with 2-3 rings have not been found to be carcinogenic in experimental animals, although many PAHs subjected to genotoxic tests based on mammalian DNA integrity (damage, mutation, chromosomal abnormalities) or on the Ames Test return a positive result (Wattiau, 2002). It has also been suggested that genotoxicity may increase with

increased molecule size up to 4-5 rings (Kanaly and Harayama, 2000). Benzo[*a*]pyrene is a potent carcinogen through most exposure routes (ingestion, inhalation and dermal exposure). It is thought that the mechanism of carcinogenicity is through an effect on DNA repair. In mammal cells, benzo[*a*]pyrene can induce DNA binding, sister chromatid exchange, chromosomal aberrations, point mutations and transformations (Juhasz and Naidu, 2000a).

Many PAHs exhibit a carcinogenic potential either through initiation or being a complete carcinogen (Table 1.2). The carcinogenic potential of condensates from emissions such as diesel and gasoline engines, coal combustion and side-stream smoke from cigarettes has been found to be predominantly due to PAHs, particularly those containing four or more rings. Although considered a potent carcinogen, the contribution of benzo[*a*]pyrene to the carcinogenic potential of the various emission condensates was relatively minor (e.g. accounting for 0.17% of the total carcinogenicity of tobacco smoke condensate) (Grimmer, 1991). This suggests an increased contribution of a variety of PAHs to carcinogenesis, not simply the presence of a single PAH carcinogen.

The carcinogenic effect of PAHs on mammalian cells is a consequence of the metabolic transformation resulting in activation to diol epoxides (highly reactive molecules that covalently bind to DNA). This activation occurs mainly in the microsomes of the endoplasmic reticulum and is catalysed by monoxygenase enzymes associated with cytochrome P-450 (Wattiau, 2002). The transformation of PAHs by cytochrome P-450 monoxygenases is believed to occur predominantly in the endoplasmic reticulum and in nuclear membranes. The PAH is oxidized by cytochrome P-450 monoxygenases to form phenols and dihydrodiols, occasionally involving the creation of highly reactive epoxide intermediates. It is the formation of these reactive epoxides that is proposed as the step that results in the carcinogenic activity of the PAH molecule (Kloppman *et al*., 1999).

As well as determining their likely environmental fate, PAH structure also indicates its toxigenic potential. PAH molecules possessing either a "bay" region (a void in the structure of the molecule bordered by the edges of three rings) or a "fjord region" (a void in the structure of the molecule bordered by the edges of four rings) have differing toxic potentials due to the site of metabolic activation created by these structural differences (Figure 1.2) (Harvey, 1991). The "bay region" diol epoxide intermediates are thought to be the ultimate carcinogen for alternant PAHs, as once formed they can covalently bind to DNA and other cellular macromolecules to initiate carcinogenesis and mutagensis (Agency for Toxic Substances and Disease Registry, 1995). It has been proposed that the PAHs exhibiting a high carcinogenic potency tend to possess a bay region, as seen in the benzo[*a*]pyrene molecule, although this theory is not supported by evidence that benzo[*e*]pyrene (the isomer of benzo[*a*]pyrene) possesses two bay regions and is practically inactive (Kloppman *et al.*, 1999).

It has been proposed that the best predictor of carcinogenic effect is based on the molecules shape as an indicator of acceptable targets for the cytochrome oxygenases. Klopman *et al.* (1999) postulated that for an enzyme to catalyse a metabolic process at a certain site, there must be sufficient reactivity and ready accessibility for the enzyme. The impact of reactivity is relatively obvious (i.e. if it is not high enough, there will be no reaction), but even if the reactivity is high, the reaction will not occur if enzyme accessibility is too low as a result of high steric hindrance. Both conditions must be met to ensure the reaction takes place. Kloppman *et al.*, (1999) found from their experiments using the potent carcinogen benzo[*a*]pyrene that the major products of cytochrome mediated metabolism were 7-8 and 9-10 epoxides, believed to be related to benzo[*a*]pyrene's carcinogenicity, supporting their theory of reactivity and enzyme accessibility (Kloppman *et al.*, 1999).

Many PAHs are thought to possess mutagenic potential to *Salmonella typhimurium* (Ames Test) following metabolic activation. The mutagenic effect of environmental PAHs has been hard to determine due to the inherent mutagenic properties of many soils, particularly when using the Ames Test (Knize *et al.*, 1987). This is compounded by the human health risk posed by soil mutagens being relatively unkown and difficult to determine. The presence of PAHs does not necessarily correlate with increased mutagenicity, despite the presence of known mutagens such as benzo[*a*]pyrene or other PAHs (Watanabe and Hirayama, 2001).

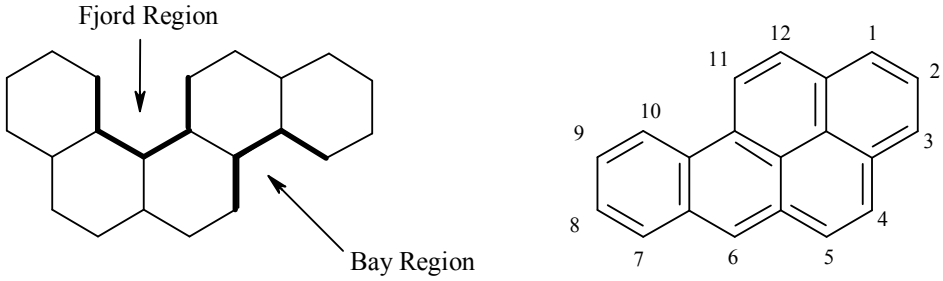
1.2.3 Sources and Extent of Contamination in the Environment

PAHs are environmentally ubiquitous compounds of both anthropogenic and biogenic origin. They are constituents of creosote and other commonly used wood treatment products, petroleum, coal tar, shale oil, and are formed as by-products from incomplete combustion of organic material. Over the past 100 years, there has been an increase in the amount of PAHs present in the environment from anthropogenic sources and atmospheric deposition from natural sources (Juhasz and Naidu, 2000). The accumulation of PAHs of anthropogenic origin in the environment is believed to be a result of deposition outstripping environmental removal mechanisms. As a consequence of their toxicity, mutagenicity and carcinogenicity, many PAHs have been identified as priority pollutants by numerous regulatory authorities including Environment Australia and the United States Environment Protection Authority (USEPA). Benzo[*a*]pyrene is considered a Priority Persistent, Bioaccumulative and Toxic Pollutant by the USEPA (USEPA, 2002).

Table 1.2: Summary of Results of Tests for Genotoxicity and Carcinogenicity for the 33 Polycyclic Aromatic Hydrocarbons Studied
(Adapted from IPCS, 1998 with information from Harvey, 1991 and USEPA, 2004)

Compound	Genotoxicity	Carcinogenicity	Carcinogenic Activity
Acenaphthene	(?)	?	0
Acenaphthylene	(?)	No studies	?
Anthanthrene	(+)	+	I
Anthracene	-	-	0
Benz[*a*]anthracene	+	+	I
Benzo[*b*]fluoranthene	+	+	C
Benzo[*j*]fluoranthene	+	+	C
Benzo[*ghi*]fluoranthene	(+)	(-)	0
Benzo[*k*]fluoranthene	+	+	C
Benzo[*a*]fluorene	(?)	(?)	0
Benzo[*b*]fluorene	(?)	(?)	I
Benzo[*ghi*]perylene	+	-	C
Benzo[*c*]phenanthrene	(+)	(+)	0
Benzo[*a*]pyrene	+	+	C
Benzo[*e*]pyrene	+	?	I
Chrysene	+	+	I
Coronene	(+)	(?)	I
Cyclopenta[*cd*]pyrene	+	+	C
Dibenz[*a,h*]anthracene	+	+	C
Dibenzo[*a,e*]pyrene	+	+	C
Dibenzo[*a,h*]pyrene	(+)	+	C
Dibenzo[*a,l*]pyrene	(+)	+	C
Fluoranthene	+	(+)	C
Fluorene	-	-	0
Indeno[1,2,3-*cd*]pyrene	+	+	C
Naphthalene	-	(?)	0
Perylene	+	(-)	0
Phenanthrene	(?)	(?)	0
Pyrene	(?)	(?)	0
Triphenylene	+	(-)	0

Carcinogenicity and Mutagenicity: + = positive, - = negative, ? = questionable ( ) = results derived from a small database
Carcinogenic Activity: 0 = inactive, I = initiator, C = complete carcinogen



(Harvey, 1997)

Figure 1.2: Bay / Fjord Regions (Benzo[*c*]chrysene) and Numbering of Rings (Benzo[*a*]pyrene) on the PAH Molecule

In Australia, PAHs are contributed to the atmosphere via point sources (e.g. coal fired power stations) and diffuse sources such as motor vehicle exhausts (predominantly naphthalene, phenanthrene and pyrene) and domestic heating using wood fired stoves (predominantly benzo[*a*]pyrene and benzo[*g,h,i*]perylene). PAH levels in air show a seasonal difference with an increase in particulate associated PAHs in winter and vapour phase PAHs in summer. The increase in particulate phase PAHs in winter is related to the increase in the use of domestic heating, traffic congestion, less pollutant dispersal due to meteorological conditions and the absence of photochemical degradation that occurs in summer (Environment Australia, 1999).

PAH contamination of soils in Australia is principally a result of industrial activities such as the manufacture of gas from coal and wood treatment using preservatives such as creosote. In manufactured gas plants, the tars and oils separated from gas during manufacture contained significant amounts of PAHs which were either disposed on-site or sold as a manufacture by-product. Hence contamination of the surrounding soil with numerous PAHs, particularly high molecular weight compounds including pyrene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and indeno[*g,h,i*]perylene was common place (Table 1.3) (NSWEPA, 2003).

The formation of PAHs via combustion differs depending on the source of material, the method of combustion and combustion parameters such as temperature and length of time. The most structurally stable PAHs (angular PAHs such as phenanthrene and chrysene) require high temperatures and extended time for formation (USEPA, 1999). The influence of combustion material and method on PAH fractions is provided in Figure 1.3, where the relative quantities of PAHs from a variety of combustion sources is shown. Similarly, the amount and type of each PAH generated by fossil fuel combustion

or industrial processes is dependent on factors including the process type, temperature and efficiency, and the fuel or fuel mixture used (Khalili *et al*., 1995).

Due to the differing proportions of the relative molecular mass PAHs in various sources in the environment, each source contributes different amounts of a particular PAH to the environment. As a consequence, the source of PAHs in environmental samples can be determined by the relative proportions of types of PAHs present in the sample. For example, coal contains a larger proportion of the lower weight PAHs including napthalene, fluorene, phenanthrene, fluoranthene and pyrene, creosote contains a greater proportion of fluoranthene and the intermediate weight PAHs (phenanthrene to benzo [*b* or *k*] fluoranthene) and crankcase oil has a higher proportion of high molecular weight PAHs (Brooks, 2004).

Studies into the degradation of high molecular weight PAHs have predominantly focused upon benzo[*a*]pyrene due to its potent carcinogenicity and environmental recalcitrance (Juhasz and Naidu, 2000a). Benzo[*a*]pyrene from anthropogenic sources usually arises in the environment via the deposition of atmospheric contaminants deriving from combustion. There has been an increase in soil benzo[*a*]pyrene concentration over the past 100-150 years. Due to its environmental stability, benzo[*a*]pyrene accumulates in marine organisms and plants (Juhasz and Naidu, 2000a). In aerobic sediments, benzo[*a*]pyrene has a half-life of 300 weeks, making it a persistent organic pollutant (Henner *et al*, 1997). Significant degradation of benzo[*a*]pyrene in the environment is thought to require 3.3 years in oil contaminated sediments versus 60 years in uncontaminated sediments (Kanaly and Harayama, 2000).

Table 1.3: PAH Levels in Soils from Contaminated Sites, Australia

PAH	PAH Concentration (mg/kg soil) in Soil Samples		
	Former Wood Treatment Site Australia[a]	**Former Gas Works Australia[a]**	**Petrochemical Site Australia[b]**
Acenaphthene	670	6.3	*nd*
Acenaphthylene	32	9.0	43
Anthracene	150	43	53
Benz[a]anthracene	290	110	33
Benzo[*a*]pyrene	110	94	15
Benzo[*b*]fluoranthene	170	120	*nd*
Benzo[*g,h,i*]perylene	37	70	*nd*
Benzo[*k*]fluoranthene	120	79	*nd*
Chrysene	430	90	*nd*
Dibenz[*a,h*]anthracene	10	10	12
Fluoranthene	1900	64	137
Fluorene	340	12	87
Indeno[1,2,3-*c,d*]pyrene	55	180	nd
Naphthalene	1.1	32	186
Phenanthrene	870	52	156
Pyrene	1400	120	99
Total PAHs	**6,600**	**1,100**	**821**

[a] = This Study [b] = Juhasz, 1997 *nd* = Not Determined

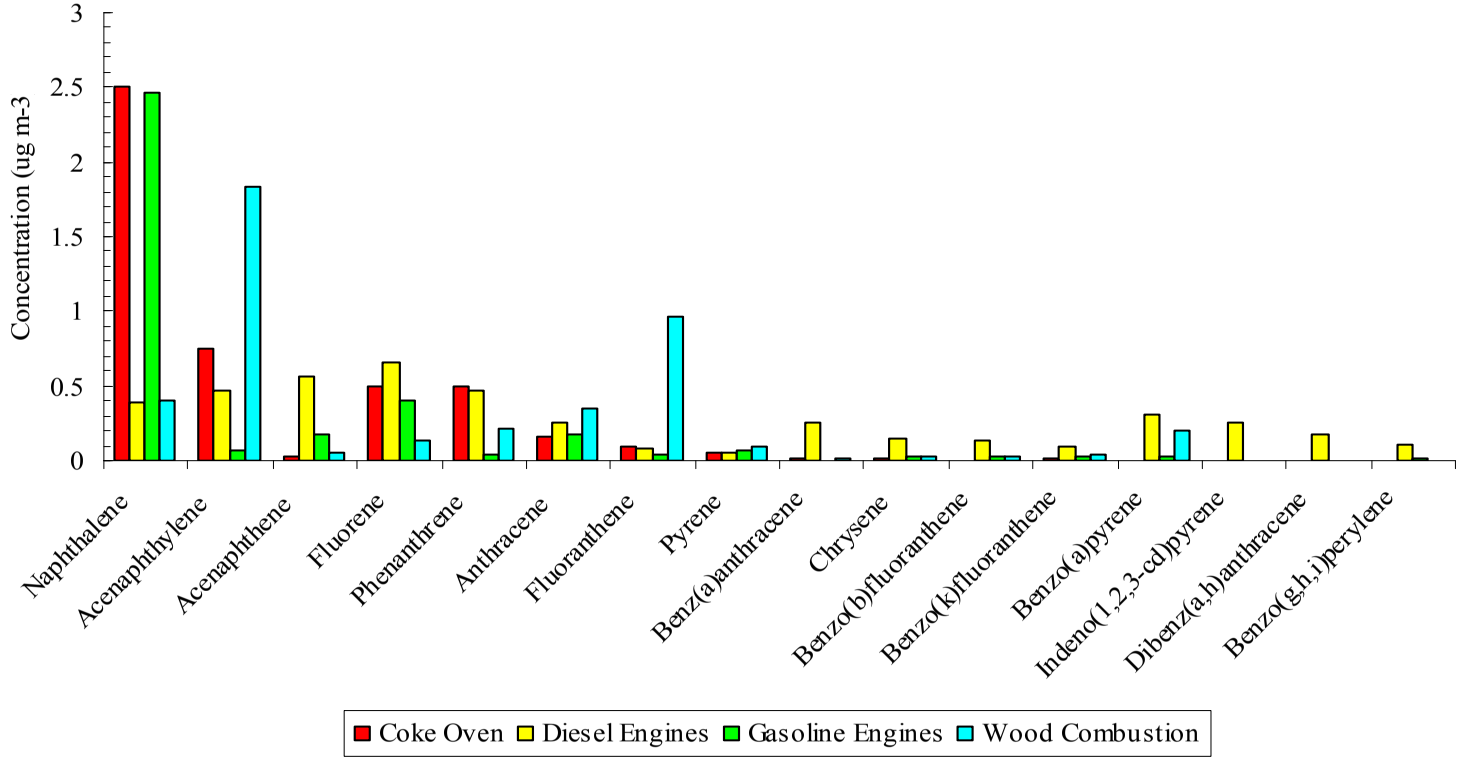


Figure 1.3: Average Concentration (μg m^{-3}) of PAHs from Various Emission Sources in Chicago (Khalili *et al*, 1995)

1.3 FATE OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE ENVIRONMENT

There are a variety of mechanisms by which PAHs are degraded in the environment, including chemo-oxidation and photooxidation, although microbial degradation is considered the primary route of degradation of PAHs in soils (Juhasz and Naidu, 2000) (Figure 1.4). PAHs have differing half lives in environmental compartments (e.g. soil, air, water) depending on their structural susceptibility to chemical, physical or biological decomposition (Table 1.4). Alteration in the structure of the PAH arsing from environmental modification usually results in changes in toxicity, mobility or other chemical characteristic of the compound. This alteration may result in binding of the PAH with soil components or destruction of the molecule via biotransformation (Mueller *et al.*, 1996). Biotransformation is the chemical alteration of a compound's molecular structure achieved by organisms (e.g. bacteria, fungi, algae) resulting in a change in the compound's complexity or loss of a characteristic property. The biotransformation rate in environmental systems is dependent on numerous physiochemical phenomena controlling bioavailability and the biokinetic phenomena pertaining to electron acceptors, nutrient supply, toxicity, inhibition and competitive substrate utilisation (Ramaswami and Luthy, 1997).

PAHs in the environment are subject to a number of processes that affect their fate. The following briefly details some of the environmental fate of PAHs prior to a more detailed analysis of microbial degradation.

1.3.1 Volatilisation

Volatilisation can occur in the atmosphere and at or near to the surface of water, soil and vegetation, allowing the destruction or appreciable modification of organic chemicals, although this is limited to compounds with higher vapour pressures

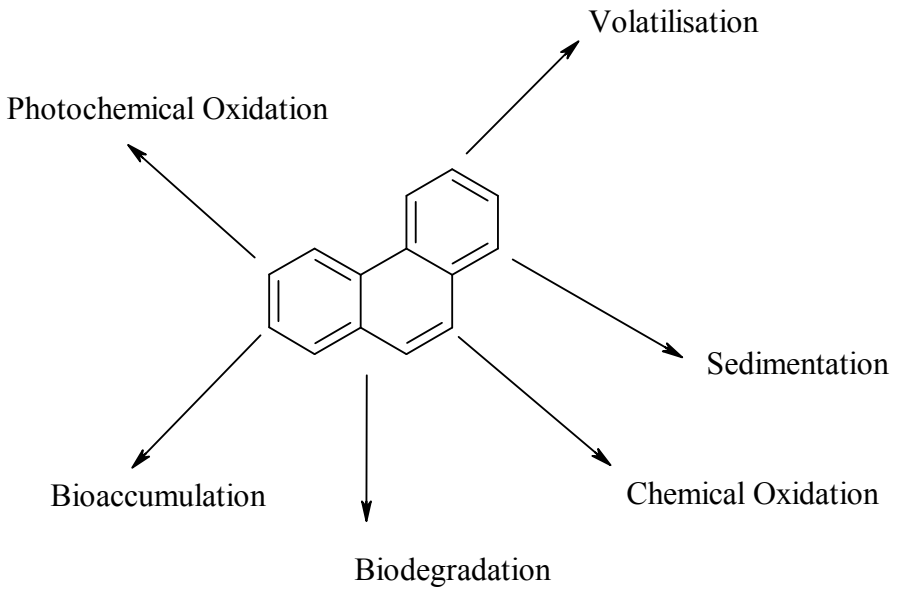


Figure 1.4: Putative Model of Fate of a PAH (Phenanthrene) in Soil (adapted from Semple *et al.*, 2003)

(Alexander, 1999). Due to their predominantly low vapour pressures, loss of PAHs from soils due to volatilisation is limited to a few PAHs (particularly naphthalene and fluorene). At temperatures commonly experienced in the environment (0-40 ºC), most PAHs are in their solid form, limiting sublimation from the solid to vapour phase. PAHs (e.g. naphthalene, fluorene, fluoranthene, pyrene) are often found in ambient air in homes, although this is more often a result of temperature increases associated with activities such as the use of heating appliances or cigarette smoking (van Winkle and Scheff, 2001).

A variety of environmental factors have been demonstrated to affect volatilisation of PAHs and consequently their fate in the environment. The presence of organic material and the growth of plants has been proposed as affecting the extent of volatilisation by enhancing adsorption of the PAH to organic material resulting in retardation of volatilisation. Alternately, root penetration resulting in soil fragentation can result in increased air flow, enhancing volatilisation (Ghenney *et al.*, 2004).

1.3.2 Photochemical Oxidation

PAHs can absorb solar radiation and undergo transformation. Specfic molecular groupings in the PAH molecule absorb infra-red electromagnetic radiation from ultra-violet and visible light causing increases in molecular rotation or vibration that may result in fragentation, oxidation or polymerisation (Connell *et al*., 1997). Partial oxidation of PAHs can occur through solar irradiation in the presence of gaseous or dissolved oxygen, although this tends to be limited to PAHs that are not angular or clustered (Kirso *et al.*, 1991). There is a paucity of research on photochemical oxidation of PAHs in environmentally relevant conditions, particularly soil, with most studies being undertaken with artificial light or in the presence of catalysts such as titanium oxide (TiO_2) (McConkey *et al.*, 2002).

Table 1.4: Suggested Half Lives of Polycyclic Aromatic Hydrocarbons in Various Environmental Compartments (IPCS, 1998)

(Half life classifications (1-8) were assigned to individual PAHs based on half life estimates in air, water, soil and sediment)

Class	Half-Life Mean (Hrs)	Range (Hrs)
1	17	10-30
2	55	30-100
3	170	100-300
4	550	300-1 000
5	1 700	1 000-3 000
6	5 500	3 000-10 000
7	17 000	10 000-30 000
8	55 000	> 30 000

Compound	Air	Water	Soil	Sediment
Acenaphthylene	2	4	6	7
Anthracene	2	4	6	7
Benz(a)anthracene	3	5	7	8
Benzo[*a*]pyrene	3	5	7	8
Benzo(*k*)fluoranthene	3	5	7	8
Chrysene	3	5	7	8
Dibenz[*a,h*]anthracene	3	5	7	8
Fluoranthene	3	5	7	8
Fluorene	2	4	6	7
Naphthalene	1	3	5	6
Perylene	3	5	7	8
Phenanthrene	2	4	6	7
Pyrene	3	5	7	8

The structure of the PAH has an impact on the potential for photochemical oxidation. In a study of the potential for photochemical oxidation in the atmosphere of structural isomers benzo[a]pyrene and benzo[e]pyrene, a significant difference in photochemical oxidation rate was observed. Benzo[e]pyrene was found not to photochemically oxidise whereas benzo[a]pyrene was found to photochemically oxidise (Takata and Sakata, 2002).

Photochemical oxidation of PAHs can be enhanced by a variety of environmental factors including pH, salinity, the presence of minerals such as smectite clay (Kong and Ferry, 2003), and temperature (Miller and Olejnik, 2001). Photochemical oxidation is considered an important process in the degradation of dissolved PAHs, for example those derived from oil spills in the ocean. Photochemical oxidation was considered an important process in PAH degradation following an oil spill in a mangrove swamp, particularly as the washing of PAH containing crude oil onto the shoreline and plant materials exposed the PAHs to sunlight (Ke *et al*., 2002).

1.3.3 Chemical Oxidation

Non-enzymatic or non-photochemical reactions are prominent in soil, but rarely result in the complete modification of a compound. Usually, slight modification occurs that results in a compound that is chemically similar to the original compound (Alexander, 1999). In soils, PAHs can be altered by any abiotic oxidants present, including metal ions (Mn and Fe) and clay minerals (including oxides and oxyhydroxides of Al, Fe, Mn and Si) (Gramss *et al.*, 1999).

1.3.4 Sedimentation

As the suggested half lives in Table 1.4 demonstrate, PAHs in sediments are more persistent than in other environmental compartments. Sediments act as a sink for deposited PAHs from industrial discharges or other emissions due to factors such as the sorption of PAHs to sediment organic material (adsorption to sediment minerals is prevented by the presence of water) and low diffusion of O_2 into organic rich sediments (Shiaris, 1989).

Sorption to sediment organic matter results in a portion of the PAHs in the sediment becoming highly refractory, resisting dissolution into the water column and microbial degradation, resulting in accumulation (Chiou *et al.*, 1998). The potential for deposition and accumulation in sediments can be predicted by the PAH's octanol-water coefficient (K_{ow}) and sediment characteristics such as organic matter content and sediment aromaticity (Arzayus *et al.*, 2001).

PAHs can accumulate in sediments in water either through gas flux or through the deposition of particles with sorbed PAHs. PAHs that enter water through gas flux tend to bind with particles of organic matter (e.g. plankton) due to their low solubility and are either deposited or ingested by other organisms and are deposited in fecal pellets. Through this process, the PAH may be cycled through the biota prior to deposition, remain suspended in the water column or accumulate in a higher organism (Arzayus *et al.*, 2001).

1.3.5 Bioaccumulation

The accumulation of PAHs in organisms is highly variable, depending on: the PAH; the environment of exposure; the organism's capacity to metabolically transform the PAH and the lipid content of the organism (in which the PAH may

accumulate). Those organisms that have little or no capacity to metabolically transform PAHs (e.g. molluscs) can accumulate high concentrations of PAHs whereas those with some metabolic capacity (e.g. fish) accumulate little or no PAHs (IPSC, 1998). The ability to metabolise PAHs can have a deleterious effect through the production of reactive metabolites resulting in enhanced toxicity. This has been proposed as a cause for the differential susceptibility to fluoranthene between amphipods and midges; with the midge (which possesses some metabolic capability to transform PAHs being more susceptible to fluoranthene, despite a lower body burden of fluoranthene (Schuler *et al.*, 2004).

Biomagnification through trophic transfer is similarly dependent on a capacity to metabolise the PAH taken up (Hofelt *et al.*, 2001). High molecular weight PAHs tend to have a higher potential to biomagnify through trophic transfers in the environment due to low water solubility and low reactivity resulting from their large size and angularity (Kanaly and Harayama, 2000). Phytoplankton are thought to play an important role in the fate and transport of persistent organic pollutants like PAHs and their consumption an important initial phase in bioaccumulation (Fan and Reinfelder, 2003).

A negative correlation between trophic level and PAH accumulation has been observed, suggesting an influence of metabolism in higher organisms. Accumulation of PAHs has been found to occur in organisms lower down in trophic levels. Crabs and lug worms in tidal flats were found to have a higher PAH burden that higher organisms such as squid and porpoises. It was proposed that the higher PAH levels were a product of these organisms' consumption of PAH contaminated sediments (Nakata *et al.*, 2003).

Uptake into plants through roots and leaves occurs in some instances, but generally PAH concentrations in plants are less than those in the soil in which they grow (Edwards, 1983).

1.4 MICROBIAL DEGRADATION

1.4.1 General Introduction

PAH degrading organisms are common members of the soil microbial population. In non-contaminated soils, fungi and bacteria capable of PAH degradation obtain the carbon and energy required for growth through the degradation of organic compounds (Atlas and Bartha, 1987). The pathways of microbial degradation tend to show broad substrate specificity and occur both aerobically and anaerobically (Harayama, 1997). Many of these organisms are lignin-degraders as lignin is the most abundant form of aromatic carbon in nature whose function is to protect polysaccharides in plants from enzymatic attack (Juhasz and Naidu, 2000). As a consequence, lignin degraders are widely distributed in nature and have been isolated from a number of sources including soil and the faeces of wood ingesting animals. The degradation of lignin is achieved via the production of enzymes that are relatively non-specific and can be exploited for the degradation of some organic pollutants (Juhasz and Naidu, 2000).

The alteration of contaminants in the environment via biodegradation tends to occur through four general mechanisms:

- Intra/Extracellular Enzyme Attack - where the contaminant may be used as a source of carbon and energy;
- Enzymatic Attack as a Protective Measure - where the contaminant is altered in a manner that allows it to be mobilised and removed from the cell's vicinity or detoxified (e.g. O-methylation);
- Enzymatic Attack for No Detectable Benefit - also known as cometabolism, where the contaminant is degraded as a incidental result of another cell process; and;

- Non-Enzymatic Attack – Achieves degradation as a result of another process. For example, a metabolic process producing a change in the environment such as the consumption of oxygen or the production of fermentation by-products altering the soil pH (Madsen, 1997)

The first and third mechanisms are the predominant routes by which fungi and bacteria achieve the degradation of PAHs. This discussion will focus predominantly on the degradation of PAHs in soil by bacteria and fungi, although algal degradation of PAHs has been reported (Figure 1.5). Algal species including *Chlamydomonas* spp., *Dunaliella* sp. and *Scenedesmus obliquus* have been reported as being able to biotransform naphthalene and *Selenastrum capricornutum* has been reported as being able to bioaccumulate naphthalene, phenanthrene and pyrene and biotransform benzo[*a*]pyrene (Semple *et al*., 1999).

This thesis predominantly focuses upon the microbial degradation of five PAHs – fluorene, phenanthrene, fluoranthene, pyrene and benzo[*a*]pyrene. The discussion will consist of a general discussion about the mechanisms of PAH degradation for both bacteria and fungi, then provide further detail on the degradation of each individual PAH. The five PAHs were selected on the basis of their classification by the USEPA as priority pollutants, their ubiquity at contaminated sites and the ranges of molecular mass they represent.

1.4.2 Bacterial Degradation of PAHs

Numerous bacterial species capable of degrading a variety of PAHs have been isolated (Table 1.5). The ability of bacteria to degrade PAHs is usually determined by i) ability of bacteria to transport the PAH into the cell (dependent largely on molecular size and water solubility), ii) the PAH being a suitable substrate for the available enzymes and iii) the PAH being an inducer for the appropriate transport system or degradative enzymes (Juhasz and Naidu, 2000a).

Bacterial degradation predominantly occurs via aerobic oxygenase mediated pathways; although an alternative pathway of degradation involving utilisation of nitrate as the alternative electron acceptor has been reported (Eriksson *et al.*, 2003). In the latter process, oxidised forms of nitrogen are reduced and organic carbon from soil material or contaminants is oxidised (Pothuluri and Cerniglia, 1998).

There has been limited investigation into microaerobic and anaerobic degradation of PAHs. Anaerobic degradation has been suggested as occurring in a similar manner to aerobic degradation, namely modification into intermediates followed by ring cleavage and the creation of metabolites for cell function (Holliger and Zehnder, 1996).

1.4.2.1 Oxygenase Enzyme Systems

A variety of oxygenase systems are used by both fungi and bacteria to achieve the degradation of PAHs. Generally ring oxidation reactions are mediated by dioxygenases in bacteria and monoxygenases in eukaryotes. Some organisms have demonstrated utilisation of both monoxygenases and dioxygenases for the degradation of PAHs, in particular *Mycobacterium* sp. in experiments using radiolabelled $^{18}O_2$ and pyrene (Cerniglia and Heitkamp, 1990).

The initial step in PAH degradation by the oxygenase enzyme system is aerobic catabolism via oxidation that incorporates two oxygen atoms into the aromatic nucleus by dioxygenase to form a dihydrodiol. This initial oxidation is considered to be the rate limiting step of a multicompetent enzyme system. This is followed by either *ortho* or *meta* cleavage of the dihydroxylated intermediates resulting in the formation of protocatechuates and catechols which are further oxygenated to tricarboxylic acid intermediates (such as succinic, fumaric, pyruvic and acetic acids and aldehydes) for use in the synthesis of cellular components

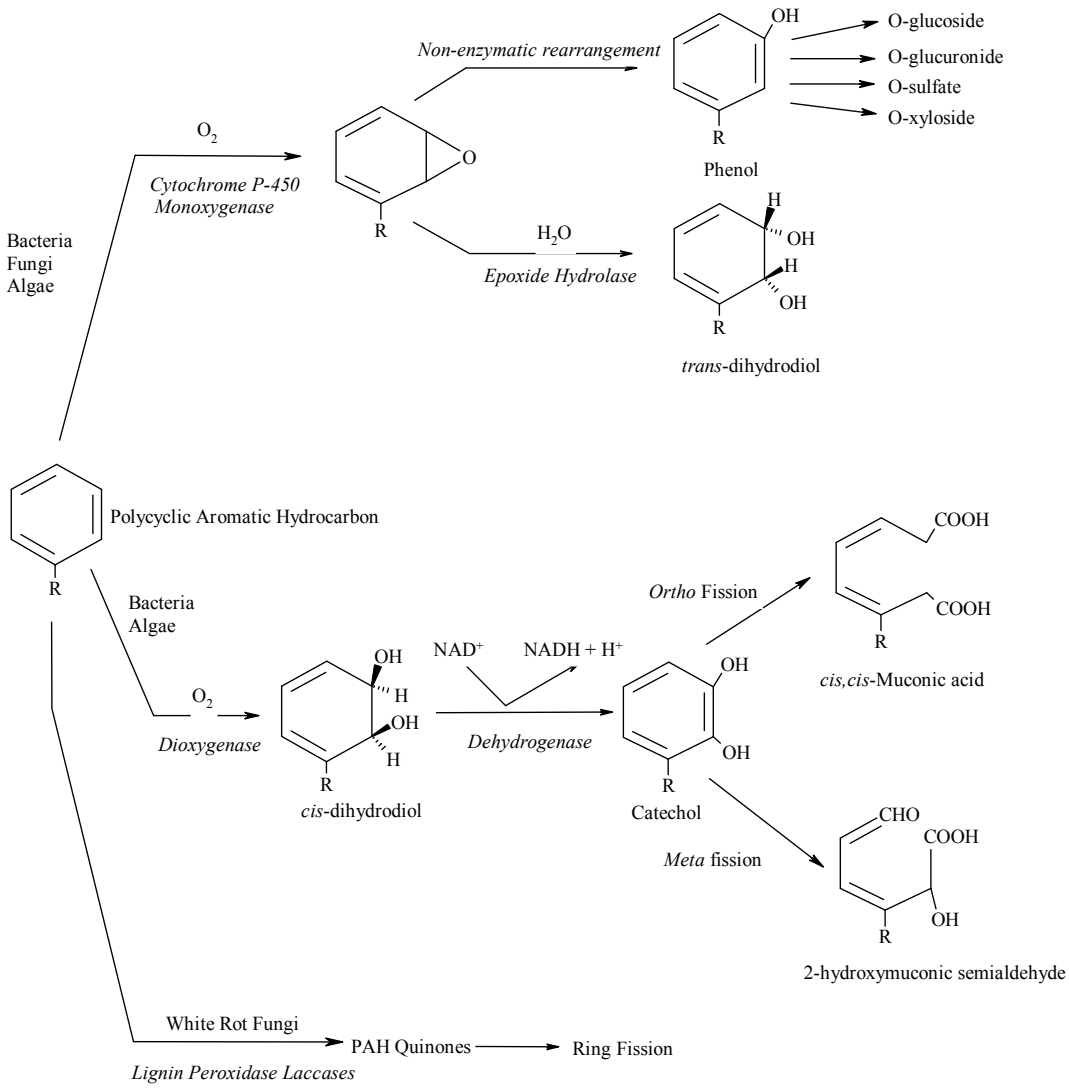


Figure 1.5: Various Mechanisms of Microbial Polycyclic Aromatic Hydrocarbon Degradation (Atlas and Cerniglia, 1995)

Table 1.5. Polycyclic Aromatic Hydrocarbons Oxidised by Different Species/Strains of Bacteria (adapted from Cerniglia, 1992, Juhasz and Naidu, 2000 and UMBBD, 2004).

Organisms	Reference
Fluorene	
Terrabactersp., Staphylococcus auriculans, Arthrobacter, sp., *Mycobacterium gilvum, Sphingomonas aromaticivorans, Sphingomonas* sp. LB216, *Leclercia adecarboxylata*	Trenz *et al.* (1994), Monna *et al.* (1993), Grifoll *et al.* (1992), Boldrin *et al.* (1993), Gaasterland *et al* .(1999), Casellas *et al.* (1997), Bastiens *et al,* 2001, Sarma *et al*. (2004)
Phenanthrene	
Aeromonas sp., A. faecalis, A. denitrificans, Arthrobacter polychromogenes, Beijernickia sp., Micrococcus sp., Mycobacterium sp., P. putida, Sp. paucimobilis, Rhodococcus sp, Vibrio sp., Nocardia sp., Flavobacterium sp., Streptomyces sp., S. griseus, Acinetobacter sp., P. aeruginosa, P. stutzeri, P. saccharophila, Stenotrophomonas maltophilia, Cycloclasticus sp., P. fluorescens, Acinetobacter calcoaceticus, Acidovorax delafieldii, Gordona sp., Sphingomonas sp., Comamonas testosteroni, Cycloclasticus pugetii, , Sp. yanoikuyae, Agrobacterium sp., Bacillus sp., Burkholderia sp., Sphingomonas sp., Pseudomonas sp., Rhodotorula glutinis, Nocardioides sp., Flavobacterium gondwanense, Halomonas meridiana, Burkholderia sp., Mycobacterium sp Strain PYR-1, Janibacter sp. Mycobacterium sp Strain RJGII-135, Sphingomonas sp Strain ZL5, Microbacterium sp and Paracoccus sp, Pichia anomala	Kiyohara *et al.* (1976, 1982, 1990), Weissenfels *et al.* (1990, 1991), Keuth and Rehm (1991), Jerina *et al.* (1976), Colla *et al.* (1959), West *et al.* (1984), Kiyohara and Nagao (1978), Heitkamp and Cerniglia (1988), Guerin and Jones (1988a, 1989), Treccani *et al.* (1954), Evans *et al.* (1965), Foght and Westlake (1988), Mueller *et al.* (1990b), Sutherland *et al.* (1990), Ghosh and Mishra (1983), Savino and Lollina (1977), Trower *et al.* (1988), Barnsley (1983b), Yang *et al.* (1994), Kohler *et al.* (1994), Stringfellow and Aitken (1995), Boonchan (1998), Juhasz (1998), Geiselbrecht *et al.* (1998), Foght and Westlake (1996), Kastner *et al.* (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Goyal and Zylstra (1996), Dyksterhouse *et al.* (1995), Allen *et al.* (1999), Aitken *et al.* (1998), Romero *et al.* (1998), Iwabuchi *et al.* (1998), Churchill *et al.* (1999), Juhasz (1991), Laurie and Lloyd-Jones (1999), Moody *et al.* (2001), Yamazoe *et al.* (2004), Miyata *et al.* (2004), Liu *et al.* (2004), Zhang *et al.* (2004), Pan *et al*. (2004)

Table 1.5 (cont.). Polycyclic Aromatic Hydrocarbons Oxidised by Different Species/Strains of Bacteria (adapted from Cerniglia, 1992, Juhasz and Naidu, 2000 and UMBBD, 2004).

Organisms	Reference
Fluoranthene	
A. denitrificans, Mycobacterium sp., P. putida, Sp. paucimobilis, Bu. cepacia, Rhodococcus sp., Pseudomonas sp., Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Acidovorax delafieldii, Gordona sp., Sphingomonas sp., P. saccharophilia, Pasteurella sp., *Bacillus cereus, Mycobacterium gilvum, Sphingomonas yanoikuyae, Pseudomonas stutzeri, Mycobacterium austroafricanum, Leclercia adecarboxylata*	Kelly and Cerniglia (1991), Walter *et al.* (1991), Weissenfels *et al.* (1991), Foght and Westlake (1988), Barnsley (1975b), Mueller *et al.* (1990a, 1990b), Ye *et al.* (1996), Kelley *et al.* (1993), Boonchan (1998), Juhasz (1998), Lal and Khanna (1996), Shuttleworth and Cerniglia (1996), Mahro *et al.* (1995), Willumsen and Karlson (1998), Sepic *et al.* (1998), Willumsen *et al.* (1998), Churchill *et al.* (1999), Chen and Aitken (1999), Sepic *et al.* (1998), Kazunga *et al.* (2001), Boldrin *et al.* (1993), Kazunga *et al.* (2001), Bogan *et al.* (2003), Sarma *et al*. (2004)
Pyrene	
A. denitrificans, Mycobacterium sp., Rhodococcus sp. , Sp. paucimobilis, Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., P. putida, Bu cepacia, P. saccharophilia, Mycobacterium gilvum, Leclercia adecarboxylata, Mycobacterium pyrenivorans	Heitkamp *et al.* (1988a), Walter *et al.* (1991), Weissenfels *et al.* (1991), Grosser *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996), Boonchan (1998), Juhasz (1998), Kastner *et al.* (1998), Lal and Khanna (1996), Mahro *et al.* (1995), McNally *et al.* (1999), Jimenez and Bartha (1996), Churchill *et al.* (1999), Juhasz *et al.* (1997), Chen and Aitken (1999), Boldrin *et al.* (1993), Sarma *et al*. (2004), Derz *et al.* (2004)
Benzo[*a*]pyrene	
Sp. paucimobilis, Beijerinckia sp., *Mycobacterium sp, Rhodococcus sp. UW1, Pseudomonas sp, Agrobacterium sp., Bacillus sp., Burkholderia cepacia, Stenotrophomonas maltophilia*	Ye *et al.* (1996), Gibson *et al.* (1975), Schneider *et al.* (1996)*, Walter *et al.* (1991), Aitken *et al.* (1998), Juhasz *et al.* (1997), Boonchan *et al.* (2000)*

** = Recorded as a substrate in the University of Minnesota – Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu/index.html)*

and energy. Complete mineralisation of the PAH results in the production of CO_2 and water (Wilson and Jones, 1993).

The degradation of PAHs via the dioxygenase pathway has been suggested by numerous studies using radiolabelled compounds and is indicated by the formation of dihydrodiols. In a study into the degradation of pyrene, a *Mycobacterium* species was found to convert ^{14}C-pyrene into 3 metabolites, 4,5-phenanthrene dicarboxylic acid, 4-phenanthrene carboxylic acid and 4,5-pyrene dihydrodiol (Schneider *et al*., 1996). Similarly, microorganisms able to degrade phenanthrene and pyrene isolated from PAH contaminated sediment and seawater produced pyrene 4,5 dihydrodiol also suggesting a dioxygenase metabolic pathway (Cullen *et al.*, 1994). In a study conducted into the degradation of phenanthrene and anthracene by *Mycobacterium* PYR-1, a novel anthracene ring fission product was isolated. This suggested the presence of different dioxygenases or a related specificity of the same dioxygenase for the initial attack on a PAH (Moody *et al,* 2001). A study of the degradation of 4-ring PAHs by pure cultures of a *Mycobacterium* sp. using $^{18}O_2$ evolution found that dioxygenases and monoxygenase enzymes catalysed the formation of dihydrodiols. HPLC analysis of the metabolites showed one major metabolite (identified as a ring fission product) and six other metabolites that accounted for 95% of the organic extractable ^{14}C residues (Heitkamp *et al*, 1988).

Whilst having broad substrate specificity, the oxygenase pathways tend to degrade compounds in order of increasing complexity. An examination of the naphthalene degrading enzyme (*nah*) system of *Pseudomonas fluoroscens* SR and other bacterial isolates found that all organisms displayed similar patterns of preferential degradation using naphthalene dioxygenase when degrading mixtures of PAHs. The greater the respective PAH differed from the naphthalene base structure, the slower the degradation rate accompanied by an increase in methyl substituents and size of the substituents (Leblond *et al.*, 2001). A similar

phenomenon has been proposed to influence the pathway of enzymatic breakdown of pyrene and benzo[*a*]pyrene (Grosser *et al.*, 1991).

NAH-A gene abundance has been found to correspond with soil PAH contamination levels. In their 1999 study, Ahn *et al* (1999) isolated 141 PAH degrading bacteria and determined phenotype by spray plate technique and the genotype by the presence of *nah*7 genes. Of the 141 isolates, 59 were randomly selected and examined for a capability to mineralise PAHs using ^{14}C - radiolabelled PAHs. 109 of the isolates were dioxygenase positive, while 87 of the 89 degraders identified by spray plate were dixoygenase positive. Two phenanthrene degraders showed no dioxygenase activity, suggesting either low or no dioxygenase activity (Ahn *et al.*, 1999). Results such as these indicate the variability of metabolic pathways to achieve degradation between organisms. These findings compound the difficulty in identifying an organism suitable for use in bioremediation - particularly where the degradative pathway may be other than that expected resulting in the generation of different and potentially more toxic metabolites.

An additional consideration is the potential for the degradative capability of the organism to be non-constitutive, (i.e. related to an extracellular mechanism such as a plasmid). Whilst numerous studies support the use of oxygenase mediated pathways, some have suggested that the ability of some bacteria to degrade PAHs may be mediated by unstable cellular mechanisms. A study into the degradative capacity of a *Micrococcus sp.* from petroliferous soil found an ability to degrade phenanthrene mainly through protocatechuate pathway evidenced by O_2 uptake and enzyme studies. *Micrococcus sp*. has been demonstrated to use naphthalene and anthracene without a lag period, suggesting that catechol-2,3 oxygenase may have a role as a constitutive enzyme. This result is to be viewed with caution as another investigation has suggested that in this particular species, the mechanism of biodegradation may be controlled by an extrachromosomal mechanism such as a plasmid. This phenomenon was suggested by the observed loss of

phenanthrene and naphthalene assimilating capability following repeated sub-culture (Ghosh and Mishra, 1983). This loss of degradative capability has also been demonstrated in a study where a strain isolated from soil lost the ability to degrade pyrene following extraction from the matrix (Kastner *et al.*, 1998).

There is a potential for PAHs to be activated during degradation to intermediates that have been identified as carcinogenic, mutagenic and teratogenic and more soluble than parent compounds. Consequently, the complete destruction of the target compound through mineralisation is considered the most desirable endpoint of bioremediation (Wilson and Jones, 1993). The ability to mineralise the compound to prevent the formation of toxic water soluble metabolites and grow on PAHs as a sole carbon source is important in bioremediation as it ensures the survival and dominance of the degrading organism (Stanley *et al.*, 1999). Mineralisation of the target compound is viewed as the ultimate objective of biodegradation as it results in complete removal rather than the potential generation of metabolites that pose another health or ecological risk.

Whilst complete mineralisation is the ideal for the degradation of high molecular weight PAHs, it is often not feasible. Incomplete degradation may not necessarily result in the formation of toxic intermediates. Ye *et al.* (1996) suggested an ability of a resting cell suspension of *Sphingomonas paucimobilis* to metabolise a variety of high molecular weight PAHs when grown on fluoranthene. The cell suspension supported the following destruction efficiencies for PAHs (all supplied at 10 μg/ml): 80% pyrene; 72.9% benz[*a*]anthracene; 31.5% chrysene; 33.3% benzo[*a*]pyrene; 12.5% benzo[*b*]fluoranthene and 7.8% dibenz[*a,h*]anthracene after 16 hours incubation. Degradation was found to increase with increased cell density. Benzo[*a*]pyrene was degraded to highly polar metabolites accompanied by a significant decrease in benzo[*a*]pyrene mutagenicity (Ye *et al.*, 1996).

1.4.2.2 Cometabolism and PAH Degradation

To date, bacteria have not been isolated with the ability to utilise benzo[*a*]pyrene as a sole carbon and energy source (Juhasz and Naidu, 2000a). Failure to isolate bacteria than can grow on 5-ring PAHs as a sole source of carbon and energy has led researchers to believe that such compounds are only degraded cometabolically (Stanley *et al.*, 1999). Benzo[*a*]pyrene degradation (transformation) has been reported to occur under cometabolic conditions where low molecular weight PAH (Molina *et al*., 1999), diesel fuel (Kanaly and Harayama, 2000), yeast extract (Schneider *et al.*, 1996) or salicylic acid were supplied as the carbon source.

Molecular structure also appears to have an impact on the potential for cometabolic degradation. An investigation into degradation of PAHs by *Sphingomonas paucimobilis* observed that the degradation rate for *benzo[a]pyrene* (consisting of five benzene rings) was the same as chrysene (consisting of four benzene rings), suggesting that ring number is not the only degradation rate determinant, as more clustered PAHs appear to be more susceptible to biodegradation than less clustered (this conclusion was derived from a comparison of benzo[*a*]pyrene to dibenz[*a,h*]anthracene). This result also indicates that a PAH's water solubility may have a variety of impacts on degradation as in this instance, the PAH of lesser solubility degraded faster than more highly soluble compounds (Ye *et al.*,1996).

1.4.2.3 Bacterial Degradation of Fluorene

Fluorene degradation by bacteria has been reported in a number of studies and it has been demonstrated to be utilised as a sole carbon and energy source and to be degraded cometabolically (Boldrin *et al.*, 1993). Fluorene may be mineralised or

metabolites (particularly 1-fluorenol, 2-fluorenone and 1-indanone) may accumulate as a result of transformation processes (Casellas *et al.*, 1998). Bacterial isolates capable of degrading fluorene are listed in Table 1.4 and include organisms from the genera *Sphingomonas* (Baastiens *et al.*, 2001), *Arthrobacter* (Casellas *et al.*, 1998), *Staphylococcus* (Monna *et al.*, 1993), *Pseudomonas* (Resnick and Gibson, 1996) and *Pseudomonas* (Grifoll *et al.*, 1995).

A number of pathways have been reported for fluorene degradation. The fluorene degradation pathway of *Pseudomonas cepacia* F297 has been reported as analogus to the degradation of naphthalene resulting in the production of phthalate. The degradation of fluorene involves aromatic ring dioxygenation, ring cleavage and the release of pyruvate. The pyruvate released was proposed as supporting growth of *Pseudomonas cepacia* F297 (Grifoll *et al.*, 1995).

Arthrobacter sp Strain F101 was proposed to degrade fluorene through three independent pathways. Two routes involved dioxygenation and meta cleavage to ultimately form salicylate for use in central metabolism while the other route involved monoxygenation at the C-9 position to form 9-fluorenone (Figure 1.6). Dioxygenation of the ketone formed was proposed, although no further oxidation was reported (Casellas *et al.*, 1997). A similar phenomenon of the generation of 9-fluorenone without further oxidation has also been observed in *Mycobacterium* sp. (Boldrin *et al.*, 1993).

1.4.2.4 Bacterial Degradation of Phenanthrene

Phenanthrene is often used as a model PAH compound for bacterial degradation studies as it is the smallest PAH molecule to possess a bay region and hence is useful for the study of the metabolism of carcinogenic PAH molecules (UMBBD, 2004). Numerous bacteria (Table 1.4) have been reported as possessing a

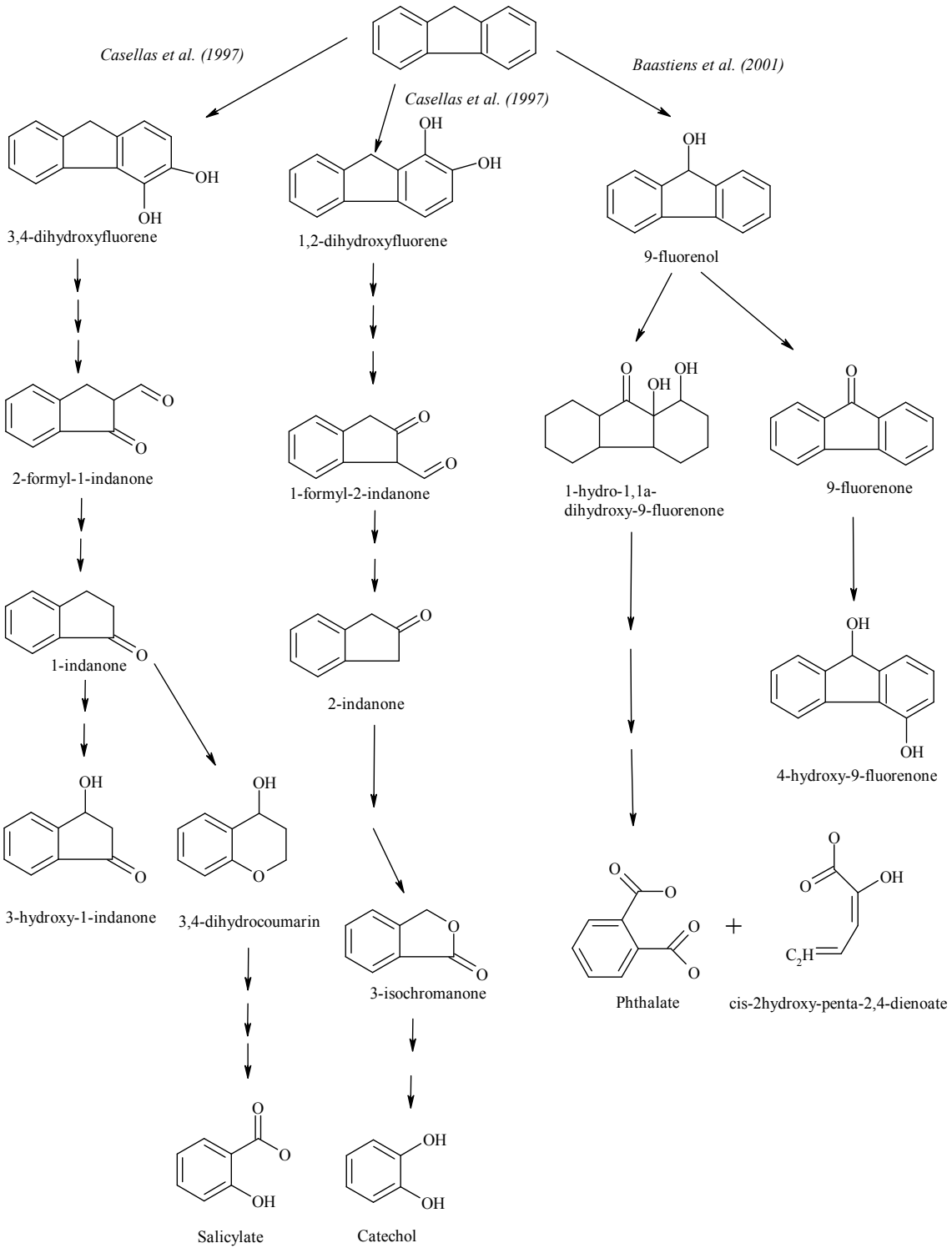


Figure 1.6: Pathways of Bacterial Fluorene Degradation (UMBBD, 2004)

capability for degrading phenanthrene, using it as a sole carbon and energy source and through cometabolism (Tongpim and Pickard, 1999).

A variety of sites on the phenanthrene molecule are subject to initial enzymatic attack depending of the bacterial species. *Pseudomonas sp*. and *Beijerinckia sp*. have been shown to oxidise phenanthrene at carbon positions 1,2 and 3,4 to form phenanthrene *cis*-1,2-dihydrodiol and phenanthrene *cis*-3,4-dihydrodiol. *Mycobacterium sp*. and *Streptomyces sp*. have been shown to initiate the enzymatic attack at positions 9 and 10, forming phenanthrene *trans*-9,10-dihydrodiol (Tongpim and Pickard, 1999).

Bacterial degradation of phenathrene has been reported as proceeding through a variety of pathways (Figure 1.7). These include via salicylate and catechol (the salicylate pathway), a naphthalene-like pathway indicating that many bacteria capable of degrading phenathrene can also degrade naphthalene, although this is not always the case. Using this pathway, phenanthrene is metabolised to salicylate which if further metabolised to catechol. Catechol is further degraded via *ortho* (using catechol-2,3-dioxygenase) or *meta* cleavage (catechol-1,2-dioxygenase), depending on the species of bacteria (Liu *et al.*, 2004).

Those bacteria not able to degrade naphthalene as well as phenanthrene tend to degrade phenanthrene via a protocatechuate (the protocatechuate pathway) (Ghosh and Mishra, 1983). Phenanthrene is converted to protocatechuate which is converted to 2-carboxy-*cis*, *cis*-muconate by protocatechuate 3,4-dioxygenase or 4-carboxy-2-hydroxymuconate semialdehyde by protocatechuate 4,5-dioxygenase (Liu *et al.*, 2004).

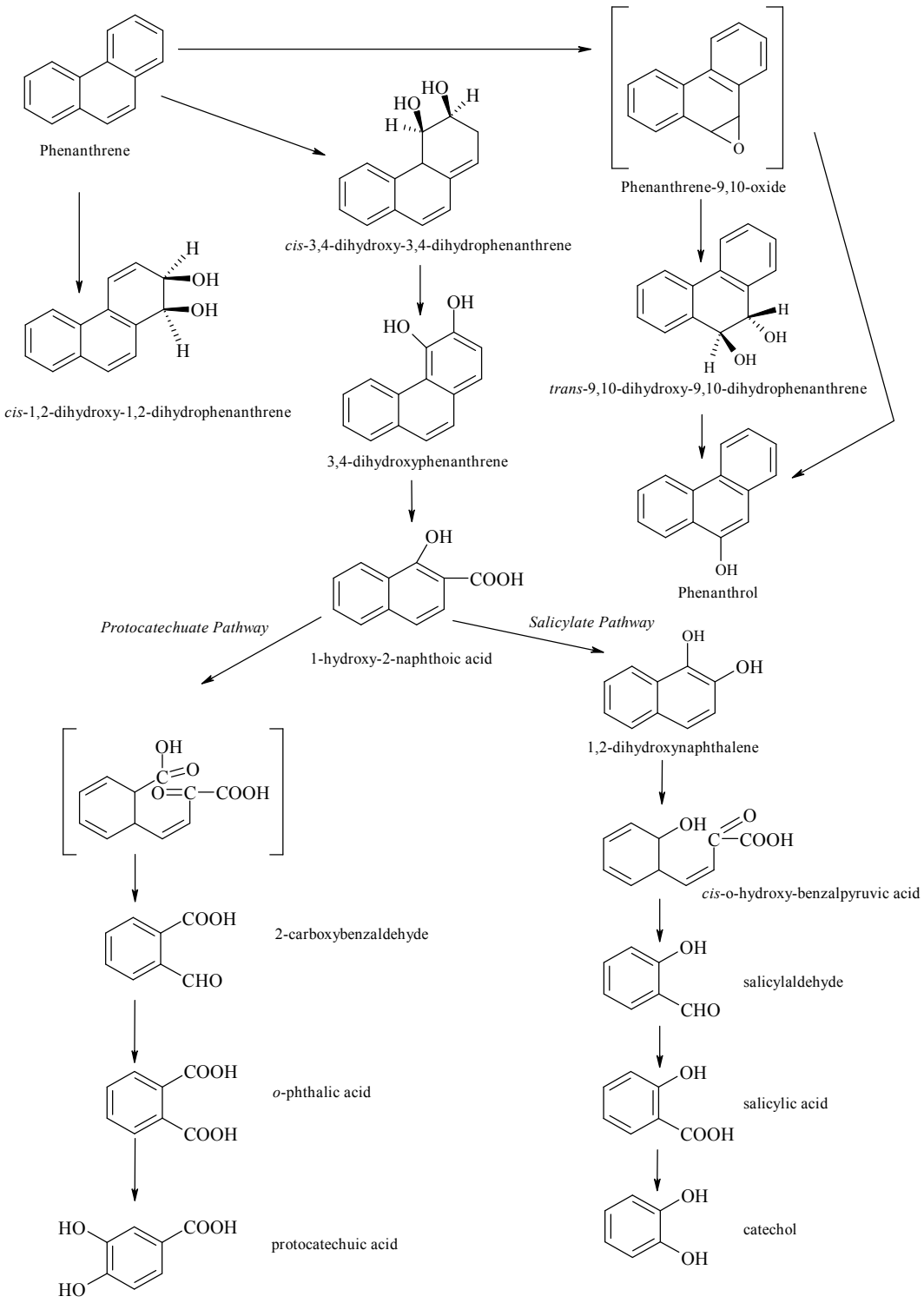


Figure 1.7: Pathways of Bacterial Phenanthrene Degradation (*adapted from Juhasz, 1997*)

1.4.2.5 Bacterial Degradation of Fluoranthene

A number of pathways have been suggested for the bacterial degradation of fluoranthene. In *Mycobacterium* sp Strain PYR-1, the molecule is initially hydroxylated in a dioxygenase mediated reaction in positions C-1 and C-2 forming an intermediate which is subject to *meta* cleavage resulting in the formation of metabolites including 9-fluorenone-1-carboxylic acid (the predominant metabolite), 9-fluorenone, phthalic and benzoic acid (Figure 1.8). The ability of *Mycobacterium* sp Strain PYR-1 to utilise these metabolites resulted in the mineralisation of fluoranthene (Kelley *et al.*, 1993). Mineralisation of fluoranthene has also be proposed to occur in *Alcaligines denitrificans* in a similar manner via the production of 8-hydroxy-7-methoxyfluoranthene as a result of dihydroxylation at positions C-7,8 and 9,10 (Weissenfels *et al.*, 1991).

An alternative pathway for the mineralisation of fluoranthene was proposed in earlier work by Kelly et al. (1991) again using *Mycobacterium* sp. The dioxygenase initiates its enzymatic attack on positions C1,2 or 2,3, forming a dihydroxylated fluoranthene intermediate, which is further oxidised at the C-1,2 or 2,3 positions. Further metabolism results in the formation of 9-fluorenone-1-carboxylic acid. 9-fluorenon-1-carboxylic acid is easily metabolised and hence does not accumulate (Kelly *et al.*, 1991).

1.4.2.6 Bacterial Degradation of Pyrene

Degradation of pyrene has been reported for a number of bacterial isolates listed in Table 1.4. Pyrene degradation has been reported as occurring as a product of cometabolism and as a sole carbon and energy source (Saraswathy and Hallberg, 2002; Juhasz *et al.*, 2000; Boonchan *et al.*, 1998; Boldrin et al., 1993: Heitkamp and Cerniglia, 1988). A number of researchers have reported a metabolic

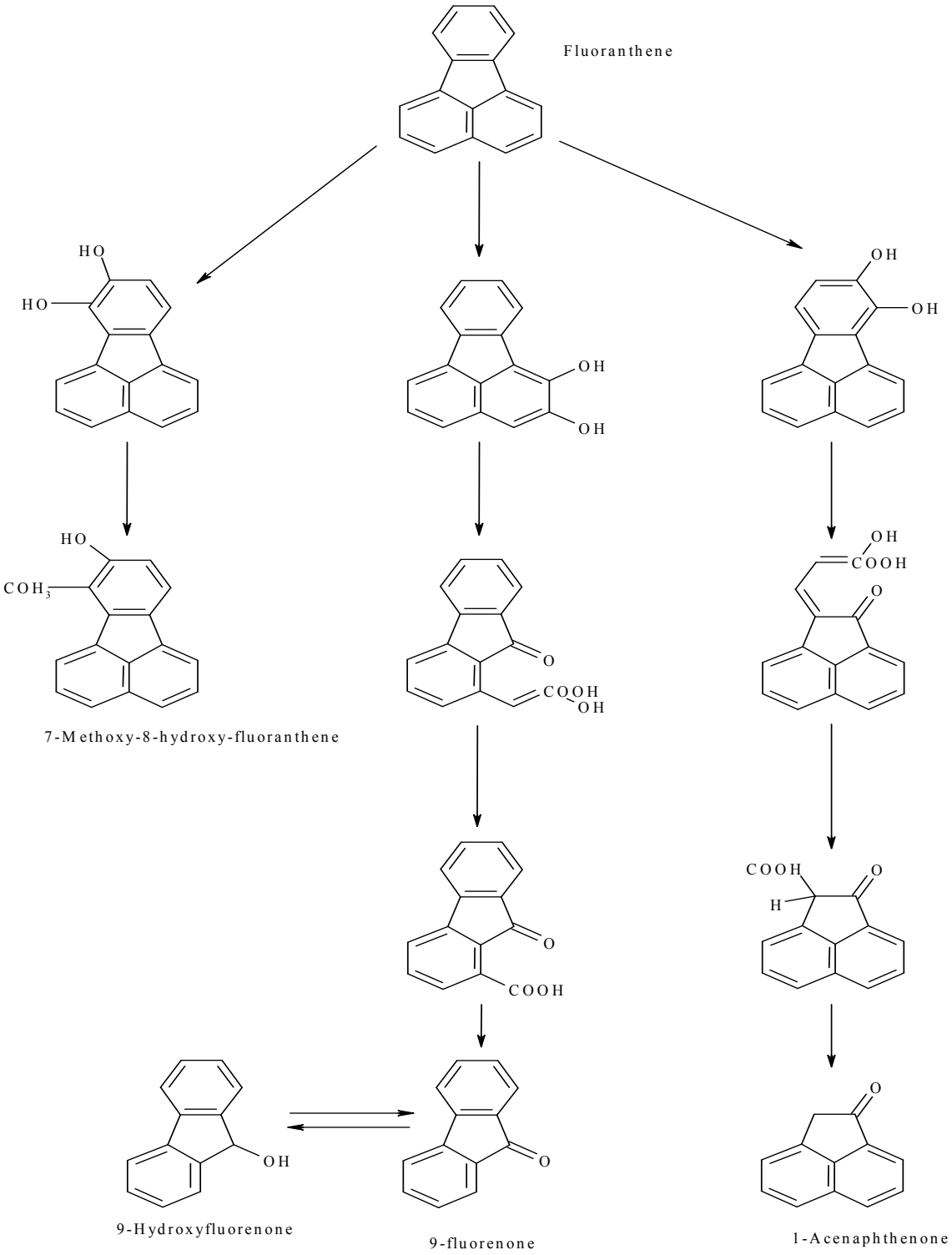


Figure 1.8: Pathways of Bacterial Fluoranthene Degradation (*adapted from Juhasz, 1997*)

similarity between the utilisation of pyrene and phenanthrene, including the ability to degrade both phenanthrene and pyrene concurrently (Molina *et al.*, 1999). Mineralisation of pyrene has been reported for some bacterial species (e.g. Heitkamp *et al.*, 1988) and the accumulation of metabolites has been reported in others (e.g. Kazunga and Aitken, 2000).

Multiple pathways for the initial ring oxidation of pyrene by *Mycobacterium* sp. have been reported. Pyrene degradation in bacteria has been reported as predominantly occurring through oxidation mediated by dioxygenase at the 4,5 position or "K-region" resulting in the formation of *cis*-4,5-dihydrodiols. The formation of *trans* and *cis*-4,5-dihydrodiols suggested the involvement of both monoxygenase and dioxygenase respectively. These degradation products are further metabolised to produce ring fission products such as 4-hydroxyperinaphthenone, 4-phenanthroic acid, phthalic acid and cinnamic acid (Figure 1.9) (Heitkamp *et al.*, 1988).

The production and accumulation of "dead end" metabolites has been shown to have the potential to accumulate in PAH contaminated systems and influence the removal of other PAHs. This has been demonstrated in studies using a variety of strains that produce the same metabolites but each having differing abilities to further degrade them. To illustrate, the study of Kazunga and Aitken (2000) demonstrated that *Pseudomonas stutzeri* strain P16 and *Bacillus cereus* strain P21 were capable of transforming pyrene primarily to *cis*-4,5-dihydro-4,5-dihydroxypyrene (PYRdHD), the first intermediate in the known pathway for the aerobic bacterial mineralisation of pyrene. *Sphingomonas yanoikuyae* strain R1 transformed pyrene to PYRdHD and pyrene-4,5-dione (PYRQ) and *Mycobacterium* PYR1 also transformed PYRdHD to PYRQ, but was able to use both as growth substrates.

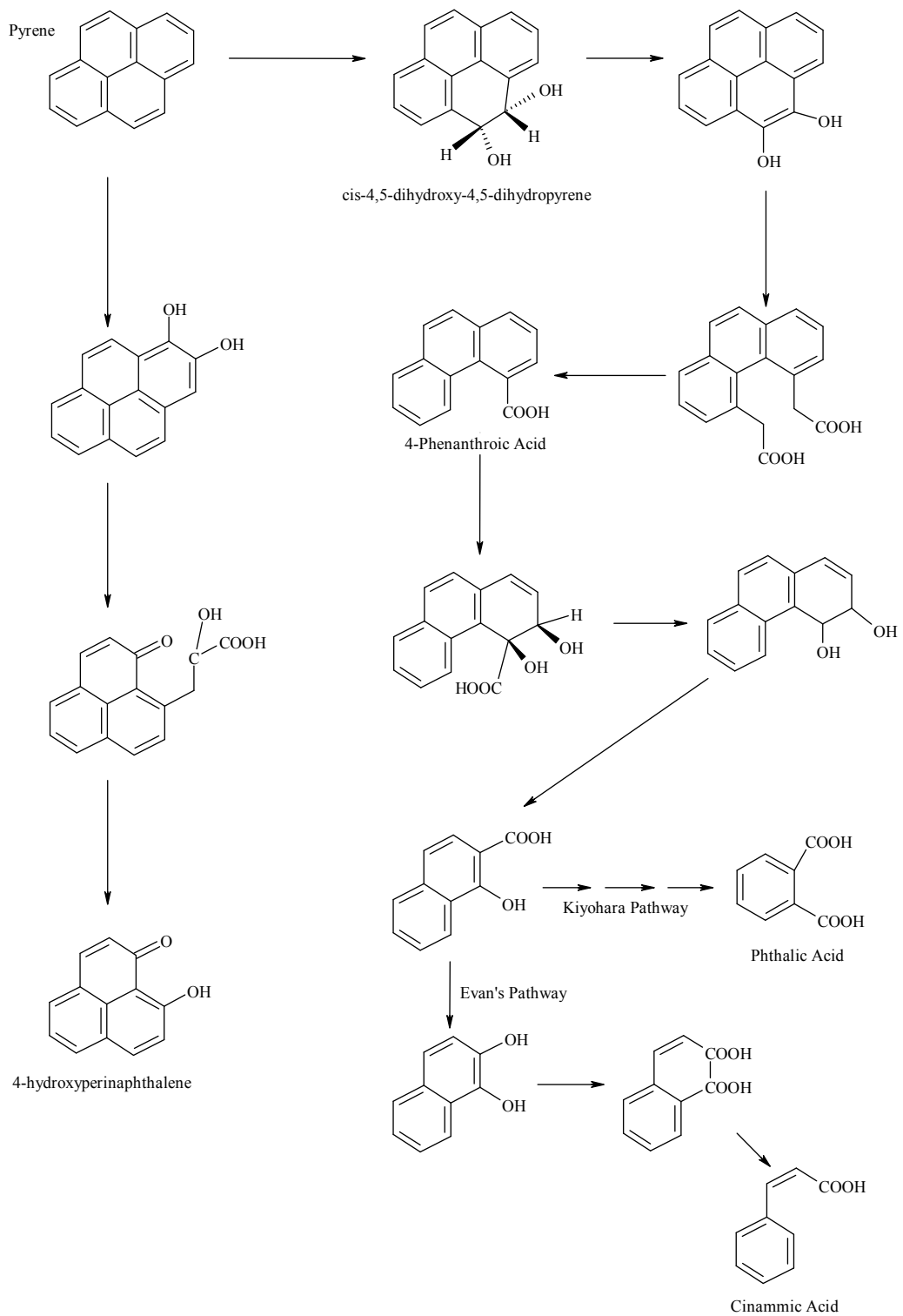


Figure 1.9: Bacterial Degradation of Pyrene by *Mycobacterium* sp. Strain PYR-1 (Cerniglia, 1992).

1.4.2.7 Bacterial Degradation of Benzo[a]pyrene

Bacterial degradation of benzo[*a*]pyrene has only been reported in a few instances by both single organisms (Table 1.4) and by bacterial consortia (Juhasz *et al.*, 2000, Boonchan *et al.*, 1998 Schneider *et al.*, 1996 and Kanaly *et al.*, 2000). The ability of bacteria able to use benzo[*a*]pyrene as a sole growth substrate has not been demonstated and benzo[*a*]pyrene degradation by bacteria has always occurred in the presence of other growth substrates (Juhasz and Naidu, 2000). Degradation of benzo[*a*]pyrene by *Beijerinckia* sp. (Gibson *et al.*, 1975), *Mycobacterium* sp (Schneider *et al.*, 1996; Moody *et al.*, 2004) and *Stenotrophomonas maltophilia* (Boonchan *et al.*, 2000 and Juhasz et al., 1998) has been reported.

Two pathways have been suggested for the degradation of benzo[*a*]pyrene by *Mycobacterium* sp. involving dioxygenase. This involves initial oxidation of the benzo[*a*]pyrene molecule forming either the *cis*-4,5-BaP-dihydrodiol, *cis*-9,10-dihydrodiol or the *cis*-7,8-dihydrodiol. These dihydrdiols undergo either ortho fission to form 4,5-chyrsene-carboxylic acid or meta fission to form *cis*-4-(8-hydroxypyren-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid finally forming the polycyclic aromatic acids 7,8-dihydro-pyrene-7-carboxylic acid or 7,8-dihydro-pyrene-8-carboxylic acid (Figure 1.10) (Schneider *et al.*, 1996).

Mycobacterium vanbaalenii PYR-1 grown in the presence of yeast extract, peptone and starch has been shown to metabolise benzo[*a*]pyrene using mono- and dioxygenases (indicated by the formation of *cis*- and *trans*- isomers) which intiate enzymatic attack at the C-4,5, C-9,10 and C-11,12 positions. The major intermediates formed were cis-4,5-dihydroxybenzo[*a*]pyrene (benzo[*a*]pyrene 4,5-dihydrodiol), *cis*-11,12-dihydroxybenzo[*a*]pyrene (benzo[*a*]pyrene *cis*-11,12-dihydrodiol), *trans*-11,12-dihydro-11,12-dihydroxybenzo[*a*]pyrene

(benzo[*a*]pyrene *trans*-11,12-dihydrodiol), 10-oxabenzo-[*def*]chrysen-9-one and hydroxymethoxy and dimethoxy derivatives of benzo[*a*]pyrene. The formation of trans-11,12-dihydrodiol suggested the involvement of Cytochrome P-450 and epoxide hydrolase (Moody *et al.*, 2004).

It has been reported that the metabolites from benzo[*a*]pyrene degradation may inhibit benzo[*a*]pyrene's further degradation, making them "dead end" metabolites. In a study using *Stenotrophomonas maltophilia* VUN 10,003, high cell number inoculations were able to achieve degradation of 10-15 mg L^{-1} [7-^{14}C] benzo[*a*]pyrene after a 21-28 day lag period, although degradation was observed to rapidly decrease after 56 days, despite a viable population being present. Most of the [7-^{14}C] benzo[*a*]pyrene (83%) was recovered as undegraded substrate or nonpolar extractable metabolites, indicating transformation rather than mineralisation. The use of medium containing polar and non-polar benzo[*a*]pyrene metabolites was found to inhibit degradation (Juhasz *et al.*, 2002).

Metabolite repression has also been shown to occur during the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by *Stenotrophomonas maltophilia*. Using a high cell density inoculum, *Stenotrophomonas maltophilia* was found to be able to degrade 10-15 mg/l of benzo[*a*]pyrene and dibenz[*a,h*]anthracene after 63 days (following a 21-28 day lag period). Degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene was observed to cease after 56 days and metabolite repression was proposed as being responsible for this cessation of degradation. This proposition was supported by the inhibition of benzo[*a*]pyrene degradation being observed in further experiments using spent medium from previous benzo[*a*]pyrene degradation experiments (Juhasz *et al.*, 2002).

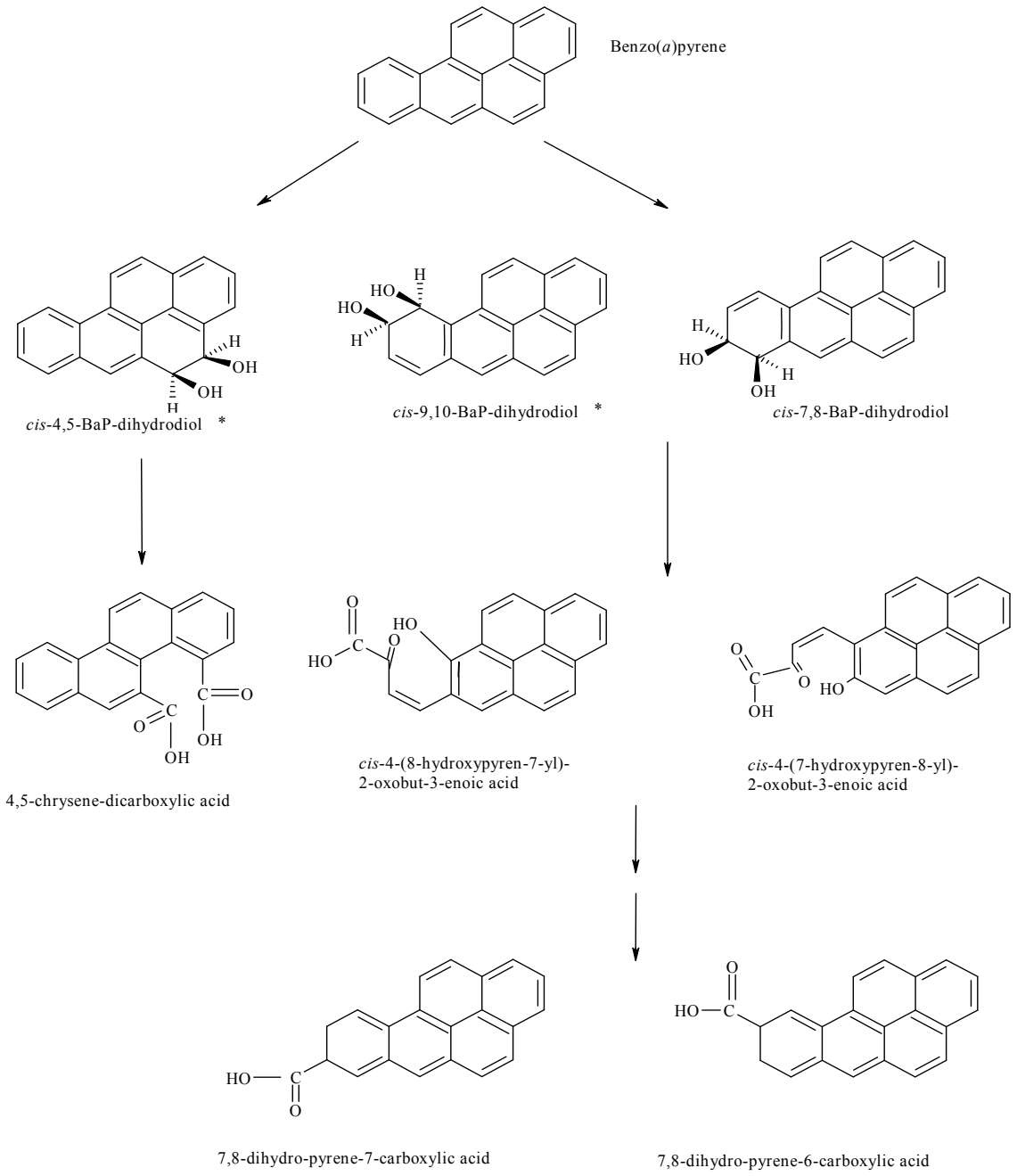


Figure 1.10: Proposed Pathway for the Degradation of Benzo[*a*]pyrene by *Mycobacterium* sp. Strain RJGII-135 (Schneider *et al*., 1996)

(Denotes hypothetical intermediates)*

1.4.3 Fungal Degradation of PAHs

As bacteria are limited in their ability to degrade high molecular weight PAHs intracellularly (due to issues associated with the transport of these compounds across cell wall) it has been suggested that organisms capable of producing extracellular enzymes with broad substrate specificity should be considered as possible candidates for the degradation of high molecular weight PAHs in soils. Wood degrading or "ligninolytic" fungi have been found to be capable of degrading PAHs due to relatively non-specific enzyme systems they produce to achieve the degradation of lignin. Except for cellulose, lignin is the most abundant biological compound found in nature, yet it is degraded by only a small number of microorganisms, primarily *Basidiomycetes*. While prokaryotes (in particular actinomycetes) are capable of transforming lignin to varying degrees, there is a general recognition that specific types of wood decaying fungi constitute the major decomposers (Buswell, 1991).

Lignin is a heterogeneous, three dimensional polymer composed of oxy-phenylpropanoid units connected by several different C-C and C-O-C linkages (Figure 1.11). Together with hemicellulose and cellulose, it is a major structural component of woody tissues. The woody vascular tissue of plants is usually comprised of 20-35% lignin to increase rigidity, and the resistance of the lignin polymer to biological degradation protects cell walls from microbial attack (Buswell, 1991). The variety of enzymes produced by fungi achieve degradation of lignin via the formation of radicals inside the lignin polymer, which results in destabilisation of bonds and finally the breakdown of the macromolecule (Hofrichter *et al.*, 1999). The enzymes that achieve degradation of lignin must have low substrate specificity to accommodate the natural irregularity of the lignin polymer and as such, it is possible that these enzymes may catalyse one-electron reactions of some aromatic and heteroaromatic compounds (Hammel *et al.*, 1986). Fungi are thought to achieve degradation of PAHs in soils due to the structural similarity of many PAHs to lignin. PAH degradation is achieved via

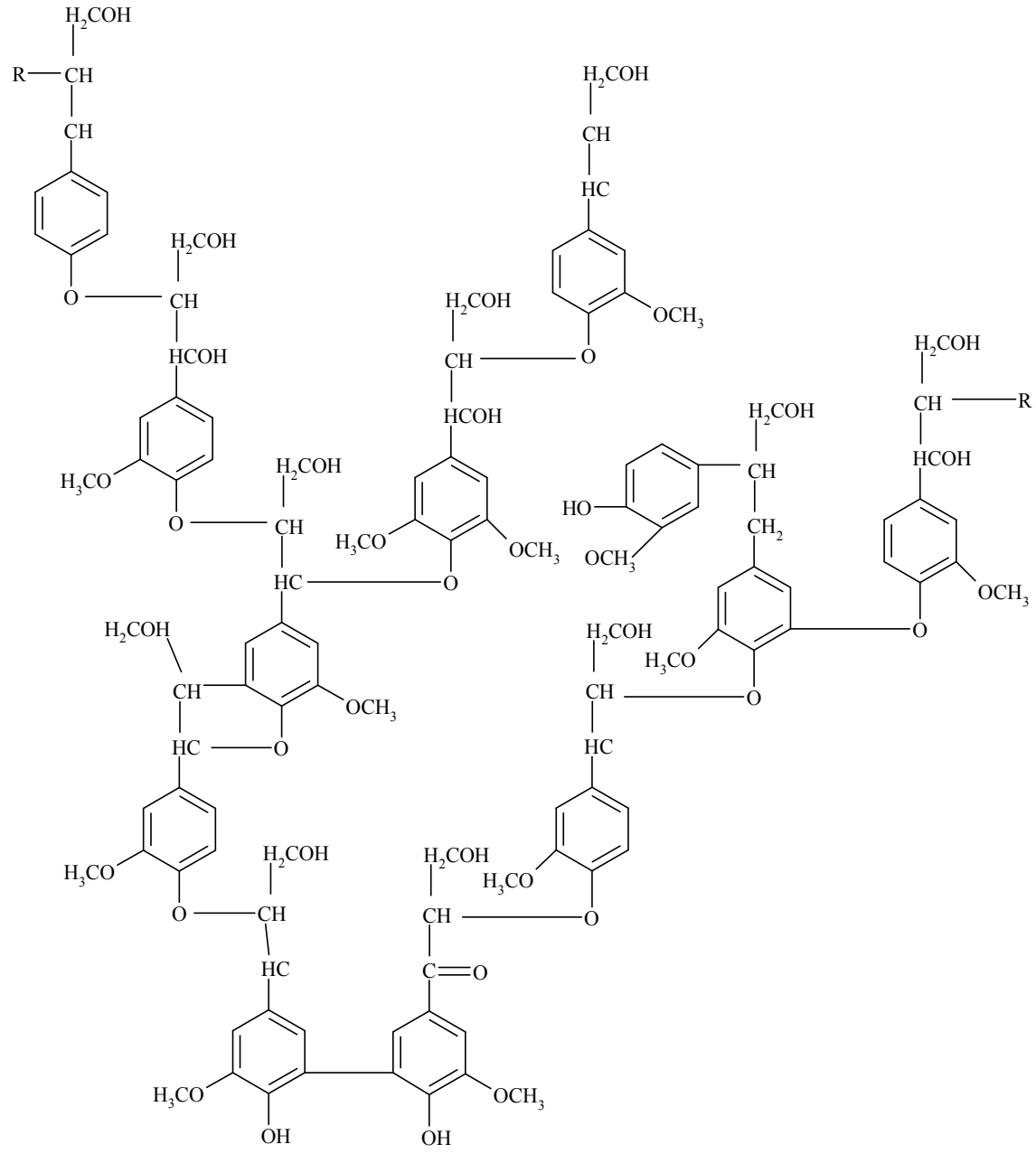


Figure 1.11: A Portion of the Lignin Polymer Structure (Barr and Aust, 1994)

R= Indicates where polymer extends beyond that represented

the production of these non specific extracellular enzymes with the process being assisted by the mechanical pressure on soil particles generated by elongating hyphae (Canet *et al.*, 1999).

This review will include a brief overview of fungal PAH degradation, details of the enzymes produced and provide examples of fungal PAH degradation of fluorene, phenanthrene, fluoranthene, pyrene and benzo[*a*]pyrene. A list of fungi able to achieve the degradation of these PAHs is contained in Table 1.6.

1.4.3.1 White Rot Fungi

The white rot fungi are a group of fungi capable of extensively degrading lignin and, are grouped together on the basis of physiology rather than taxonomy. Members of the white rot fungi group consists mainly of the *Basidiomycete* genera, but also include a few *Ascomycete* genera. The ability to catabolise cellulose and hemicellulose, the polysaccharides forming the main component of lignocellulose, is a common primary metabolic process amongst white rot fungi (Pointing, 2001).

Xenobiotic oxidation by white rot fungi is not rapid or efficient, but very non-specific facilitating the degradation of a wide variety of pollutants (Hammel, 1995). The lignin degrading enzymes produced by white rot fungi have been found to achieve the degradation of munitions waste, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bleach plant effluent, synthetic dyes, synthetic polymers and wood preservatives (Pointing, 2001).

Table 1.6. Polycyclic Aromatic Hydrocarbons Oxidised by Different Species of Fungi (adapted from Juhasz and Naidu, 2000).

Organism	Reference
Fluorene *Pleurotus ostreatus, Phanerochaete sp., Cunninghamella elegans, Laetiporus sulhureus, Penicillium sp., Trametes versicolor*	Sack et al. (1997), Bezalel *et al.* (1996), Bogan et al. (1996), Collins and Dobson (1996), George and Neufeld (1989), Pothuluri *et al.* (1993), Sack and Gunther (1993)
Phenanthrene *C. elegans, P. chrysosporium, P. laevis, Pleurotus ostreatus, T. versicolor, Bjerkandera adjusta, Pleurotus ostreatus, Cylindrocladium simplex, Monosporium olivaceum, Curvularia lunata, Curvularia tuberculata, Laetiporus sulphureus, Daedaela quercina, Flamulina velutipes, marasmiellus sp., Penicullium sp., Kuehneromyces mutabilis, Laetiporus sulphureus, Agrocybe aegerita, Aspergillus niger, Syncephalastrum racemosum, Phlebia lindteri, Cyclothirium sp.*	Cerniglia and Yang (1984), Cerniglia *et al.* (1989), Morgan *et al.* (1991), Sutherland *et al.* (1991), Bumpus (1989), Hammel *et al.* (1992), Bezelel *et al.* (1996), Brodkorb and Legge (1992), Schutzendubel *et al.* (1999), Lisowska and Dlugonski (1999), Bogan and Lamar (1996), Sack and Gunther (1993), Sack *et al.* (1997a), Collins and Dobson (1996), Casellas *et al.* (1996), Sutherland *et al.* (1993), Mori et al. (2003), da Silva *et al.* (2003)
Fluoranthene *C. elegans, C. blackesleeana, C. echinulata, Bjerkandera adjusta, Pleurotus ostreatus, Sporormiella australis, Cryptococcus albidus, Cicinobolus cesatii, Pestalotia palmarum, Beauveria alba, Aspergillus terreus, Mortierella ramanniana, Rhizopus arrhizus, Laetiporus sulphureus, Daedaela quercina, Flamulina velutipes, marasmiellus sp., Penicullium sp.*	Pothuluri *et al.* (1990, 1992a), Schutzendubel *et al.* (1999), Salicis *et al.* (1999), Sack and Gunther (1993)
Pyrene *C. elegans, P. chrysosporium, Penicillium sp., P. janthinellum, P. glabrum, P. ostreatus, Syncephalastrum racemosum, Bjerkandera adjusta, Pleurotus sp., Dichomitus squalens, Flammulina velutipe, Trammetes versicolor, Kuehneromyces mutabilis, Laetiporus sulphureus, Agrocybe aegerite, Naematoloma frowardii, Cyclothirium sp.*	Cerniglia *et al.* (1986), Hammel *et al.* (1986), Launen *et al.* (1995), Bezelel *et al.* (1996), Schutzendubel *et al.* (1999), Martens and Zadrazil (1998), Sack *et al.* (1997b), Wunder *et al.* (1997a), Sack and Fritsche (1997), Lang *et al.* (1996), Stanley *et al.* (1999), Boonchan et al. (1998), da Silva *et al*. (2003)
Benzo[*a*]pyrene *Cunninghamella elegans, Phanerochaete laevis, Pleurotus ostreatus, P. Chrysosporium, Aspergillus ochraceus, Trametes versicolor, Pycnoporus cinnabarinus, Bjerkandera* sp. strain BOS55, *Penicillium janthinellum, Saccharomyces cerevisiae, Neurospora crassa, Candida lipolytica, Syncephalastrum racemosum, Nematoloma frowardii, Stropharia coronilla, Fusarium solani, Cyclothirium sp.*	Cerniglia and Gibson (1979), Bogan and Lamar (1996), Bezalel *et al.* (1996), Barclay *et al.* (1995), Datta and Samanta (1988), Collins *et al.* (1996), Rama *et al.* (1998), Kotterman *et al.* (1998), Stanley *et al.* (1999), Wiseman and Woods (1979), Lin and Kapoor (1984), Cerniglia and Crow (1981), Launen *et al.* (1995), Sack *et al.* (1997), Steffen *et al.* (2003), Verdin *et al*. (2003), da Silva *et al*. (2003)

The broad spectrum of pollutants that fungal enzymes can degrade has prompted extensive research into their bioremediation potential. White Rot Fungi have been extensively focused upon in studies of fungal degradation of PAHs due to their extracellular ligninolytic enzyme production. The induction of these enzymes appears to be independent of the concentration of the target pollutants present, therefore fungal degradation can occur at low pollutant concentrations (Canet *et al.*, 2001). White rot fungi have also been found to enhance biodegradation through the production of biosurfactants. In a study of white rot fungal degradation of pyrene, it was found that biosurfactant formation by *Phanerochaete chrysoporium* may have resulted in solubilisation of pyrene, thereby enhancing degradation (Song, 1999). A similar result was obtained using *Hypholoma fasciculare* where zones of clearing around colonies when grown on PAHs were proposed to have been formed due to the production of biosurfactants not biodegradative enzymes (Anderson *et al.*, 2000).

White rot fungi can degrade PAH via lignolytic and non-lignolytic pathways (Harayama, 1995). There is substantial evidence that lignolytic enzymes are involved in PAH mineralization, although some non-lignin modifying enzyme mediated PAH mineralisation by white rot fungi grown under non-lignolytic conditions has been reported. Most reports of PAH degradation by white fungi has been attributed to the secretion of one or more of three extracellular enzymes, although degradation has also be reported as being achieved via other enzyme systems. The primary extracellular systems of white rot fungi comprise two glycosylated heme-containing peroxidases (Lignin Peroxidase [LiP] and Manganese Dependent Peroxidase [MnP]) and a copper-containing phenoloxidase (Laccase) (Pointing, 2001). The production of these enzymes occurs during secondary metabolism and is induced by limited nutrient levels, particularly nitrogen, carbon and sulphur, although some fungi have been observed to produce Lignin Peroxidase, Manganese Peroxdidase and Laccase under conditions of nitrogen sufficiency (Moreira *et al.*, 2003). Particular species of white rot fungi may also express multiple systems. In a study of 46 wood pulp

bleaching fungi screened for production of LiP, MnP and manganese independent peroxidase (MiP) to achieve the degradation of polychlorinated dibenzo-p-dioxins, 38 of the isolates that could produce MnP and 22 could also produce LiP (Manji and Ishihara, 2004).

1.4.3.2 Deuteromycete

Penicillium species are routinely isolated as competent degraders of PAHs. Of the 41 fungal isolates from from PAH contaminated sediment, 10 degraded pyrene, including 2 *Zygomycetes*, *Diematiceae, Sphaeropsidae* and 6 *Deuteromycetes*, of which, *Penicillium janthinellum* was found to be the best pyrene degrader of the *Penicillium* isolates (Ravelet *et al.*, 2000).

The performance of many PAH degrading fungi is highly variable. Unlike the investigation mentioned previously, a study using indigenous fungi isolated from soil from a former gasworks site in submerged cultures with pyrene as the sole carbon source yielded five strains that were capable of degrading pyrene. These included one strain of *Trichoderma harzianum* and four strains with characteristics of *Penicillium* (*simplicissimum*, *janthinellum*, *funiculosum* and *terrestre*). Unlike previous studies, *P. janthinellum* was reported as the slowest degrader. Although PAH degradation is not often found to provide carbon or energy for growth in fungi, the degradation of pyrene was found to directly correlate with an increase in biomass in all the fungal strains studied (Saraswathy and Hallberg, 2002).

As with bacterial degraders of PAHs, some fungi have been found to lose degradative ability. In a study of non-basidiomycete fungi isolated from PAH contaminated soil, 50% of isolates were able to metabolise pyrene/benzo[*a*]pyrene. The most common was a *Penicillium* and *P. janthinellum* achieved the most rapid degradation. Upon re-screening, some

fungi lost ability to oxidise pyrene which was not related to the viability of the fungus as all fungi studied grew well. Attempts to induce activity again by replating and incubation with various compounds were unsuccessful. This phenomenon was not observed to occur with active isolates whose growth was maintained on PAHs (Launen *et al.*, 1995).

Although this variation has hampered the ability to achieve a consistent level of degradation, fungal enzymes show excellent potential in achieving the degradation of recalcitrant PAHs and other environmental pollutants. This discussion will now elaborate on the enzyme systems that white rot fungi use to achieve degradation, particularly focusing on PAHs.

1.4.3.3 Manganese Peroxidases

Manganese peroxidases (MnP) are haemo-glycoproteins whose synthesis by fungi is induced by the presence of manganese $^{2+}$ (Mn^{2+}) (Harayama, 1997). In nature, Mn^{2+} required for the induction and activity of MnP is supplied in the lignocellulose, which contains high levels of Mn^{2+} (Hofrichter *et al.*, 1999). MnP catalyses H_2O_2 dependent oxidation of Mn^{2+} to Mn^{3+} using phenolic compounds as a substrate and H_2O_2 as the terminal electron acceptor (Paszczynski and Crawford, 1995). Most white rot fungi produce MnP under conditions of nutrient limitation and require manganese for the expression of the *mnp* gene (Manji and Ishihara, 2004). MnP is thought to play the crucial role in the primary attack on lignin because it generates the strong oxidant Mn^{3+}; this oxidant acts as a diffusible redox mediator which attacks certain aromatic moieties of the lignin polymer. Due to its broad substrate specificity, MnP has been considered for a variety of industrial uses such as the bleaching and delignifying of eucalyptus Kraft pulp (Moreira *et al.*, 2003).

MnP is thought to be one of the primary extracellular peroxidases produced during PAH metabolism, and extensive research has focused on this enzyme (Pointing, 2001). MnP are thought to predominantly achieve the degradation of PAHs with lower ionisation potentials and higher molecular weight. This has been demonstrated by the conversion of compounds such as benzo[*a*]pyrene, benzo[*g,h,i*]perylene and indeno[1,2,3-*c,d*]pyrene to larger extents than the higher ionization potential lower molecular mass PAHs such as phenanthrene and fluoranthene (Steffen *et al.*, 2003). The action of MnP on lower ionisation potential, high molecular weight PAHs was also demonstrated by the concurrent removal of 16 different PAHs in a cell fee reaction mixture with crude enzyme or purified MnP. The ability to degrade these more recalcitrant higher molecular weight PAHs has led to the intensive investigation of MnP producing strains as potential inoculants for the bioremediation of high molecular weight PAHs (Steffen *et al.*, 2003).

The impact of Mn^{2+} supplementation and nutrient limitation has been routinely found in MnP mediated PAH degradation. The ability of *Phanerochaete laevis* to transform PAHs in liquid culture was investigated in relation to its complement of extracellular lignolytic enzymes. The effect of nutrient limitation on enzyme production was demonstrated by the increased production of MnP in nitrogen limited media and its regulation by the amount of Mn^{2+} in the media. *Ph. laevis* was found to have the capability to extensively transform anthracene, phenanthrene, benz[*a*]anthracene and benzo[*a*]pyrene (Bogan and Lamar, 1996). MnP was the predominant lignolytic activity detected in the supernatants of thirteen deuteromycete lignolytic fungal strains investigated for their capacity to degrade PAHs and produce lignolytic enzymes during growth. This indicated that a wide range of fungi utilize this enzyme system for PAH degradation (Clemente *et al.*, 2001).

The operation of the MnP enzyme system on PAHs does not necessarily occur independently. It is thought that in some fungi (*Deuteromycetes* and

Zygomycetes), partial oxidation occurs through the MnP enzyme system prior to the involvement of intracellular reactions including Cytochrome P-450 that interlock and enable efficient benzo[*a*]pyrene degradation (Steffen *et al.*, 2003). The presence of organic acids has also been found to contribute to the degradation of PAHs in the basidiomycetous fungus *Nematoloma forwardii* (Hofrichter *et al.*,1999).

1.4.3.4 Laccase

Laccase is the most widespread enzyme among the white rot fungi (Baldrian, 2004). Laccases are copper dependent phenol oxidases that are widespread in plants and fungi (*Ascomycetes* and *Deuteromycetes)* whose physiological function is mainly connected with lignin transformation. A characteristic of laccase molecules is their prosthetic group, which contains four copper atoms that differ in their redox potential, accessibility to solvents and spectra. A laccase mediated reaction is characterized by a two step mechanism, initially involving the formation of a free radical from an organic substrate and the subsequential reduction of O_2 (Paszczynski and Crawford, 1995). The organic substrate (e.g. phenols, lignin) and molecular oxygen is reduced directly to water without involvement of a mediator (Filazzola *et al.*, 1999). It has been proposed that laccases are less important in the oxidation of PAHs than the peroxidases (Harayama, 1997), although the specific effect of laccases may be difficult to determine as most *Basidiomycetes* with lignolytic enzymatic activity secrete laccase and MnP simultaneously (Saparrat *et al.*, 2002)

Laccase activity was found to be increased by the presence of Cu^{2+} ions and inhibited by the presence of Mn^{2+} ions. The addition of Cu^{2+} to purified enzyme stimulated the laccase activity and a similar result has been found in the white rot fungi *Pleurotus ostreatus* and *Lentinus edodes*, although no laccase inhibition was observed in the presence of Mn^{2+} (Baldrian, 2004).

Laccase is thought to contribute to the degradation of PAHs in a variety of ways. It has been proposed that laccase is a mediator in the oxidation of non-phenolic substrates and involved in a later degradation pathway of PAH metabolites but not in the initial attack on the molecules (Bezalel *et al.*, 1996). Laccase can also contribute to the detoxification of PAHs through the formation of polymeric products (Filazzola *et al.*, 1999).

As with MnP, laccase is thought to achieve degradation through an interlocking of enzyme systems. In an experiment using spent mushroom compost (from oyster mushroom cultivation) to achieve the degradation of PAHs in aged creosote soils, an 86% removal of total PAHs (89% of the 3 ring, 87% of 4 ring and 48% of 5 ring PAHs) was achieved after 7 weeks incubation at ambient temperature. The *Pleurotus ostreatus* present in the compost was proposed as achieving degradation of the PAHs through a combined intra and extracellular pathway, intiated by Cytochrome P-450 (Cyt-P450) followed by laccase (Eggen, 1999).

1.4.3.5 Lignin Peroxidases

LiP catalyses H_2O_2 dependent oxidation of phenolic and nonphenolic compounds in the presence of endogenously generated H_2O_2, and veratryl alcohol to achieve a one-electron oxidation of lignin to generate aryl cation radicals. (Manji and Ishihara, 2004). LiP is not usually found to be expressed in cultures of white rot fungi incubated with PAHs, although it has the potential to achieve partial degradation of PAHs. Purified LiP of *Nematoloma forwardii* has been shown to oxidise anthracene and pyrene in the presence of veratryl alcohol (Pointing, 2001). The conditions leading to the production of LiP are not well understood (Saparrat *et al*., 2002). The presence of high levels of reactive oxygen species (ROS) is considered to be a prerequisite for LiP expression (Belinky *et al.*, 2003).

The potential for the oxidation of substrates via LiP catalysis tends to correlate with their ionisation potential. LiP has been shown to catalyse reactions (presumed to be oxidative ones) with PAH having ionisation potentials of $\leq$ 7.55 eV (Bogan *et al.*, 1996). Experiments using pyrene as a substrate in medium containing $H_2{}^{18}O$ showed that pyrene-1,6-dione and pyrene-1,8-dione are the major oxidation products (84% of total) and that the quinone oxygens come from water. The quinones formed were not substrates for LiP, hence the production of mutagens such as pyrene-1,6-dione and pyrene-1,8-dione as well as some other PAH quinones, that are not subject to further degradation, is of particular concern when exploiting this enzyme pathway (Hammel *et al.*, 1986)

Adding to the uncertainty surrounding LiP induction and degradation of PAHs is the findings of an investigation into the influence of Mn^{2+} and nitrogen containing nutrients on the biodegradation of anthracene using the ligninolytic fungus *Bjerkandera* sp. BOS55. Nitrogen was added in the form of an amino acid mixture or peptone and was found to result in 10 to 14 fold increases in the extracellular peroxidase titres compared to those obtained in nitrogen limited medium. The peptone supplement only increased the rate of anthracene elimination by 2.5 fold. In the absence of Mn^{2+}, the MnP titre was decreased and the LiP titre was increased and accompanied by a large improvement in the degradation of anthracene. In this study, the white rot fungus *Bjerkandera* sp. BOS55 produced more lignolytic peroxidases when grown in N-sufficient media. The absence of Mn was shown to repress MnP and stimulate LiP. Because the occurrence of LiP was associated with the highest anthracene degradation rates, it was considered a more efficient peroxidase towards anthracene than MnP (Kotterman *et al.*, 1995).

1.4.3.5 Cytochrome P-450 Oxygenases

As in mammalian metabolism, cell associated Cytochrome P-450 monoxygenases (P-450) are also important in fungal biodegradation of PAHs, and are indicated by the formation of trans-hydrodiols (Juhasz and Naidu, 2000a). Similar to mammalian metabolism, fungal degradation of PAHs via P-450 can also result in metabolic activation of PAHs leading to genotoxicity. Fungi able to oxidise benzo[*a*]pyrene via P450 include *Aspergillus ochraceus, A. fumgatus*, *Cunninghamella elegans*, *Chrysosporium pannorum*, *Morierella verrucosa*, *Neurospora crasssa* and *Penicillium* spp. (Datta *et al.*, 1983, Venkateswarlu *et al.*, 1999, Cerniglia and Gibson, 1979, Lin and Kapoor, 1984 and Stanley *et al.*, 1999). Unlike the degradation via lignolytic enzymes, fungal metabolism via this pathway is highly regio- and stereoselective (Cerniglia, 1997). The evidence for the degradation of PAHs via non-ligninolytic pathways has been found through the ability of fungi that are unable to produce lignin peroxidases (e.g. *Pleurotus ostreatus*) to mineralise PAHs more thoroughly than peroxidase producing fungi (e.g. *P. chrysoporium*). *Pleurotus ostreatus* expresses both laccase and Manganese Independent Peroxidase (MiP), but a reduction in PAH metabolite formation was observed when the P-450 pathway was inhibited, providing evidence for its involvement in PAH degradation (Harayama, 1997). It has been proposed that both the intracellular (P-450) and extracellular (MnP) processes can work in conjunction to achieve PAH degradation (Steffen *et al.*, 2003).

Non-white rot fungi have also been proposed as being able to degrade PAHs via P-450. The non white rot fungi (*Fusarium solanii*) isolated from fuel contaminated soil was screened for growth on benzo(*a*) pyrene as a sole carbon source. It was found that spores could germinate in the presence of benzo[*a*]pyrene and that growing mycelium mineralised ^{14}C benzo[*a*]pyrene. The hyphae were found to accumulate benzo[*a*]pyrene or its metabolites suggesting an intercellular rather than extracellular enzyme pathway. The

involvement of P-450 was again indicated by the decrease in $^{14}CO_2$ released in presence of a cytochrome inhibitor (Rafin *et al.*, 2000).

The involvement of P-450 in the degradation of benzo[*a*]pyrene has been implied in an investigation of the involvement of extracellular oxidative enzymes (laccase, lignin peroxidase and manganese-dependent peroxidase) by three mitosporic fungi (*Deuteromycetes*) isolated from polluted soils. In these three fungal strains (*Trichoderma viridev* (now *Gliocladium virens*), *Fusarium solani* and *Fusarium oxysporum*) no apparent correlation between benzo[*a*]pyrene degradation and the tested lignolytic enzymes production could be shown in the culture conditions used (Verdin *et al.*, 2003).

The observation that no LiP or MnP activities were detected in the different fungal strains either in the presence or absence of benzo[*a*]pyrene provides some evidence that extracellular peroxidases were not involved in benzo[*a*]pyrene degradation in the three isolates used. This indicates the possible involvement of an alternative enzyme system to ligninase; further suggested by the results of a preliminary experiment assessing crude enzymatic extract of *F.solani* grown on synthetic medium containing benzo[*a*]pyrene showing the presence of P-450 (Verdin *et al.*, 2003).

1.4.3.6 Other Enzyme Systems

Other enzyme systems (e.g. Manganese Independent Peroxidase, Glyoxal Dismutase, superoxide dismutase) either achieve oxidation by themselves or assist in the production of mediators for the degradation of compounds. Unlike the peroxidase and cytochrome enzyme systems, there has been limited research into the contribution of other enzyme systems and their involvement appears to be limited to a few fungal species (Manji and Ishihara, 2004). Most of these enzymes are associated with the other lignin modifying enzymes but are unable to degrade lignin alone (Pointing, 2001). Since not all fungi produce the same

lignin degradation enzymes, mechanisms producing the oxygen radical may have special importance in those fungi lacking MnP activities or extracellular oxidases (Guillen *et al*., 2000).

The production of H_2O_2 as an electron acceptor is a vital part in the operation of MnP and LiP. In an assessment of the rate of PAH degradation by *Bjerkandera* sp ST B0S55, attempts to increase peroxidase enzyme production did not increase biodegradation or oxidation. PAH oxidation was only enhanced when H_2O_2 generation was increased by the addition of glucose, indicating that the extracellular oxidation of PAHs by white rot fungi is the combined effect of peroxidases and H_2O_2 production (Kotterman *et al.*, 1998).

As with the section concerning bacterial degradation, the following discussion focuses on the fungal degradation of the five PAHs fluorene, phenanthrene, fluoranthene, pyrene and benzo[*a*]pyrene. Unlike the bacterial degradation section, degradation pathways will only be shown for benzo[*a*]pyrene as this forms the central focus of the discussion on fungal PAH degradation.

1.4.3.7 Fungal Degradation of Fluorene

Pleurotus ostreatus has been shown to achieve degradation of fluorene via oxidation at the aliphatic bridges instead of at the aromatic ring. The products of this oxidation were 9-fluorenol and 9-fluorenone, considered dead end metabolites for P-450 mediated reactions (Bezalel *et al.*, 1996).

1.4.3.8 Fungal Degradation of Phenanthrene

A number of fungal isolates have the ability to degrade phenanthrene using a variety of mechanisms. *Pleurotus ostreatus* degrades phenanthrene via P-450, predominantly forming *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene

(phenathrene *trans*-9,10-dihydrodiol) and 2,2-diphenic acid (Bezalel, 1996). 2,2-diphenic acid is also formed during lignolytic degradation of phenanthrene by *Phanerochaete chrysosporium*. In nutrient limited lignolytic cultures, *P. chrysosporium* oxidised phenanthrene at the 9,10 positions to form phenanthrene-9,10-quinone, followed by ring fission to form 2,2-diphenic acid (Hammel, *et al* 1992). The quinones formed through phenanthrene degradation have also been reported to accumulate using cultures of *P. chrysosporium* (Kennes and Lema, 1994). This result emphasises the variability of fungal degradation. In a study of fungi isolated from soil from polluted industrial areas, two fungi (designated Strain IM 1063 and IM 6325) were able to metabolise phenanthrene to form 9-phenanthrol (Szweczyk *et al.*, 2003).

1.4.3.9 Fungal Degradation of Fluoranthene

Cunninghamella elegans has been shown to achieve the degradation of fluoranthene forming five metabolites (percentages in parentheses indicates relative proportions of metabolites formed): 9-hydroxy-fluoranthene-*trans*-2,3-dihydrodiol (3.4%); 3-(8-hydroxy-fluoranthene)-β-gluca-pyranoside (9.5%); 8-hydroxy-fluoranthene-*trans*-2,3-dihydrodiol (17.7%); *trans*-2,3-dihydroxy-2,3-dihydrofluoranthene (3.2%) and 3-fluoranthene-β-glucapyranoside (42.7%). The formation of these metabolites was a product of P-450 metabolism (Pothuluri *et al*., 1992).

1.4.3.10 Fungal Degradation of Pyrene

Pleurotus ostreatus has been shown to metabolise pyrene predominantly to the *trans*-4,5-dihydrodiol. The initial area of enzymatic attack was the K-region of the molecule forming an epoxide which was hydrated to form the *trans*-4,5-dihydrodiol. It was suggested that this regio and stereoselective enzymatic reaction is a product of P450 monoxygenase (Bezalel *et al.*, 1996).

The degradation of pyrene and benzo[*a*]pyrene by *Aspergillus terreus* has provided more evidence for the involvement of P450 monoxygenases. The formation of hydroxypyrene (a transient metabolite) which was converted to the sulfate conjugate pyrenylsulfate and benzo(*a*)pyrenylsulfate suggested hydroxylation by P450 monoxygenase followed by conjugation with sulfate ion (Capotorti *et al.*, 2004).

Lignolytic pyrene degradation has been reported as occurring via aromatic ring cleavage (Da Silva *et al.*, 2003).

1.4.3.11 Fungal Degradation of Benzo[a]pyrene

The potential for fungi to degrade benzo[*a*]pyrene has been demonstrated on a number of occasions, with most studies reporting the transformation of benzo[*a*]pyrene to polar metabolites (see Table 1.6). Several enzyme systems have been reported as being involved in the fungal degradation of benzo[*a*]pyrene and it is commonly thought that like bacteria, fungi require an alternative carbon source as benzo[*a*]pyrene does not provide carbon for growth. Recently, this has been disputed where visual examination of the mycelial growth of *Marasmiellus troyanus* in benzo[*a*]pyrene containing microcosms appeared more profuse than in controls, both of which were supplied with alternative nutrient sources (Nemergut *et al.*, 2000). This proposition should be considered with caution as fungal growth can also be associated with starvation.

MnP has been implicated in the fungal degradation of benzo[*a*]pyrene by *Phanerochaete laevis* HHB-1625. When grown in buffered media containing glucose and ammonium tartrate, 9.3% and 50% of the radioactivity of the 1-1.5 μM (7,10-^{14}C) benzo[*a*]pyrene added was recovered in the water and organic fractions respectively without the accumulation of quinones (Figure 1.12). MnP activities were shown to be maintained at high levels throughout the experiment (Bogan and Lamar, 1996).

S. coronilla was found to be capable of metabolising and mineralising benzo[*a*]pyrene in liquid culture, when Mn^{2+} (as $MnCl_2.4H_2O$) was supplied at a concentration of 200 μM. *S. coronilla* converted 80% (50 mg/l[1] to 10 mg/l) of the benzo[*a*]pyrene in Mn^{2+} supplemented cultures within 28 days, while only 20% (50 mg/l to 40 mg/l) disappeared in non-supplemented cultures. In the presence of Mn^{2+}, the benzo[*a*]pyrene concentration dropped rapidly during the first week, whereas the slow decrease in the absence of additional Mn^{2+} was nearly linear throughout the test. *S. coronilla* mineralised approximately 12% of the added [^{14}C] benzo[*a*]pyrene in Mn^{2+} supplemented cultures within 6 weeks, whereas only 1% was evolved as $^{14}CO_2$ in non-supplemented cultures. In addition, approximately 40% of the label was detected in the aqueous fraction and less than 4% was detectable in the ethanolic fraction when Mn^{2+} was supplemented to culture medium (Steffen *et al.*, 2003).

P450 monoxygenase has been implicated in the fungal degradation of benzo[*a*]pyrene. The P450 monoxygenase appeared to initiate enzymatic attack at the C-7,8 and C-9,10 positions. Metabolites including *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, benzo[*a*]pyrene 1,6-quinone and benzo[*a*]pyrene 3,6-quinone were formed by *Cunninghamella elegans* incubated with benzo[*a*]pyrene in Sabouraud dextrose Broth (Cerniglia and Gibson, 1979). A similar enzyme system has been shown to be used in the degradation of benzo[*a*]pyrene in cultures of *Aspergillus ochraceus* grown in the presence or absence of inducers (3-methyl cholanthrene, Phenobarbital and progesterone) (Datta and Samanta, 1988).

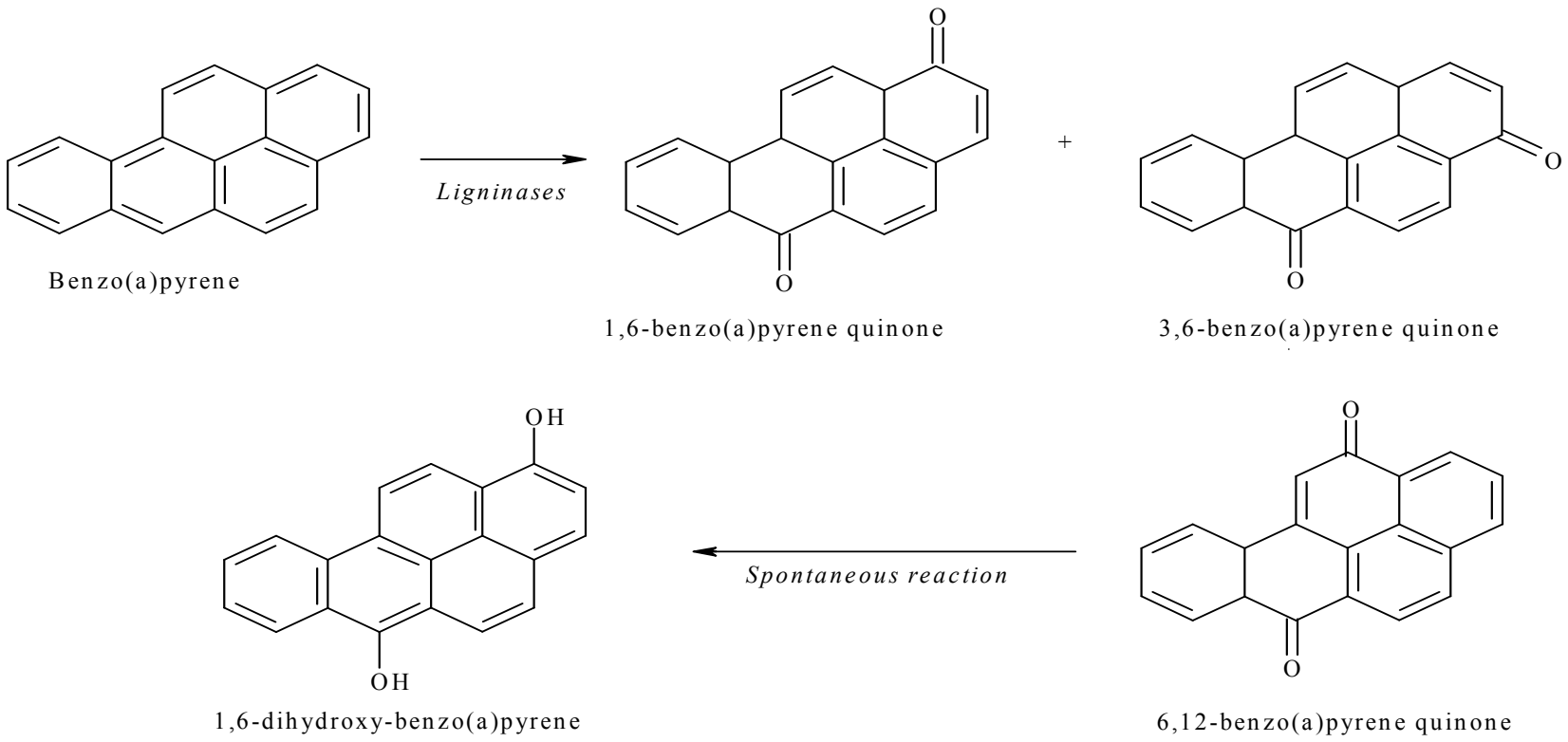


Figure 1.12: Oxidation of Benzo[*a*]pyrene by Fungal Ligninases (adapted from Hammerli *et al*., 1986)

(Only 1,6-dihydroxy-benzo[a]pyrene showns as formed, but each quinone would form its respective hydroxylated product)

1.4.4 Cocultures and the Removal of the Metabolic Bottleneck

Historically, many studies investigating the potential for the degradation of PAHs have involved the use of single organisms. Whilst this allows a better elucidation of the metabolic pathways involved in degradation for particular organisms, it is highly reductionist and ignores the cooperative action of microbial consortia that occurs in the degradation of environmental contaminants under natural conditions (Buswell, 1991). Traditionally a variety of methodological obstacles have prevented investigators from simultaneously documenting identity and activity in real world habitats such as soil. This is a product of an incomplete understanding of the microhabitat, physiochemical characteristics, a large reservoir of inactive but potentially responsive cells in environmental samples and the propensity for microbial communities to change after removal from their in situ context (Padmanabhan *et al.*, 2003). A more extensive understanding of the cooperative roles of members of microbial consortia and ecosystem interactions has been achieved through improvements in analytical technology. This has revealed some of the complexity of these interactions.

In recognition of the importance of the ecological interaction of a number of organisms, research has increasingly focused upon the use of microbial cocultures or consortia. Microbial cocultures and consortia are thought to provide the greatest likelihood of consistent degradation of high molecular weight PAHs. A consortium is a collection of organisms with some functional association, for example, they may provide growth factors for one species or eliminate inhibitory compounds that affect another (Alexander, 1997). Mixed cultures have been observed to perform better than individual isolates at degrading high molecular weight PAHs, whereas isolates perform better at degrading more water soluble PAHs. It is the broader and complimentary enzymatic capabilities of mixed cultures than individual isolates that is suggested as contributing the greater ability to degrade complex mixtures (Trzesicka-Mlynarz and Ward, 1995).

Cocultures are particularly of benefit in the fungal metabolism of PAHs. The accumulation of metabolites following fungal metabolism is a concern due to the formation of potently carcinogenic epoxides and dihydrodiols following the oxidation of benzo[*a*]pyrene by monooxygenases (Juhasz and Naidu, 2000a).

1.4.4.1 Defined Fungal and Bacterial Cocultures

The likelihood of assembling an effective consortium for bioaugmentation to achieve the degradation of a recalcitrant contaminant is greater if the diversity of the consortium is very low and the components are defined (Van der Gast *et al.*, 2002). Defined fungal and bacterial cocultures, comprising one of each organism, have been proposed as one way to overcome the many barriers to the degradation of high molecular weight PAHs (Boonchan *et al.*, 2000). Barriers to degradation include the inability of bacteria to transport the PAH into the cell due to molecular size, the PAH not being a substrate for the available enzymes or not being an inducer for the appropriate transport or degradative enzymes (Juhasz and Naidu, 2000).

In co-culture degradation of high molecular weight PAHs, it has been proposed that the process is initiated by the release of fungal extracellular enzymes that break down molecules that are too large to pass through bacterial cell walls, accomplishing a partial oxidative degradation of the PAH (Hammel, 1995). This initial ring oxidation increases the potential for degradation and mineralisation by bacteria as the oxidised metabolites have increased water solubility and reactivity thereby eliminating this initial ring oxidation as the rate-limiting step for bacteria (Cerniglia, 1997) (Figure 1.13). This process not only addresses the inability of the bacteria to transport the molecule into the cell, it also prevents the accumulation of the fungal metabolites. Previously fungal metabolites have been reported to accumulate and exhibit an inhibitory effect on degradation (Barclay *et al.*, 1995).

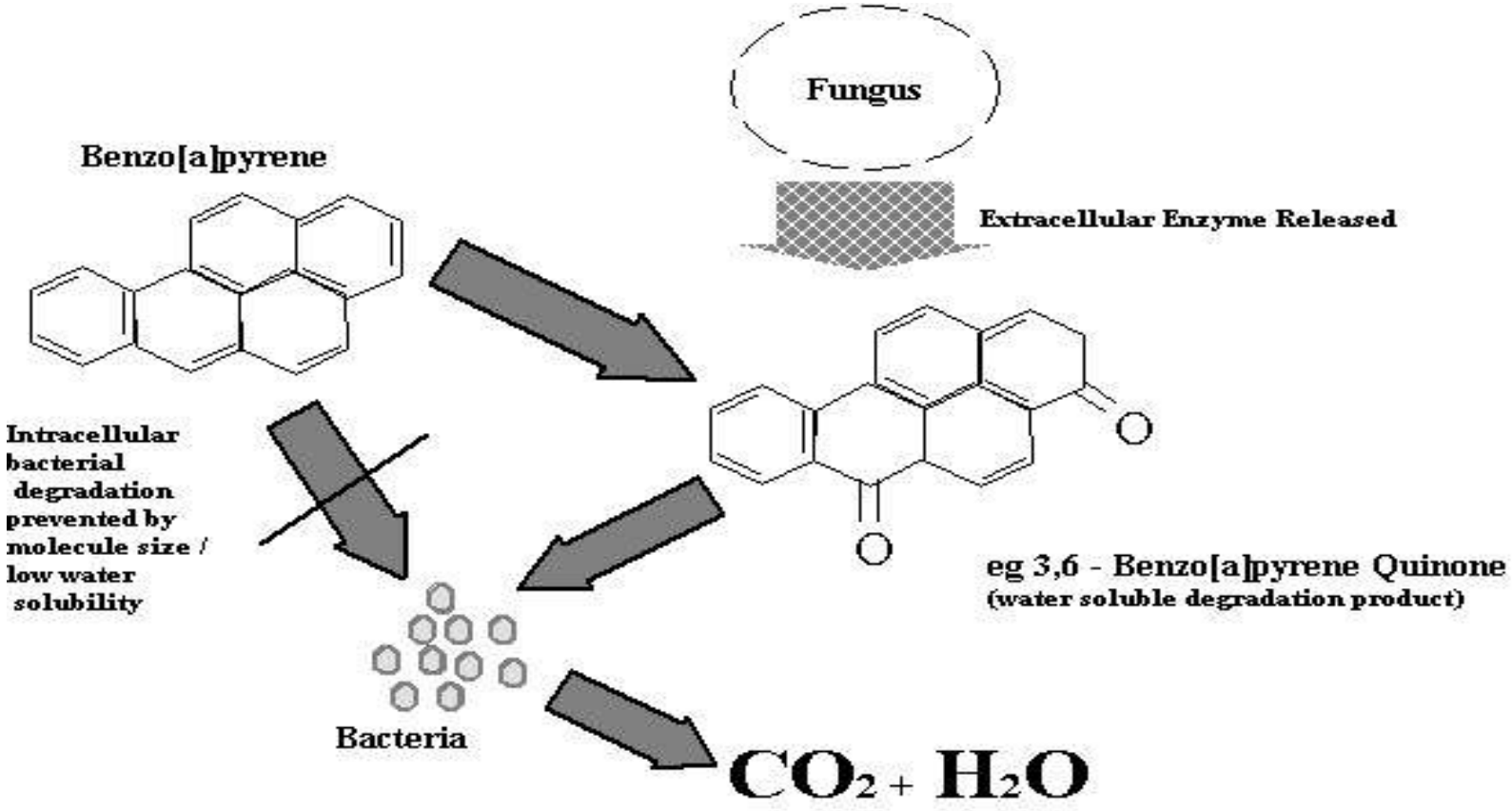


Figure 1.13: Idealised Fungal / Bacterial Co-culture Degradation of a High Molecular Weight PAH (benzo[*a*]pyrene).

Boonchan *et al*. (2000) first reported the degradation of high molecular weight PAHs by defined fungal and bacterial cocultures. Using *Stenotrophomonas maltophilia* (isolated from creosote contaminated soil) and *Penicillium janthinellum* (isolated from manufactured gas plant soil), they were able to achieve degradation of high molecular weight PAHs in liquid culture and soil microcosms. The co-culture could achieve the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in liquid culture (59% and 35% of the 50 mg/l added over 56 days), accompanied by demonstrable growth of the bacterial component. Growth on these compounds was not observed in axenic bacterial cultures despite some degradation of both compounds occurring (6-12% of the 50 mg/l added over 56 days incubation). In soils, 44 – 80% of the added benzo[*a*]pyrene and dibenzanthracene (50 mg/kg) was degraded after 100 days, without an initial lag period. Experiments using ^{14}C radiolabelled benzo[*a*]pyrene and the defined co-culture in liquid culture and soil microcosms recovered 25.5% (after 56 days incubation) and 53.2% (after 100 days incubation) of the added benzo[*a*]pyrene as $^{14}CO_2$ (Boonchan et al., 2000).

Some studies had previously indicated a co-operative degradation of high molecular weight PAHs by fungi and indigenous microflora (e.g. Kotterman *et al*., 1998) and bacterial consortia (e.g. Dries and Smets, 2002). The study of Boonchan *et al*. (2000) was the first study to use a defined bacterial and fungal co-culture to achieve the degradation of high molecular weight PAHs. Defining the species involved allowed for greater consistency in inoculum development. This increased the potential for development of a consistent and viable inoculum for bioremediation purposes.

1.4.4.2 Potential Impacts of Co-culture Components on Each Other

The co-culture components can have potential for a variety of beneficial (synergism or co-development) or detrimental impacts on each other. When using

cultures consisting of a variety of organisms, the factors that may determine selection during succession of one microbial species over another must be considered and include:

- The availability of nutrients for succeeding species;
- Changes in the concentration of inorganic nutrients;
- Competition for limited resources;
- Predation; and;
- Parasitism.

(Alexander, 1997)

These factors do not tend to occur in isolation and the impact upon each other affects the survival and activity of each organism in the co-culture.

Nutrient availability significantly influences the microbial degradation of organic contaminants. Resource ratio theory predicts that different communities are selected as a result of competition for limiting resources. The outcome of competition is determined by differences in species specific substrate affinity, the maximum growth rate and the mortality rate (Roling *et al.*, 2004).

In pristine soils, the application of fertilisers and other nutrient sources has been shown to cause shifts in both the active bacterial community structure and the active fungal community structure. Fertiliser application has been shown to cause a quick response in the bacterial community, decreasing over time – potentially due to the rapid utilisation of the readily available inorganic N and P, followed by predation (Girvan *et al.*, 2004). As nutrients are often provided during bioaugmentation or biostimulation, their effects upon microbial diversity and the levels of other nutrients required for degradation should be considered. This is particularly the case for fungal-bacterial cocultures where the optimum conditions for fungal degradation of PAHs (nutrient depletion - see Section 1.4.3.3) are not those for bacterial degradation (provision of adequate N and P).

The provision of readily available sources of nutrition may stimulate growth of other organisms preventing the establishment of the co-culture. The presence of alternative carbon sources may also have an indirect effect on the degradation of higher molecular weight PAHs. Whilst numerous studies have shown that the presence of other lower molecular weight PAHs may assist cometabolic degradation of high molecular weight PAHs, this may result in increased oxygen consumption in soil, compromising aerobic degradation. Lower molecular weight PAHs may also provide a carbon source for other heterotrophic bacteria that may out-compete organisms that degrade high molecular weight PAHs (Juhasz and Naidu, 2000a).

Studies have yielded equivocal results on the impact of the addition of nutrients based either on the rate or extent of degradation as a result of the differing requirements of the organisms mediating degradation. Nutrient limitation is of importance in contaminated sites where organic carbon is often present at greater concentrations than the nutrients required to achieve its degradation. This differs from natural environments, where organic carbon is usually limiting and other compounds (nitrogen, phosphorus, potassium) are abundant, (Alexander, 1999).

A reduced or eliminated lag period prior to the commencement or an increased rate of degradation of PAHs has been achieved through the addition of nutrient amendments such as yeast extract, peptone, glucose, mineral fertilisers, agricultural by-products (e.g. sawdust) and molasses (Ye *et al.*, 1996; Trzesicka-Mlynarz and Ward, 1995; Mishra *et al.*, 2001). This increased rate of degradation can be attributed to either increasing abundance of degrading organisms or provision of nutrients required for catabolism. This specificity in nutrient demand was demonstrated in a study examining the factors affecting the degradation of phenanthrene in soil, phosphorus was found to enhance degradation, potassium had no effect and nitrate was found to reduce degradation (Manilal and Alexander, 1991).

There is an additional effect of amendments on degradation. Nutrient addition to soil has been shown to contribute to degradation rates via increased soil aeration and water holding capacity as a result of increased plant growth (Mishra *et al.*, 2001). A study investigating the influence of sawdust addition on biodegradation in an enriched consortium using PAHs as sole carbon source observed a slight enhancement of biodegradation with sawdust. It was proposed that the sawdust acted more on soil aeration than providing a nutrient source to the population inoculated into the soil (Bidaud and Tran-Minh, 1998).

The importance of the presence of oxygen or other gases has a significant impact on the degradative pathway and the rate and extent of degradation. Some PAHs (e.g. phenanthrene) have been shown not to degrade under anaerobic conditions. As the oxygenase degradative pathway consumes oxygen, it has been proposed that the addition of O_2 can enhance biodegradation in conjunction with the addition of nutrients (Durant *et al.*, 1995).

The addition of compounds to achieve enzyme induction has also provided a variety of results. Pregrowing bacteria on PAHs or the addition of inducers (e.g. salicylate) has yielded increased rates of degradation (Leblond *et al*., 2001; Dagher, *et al.* 1997; Trzesicka-Mlynarz and Ward, 1995; Cerniglia and Heitkamp, 1990), although these findings have been disputed in other studies that have found that induction is not required prior to degradation (Cullen *et al*., 1994).

Fungi and bacteria have shown a variety of effects upon one another in soils. Soil bacteria have been shown to inhibit the growth of *Ph. chrysosporium* under laboratory conditions, whilst in other studies, the addition of white rot fungi has been shown to inhibit indigenous soil bacteria (Pointing, 2001). The inability of fungi to establish has been found in a number of studies investigating the impact of fungal bioaugmentation for the degradation of PAHs. In a study measuring three white rot fungi in PAH contaminated sterile and non-sterile soil, only

Hypholoma fasciculare grew in the non-sterile soil, while *Pleurotus ostreatus*, *Ph. chrysosporium*, and *H. fasciculare* all grew in sterile soil. *H. fasciculare* did not degrade any PAHs, *Pleurotus ostreatus* and *Ph. chrysosporium* were found to exhibit PAH degrading capabilities after 10 weeks in the sterile soil, no PAH degradation occurred in the non-sterile soil. The growth of the fungi in sterile soils appeared to be due to the decreased competition from indigenous microflora and modification of physical and chemical environment (increased concentration of soluble nutrients / organic material (Anderson *et al.*, 2000).

Fungi can also exert an impact upon the bacterial component of the soil biota. In contrast with the failure of *Pleurotus ostreatus* to establish in non-sterile microcosms, fungal inoculation of PAH contaminated soil was found to reduce the number of bacterial colony forming units significantly and permanently. This study showed a variety of effects on bacteria due to the presence of fungi; specifically *Pleurotus ostreatus* killed indigenous soil bacteria that degraded PAHs, whereas *Hypholoma* and *Stropharia* fungal species appeared to promote the development of opportunistic bacteria (Gramss *et al.*, 1999).

The effect of coculture components on each other is by no means consistent. The effect of white rot fungi on soil microorganisms and the mineralisation of ^{14}C radiolabelled pyrene in solid state fermentation was investigated using *Dichomitus squalens* and a *Pleurotus* sp. The two fungi showed contrasting ecological behaviour in competition with the soil microflora. The *Pleurotus* sp. was highly resistant to microbial competition and had the ability to penetrate the soil wherease *D. squalens* was less competitive and did not colonise the soil. The survival of each fungus was dependent on the duration of pre-incubation on a lignocellulosic substrate (straw). Several factors were proposed as being responsible for the higher competitiveness of *Pleurotus* sp with soil microflora compared to *D. squalens*, including the production of antibiotics, bacteriolytic enzymes or metabolites with toxic effects on the soil microflora. The potential for a reduction of bacterial numbers following a lowering of pH in the soil caused

by *Pleurotus* sp. was rejected as the pH of straw cultures of *D. squalens* were approximately the same (In der Wiesche *et al.*, 1996).

Changes in community profiles are not necessarily related to competition for resources, and one particular species need not dominate. This phenomenon was demonstrated using a small unit rRNA based cloning approach to examine 29 soil samples from four geographically distinct locations taken from the surface, vadose zone and saturated subsurface (Zhou *et al.*, 2002). An attempt was made to define the key determinants that drive microbial community structure in soil and found that differing ecological structures in the microbial communities occurred. Microbial communities in low carbon, saturated, subsurface soils showing competitive structures, microbial communities in low carbon surface soils showed remarkably uniform distributions and all species were equally abundant. The uniform distribution of species in the community implied that competition does not shape its structure. Four mechanisms were suggested as potentially producing a noncompetitive diversity pattern: (i) superabundant resources – if more than enough resources are available, there is no competition and communities evolve to high diversity (ii) resource heterogeneity – if resources are available in many different forms, populations can avoid competition by specialisation (iii) spatial isolation – if the habitat is subdivided into may separate pockets of resources, populations can avoid competition by physical isolation and (iv) nonequilibrium conditions – more populations can be maintained under fluctuating environmental conditions. It was proposed that a diversity of carbon sources can result in the establishment of a a non-competitive community (Zhou *et al.*, 2002).

1.5 BIOREMEDIATION AND THE ENHANCEMENT OF PAH BIODEGRADATION

The intent of this investigation is to develop a co-culture to achieve degradation of high molecular weight PAHs. This requires consideration of the numerous factors that influence both the potential of the organism to degrade the compound and the ability of the compound to be degraded (Table 1.7).

1.5.1 Bioaugmentation

Bioaugmentation is considered to be warranted for sites that are extensively contaminated and have low populations of indigenous contaminant degrading microorganisms (< 10^5 CFUs/g), resulting in low rates of biodegradation, or to increase the rate of biodegradation. In instances where the indigenous degrading population is sufficient and has demonstrated an ability to degrade the contaminant of concern, bioaugementation is often considered unnecessary. Degradation by indigenous microorganisms has been shown to be achieved on a number of occasions (e.g. Durant *et al.*, 1995) (Canet *et al.*, 2001).

Established communities have a high degree of stability and the resistance of established soil communities to introduced organisms is an important factor in the long-term success of bioaugmentation **(**Atlas and Bartha, 1987). The impact of competition with other organisms has been demonstrated previously in a study of *Mycobacterium* ecology in contaminated soils. It was found that sterile contaminated soils inoculated with a known pyrene degrading organism resulted in greater degradation of PAHs compared to non-sterile inoculated soils. This observation suggested that competition with indigenous soil flora may be a limiting factor in bioaugmentation strategies (Cheung and Kinkle, 2001).

Bioaugmentation has been attempted with varying success. Indigenous populations that make up a soil microbial community are responsible for a biotic balance that acts in a way to prevent the establishment of introduced species. Homeostasis allows a community to resist changes in an environment subject to abiotic and biotic modifications - although significant abiotic perturbations (such as extensive site contamination) can cause an appreciable change in the community. The introduction of different species is rarely sufficient to alter the community and often the community prevents new species from becoming established. Bioaugmentation must not only address the ability to grow on and degrade a pollutant, but also cope with the factors associated with the community homeostasis. Therefore, the organism selected for bioaugmentation must have capabilities for growth and establishment under conditions of competition, predation and parasitism (Alexander, 1997).

In some instances, the removal and reintroduction of organisms from a contaminated site has increased the chance of inoculum survival, growth and degradation. A bioaugmentation study was undertaken to evaluate the addition of a bacterial (consortium) inoculum sourced from the site. The inoculum was introduced to stimulate bioremediation of oily sludge contaminated soil at oil refinery where indigenous bacteria population of hydrocarbon degraders was low (10^3-10^4 cells/g). It was found that the addition of the bacterial consortium and the addition of nutrients resulted in a 91.9 - 94.8% removal of aromatic hydrocarbons in one year compared with 12.9% removal in an uninoculated control plot without nutrients. Not only did the introduced organisms achieve degradation of the compounds, but the population of introduced species was found to be stable after 1 year (Mishra *et al.*, 2001).

Table 1.7: Considerations for Process Design in Bioremediation (Madsen, 1997 and Juhasz and Naidu, 2000).

Contaminant	**Site**	**Inocula**	**Engineering**
toxicity	geology	pure or mixed culture	water flow
molecular structure	hydrology	growth conditions	aeration
bioavailability	soil type	supplements required	chemical amendments
volatility	climate	limited metabolic capabilities	physical mixing
susceptibility to microbial attack	legal - political situation	Non-adapted population	surfactants

A similar result was observed in a study conducted into the mineralisation of pyrene, benzo[*a*]pyrene and carbazoles in soils with different history of hydrocarbon exposures, amounts of total organic carbon, microbial biomass, and microbial activity from 3 coal gasification sites. Using cultures enriched on pyrene and carbazole from the site and reintroduced into the soil, mineralisation was enhanced (to a level of 55% for pyrene of the 37.8 ng/g ^{14}C labelled pyrene added) compared to indigenous degradation (around 1%) (Grosser *et al.*, 1991).

A significant hurdle to overcome in creating a multispecies inoculum for bioaugmentation is to ensure stable proportions of each species are maintained and the inoculum performs reliably. This population stability is not usually a characteristic of microbial communities, particularly those that inhabit contaminated sites or industrial wastes. Previous studies of microbial communities in bioreactors indicate that such communities are dynamic and unstable, making the maintenance of a mixed culture inoculum difficult. It has been proposed that to successfully bioaugent a contaminated site requires the careful selection of consortia based on an improved understanding of the composition of indigenous microbial communities. This is counter to the conventional approach that selects strains chiefly on the basis of specific traits (such as the ability to catabolise targeted substrates) followed by consideration of their natural abundance in the target habitat (Van der Gast *et al.*, 2002).

It can be particularly difficult to develop viable fungal inocula for bioaugementation. White rot fungi consistently demonstrate the ability to degrade PAHs under laboratory conditions, but this infrequently translates to an ability to degrade PAHs in soils. Soil conditions must be favourable for growth of white rot fungi. Soil moisture content, C/N ratio and temperature have been demonstrated to affect colonisation rates of white rot fungi in soil (Pointing, 2001). Many white rot fungi possess a strong lignolytic capacity and the lignolytic systems of white rot fungi show considerable variation (Wunch *et al.*,

1997) (see Section 1.4.3). This variation has led some researchers to propose that the degradative capabilities of white rot fungi are too complex and unpredictable for consistent and reliable degradation of environmental contaminants under field conditions, particularly when inoculating from liquid culture into soil (Paszczynski and Crawford, 1995).

The range of enzymes produced under differing conditions either in the laboratory or in the field can result in the formation of intermediates with varying levels of toxicity or cause metabolic bottlenecks (Bogan and Lamar, 1996). This issue is compounded by the lack of clarity in the role of each of white rot fungi's enzymes in degradation and the conditions that lead to the production of a particular enzyme (Moreira *et al.*, 2003). Concerns have also been expressed in the ability of white rot fungi to colonise soil and therefore its viability as an inoculant for bioremediation. Since these fungi specialise in colonising compact wood (timber and stumps), they cannot compete in soil for a prolonged time, limiting their contribution to the removal of recalcitrant PAHs under natural conditions (Steffen *et al.*, 2003). This limited understanding has been further compounded by the substantial variation in the methods used to study fungal degradation. This phenomenon makes comparison between studies difficult and limit the ability to draw general conclusions from the results, even in circumstances where the same organism has been used (Canet *et al.*, 1999)

The results of fungal bioaugmentation have been equivocal. Inoculation of *Ph. chrysosporium* into a benzo[*a*]pyrene contaminated soil composting system was found to increase the rate of bound residue formation although it was found to be ineffective in significantly enhancing the extent of benzo[*a*]pyrene removal during the 95 day study. Removal efficiencies were 62.8% for the fungal inoculated, 65.6 % uninoculated and 49.3 % in the killed composting systems, although there was limited mineralisation and decreases in concentration were mostly achieved through bound residue formation. The authors of the study determined that bioaugmentation with *Ph. chrysosporium* may not be effective in

enhancing BaP removal over systems amended with organic material. They were also concerned that bioaugementation had the potential to result in nutrient limitation, and that the initial uptake of nutrients by the introduced organism may result in a limited extent of biotransformation (Mcfarland and Jin, 1995).

1.5.2 Influences on Biodegradation and Bioaugmentation

The parameters leading to failure of bioaugmentation are similar to those that determine the potential succession of one microbial species over another in soils. These effects may operate solely or in combination to impact on biodegradation. Factors include:

- Bioavailability:
    - Strong binding to soil matter decreases bioavailability (Leblond *et al.*, 2001). Soil is a very effective adsorbent of PAHs and PAH metabolites and as a consequence, the sorption of PAHs to the soil matrix is an important factor in determining the fate of PAHs in soils (Hwang and Cutright, 2003). Broadly categorised, the two sites to which PAHs bind in soil are colloidal mineral surfaces and organic matter (Smith *et al*., 1999). Bacteria appear to be only able to achieve degradation of the target compounds when they are dissolved in the aqueous phase, but the average aqueous concentration of a pollutant has been demonstrated as not to be a good indicator of bioavailability (Bsoma et al., 1997). Low bioavailability of a compound is a major factor in preferential degradation, although the potential for desorption is not the only limiting factor in PAH degradation.
    - The impact of bioavailability is demonstrated by the commonly observed phenomenon in land treatment units of a two phase pattern of degradation characterised by an initial fast phase followed by a slow phase. It has been suggested that the initial

fast phase is mediated by bacterial utilisation of bioavailable compounds and is governed by enzyme kinetics, wherease the slower phase is governed by the rate of dissolution from soil particles (Kaplan, and Kitts, 2004).

- Limiting Nutrients:
    - The inoculum must obtain N, P and oxygen and other inorganic nutrients to survive in the environment. Nutrients are often limiting, particularly in situations where organic pollution is extensive and the inoculum is in competition with indigenous organisms. A slow growing inoculum is often a poor competitor for faster growing native organisms.
    - An excess of nutrients may limit fungal enzyme production, impeding degradation.
- Predation or Parasitism:
    - PAH degrading organisms can also be subject to predation by other organisms. An attempt at enrichment of PAH degrading bacteria in the subsurface of contaminated zones found elevated protozoan numbers in sediment samples displaying high PAH degradation, thereby suggesting a food chain was established based on PAH degrading bacteria (Ghiorse *et al.*, 1995).
- Inability of Bacteria to Move through Medium:
    - Mixing in microcosms allows bacteria to come into contact with the substrate. This is a particularly significant issue in the field. Even where plants or organisms have penetrated the soil, it is often the case that the substrate is located at a distance from the sites where bacteria exists. The lack of movement is a result of (a) physical filtration where the cells are physically blocked by solids and (b) adhesion of cells on soil particles.
- Co-contaminants and Toxicity:
    - The cometabolic degradation of high molecular weight PAHs in a mixture with lower molecular weight PAHs may be a result of

increased metabolic activity in response to the presence and degradation of the lower molecular weight PAHs (Juhasz and Naidu, 2000a). It is also considered that a mix of PAHs can induce enzyme activity more strongly as some PAHs are poor inducers of their own degrading enzymes (Bouchez *et al.*, 1995). This has been demonstrated in experiments using a bacterial consortium from petrochemical sludge revealed that individual rates of degradation were enhanced when 5 PAHs were added to samples simultaneously (Yuan *et al.*, 2003). Use of other more available carbon sources.

  - Whilst studies have suggested a cometabolic enhancement of PAH degradation, other results have demonstrated little, no or an inhibitory effect of the presence of additional carbon sources (Ye *et al.*, 1996).
  - Inhibition of metabolic pathways due to toxic metabolites

- Concentration of Organic Substrate is too Low to Support Multiplication:
  - There is a need for carbon source to support growth. An organism is unlikely to succeed when the organism cometabolises the pollutant unless another carbon source is provided to support growth.
- Environmental Parameters
  - Temperature
  - pH needs to be maintained at near the natural level of the site of concern to reduce the impact of pH on degradation.
  - Salinity: The effects of salinity/pH may inhibit indigenous microflora and an introduced inoculum (Kastner *et al.*, 1998).

(adapted from Alexander, 1999)

1.6 Research Directions

The research presented in this thesis was prompted by the finding that a defined fungal-bacterial co-culture could achieve the mineralisation of benzo[*a*]pyrene (Boonchan, 1998). The co-culture consisted of *Penicillium janthinellum* and *Stenotrophomonas maltophilia* isolated from manufactured gas plant and creosote contaminated soils respectively. The study demonstrated that axenic cultures of the bacteria could utilise pyrene in basal salts medium as a sole carbon and energy source and that axenic cultures of the fungus could achieve a partial degradation of benzo[*a*]pyrene and other PAHs in broth cultures. When the bacterial and fungal cultures were combined, the co- culture was able to mineralise benzo[*a*]pyrene in both soil and liquid cultures with an accompanying reduction in toxicity.

As the literature shows, the potential for microorganisms to be exploited for their biodegradative capabilities for high molecular weight PAHs is yet to be demonstrated consistently. The opportunities presented by the use of cocultures of bacteria and fungi and their broader enzymatic capabilities have yet to be fully explored but preliminary studies such as the research of Boonchan *et al.* (2000) suggest the possibility of the use of cocultures to achieve reproducible and efficient degradation of high molecular weight PAHs.

The work presented in this thesis examines the microbial degradation of high molecular weight PAHs and the effect of co-culture combinations on the rate and extent of PAH degradation. The objectives of the study were to:

1. Enrich, isolate and identify microorganisms capable of degrading high molecular weight PAHs – specifically benzo[*a*]pyrene;
2. Determine the PAH degradative capabilities of the microorganisms in liquid and soil matrices; and

3. Determine the PAH degradative capabilities of the microorganisms in liquid and soil matrices in a variety of coculture combinations.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

Bacterial strains used in this work (other than those isolated from environmental sources) were either provided by Victoria University (Melbourne) or Flinders University of South Australia. Environmental isolates were obtained from a variety of sources using the methods contained in Section 2.5.1. Details of bacterial strains are shown in Table 2.1. Stocks of organisms were stored at 4^{o}C on R2A slants and maintained on BSMY agar plates with 250 mg/l pyrene unless otherwise stated.

2.2 FUNGAL STRAINS

Two fungal strains were used in this work. *Penicillium janthinellum* was provided by Victoria University and given the Victoria University strain number of VUN 10,201. *Phanerochaete chrysosporium* was provided by Flinders University of South Australia having been obtained from the American Type Culture Collection (ATCC) and was strain number (ATCC 24725). Fungal strains were maintained on Potato Dextrose Agar (PDA).

Table 2.1: Bacterial Strains used in this study.

Bacterial Strain	Source	Reference	Experiments
VUN 10,010	Victoria University	(Boonchan, 1998)	L, S, CC
Mycobacterium 1B	Flinders University of SA	(Dandie *et al*., 2004)	L, S, CC
Mycobacterium sp. Strain BS5	Soil from a former manufactured gas plant	This work	E, L, S, CC
Mycobacterium sp. Strain KA5	Soil from agricultural area (Kanmantoo)	This work	E, L, CC
Ralstonia pickettii	Termite mound matrix	This work	E, L
Stenotrophomonas maltophilia	Kangaroo Faeces	This work	E, L
Mycobacterium sp. Strain KF4	Kangaroo Faeces	This work	E, L

E = Enriched and isolated as part of this work, L = Degradation experiments using liquid medium, S = Degradation experiments using soil medium, CC = used in fungal-bacterial coculture combination

2.3 GENERAL METHODS

General weight measurements were made using TS4KD Precision Standard Balance (Ohaus) and smaller quantities and measurements for the preparation of standards were made using Mettler AE260 Deltarange analytical balance (FSE). All media was prepared using ultra pure water produced using a Millipore Milli Q Ultra Pure Water System (Millipore). pH was determined using an Activon Φ50 pH meter (Beckman Instruments). Cultures and extracts were centrifuged using either a Beckman CS-15 Centrifuge (medium scale) or Sorvall Superspeed RC2B or RC-5 Ultracentrifuge (high speed). Liquid inocula were incubated in a 30^{o}C room at 150 RPM on Ratek Instruments Australia Orbital Mixers (Adelab) or Heidelab Unimax 2010 Orbital Mixer. Incubation of plates occurred in Scientific Equipment Manufacture Pty Ltd (Australia) temperature controlled cabinets. Media were autoclaved at 121^{o}C for 15 minutes. Filter sterilised solutions were filtered using 0.20 μm disposable filters (Pallman Life Sciences), solutions containing organic solvents were prepared using 0.20 μm hydrophobic PTFE filters (Gelman Sciences). Glassware and other equipment was washed with Pyroneg (Suma) and triple rinsed with deionsed water. Glassware and equipment exposed to PAHs were rinsed with acetone and acid washed overnight in 1% HNO_3 , prior to washing in Pyroneg.

2.4 MATERIALS

2.4.1 Suppliers

Chemicals, solvents, reagents and microbiological media were obtained from Sigma-Aldrich (Australia), Fluka, Crown Scientific (Australia), Oxoid, BDH Laboratory Supplies (England) and MP Biochemicals (Germany) unless otherwise specified. All chemicals, solvents and reagents were of analytical grade unless otherwise specified.

All solvents used were of Omnisolv or HPLC grade. Dichloromethane (DCM) and Acetone were obtained from Merck Pty Ltd. Pentane was obtained from Chem-Supply (Southern Cross Scientific). Dimethylformanide (DMF) was obtained from Siga-Aldrich.

2.4.2 Stock Solutions

Ethidium Bromide: Ethidium bromide (1 g) was dissolved in 100 ml Milli-Q water and stored at 4^{o}C in the dark.

10% Glucose: Glucose (100 g) was dissolved in 1 l Milli-Q water, autoclaved and stored at room temperature.

INT Solution: Iodonitrotetrazolium Chloride (p-iodotetrazolium violet 2-[4-iodophenyl]-3-[4-nitrophenyl-5-phenyltetrazolium chloride) (250 mg) was dissolved in 2 ml of dimethylformanide and made up to 25 ml using Milli-Q water.

10% Methanol: Methanol (100 ml) was mixed with 900 ml Milli Q water and stored at room temperature.

0.1 M NaOH: Sodium Hydroxide (4 g) was dissolved in 1 l Milli-Q water and stored at room temperature.

10% NH_4NO_3: Ammonium nitrate (100 g) was dissolved in 1 l Milli-Q water and autoclaved. The solution was stored at room temperature.

PAH Stock Solutions: The respective PAH was prepared in either dimethylformanide (DMF) for liquid culture / agar based experiments or dichloromethane (DCM) for soil experiments. Concentrations differed depending

on the PAH – phenanthrene and pyrene stock solutions (20 mg/ml); fluorene (10 mg/ml) and chrysene, anthracene, benzanthracene, dibenz[a,h]anthracene, fluoranthene and benzo[*a*]pyrene (5 mg/ml). PAHs for multiple substrate utilisation experiments (chrysene, anthracene, benzanthracene, dibenz[a,h]anthracene, benzo[g,h,i]pyrene and benz[e]acenaphthylene were prepared at 1 mg/ml. PAH stock solutions were stored at 4^{o}C in the dark.

For soil experiments, a 5-PAH stock solution was prepared in DCM consisting of fluorene (10 mg/ml), phenanthrene (25 mg/ml), fluoranthene (5 mg/ml), pyrene (25 mg/ml) and benzo[*a*]pyrene (5 mg/ml). The 5-PAH solution was intended to limit the amount of DCM added to the microcosm.

0.5x TBE Buffer: 5.4 g/l Trizma Base, 2.75 g/l Boric Acid and 40 mM EDTA

2.4.3 Media Composition

All media was prepared using Milli-Q water. All media recipes are per litre of Milli-Q water unless otherwise stated. Media was sterilised via autoclaving at 121^{o}C for 15 minutes unless otherwise stated.

Basal Salts Medium (BSM)

K_2HPO_4	0.4 g
KH_2PO_4	0.4 g
$(NH_4)_2SO_4$	0.4 g
NaCl	0.3 g

Where required, basal salts medium was supplemented with the following nutrients per litre:

BSMY - Yeast Extract (0.005%)	0.05 g
BSMY[3] - Yeast Extract (0.03%)	0.3 g

Where solid media was required, 15 g Bacteriological Agar #1 (Oxoid) was added prior to autoclaving. Vitamin, Trace Element and Mg/Ca Solutions (0.2 µm filter sterilised) were added to BSM, BSMY, BSMY[3] and BSMG at 5 ml/l following autoclaving and cooling.

Vitamin Solution

Biotin	20 mg
Folic acid	20 mg
Thiamine HCl (B_1)	50 mg
D-calcium pantothenate	50 mg
Vitamin B_{12}	50 mg
Riboflavin (B_2)	50 mg
Niacin (nicotinic acid)	200 mg
Pyridoxal HCl	30 mg
p-aminobenzoic acid	20 mg

Trace Element Solution

$FeSO_4.7H_2O$	200 mg
$ZnSO_4.7H_2O$	10 mg
$MnCl_2.4H_2O$	3 mg
$CoCl_2.2H_2O$	20 mg
$CuCl_2.2H_2O$	1 mg
$NiCl_2.6H_2O$	2 mg
$NaMoO_2.2H_2O$	500 mg
H_2BO_3	30 mg

Magnesium and Calcium Solution

$MgSO_4.7H_2O$	0.4 g
$CaCl_2.2H_2O$	0.4 g

Luria Bertani Broth

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

pH adjusted to 7 with 10M NaOH.

Malt-Yeast Peptone Dextrose Medium (MYPD)

Malt Extract	3 g
Yeast Extract	3 g
Peptone	5 g
Dextrose	10 g

If a bacteriostat was required, Rose Bengal was added to a final concentration of 0.03% w/v. Where solid media was required, 15 g Bacteriological Agar #1 (Oxoid) was added prior to autoclaving.

Nutrient Broth

This medium was prepared by adding 13 g Nutrient Broth (Oxoid) to one litre of Milli-Q water.

Nutrient Agar

This medium was prepared by adding 28 g of Nutrient Agar (Oxoid) to one litre of Milli-Q water.

Phosphate Buffered Saline (10x) (PBS)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Adjust to pH 7.4. Prior to use, the PBS 10x Stock was diluted 10 times prior to autoclaving.

Potato Dextrose Agar (PDA)

This medium was prepared by adding 39 g Potato Dextrose Agar (Oxoid) to one litre Milli-Q water.

R2A Broth

Yeast Extract	0.5 g
Tryptone	0.25 g
Peptone	0.75 g
Dextrose	0.5 g
Starch	0.5 g
K_2HPO_4	0.3 g
$MgSO_4$	0.024 g
Sodium Pyruvate	0.3 g

If solid media was required, R2A Agar (Oxoid) was used.

Trypticase Soy Broth Agar (TSBA)

Trypticase Soy Broth	30 g
Agar	15 g

2.5 MICROBIOLOGICAL METHODS

2.5.1 Enrichment of PAH Degrading Bacteria

Contaminated soil for PAH degrading isolate enrichments was obtained from a former manufactured gas plant site in Glenelg, South Australia. The soil was taken from approximately 200 mm below the surface where discoloured soil with a strong hydrocarbon odour was encountered. 0.5 kg of soil was collected in 500 ml sterilised jars and stored at 4^{o}C until required. An analysis of the soil is shown in Table 3.1 (pg 137).

Uncontaminated soil for PAH degrading isolate enrichments was obtained from non-PAH exposed agricultural soil at Kanmantoo, South Australia. The soil was taken from a depth of 500 mm below the surface on the edge of area under cultivation. 0.5 kg of soil was collected in 500 ml sterilised jars and stored at 4^{o}C until required. An analysis of the soil is shown in Table 3.1 (pg 137).

Other enrichment sources were:

- the mound matrix from a termite mound;
- decaying wood within the termite mound structure; and
- Western Grey Kangaroo Faeces;

A minimum of 20 g (except for kangaroo faeces - of which 6 g was collected) of each enrichment source was collected in 250 ml sterile containers and stored at 4^{o}C until required. 20 g of each enrichment source was added to 100 mls PBS in 250 ml Schott Bottles and placed on a laboratory shaker for shaking overnight. Following shaking, the samples were left to settle for a minimum of 1 hour. 5 ml of the supernatant was then added to 45 ml of the following media:

- Basal Salts Medium;
- Basal Salts Medium + 0.05 g/l;
- Basal Salts Medium + 0.3 g/l Yeast; and
- R2A Broth.

All basal salts based media had either a mix of 4 PAHs (dibenzanthracene, benzo[*a*]pyrene, chrysene and fluoranthene – all at 50 mg/l) or 5 PAHs (fluorene – 100 mg/l, phenanthrene and pyrene – 250 mg/l, and fluoranthene and benzo[*a*]pyrene – 50 mg/l) added. Enrichments were incubated at 30^{o}C at 150 rpm for up to 10 weeks. These samples were further enriched using 5 ml of the original enrichment added to 45 ml BSMY, BSM or BSMG containing the 5 PAH enrichment suite.

Following incubation of the second enrichment, 1 ml from all bacterial enrichments was transferred to serum bottles containing 9 ml BSMY with pyrene (250 mg/l) or benzo[*a*]pyrene (50 mg/l) as a growth substrate.

2.5.2 Isolation of PAH Degrading Bacteria

Following enrichment, 0.1 ml of the bacterial enrichments were spread plated onto BSM, BSMY and BSMY^{3} plates containing either pyrene (250 mg/l) or benzo[*a*]pyrene (50 mg/l). Plates were incubated at 30^{o}C and routinely checked for PAH clear zones around colonies. Bacterial colonies showing clearing were transferred to BSMY plates containing pyrene (250 mg/l) until pure colonies were

isolated. Pure colonies were routinely subcultured onto BSMY plates containing pyrene or R2A plates.

2.5.3 Substrate Range of PAH Degrading Bacteria

20 µl of bacterial isolates were inoculated into 96 well Microtitre Plates containing 170 µl BSM and 10 µl of the particular substrate tested (Table 2.2). Substrates were added to a final concentration of 50 mg/l and made up in either BSM or DMF depending on solubility. Wells containing BSM without substrates and R2A media were used as controls. All substrates were sterilised prior to use by either 0.2 µm filtration or autoclaving.

Substrates used included:

- carbohydrates;
- potential PAH degradation metabolites or pathway intermediates;
- PAHs other than those tested in further experiments;
- various compounds commonly found in conjunction with PAHs in the environment (hydrocarbons, phenols); and
- compounds used in bioremediation projects (surfactants).

2.5.4 Preparation of Inocula

2.5.4.1 Bacteria

Bacterial inocula for degradation or growth experiments were prepared in 500 ml conical flasks containing 200 ml BSMY (or BSM for substrate range experiments) and pyrene (250 mg/l) as a carbon and energy source. Cultures were incubated at 30^{o}C at 150 rpm until the pyrene in the medium was cleared (usually 7-14 days).

Table 2.2: Range of Substrates Tested as Sole Carbon and Energy Sources for Bacterial Isolates

Substrate			
Carbohydrates	**Metabolites**	**PAHs**	**Miscellaneous**
Lactose	Succinate	Acenaphthylene	Triton X-100
Sucrose	Pyruvate	Anthracene	Tween 20
Maltose	Salicylic Acid	Benzanthracene	Tergitol 10
Arabinose	Catechol	Dibenzanthracene	Dowfax
Sorbitol	Protocatechuic acid	Chrysene	Cresol
d-Glucose	Maleic Acid	Naphthalene	pentachlorophenol
Mannitol	Cinnamic Acid	Benzo[*g,h,i*]pyrene	4-chlorophenol
Adonitol	d-pantothenic acid		2,4,5 trichlorophenol
Raffinose	phthalic acid		Tween 80
Rhamnose	benzoic acid		Benzene
			Toluene
			Xylene
			Pentane
			Hexane
			Octane
			Diesel

The evaluation of growth was undertaken using INT method (Juhasz, *pers. comm*.). 60 µl of a 10 mg/ml solution of INT was added to the wells and incubated at 30^{o}C. Growth was determined by the formation of a red colouration of varying intensity after 24 hours incubation compared to control wells containing only BSM or R2A.

Cells were harvested by centrifugation at 16770 *x g* for 10 minutes at 4^{o}C. Cell pellets were washed twice in 20 ml BSMY or BSM (depending on the medium used in the experiment to follow), re-centrifuged after each wash, then resuspended in 20 ml BSMY or BSM to achieve a 10-fold concentration in cell biomass.

2.5.4.2 Fungi

Fungal inocula for degradation experiments were prepared by growing the respective fungal isolate on PDA plates for 7-10 days. Following sufficient growth, 10 ml of MYPD was pipetted onto the plate and the plate gently agitated to suspend the spores. The spore suspension was then used to inoculate 200 ml sterile MYPD in a 500 ml conical flask. Inoculated flasks were incubated for 48 hours at 30^{o}C at 150 rpm. After 48 hours incubation, the mycelial pellets formed were filtered through Whatman #1 filter paper and washed twice using BSMY.

Killed bacterial and fungal inocula for experimental controls were prepared in the same manner above with the addition of 0.2% $HgCl_2$ to the cultures 24 hours prior to the commencement of the particular experiment.

2.5.5 Analysis of Biomass

2.5.5.1 Bacterial

Microbial numbers in liquid and soil cultures were determined by the most probable number technique. Ten-fold serial dilutions of soil and culture fluids were made in PBS to a dilution of 10^{-10}. Sterile media (180 µl) was dispensed into 96-well reusable microtitre plates (Greiner Bio-One) with disposable lids. Five replicate wells were inoculated with 20 µl the cell/soil suspensions. Plates

were incubated for 7-14 days, depending on the organism and media used. After incubation, growth was scored by observing the presence or absence of turbidity in wells. The viable count was estimated from the results using statistical tables (Cochran, 1950).

Some soil experiments were analysed by two Most Probable Number methods. 1 g of soil was diluted in 9 ml PBS, vortexed for 1 minute and serially diluted in 1 ml aliquots of PBS to a final dilution of 10^{-10}. A total soil bacterial MPN was determined using R2A medium and a PAH degrading bacteria MPN was determined as follows. PAH degrading bacterial numbers were determined using an adapted MPN method developed for the selective enumeration of PAH degrading bacteria. 10 µl of a PAH mix containing 1 g/l fluorene, anthracene and dibenzothiophene and 100 g/l phenanthrene (dissolved in pentane) was pippetted into microtitre plates and the pentane allowed to evaporate leaving a deposit of PAH crystals on the bottom of the well. 180 µL of BSMY was added to the well and was inoculated with 20 µL of the respective dilution. Positive wells developed a brown colour; the most probable number was estimated using statistical tables (Wrenn and Venosa, 1996).

2.5.5.2 Fungal

Dry weight was used to determine the fungal biomass in liquid culture experiments. Cultures were filtered using Whatman #1 filter paper that had been pre-dried at 105^{o}C. Mycelia on the filter paper was rinsed using Milli-Q water and dried at 105^{o}C for 24 hours and then weighed. Blanks containing only media and the respective PAH were filtered and dried in the same manner to determine their contribution to the final weight of the mycelia.

Increases in fungal biomass were also determined using a most probable number method similar to the method above for bacteria using MYPD + Rose Bengal

(0.3% w/v) as the medium. Positive wells were identified by obvious fungal growth.

2.6 IDENTIFICATION OF PAH DEGRADING BACTERIA

2.6.1 FAME Analysis

Bacterial cells were grown on Tryptic Soy Broth Agar plates (TBSA) (BBL, Australia) at 28ºC for 24h for faster growing isolates and 48 h for slower growing isolates before being harvested for analysis. FAME analysis was performed by Mr. Bruce Hawke, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Land and Water, Adelaide, as an initial identification of isolated strains. Briefly, extracts were prepared by saponification in NaOH in methanol, methylation in HCl in methanol and extracted into the organic phase using hexane in methyl-tert butyl ether (MTBE). Extracts were washed in dilute NaOH prior to analysis. Fatty acid profiles were compared with those of the MIDI Microbial Identification System database (MIDI, Delaware, USA).

2.6.2 16s rRNA Analysis

2.6.2.1 DNA Extraction

Bacterial isolates were grown on LB Agar plates at 30^{o}C for 48 hours. Single colonies were suspended in 200 µl PBS. Total DNA was extracted from the suspended bacterial cells using the DNeasy® Plant Mini Kit (Qiagen, California USA) as per the manufacturer's instructions.

2.6.2.2 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a Perkin Elmer Thermal Cycler PE 9700 (Perkin Elmer, USA). 16S ribosomal DNA was amplified using the following primer sets (Geneworks, Hindmarsh SA):

- fD1 (5'-AGA GTT TGA TCC TGG CTC AG 3') (Weisberg et al., 1991)
- rD1 (5'-AAG GAG GTG ATC CAG CC 3') (Weisberg et al., 1991)
- 27f (5' AGA GTT TGA TCM TGG CTC AC 3')
- 765r 5' TAC GGY TAC CTT GTT ACG ACT T 3')

Amplification using Primer Set fD1 – rD1 (Kanmantoo Isolate)

The amplification reaction was composed of: 10 mM Tris-HCL (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM dNTP; 200 uM of each respective primer (fD1 and rD1); 0.75 U Taq DNA polymerase (Roche) and 5 µl template DNA. Total reaction volume was 25 µl. Cycling conditions were: 1 cycle at 95^{o}C for 10 minutes (for activation of Taq); 35 cycles at 95^{o}C for 30 seconds, 47^{o}C for 30 seconds and 72^{o}C for 2 minutes and a final extension cycle at 72^{o}C for 10 minutes.

Amplification using Primer Set 27f – 765r (Buckle St Isolate and Kangaroo #1)

The amplification reaction was composed of: 37 µl sterile H_2O; 5 µl Taq Polymerase buffer with 2mM $MgSO_4$; 1 µl dNTP (at 100 mM); 2 µl of each respective primer (27f and 765r at 2 mM); 1 µl Taq DNA polymerase (5 U/µl) (NEB) and 2 µl template DNA. Total reaction volume was 50 µl. Cycling conditions were: 1 cycle at 95^{o}C for 2 minutes (for activation of Taq); 40 cycles at 95^{o}C for 30 seconds, 47^{o}C for 30 seconds and 72^{o}C for 2 minutes and a final extension cycle at 72^{o}C for 10 minutes.

2.6.2.3 Agarose Gel Electrophoresis of DNA and Visualisation

Agarose gel electrophoresis was used to detect PCR products. Gels were composed of 2% w/v agarose in 0.5x TBE buffer and run at 100V for 50-60 minutes in the same buffer using an EC105 power pack (E-C Apparatus Corporation). DNA samples were run alongside 5µl 1 kB DNA molecular weight marker (Promega) to estimate product size. Gels were stained with ethidium bromide. DNA was visualised using a Bio-Rad UV Transilluminator 2000. Photographs of agarose gels were taken using the Bio-Rad DigiDoc system (Bio-Rad Laboratories Pty. Ltd., Regents Park, NSW). Gel photographs were analysed using Adobe PhotoDeluxe.

2.6.2.4 PCR Product Purification and Sequencing

The PCR products obtained were purified using Promega (Madison, USA) Wizard ® PCR Preps DNA Purification System Kit (for fD1 and rD1) or MoBio UltraClean PCR clean up kit (Geneworks) (for 27f and 765r). The protocol for the Ultraclean PCR Clean Up Kit was modified slightly by using water instead of 1 mM Tris buffer to improve the elution step. Purified DNA samples were sequenced by the Flinders University of South Australia/Flinders Medical Centre DNA Sequencing Core Facility (Department of Haemotology, FMC, Adelaide). DNA was sequenced using both forward and reverse primers – fD1 and rD1 or 27f. Forward and reverse sequences were aligned manually and a partial consensus sequence obtained. The consensus sequence was entered into the National Centre of Biotechnology Information (NCBI) database using the nucleotide BLAST and the nearest relatives identified. Phylogenetic trees of bacterial strains were constructed using Phylip Neighbour Joining method on Ribosomal Database Project II (RDP).

2.7 MICROBIAL DEGRADATION OF PAHS

2.7.1 Degradation of PAHs in Liquid Culture

The following provides details of all liquid culture experiments using non-radiolabelled PAHs. Unless otherwise stated, experiments were conducted in 30 ml serum bottles capped with neoprene stoppers and sealed with aluminium crimp seals (Alltech, Australia). Unless otherwise stated, all experiments using basal salts medium supplemented with yeast, basal salts medium and distilled water experiments were supplemented with vitamins, trace elements and Mg/Ca solution. Liquid culture experiments were incubated at 30^{o}C and 150 rpm in a shaking incubator (Ratek, Victoria) in the dark. Aeration was provided using 0.2 μm filtered air for experiments running in excess of 14 days. All experiments were conducted in triplicate for each sample point. Experiments were sampled at day 0, 2, 4, 8, 11 and 14 for fluorene and phenanthrene, day 0, 2, 5, 10, 15 and 20 for fluoranthene and pyrene and day 0, 5, 15, 20, 35 and 50 (bacterial) and day 0, 30, 60 and 90 (fungal) for benzo[*a*]pyrene. Sampling was conducted as per the methods in Section 2.5.5.1 (Bacterial), 2.5.5.2 (Fungal) and 2.8.1.1 (PAH).

2.7.1.1 Axenic Bacterial

All liquid culture bacterial experiments were conducted using 9.8 ml of the basal salts medium with 0.05 g/l yeast extract. 100 μl of the respective PAH in DMF was added to give the desired final concentration (fluorene 100 μg/ml, phenanthrene and pyrene 250 μg/ml and fluoranthene and benzo[*a*]pyrene 50 μg/l). Culture fluids were inoculated with 100 μl of the bacterial inoculum (final cell population of approximately 10^6 cells/ml) prepared as per Section 2.5.4.1 and incubated at for up to 50 days.

2.7.1.2 Axenic Fungal

Fungal experiments were conducted using 9.8 ml of MYPD medium or basal salts medium. 100 µl of benzo[*a*]pyrene in DMF was added to give a final concentration of 50 µg/ml. Culture fluids were inoculated with 0.25 g (wet weight) of fungal mycelial pellets prepared as per Section 2.5.4.2 and incubated for up to 90 days.

2.7.1.3 Co-culture

Co-culture experiments were conducted in 9.9 ml of basal salts medium with 0.05 g/l yeast extract. 100 µl of a 5 PAH stock solution was added to give a final concentration of: 100 µg/ml fluorene, 250 µg/ml phenanthrene and pyrene and 50 µg/m1 fluoranthene and benzo[*a*]pyrene in DMF. Culture fluids were inoculated with 0.25 g (wet weight) fungal mycelial pellets or 100 µl of bacterial inoculum (Section 2.5.4) and incubated for 90 days.

2.7.2 Microbial Degradation of PAHs in Spiked Soil Microcosms

2.7.2.1 Soil

Uncontaminated soil for spiked PAH microcosm experiments was sourced from a field used for wheat cultivation in a farming area previously unexposed to PAHs in Kanmantoo, South Australia. A chemical analysis of the soil is shown in Table 2.2. The soil type (determined from the Handbook of Australian Soils) is a Solonised Brown Soil (formerly known as a Mallee Soil) or Calcarosol (Australian Soil Classification) which consists predominantly of calcium and magnesium carbonates. These soils generally have a low organic content. Particle size proportions ranges are: Coarse Sands (26-57%), Fine Sands (36-

51%), Silt (1%) and Clay (7-22%), Gravel (6%). The cation exchange capacity ranges between 3-9 (low), bulk density is 1.6 g/cm^3. Chemical and physical properties vary slightly with increasing depth (eg pH increases from 7 – 9).

Soil for microcosm experiments was 2 mm sieved during collection, immediately transferred to Flinders University and stored at 4^{o}C. An analysis of the soil was conducted to assess levels of PAHs, metals and nutried levels by Collex Laboratories (Kilburn, South Australia). Details of the analysis are provided in Table 3.1 (Section 3.2.2).

2.7.2.2 Soil Dry Weight and Water Holding Capacity

Soil water holding capacity was determined using Australian Standard 3743-1996 Method for the Measurement of Air Filled Porosity and Total Water Holding Capacity. A known volume of soil was saturated by alternating immersion in water for 10 minutes with 2-5 minutes drainage three times. The soil was allowed to drain for 30 minutes then placed in a pre-weighed heat resistant dish and weighed. The soil was then dried for 24 hours at 105^{o}C and the final dry weight was measured.

Water Holding Capacity (WHC) was determined using the following equation:

$$WHC = \frac{\text{(Mass of dish and soil wet – Mass of dish and soil dry) x 100}}{\text{Total Volume of Mix}}$$

2.7.2.3 Soil pH

10 g of air dried soil was sieved using a 2 mm sieve and placed in duplicate 100 ml Schott bottles. 25 ml of either Milli-Q water or a KCl solution was added, the bottle capped and shaken for 1 minute on a laboratory shaker. The bottle was allowed to stand for 1 hour to allow the sediment to settle, the solution re-stirred and the pH measured using a pH probe.

2.7.2.4 Determination of Soil Organic Matter Content

5 g of soil was placed in a ceramic crucible and weighed. The soil and crucible were heated in an oven overnight at 105^{o}C and reweighed. The dried soil was then placed in a furnace and heated at 375 ^{o}C or 850^{o}C overnight. The crucibles were then weighed to calculate the loss on ignition. The loss on ignition was determined by the following equation:

$$\text{LOI (\%)} = \frac{\text{(Weight following Ignition-Dried Weight)}}{\text{(Weight following Ignition -Initial weight)}} \times 100$$

Organic matter content was determined using standard curves (Ball, 1964) as below:

LOI at 375ºC	Organic Carbon = (0.458 x LOI %) – 0.40
LOI at 850ºC	Organic Carbon = (0.476 x LOI %) – 1.87

2.7.2.5 Amendments

A nutrient ratio of 100 C: 10 N: 1P (Alexander, 1999) was supplied to spiked soils prior to the commencement of degradation studies. A 1% solution of NH_4NO_3 was used to supply nitrogen and the basal salts medium provided sufficient phosphorus to meet the ratio requirements.

To determine the impact of nutrient provision on the effectiveness of sequential inoculation, an experiment was conducted using differing nutrient sources provided in conjunction with the inocula. The nutrient sources trialled were: basal salts medium supplemented with 0.05 g/l yeast extract and a 10% glucose solution added to provide a final glucose concentration of approximately 1%. Distilled water was used to assess the absence of nutrient addition. All nutrient solutions were supplemented with vitamin, trace element and Mg/Ca solutions.

2.7.2.6 Soil Spiking

Microcosms containing 100 g of 2 mm sieved soil from agricultural land previously unexposed to PAHs were inoculated with varying combinations of the test organisms. A 10 g portion of the soil was spiked with a solution containing a range of PAHs in DCM to give a final soil concentration of 50 mg/kg for fluoranthene and benzo[*a*]pyrene, 100 mg/kg for fluorene and 250 mg/kg for phenanthrene and pyrene. The DCM in the spiked soil was allowed to evaporate in a fume hood for 2 hours prior to being mixed in with the remaining soil.

The spiked soil was made up to 50% of water holding capacity using the appropriate medium, inocula and nutrient solution. Samples of soil were taken at regular intervals to determine microbial growth and PAH concentration. Microcosms were incubated at 25^{o}C in the dark and stirred for aeration at 20 day intervals.

2.7.2.7 Inoculation

Bacterial, fungal or co-culture inocula were prepared as per Sections 2.5.5.1 and 2.5.5.2 and added to soil amendments used to make up the appropriate water holding capacity for microcosm experiments. Bacterial inoculum was added to give a final cell concentration of approximately 10^6 cells/g. Fungal inoculum was added to give a final concentration of 25 g/kg (wet weight) of fungal mycelial pellets.

To determine the importance of inoculation protocol on PAH degradation, fungus and bacteria were incubated either concurrently or in sequence (either fungi prior to bacteria or vice versa). Sequential inoculation was undertaken at the 50 day mark of the incubation period.

Soil samples were taken at day 0, 20, 40, 50 (in the case of sequential inoculation), 60, 80 and 100. Samples were analysed for microbial biomass using the methods contained in Section 2.5.5 and for PAH concentration as per the method in Section 2.8.1.2.

2.7.3 Mineralisation of Pyrene and Benzo[a]pyrene in Liquid Culture and Soil Microcosms

2.7.3.1 Liquid Culture

All bacterial isolates were assessed for their ability to mineralise [4, 5, 9, 10-^{14}C] pyrene (55 mCi/mmol) (Sigma-Aldrich) in duplicate biometer flasks (Konte). Unlabelled pyrene was added to 20 ml of BSMY at a concentration of 250 μg/ml and 10 μl of [4, 5, 9, 10-^{14}C] pyrene diluted in DMF was added. 200 μl of bacterial inoculum prepared as per Section 2.5.4.1 was inoculated and the cultures were incubated at 30^{o}C at 150 rpm for 10 days. Samples were taken at 2, 8 and 24 hours then daily for 10 days. Fungal isolates were assessed for their ability to mineralise 7-^{14}C benzo[*a*]pyrene in duplicate biometer flasks. Unlabelled benzo[*a*]pyrene was added to 20 ml basal salts medium, MYPD and distilled water at a concentration of 50 μg/ml and 10 μl of 7-^{14}C benzo[*a*]pyrene was added. The cultures were incubated for 90 days in MYPD and BSM and routine samples were taken at 0, 30, 60 and 90 days. $^{14}CO_2$ evolution and distribution of ^{14}C in the culture medium, biomass and gaseous phase was determined as per Section 2.8.3.

2.7.3.2 Degradation of Radiolabelled Benzo[a]pyrene in Spiked Soil Microcosms

Experiments to determine the extent of benzo[*a*]pyrene mineralisation in spiked soils were conducted in 250 ml conical flasks using 100 g of spiked soil prepared under the conditions mentioned previously. 10 μl of 7-^{14}C benzo[*a*]pyrene (26.6

mCi/mmol) diluted in DMF was added to the solution containing the inoculum and medium to make up water holding capacity prior to addition to the soil. A 5 ml test tube (Gibco) containing 4 ml of 0.1M NaOH was suspended in the flask to trap $^{14}CO_2$ evolved during the experiment. Routine samples were taken as per the method in Section 2.7.1.4. The flasks were sealed with a No. 52 suba seal and parafilm and statically incubated at 30^{o}C in the dark for 100 days.

2.8 ANALYTICAL METHODS

2.8.1 Extraction of PAHs from Culture Medium

2.8.1.1 Liquid

Prior to extraction, an internal standard (benzo[b]fluorene, 0.1 ml of 1000 μg/ml) was added to the culture fluid in each serum bottle. Each extraction consisted of addition of 3 ml of DCM, vigorous shaking of the serum bottles and sonication for 30 minutes. This was repeated 3 times, with the culture fluid and DCM being frozen (- 20ºC) between each extraction step to facilitate the separation of the organic and aqueous phases. The DCM was removed from the serum bottles after thawing at room temperature. The rigorous extraction procedure was required to ensure recovery of all PAH from the serum bottles. Approximately 1 ml of the DCM extract was then filtered and dried through a column containing anhydrous sodium sulphate and glass wool into a 2.0 ml GC vial (Varian) and stored at –20ºC before analysis by GC-FID (Boonchan, 1998).

2.8.1.2 Soil

Recovery of PAHs from spiked soil was achieved using an ultrasonic extraction method. 1 g (w/w) of soil was mixed with 1 g anhydrous sodium sulphate in a Teflon centrifuge tube (Nalgene, New York). 100 μl of internal standard (1000

µg/ml benzo[b]fluorene) was added to the samples prior to the addition of 5 ml DCM. The sample was then vortex mixed for 30 seconds and stored on ice to chill. Samples were then sonicated for a total of 4 minutes in 5 second pulses using a Ultraheat Systems XL 2020 Ultrasonicator with a a 1/8 inch tapered microtip attached to a ½ inch horn. Following ultrasonication, samples were stored at -20^{o}C overnight to allow settling of soil particles. Approximately 1 ml of the DCM extract was then filtered and dried through a column containing anhydrous sodium sulphate and glass wool into a 2.0 ml GC vial (Varian) and stored at –20ºC before analysis by GC-FID (Juhasz, *pers. comm*.).

2.8.2 Determination of PAH Concentration by Gas Chromatography

PAH concentration in liquid and soil DCM extracts was determined using a Varian Star CP-3800 Gas Chromatograph equipped with a flame ionisation detector. The following conditions were standard for all analyses:

Column:	EC-5 ECONO-CAP capillary column (30 m x 0.25 mm, Alltech, Australia)
Carrier Gas:	Nitrogen
Injector Temperature:	320ºC
Detector temperature:	330ºC
Column Flow Rate:	1.5 ml/minute
Split Flow Ratio:	Splitless

For analysis of PAH extracts, the oven temperature was programmed at 200^{o}C for 1 minute, followed by a linear increase of 10^{o}C/minute to 300^{o}C and held for 10 minutes.

The concentration of PAHs was calculated using benzo(b)fluorene (1000 µg/ml) as the internal standard. Standard solutions (1 ml) were prepared with the

internal standard and PAHs ranging in concentration from 10 – 300 mg/ml. Standards were analysed by GC-FID and the peak ratio of the PAHs to the internal standard was calculated. The concentration of PAHs versus the peak ratio was plotted and a line of best fit obtained (R^2= >0.95). The concentration of the PAH was calculated using Varian Star Chromatography Workstation Vs.6.2 which uses the ratio between the PAH and internal standard peaks and the respective PAH line of best fit.

2.8.3 Detection of Radioactivity

The $^{14}CO2$ from radiolabelled experiments using [4,5,9,10-^{14}C] pyrene and [7-^{14}C] benzo[*a*]pyrene was collected in a 0.1M NaOH trap (5 ml). At the appropriate sampling time, the entire volume of NaOH was removed for analysis and replaced by fresh NaOH. At the completion of the liquid media experiments, 10M HCl (0.5 ml) was added to the medium to liberate any residual $^{14}CO2$. Duplicate 1 ml aliquots of the NaOH were added to 9 ml Readysafe liquid Scintillation cocktail (Beckman-Coulter, USA) and analysed for radioactivity on a Beckman LS 3801 Scintillation Counter using the following conditions:

Measuring Parameters:	^{14}C
Count Time:	300 seconds
Repeats:	2
Cycles:	2
Replicates:	2

To obtain a mass balance of radioactivity remaining, the culture fluid was centrifuged at 5,000 rpm for 10 minutes. Duplicate 1 ml aliquots of the culture supernatant were removed and added to 9 ml Readysafe scintillation cocktail for analysis of radioactivity. Undegraded PAH was removed from the culture fluid by extraction with DCM (5 ml) and duplicate 1 ml aliquots were added to 9 ml Readysafe liquid scintillation cocktail for analysis of radioactivity. The cell

debris was resuspended in 5 ml Milli-Q water and 1 ml duplicate aliquots of the cell suspension were added to 9 ml Readysafe liquid scintillation cocktail for analysis of radioactivity.

CHAPTER 3

ENRICHMENT AND ISOLATION OF PAH-DEGRADING ORGANISMS FROM CONTAMINATED AND NON-CONTAMINATED ENVIRONMENTS

3.1 INTRODUCTION

Traditionally, organisms for bioremediation purposes have been sourced from environments contaminated with the target pollutant(s) on the assumption that there is a better likelihood for these organisms to have potential to degrade these compounds. This has been undertaken in numerous studies on a variety of compounds including: PAHs (Kanaly *et al*., 2000; Boonchan *et al*., 2000; Juhasz *et al*., 1998; Ghiorse *et al*., 1995; Kazunga and Aitken, 2000); nitroaromatic compounds (Snellinx *et al*., 2003) and spent metal working fluids (van der Gast *et al*., 2002). It is thought that the contaminated environment exerts a selective pressure that selects for organisms with the metabolic capability to degrade the target compound as a carbon and energy source and that the likelihood of isolating an organism with degradative capabilities from these sources is greater (Margesin *et al.*, 2003).

The isolation of organisms for the degradation of "xenobiotics" from environments that have previously had little or no exposure to a target compounds is less usual and may be more appropriate in instances where the target compound is not a potential carbon source (Juhasz and Naidu, 2000). As many of the organisms that achieve degradation have an ecological role in uncontaminated environments such as the degradation of lignin, their distribution in the environment is widespread. Consequently, potential sources in the natural environment for organisms with degradative capabilities are likely to be those where lignin degradation occurs (for example, leaf detritus and the gastrointestinal tract of herbivores). In the work presented here, the sources of bacterial isolates from uncontaminated environments were selected on this basis.

Following enrichment, organisms are usually isolated using the target compounds. This is achieved by testing growth and removal of the target compound on liquid and solid basal salts media containing the target compound

as a sole carbon source and supplements for growth and degradation. A variety of methods have been developed for assessing degradative capabilities, usually indicated by the formation of zones of clearing around colonies or the clearing of medium containing the compound. Such methods include spraying an ethereal solution of the target compound onto or incorporating the target compound into mineral salts agar on which the organism of interest has been inoculated (Kiyohara *et al*., 1982)

The identification of microorganisms once isolated was formerly dependent exclusively on the assessment of phenotypic properties from the results of physiological and biochemical tests and morphology on differing media. This approach has given way to more definitive phenotypic approaches such as Fatty Acid Methyl Ester (FAME) analysis or genotypical analysis. rDNA (ribosomal DNA) approaches are now routinely used to determine the identity of microorganisms and more recently, community structures (Macrae, 2000).

As xenobiotics are rarely, if ever, found in isolation in the environment an assessment of the capability of the respective isolates to survive and grow in the presence of a range of compounds is necessary. In the work presented in this chapter, isolates were tested against a range of compounds, including carbohydrates, hydrocarbons, PAHs, surfactants and cresols. The selection of the various substrates was made on the basis of the bacterial isolates demonstrating the ability to grow in the presence of: pollutants commonly found in contaminated environments in conjunction with PAHs (hydrocarbons, phenols), intermediates in the various pathways for the metabolism of PAHs or compounds used in bioremediation processes to enhance growth (carbohydrates) or the bioavailability (surfactants) of PAHs in soils.

The general aims of the research presented in this chapter were to:

1. Enrich bacteria able to grow on PAH compounds from PAH contaminated sources (manufactured gas plant soil) and non-PAH contaminated sources (Kanmantoo agricultural soil, termite mound matrix and kangaroo faeces);

2. Isolate pure cultures from the PAH enrichments with the ability to grow on PAH compounds;

3. Determine the range of substrates able to be utilized by the bacterial isolates for growth; and

4. Identify the PAH degrading bacteria at the genus and species levels using FAME analysis and 16S rRNA gene sequencing.

3.2 ENRICHMENT FROM VARIOUS ENVIRONMENTAL SOURCES

The use of a number of environmental sources, both contaminated and uncontaminated was undertaken to provide a number of alternative bacterial isolates to test the coculture process. As the previous development of a coculture relied on the use of two organisms that had not been isolated from the same source, it was considered that potential coculture components could be collected from both contaminated and non-contaminated sources.

3.2.1 Manufactured Gas Plant Soil

A number of soil samples were taken from the site of a former manufactured gas plant in Glenelg, South Australia. The production of gas from coal commenced in 1870 on the site (Figure 3.1) and all structures comprising the manufactured gas plant were removed in the 1960's. Since that time, the site has been used as a Council Depot and only temporary structures remain. Soil was taken from three locations on the site: the southern fence line, northern edge of the former gas holder and the south western corner of the site, near the former tar-pit (Figure 3.2). All samples were taken at a depth of 0.5 m where the soil was sandy and had a strong hydrocarbon odour. Lenses of tarry material, broken brick and other debris were dispersed throughout. An analysis of the soil is provided in Table 3.1.

3.2.2 Uncontaminated Agricultural Soil

Soil was collected from the boundary of a field used for the cultivation of wheat and pea straw in Kanmantoo, South Australia (Figure 3.3). The field has always been used for agriculture and no industrial activity is undertaken in the near vicinity. The soil is silty and mixed with small rock particles. A small amount of organic matter (roots and other woody material) was present in the soil. Soil was collected from a depth of 0.2 m. An analysis of this soil has been provided in Table3.1.

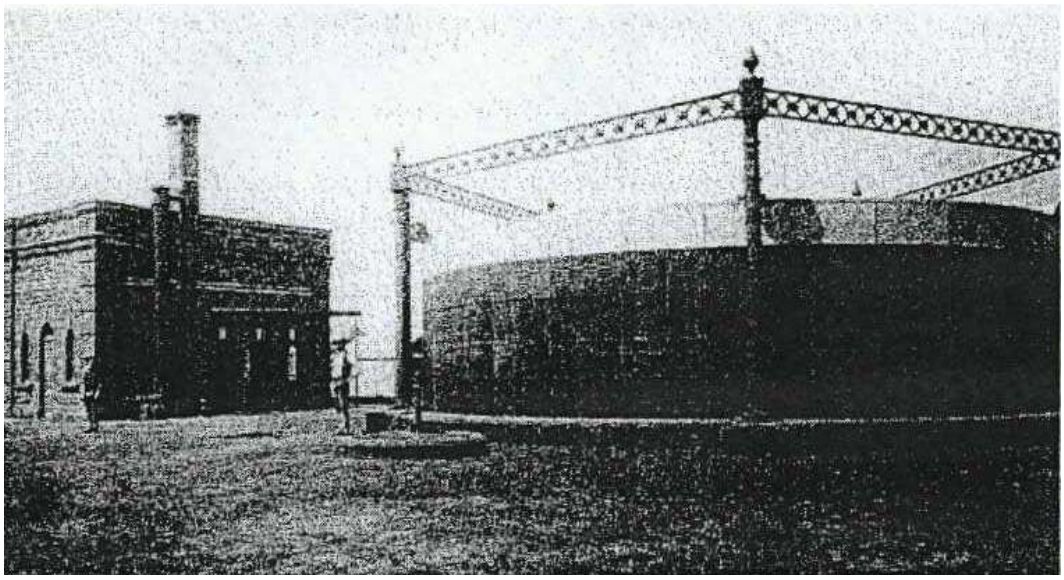


Figure 3.1: Photograph of the former Manufactured Gas Plant at Buckle Street, Glenelg (c. 1900) showing gas holder structure and site building (photograph taken looking west - north-west).

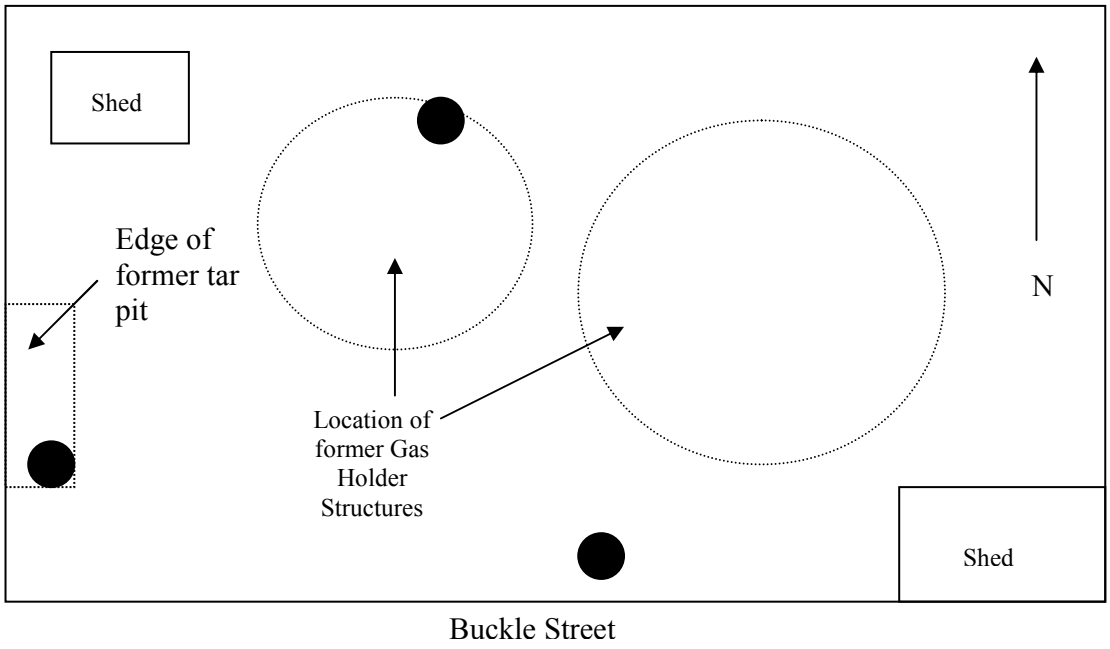


Figure 3.2: Schematic diagram of former Manufactured Gas Plant at Buckle Street, Glenelg. Gas holder structures and other site structures are no longer visible (indicated by dotted line), existing structures (indicated by black line). ● indicated locations where soil was sampled for this work.

Table 3.1: Analysis of Soils used for Enrichments (Former Manufactured Gas Plant Soil and Kanmantoo Soil) and Microcosm Trials (Kanmantoo Soil).

Analyte	Kanmantoo Soil	Former Manufactured Gas Plant Soil	Relevant Levels		
			Health [a]	Eco [a]	Backgrnd [b]
Nutrients - % (w/w)					
Total Kjeldahl Nitrogen	0.16	0.42	-	-	*0.01 – 0.10 (0.04 - 0.01)*
Boron	<0.001	<0.001	3000	-	-
Calcium	0.18	0.44	-	-	-
Copper	<0.01	<0.01	-	-	-
Iron	2.2	2.7	-	-	-
Potassium	0.47	0.22	-	-	*0.18 – 0.67*
Magnesium	0.38	0.20	-	-	-
Manganese	0.03	0.02	1500	500	800
Sodium	0.02	0.02	-	-	-
Phosphorus	0.02	0.02	-	-	*0.02 (0.01)*
Sulphur	<0.01	0.41	-	600	*0.01*
Zinc	<0.01	0.02	-	-	-
Heavy Metals – mg/kg					
Arsenic	<1.0	4.8	100	20	1-50
Cadmium	<1.0	<1.0	20	3	1
Chromium	<1.0	77	100	1	-
Copper	<1.0	49	1000	100	2-100
Lead	<1.0	11000	300	600	2-200
Mercury	<1.0	<1.0	10	-	-
Molybdenum	20	11	<1.0	-	-
Nickel	<1.0	38	600	60	5-500
Zinc	<1.0	200	7000	200	10-300
PAHs – mg/kg					
Naphthalene	<0.10	32	-	-	-
Acenaphthylene	<0.10	9.0	-	-	-
Acenaphthene	<0.10	6.3	-	-	-
Fluorene	<0.10	12	-	-	-
Phenanthrene	<0.10	52	-	-	-
Anthracene	<0.10	43	-	-	-
Fluoranthene	<0.10	64	-	-	-
Pyrene	<0.10	120	-	-	-
Benz(a)anthracene	<0.10	110	-	-	-
Chrysene	<0.10	90	-	-	-
Benzo(b)fluoranthene	<0.10	120	-	-	-
Benzo(k)fluoranthene	<0.10	79	-	-	-
Benzo[*a*]pyrene	<0.10	94	1	-	-
Indeno(1,2,3-CD)pyrene	<0.10	180	-	-	-
Dibenz(a,h)anthracene	<0.10	10	-	-	-
Benzo(g,h,I)perylene	<0.10	70	-	-	-
Total PAH	<0.10	1100	20	-	-
Total Petroleum Hydrocarbons	-	7800	-	-	-

- = No level

[a] = Health and Ecological Investigation Levels from the National Environment Protection (Assessment of Site Contamination) Measure - Schedule B (1) Guideline on Investigation Levels for Soil and Groundwater

[b] = *Italicised text* refers to general ranges for Solonized Brown Soils/Calcarosol and are applicable to Kanmantoo soil only. Values in parentheses are ranges from a Solonized Brown Soil/Calcarosol from NSW

3.2.3 Termite Mound

A portion was removed from the western side of a termite mound that had been constructed around the decaying stump of a eucalyptus tree (Figure 3.4). The mound was active, with areas of recent mound development and the presence of a number of termites in the portion of the mound collected. Any termites remaining in the mound matrix were removed prior to the enrichment process.

3.2.4 Kangaroo Faeces

Six pellets (of a total wet weight of approximately 20 g) of fresh kangaroo faeces were collected from the soil surface at Upper Sturt, South Australia (Figure 3.5). The faeces were from a Western Grey Kangaroo (*Macropodus fuliginosus*) that is locally abundant in the Upper Sturt area.

3.2.5 Broth Enrichments

Following initial shaking, the respective soil samples were allowed to settle prior to 5 ml of the supernatant being removed to begin broth enrichments. Samples were initially enriched using 45 ml of either a 4 PAH mixture (dibenzanthracene, anthracene, benzo[*a*]pyrene, chrysene and fluoranthene all at 50 mg/l) or 5 PAH mixture (fluorene – 100 mg/l, phenanthrene – 250 mg/l, fluoranthene – 50 mg/l, pyrene – 250 mg/l and benzo[*a*]pyrene – 50 mg/l) in BSM, BSMY and BSMY^3. The enrichments were incubated over one month and routinely checked for evidence of growth or transformation of the PAH mixtures (Table 3.2). Following this initial phase of enrichment with a PAH mixture, the enriched samples were inoculated into BSMY and pyrene or BSMY and benzo[*a*]pyrene.

Figure 3.3: Kanmantoo agricultural land used for the cultivation of pea straw and wheat. Soil from this location was used in enrichment experiments and as a medium for soil based experiments. Arrow indicates the location from which soil was collected.

Figure 3.4: Cross section of termite mound matrix used for enrichments.

Figure 3.5: Western Gray Kangaroo (*Macropodus fuliginosus*) faeces used for enrichments.

Whilst no change was observed in BSMY and benzo[*a*]pyrene enrichments, clearing of the media was observed to occur in BSMY and pyrene enrichments. The enrichment from the termite mound, kangaroo faeces and the manufactured gas plant soil were all found to produce clearing in the media resulting in a golden – yellow colour change in the media. Each came from a different initial enrichment, with the termite mound from BSM with the 5 PAH mixture, the Kangaroo faeces from the BSM and 4 PAH mixture and the manufactured gas plant soil enrichment and Kanmantoo soil isolate from the BSMY and 5 PAH mixture. The manufactured gas plant soil enrichment was observed to form a golden "bread-crumb" like biomass in the BSMY and pyrene enrichments after 7-10 days incubation.

3.3 ISOLATION OF BACTERIAL ISOLATES ON POLYCYCLIC AROMATIC HYDROCARBONS

Following enrichment, each solution (with multiple PAHs and single PAHs) was plated on BSMY agar with pyrene or BSMY agar with benzo[*a*]pyrene and R2A agar at various dilutions. Numerous white colonies were observed on plates at lower dilutions although there were not associated with the formation of zones of clearing.

Two colonies producing zones of clearing on BSMY and pyrene plates were observed from enrichments of the manufactured gas plant soil. These two colonies were transferred to new BSMY and pyrene (250 mg/l) plates and R2A agar. One formed slow growing orange colonies on R2A agar, the other formed small crenalated light brown colonies. Subsequent plating achieved the isolation of a single colony that was inoculated into BSMY and pyrene broths to confirm its ability to degrade pyrene in liquid culture. Further confirmation of pyrene degrading ability was achieved through the inoculation of the orange coloured colonies onto BSMY and pyrene plates (Figure 3.6). Inoculation into broths

Table 3.2: Summary of observations from enrichment process for environmental isolates

Enrichment Source	Enrichment Medium	Observations
Manufactured Gas Plant Soil	4 PAH Mix (BSMY)	Clearing Colour change in medium (golden/yellow) "bread crumb" biomass formed
Kanmantoo Soil	5 PAH Mix (BSMY)	Clearing
Termite Mound Matrix	5 PAH Mix (BSM)	Clearing Colour change in medium (golden/yellow)
Kangaroo Faeces	4 PAH Mix (BSM)	Clearing Colour change in medium (golden/yellow)

resulted in the formation of the same golden colouration in the media and "bread crumb" like biomass observed in the initial phases of the enrichment process. The inoculation of the light brown colonies failed to achieve the degradation of pyrene in pyrene broths and was not further investigated.

The isolation of pyrene degrading bacteria from the other environmental sources followed a similar course, with isolation being achieved through repeated plating on BSMY and pyrene and the transfer of colonies that formed zones of clearing onto both BSMY and pyrene plates and R2A. Pyrene degrading ability was confirmed through the clearing of the media following inoculation into BSMY and pyrene broths.

The isolate from the manufactured gas plant soil was the only isolate shown to form zones of clearing around the small colonies grown on BSMY plates containing benzo[*a*]pyrene (Figure 3.7). This highly encouraging result prompted the repeated testing of this isolate in both broths and BSMY plates containing benzo[*a*]pyrene. Whilst some zones of clearing were formed when further plated, the isolate did not achieve degradation of benzo[*a*]pyrene in broths, even when pregrown on benzo[*a*]pyrene plates. The other isolates did show some growth on BSMY and benzo[*a*]pyrene plates, although none produced zones of clearing.

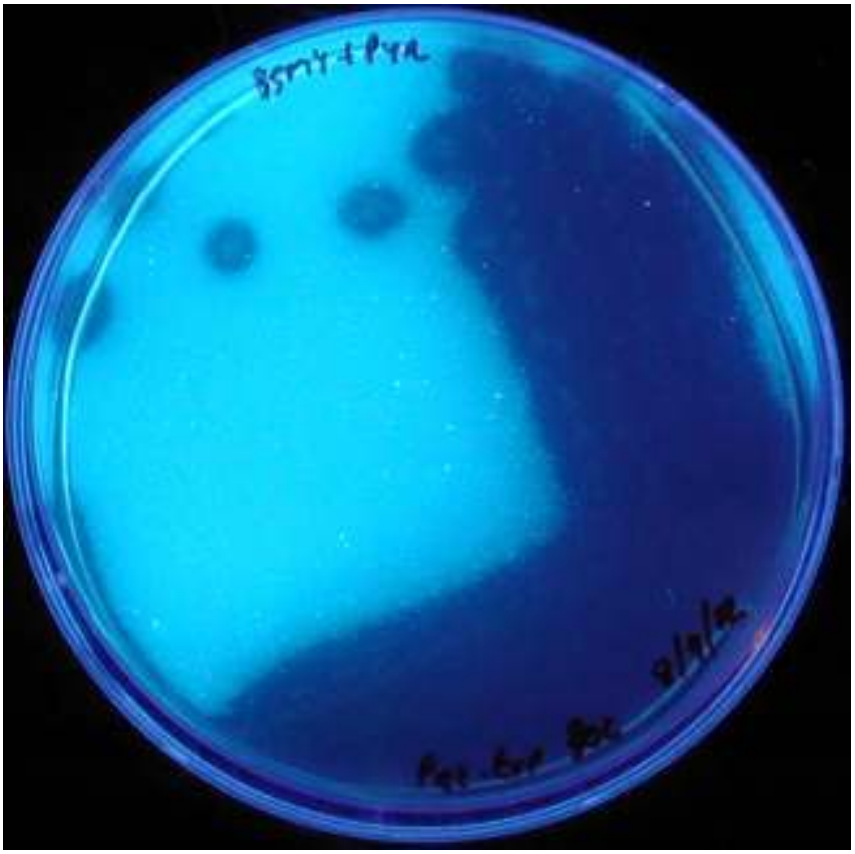


Figure 3.6: Example of zones of clearing formed around colonies isolated from manufactured gas plant soil enrichments on BSMY agar plates containing 250 mg/l pyrene. The blue fluorescence is produced by the pyrene in the agar when placed on a UV transilluminator (302 nm).

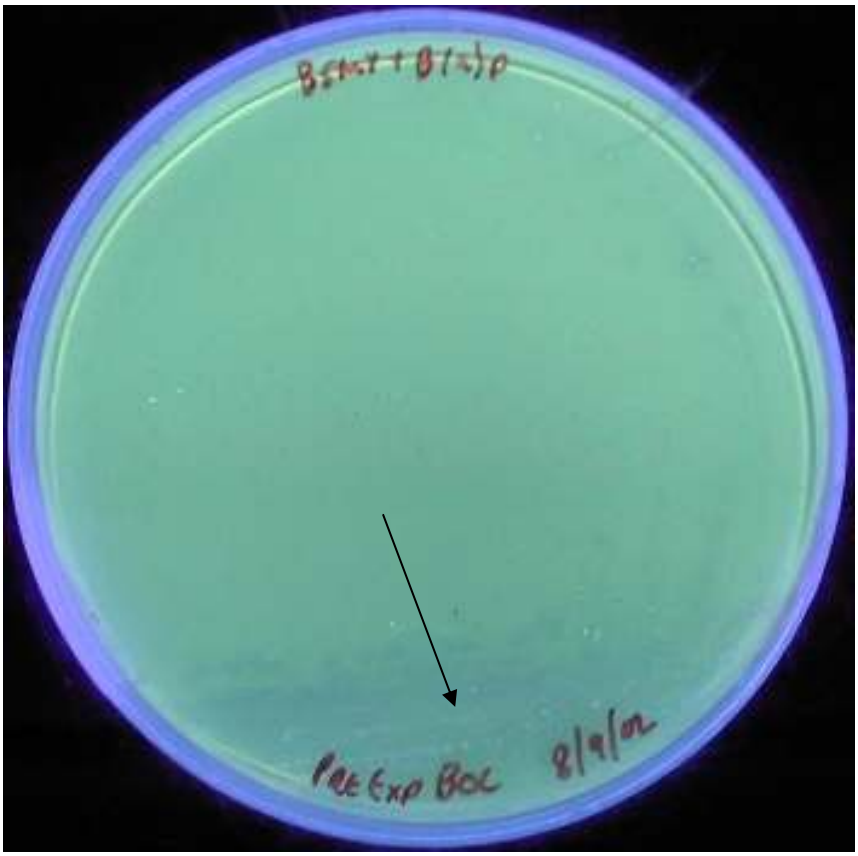


Figure 3.7: Zones of clearing formed around colonies isolated from manufactured gas plant soil enrichments on BSMY agar plates containing 50 mg/l benzo[*a*]pyrene. The green fluorescence is produced by the benzo[*a*]pyrene in the agar when placed on a UV transilluminator (302 nm). Arrow indicates zones of clearing around colonies in the densest streak on the plate.

3.4 IDENTIFICATION AND CHARACTERISATION OF PAH DEGRADING ORGANISMS

3.4.1 Morphology and Substrate Utilisation

The various morphologies of the bacterial isolates during growth on R2A agar are shown in Table 3.3.

All bacterial isolates tested were able to utilize a broad range of carbohydrates (Table 3.4). Utilization of the potential metabolites tested was generally similar between isolates with protocatechuic acid, cinammic acid, d-pantothenic acid and phthalic acid not being utilized by the isolates. The Kangaroo #2 isolate utilized maleic acid, which was unable to be utilized by the other isolates tested.

Greater variation between isolates was observed in substrate utilization experiments using surfactants, phenols, hydrocarbons and PAHs (Table 3.5). The Kanmantoo bacterial isolate and manufactured gas plant isolate had the broadest ability to utilize surfactants, although the best growth in the presence of surfactants was observed by the Kangaroo #1 isolate in the presence of Tergitol NP-10. None of the isolates utilized phenols. All bacterial isolates showed a broad ability to utilize hexadecane, heptane, hexane and diesel. Most were generally able to utilize benzene and toluene, although the Kanmantoo isolate was the only isolate able to utilize xylene to any extent. No isolates were able to utilize acenaphthylene and naphthalene. The Kangaroo #2 isolate had the ability to degrade the broadest range of PAHs and both Kangaroo Isolates showed an ability to utilize benzo[*g,h,i*]perylene.

Table 3.3: Morphology of the Bacterial Isolates. Description of colony morphology is based on growth on R2A after 48 hours.

Characteristic	Manufactured Gas Plant Isolate	Kanmantoo Soil Isolate	Termite Mound Isolate	Kangaroo Faeces Isolate #1	Kangaroo Faeces Isolate #2
Gram Stain	+	+	-	+	-
Growth on R2A[a]	Low	Med	High	Low	High
Growth on Nutrient Agar[a]	Low	Med	High	Low	High
Size (mm)	< 2	< 2	2	2	2
Shape	Circular	Circular	Circular	Circular	Circular
Elevation	Convex	Convex	Convex	Umbonate	Umbonate
Surface	Smooth / mucoid	Smooth / mucoid	Smooth / dull	Smooth / mucoid	Dry/rough
Edge	Entire	Entire	Entire	Entire	Lobate
Pigent	Orange/Yellow	Yellow	Cream	Cream	Cream
Opacity	Opaque	Opaque	Translucent	Translucent	Translucent

[a] = Determined by extent of colony growth on streaks (low = growth only on first streak, medium = growth on second streak, high = growth on third and fourth streak).

Table 3.4: Growth of Bacterial Isolates on various carbohydrates and metabolites. 10 µl of the respective compound was added to 170 µl BSM in a 96-well microtitre plate as the sole carbon and energy source at a concentration of 50 mg/l and inoculated with 20 µl of the particular bacterial strain. Microorganisms were incubated for 24 and 48 hours at 30^{o}C. Growth was indicated by the intensity of a red colouration formed after the addition of 60 µl of INT and compared to R2A and BSM controls (refer Section 2.5.3).

Substrate	***Manu. Gas Plant Isolate***	***Kanmantoo Isolate***	***Termite Mound Isolate***	***Kangaroo #1 Isolate***	***Kangaroo #2 Isolate***
CARBOHYDRATES					
Lactose	+++	+++	+++	+++	+++
Sucrose	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++	+++
Arabinose	+++	+++	+++	+++	+++
Sorbitol	+++	+++	+++	+++	+++
Adonitol	+++	+++	+++	+++	+++
Mannitol	+++	+++	+++	+++	+++
Rhamnose	+++	+++	+++	+++	+++
Raffinose	+++	+++	+++	+++	+++
Mannose	+++	+++	+++	+++	+++
Glucose	+++	+++	+++	+++	+++
METABOLITES					
Succinate	++	++	+++	+++	+++
Pyruvate	++	++	+++	+++	+++
Salicylate	++	++	+++	+++	+++
Catechol	+	++	+++	+++	+++
Protocatechuic Acid	-	-	-	-	-
Maleic Acid	-	-	-	-	++
Cinammic Acid	-	-	-	-	-
d-pantothenic acid	-	-	-	-	-
phthalic acid	-	-	-	-	-
Sodium Benzoate	++	+++	++	+++	+++
p-aminobenzoic acid	++	++	++	+++	++
3-chlorobenzoate	-	+	+	++	-

- = no growth + = low growth ++ = medium growth +++ = high growth

Table 3.5: Growth of Bacterial Isolates on various surfactants, phenols, hydrocarbons and PAHs. Compounds were added to 170 µl BSM in a 96-well microtitre plate as the sole carbon and energy source at a concentration of 50 mg/l and inoculated with 20 µl of the particular bacterial strain. Microorganisms were incubated for 1 and 24 hours at 30^{o}C. Growth was indicated by the intensity of a red colouration formed after the addition of 60 µl of INT and compared to R2A and BSM controls (refer Section 2.5.3).

Substrate	***Manu. Gas Plant Isolate***	***Kanmantoo Isolate***	***Termite Mound Isolate***	***Kangaroo #1 Isolate***	***Kangaroo #2 Isolate***
SURFACTANTS					
Triton X-100	++	++	-	+	-
Tween 20	++	++	-	+	-
Tween 80	++	+	+	-	-
Tergitol NP-10	-	++	++	+++	-
Dowfax	-	-	-	-	-
PHENOLS					
Cresol	-	-	-	-	-
Pentachlorophenol	-	-	-	-	-
4-chlorophenol	-	-	-	-	-
3-chlorophenol	-	-	-	-	-
2,4,5-trichlorophenol	-	-	-	-	-
HYDROCARBONS					
Benzene	++	++	++	++	+++
Toluene	++	++	++	++	++
Xylene	+	++	-	+	-
Hexadecane	+++	+++	+++	+++	+++
Heptane	+++	+++	+++	+++	+++
Hexane	+++	+++	+++	+++	+++
Diesel	+++	+++	+++	+++	+++
PAHs					
Naphthalene	-	-	+	-	-
Acenaphthylene	-	-	-	-	-
Anthracene	++	-	++	+	++
Benzanthracene	++	++	++	++	++
Dibenzanthracene	++	++	++	++	++
Benz[*e*]acenaphthylene	-	++	-	+	++
Benzo[*g,h,i*]perylene	-	-	++	+++	+++
Chrysene	-	++	+	+	++

- = no growth + = low growth ++ = medium growth +++ = high growth

3.4.2 Fatty Acid Methyl Ester (FAME) Analysis

Only the two gram negative bacterial isolates isolated from the termite mound and kangaroo faeces were successfully identified using FAME analysis. Both organisms grew to levels sufficient for FAME analysis during the required incubation period (24-48 hours), producing confluent growth on three of four streaks and well defined and sufficiently large colonies on the fourth streak. The other gram positive isolates did not grow sufficiently in the desired period to provide enough colonies for effective identification. Attempts to concentrate extracts did not yield sufficient material for identification. Modified methods are available for idenfication of gram positive isolates that are likely to be *Mycobacterium* sp. utilizing alternative media (Middlebrook 7H10 culture media and glycerol) and an increased incubation time and temperature. The isolates not identified using FAME analysis were not subjected to this alternative FAME analysis as the 16SrRNA method of identification was also being used.

The bacterial isolate from the termite mound matrix grew well on TBSA over 24 hours. FAME analysis of the gram negative bacterial isolate from the termite mound (Figure 3.8) obtained a 0.694 similarity to *Ralstonia picketii*.

The gram negative bacterial isolate from kangaroo faeces (Kangaroo #2) grew the best on TBSA of all the bacterial isolates over 24 hours. FAME analysis (Figure 3.9) obtained a 0.672 similarity to *Stenotrophomonas maltophilia*.

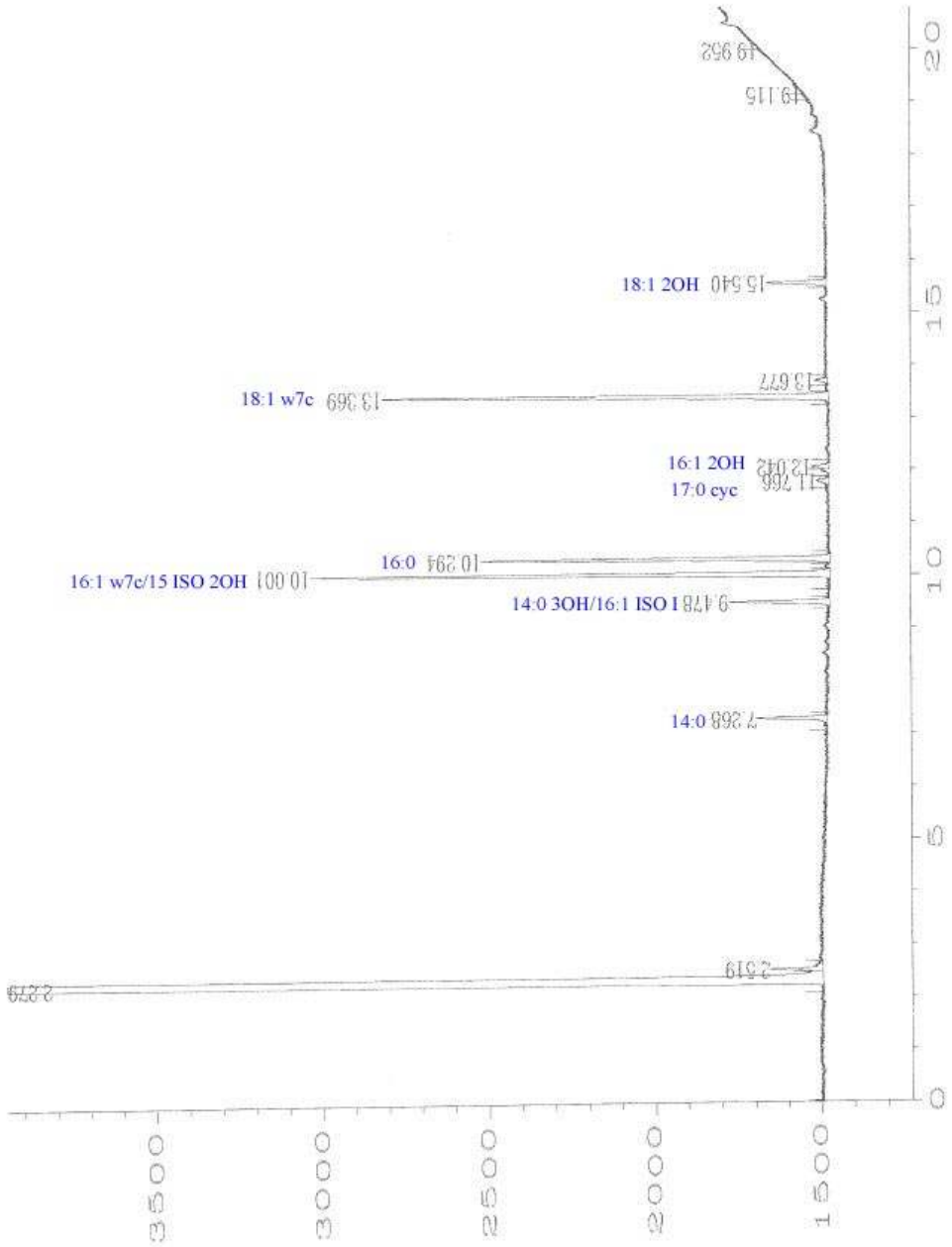


Figure 3.7: Chromatogram from FAME analysis of the bacterial isolate from the termite mound matrix, identified as *Ralstonia pickettii*. Major fatty acid peaks are labelled in blue text.

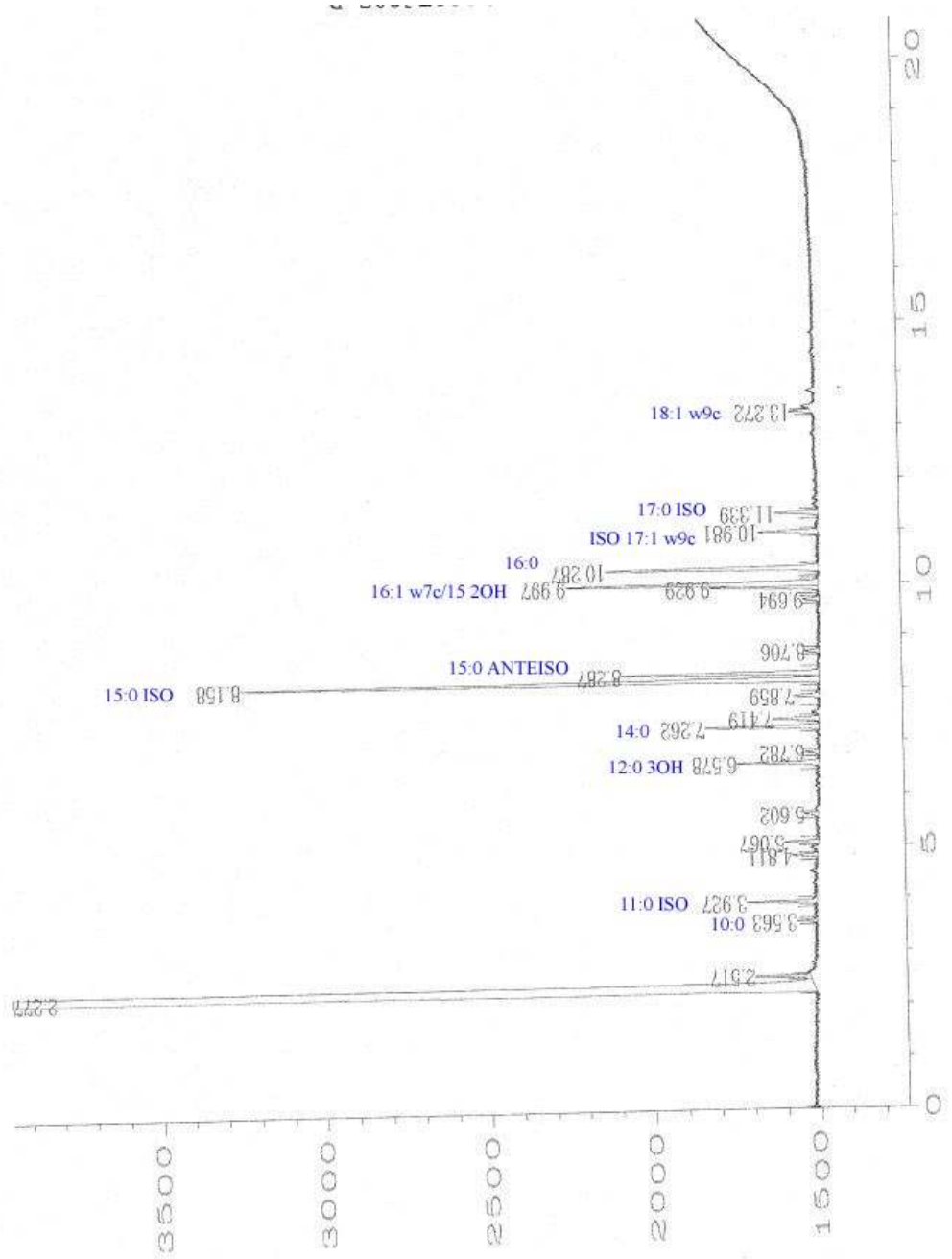


Figure 3.8: Chromatogram from FAME analysis of the bacterial isolate from kangaroo faeces, identified as *Stenotrophomonas maltophilia*. Major fatty acid peaks are labelled in blue text.

3.4.3 Determination of 16SrRNA Gene Sequences

Identification of the other bacterial isolates (manufactured gas plant isolate, Kangaroo isolate #1 and Kanmantoo isolate) was performed using 16SrRNA. Initially the PCR primers sets used were fD1 (5'-AGA GTT TGA TCC TGG CTC AG) / rD1 (5'-AAG GAG GTG ATC CAG CC) (Weisberg et al., 1991) and 704f 5'-GTA GCG GTG AAA TGC GTA GA / 765r 5'- CTG TTT GCT CCC CAC GCT TTC (Damiani *et al*., 1997). Initial attempts to PCR the DNA extracted from bacterial isolates using the 704f -765r primer set were unsuccessful and this primer set was not used further.

A PCR product of 1.4 kb was amplified from the Kanmantoo isolate using the fD1-rD1 primer set. The identity of this sequence was determined using BLASTN 2.2.10 (Basic Local Alignment Search Tool) similarity search accessed through NCBI (National Centre of Biotechnology Information) database. Results of this identity search revealed that the sequence obtained from the Kanmantoo isolate showed highest similarity to *Mycobacterium* spp. (Table 3.6).

Attempts to PCR the manufactured gas plant isolate and Kangaroo isolate #1 using fD1/rD1 primer set resulted in insufficient PCR product to be utilized for sequencing. The sequence length for the manufactured gas plant isolate using the fD1/rD1 primer set was 587 bp and no sequence was achieved for the Kangaroo isolate #1.

An alternative method and primer set (27f-765r) achieved better results for the manufactured gas plant isolate and Kangaroo #1 isolate. PCR products of 715 bp and 370 bp were obtained from the manufactured gas plant isolate and Kangaroo #1 isolate respectively, and subjected to a BLASTN 2.2.10 (Basic Local Alignment Search Tool) similarity search accessed through NCBI (National Centre of Biotechnology Information) database. Similar to the Kanmantoo

isolate, results of these identity searches revealed that the sequences obtained from the manufactured gas plant isolate and kangaroo #1 showed highest similarities to *Mycobacterium* spp. (Table 3.5).

The isolates were given strain identifiers based on their enrichment source and the PAH mixture used to enrichment them. The Kanmantoo isolate was designated *Mycobacterium* sp. Strain KA5 (Kanmantoo Agricultural, 5 PAH mixture. The manufactured gas plant isolate was designated *Mycobacterium* sp. Strain BS5 (Buckle Street – site of the former manufactured gas plant – 5 PAH mixture). The kangaroo #1 isolate was designated *Mycobacterium* KF4 (kangaroo faeces, 4 PAH mixture).

3.5 DISCUSSION

The intention of the work detailed in this Chapter was to provide additional isolates for assessment of PAH degrading ability and for use in various coculture combinations for further experiments. In this work, this method yielded both gram positive and gram negative bacterial isolates with the ability to utilize a variety of substrates, a phenomenon of note due to the variety of sources from which the isolates were obtained.

Pyrene is not routinely used for the isolation of PAH degrading bacteria as phenanthrene is more commonly used (e.g, Kasai *et al*., 2003). Pyrene was selected as the primary focus of the investigation to obtain organisms with an ability to degrade the higher molecular weight PAHs. The PAH selected for enrichment has been reported as selecting for bacteria that have varying capabilities to degrade a range of PAHs. Daane *et al*. (2001) reported that isolates enriched on phenanthrene were able to metabolise a greater number of PAHs than those enriched on naphthalene. Enrichments undertaken in the work

Table 3.6: 16S rRNA Identification of Bacterial Isolates. Top ten aligned sequences between bacterial isolates and members of the BLAST sequence database.

Isolate	Primer Set	Aligned Sequences from BLAST	% Similarity
Buckle Street (Strain BS5)	27f-765r	*Mycobacterium* sp. PYR GCK	98
		Mycobacterium gilvum isolate VM0583	98
		Mycobacterium gilvum isolate VM0442	98
		Mycobacterium gilvum isolate VM0552	98
		Mycobacterium gilvum isolate VM0504	98
		Mycobacterium gilvum isolate VM0505	98
		Mycobacterium sp. strain BB1	98
		Mycobacterium sp. HE5	98
		Mycobacterium mucogenicum strain ATCC 49649	98
		Mycobacterium mucogenicum strain ATCC 49649	98
Kanmantoo (Strain KA5)	fD1-rD1	*Mycobacterium monacense* B9-21-178	94
		Mycobacterium sp. KMS	94
		Mycobacterium sp. JLS	94
		Mycobacterium gilvum isolate VM0442	93
		Mycobacterium gilvum isolate VM0552	93
		Mycobacterium gilvum isolate VM0504	93
		Mycobacterium gilvum isolate VM0505	93
		Mycobacterium sp. PYR GCK	93
		Mycobacterium petroleophilum	93
		Mycobacterium chlorophenolicus (PCP-1)	93
Kangaroo #1 (Strain KF4)	27f-765r	*Mycobacterium* sp. JLS	95
		Mycobacterium monacense B9-21-178	95
		Mycobacterium vaccae isolate VM0588	95
		Mycobacterium vaccae isolate VM0587	95
		Mycobacterium sp. KMS	95
		Mycobacterium sp. MCS	95
		Mycobacterium doricum strain DSM 44339	94
		Mycobacterium doricum	94
		Mycobacterium duvalii	94
		Mycobacterium duvalii strain CIP 104539	94

presented in this chapter were undertaken using a spectrum of PAHs with the intention of selecting bacteria able to survive and grow in the presence of a number of PAHs.

Although employed in the work presented here, the use of multiple PAHs during enrichment is not required to enrich and isolate organisms with broad substrate specificity. Zhang *et al*. (2004) isolated three bacteria (*Pseudomonas* sp., *Microbacterium* sp. and *Paracoccus* sp.) from Greek soils contaminated with PAHs arising from wood processing, steel manufacture and oil refining respectively using phenanthrene as the enrichment substrate. Each strain could degrade a variety of compounds, with the *Paracoccus* sp. degrading the widest range of compounds that included PAHs (anthracene, phenanthrene, fluorene, fluoranthene, chrysene and pyrene), cresol and n-alkanes. None of the strains isolated could utilize simple aromatic hydrocarbons such as toluene and xylene or five ringed PAHs.

It was interesting to note the variability in the isolates obtained from the enrichments from various sources. The use of pyrene in enrichment cultures allowed for the isolation of both gram positive and negative bacteria. The culture or isolation conditions during enrichment should attempt to ensure that there is no bias in the technique towards faster growing organisms or a particular physiological parameter (e.g. gram negative over gram positive) at the expense of more capable degraders (Ahn *et al.*, 1999). It has been proposed that this is a result of factors such as the hydrophobicity of the cell wall of gram positive isolates providing an advantage in degrading higher molecular weight PAHs and hence favouring their enrichment and isolation. This phenomenon may have been observed to some extent in the work presented here, given that both gram types were isolated; but *Mycobacterium* spp. were the predominant isolates.

3.5.1 Enrichment and Isolation from Contaminated Soils

Enrichment of bacteria from contaminated soils or sludges using shaking enrichments is only one of many methods to isolate PAH utilizing bacteria and the enrichment method has shown some relationship to the type of bacteria isolated. Bastiaens *et al*. (2000) found that enriching bacteria from PAH-contaminated soil and sludge using different methods (shaking enrichments in liquid medium with solid PAH and recovery from hydrophobic membranes containing sorbed PAHs) recovered mainly *Sphingomonas* spp. from shaking enrichments and *Mycobacterium* spp. from the membrane enrichments. Although only one enrichment method was used in the experiments detailed in this chapter, a variety of bacteria (gram positive and negative) were isolated using a similar method.

It has been proposed that contaminated environments enrich xenobiotic utilizing bacteria *in situ* where high levels of contamination reduce bacterial diversity (Margesin *et al*., 2003). In experiments assessing the diversity of *Sphingomonas* spp. in contaminated soils, Leys *et al*. (2004) found *Sphingomonas* spp. present in all the soils tested, despite these having been collected from different locations and with varying geological and chemical properties. It was proposed that the *Sphingomonas* spp. were important colonizers of contaminated soils. Once again, the enrichment method was suggested as having an impact; the authors suggesting that the high PAH concentration in the enrichments may have selected for *Sphingomonas* spp. at the expense of other organisms present in lower populations.

The distinction in the proportion of xenobiotic degrading bacterial species in contaminated and non-contaminated soils did not appear to be significant in the work presented here. Margesin *et al*. (2003) reported a significantly higher percentage of genotypes of gram negative bacteria involved in the degradation of

xenobiotic compounds in contaminated soil (50-70%) than in pristine soil (0-12.5%) as determined by hybridised gene probes in a number of soil samples. This result suggested that gram negative bacteria are enriched in contaminated soil. This difference in genotype frequency between the contaminated and uncontaminated soils was not observed for gram positive bacteria, with genotypes for gram positive bacteria detected at 41.7-70% in contaminated soils and 37.5-50% in pristine soils.

3.5.2 Enrichment and Isolation from Uncontaminated Sources

Enrichment and isolation of PAH degrading bacteria from environmental sources with no prior history of PAH contamination has not been undertaken routinely, although it is generally accepted that PAH degrading bacteria are ubiquitious in the environment. PAH degrading bacteria have been isolated from a wide range of uncontaminated sources, including the rhizosphere of salt marsh plants and the burrows of benthic animals (Daane *et al*., 2001; Chung and King, 2001).

Widada *et al*. (2002) enriched (on naphthalene or phenanthrene) and isolated a number of PAH degrading bacteria from both contaminated sources (soil, wastewater and sludge) and uncontaminated sources (pristine soils, river water and sediments). *Ralstonia* sp. was isolated from both contaminated (sludge oil, Thailand) and uncontaminated sources (pristine soil, Japan). Similarly, a *Ralstonia* sp. was isolated in the work presented in this Chapter from an uncontaminated source (termite mound matrix). Phenanthrene degrading cultures have also been isolated from Italian agricultural soil with no prior history of exposure to PAHs (Andreoni *et al*., 2003).

The isolation of PAH degrading bacteria from animal faeces has been previously reported by Juhasz and Naidu (2000). Faeces from animals with a woody plant diet (hippopotamus, koala and red panda) and a carnivore (jaguar) were used for

enrichment on a variety of PAHs, polychlorinated biphenyls, heterocyclic compounds, pentachlorophenol and organochlorine pesticides. The microbial communities from the tertiary enrichments from the hippopotamus, red panda and koala sources were able to grow on phenanthrene and pyrene, as well other compounds.

3.5.3 Identification of Isolates

As the overarching intent of this work was to supply bacteria for use in defined coculture combinations with fungi, identification of the isolates was necessary. A variety of methods were used (FAME analysis and 16SrRNA genotypic identification) as a number of difficulties were experienced in the identification process. Bacteria satisfactorily identified during the initial FAME analysis were not subjected to further investigation using 16SrRNA to reduce the time and resources dedicated to identification. Whilst FAME analysis has limitations (related to the strict growth requirements related to media, incubation time and temperature) and is significantly limited by the bacteria having to possess a fatty acid profile contained in the MIDI database (currently around 100,000 strains) to allow identification, the presumptive initial identification was considered sufficient for the purposes of this work (Sasser, 2001). The FAME analysis identified two gram negative bacteria as species previously reported to degrade xenobiotics in soils (See Sections 3.5.5 and 3.5.6). As was observed in the work presented here, the slower growing gram positive isolates were not conducive to the use of this analysis and were subjected to 16SrRNA analysis.

Although not successful here, FAME analysis has been successfully used to identify gram positive bacteria. Khan *et al*. (2002) used FAME, 16SrDNA, DNA-DNA hybridization and other methods to determine the taxonomic relationship between *Mycobacterium* sp. Strain PYR-1 (isolated from petroleum contaminated sediments) and other *Mycobacterium* sp. When compared,

Mycobacterium sp. Strain PYR-1 and *Mycobacterium austroafricanum* showed strong similarity although they were not identical. The other phenotypic and genotypic analyses, in conjunction with FAME, indicated that *Mycobacterium* sp. Strain PYR1 was a novel species of the genus and was designated *Mycobacterium vanbaalenii* sp. nov.

The presumptive identification was considered sufficient as the bacteria were subjected to further analysis and some were to be eliminated from the investigation following the assessment of the PAH degrading capabilities in both liquid and soil media.

3.5.4 *Mycobacterium* spp.

In the work presented in this Chapter, three of the environmental isolates were identified as *Mycobacterium* spp. These were designated *Mycobacterium* sp. Strain BS5 (isolated from manufactured gas plant soil), *Mycobacterium* sp. Strain KA5 (isolated from kanmantoo agricultural soil) and *Mycobacterium* sp. Strain KF4 (isolated from kangaroo faeces).

Mycobacterium sp. Strain BS5 was very slow growing, affecting its ability to be identified using FAME analysis and potentially an influence on growth in the substrate range tests. A highly encouraging result was the observation of clearing of BSMY agar plates containing benzo[*a*]pyrene (50 mg/l). No other isolates were observed to be able to produce zones of clearing on plates containing benzo[*a*]pyrene. A potential explanation for the zones of clearing surrounding *Mycobacterium* sp. Strain BS5 colonies on BSMY plates containing benzo[*a*]pyrene may have been the production of biosurfactants. These may have solubilised the benzo[*a*]pyrene causing clearing. Solubility enhancing biosurfactants and bioemulsifiers have been shown to be produced by a variety of known PAH degraders including *Mycobacterium sp*, *Pseudomonas aeruginosa*,

Pseudomonas putida and *Sphingomonas spp.* (Dagher *et al.*, 1997) (Wick *et al.*, 2001).

As observed in the work presented in this chapter, *Mycobacterium* spp. are routinely isolated following enrichment and isolation of PAHs from environmental sources. It has been proposed that gram positive bacteria (particularly *Mycobacterium* sp.) play a more important role in the degradation of high molecular weight PAHs in the environment than gram negative bacteria (Brezna *et al*., 2003). This greater ability to degrade higher molecular weight PAHs has been attributed to their hydrophobic cell wall and the multiple dioxygenases possessed by *Mycobacterium* sp. The possession of multiple dioxygenases is not solely limited to *Mycobacterium* sp. and has also been reported in *Sphingomonas* sp. and *Cycloclasticus* sp. (Demaneche *et al*., 2004; Kasai *et al*., 2003).

The slow growth and variation in morphology of the *Mycobacterium* sp. isolates in this Chapter have been observed previously. Sutherland *et al*. (2002) reported difficulty in isolating a *Mycobacterium* sp. capable of degrading endosulfan from enrichments due to the slow growth of the "active" organism in comparison to the rest of the consortium. An extended period of growth was required in this study to allow the formation of colonies that were obvious and able to be harvested for replating. Although *Mycobacterium* sp. are renowned for being a slow growing species in soils, their growth on PAHs is more rapid than other bacteria (Harayama, 1997).

Mycobacterium sp. isolates from contaminated soil have previously been shown to have broad substrate specificity for PAHs (eg Grosser *et al.*, 1991 and Sepic *et al.*, 1997). Churchill *et al*. (1999) isolated *Mycobacterium* sp. strain CH1 using methods similar to those used in this chapter (isolation on pyrene coated agar plates) from PAH contaminated freshwater sediments. The authors found it to be capable of mineralizing phenanthrene, pyrene and fluoranthene and utilizing both

solid and liquid alkanes as a sole carbon and energy source. Similarly, Bogan *et al*. (2003) isolated *Mycobacterium austroafricanum* from manufactured gas plant soil that was capable of growth on phenanthrene, fluoranthene and pyrene and could mineralize dodecane and hexadecane. *Mycobacterium austroafricanum* was isolated on agar plates using pyrene (or another PAH) as sole carbon and energy source. It has been reported that *Mycobacterium vanbaalenii* PYR-1 can metabolise a wide variety of PAHs, including naphthalene, biphenyl, anthracene, phenanthrene, pyrene, fluoranthene and benzo[*a*]pyrene (Moody *et al.*, 2003).

The enrichment of degrading bacteria using a mixture of PAHs has been successfully used previously. Molina *et al*. (1999) used a 16 PAH mixture to enrich bacteria from PAH contaminated sediments from the Buffalo River, New York. Isolation on agar plates using pyrene as a sole carbon and energy source yielded a *Mycobacterium* sp. (termed MR-1).

Isolation on different PAHs has yielded differing organisms. Ho *et al*. (2000) used fluoranthene and pyrene as a sole carbon and energy source on spray plates to isolate PAH degrading bacteria. Of the 28 strains isolated using fluoranthene, all were found to be gram negative, many of which were *Sphingomonas* spp. Of the 21 strains isolated using pyrene, all were found to be gram positive, many of which were *Mycobacterium* spp. This specific selection of gram type arising from the isolation method was not observed to occur in the work presented here, where both gram positive and gram negative bacteria were isolated using the same method of isolation on pyrene plates.

Zhang *et al*. (2004) was also able to isolate differing gram type bacteria using the same enrichment and isolation methods. Using PAH contaminated soil from wood processing, steel mill and oil refinery sites, they were able to enrich and isolate on phenanthrene, *Pseudomonas* sp., *Microbacterium* sp. and *Paracoccus* sp. Each possessed a different ability to degrade PAHs, with the *Pseuodmonas* sp. only able to degrade phenanthrene and naphthalene, the *Microbacterium* sp.

only able to degrade phenanthrene and the *Paracoccus* sp. able to degrade anthracene, phenanthrene, fluorene, fluoranthene, pyrene and chrysene.

3.5.5 *Ralstonia pickettii*

The selection of a termite mound as a source of PAH utilizing bacteria was based on the belief that bacteria able to utilize complex polysaccharides would be found in this environment. The termite gut harbours both resident and transient bacteria that achieve polysaccharide hydrolysis resulting in nutrition for the termite (Brennan *et al*., 2004). This symbiotic relationship between termites and bacteria also extends to other microorganisms such as fungi, that have a beneficial influence on nutrition, survival and nest construction. Moreover, bacteria isolated from termites have been shown previously to be capable of the degradation of biphenyls and polychlorinated biphenyls (Breznak, 2004).

Ralstonia pickettii has previously been identified under the basonym *Pseudomonas pickettii* and synonym *Burkholderia pickettii* (American Society of Microbiology, 1996). They have been isolated from a number of sources including hydrocarbon contaminated environments, clinical isolates from humans and have been found to be able to modify a number of compounds including carbazole (Schneider *et al*., 2000), benzene (to phenol then to catechol) and toluene (to *m*-cresol then to 3-methycatechol) (Tao *et al*., 2004) . In the work presented in this chapter, *Ralstonia pickettii* was observed to grow in the presence of benzene and toluene. *Ralstonia* spp. have been found to possess toluene para or 3-monooxygenases through which they achieve degradation of compounds. Extensive study has been made of the naphthalene catabolic genes (*nag*) that encode the enzymes for naphthalene degradation in *Ralstonia* sp. Strain U2 (Jones *et al*., 2003; Dionisi *et al*., 2004). The *Ralstonia pickettii* isolated during the work described in this chapter was the only isolate to grow in the presence of naphthalene, although this growth was minimal.

3.5.6 *Stenotrophomonas maltophilia*

S. maltophilia is a gram-negative aerobic rod and has been investigated both for its ability to degrade xenobiotics and its potential to cause opportunistic infections in humans (Dungan *et al*., 2003). *S. maltophilia* has been isolated previously as a PAH degrading bacterial isolate and as part of a bacterial community. Juhasz *et al*. (2000) isolated a community, identified as Community Five, that was comprised of three strains of *S. maltophilia* from a former gas manufacturing plant soil that was able to grow on pyrene as a sole carbon and energy source. Juhasz *et al*. (2000a) also isolated *S. maltophilia* (identified as VUN 10,003 and previously classified as *Burkholderia cepacia*) from the same soil. The VUN 10,003 isolate was able to degrade pyrene, fluoranthene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene to varying extents both as single PAHs and in a PAH mixture.

Boonchan *et al*. (1998) also isolated a *Stenotrophomonas maltophilia* (identified as VUN 10,010 and used in the further work undertaken as part of this investigation) from the same soil as Juhasz *et al*. (2000). This isolate was similarly able to degrade a number of PAHs with up to seven rings.

3.6 CONCLUSIONS

The work undertaken in this chapter resulted in the successful enrichment and isolation of PAH degrading bacteria from both contaminated and non-contaminated sources. The isolates were designated as *Mycobacterium* sp. strain BS5, *Mycobacterium* sp. strain KA5, *Mycobacterium* sp. strain KF4 following identification using 16SrRNA analysis and *Ralstonia picketii* and *Stenotrophomonas maltophilia* following FAME analysis. All isolates showed the capability to grow on a range of substrates including PAHs and aromatic

hydrocarbons. One isolate, *Mycobacterium* sp. strain BS5, was observed to produce zones of clearing on BSMY plates containing 50 mg/l benzo[*a*]pyrene indicating an ability to degrade benzo[*a*]pyrene or to produce biosurfactant compounds.

The following chapter describes the analysis of PAH degradative capacity of two fungal and seven bacterial isolates (including the five isolates) in liquid medium when inoculated individually and as part of a fungal-bacterial coculture.

CHAPTER 4

PAH DEGRADATION IN LIQUID MEDIA

4.1 INTRODUCTION

The study of PAH degradation in liquid media is commonly undertaken to determine the degradative potential of selected organisms, including rates and extent of PAH degradation, transformation pathways and the influence of environmental parameters on these processes. Degradation experiments in liquid culture are conducted under controlled conditions commonly using a minimal media, such as basal salts medium, where the compound of interest is supplied as a carbon and energy source. Following the desired incubation period, the media is sampled to determine the extent of degradation and the growth of the inoculum.

Traditionally, liquid culture experiments have been undertaken using single isolates and single carbon sources. Whilst this allows an effective assessment of the isolate's ability to degrade a single compound, it is not truly reflective of the environment an isolate selected for bioaugmentation is likely to encounter upon release into the environment. PAHs are rarely, if ever, found in isolation and as such it is necessary to assess the performance of the isolate against a suite of PAHs of varying ring number and structure.

The liquid culture experiments described in this chapter were performed to determine the PAH degradative performance of the organisms in order to select the best performing strains for further study. Initially, two fungal isolates were screened, namely *P. janthinellum* (isolated from contaminated soil and provided by Victoria University of Technology) and *Ph. chrysosporium* (ATCC 24725). Fungal isolates were initially examined for their ability to degrade benzo[*a*]pyrene as a carbon and energy source and cometabolically. Following this initial assessment, fungal isolates were assessed for their ability to degrade a PAH mixture. As the purpose of this investigation was to determine a viable coculture combination for the degradation of high molecular weight PAHs,

analysis of metabolites formed by the respective isolates was not undertaken and considered beyond the scope of the work to be presented here.

The bacterial strains isolated in Chapter 3 and two others used in previous studies were assessed for the ability to degrade single PAHs (fluorene, phenanthrene, fluoranthene, pyrene and benzo[*a*]pyrene) in addition to a PAH mixture. Bacterial isolates used included:

- VUN 10,010 (provided by Victoria University of Technology, isolated from contaminated soil and enriched on pyrene);
- *Mycobacterium* 1B (provided by Dr. Catherine Dandie, Flinders University of South Australia, isolated from a PAH degrading microbial culture);
- *Mycobacterium* sp. Strain BS5 (isolated from contaminated soil from a former gasworks site and enriched on pyrene);
- *Mycobacterium* sp. Strain KA5 (isolated from non-contaminated agricultural soil and enriched on pyrene);
- *R. pickettii* (isolated from the mound matrix of a termite mound and enriched on pyrene);
- *Mycobacterium* sp. Strain KF4 (isolated from Kangaroo faeces and enriched on pyrene); and
- *S. maltophilia* (isolated from Kangaroo faeces and enriched on pyrene).

Following the assessment of individual isolates ability to degrade PAHs in liquid medium, coculture combinations of the best performing bacterial and fungal isolates were assessed in various coculture combinations for their ability to degrade the PAH mixture.

Radiolabelled experiments (using [4,5,9,10-^{14}C] pyrene or [7-^{14}C] benzo[*a*]pyrene) were also conducted to determine if an isolate or coculture had the capability of mineralizing the respective compound. Mineralization or the

complete destruction of the compound is the ultimate goal in PAH degradation as it eliminates the potential problem of toxic metabolite generation.

The general aim of the experiments reported in this chapter was to examine the potential of the bacterial and fungal isolates in axenic and coculture combinations to degrade high molecular weight PAHs in liquid culture. The specific aims were to:

1. Determine the ability of fungal and bacterial isolates to degrade single PAHs and PAH mixtures in liquid culture;
2. Determine the ability of fungal and bacterial isolates to mineralize [4,5,9,10-^{14}C] pyrene or [7-^{14}C] benzo[*a*]pyrene;
3. Determine the ability of various bacterial - fungal coculture combinations to degrade high molecular weight PAHs; and
4. Select appropriate coculture combinations for further investigation in spiked soil experiments.

4.2 DEGRADATION OF PAHS IN LIQUID CULTURE BY AXENIC MICROBIAL ISOLATES

To determine the degradative capabilities of the isolates, experiments were conducted using benzo[a]pyrene in BSM and MYPD for fungal isolates and single PAHs in BSMY inoculated with axenic cultures of bacteria. The duration of the experiments varied depending on the PAH being supplied with fungal experiments being conducted over 90 days and bacterial experiments being conducted over 14 days for fluorene and phenanthrene; 20 days for fluoranthene and pyrene and 50 days for benzo[*a*]pyrene.

The fungal component is intended to partially oxidize benzo[*a*]pyrene through the release of extracellular enzymes. This allows the partially oxidized

benzo[*a*]pyrene (with its corresponding increased water solubility) to be transferred across the bacterial cell membrane for further degradation by the bacterial component of the coculture. The focus of fungal experiments presented here was to assess fungal degradation of benzo[*a*]pyrene (50 mg/l) over a 90 day incubation period (sampled at day 0, 30, 60 and 90). Fungal isolates were grown for inoculation as per the method in Section 2.5.4.2 and biomass was analysed as per method 2.5.5.2.

The bacterial isolates for these experiments were grown as per the method in Section 2.5.4.1. Degradation was demonstrated by a reduction in PAH concentration compared to uninoculated and killed controls. Bacterial growth on the test substrate was determined as per the method in Section 2.5.5.1.

4.2.1 Abiotic PAH Removal

Abiotic controls were conducted for all liquid culture experiments and PAH concentration is provided as a comparison to the inoculated experiment on the relevant figure. Abiotic benzo[*a*]pyrene removal in abiotic controls using both BSM and MYPD media was not observed.

In experiments using BSMY as the medium, a significant decrease (52.4 mg/l; 52.9% of the initial concentration) in the concentration of fluorene in the abiotic control was observed over the 14 day incubation period. This resulted in a final fluorene concentration of 46.6 ± 6.5 mg/l. The reduction in fluorene concentration appeared to be the result of abiotic processes (e.g. volatilization) as sterility was maintained over the incubation period.

In BSMY based experiments, the other PAHs were subject to fluctuations over the incubation period, but final concentrations remained within 1 mg/l of the initial concentration.

4.2.2 Degradation of Benzo[*a*]pyrene in BSM and MYPD by Fungal Isolates

Experiments assessing the ability of *P. janthinellum* and *Ph. chrysosporium* were conducted using two different media, BSM and MYPD. BSM was used as a culture medium to determine if transformation of 50 mg/l benzo[*a*]pyrene would occur through the generation of enzymes in a nutrient poor medium.

Although it was not to be used in further liquid culture experiments, MYPD was initially used as a comparative culture medium to determine if degradation of 50 mg/l benzo[*a*]pyrene occurred cometabolically. The MYPD medium supplies an alternative carbon and energy source for the fungi being assessed, allowing growth of the fungal inocula without having to rely on benzo[*a*]pyrene as a source of carbon and energy.

When BSM was used as the growth medium, neither *P. janthinellum* or *Ph. chrysosporium* were able to degrade benzo[*a*]pyrene following the 90 day incubation period (Figure 4.1 and Figure 4.3). Dry weight analysis of the fungal biomass revealed a reduction for *P. janthinellum* of 0.2 mg/ml to 0.02 mg/ml and for *Ph. chrysosporium* of 0.2 mg/ml to 0.06 mg/ml.

In MYPD, degradation of benzo[*a*]pyrene was observed. In *P. janthinellum* inoculated experiments, 3.8 ± 2.6 mg/l (9.5%) of the 50 mg/l of benzo[*a*]pyrene added was removed over the 90 day incubation period (Figure 4.2). Benzo[*a*]pyrene removal occurred at Day 60 following an increase in fungal biomass that peaked at Day 60 at 4 mg/ml.

Ph. chrysosporium inoculated experiments in MYPD performed better than *P. janthinellum* (Figure 4.4). A total of 9.8 ± 1.6 mg/l (22.8%) benzo[*a*]pyrene was removed over the 90 incubation period. Analysis of fungal biomass showed a

peak in biomass at Day 30 of 3.7 mg/ml. As with the *P. janthinellum* inoculated experiments, *Ph. chrysosporium* biomass reduced slightly after Day 60 (1.4 mg/ml), although in this instance, did not fall below inoculation levels.

Killed controls conducted for all experiments demonstrated that the removal of benzo[*a*]pyrene was unlikely to be due to binding to fungal mycelia. Over the 90 day incubation period, recovery of benzo[a]pyrene in the killed controls was greater than 99%.

4.2.2 Bacterial Degradation of Single PAHs

Experiments assessing the ability of the various bacterial isolates to degrade individual PAHs were conducted using BSMY. The orginal intention was to conduct these experiments using BSM as used in the initial fungal experiments, but the results of the fungal experiments indicated that fungal degradation did not occur using this medium. As some benzo[*a*]pyrene degradation had occurred using MYPD, it was considered that an appropriate medium should have a small quantity of alternative substrate present. The addition of 0.05 g/l yeast extract to the BSM was employed to provide a limited quantity of an alternative easily degradable substrate without substantially influencing bacterial degradation of the target PAH compounds.

A summary of the results of axenic bacterial degradation experiments using single PAHs is presented in Table 4.1. Graphical representation of the varying capacity of the isolated organisms to degrade three- and four-ring PAHs as carbon and energy sources are presented in Figures 4.5-4.10.

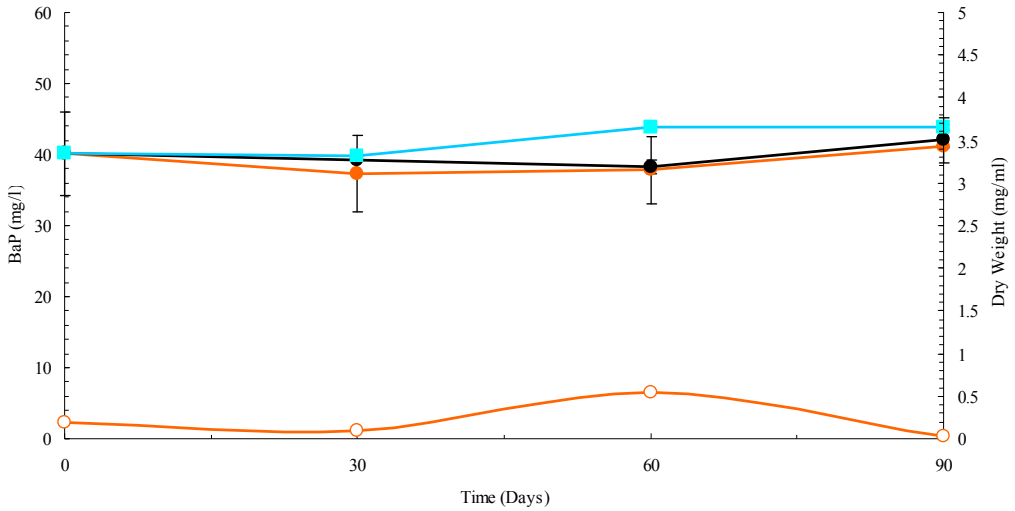


Figure 4.1: Time course for benzo[*a*]pyrene degradation by *P. janthinellum* in BSM. *P. janthinellum* inoculated (●), killed *P. janthinellum* (●) and abiotic controls (■). Dry weight analysis of microbial biomass (○) is also shown. Data points are the mean of triplicate experiments.

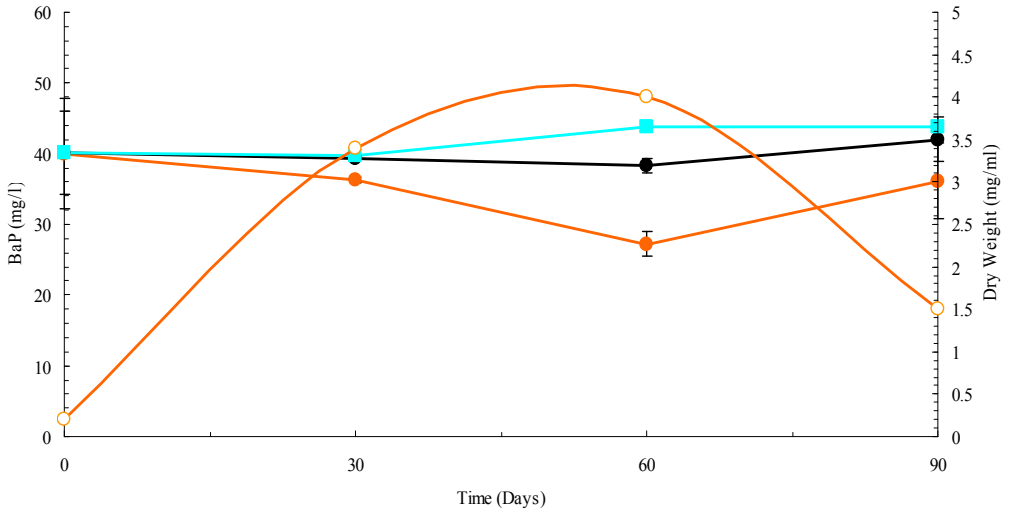


Figure 4.2: Time course for benzo[*a*]pyrene degradation by *P. janthinellum* in MYPD. *P. janthinellum* inoculated (●), killed *P. janthinellum* (●) and abiotic controls (■). Dry weight analysis of microbial biomass (○) is also shown. Data points are the mean of triplicate experiments.

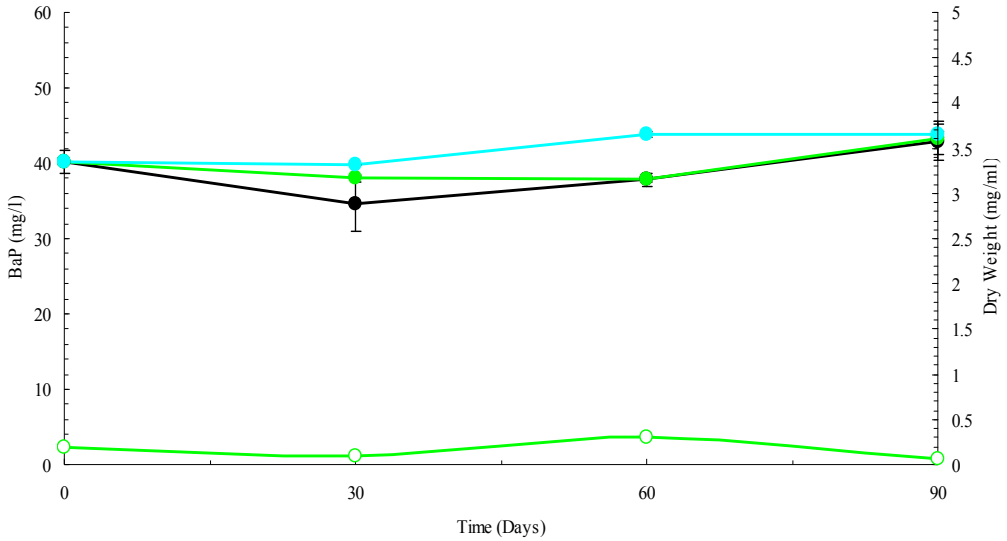


Figure 4.3: Time course for benzo[*a*]pyrene degradation by *Ph. chrysosporium* in BSM. *Ph. chrysosporium* inoculated (●), killed *Ph. chrysosporium* (●) and abiotic controls (■). Dry weight analysis of microbial biomass (○) is also shown. Data points are the mean of triplicate experiments.

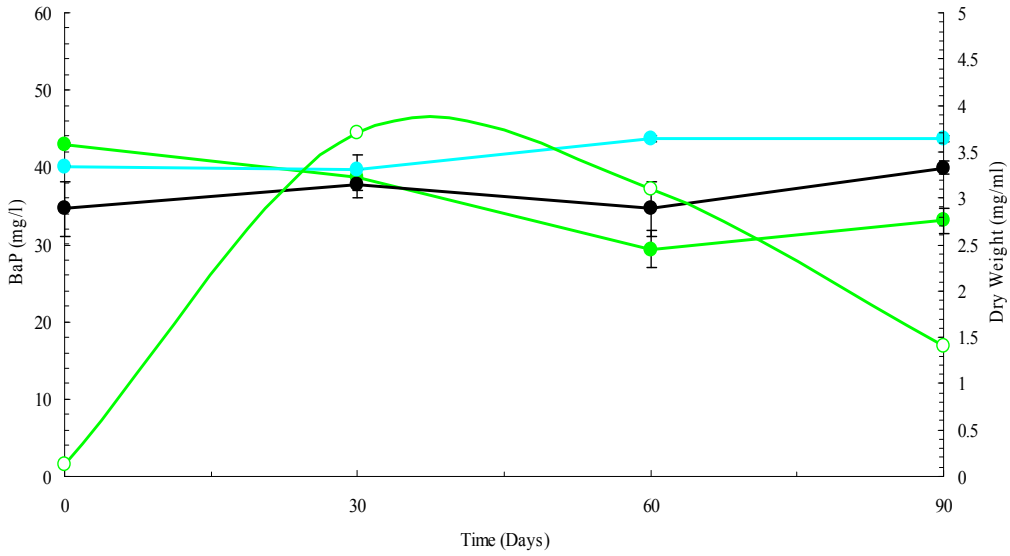


Figure 4.4: Time course for benzo[*a*]pyrene degradation by *Ph. chrysosporium* in MYPD. *Ph. chrysosporium* inoculated (●), killed *Ph. chrysosporium* (●) and abiotic controls (■). Dry weight analysis of microbial biomass (○) is also shown. Data points are the mean of triplicate experiments.

All isolates showed either no or limited capacity to remove the 100 mg/l fluorene in the medium beyond the abiotic control, in which 52.9% of fluorene was removed by the conclusion of the experiment. This suggests that the loss of fluorene from the culture was a result of abiotic factors (e.g. volatilization) rather than the inoculation of the particular bacterial isolate.

As no bacterial isolate achieved the degradation of fluorene or benzo[*a*]pyrene in the experiments using single PAHs and axenic bacterial inocula, the results presented in this section refer only to experiments using phenanthrene, fluoranthene and pyrene as single PAHs.

4.2.2.1 Phenanthrene

All seven isolates were capable of phenanthrene removal; however, isolates showed variation in lag periods preceding degradation and the rate and extent of degradation over the incubation period.

In BSMY medium containing 250 mg/l phenanthrene inoculated with *Mycobacterium* sp. Strain BS5, *Mycobacterium* sp. Strain KA5, *Mycobacterium* sp. Strain KF4 and *R. pickettii*, degradation lag periods were not observed (Figure 4.5). For *Mycobacterium* sp. Strain BS5 and *Mycobacterium* sp. Strain KA5, degradation was rapid with > 98% of phenanthrene being removed by day 4, while complete removal of phenanthrene in *Mycobacterium* sp. Strain KF4 inoculated cultures required 8 days of incubation. While no degradation lag period was observed for *R. pickettii*, phenanthrene degradation ceased by day 4, resulting in a 46% removal.

Table 4.1: Percentage Removal of Single PAHs by Bacterial Isolates in BSMY Media (Values represent the mean and standard deviation of triplicate analysis)

		% PAH Decrease[a]								Time
PAH	**Concentration (mg/l)**	**VUN 10,010**	***Myco.* 1B**	***Myco* sp. Strain BS5s**	***Myco* sp. Strain KA5**	***R. pickettii***	***Myco* sp. Strain KF4**	***S. maltophilia***	**Abiotic Control**	**(Days)**
FLU	100	-	-	-	-	-	-	-	52.9 ± 6.6	14
PHE	250	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	54.5 ± 4.7	100 ± 0.0	83.9 ± 2.2	-	14
FLA	50	100 ± 0.0	100 ± 0.0	24 ± 3.8	59.6 ± 13.5	100 ± 0.0	19.9 ± 3.9	87.3 ± 11.3	-	20
PYR	250	100 ± 0.0	100 ± 0.0	94.3 ± 6.2	100 ± 0.6	100 ± 0.0	98.5 ± 2.6	96 ± 5.6	-	20
BaP	50	-	-	-	-	-	-	-	-	50

[a] Decrease calculated relative to abiotic control

[b] A decrease of 52.9 ± 6.6 % was observed in abiotic fluorene controls presumably due to volatilization.

- No degradation observed beyond the abiotic control

A four day lag period was observed in cultures inoculated with VUN 10,010. Removal substantially increased at day 4, reducing the concentration of phenanthrene in the medium from 220.9 mg/l to 54.8 mg/l. *Mycobacterium* 1B experienced a similar lag period after which phenanthrene was rapidly removed, with the concentration reducing from 237 mg/l on day 4 to 19.3 mg/l on day 11.

During experiments using phenanthrene, a colour change was observed in the media that resulted in DCM extracts that ranged in colour from orange to pink. Over the duration of the experiment, the colouration in the medium and extract was observed to disappear for all isolates except *R. pickettii*. In *R. pickettii* inoculated experiments, this colour change (and the resultant orange coloured extract) remained and coincided with the cessation of phenanthrene removal, suggesting that the formation of this coloured metabolite may have inhibited degradation.

In cultures inoculated with *S. maltophilia*, VUN 10,010 and *Mycobacterium* 1B, degradation lag periods of between 2 and 4 days were observed. Following this lag period, rapid removal of phenanthrene proceeded. Removal of phenanthrene below detection limits occurred by day 8 in *S. maltophilia* inoculated cultures, whereas in the same period, 80% removal occurred in VUN 10,010 and *Mycobacterium* 1B inoculated cultures. In *S. maltophilia* inoculated experiments, an increase in phenanthrene concentration to 43.5 mg/l (17%) was observed at the final sample point after 14 days incubation, although at day 8 only 2 mg/l (0.8%) remained. This was due to a large standard deviation between the triplicate samples of 55.6 mg/l (21.8%) as a result of the failure in one of the three replicates to remove phenanthrene. The exclusion of this failed replicate gave a day 14 phenanthrene concentration of 4.2 mg/l (1.6%).

Phenanthrene removal corresponded with an increase in microbial numbers in cultures inoculated with *Mycobacterium* sp. Strain BS5, *Mycobacterium* sp.

Strain KA5, *Mycobacterium* sp. Strain KF4, *R. pickettii* and *S. maltophilia* (Figure 4.6). Microbial numbers increased by an order of magnitude during phenanthrene degradation, however, following removal of phenanthrene, decreased to initial numbers. In cultures inoculated with VUN 10,010 and *Mycobacterium* 1B, microbial numbers decreased following inoculation and did not recover until after the degradation lag period. After this period, microbial numbers fluctuated for the remainder of the experiment, recovering to initial numbers at the conclusion of the experiment ($2.2x10^5$ cells/ml).

4.2.2.2 Fluoranthene

The greatest variation in degradative capacity was observed in experiments using fluoranthene (50 mg/l) (Figure 4.7). VUN 10,010, *Mycobacterium* 1B and *R. picketii* were all competent degraders of fluoranthene, achieving a 100% removal over the incubation period. *S maltophilia* was also a competent degrader achieving an 87.3% removal over the incubation period. The other bacterial isolates had a significantly lesser capacity to remove fluoranthene, with *Mycobacterium* sp. Strain KA5 achieving 59.6%, *Mycobacterium* sp. Strain BS5 achieving only 24%, and the *Mycobacterium* sp. Strain KF4 isolate achieving only 19.9% after 20 days incubation.

Fluoranthene removal in all experiments was preceded by an extended lag period. *R. pickettii* and *S. maltophilia* achieved removal of fluoranthene after the shortest period, commencing 5 days after inoculation. Reductions in concentration of fluoranthene in VUN 10,010 and *Mycobacterium* 1B inoculated cultures occurred after 10 day lag periods. Those isolates achieving the least removal of fluoranthene (*Mycobacterium* sp. Strain BS5 and *Mycobacterium* sp. Strain KF4) did so incrementally, making the determination of a distinct lag period difficult. This observation indicates that the lack of fluoranthene removal may have been more a product of slower degradation rather than a lack of degradative ability.

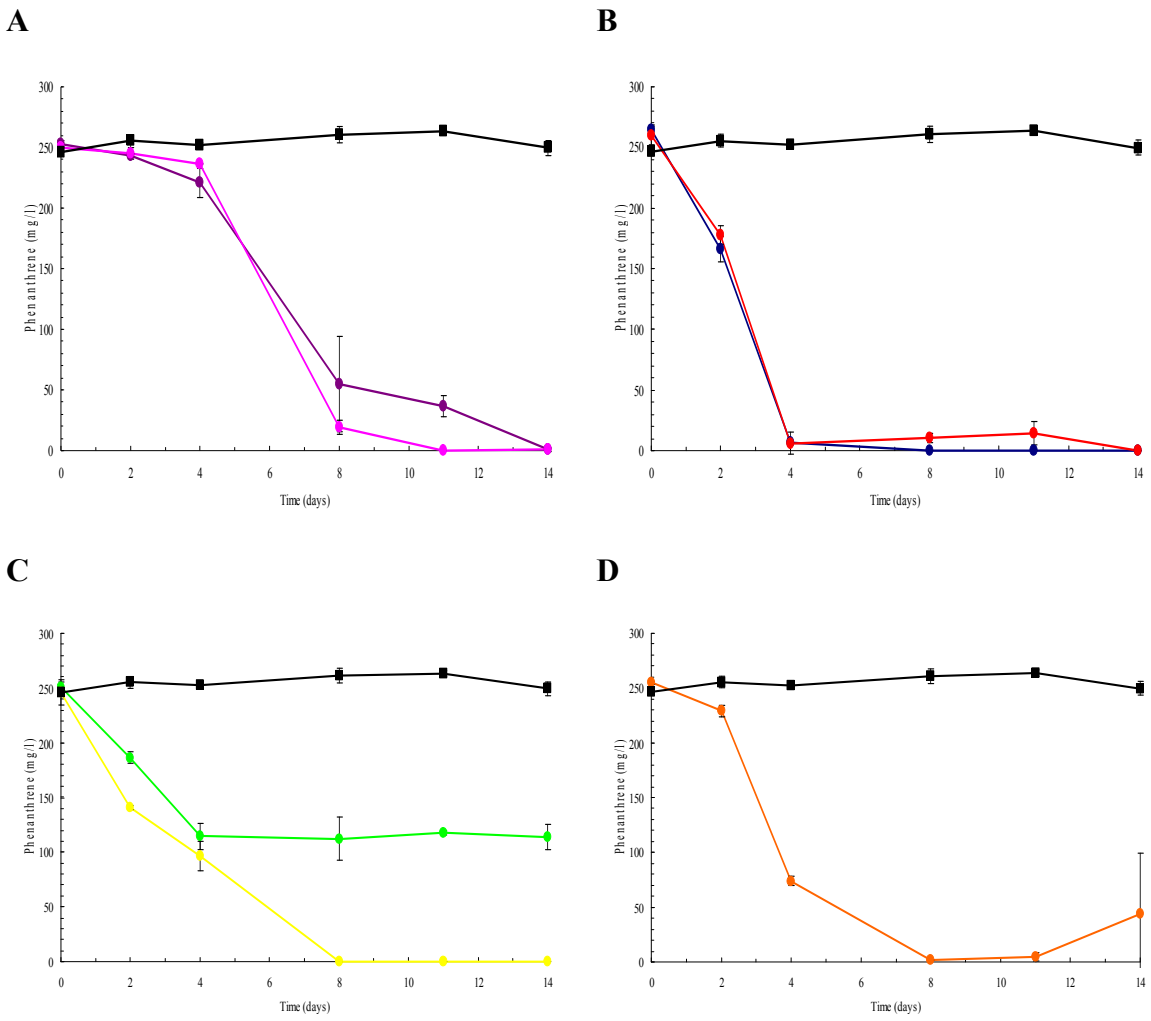


Figure 4.5: Phenanthrene concentration in BSMY inoculated with axenic cultures of: A – VUN 10,010 (●) and *Mycobacterium* 1B (●); B –- *Mycobacterium* sp. Strain BS5 (●) and *Mycobacterium* sp. Strain KA5 (●) C - - *R picketii* (●) and *Mycobacterium* sp. Strain KF4 (●) and D – *S. maltophilia* (●). Phenanthrene concentration in abiotic controls is also shown (■).

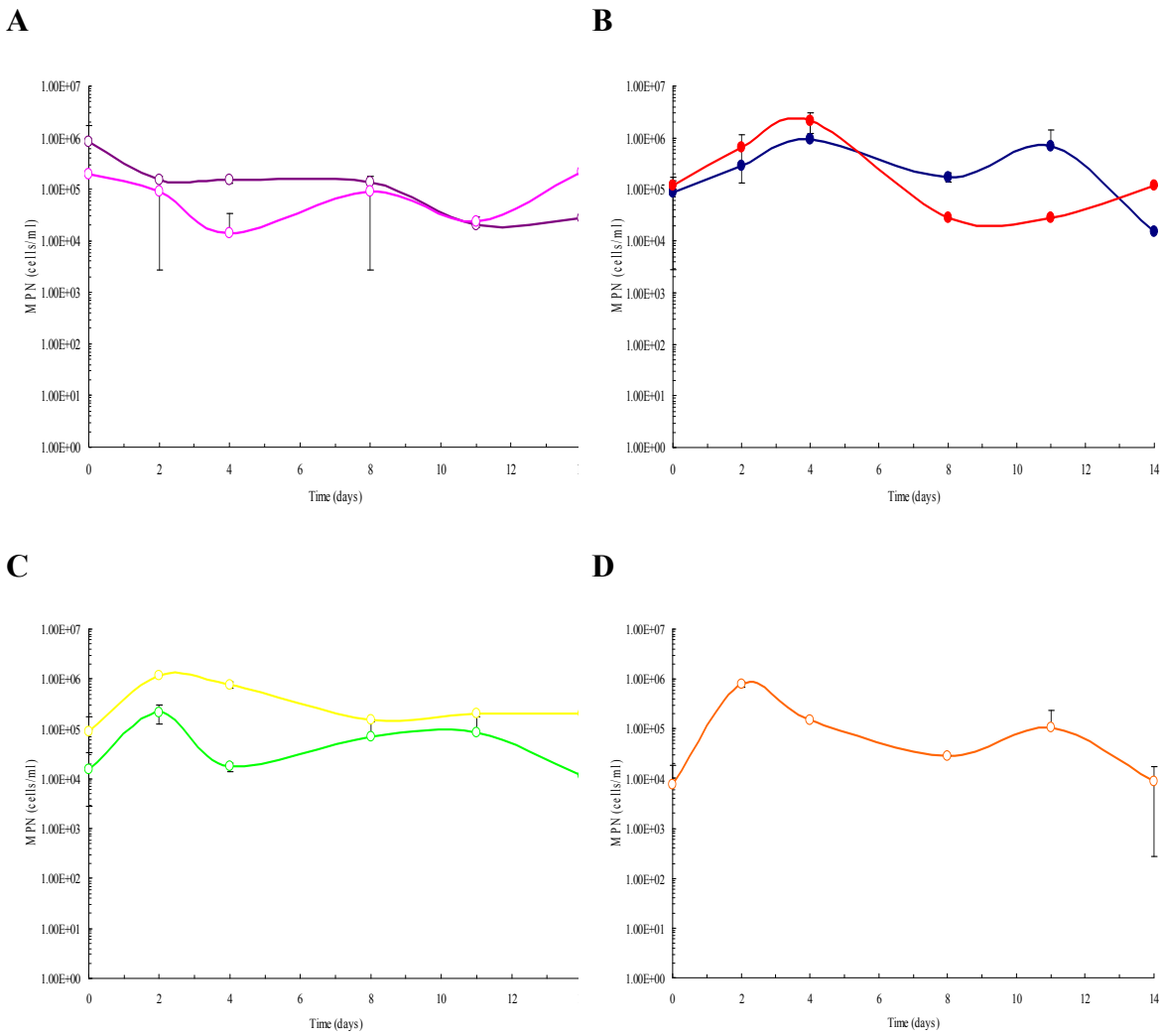


Figure 4.6: Changes in microbial numbers in experiments using phenanthrene as a carbon source in BSMY inoculated with axenic cultures of: A – VUN 10,010 (○) and *Mycobacterium* 1B (○); B – *Mycobacterium* sp. Strain BS5 (○) and *Mycobacterium* sp. Strain KA5 (○); C - *R picketii* (○) and *Mycobacterium* sp. Strain KF4 (○) and D - *S. maltophilia* (○).

Reductions in fluoranthene concentration coincided with microbial growth in experiments inoculated with VUN 10,010, *Mycobacterium* sp. Strain BS5, *Mycobacterium* sp. Strain KA5, *R. pickettii* and *S. maltophilia* (Figure 4.8). A relatively stable microbial population was maintained in experiments inoculated with VUN 10,010 at around $2.4x10^5$ cells/ml. This contrasted with *Mycobacterium* 1B inoculated experiments, where growth was more variable. Following inoculation, the biomass in *Mycobacterium* 1B inoculated experiments increased from $2.4x10^5$ cells/ml to $1.5x10^6$ cells/ml at day 2. Following this increase, a reduction in biomass from $7.4x10^5$ cells/ml to $1.5x10^5$ cells/ml was observed, corresponding with increased fluoranthene removal.

The low numbers of *Mycobacterium* sp. Strain BS5 inoculated at the commencement of the experiment ($1.1x10^3$ cells/ml) may explain the extended lag period and limited removal of 11 mg/l (19.5%) of fluoranthene over the incubation period. Microbial numbers were observed to increase near the end of the experiment ($1.1x10^5$ cells/ml), corresponding with increased fluoranthene removal.

Growth was not always observed to coincide with degradation. The poorest degrader, *Mycobacterium* sp. Strain KF4 was observed only to remove 10.8 mg/l (19.9%) over the incubation period although this failure to degrade fluoranthene was not a result of a lack of growth of the inoculum. An increase in microbial population occurred following inoculation ($2.8x10^4$ cells/ml), peaking at day 5 ($1.7x10^6$ cells/ml). It does not appear that the failure to remove fluoranthene in these experiments was a result of inhibition via degradation intermediate formation, as the growth of the microbial population did not correspond with an increase in degradation. Initial growth may have been as a result of the utilisation of the yeast extract component of the medium.

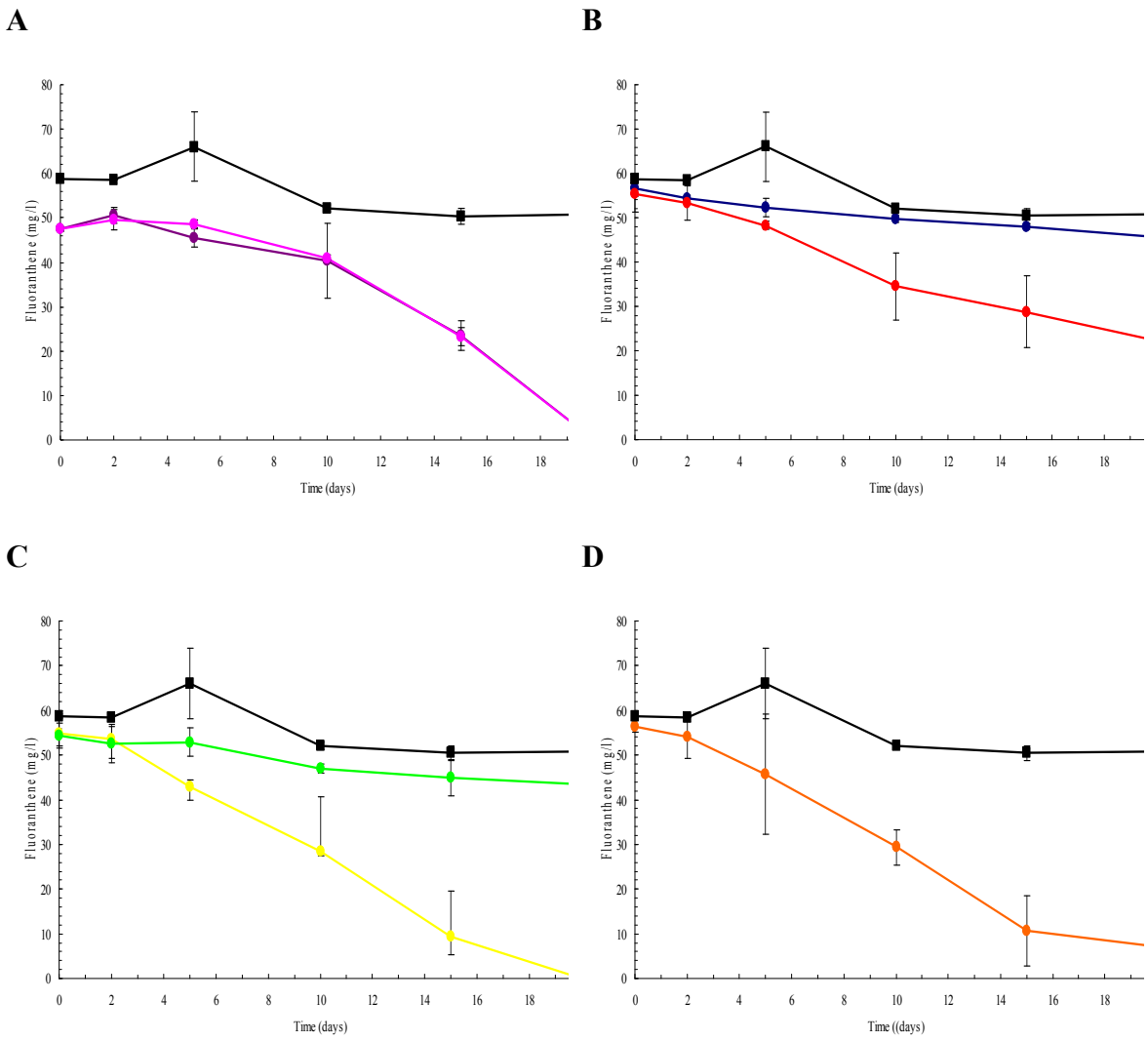


Figure 4.7: Fluoranthene concentration in BSMY inoculated with axenic cultures of: A – VUN 10,010 (●) and *Mycobacterium* 1B (●); B - *Mycobacterium* sp. Strain BS5s (●) and *Mycobacterium* sp. Strain KA5 (●); C - *R picketii* (●) and *Mycobacterium* sp. Strain KF4 (●) and D – *S. maltophilia* (●). Fluoranthene concentration in abiotic controls is also shown (■).

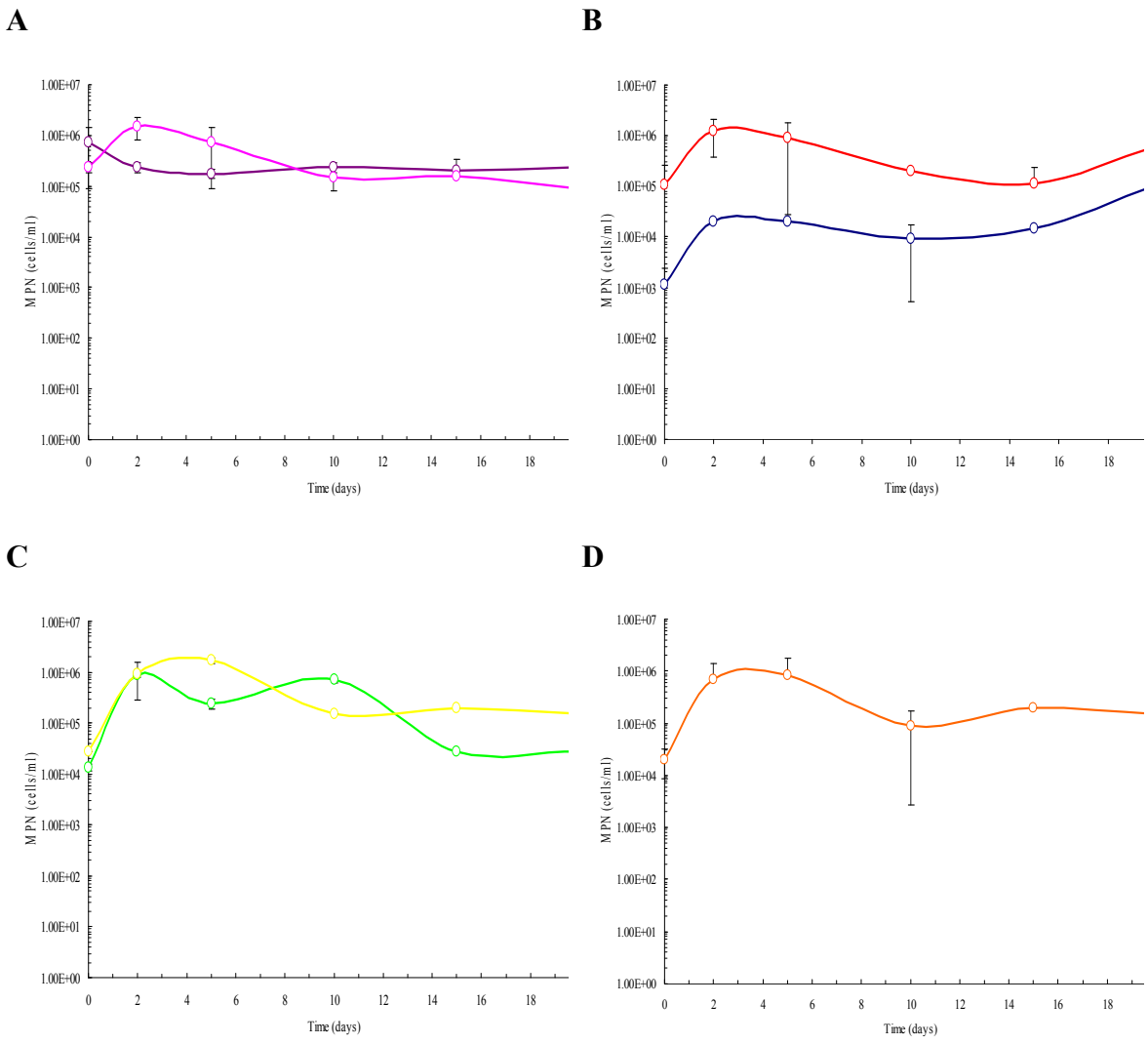


Figure 4.8 Changes in microbial numbers in experiments using fluoranthene as a carbon source in BSMY inoculated with axenic cultures of: A – VUN 10,010 (O) and *Mycobacterium* 1B (O); B - *Mycobacterium* sp. Strain BS5s (O) and *Mycobacterium* sp. Strain KA5 (O); C - *R picketii* (O) and *Mycobacterium* sp. Strain KF4 (O) and D - *S. maltophilia* (O).

4.2.2.3 Pyrene

All isolates were competent pyrene degraders, reflecting the fact that all were isolated on pyrene following enrichment. VUN 10,010, *Mycobacterium* 1B, *Mycobacterium* sp. Strain KA5 and *R. picketii* all achieved a reduction in concentration below detection limits after 20 days incubation in BSMY containing 250 mg/l pyrene (Figure 4.9). The other isolates were able to achieve a similar reduction, with most removing between 94.3% (*Mycobacterium* sp. Strain BS5) and 98.5% (*Mycobacterium* sp. Strain KF4) over the incubation period.

In contrast to the experiment using fluoranthene as the target compound, long lag periods were not observed in the pyrene based experiments. This may have been a product of the bacterial isolates used in the experiments being grown on pyrene prior to inoculation. Removal of pyrene occurred immediately in experiments inoculated with *Mycobacterium* 1B (13.8% removed by day 2) and *Mycobacterium* sp. Strain KA5 (8.5% removed by day 2). Removal in cultures inoculated with VUN 10,010, *Mycobacterium* sp. Strain BS5, *R picketii*, *Mycobacterium* sp. Strain KF4 and *S. maltophilia* occurred substantially after day 2. In all experiments except those inoculated with *Mycobacterium* sp. Strain BS5 and *Mycobacterium* sp. Strain KA5 in which 60% and 66% respectively was removed, a significant proportion of pyrene had been removed by day 10 (between 80% for *S. maltophilia* and 93.5% *Mycobacterium* sp. Strain KF4). The removal of pyrene in *Mycobacterium* sp. Strain BS5 inoculated experiments was observed to occur over a more extended period and did not reach the same extent as the VUN 10,010 or *Mycobacterium* 1B inoculated experiments. The *Mycobacterium* sp. Strain BS5 inoculated experiments had a mean residual concentration of 15.2 mg/l (5.7%).

The increase in pyrene removal at day 2 corresponded with an increase in microbial population in all experiments, although the peak populations varied between isolates (Figure 4.10). In *Mycobacterium* sp. Strain BS5 inoculated experiments, the population declined to $8.9x10^3$ cells/ml following the removal of pyrene to a level below the initial population at inoculation. The VUN 10,010, *R. pickettii* and *S maltophilia* isolates reached their peak population at day 15 of $2.8x10^6$ cells/ml, $1.2x10^6$ cells/ml and $1.5x10^7$ cells/ml respectively.

4.2.2.4 Summary of Degradation of Single PAHs by Fungal and Bacterial Isolates

Both fungal isolates were subjected to further assessment of their capability to degrade a PAH mixture as both an axenic and coculture inoculum. Both *P. janthinellum* and *Ph. chrysosporium* were found to be able to degrade benzo[a]pyrene in MYPD medium, although not in BSM medium.

The assessment of degradative performance of the bacterial isolates was used as a screen to select bacteria for further assessment of their PAH degradative capability as an axenic culture and in coculture inoculum. VUN 10,010, *Mycobacterium* 1B, *Mycobacterium* sp. Strain BS5 and *Mycobacterium* sp. Strain KA5 isolates were selected for further study. *Mycobacterium* sp. Strain BS5 was selected as a bacterial isolate from contaminated soil and *Mycobacterium* sp. Strain KA5 isolate as a bacterial isolate from a non-contaminated environment and an indigenous organism from the soil to be used in later soil microcosm experiments.

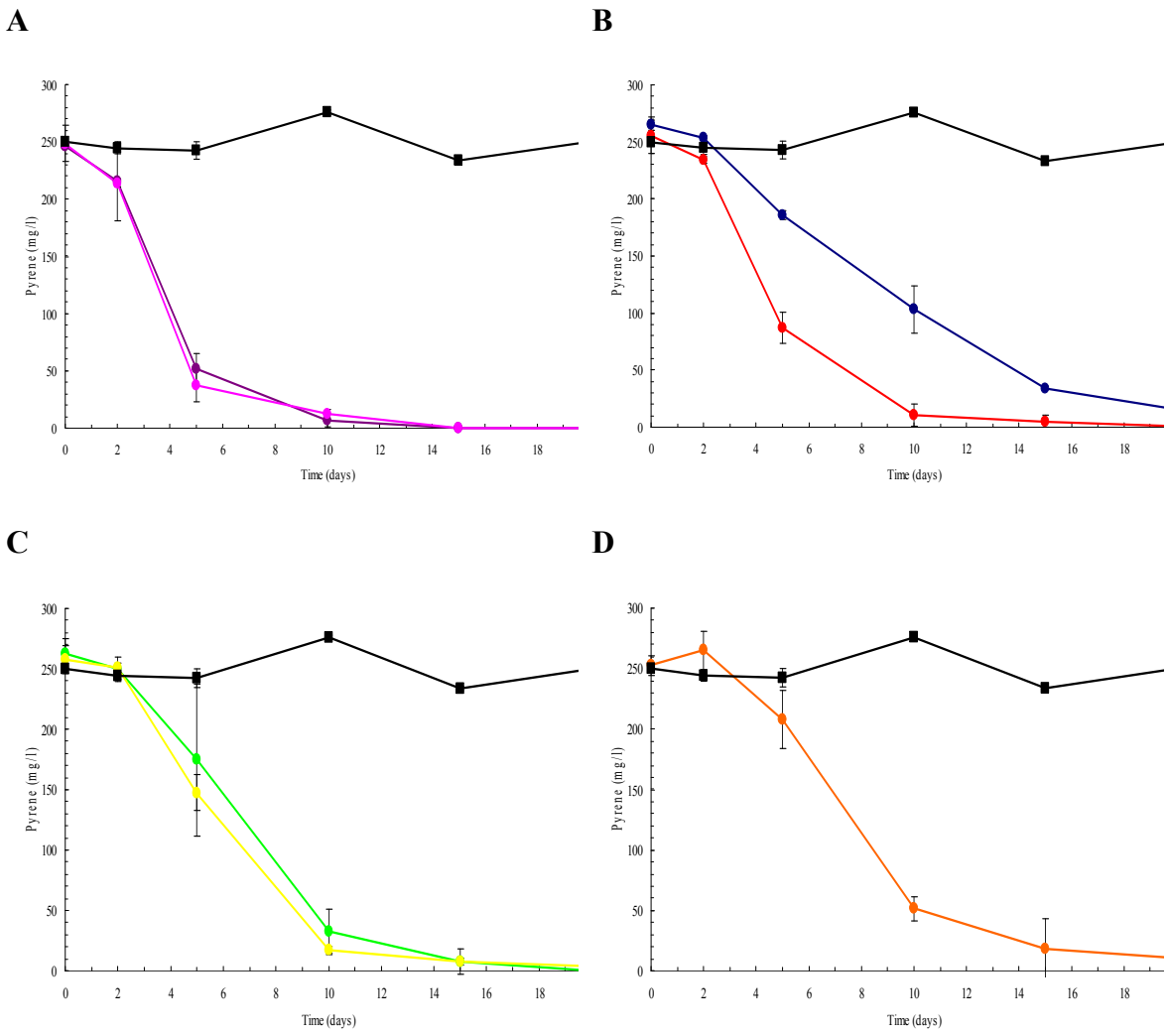


Figure 4.9: Pyrene concentration in BSMY inoculated with axenic cultures of: A – VUN 10,010 (●) and *Mycobacterium* 1B (●); B - *Mycobacterium* sp. Strain BS5s (●) and *Mycobacterium* sp. Strain KA5 (●); C – *R. picketii* (●) and *Mycobacterium* sp. Strain KF4 (●) and D – *S. maltophilia* (●). Pyrene concentration in abiotic controls is also shown (■).

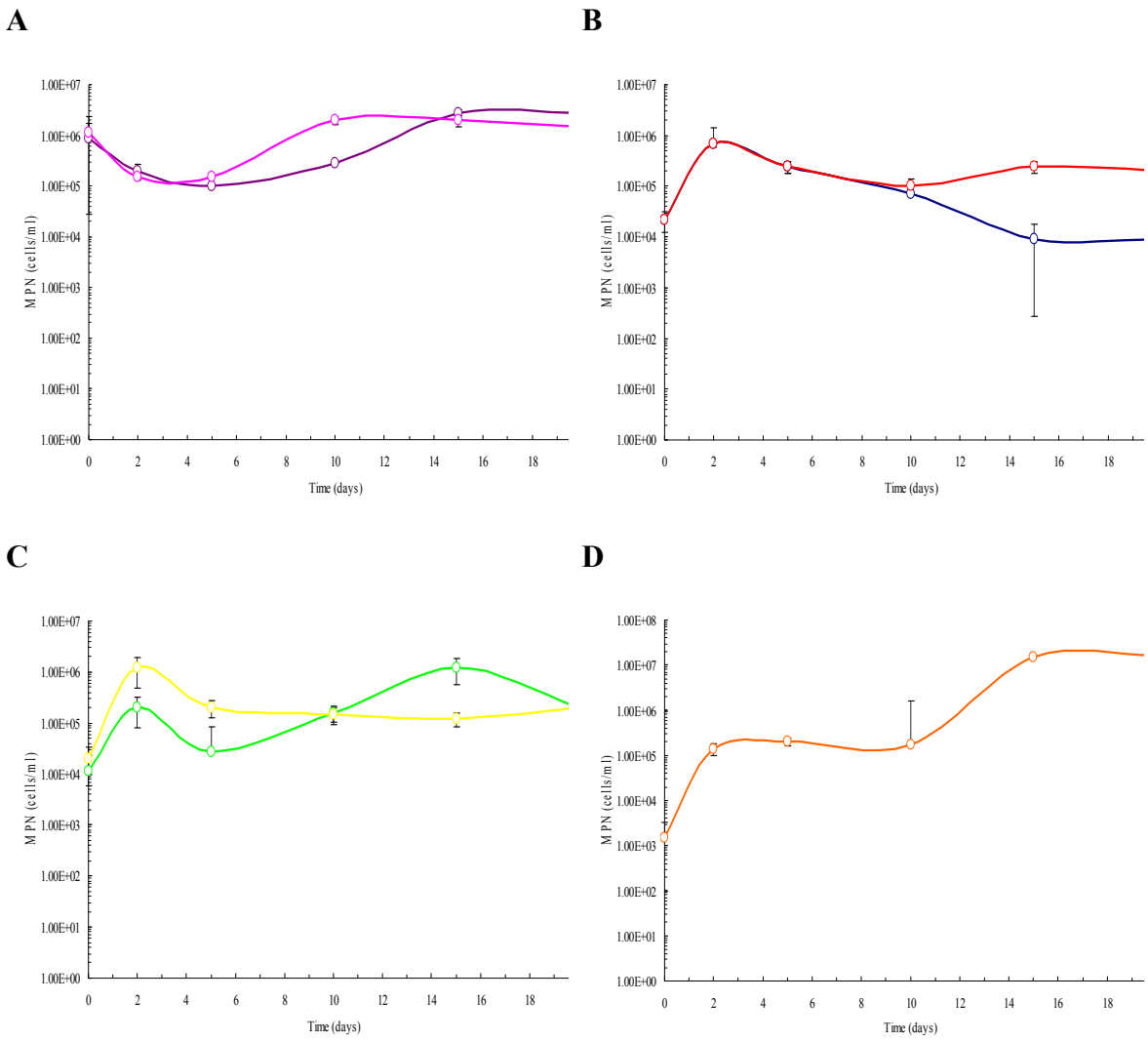


Figure 4.10 Changes in microbial numbers in experiments using pyrene as a carbon source in BSMY inoculated with axenic cultures of: A – VUN 10,010 (○) and *Mycobacterium* 1B (○); B - *Mycobacterium* sp. Strain BS5s (○) and *Mycobacterium* sp. Strain KA5 (○); C - *R picketii* (○) and *Mycobacterium* sp. Strain KF4 (○) and D - *S. maltophilia* (○).

R. pickettii was not selected for further assessment due to its poor performance in degrading phenanthrene. *Mycobacterium* sp. Strain KF4 isolate was not selected for further study due to its poor performance in removing fluoranthene. *S. maltophilia*, whilst a competent degrader of the PAHs tested except fluorene and benzo[*a*]pyrene, was observed to lose the capacity to degrade PAHs following repeated subculturing. This was confirmed during repeated attempts to grow *S. maltophilia* on pyrene for further study.

4.2.4 Axenic Degradation of ^{14}C Radiolabelled [4,5,9,10-^{14}C] Pyrene and [7-^{14}C] Benzo[*a*]pyrene

Radiolabelled experiments were conducted as per Section 2.7.1.4 and analysed as per Section 2.8.3. Despite benzo[*a*]pyrene degradation being observed to occur using these fungal isolates in MYPD, the medium was not used for further assessment. This decision was based on the goal of this work being to develop a viable coculture process. MYPD was considered unsuitable for further experiments due to the potential for the bacterial component to utilize a more easily degradable substrate and hence would not achieve the degradation of the target compounds. The yeast extract component of BSMY was considered to be at a concentration that would not substantially influence the degradation of the target PAH.

4.2.4.1 Pyrene Mineralisation

Figures 4.11 and 4.12 present the results from BSMY liquid culture biometers using ^{14}C radiolabelled pyrene as a carbon and energy source. Figure 4.11 shows the cumulative evolution of $^{14}CO_2$, indicating mineralization, for the respective isolates. Although the time before onset of $^{14}CO_2$ evolution in bacterial

inoculated biometers differed between isolates, the extent was similar for each, ranging between 79% for the *Mycobacterium* sp. Strain KA5 Isolate and 70% for the *Mycobacterium* sp. Strain BS5.

VUN 10,010 was found to have the shortest lag-period prior to the onset of $^{14}CO_2$ evolution, commencing at 48 hours and plateauing at 76.8% of ^{14}C added being evolved as $^{14}CO_2$ at 144 hours. *Mycobacterium* sp. Strain KA5 achieved the greatest extent of mineralization of pyrene, with 79.8% of the ^{14}C added being recovered as $^{14}CO_2$, albeit with a slightly longer lag period. *Mycobacterium* 1B and *Mycobacterium* sp. Strain BS5 achieved similar extents of $^{14}CO_2$ evolution, although the lag-periods for each were longer. *Mycobacterium* sp. Strain BS5 had the most gradual increase in $^{14}CO_2$ generation and may have reached a greater extent if the experiment duration was longer.

A mass balance analysis conducted at the conclusion of the [4,5,9,10-^{14}C] pyrene experiments (Figure 4.12), further indicated that all the bacterial isolates tested were competent pyrene degraders. ^{14}C in the aqueous fraction ranged between 2.2% (*Mycobacterium* 1B) to 2.7% (*Mycobacterium* sp. Strain BS5 isolate) and in the cellular debris between 1.7% (*Mycobacterium* 1B) and 10.6% (VUN 10,010).

$^{14}CO_2$ was not evolved in the fungal inoculated biometers using BSM media. After the 240 hour incubation period, a majority of the ^{14}C added was recovered as undegraded PAH. In *P. janthinellum* and *Ph. chrysosporium* inoculated experiments, 62.9% and 80.4% was recovered as undegraded PAH respectively. Water soluble metabolites were only constituted 0.5% for *P. janthinellum* and 0.6% for *Ph. chrysosporium* of total ^{14}C recovered. A considerable amount of ^{14}C was found in the cellular debris, totaling 36.4% for *P. janthinellum* and 18.9% for *Ph. chrysosporium*.

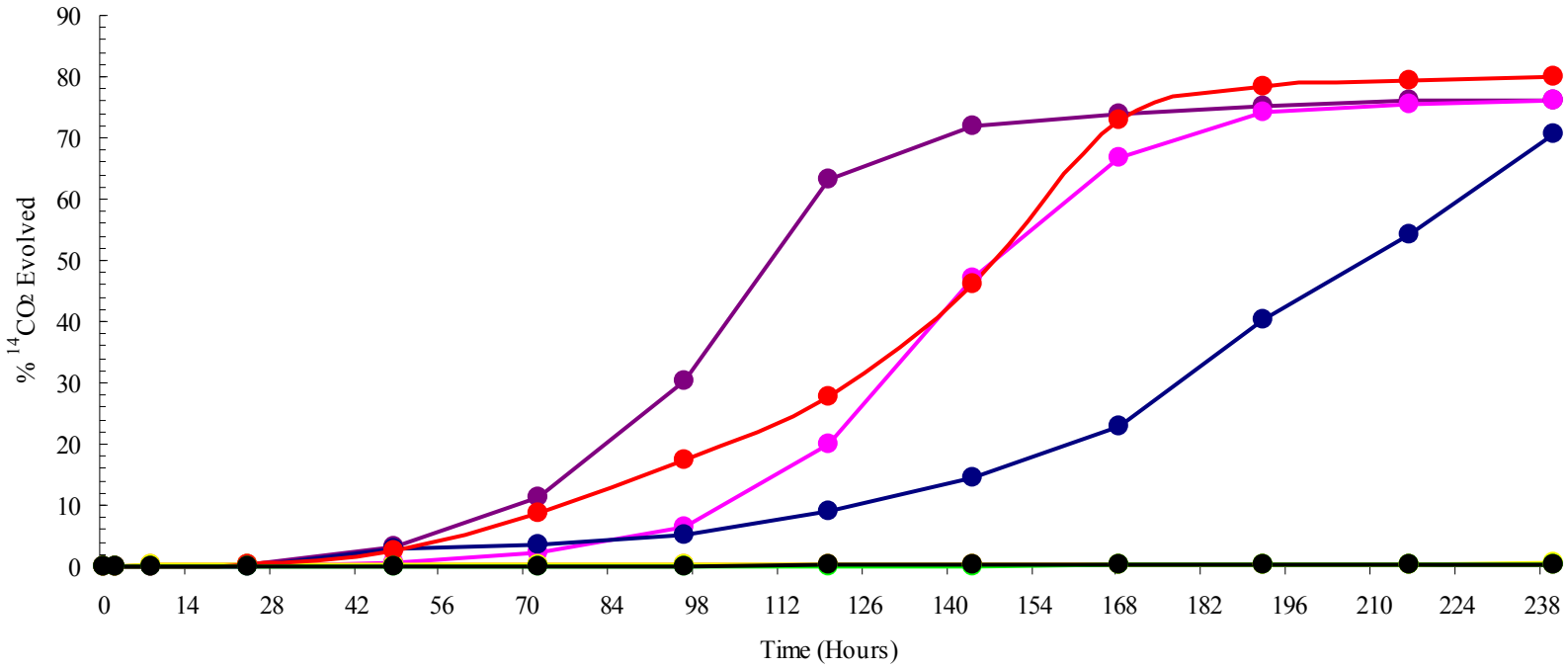


Figure 4.11: Cumulative Recovery of $^{14}CO_2$ Generated in Axenic Inoculated in BSMY Biometers Containing [4,5,9,10-^{14}C] Pyrene. VUN 10, 010 (●), *Mycobacterium* 1B (●), *Mycobacterium* sp. Strain BS5s (●), *Mycobacterium* sp. Strain KA5 (●), *P. janthinellum* (●), *Ph. chrysosporium* (●), Abiotic control(●) and Killed control (●).

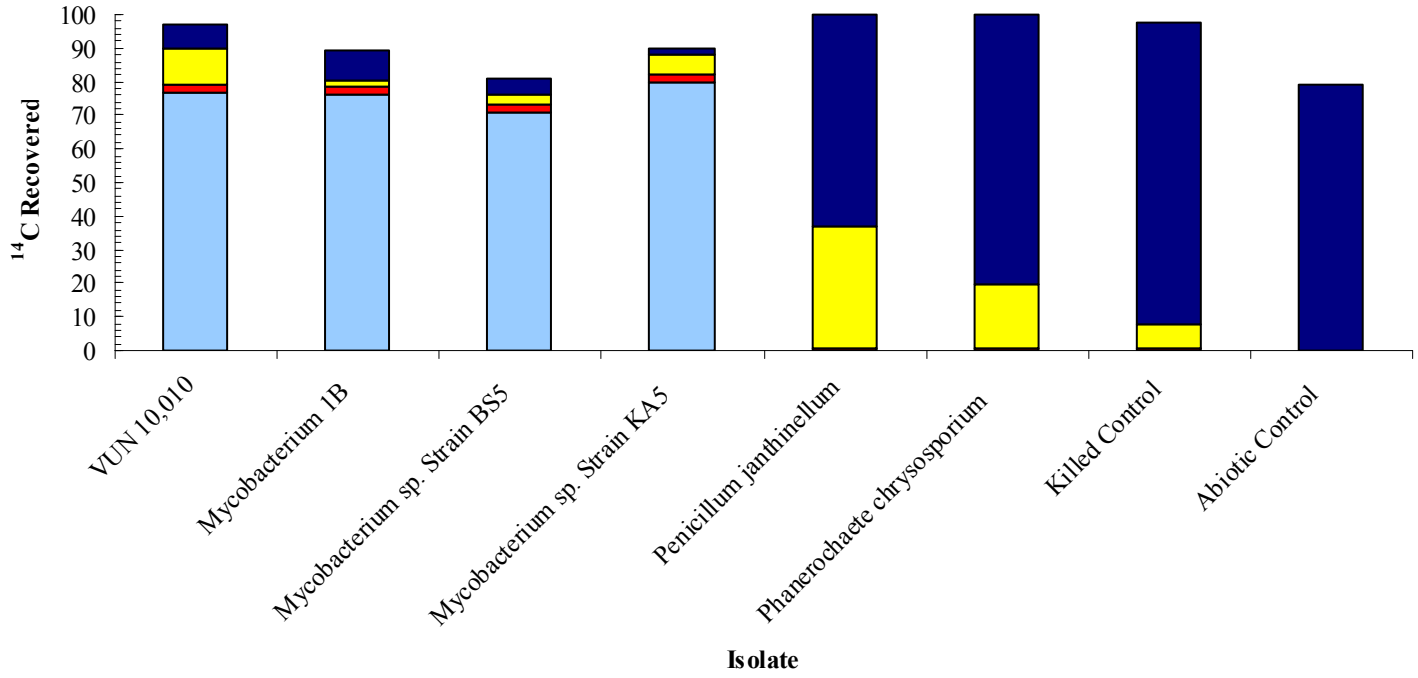


Figure 4.12: Mass Balance from Axenic Inoculated BSMY Biometers Containing [4,5,9,10-^{14}C] Pyrene. $^{14}CO_2$ (), Water soluble metabolites (), Cellular Debris () and undegraded (). Recoveries ranged from a maximum of >99% to a minimum of 79.1%. Data points shown are from duplicate biometers.

4.2.4.2 Benzo[a]pyrene Mineralisation

A number of axenic liquid culture biometer experiments using [7-^{14}C] benzo[*a*]pyrene in BSMY for the bacterial isolates and BSMY and BSM for the fungal isolates were conducted. None of these cultures was found to evolve $^{14}CO_2$, indicating that mineralization was not occurring. Mass balance analysis also found no significant quantities of water soluble metabolites, suggesting that benzo[*a*]pyrene degradation had not occurred.

4.3 AXENIC DEGRADATION OF A PAH MIXTURE IN BSMY

The following experiments were conducted using a PAH mixture comprised of the same PAHs at the same concentrations as used in the previous series of experiments. It was necessary to determine the capability of each of the isolates to degrade PAH mixtures as PAHs are rarely found in the environment as single compounds. As a PAH mixture was being used, an extended incubation period of 90 days was adopted.

4.3.1 Abiotic Controls

As with the single PAH experiments, some PAH loss was observed in abiotic cultures containing the PAH mixture (Figure 4.13). As anticipated and previously observed, abiotic loss of fluorene was the greatest, at around 50.1 mg/l (52%). Some loss of the other PAHs was observed: 36.6 mg/l (14%) for phenanthrene; 4.3 mg/l (8.4%) for fluoranthene and 7.5 mg/l (3%) for pyrene. The "losses" of PAHs may have been due to volatilisation variations in the

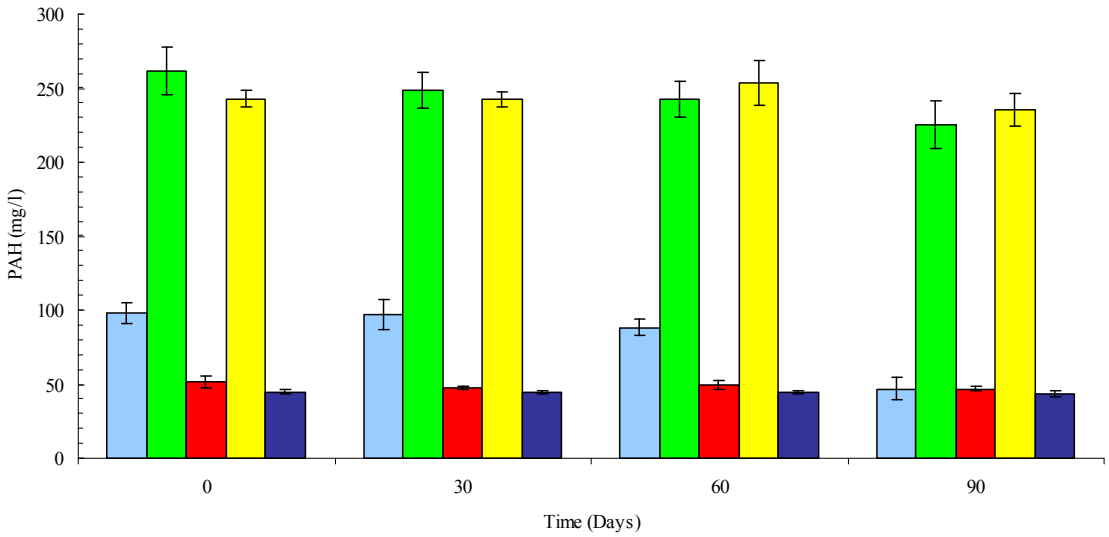


Figure 4.13: Changes in PAH concentration in BSMY abiotic controls supplemented with a PAH mixture containing: fluorene ( ) (100mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for six replicates.

transfer of the PAH mixture to the cultures through pipetting or sorption to the glass of the serum bottles after the 90 days incubation (more likely for the higher molecular weight PAHs). The five PAH spike was combined into a single solution to limit the amount of DMF added to the cultures to limit any toxic effect of the solvent.

4.3.2 Fungal Axenic Cultures

Although not previously assessed for the degradation of single PAHs except benzo[*a*]pyrene, the fungal isolates were examined for their ability to degrade PAHs in the presence of the PAH mixture. These experiments were conducted to determine the contribution of the fungal component to the subsequent coculture experiments using the same experimental conditions.

A limited amount of PAH degradation was found to occur in both *P. janthinellum* (Figure 4.14) and *Ph. chrysosporium* (Figure 4.15) cultures over the 90 day incubation period. There was little variance in the residual concentrations of PAHs in fungal inoculated experiments. Residual concentrations of 19.8 mg/l (19.3%) and 19.4 mg/l (19.3%) for fluorene; 187.4 mg/l (74%) and 188.2 mg/l (72.4%) for phenanthrene; 44.7 mg/l (86%) and 43.3 mg/l (89%) for fluoranthene and 227.6 mg/l (87%) and 231.6 mg/l (92.6%) for pyrene were achieved in *P. janthinellum* and *Ph. chrysosporium* inoculated experiments respectively. A reduction in concentration of 2.1mg/l (5.1%) of benzo[*a*]pyrene was observed in axenic *Ph chrysoporium* cultures although this was not outside of the margin of error observed in abiotic experiments and as such may not be truly indicative of degradation.

In both fungal inoculated experiments, a reduction in biomass was observed through dry weight analysis. A four fold reduction in *P. janthinellum* dry weight ($5.1x10^{-6}$ mg/ml to $1.2x10^{-6}$ mg/ml) and six fold reduction in *Ph. chrysosporium* dry weight ($4.9x10^{-6}$ mg/ml to $8x10^{-7}$ mg/ml) occurred over the 90 day incubation period.

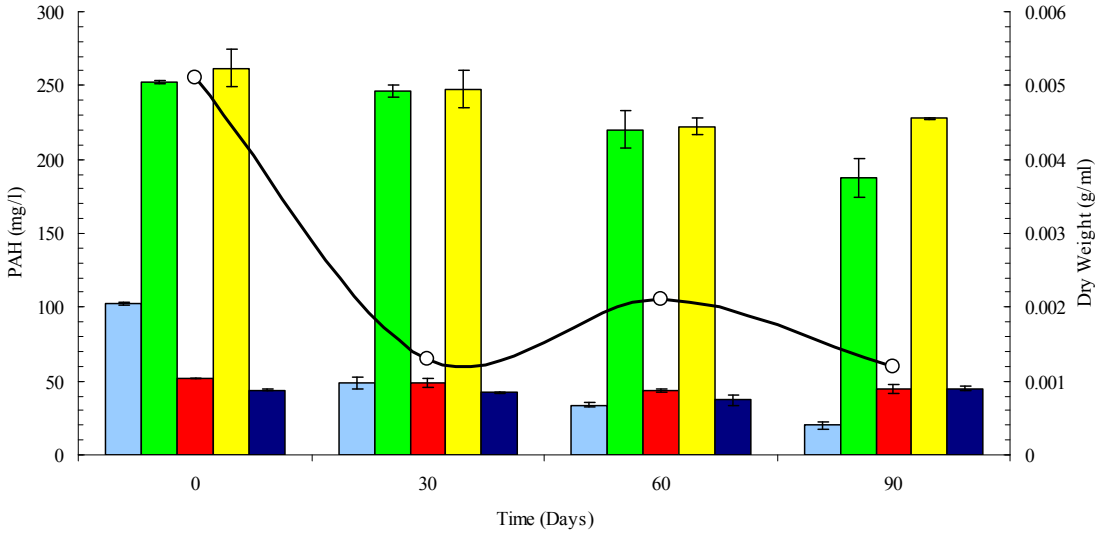


Figure 4.14: Changes in PAH concentration in BSMY axenic *P. janthinellum* inoculated cultures supplemented with a PAH mixture containing: fluorene (■) (100mg/l); phenanthrene (■) (250 mg/l); fluoranthene (■) (50 mg/l); pyrene (■) (250 mg/l) and benzo[*a*]pyrene (■) (50 mg/l). Changes in fungal biomass (○). Data points represent the mean and standard deviation for three replicates.

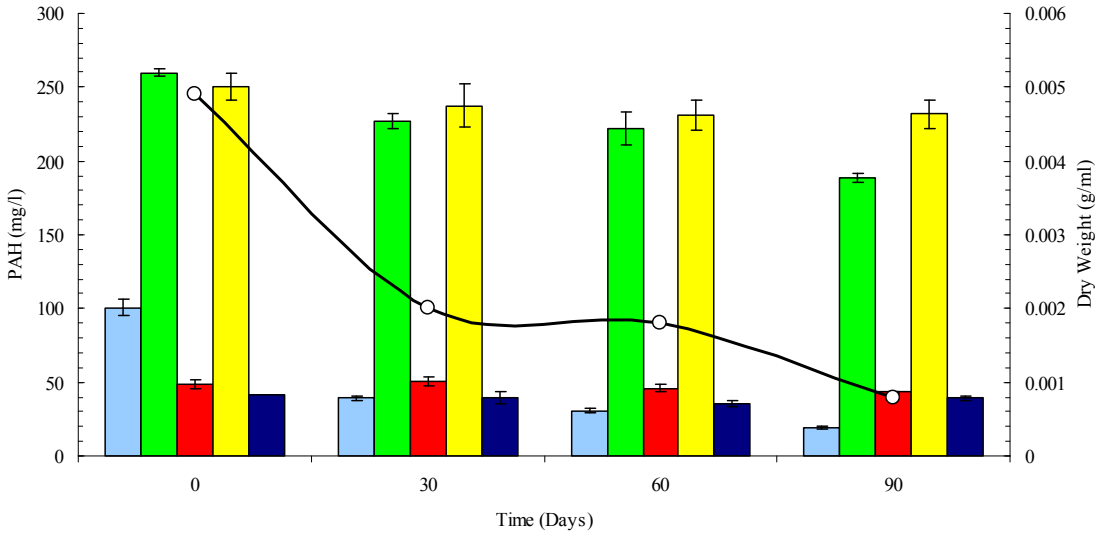


Figure 4.15: Changes in PAH concentration in BSMY axenic *Ph. chrysosporium* inoculated cultures supplemented with a PAH mixture containing: fluorene (■) (100mg/l); phenanthrene (■) (250 mg/l); fluoranthene (■) (50 mg/l); pyrene (■) (250 mg/l) and benzo[*a*]pyrene (■) (50 mg/l). Changes in fungal biomass (○). Data points represent the mean and standard deviation for three replicates.

4.3.3 VUN 10,010 Axenic Cultures

Despite the extended incubation period, the rate and extent of degradation was found to be limited by the presence of five PAHs (Figure 4.16). An example of the impact of multiple PAHs on removal was the degradation of 104.9 mg/l (43%) of the initial pyrene concentration compared to being degraded below detection limits when pyrene was present alone. A similar phenomenon was observed for fluorene, phenanthrene and fluoranthene. The incomplete removal of these PAHs when present in a mixture resulted in residual concentrations of 4.9 mg/l (4.4%), 20.2 mg/l (7.6%) and 8.4 mg/l (17.4%) respectively. This inhibition phenomena was found to occur for all PAHs and no benzo[*a*]pyrene degradation occurred. Concurrent degradation of PAHs occurred as a reduction in concentration was observed for all PAHs under five-rings at each sample point. The extended incubation period over which these experiments were conducted (90 days compared to 14-50 days for the single PAH experiments) did not appear to influence the extent of degradation.

VUN 10,010 cell numbers were observed to decline following inoculation ($1.4x10^6$ cells/ml to $5.4x10^4$ cells/ml) then to increase to $1x10^5$ cells/ml during the middle stages of the incubation period (between days 30-60) corresponding with degradation. This population was maintained over the remainder of the incubation period.

4.3.4 *Mycobacterium* 1B Axenic Cultures

As with the VUN 10,010 axenic culture degradation in the presence of multiple substrates, *Mycobacterium* 1B's capability to degrade five PAHs when present as a mix appeared to be inhibited (Figure 4.17). In the presence of single PAHs, *Mycobacterium* 1B was able to degrade phenanthrene, fluoranthene

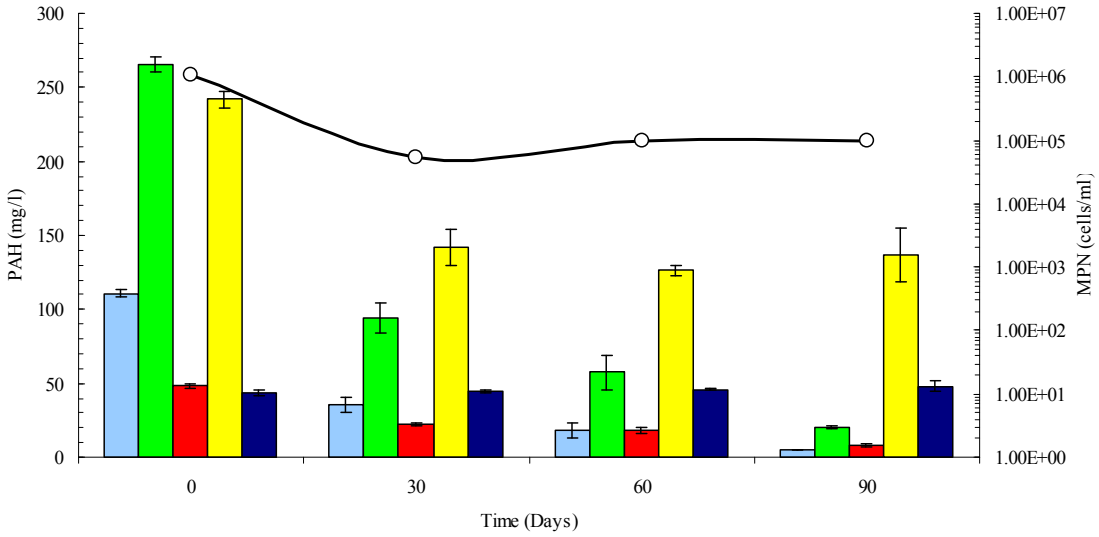


Figure 4.16: Changes in PAH concentration in BSMY axenic VUN 10,010 inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in bacterial biomass (○). Data points represent the mean and standard deviation for three replicates.

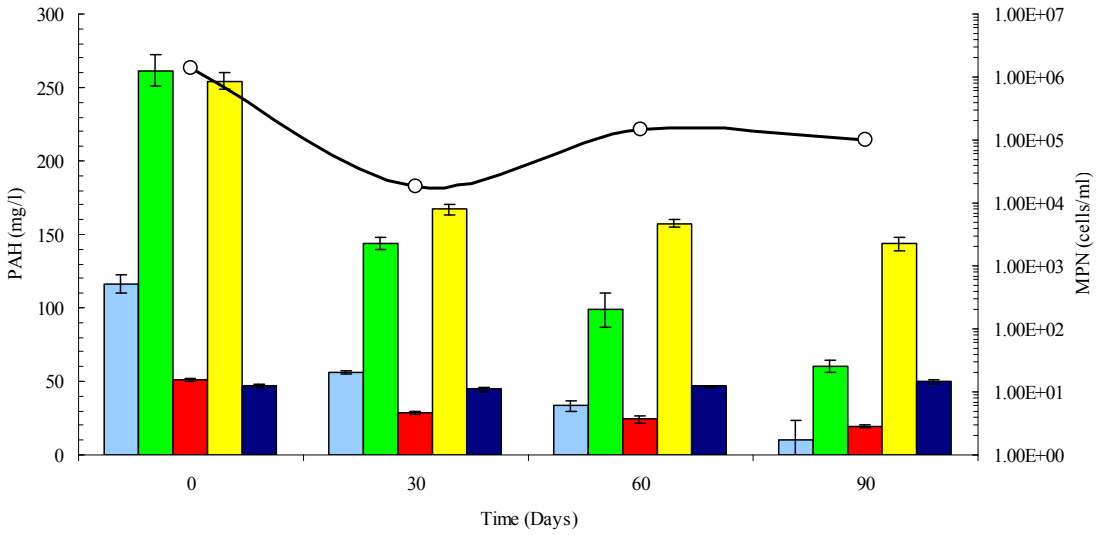


Figure 4.17: Changes in PAH concentration in BSMY axenic *Mycobacterium* 1B inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in bacterial biomass (○). Data points represent the mean and standard deviation for three replicates.

and pyrene below detection limits. In the presence of the PAH mixture, residual concentrations of 10.2 mg/l (8.7%), 60 mg/l (22.9%), 19.4 mg/l (38%) and 143.5 mg/l (56.3%) respectively remained after the 90 day incubation period. As with VUN 10,010, no degradation of benzo[*a*]pyrene was observed.

A greater reduction in microbial population was observed following inoculation of *Mycobacterium* 1B. The population declined from $1.4x10^6$ cells/ml to $1.8x10^4$ cells/ml by day 30, despite a reduction in PAHs occurring. An increase in numbers was observed following this initial decline and remained between $1.5x10^5$ cells/ml and $1x10^5$ cells/ml for the remaining incubation period.

4.3.5 *Mycobacterium* sp. Strain BS5 Axenic Cultures

Mycobacterium sp. Strain BS5's degradative capability for fluorene and fluoranthene in the PAH mixture was found to be greater than that observed when these PAHs were present in isolation, although this appears to have been more a result of the extended incubation period than enhancement due to the presence of other PAHs (Figure 4.18). A residual concentration of 8.1 mg/l fluorene remained after the 90 day incubation period, resulting in a 93% removal in the presence of multiple PAHs compared to 47.6% removal in the presence of the single PAH. Similarly, fluoranthene removal was enhanced in the presence of a PAH mixture, with a 61% removal in the presence of multiple PAHs compared to 24% in the presence of the single PAH. As demonstrated in the radiolabelled pyrene experiments, *Mycobacterium* sp. Strain BS5 achieved degradation at a slower rate and hence the extended duration of the experiment may have been a factor in the observed improvement in PAH degradation. The influence of the extended experiment duration was not observed in other axenic bacterial isolate experiments.

As with the other bacterial isolates, a reduction in the extent of phenanthrene and pyrene degradation was observed when multiple PAHs were present, with residual concentrations of 37.8 mg/l (14.4%) and 150.4 mg/l (59.8%) remaining after the 90 day incubation period. No benzo[*a*]pyrene degradation was observed during the experiment.

The axenic *Mycobacterium* sp. Strain BS5 culture did not experience a reduction of numbers to the same extent as other bacterial isolates following inoculation. A gradual reduction in cell numbers was observed to occur throughout the incubation period, from the starting level of $1.4x10^6$ cells/ml to $1.4x10^4$ cells/ml at day 90.

4.3.6 *Mycobacterium* sp. Strain KA5 Axenic Cultures

As with VUN 10,010 and *Mycobacterium* 1B, *Mycobacterium* sp. Strain KA5 did not achieve the same extent of degradation as in the presence of single PAHs (Figure 4.19). *Mycobacterium* sp. Strain KA5's degradative capacity appeared to be substantially reduced in the presence of the PAH mixture. At the conclusion of the experiment, residual concentrations of 23 mg/l (21.3%), 73.5 mg/l (28%), 34.8 mg/l (70.9%) and 209.1 mg/l (85.3%) of fluorene, phenanthrene, fluoranthene and pyrene remained respectively. No degradation of benzo[*a*]pyrene was observed to occur.

A reduction in microbial population from inoculation levels of $1.8x10^6$ cells/ml to $5.8x10^4$ cells/ml at day 30 was observed. The population recovered to $1.3x10^5$ cell/ml by day 60 and remained around this number for the remainder of the incubation period. The increase in population corresponded with the commencement of PAH degradation.

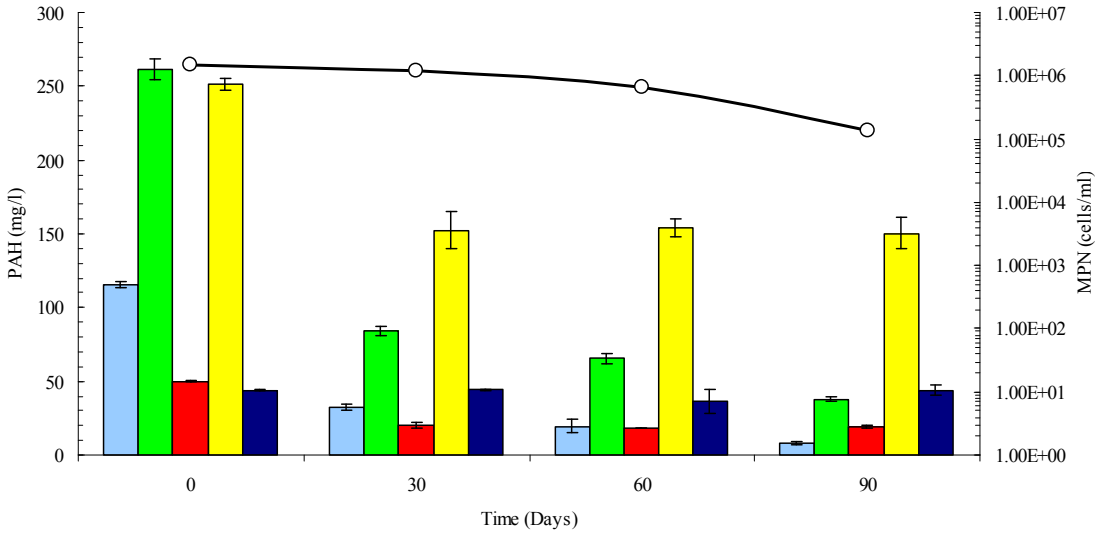


Figure 4.18: Changes in PAH concentration in BSMY axenic *Mycobacterium* sp. Strain BS5 inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in bacterial biomass (○). Data points represent the mean and standard deviation for three replicates.

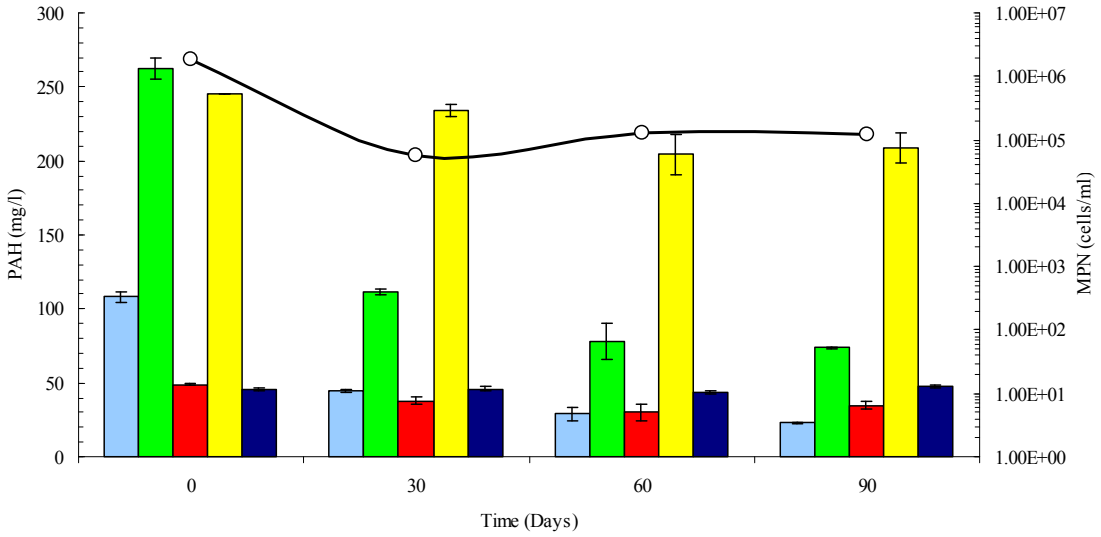


Figure 4.19: Changes in PAH concentration in BSMY axenic *Mycobacterium* sp. Strain KA5 inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in bacterial biomass (○). Data points represent the mean and standard deviation for three replicates.

4.3.7 General Observations of Axenic Isolate's Degradation of the PAH Mixture

As fungal isolates were not initially assessed for their ability to degrade the PAHs used in the PAH mixture individually, only a comparison of the rate and extent of PAH degradation for individual PAHs and the PAH mixture for axenic bacterial isolates is shown Table 4.2. Rates were determined by dividing the amount of PAH degraded at the conclusion of the experiment by the average biomass over the incubation period and dividing that figure by the number of days over which the experiment was conducted. Lag periods were not incorporated into this calculation as the 30 day intervals between sampling in experiments using the PAH mixture did not allow the accurate assessment of any lag period occurring. Due to the variation in the time and bacterial biomass component of this calculation, rate comparisons made were of a general nature as these factors had an influence on the rate determination.

The rate of PAH degradation was not related to the extent of degradation. Rates of PAH degradation in experiments using the PAH mixture ranged from 6 to 73 times less than those in experiments where a single PAH was present. There was a significant difference ($p=<0.05$) between the extent of degradation between individual PAHs and the PAH mixture for each of the bacterial isolates assessed.

Unlike experiments using single PAHs, fluorene removal was substantially greater in all experiments using a PAH mixture than that observed in the abiotic control which had a residual concentration of 46.9 mg/l of fluorene remaining at day 90. This removal may have been a product of the presence of other PAHs inducing the requisite enzymes for fluorene degradation or the degradation of fluorene occurring cometabolically during the degradation of the other PAHs.

The greatest rate of degradation for an individual PAH overall (162 mg/10^6 cells/l/day) was for phenanthrene by *Mycobacterium* 1B. Interestingly, *Mycobacterium* sp. Strain BS5s had the most rapid rate of degradation of fluoranthene (19 mg/10^6 cells/l/day) and pyrene (73 mg/10^6 cells/l/day) when these PAHs were present as single PAHs; although these rates must be considered in light of the average lower biomass in these experiments.

VUN 10,010 was the best performing isolate overall in terms of rate of degradation in experiments using the PAH mixture. The most rapid rate of degradation observed using the PAH mixture was again for phenanthrene (8 mg/10^6 cells/l/day) and achieved by VUN 10,010. The high rates of PAH degradation by VUN 10,010 in experiments using the PAH mixture may have been a product of it having the lowest average biomass (3.4 mg/10^6 cells/l/day) of all the isolates tested.

4.4 COCULTURE DEGRADATION OF THE PAH MIXTURE

The experiments using axenic cultures of fungi and bacteria demonstrated the impact of multiple PAHs on degradation. All bacterial isolates achieved lower reductions in PAH concentration despite a longer incubation period. As a consequence, a series of experiments were undertaken to test the theory that fungal-bacterial cocultures possess both a greater capacity than single organisms to degrade high molecular weight PAHs and PAH mixtures and the ability to degrade benzo[*a*]pyrene.

Table 4.2: Comparison of Degradation of Single PAH and PAH Mixture of Axenic Bacterial Isolates

Inoculum	PAH	Incubation Time (days)		Degradation Lag Period (days)[a]		Average Biomass over Incubation Period (10^5 cells/ml)		PAH Degraded (mg/l)		Rate (mg / 10^6 cells/l/day)	
		S	**M**	**S**	**M**	**S**	**M**	**S**	**M**	**S**	**M**
VUN 10,010	FLU	14	90	-	-	2.0	3.4	-	105.8	-	3.4
	PHE	14	90	2	-	2.2	3.4	252.4	245.3	82	8
	FLA	20	90	5	-	3	3.4	47.2	39.9	7.9	1.3
	PYR	20	90	-	-	12	3.4	246.6	104.9	10.3	3.4
	BaP	50	90				3.4	-	-	-	-
Mycobacterium 1B	FLU	14	90			1.7	4.2	-	106.4	-	2.8
	PHE	14	90	4	-	1.1	4.2	249.3	201.6	162	5.3
	FLA	20	90	5	-	5	4.2	47.6	31.6	4.8	0.8
	PYR	20	90	-	-	12	4.2	248.4	111	10.4	2.9
	BaP	50	90		-		4.2	-	-	-	-
Mycobacterium sp. Strain BS5	FLU	14	90	2	-	3.3	8.6	-	107.2	-	1.4
	PHE	14	90	-	-	3.7	8.6	264.9	223.9	51	2.9
	FLA	20	90	5	-	0.3	8.6	11	30.5	19	0.4
	PYR	20	90	-	-	1.7	8.6	249.6	100.8	73.4	1.3
	BaP	50	90	-	-		8.6	-	-	-	-
Mycobacterium sp. Strain KA5	FLU	14	90	2	-	2.5	5.4	-	85	-	1.7
	PHE	14	90	-	-	5.1	5.4	260.2	188.9	36.4	3.9
	FLA	20	90	2	-	5.3	5.4	33	14.3	3.1	0.3
	PYR	20	90	-	-	2.5	5.4	254.9	36	51	0.7
	BaP	50	90	-	-		5.4	-	-	-	-

S = Single PAH, M = PAH Mixture
FLU = fluorene, PHE = phenanthrene, FLA = fluoranthene, PYR = pyrene, BaP = benzo[*a*]pyrene

[a] Lag period in PAH mixture experiments could not be determined due to 30 day sample intervals.

Using the same fungal and bacterial strains assessed in experiments using the PAH mixture, a variety of coculture combinations were evaluated under equivalent conditions (Section 2.7.1.1 and 2.7.1.2) over a 90 day incubation period. PAH removal and microbial numbers were monitored at day 0, 30, 60 and 90. Experiments were conducted using BSMY as the media. The selection of media for experiments using both fungi and bacteria as inoculants presented difficulties due to the differing nutritional requirements of the coculture components. BSMY was selected as it is a minimal media providing a limited quantity of carbon and nitrogen sufficient for bacterial degradation to be achieved.

Both fungal and bacterial inoculants were prepared as per Section 2.5.4.1 & 2. Degradation was determined by a decrease in PAH concentration compared to abiotic and killed controls. Only changes in bacterial biomass were assessed as previous experiments had shown that an overall decline in fungal biomass occurred in the presence of single PAHs and the PAH mixture in BSMY. Visual assessment of the fungal mycelia provided an indication of the fungal component's viability as upon death, the fungal mycelia either darkened or disintegrated during continued incubation.

These experiments used the same PAH mixture added in axenic liquid culture experiments and were incubated for 90 days. Bacterial growth was assessed using an MPN method for determining PAH degraders. Abiotic and $HgCl_2$ killed controls were run in conjunction with the experiments.

4.4.1 Coculture of VUN 10,010 and *P. janthinellum*

When inoculated as a coculture, VUN 10,010 and *P. janthinellum* were more effective at removing PAHs from BSMY than their axenic counterparts (Figure

4.20). Over the 90 day incubation period, a 91% reduction in total PAH concentration was observed in VUN 10,010 and *P. janthinellum* cocultures, compared to 64% and 15% reductions in total PAH concentration when VUN 10,010 and *P. janthinellum* were inoculated individually. A decrease in the lag period prior to PAH degradation was also observed. For example, fluoranthene was reduced by 50.6 mg/l (94.5%) in the first 30 days of the experiment, compared to a 26.3 mg/l (54%) reduction in axenic VUN 10,010 inoculated experiments. At the end of the incubation period, all three- and four-ring PAHs were degraded below detection limits, however no significant reduction in benzo[*a*]pyrene concentration was observed. Over the time course of the experiment, the three- and four-ring PAHs were removed concurrently, although the rate of removal varied between compounds.

Following inoculation, VUN 10,010 microbial numbers decreased over the initial 30 day period, declining from $5.4x10^5$ cells/ml to $1.8x10^5$ cells/ml. Following this initial lag period, further slight reductions in number of VUN 10,010 occurred over the remaining incubation period, resulting in a population size of $1.6x10^5$ cells/ml at day 60 and $1x10^5$ cells/ml at day 90.

4.4.2 Coculture of VUN 10,010 and *Ph. chrysosporium*

A slower removal rate of three- and four-ring PAHs was observed in VUN 10,010 and *Ph. chrysosporium* inoculated coculture experiments when compared to experiments inoculated with VUN 10,010 and *P. janthinellum* (Figure 4.21). For example, fluoranthene removal in the first 30 days was 35.8 mg/l (66%) compared to 94.5% using the previous coculture combination. The extent of removal was less than the VUN 10,010 and *P. janthinellum* coculture inoculated experiments, with a total PAH removal of 64% compared to 91%. Residual PAH concentrations at the end of the incubation period were: 3.5 mg/l (3.2%) for fluorene; 26.8 mg/l (10.6%) for phenanthrene; 11.7 mg/l (21.7%) for fluoranthene and 139.6 mg/l (54.8%) for pyrene. Pyrene degradation was found to be the most

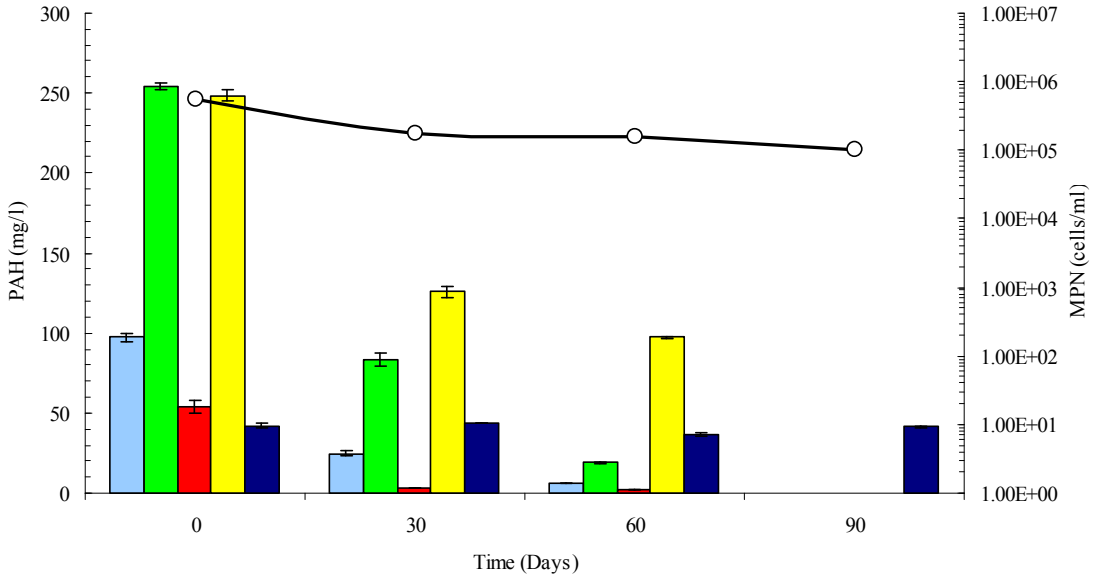


Figure 4.20: Changes in PAH concentration in BSMY VUN 10,010 and *P. janthinellum* inoculated cultures supplemented with a PAH mixture containing: fluorene (■) (100 mg/l); phenanthrene (■) (250 mg/l); fluoranthene (■) (50 mg/l); pyrene (■) (250 mg/l) and benzo[*a*]pyrene (■) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

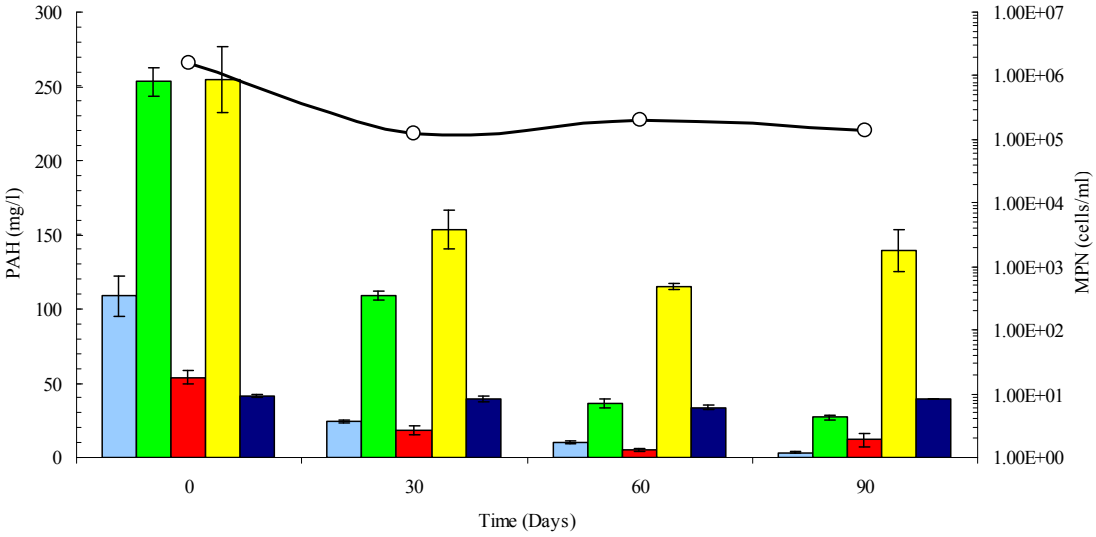


Figure 4.21: Changes in PAH concentration in BSMY VUN 10,010 and *Ph. chrysosporium* inoculated cultures supplemented with a PAH mixture containing: fluorene (■) (100 mg/l); phenanthrene (■) (250 mg/l); fluoranthene (■) (50 mg/l); pyrene (■) (250 mg/l) and benzo[*a*]pyrene (■) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

inhibited in this coculture combination. The results of this coculture combination experiment were similar to those achieved for the axenic VUN 10,010 experiments, indicating that the *Ph. chrysosporium* component did not contribute to the degradation of the three- and four-ring PAHs.

A small amount of benzo[*a*]pyrene removal was observed in the VUN 10,010 and *Ph. chrysosporium* inoculated experiments. A reduction in benzo[*a*]pyrene concentration of 2.4 mg/l (5.7%) was achieved by Day 90.

VUN 10,010 growth in coculture with *P chrysosporium* was not subject to the same level of fluctuation as that in the *P. janthinellum* coculture. As observed previously, a reduction in VUN 10,010 population was observed following inoculation, from $1.6x10^6$ cells/ml to $1.2x10^5$ cells/ml at day 30 that recovered to $2.1x10^5$ cells/ml at day 60 and declined slightly to $1.4x10^5$ cells/ml at day 90.

4.4.3 Coculture of *Mycobacterium* 1B and *P. janthinellum*

Similar to the VUN 10,010 and *P. janthinellum* coculture, the coculture of *Mycobacterium* 1B and *P. janthinellum* enhanced PAH removal beyond that observed in axenic cultures of each. Removal was more akin to that achieved by axenic *Mycobacterium* 1B in the presence of single PAHs (Figure 4.22). Total PAH removal in the coculture experiments was 89% compared with 54% removal in axenic *Mycobacterium 1B* inoculated experiments and 15% removal in axenic *P. janthinellum* inoculated experiments using the PAH mixture. The removal of PAHs using the *Mycobacterium* 1B and *P. janthinellum* coculture combination was slower than that in the coculture combinations using VUN 10,010 as the bacterial component.

The extent of degradation of the three- and four-ring PAHs was greater than that of the VUN 10,010 and *Ph. chrysosporium* coculture. A reduction in concentration below detection limits for fluorene, phenanthrene and fluoranthene and a residual pyrene concentration of 21.53 mg/l (8.4%) was achieved by Day 90. Concurrent PAH degradation was observed to occur, with reductions in concentration for three- and four-ring PAHs recorded at all sample points, although the greatest reductions in pyrene concentration occurred when the concentration of the more easily degraded PAHs had been reduced. No benzo[*a*]pyrene degradation was observed using the coculture combination.

Mycobacterium 1B microbial numbers were observed to decrease following inoculation, falling from $1.1x10^6$ cells/ ml to $1.7x10^5$ cells/ml at day 30. An increase in degradation corresponded with an increase in the *Mycobacterium* 1B population, increasing from day 30 to day 60 ($1.9x10^5$ cells/ml) to $2.1x10^5$ at day 90.

4.4.4 Coculture of *Mycobacterium* 1B and *Ph. chrysosporium*

The *Mycobacterium* 1B and *Ph. chrysosporium* coculture (Figure 4.23) achieved an extent of degradation similar to that of the *Mycobacterium* 1B and *P. janthinellum* coculture. A comparison between the axenic *Mycobacterium* 1B and this coculture showed degradation of total PAHs of 54% and 81% respectively. The differences between the two cocultures using *Mycobacterium* 1B as the bacterial component was not as great as those observed in the cocultures using VUN 10,010 as the bacterial component. Only a 7.7% difference in the total PAHs degraded was observed between the two *Mycobacterium* 1B cocultures, compared to a 27% difference between the VUN 10,010 cocultures.

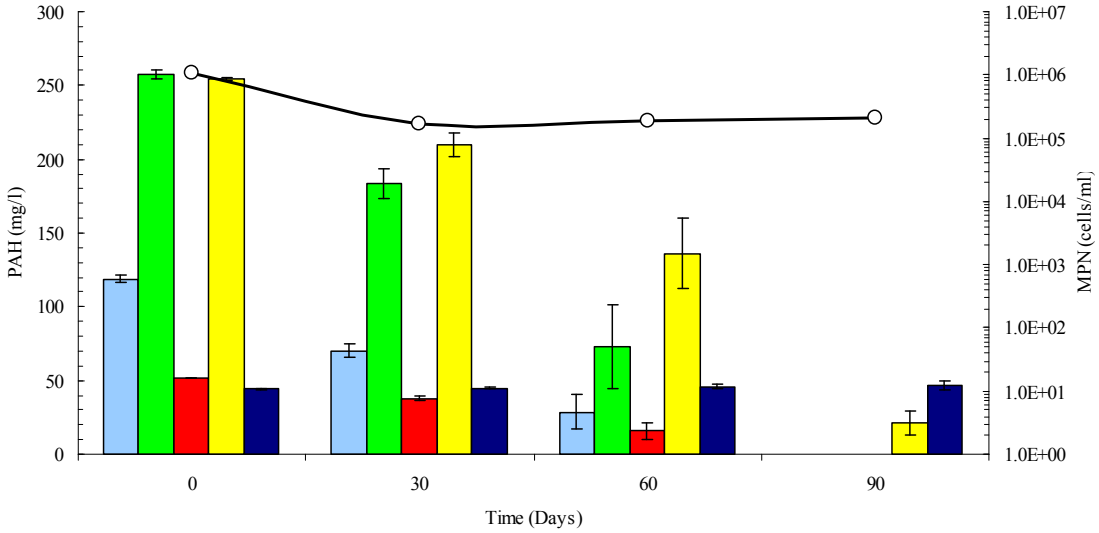


Figure 4.22: Changes in PAH concentration in BSMY *Mycobacterium* 1B and *P. janthinellum* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

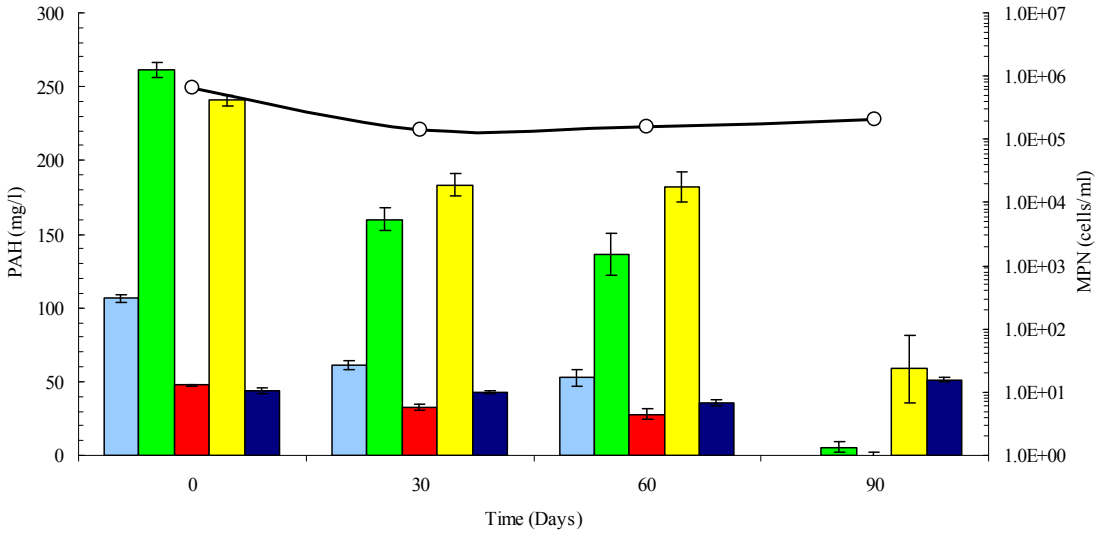


Figure 4.23: Changes in PAH concentration in BSMY *Mycobacterium* 1B and *Ph. chrysosporium* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

Interestingly, the *Mycobacterium* 1B and *Ph. chrysosporium* coculture initially achieved a more rapid removal of the three- and four-ring PAHs than the *Mycobacterium* 1B and *P. janthinellum* coculture. The greatest difference in rate of removal at day 30 between the two coculture combinations was for phenanthrene, where 101.5 mg/l (38.8%) was removed compared to 73.5 mg/l (28.6%), and pyrene, where 57 mg/l (23.7%) was removed compared to 44.7 mg/l (17.6%).

As with the VUN 10,010 cocultures , the extent of degradation in the coculture using *Ph. chrysosporium* was less than that observed in the *P. janthinellum* coculture. Residual concentrations remaining at the conclusion of the experiment were 5.38 mg/l (2.5%) for phenanthrene, 0.5 mg/l (1%) for fluoranthene and 58.5 mg/l (24.3%) for pyrene. No reduction in benzo[*a*]pyrene was observed over the 90 day incubation period.

Following inoculation, a reduction in microbial numbers was observed for *Mycobacterium* 1B with numbers falling from $6.6x10^5$ cells/ml to $1.4x10^5$ cells/ml at day 30. As observed in the *Mycobacterium* 1B and *P. janthinellum* cocultures, the *Mycobacterium* 1B population was observed to grow following this initial decline, increasing to $1.6x10^5$ cells/ml at day 60 to $2.1x10^5$ cells/ml at day 90. Growth of the *Mycobacterium* 1B population corresponded with the increased removal of the three- and four-ring PAHs.

4.4.5 Coculture of *Mycobacterium* sp. Strain BS5s and *P. janthinellum*

The *Mycobacterium* sp. Strain BS5s and *P. janthinellum* coculture combination also demonstrated a better PAH removal than axenic bacterial and fungal cultures (Figure 4.24). This coculture combination achieved an 80% reduction in total PAHs compared to a 58% reduction in the axenic *Mycobacterium* sp. Strain BS5 cultures. At the end of the 90 day incubation period, residual concentrations were

2.6 mg/l (2.3%) for fluorene, 10.6 mg/l (4.3%) for phenanthrene, 3.9 mg/l (8%) for fluoranthene and 56.9 mg/l (23.2%) for pyrene. No benzo[*a*]pyrene removal was observed over the incubation period. The commencement of substantial PAH removal occurred following a 60 day lag period, similar to the experiments using axenic *Mycobacterium* sp. Strain BS5 as an inoculant.

As with the other bacterial inoculants, *Mycobacterium* sp. Strain BS5 cell numbers were observed to decline following inoculation from $6.4x10^5$ cells/ml to $1.2x10^5$ cells/ml at day 30. The population recovered as degradation of the three- and four-ring PAHs increased, reaching $5.4x10^5$ cells/ml at day 60, although it later declined to $1.6x10^5$ cells/ml at day 90.

4.4.6 Coculture of *Mycobacterium* sp. Strain BS5 and *Ph. chrysosporium*

Unlike the other bacterial inoculants, the difference in rate and extent of PAH degradation between cocultures using the same bacterial inoculant and differing fungal inoculants was substantially less for cocultures where the *Mycobacterium* sp. Strain BS5 was the bacterial component. The coculture of *Mycobacterium* sp. Strain BS5 and *Ph. chrysosporium* (Figure 4.25) achieved a total PAHs removal of 76.4% similar to the extent of degradation of the *Mycobacterium* sp. Strain BS5 and *P. janthinellum* coculture removal of total PAHs (80%). The residual concentration of the three- and four-ring PAHs at the end of the incubation period was also similar, with the greatest difference being the extent of pyrene degradation, with a residual concentration of 76.3 mg/l (30.7%) compared to 56.9 mg/l (23.2%) in the *Mycobacterium* sp. Strain BS5 and *P. janthinellum* inoculated coculture.

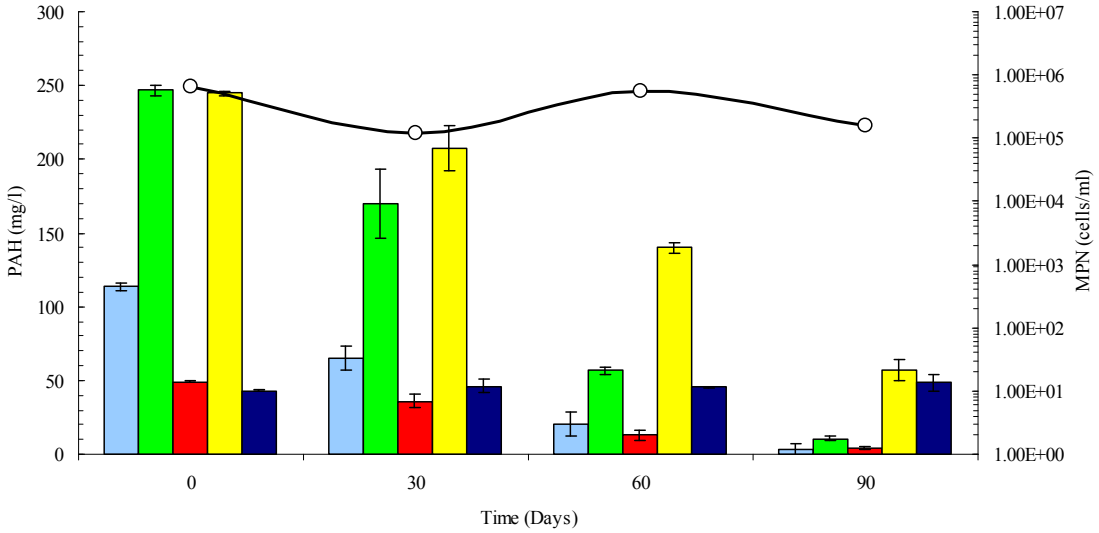


Figure 4.24: Changes in PAH concentration in BSMY *Mycobacterium* sp. Strain BS5 and *P. janthinellum* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

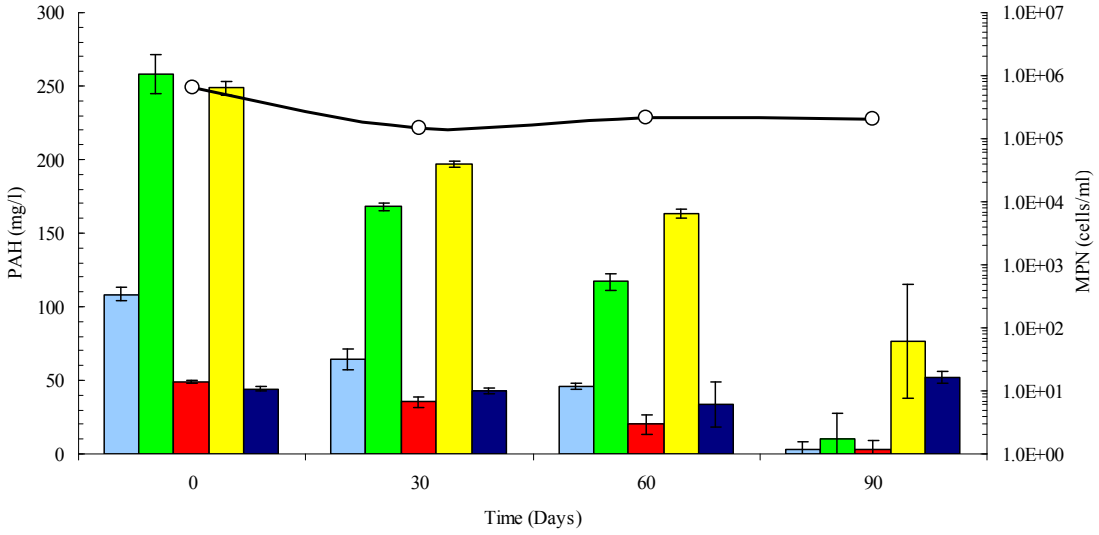


Figure 4.25: Changes in PAH concentration in BSMY *Mycobacterium* sp. Strain BS5 and *Ph. chrysosporium* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

This observation suggested that the fungal component of cocultures using the *Mycobacterium* sp. Strain BS5 as the bacterial component did not exert as great an influence as observed in cocultures using VUN 10,010 and *Mycobacterium* 1B. As in the other coculture experiments, no benzo[*a*]pyrene degradation was observed to occur.

A reduction in *Mycobacterium* sp. Strain BS5 cell numbers was observed following inoculation from $6.4x10^5$ cells/ml to $1.4x10^5$ cells/ml at day 30. Unlike the *Mycobacterium* sp. Strain BS5 and *P. janthinellum* coculture, there was not as large an increase in cell numbers as PAH degradation increases, with levels remaining relatively stable between day 60 ($2.2x10^5$ cells/ml) and day 90 ($2x10^5$ cells/ml).

4.4.7 Coculture of *Mycobacterium* sp. Strain KA5 and *P. janthinellum*

PAH removal was improved by the coculture combination of *Mycobacterium* sp. Strain KA5 and *P. janthinellum* (Figure 4.26) compared to axenic cultures, although the extent was not as great as for the other bacterial isolates combined with *P. janthinellum*. Total PAH removal was 70% for the coculture compared to 37% in the axenic *Mycobacterium* sp. Strain KA5 cultures. Residual concentrations were 2.3 mg/l (0.9%) for phenanthrene, 2.3 mg/l (4.7%) for fluoranthene and 131.4 mg/l (54.2%) for pyrene. No benzo[*a*]pyrene degradation occurred.

Unlike the other bacterial components used in coculture combinations, the *Mycobacterium* sp. Strain KA5 showed no initial decline in cell numbers following inoculation, remaining at $2.3x10^5$ cells/ml to day 30. At day 60, the commencement of increased degradation was accompanied by an increase in cell

numbers to $5.7x10^5$ cells/ml. A reduction in cell numbers to the inoculation levels occurred at day 90.

4.4.8 Coculture of *Mycobacterium* sp. Strain KA5 and *Ph. chrysosporium*

The *Mycobacterium* sp. Strain KA5 and *Ph. chrysosporium* coculture was found to achieve a marginally better removal of PAHs than *Mycobacterium* sp. Strain KA5 and *P. janthinellum* coculture. This contradicted the finding in the other experiments, where using *P. janthinellum* as the fungal component achieved greater reductions in PAH concentration. These coculture combinations achieved an almost identicial reduction in total PAHs, with this *Ph. Chrysosporium* coculture combination achieving a 72% reduction in total PAH concentration compared to the 70% reduction in the *P. janthinellum* coculture (Figure 4.27). The reduction in PAH concentration at day 30 was more rapid than the *Mycobacterium* sp. Strain KA5 and *P. janthinellum* coculture, achieving a 68 mg/l (63%) reduction in fluorene, 159 mg/l (61%) reduction in phenanthrene, 15.1 mg/l (31.7%) reduction in fluoranthene and 15.3 mg/l (6.4%) reduction in pyrene. This initial removal of PAHs had slowed by day 60, and by this time reductions in PAH concentration were less than that of the other coculture using *Mycobacterium* sp. Strain KA5 as the bacterial component. Fluorene was not detectable by the end of the incubation period and residual concentrations were slightly less than the other *Mycobacterium* sp. Strain KA5 coculture, being 1.8 mg/l (0.7%) for phenanthrene, 1.8 mg/kg (3.8%) for fluoranthene and 113.7 mg/l (47.4%) for pyrene. No benzo[*a*]pyrene degradation was observed by the end of the incubation period.

MPN analysis indicated a larger inoculum size of $1.3x10^6$ cells/ml in the *Mycobacterium* sp. Strain KA5 and *Ph. chrysosporium* coculture. As with other experiments, a reduction in numbers at day 30 was observed, reducing to $1.5x10^5$ cells/ml. Following this initial reduction, the population level remained relatively stable, at $1.6x10^5$ cells/ml at day 60 and $2x10^5$ cells/ml at day 90.

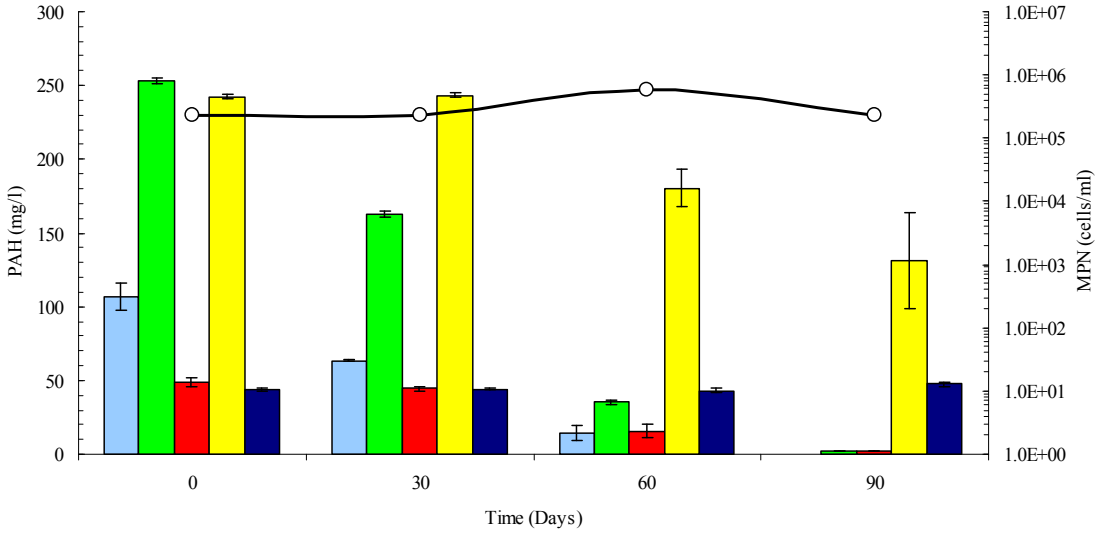


Figure 4.26: Changes in PAH concentration in BSMY *Mycobacterium* sp. Strain KA5 isolate and *P. janthinellum* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

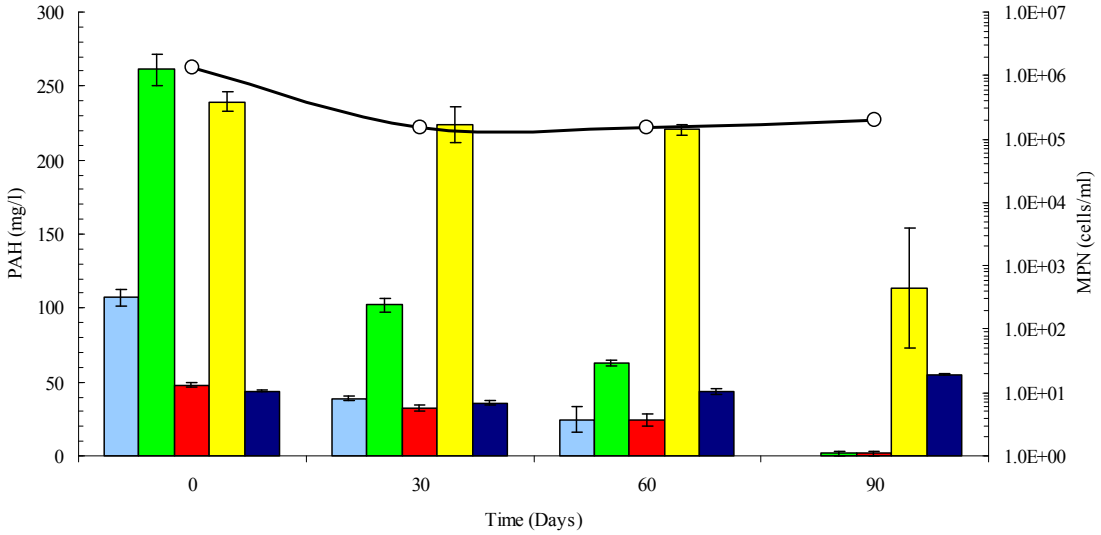


Figure 4.27: Changes in PAH concentration in BSMY *Mycobacterium* sp. Strain KA5 Isolate and *Ph. chrysosporium* inoculated cultures supplemented with a PAH mixture containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Changes in microbial PAH degrading biomass (○). Data points represent the mean and standard deviation for three replicates.

4.4.9 General Observations of Coculture Degradation and Growth

In general, the presence of the fungal component enhanced degradation of all PAHs when compared to axenic bacterial cultures (Table 4.3), although benzo[*a*]pyrene remained largely undegraded. This indicated that the fungal component stimulated the enhanced degradation of fluorene, phenanthrene, fluoranthene and pyrene in liquid culture despite each fungal component not achieving substantial degradation in axenic cultures. The differences being observed in the rate and extent of degradation in experiments using the same bacterial component and differing fungal components further indicated an influence.

Cocultures with *P. janthinellum* as the fungal component generally performed better compared to *Ph. chrysoporium* in terms of extent of removal. The VUN 10,010 and *P. janthinellum* coculture was the best performer of all the coculture combinations trialled in terms of extent of removal, removing 91% total PAHs. The other coculture combinations, using *P. janthinellum* as the fungal component, in order of performance were *Mycobacterium* 1B (89%), *Mycobacterium* sp. Strain BS5 (80%) and *Mycobacterium* sp. Strain KA5 (70%).

In experiments using *Ph. chrysosporium*, VUN 10,010 achieved the least degradation. In these experiments, *Mycobacterium* 1B was the best performing bacterial isolate in conjunction with *Ph. chrysosporium*, removing 81% total PAHs. The other coculture combinations in order of extent of removal were *Mycobacterium* sp. Strain BS5 (76%), *Mycobacterium* sp. Strain KA5 (72%) and VUN 10,010 (64%).

Multivariate statistical analysis using Tukeys HSD test of the differences between axenic bacteria inoculated cultures and cocultures found a significant difference ($p=<0.05$) between cocultures and axenic bacterial cultures for the removal of

higher molecular weight PAHs (particularly pyrene). No significant differences were observed for fluorene or phenanthrene. Differences between the two fungal components similarly became significant for the higher molecular weight PAHs. A significant difference between *P. janthinellum* and *Ph. chrysosporium* was observed for coculture degradation for pyrene and benzo[*a*]pyrene for all bacterial inoculants. This significant difference for benzo[*a*]pyrene should be viewed in light of the limited extent of benzo[a]pyrene degradation, although the standard deviation was small for both ($\pm < 0.1$ mg/l).

In all experiments except the *Mycobacterium* sp. Strain KA5 and *P. janthinellum* coculture, there was an initial decline in bacterial numbers following inoculation. In some instances, the microbial population recovered to, or close to, the level at inoculation. In cocultures with *P. janthinellum* as the fungal component, microbial numbers were observed to recover and grow, coinciding with increased PAH removal. Excepting VUN 10,010, cocultures in which *Ph. chrysosporium* was the fungal component were not observed to achieve an increase in microbial numbers, despite degradation being observed. This may explain the lesser removal generally observed in cocultures in which *Ph. chrysosporium* was the fungal component. The VUN 10,010 and *Ph. chrysosporium* coculture, despite the bacterial component being observed to grow, achieved the least degradation of all coculture combinations assessed. This suggests the influence of another source of carbon contributing to the growth of the bacteria, such as the yeast extract in the BSMY.

Table 4.3: Comparison of degradation rates of PAH mixture in BSMY between axenic bacterial isolates and coculture combinations over a 90 day incubation period.

Inoculum	PAH	PAH Degraded (mg/l)[a]			PAH Degraded (mg/l/day)[b]		
		Ax	Pj	Pc	Ax	Pj	Pc
VUN 10,010	FLU	105.8	97.4	105.3	1.2	1.1	1.2
	PHE	245.3	254.1	226.3	2.7	2.8	2.5
	FLA	39.9	53.5	42.2	0.4	0.6	0.5
	PYR	104.9	248.6	115.2	1.2	2.8	1.3
	BaP	-	0.9	2.4	-	<0.1	<0.1
Mycobacterium 1B	FLU	106.4	119	106.4	1.2	1.3	1.2
	PHE	201.6	257.3	255.9	2.2	2.9	2.8
	FLA	31.6	51.6	47	0.4	0.6	0.5
	PYR	111	233	182	1.2	2.6	2
	BaP	-	-	-	-	-	-
Mycobacterium sp. Strain BS5	FLU	107.2	111.1	105.7	1.2	1.2	1.2
	PHE	223.9	236.5	248.1	2.5	2.6	2.8
	FLA	30.5	45.4	45.8	0.3	0.5	0.5
	PYR	100.8	187.9	172.5	1.1	2.1	1.9
	BaP	-	-	-	-	-	-
Mycobacterium sp. Strain KA5	FLU	85	106.9	107	0.9	1.2	1.2
	PHE	188.9	250.8	259.3	2.1	2.8	2.9
	FLA	14.3	46.5	45.9	0.2	0.5	0.5
	PYR	36	111	125.9	0.4	1.2	1.4
	BaP	-	-	-			

Ax = Axenic Bacterial Isolate, **Pj** = Coculture with *Penicillium janthinellum* as fungal component, **Pc** = Coculture with *Phanerochaete Chrysosporium* as fungal component.
FLU = fluorene, PHE = phenanthrene, FLA = fluoranthene, PYR = pyrene, BaP = benzo[*a*]pyrene
a Reduction in concentration compared to initial concentration
b rate determined by dividing the total amount degraded compared to initial concentration by the number of days of the incubation period or period taken to degrade the PAH below detection limits.

4.4.10 Coculture Degradation of ^{14}C Radiolabelled [4,5,9,10-^{14}C] Pyrene and [7-^{14}C] Benzo[*a*]pyrene

Coculture combinations were also tested for their ability to mineralise [4,5,9,10-^{14}C] pyrene and [7-^{14}C] benzo[*a*]pyrene. Experiments were conducted under the same conditions as those conducted using axenic inoculants. The recovery of ^{14}C following assessment of mass balance in the experiments using [4,5,9,10-^{14}C] pyrene ranged between 86.1% and 96.5%. Despite using the same methodology to extract ^{14}C in experiments using [7-^{14}C] benzo[*a*]pyrene, they achieved lower recoveries of 68%.

4.4.10.1 [4,5,9,10-^{14}C] Pyrene

Results for coculture inoculated experiments are shown in Figure 4.28. Pyrene mineralization was observed to occur at a similar rate and extent as that in experiments using axenic bacterial isolates. *Mycobacterium* sp. Strain KA5 in coculture with *Ph. chrysosporium* was the best performing coculture overall, with 80.2% of the ^{14}C added evolved as $^{14}CO_2$, although this was only marginally better than *Mycobacterium* 1B in coculture with *Ph. chrysosporium* which achieved the evolution of 80% $^{14}CO_2$. *Mycobacterium* 1B was the best performed bacterial isolate in coculture with *P. janthinellum*, with 79.6% of the ^{14}C added evolved as $^{14}CO_2$. Excluding the most efficient degraders, $^{14}CO_2$ evolution for the other coculture combinations ranged between 69% (*Mycobacterium* sp. Strain BS5s and *Ph. chrysosporium*) and 77.8% (*Mycobacterium* sp. Strain KA5 and *P. janthinellum*).

Mass balance at the conclusion of the experiment found that there was limited generation of water soluble metabolites, ranging between 2.5% (both *Mycobacterium* 1B cocultures and *Mycobacterium* sp. Strain BS5s and *P.*

janthinellum) and 3.4% (VUN 10,010 and *Ph. chrysosporium*). The largest amount of ^{14}C (17.5%) in the biomass was found in experiments inoculated with the *Mycobacterium* sp. Strain BS5 and *P. janthinellum* coculture, with other experiments ranging between 1.1% (*Mycobacterium* sp. Strain KA5 and *Ph. chrysosporium*) and 16.2% (VUN 10,010 and *Ph. chrysosporium*).

4.4.10.2 [7-^{14}C] Benzo[a]pyrene

Unlike previous [7-^{14}C] benzo[*a*]pyrene experiments using axenic fungi, which were conducted using BSM media, the coculture experiments were conducted using BSMY media to supply carbon and nitrogen for the bacterial component. Consistent with the findings in the non-radiolabelled liquid culture experiments, no mineralization of benzo[*a*]pyrene occurred in experiments inoculated with the various coculture combinations. Mass balance analysis found that after the extended incubation period, partial transformation had occurred in the microcosm inoculated with VUN 10,010 and *P. janthinellum*, with (8.4%) of the ^{14}C radiolabel in the aqueous phase. Despite some transformation, ^{14}C remained mostly undegraded, either attached to the biomass (76%) or undegraded in the media (15.6%). Comparison to the killed control found 0.4% of the ^{14}C in the aqueous phase, 13.6% attached to the biomass and the remaining ^{14}C as undegraded [7-^{14}C] benzo[*a*]pyrene.

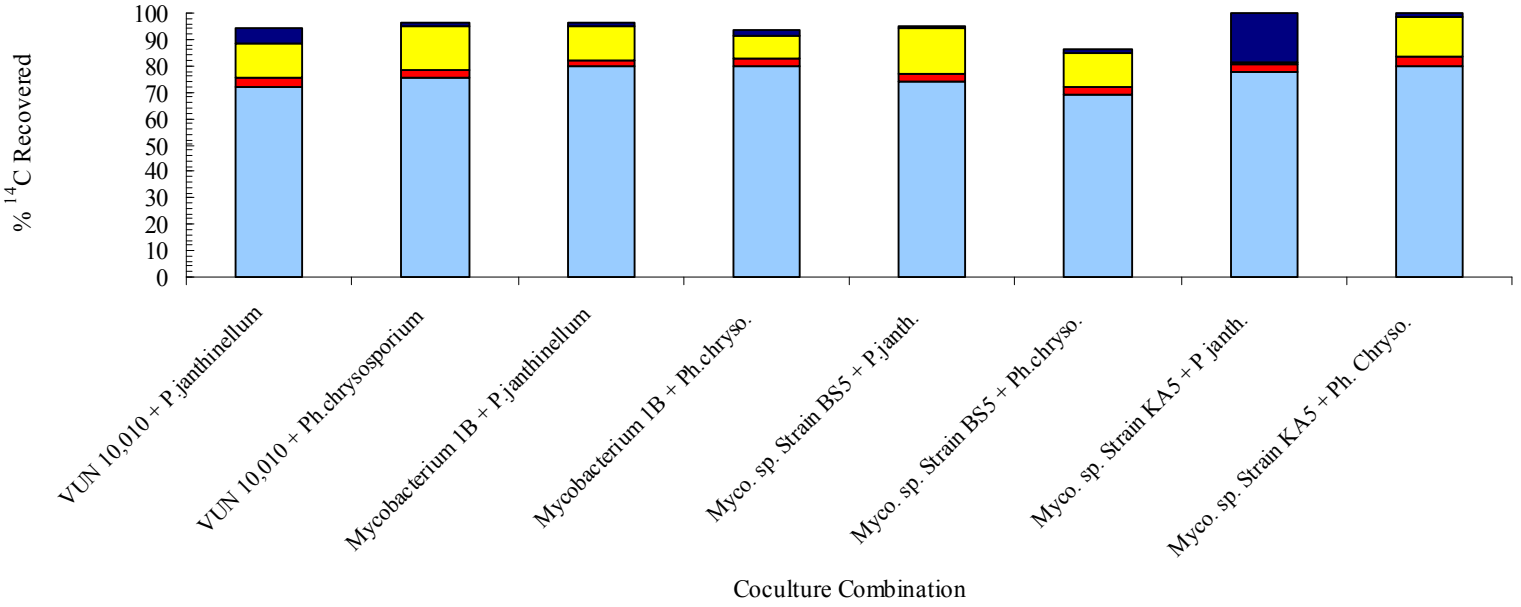


Figure 4.28: Mass balance from coculture inoculated [4,5,9,10-^{14}C] pyrene in BSMY biometers. $^{14}CO_2$ (), Water soluble metabolites (), Cellular Debris () and undegraded (). Recoveries ranged from a maximum of >99% to a minimum of 86.1%. Data points shown are from duplicate biometers.

4.5 DISCUSSION

Biologically mediated degradation is the primary route of PAH removal in the environment. Degradation of three- and four-ring PAHs has been repeatedly demonstrated using a variety of organisms in liquid culture, yet the degradation of five ring, particularly benzo[*a*]pyrene, and other higher molecular weight PAHs is yet to be consistently achieved. The chemical nature of these compounds and their interaction with the environment make them highly resistant to chemical and biological breakdown leading to their persistence in the environment.

The experiments described in Section 4.2 of this Chapter investigated the PAH degrading capability of axenic fungal and bacterial cultures. The axenic fungal experiments, conducted only on benzo[*a*]pyrene, showed limited degradation. The experiments showed many of the axenic bacterial isolates had the ability to degrade a range of PAHs consistent with their enrichment on the same range of PAHs and isolation using pyrene.

4.5.1 Fungal Degradation of Benzo[*a*]pyrene

The major focus of liquid culture experiments involving fungal isolates was to determine their ability to transform benzo[*a*]pyrene (as this was proposed as their primary role in the coculture combination). Although not demonstrated to any great extent in the experiments presented here, ligninolytic and non-ligninolytic fungi have shown an ability to degrade PAHs, such as phenanthrene and pyrene, and achieve their mineralization (e.g. Sack *et al*., 1997; Hammel *et al*., 1992, Moen and Hammel, 1994).

In this work, the fungal component of the coculture was intended to achieve a partial oxidation of benzo[*a*]pyrene to allow the further degradation of benzo[*a*]pyrene transformation products by the bacterial component. It was interesting to note in these experiments that the most substantial degradation of benzo[*a*]pyrene (22.9%) was achieved by *Ph. chrysosporium* in MYPD media. *Ph. chrysosporium* has been shown previously to achieve degradation of benzo[*a*]pyrene in liquid media, but this is usually in N-limited basal salts medium or when provided with an alternative carbon source (e.g. Wunch *et al*., 1997; Field *et al*., 1992).

P. janthinellum was found to achieve limited benzo[*a*]pyrene degradation (9.6%) in MYPD, consistent with observations in experiments conducted by Boonchan *et al*. (2000) using nutrient rich medium. This partial degradation was encouraging as it indicated that *P. janthinellum* and *Ph. chrysosporium* was able to achieve some degradation of benzo[*a*]pyrene, albeit cometabolically in MYPD media.

The fungi assessed for degradation in liquid culture were found to grow during the experiment in MYPD, although this growth was not maintained, tending to confirm previous observations that the degradation of PAHs by fungi does not provide energy for growth (Boonchan *et al*., 2000). This has important implications for future experiments requiring the provision of alternative nutrient sources to produce high initial levels of fungal mycelia to be inoculated to produce sufficient degradative enzymes and accommodate reductions in biomass.

Although benzo[*a*]pyrene degradation was achieved in MYPD media, its use in further experiments assessing a coculture's ability to degrade high molecular weight PAHs was precluded due to it providing an alternative carbon source for the bacterial component. The selection of media and supplements for coculture degradation experiments must be carefully considered as each component has differing nutritional requirements that can have a negative impact on the other component. An alternative example is the provision of nitrogen required for

bacterial degradation. As nitrogen deficiency has previously been shown to induce the production of fungal enzymes used in the degradation of PAHs, the provision of this nutritional requirement of bacteria may detrimentally influence fungal enzyme production, reducing degradation.

The differences in nutrional requirements of the components of the coculture are problematic, particularly when attempting to achieve the degradation of high molecular weight PAHs. The supplements must be balanced so that their provision does not deleteriously influence degradation by the other component. In controlled conditions such as those of liquid culture experiments, this is difficult and in field conditions in soils, the difficulty is compounded by the presence of indigenous organisms.

Carmichael and Pfaender (1997) tried a range of organic and inorganic supplements (e.g. salicylic acid, nutrient broth, M9 buffer and various surfactants) to soils in an attempt to improve the mineralisation of ^{14}C phenanthrene and pyrene. It was found that the addition of supplements actually reduced the mineralisation of ^{14}C phenanthrene and pyrene. A number of reasons were proposed for the reduction of mineralisation, including increases in the population of non-PAH degrading bacteria as a product of the addition of the supplement or the preferential use of the particular supplement as a carbon and energy source. The addition of supplements with components of nitrogen was also observed to suppress mineralisation of the PAHs present. Whilst these experiments were conducted in soil, the suppression of PAH mineralisation illustrates that the provision of any supplements through the particular media selected must be carefully considered.

Atagana *et al*. (2003) observed a similar phenomenon and related it both to the level of nitrogen provided and the molecular mass of the respective PAH. In experiments assessing the impact of nitrogen supplementation on the remediation of creosote contaminated soils, it was found that the provision of nitrogen at 25

C: 1 N below the ratio of 100 C: 10 N: 1 P was most effective in reducing the creosote contamination. It was also observed that the influence of nitrogen supplementation reduced as the molecular mass and structural complexity of the PAH increased.

The variability in the types of fungal enzymes produced as a product of the type of nutrients supplied was considered when selecting the type of medium to conduct further experiments. As discussed in Section 1.4.3, the production of extracellular enzymes is often induced by nutrient limitation, although this was not observed here where benzo[*a*]pyrene removal was observed to occur in MYPD medium. Kotterman *et al*. (1995) observed an increase in anthracene degradation by *Bjerkandera* sp. BOS55 when Mn was limiting and nitrogen sufficient liquid media was used and proposed that the improved degradation was being achieved by the extracellular release of LiP. This demonstrates the influence of supplements not only on enzyme production, but also the influence of the type of enzyme on the transformation of particular PAHs.

Although the above examples relate largely to experiments using soil as the medium, they illustrate the influence of the various nutritional requirements on both the extent and type of PAH degradation occurring. As a consequence of the failure of the two fungal components to degrade benzo[*a*]pyrene in BSM and the unsuitability of using MYPD as the medium for further experimentation due to it providing an easily utilised carbon source for both the fungal and bacterial components, it was decided to proceed with liquid culture experiments using BSMY as the medium.

4.5.2 Bacterial Degradation of Single PAHs

All bacterial isolates showed broad substrate specificity and could effectively degrade PAHs up to and including those containing four fused benzene rings.

The ability of organisms isolated and enriched on pyrene to degrade a number of PAHs has been reported previously. Ho *et al* (2000) enriched 21 bacterial isolates on pyrene plates, all of which were able to degrade pyrene, fluoranthene and phenanthrene without induction, yet only 3 were able to degrade fluorene (Ho *et al*., 2000).

The variation in degradative capability between bacterial isolates has been identified in numerous investigations and has been suggested to be a product of the particular bacterial species' physiology such as cell surface binding, membrane transport and the affinity for PAHs of the various intracellular enzymes (Miyata *et al.*, 2004). It has also been proposed that the degradation of multiple PAHs can be mediated by the same gene, for example the *nahA* gene has been found to mediate the degradation of phenanthrene, fluorene and anthracene (Ahn *et al*., 1999). It is interesting to note the broad substrate specificity of the bacterial isolates, despite being isolated from different and (in some instances), uncontaminated environments and possessing physiological differences such as cell membrane structures (gram negative and gram positive).

Mycobacterium sp. are routinely identified as degraders of PAHs. *Mycobacterium sp*. have been reported as being able to degrade phenanthrene, fluoranthene and pyrene in a number of studies (Heitkamp *et al*., 1988; Sepic *et al*., 1997; Bogan *et al*., 2003). The ability to degrade PAHs has also been reported to be influenced by the presence of co-substrates. In Sepic *et al*. (1997), 16 bacterial isolates from activated sludge were assessed for their PAH degrading abilities. *Mycobacterium sp*. were found to be competent degraders, with the greatest degradation being observed when the PAHs were present as a sole carbon and energy source. This observation was confirmed in this study, with the rates and extents of PAH degradation being greater in experiments using sole PAHs in the presence of axenic isolates than that in the presence of the PAH mixture.

The similarities in performance between *Mycobacterium* 1B and VUN 10,010 were not surprising as *Mycobacterium* 1B was isolated from VUN 10,010 by Dandie *et al*. (2004). In the investigation conducted by Dandie *et al*. (2004), *Mycobacterium* 1B was able to degrade fluoranthene, but not fluorene and VUN 10,010 was observed to degrade fluorene but not fluoranthene. The findings presented in this work contradict those for *Mycobacterium* 1B in the Dandie et al. (2004) study, with substantial removal of fluorene and fluoranthene by both *Mycobacterium* 1B and VUN 10,010 when these compounds were present in the PAH mixture. Whilst this does not agree with the results obtained by Dandie *et al*. (2004), it is in agreement with work conducted by Juhasz (*in press*) that found *Mycobacterium* sp. 1B could utilise fluorene as a growth substrate.

The axenic bacterial isolates used in this study were not able to degrade fluorene beyond (e.g. *Mycobacterium* sp. Strain BS5) or much beyond (e.g. *Mycobacterium* sp. Strain KA5) the abiotic removal observed in uninoculated experiments. This phenomenon was not observed in the coculture experiments, where the presence of the fungal component may have achieved a partial degradation of fluorene, allowing bacterial degradation to occur. This may indicate a phenomena observed in previous studies using *Sphingomonas* sp. and fluorene as a substrate in which it was found that the metabolites of fluorene degradation are stronger inducers of fluorene degradation that fluorene itself (Bastiaens *et al*., 2001). Fluorene metabolites have also been shown to repress fluorene degradation, as was the case when 9-fluorenone (a fluorene degradation metabolite) was shown to prevent further degradation of fluorene by *Arthrobacter* strain F101 (Casellas *et al*., 1998).

The colour change in media resulting in a pink-orange extract found during the degradation of phenanthrene by bacterial isolates has been reported previously. Common to bacterial pathways for phenanthrene degradation is the formation of 1-hydroxy-2-naphthoic acid, which often accumulates, producing an orange colour in liquid medium. It has been proposed that phenanthrene degradation is

biphasic, with 1-hydroxy-2-napthoic acid accumulating while phenanthrene is initially degraded to form pyruvate. When phenanthrene is exhausted, metabolism of 1-hydroxy-2-naphthoic acid commences (Guerin and Jones, 1988). Goyal and Zylstra (1996) observed the media in which *Comamonas testosterone* was incubated with phenanthrene become yellow/brown at the late stationary phase of incubation.

The formation of a brown colour in media during PAH degradation has been found in some instances to be due to the formation of catechol. Park *et al.* (2004) incubated *Pseudomonas putida* NCIB 9816-4 with naphthalene crystals in liquid media, during which a brown colouration developed in the media. The naphthalene degradation pathway is used by some bacteria in the degradation of phenanthrene, resulting in the formation of catechol, giving the media its brown colouration. Although metabolites were not analysed in this work, the darkening of the media and repression of phenanthrene degradation which occured in the *R. pickettii* inoculated liquid media experiments may have been due to such a phenomenon.

All bacteria used in the work presented in this chapter were highly competent pyrene degraders. As all were enriched in the presence of multiple PAHs and isolated on pyrene, it is not surprising that many showed a broad substrate specificity and the ability to not only degrade pyrene, but mineralize it. Boonchan (1998), in experiments using ^{14}C pyrene and VUN 10,010 as an inoculum, reported 70.1% of the ^{14}C added being evolved as $^{14}CO_2$. This compares with 76.8% of the ^{14}C added being evolved as $^{14}CO_2$ by VUN 10,010 in this study and 70% $^{14}CO_2$ being evolved in experiments inoculated with *Mycobacterium* sp. Strain BS5, the least competent pyrene degrader of all the bacterial strains tested. This also compares favourably with $^{14}CO_2$ yields of other bacteria incubated with ^{14}C pyrene, for example 63% mineralization by *Mycobacterium* sp. (Molina *et al.*, 1999). The ability to mineralize the target PAH is highly desirable as it shows the organism tested is capable of the total

destruction of the compound, resulting in the formation of CO_2 and water. Complete removal of the compound eliminates the possibility of formation of metabolites that may accumulate (preventing further degradation) or are potentially more toxic than the parent compound.

PAH mineralisation has been observed in other studies using pure bacterial strains (*Mycobacterium sp.*, *Stenotrophomonas sp.*, *Sphingomonas sp.*, *Rhodococcus sp.* and *Pseudomonas sp.*) grown using naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene as sole carbon and energy sources. When CO_2, biomass, and water soluble metabolites were evaluated, the carbon balances correlated with O_2 consumption. Mineralisation was higher and biomass yields lower for the higher molecular weight PAHs. *Pseudomonas sp.* produced more CO_2 and less biomass than *Rhodococcus sp.*, although metabolites were low and approximately equal for both. Following these results, it was proposed that the low biomass yields in PAH biodegradation is a result of the high maintenance energy requirements of low growth rates (Bouchez *et al.*, 1996).

Although not observed in this work, the mineralization of benzo[*a*]pyrene has been achieved by axenic bacterial isolates (e.g. Chen and Aitken, 1999 and Juhasz, 1998). The partial transformation of benzo[*a*]pyrene has also been achieved by axenic bacteria including *Rhodococcus* sp. strain UW1, *Burkholderia cepacia* strains, *Mycobacterium* strains, as well as a mixed culture containing *Pseudomonas* and *Flavobacterium* species (Walter *et al*., 1991; Juhasz *et al*., 1996; 1997; Schneider *et al*, 1996; Trzesicka-Mlynarz and Ward, 1995). Stanley *et al*. (1999) found *S. maltophilia* strains were able to transform benzo[*a*]pyrene, but in this study, the *S. maltophilia* isolated from kangaroo faeces was not able to transform benzo[*a*]pyrene. This demonstrates the variation in capacity of the *S. malthophilia* to achieve degradation of PAHs and further suggests that the ability to achieve degradation of PAHs in some bacteria may be mediated by a plasmid or some other non-constitutive mechanism. The loss of the capacity to degrade

PAHs in the *S. maltophilia* isolate used in this work may be explained by the ability to degrade PAHs may have been mediated by a non-constitutive mechanism such as a plasmid (Liu *et al*., 2004).

Other studies that have isolated bacteria from the environment have also observed a loss of degradative capability following subculturing. For example, Zhang *et al.* (2004) isolated 18 bacterial strains from contaminated soil samples, eight of which lost their ability to degrade PAHs after four months subculturing (Zhang *et al*., 2004). Several authors have reported the loss of degradative ability if selective pressure is not maintained (Wattiau, 2002).

A variety of growth patterns were observed in PAH degradation experiments, with most showing a reduction in bacterial numbers following inoculation. Growth as a result of PAH degradation has been previously shown to be variable. It is considered that the low biomass yields resulting from PAH degradation may be a product of the high maintenance energy requirements of the low growth rates observed during degradation of high molecular weight PAHs (Bouchez *et al*., 1996).

4.5.3 Fungal Degradation of a PAH Mixture

The limited fungal degradation observed in experiments conducted in this work using a PAH mixture was expected. Fungi (*Ph. chyrsosporium*, *Trametes versicolor* and *Pleurotus ostreatus*) in soil have been shown to be inhibited by the presence of three- and four-ring PAHs in soils, specifically anthracene, phenanthrene and pyrene, although the mechanism of this inhibition was unclear (Novotny *et al.*, 1999). This is despite these organisms and many others being shown to be able to degrade these PAHs when present individually (see Table 1.6).

Although not observed here, fungal degradation of a PAH mixture (phenanthrene, pyrene, anthracene and benzo[*a*]pyrene) has been reported to result in the improved degradation of pyrene (da Silva *et al*., 2003). Degradation of pyrene by *Cyclothirium sp*. increased from 48% in experiments using the single PAH to 60% in experiments using the PAH mixture. Increased metabolic activity due to the presence of more easily degradable PAHs was reported as a potential cause for the improved degradation, although this proposition would contradict the view that PAH degradation is largely incidental and does not provide carbon and energy for growth or activity. Benzo[*a*]pyrene removal in the PAH mixture (48% of the added 10,000 mg/L) appeared to have occurred, although it was found to be largely adsorbed by the fungal mycelia, with only 29% degraded. da Silva *et al*. (2003) conducted these experiments in Sabouraud Dextrose Broths (SDB) which provided an additional carbon source for the fungi used. As was observed in the experiments presented in this chapter, the provision of an alternative carbon source (MYPD) for *Ph. chrysosporium* and *P. janthinellum* assisted degradation of high molecular weight PAHs. As previously stated, however the use of such media for coculture experiments was not considered appropriate due to its potential influence on the bacterial component of the coculture.

The fungal isolates achieved only minor amounts of degradation of the the PAH mixture in BSMY medium. The results of these experiments indicated that there is a potential influence of the presence of three- and four-ring PAHs on the ability of the fungal component's ability to partially transform the higher molecular weight PAHs present in the PAH mixture. The removal of these compounds was the role of the bacterial component of the coculture prompting the experiments assessing the ability of the bacterial isolates to degrade the three- and four-ring components of the PAH mixture.

4.5.3 Bacterial Degradation of a PAH Mixture

It was encouraging to observe that many of the bacterial isolates showed a capability to degrade the PAH mixture, although not to the same extent as single PAHs. These results show broad substrate specificity for PAHs in all the bacterial isolates, potentially a product of their initial enrichment in the presence of multiple PAHs. Interactions between PAHs when present in a mix have been reported previously, particularly the potential to compete for active sites on degradative enzymes (Ye *et al*., 1996).

The varying effect arising from the presence of multiple PAHs has been described previously. Bouchez *et al*. (1999) assessed the degradation of single PAHs and PAH mixtures by single isolates and a bacterial consortia. It was found that some PAH pairings (e.g. anthracene and phenanthrene) enhanced the rate of degradation by single isolates whereas others diminished the rate. For example, the presence of fluorene in the PAH pairings was observed to reduce the rate of degradation of phenanthrene. The presence of multiple PAHs similarly impeded degradation in most instances in this work, reducing the rate in the presence of multiple PAHs by factos of 6 to 73 times that in the presence of single PAHs.

The presence of multiple PAHs has also been shown to improve degradation of certain PAHs. Bauer and Capone (1988) proposed that the enhanced degradation of other PAHs after exposure to a single PAH suggests that the populations selected have either broad substrate specificity for PAHs, common pathways of PAH degradation or both. Referring to other studies, they indicated that this may be related to: (1) certain PAHs being more effective at inducing a single oxygenase system (2) more than one oxygenase system existing in individual species of bacteria and each system having a limited ability to act on a variety of PAHs or (3) the existence of multiple oxygenase systems with each variably

induced by different PAHs. This improved degradation in the presence of multiple PAHs would appear to have been the case in the work presented in this Chapter, with enhanced fluorene removal occurring in the presence of multiple PAHs, whereas when present as a single PAH, no fluorene removal occurred.

Pre-exposure to other PAHs was also found to enhance degradation, particularly of naphthalene. Several aromatic compound degrading microorganisms are known to possess different ring hydroxylating dioxygenases within the same strain (Brezna *et al*., 2003). The ability to degrade multiple PAHs simultaneously has been reported for *Mycobacterium vanbaalenii* - (Moody *et al.*, 2003) although *Mycobacterium austroafricanum* has been reported as having a limited capacity to mineralise multiple PAHs (Bogan et al., 2003).

The formation of metabolites may have repressed the degradation of other PAHs in experiments using axenic bacterial isolates and the PAH mixture. Although *R. pickettii* was the only bacterial isolate that appeared to be inhibited during experiments using phenanthrene as a carbon and energy source, a reduction in the rate and extent of removal was observed in experiments for all bacterial isolates in the presence of a PAH mixture.

Metabolite repression has been shown to occur during degradation of pyrene (Kazunga *et al*., 2002) and benzo[*a*]pyrene (Juhasz *et al*., 2002). It has been suggested that the metabolites that form and accumulate result in the repression of further degradation. Kazunga *et al*. (2002) observed that phenanthrene and benzo[*a*]pyrene degradation was repressed by the presence of metabolites from pyrene degradation, namely *cis*-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione, although this repression varied between the bacterial strains used in the experiment from strong inhibition to no effect.

Similar to the results in this study and others (e.g. Sepic *et al.*, 1997), no benzo[*a*]pyrene degradation was observed using either isolate (*Mycobacterium*

1B or *S. maltophilia*), when the benzo[*a*]pyrene was present either in isolation or in the presence of a cosubstrate such as pyrene (Dandie *et al*., 2004). Some degradation of benzo[*a*]pyrene has been observed using single organisms (e.g. *Mycobacterium austroafricanum*), although this resulted in the formation of organic soluble metabolites rather than achieving its mineralization (Bogan *et al*., 2003). In the experiments using axenic bacterial isolates presented here, no partial or complete degradation of benzo[*a*]pyrene was observed. *Sphingomonas paucimobilis* has been reported as being able to achieve the degradation of benzo[*a*]pyrene in the presence of other PAHs and with the addition of yeast extract as (Ye *et al.*, 1996). Using similar conditions in these experiments (yeast extract and other PAHs), benzo[*a*]pyrene degradation did not occur.

This demonstrated that despite the provision of alternative carbon sources, the bacterial isolates used were incapable of achieving the degradation of benzo[*a*]pyrene and that the factors that prevent benzo[*a*]pyrene degradation in bacteria (e.g. the inability to transport benzo[*a*]pyrene across the cell wall or the absence of enzymes capable of its transformation) remained.

In all instances, the rate and extent of axenic bacterial degradation of three- and four-ring PAHs was reduced when the PAHs were present in a mixture, despite an extended incubation period. The inhibition of three- and four-ring PAH degradation when provided as a mixture further supported the investigation of the potential for the fungal and bacterial coculture combinations to not only enhance the rate and extent of degradation of three- and four-ring PAHs, but also to achieve the degradation of benzo[*a*]pyrene.

4.5.5 Coculture Degradation of a PAH Mixture

The final component of this series of experiments was a trial of varying coculture combinations in liquid culture for their ability to degrade the PAH mixture. The addition of the fungal component significantly enhanced degradation for all bacterial isolates assessed. Although rates and extents differed, the removal of PAHs from the culture medium was enhanced by the use of both fungal and bacterial strains.

It has been routinely identified that the degradation of PAHs by bacterial isolates generally occurs in order of molecular complexity (Ye *et al*., 1996). This was generally observed in the experiments presented here, with the extent of degradation greatest for the lower molecular weight PAHs. In the presence of the fungal component of the coculture (e.g. *Mycobacterium* sp. Strain BS5 and *P. janthinellum*), this was not observed to the same extent, with the degradation of fluoranthene occurring at a similar rapidity as phenanthrene. This would indicate that there are other factors, and not just molecular complexity, affecting the degradation of PAHs in coculture combinations. For example, the induction of the enzymes or multiple enzymes necessary for degradation varies between PAHs, and the presence of multiple PAHs may have produced a stronger induction of the necessary enzymes than the single PAH or the utilisation of metabolites from one or other of the coculture components.

Although the extent of PAH degradation in coculture experiments using *P. janthinellum* was better than that observed in axenic bacterial or fungal liquid culture experiments, no benzo[*a*]pyrene degradation was observed in any of the coculture combinations assessed. The lack of benzo[*a*]pyrene degradation in coculture experiments was disappointing. Boonchan (1998) using the same coculture combination observed a 29.5 mg/l (59%) reduction in benzo[*a*]pyrene after 56 days incubation. Only minimal benzo[*a*]pyrene degradation was

observed in one experiment (inoculated with VUN 10,010 and either *P. janthinellum* or *Ph. chrysosporium*), which was significantly less than that reported by Boonchan (1998).

The lack of fungal degradation was particularly concerning as the partial transformation of benzo[*a*]pyrene is the key to the success of the coculture concept. Despite undertaking the growth and inoculation of both the fungal and bacterial components using methods similar to those used by Boonchan (1998), similar levels of PAH degradation were not achieved. Variability in the PAH degradative performance of the same fungi under similar conditions is not uncommon and emphasises the need to better characterise the conditions that lead to the production of the requisite enzymes for PAH degradation.

Despite the lack of extensive degradation or mineralisation of benzo[a]pyrene, an encouraging result was the finding of 8.4% of the radiolabel in the aqueous phase in biometers inoculated with VUN 10,010 and *P. janthinellum*. This indicated the potential for the formation of water soluble metabolites by *P. janthinellum* in the presence of VUN 10,010 and in media with low concentrations of yeast extract available for bacterial degradation. As the process of forming water soluble metabolites is considered the initial and crucial step in coculture degradation, this result indicated a potential for the process to occur. Boonchan (1998) used the same coculture in experiments resulting in 25.5% of the added radiolabel was evolved as $^{14}CO_2$ and 1.7% was found in the aqueous phase.

4.6 CONCLUSIONS

Despite a failure to achieve the degradation of benzo[*a*]pyrene to any significant extent in liquid culture, the various coculture combinations did demonstrate an ability to degrade the PAH mixture to a greater extent than axenic cultures. The observation that some transformation of [7-^{14}C] benzo[*a*]pyrene was occurring

when media was inoculated with a coculture comprised of VUN 10,010 and *P. janthinellum* suggested that the process was potentially feasible for achieving the degradation of high molecular weight PAHs. The use of this coculture combination required the consideration of methods to enhance the PAH degradative performance of the fungal component. As the VUN 10,010 and *P. janthinellum* coculture showed the most promise, it was selected for further experimentation.

Liquid culture is an effective method of determining degradative capability of organisms, but it is not reflective of the conditions that prevail in soils. As a consequence, this investigation was further developed to undertake a series of experiments using the same PAHs in a well defined soil using the *P. janthinellum* and VUN 10,010 coculture. Soil experiments also assessed the influence of a variety of inoculation protocols and nutrient amendments on coculture PAH degradation to address some of the issues related to fungal PAH degradation observed in experiments using liquid medium.

CHAPTER 5

TRIALS OF COCULTURE PAH DEGRADATION IN SOIL

5.1 INTRODUCTION

The results presented in the previous chapter demonstrated the enhanced capacity of coculture combinations to degrade a mixture of PAHs in a liquid medium compared to axenic cultures. Whilst liquid culture allows a strictly controlled medium to determine a microorganism's ability to degrade PAHs, it is not reflective of the conditions found in soils which can have a significant influence on this capability. In some instances, the transfer from liquid to soil has no significant impact on the organism's degradative ability (e.g. *Mycobacterium austroafricanum* - Bogan *et al*., 2003), whereas in others, the soils physiochemical properties (e.g. organic matter) significantly affect an organism's ability to establish and achieve degradation in the soil environment (Godbout *et al*., 1995 and Van Veen *et al*., 1997).

In the development of a viable inoculum for bioremediation purposes, the assessment of degradation in a well characterized soil allows for the determination of degradative capability in more relevant physical, chemical and biological conditions. Soil microcosms have been used in a number of past studies to assess PAH degradation, using both historically contaminated soils (e.g. Baud-Grasset and Vogel, 1995; Baker-Lee *et al*., 1995) and spiked soils (e.g. Boonchan *et al*., 2000; Gramss *et al.*, 1999; Smith *et al*., 1997).

In recognition of the failure of the *P. janthinellum* to achieve substantial transformation of benzo[*a*]pyrene in liquid culture experiments, a focus of the soil based experiments was maximising the contribution of the fungal component to the coculture process. This was achieved using sequential inoculation protocols and the provision of soil amendments (distilled water and 1% glucose) intended to induce enzyme production through nitrogen limitation (distilled water) or provide additional biomass by the provision of an easily utilisable carbon source. The selection of these amendments was based on previous

findings related to fungal extracellular enzyme induction from limited nutrients (See Section 1.4.3) and the findings of benzo[*a*]pyrene transformation in liquid culture experiments using MYPD medium.

The aim of this chapter was to assess the capacity of the *P. janthinellum* and VUN 10,010 coculture (selected for further investigation on the basis of this coculture's performance in liquid culture) to degrade high molecular weight PAHs in soil. This was achieved by preparing microcosm experiments with Kanmantoo soil, a South Australian agricultural soil low in organic carbon and nutrients and previously unexposed to PAHs. Spiked soil was prepared as per the method in Section 2.7.2.6 and the following investigations undertaken:

1. Natural attenuation by indigenous microorganisms present in the Kanmantoo soil;
2. Bioaugmentation with *P. janthinellum* and VUN 10,010 using different inoculation protocols (concurrent and sequential); and
3. The influence of amendments (distilled water and 1% glucose) on enhanced natural attenuation and sequentially inoculated coculture combinations.

5.2 Assessment of Degradation in Soil Microcosms.

Soil microcosm experiments were prepared using autoclaved well characterized agricultural soil (see Table 3.1 p. 137) provided with amendments as per Section 2.7.2.5. Microcosms were inoculated with individual PAH degrading bacteria, fungi and bacterial-fungal co-culture combinations as per Section 2.7.2.7, incubated at 25°C and their degradative performance monitored over a 100-day period. Aeration was provided by routine mixing of the microcosms. Control microcosms were either uninoculated or inoculated with 0.2% $HgCl_2$ killed fungi and bacteria and used for comparison with inoculated microcosms.

Bacterial numbers in microcosms were determined using a Most Probable Number Protocol as per Section 2.5.5.1. Fungal growth, when analysed, was determined using an MPN method as per Section 2.5.5.2.

An assessment of the distribution of the PAHs in the soil microcosms showed a variance between samples of uninoculated soil of less than 8% (Table 5.1) indicating that the soil spiking method achieved a thorough distribution of PAHs through the soil.

Table 5.1: Variance Between Samples in Soil Microcosm Experiments.

PAH	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	% Avg Variance
Fluorene	77.4	85.4	73.5	82	93.5	90.7	7.3
Phenanthrene	212.5	219.6	214	220.9	237.7	243.7	6.1
Fluoranthene	42	41.7	42.4	40.7	45.4	46.1	3.11
Pyrene	215.9	210.3	218.6	206.1	227.8	231.8	4.2
Benzo[*a*]pyrene	40.1	35.5	40.9	41.2	37.5	33.8	6.7

5.2.1 Loss of PAHs in Killed Control Microcosms

In the killed controls, small but significant decreases in PAH concentration were observed. In many microcosms, fungal growth was observed on the surface of the soil during the incubation period, despite being autoclaved and poisoned with $HgCl_2$. Killed controls were conducted for all experiments and due to the similarity of results, the data presented in Table 5.2 relates to killed controls supplemented with BSMY.

The loss of fluorene (51.5% decrease) was expected as it was observed in abiotic liquid culture experiments, indicating the influences of abiotic losses (e.g. due to

volatilisation). The loss of phenanthrene (29.8% decrease) may also have been due to volatilisation, as has been observed previously to occur in soils (Hawthorne *et al*., 2000). These losses due to volatilisation may have been promoted by the routine mixing of the soil as has previously been reported (Thibodeaux *et al*., 2002).

The reported reduction in concentration of the other PAHs - fluoranthene (19.9% decrease), pyrene (16.4% decrease) and benzo[*a*]pyrene (3.9% decrease) - appears to have been due to sampling variation rather than a loss of PAHs. This is reflected by the variability in PAH concentration over the incubation period.

Losses may also have been due to the failure of autoclaving and addition of $HgCl_2$ to kill all indigenous organisms achieving only a reduction in microbial numbers rather than sterilising the soil. The use of other sterilising methods, such as γ - radiation sterilisation also does not necessarily ensure the destruction of all soil microorganisms (Melcher *et al*., 2002). The failure of the soil sterilisation process may have allowed for the growth of low numbers of indigenous PAH degrading organisms over the incubation period that affected the degradation of the PAHs added to the soil, and may also account for the high degree of variability between killed control microcosms.

5.2.2 Natural Attenuation in Uninoculated Control Microcosms

Uninoculated PAH spiked soil microcosms were used for all experiments to assess PAH degradation via natural attenuation processes. This was necessitated by the isolation of a competent PAH degrader from the Kanmantoo Soil (*Mycobacterium sp. Strain KA5*) and the reductions in PAH concentration observed in controls inoculated with $HgCl_2$ killed inoculum. The existence of an

Table 5.2: Concentrations of fluorene, phenanthrene, fluoranthene, pyrene and benzo[a]pyrene in autoclaved and $HgCl_2$-killed inoculum microcosms supplemented with BSMY containing PAH spiked Kanmantoo soil.

	PAH Concentration (mg/kg)[a]						
PAH	Initial Conc.	20 Days	40 Days	60 Days	80 Days	100 Days	% PAH Decrease[b]
Fluorene	79.5 ± 12.1	65.8 ± 6.6	54 ± 6.3	47.4 ± 10.6	44.1 ± 2.2	38.6 ± 7.3	51.5
Phenanthrene	212.1 ± 23.8	191.9 ± 14.4	167.7 ± 20	155.8 ± 26.7	149 ± 8.7	149 ± 18	29.8
Fluoranthene	63.9 ± 22.8	59.4 ± 2.4	53.9 ± 1.9	34.6 ± 3.9	51.9 ± 2.1	51.2 ± 2.5	19.9
Pyrene	216.9 ± 22.8	209.2 ± 11.3	190.2 ± 14.4	185.1 ± 16.4	181 ± 10.1	181.3 ± 11.2	16.4
Benzo[*a*]pyrene	46.3 ± 2.2	49.8 ± 1.9	47.2 ± 1.9	28.6 ± 1.6	45.5 ± 2.1	44.5 ± 2.2	3.9
Total PAH	618.7	576.1	513	451.1	471.5	464.6	24.9

[a]Data reported is average of triplicate samples
[b]The percentage PAH decrease over the incubation period was calculated with reference to the initial concentration.

indigenous PAH degrading population in the soil had implications for further experiments using the Kanmantoo soil.

5.2.2.1 Influence of BSMY on PAH Degradation by Indigenous Microorganisms

The Kanmantoo indigenous PAH degrading microbial population was observed to grow from undetectable to $3.4x10^6$ cells/g soil by day 60 in PAH spiked soil supplemented with BSMY. The growth of this PAH degrading microbial community coincided with a reduction in the concentrations of fluorene, phenanthrene, fluoranthene and pyrene below detection limits after 80 days incubation. In addition to a reduction in three- and four-ring PAH compounds, the concentration of benzo[a]pyrene was reduced by 18.6 mg/kg (46.4%) after 100 days incubation, although the standard deviation between triplicate samples was 6.3 mg/kg (29.3%)(Figure 5.1).

5.2.2.2 Influence of Distilled Water on PAH Degradation by Indigenous Microorganisms

Limited PAH degradation was observed in microcosms supplemented with distilled water and vitamins/trace elements (Figure 5.2). In comparison to the reductions in PAH concentrations observed in killed control microcosms, only small decreases in fluoranthene (9.1 mg/kg, 17.8%) and benzo[*a*]pyrene (10.9 mg/kg, 24.9%) concentration were observed. The limited variation between microcosms at day 100 indicated that the loss of PAHs was less likely to be a product of sampling variation.

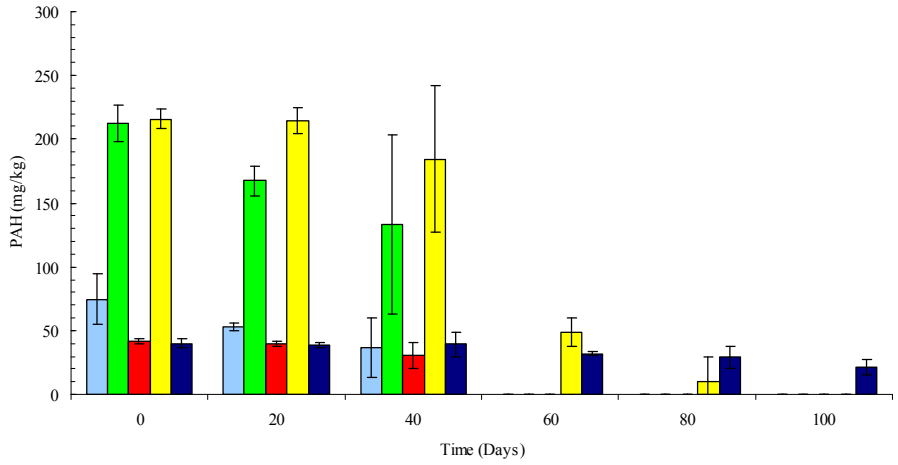


Figure 5.1: Changes in PAH concentration in uninoculated PAH spiked soil microcosms assessing natural attenuation supplemented with BSMY containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

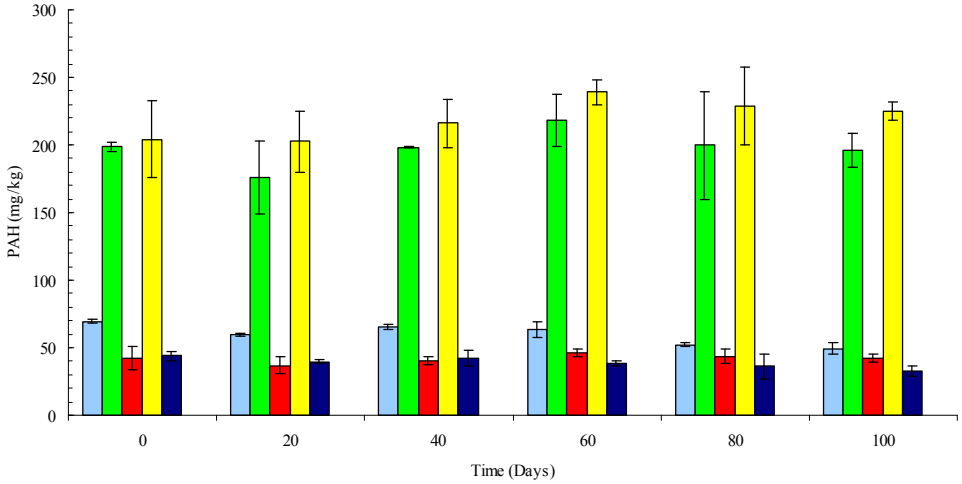


Figure 5.2: Changes in PAH concentration in uninoculated PAH spiked soil microcosms assessing natural attenuation supplemented with distilled water containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

Analysis of microbial growth in these microcosms suggested that the decrease in PAH concentration was mediated by indigenous fungi present in the soil. Fungi were observed to grow in the early phases of incubation in these microcosms, peaking at $1.25x10^3$ cells/g at day 20 before reducing in numbers to undetectable at the conclusion of the incubation period. There was no corresponding growth in PAH degrading bacteria over this period. PAH degradation occurred to the greatest extent later in the incubation period, indicating that the reduction in fungal cell numbers was accompanied by degradation of fluoranthene and benzo[*a*]pyrene. These observations suggest a "passive" fungal mediated degradation not accompanied by growth, pointing to the potential generation of fungal extracellular enzymes being induced by conditions where nutrients are limited and cell die off is occurring.

5.2.2.3 Influence of 1% Glucose on PAH Degradation by Indigenous Microorganisms

A similar degradation profile to microcosms amended with distilled water and vitamins/trace elements was observed in uninoculated microcosms amended with 1% glucose (Figure 5.3). A reduction in concentration beyond that occurring in $HgCl_2$-killed controls was observed for fluoranthene (9.3 mg/kg, 18.2%) and benzo[*a*]pyrene (3.3 mg/kg, 6.7%), albeit to a lesser extent than that observed in the distilled water microcosm experiments.

Fungal mediated degradation is again proposed for the reduction in PAH concentration observed in these microcosms. Fungal cell numbers peaked at day 40 ($1x10^3$ cells/g) and reduced to undetectable levels for the remainder of the incubation period. PAH degradation was observed to occur later in the incubation period, when the more easily degradable glucose became exhausted, indicating that degradation was associated with a reduction in fungal cell numbers.

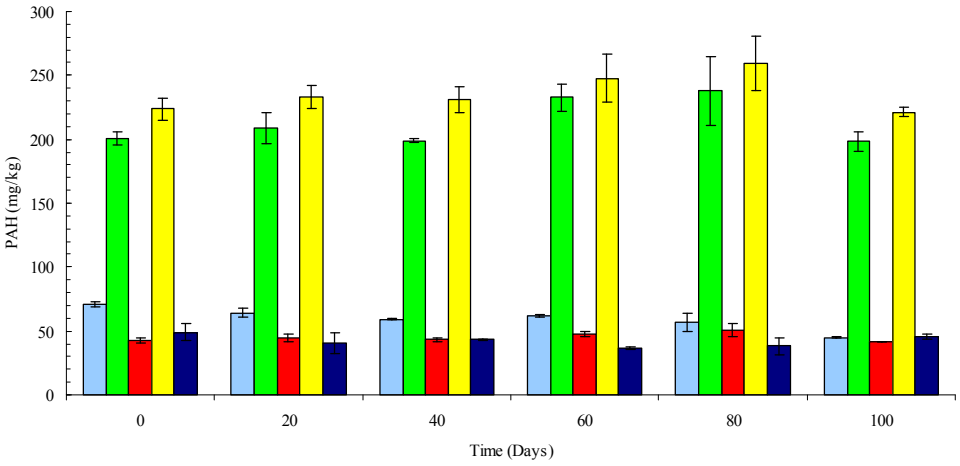


Figure 5.3: Changes in PAH concentration in uninoculated PAH spiked soil microcosms assessing natural attenuation supplemented with 1% glucose containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

5.2.3 PAH Degradation in Inoculated Microcosms

The research outlined in the previous chapter demonstrated that fungal-bacterial coculutres consisting of *P. janthinellum* and VUN 10,010 were the best performing PAH degrading combination assessed. In liquid medium, fluorene (100 mg/l), phenanthrene (250 mg/l), fluoranthene (50 mg/l) and pyrene (250 mg/l) could be degraded to below detection limits after 90 days incubation. In addition, ^{14}C benzo[*a*]pyrene mineralisation experiments indicated that transformation of benzo[*a*]pyrene to water soluble products was possible.

The above results prompted the use of these organisms for the assessment of PAH degradation in soil using various inoculation protocols and soil amendments. The following sections detail microcosm studies investigating PAH degradation in Kanmantoo spiked soil using:

(a) Axenic culture of VUN 10,010 using BSMY to make up water holding capacity;
(b) Axenic culture of *P. janthinellum* using BSMY to make up water holding capacity;
(c) *P. janthinellum* and VUN 10,010 coculture using BSMY to make up water holding capacity;
(d) Sequential inoculation of coculture components using BSMY to make up water holding capacity; and
(e) Sequential inoculation using differing soil amendments to make up water holding capacity (BSMY, distilled water and 1% glucose).

5.2.3.1 PAH Degradation in Axenic VUN 10,010 Soil Microcosms

A rapid reduction in the concentration of three- and four-ring PAHs was observed in PAH spiked soil microcosms inoculated with an axenic culture of VUN 10,010 (Figure 5.4). Fluorene and fluoranthene were removed below detection limits by

day 20, phenanthrene by day 40 and pyrene by day 60. A 4.7 mg/kg (13.3%) reduction in the concentration of benzo[*a*]pyrene was observed at the end of the 100 day incubation period.

PAH degrading microbial numbers were maintained over the majority of the incubation period. An initial increase in the number of PAH degrading microbial numbers was observed at day 20, increasing from $4.5x10^5$ to $1.1x10^7$ cells/g. This population was maintained to day 80, and then fell to $1.4x10^6$ at day 100. A reduction in PAH concentration (particularly fluoranthene and fluorene) corresponded with the increase in population. Reductions in benzo[*a*]pyrene occurred at later periods of the experiment, when the more easily degraded PAHs had been removed.

5.2.3.2 PAH Degradation in Axenic P. janthinellum Microcosms

Axenic fungal microcosms (Figure 5.5) displayed a slower initial rate of reduction for all PAHs, compared to axenic VUN 10,010 soil microcosms. Fluorene was removed below detection limits by day 40, fluoranthene and phenanthrene by day 60 and pyrene by day 80. This compares poorly with the VUN 10,010 inoculated microcosms where phenanthrene and fluoranthene were degraded below detection limits and pyrene was degraded by 92.8% by day 40. A reduction in concentration of 12.9 mg/kg (31.6%) for benzo[*a*]pyrene was observed by day 100. The removal of benzo[*a*]pyrene occurred in a linear fashion over the incubation period, increasing as the more easily degraded PAHs with fewer than 5 rings were removed.

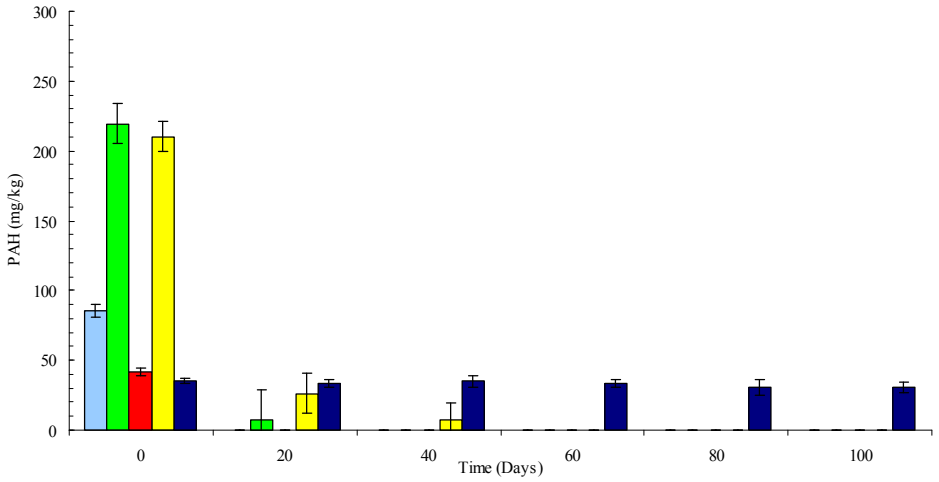


Figure 5.4: Changes in PAH concentration in PAH spiked soil microcosms supplemented with BSMY inoculated with an axenic culture of VUN 10,010 containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

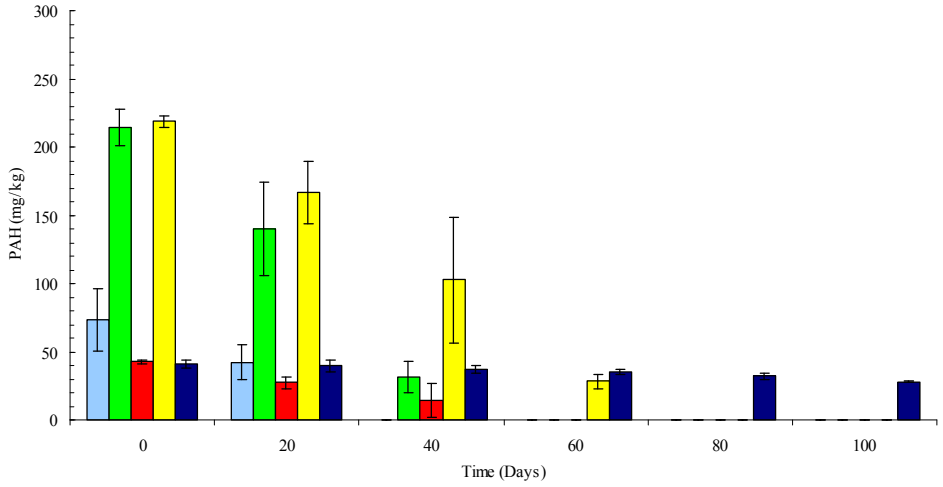


Figure 5.5: : Changes in PAH concentration in PAH spiked soil microcosms supplemented with BSMY inoculated with an axenic culture of *P. janthinellum* containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

Although only inoculated with *P. janthinellum*, a substantial PAH degrading microbial population grew in the microcosms over the incubation period. Microbial PAH degraders increased from $8.6x10^2$ cells/g at day 0 to $4.8x10^7$ cells/g by day 60. This increase in microbial PAH degraders corresponded with the removal of fluorene, phenanthrene, fluoranthene and pyrene. A reduction in benzo[*a*]pyrene concentration followed the consumption of these more easily degraded PAHs, although the population of indigenous microbial degraders was maintained.

5.2.3.3 PAH Degradation in Coculture Microcosms

Following the inoculation of VUN 10,010 and *P. janthinellum* concurrently, all PAHs under five rings were degraded to below detection limits over the 100 day incubation period (Figure 5.6). Fluorene and fluoranthene were degraded below detection limits by day 20, phenanthrene by day 40 and pyrene by day 60. A reduction in concentration of 11 mg/kg (29.4%) benzo[*a*]pyrene was achieved by day 100.

The removal of three- and four-ring PAHs corresponded with an increase in microbial PAH degraders, from $1.2x10^5$ cells/g at inoculation to $7.7x10^8$ cells/g at day 60. The microbial PAH degrading population reduced in size after the removal of the more easily degradable PAHs, resulting in a final PAH degrading microbial population size of $1.1x10^6$ cells/g at day 100.

The coculture inoculation was observed to combine the two degradation profiles observed in axenic experiments. A similar degradation profile to that of the axenic VUN 10,010 microcosm was observed during the initial phases of incubation in the coculture microcosms. Once the PAHs under 5 rings were removed, a reduction in concentration of benzo[*a*]pyrene was observed to proceed similar to that of the axenic *P. janthinellum* microcosm. This indicates that the

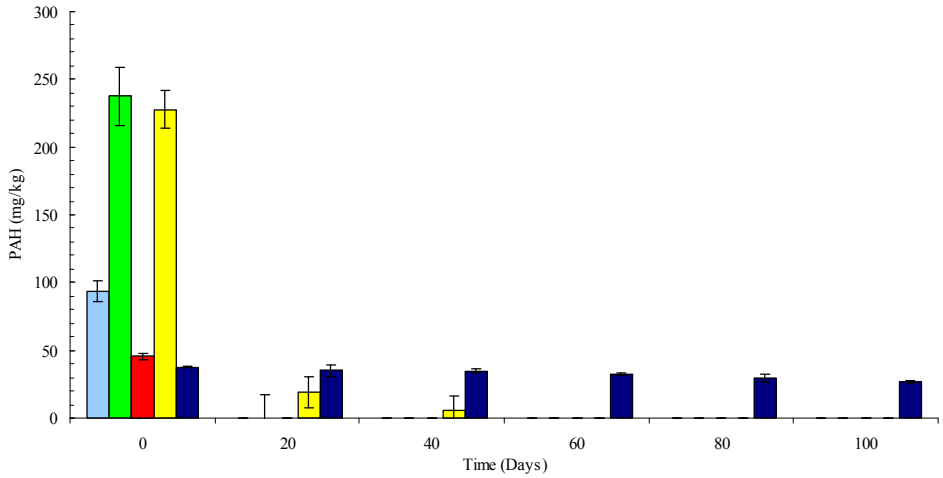


Figure 5.6: Changes in PAH concentration in PAH spiked soil microcosms supplemented with BSMY inoculated with a coculture of VUN 10,010 and *P. janthinellum* containing: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

coculture combination can achieve removal of three- and four-ring PAHs and the removal of benzo[*a*]pyrene following the low molecular weight PAH removal.

5.2.4 Assessment of PAH Degradation Using Sequential Inoculation

Following the partial removal of benzo[*a*]pyrene by bacterial-fungal cocultures, attempts were made to optimise the process by determining if varying the inoculation protocol would have an impact on benzo[*a*]pyrene degradation. The inoculation protocol compared the previously demonstrated co-inoculation (with VUN 10,010 and *P. janthinellum* inoculated into the soil at the commencement of the experiment) and sequential inoculation (with VUN 10,010 inoculated at the commencement of the experiment, then 50 days later *P. janthinellum* and vice versa). 50 days was selected as the time point for inoculation based on the results of the uninoculated experiments, where the indigenous PAH degrading population was observed to grow to sufficient numbers to affect PAH degradation after this time period.

5.2.4.1 Inoculation of VUN 10,010 then P. janthinellum

In experiments where VUN 10,010 was inoculated prior to *P. janthinellum* (Figure 5.7), a rapid loss of three- and four-ring PAHs was observed. By day 60, benzo[*a*]pyrene was the only detectable PAH in the Kanmantoo soil microcosms. The variation in the reduction in PAH concentration between replicate microcosms, as indicated by the standard deviation, was limited (± 1.8 mg/kg). PAH degrading microbial numbers were maintained around 10^6 cells/g for the duration of the experiment. PAH degrading microbial numbers were not affected following the inoculation of *P. janthinellum* at day 50. Following the inoculation of *P. janthinellum*, a limited reduction in benzo[*a*]pyrene concentration was

observed, with 5 mg/kg (14.8%) benzo[*a*]pyrene removed over the incubation period.

5.2.4.2 Inoculation of P. janthinellum then VUN 10,010

Using the alternative sequential inoculation protocol (*P. janthinellum* followed by VUN 10,010 at day 50) (Figure 5.8), PAH degradation profiles were similar to that of axenic fungal microcosms, with limited degradation of PAHs prior to day 40-50. Growth of an indigenous bacterial PAH degrading population was observed in these microcosms prior to inoculation of the bacterial component. All PAHs were present at day 40, with the removal of the three- and four-ring PAHs being completed at day 60. It was difficult to determine the relative contributions of the indigenous PAH degrading population and inoculated VUN 10,010 to PAH removal.

A reduction in the concentration of benzo[*a*]pyrene commenced following the inoculation of VUN 10,010 at day 50. Benzo[*a*]pyrene was degraded from 24.6 mg/kg at day 50 to 9.7 mg/kg at day 100. The high degree of variability between microcosms, resulting in a standard deviation of ± 11.3 mg/kg. This variability was a result of one of the three replicate microcosms failing to achieve the degradation of benzo[*a*]pyrene. This observation is inconsistent with the findings of the two other microcosms, in which benzo[*a*]pyrene was degraded to below detection limits.

The failure of the single microcosm is not explained by the level of microbial PAH degraders present in the microcosm, as these were similar to the other microcosms in which degradation of benzo[*a*]pyrene was observed to occur. This microcosm was consistently observed to achieve a smaller removal of PAHs than the other two microcosms although the cause of this failure was not apparent.

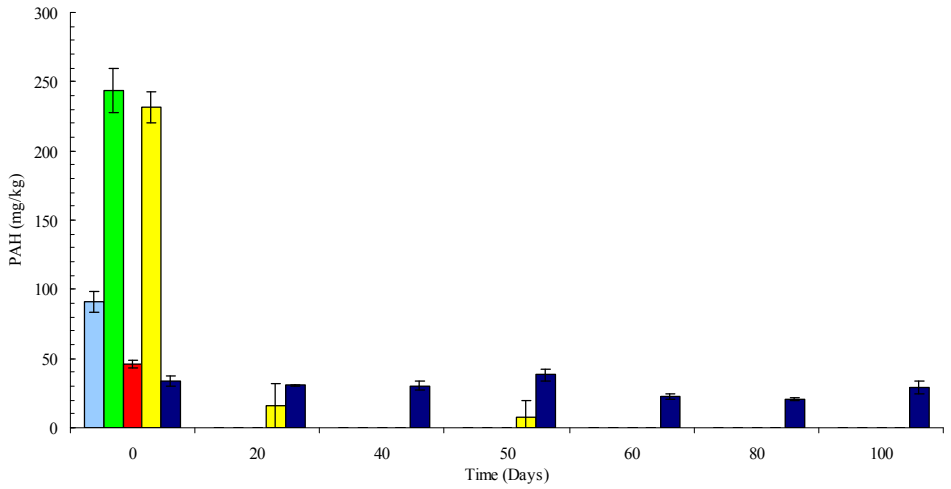


Figure 5.7: Changes in PAH concentration in PAH spiked soil microcosms supplemented with BSMY sequentially inoculated with VUN 10,010 followed by *P. janthinellum* at day 50. Spiked soil contained: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

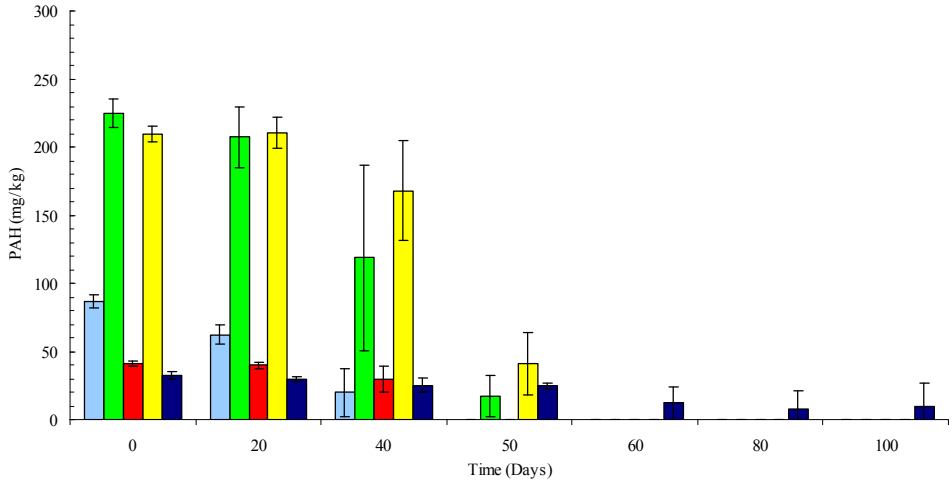


Figure 5.8: Changes in PAH concentration in PAH spiked soil microcosms supplemented with BSMY sequentially inoculated with *P. janthinellum* followed by VUN 10,010 at day 50. Spiked soil contained: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

5.2.5 Assessment of PAH Degradation Using Sequential Inoculation with Amendments

Substanial decreases in benzo[*a*]pyrene concentration were observed in microcosms sequentially inoculated with *P. janthinellum* then VUN 10,010. In order to optimise the fungal-bacterial degradation process, a comparison was made using the same inoculation strategy to determine the effect of amendments on the rate and extent of PAH degradation.

The effect of amendments was assessed by using two additional solutions to make up the water holding capacity of the soil to asses the impact of the provision of amendments on the initial fungal population. The solutions added to make up water holding capacity were a 10% glucose solution to a final concentration in the soil of 1%, and distilled water. The glucose solution was added in an attempt to increase the fungal biomass and stimulate the production of extracellular enzymes. Glucose was selected as an easily available compound that would rapidly increase fungal numbers and be consumed in a relatively short period. Distilled water was added to provide water but no additional substrate to determine the effect of nitrogen limitation, a known inducer of fungal degradative enzymes. Nitrogen for bacterial degradation was provided by the addition of ammonium nitrate solution when the bacterial component was inoculated. The same inoculation protocol was used as in the previous section, namely the inoculation of fungi at the commencement of the experiment, followed by the inoculation of bacteria after 50 days incubation. Controls consisted of uninoculated and killed microcosms.

5.2.5.1 Sequential Inoculation in Microcosms Amended with Distilled Water

In microcosms sequentially inoculated with *P. janthinellum* then VUN 10,010 amended with distilled water (Figure 5.9), PAH degradation was similar to that in axenic *P. janthinellum* inoculated microcosms. Small reductions in PAH concentration were observed prior to the inoculation of the bacterial component of the coculture at day 50. By day 50, fluorene and fluoranthene were degraded below detection limits, phenanthrene by 239.8 mg/kg (96.2%) and pyrene by 179.9 mg/kg (74.82%). Following bacterial inoculation, three- and four-ring PAHs were rapidly degraded to below detection limits and benzo[*a*]pyrene was degraded by 8.8 mg/kg (17.3%).

Microbial PAH degraders in the *P. janthinellum* and VUN 10,010 microcosms rose to $2.4x10^6$ cells/g following inoculation at day 50 and decreased to $1.7x10^5$ cells/g by day 100, corresponding with the removal of the three- and four-ring PAHs.

5.2.5.3 Sequential Inoculation in Microcosms Amended with 1% Glucose

The addition of glucose as an amendment was intended to initially increase the fungal numbers in the microcosms followed by a period of die back and enzyme release following the consumption of the glucose. In *P. janthinellum* and VUN 10,010 (Figure 5.10), PAH degradation was impaired and the bacterial component of the coculture did not establish or grow until day 100. In these microcosms, no reduction in PAH concentration was achieved and marginal removal beyond that in the killed controls was observed. The *P. janthinellum* and VUN 10,010 inoculated microcosms only achieved a 2.5 mg/kg (6.5%) reduction in fluorene, a 14.5 mg/kg (28.3%) reduction in fluoranthene and a 5.5 mg/kg (2.8%) reduction in benzo[*a*]pyrene.

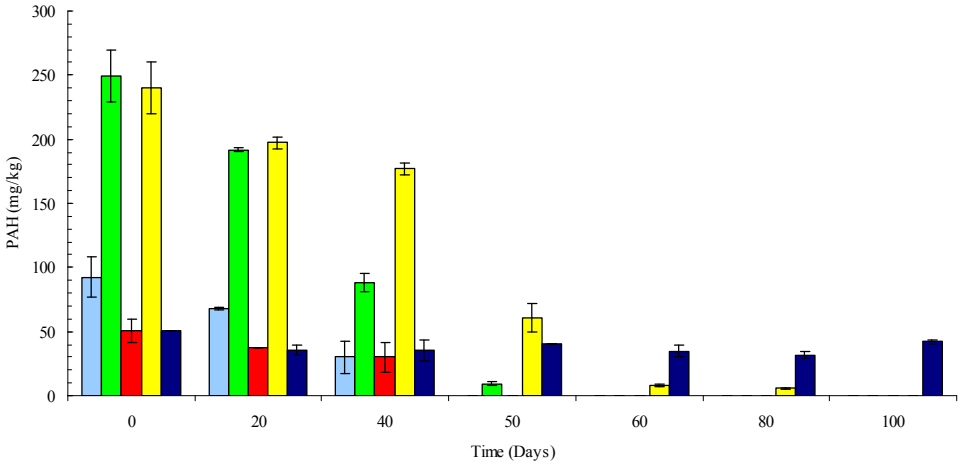


Figure 5.9: Changes in PAH concentration in PAH spiked soil microcosms supplemented with distilled water sequentially inoculated with *P. janthinellum* followed by VUN 10,010 at day 50. Spiked soil contained: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

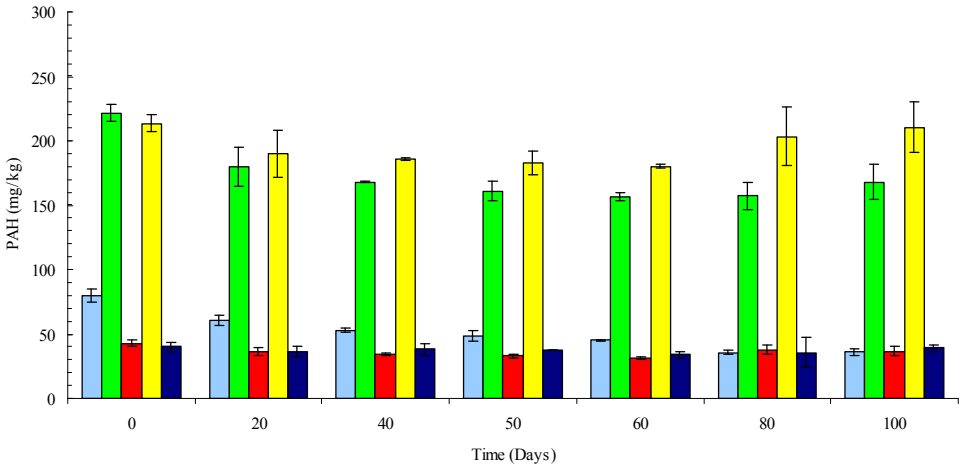


Figure 5.10: Changes in PAH concentration in PAH spiked soil microcosms supplemented with 1% glucose sequentially inoculated with *P. janthinellum* followed by VUN 10,010 at day 50. Spiked soil contained: fluorene ( ) (100 mg/l); phenanthrene ( ) (250 mg/l); fluoranthene ( ) (50 mg/l); pyrene ( ) (250 mg/l) and benzo[*a*]pyrene ( ) (50 mg/l). Data points represent the mean and standard deviation for triplicate samples.

Although provided with an easily degradable substrate, the microbial PAH degrading population in the glucose amended microcosms remained substantially lower than that in the distilled water microcosms, with only $8.5x10^2$ cells/g detected at day 100 despite inoculation at day 50.

5.2.6 General Obesrvations on Degradation in Spiked Soil Microcosms

The rates of PAH degradation achieved in the previous microcosm experiments are shown in Table 5.3. Rates were determined by dividing the amount of PAH degraded by the number of days over which the experiment was conducted or the amount of days it took for the PAH to be degraded below detection limits.

In the initial concurrently inoculated experiments supplemented with BSMY, the coculture microcosms were observed to have the highest rate for fluorene (4.7 mg/kg/day), phenanthrene (5.9 mg/kg/day), fluoranthene (2.3 mg/kg/day) and pyrene (3.8 mg/kg/day), although not significantly greater than that of the axenic bacterial microcosm. The highest rate of benzo[*a*]pyrene degradation was observed in the sequentially inoculated *P. janthinellum* and VUN 10,010 microcosm (0.2 mg/kg/day). Bioaugmentation using *P. janthinellum* and VUN 10,010 cocultures increased the rate of PAH degradation when compared to uninoculated controls.

The addition of distilled water and 1% glucose was found to reduce rates of degradation for all PAHs. The addition of 1% glucose was observed to inhibit PAH degradation or substantially reduce the rate and extent of PAH degradation. The microcosms amended with distilled water were not observed to be impacted to the same extent, although a reduction in PAH degradation rate was observed for all sequentially inoculated coculture combinations used.

Table 5.3: Comparison of rates of PAH degradation in PAH spiked Kanmantoo soil microcosms for the various inoculation protocols and amendments.

	PAH Degradation Rate (mg/kg/day)				
Organism	**FLU**	**PHE**	**FLA**	**PYR**	**B[a]P**
Uninoculated Controls					
Uninoculated Soil with BSMY	1.3	3.5	0.7	2.2	0.2
Uninoculated Soil with Distilled Water	-	-	0.1	-	0.1
Uninoculated Soil with Glucose (1%)	-	-	-	-	-
Axenic Coculture Components					
Axenic *P. janthinellum*	1.8	3.6	0.7	2.7	0.1
Axenic VUN 10,010	4.3	5.5	2.1	3.5	0.1
Concurrently Inoculated Coculture					
VUN 10,010 + *P. janthinellum*	4.7	5.9	2.3	3.8	0.1
Sequentially Inoculated Cocultures					
VUN 10,010 then *P. janthinellum*	4.5	12.1	2.3	3.9	0.1
P. janthinellum then VUN 10,010	1.7	3.7	0.8	3.5	0.2
Sequentially Inoculated Cocultures with Amendments					
P. janthinellum then VUN 10,010 with Distilled Water	1.9	4.1	1	2.4	0.1
P. janthinellum then VUN 10,010 with Glucose (1%)	0.4	-	0.1	-	-

No statistical difference was found between the various inoculants in the initial experiments (axenic VUN 10,010, *P. janthinellum*, the *P. janthinellum* VUN 10,010 coculture and uninoculated microcosms). As was observed in liquid culture experiments, statistical differences between the various inoculation protocols and inoculants were only apparent for the high molecular weight PAHs, in this instance benzo[*a*]pyrene, in sequentially inoculated microcosms. Using Tukeys multivariate analysis, a significant difference ($p=<0.05$) was found between the extent of benzo[*a*]pyrene degradation in *P. janthinellum* then VUN 10,010 sequentially inoculated microcosms and microcosms using the alternative sequential inoculation protocol (VUN 10,010 then *P. janthinellum*) and those concurrently inoculated with *P. janthinellum* and VUN 10,010.

A statistically significant difference in the extent of degradation was maintained regardless of inoculant type between microcosms amended with either BSMY or distilled water and those amended with 1% glucose. Unlike other experiments using cocultures, this difference was not limited to the higher molecular weight PAHs, but occurred for all PAHs in the mixture. As benzo[*a*]pyrene was not substantially degraded, there was no significant treatment effect between the various amendments.

A comparison of extent and rate of PAH degradation in liquid and soil media is shown in Table 5.4. To make the comparison, the rates were determined by dividing the amount of PAH removed over the incubation period or the period taken for the PAH to be degraded below detection limits. Liquid abiotic controls and uninoculated soil natural attenuation controls were excluded from the

Table 5.4: Comparison of Extent and Rate of PAH Degradation in Liquid (BSMY) and Kanmantoo Soil Medium inoculated with a coculture and axenic cultures of *P. janthinellum* and VUN 10,010.

		PAH Degraded[a]			
Inoculum	**PAH**	**Liquid Medium[b] (mg/l)**	**Soil Medium[c] (mg/kg)**	**Liquid Medium[b, d] (mg/l/day)**	**Soil Medium[c, e] (mg/kg/day)**
VUN 10,010 and *P. janthinellum*	FLU	97.4	93.5	1.1	4.7
	PHE	254.1	237.7	2.8	5.9
	FLA	53.5	45.4	0.6	2.3
	PYR	248.6	227.8	2.8	3.8
	BaP	0.9	11	<0.1	0.1
P. janthinellum	FLU	82.6	73.5	0.9	1.8
	PHE	65.3	214	0.7	3.6
	FLA	7	42.4	<0.1	0.7
	PYR	34.1	218.6	0.4	2.7
	BaP	-	12.9	-	0.1
VUN 10,010	FLU	105.8	85.4	1.2	4.3
	PHE	245.3	219.6	2.7	5.5
	FLA	39.9	41.7	0.4	2.1
	PYR	104.9	210.3	1.2	3.5
	BaP	-	4.7	-	0.1

FLU = fluorene, PHE = phenanthrene, FLA = fluoranthene, PYR = pyrene, BaP = benzo[*a*]pyrene
a Reduction in concentration compared to initial concentration
b Incubation period was 90 days.
c Incubation period was 100 days
d Rate determined by averaging PAH removal over incubation period (as removal below detection limits did not occur)
e Rate determined by taken to remove PAH below detection limits

comparison due to the presence of indigenous degrading organisms in the uninoculated soil controls contributing to PAH removal.

Significant differences ($p=<0.05$ determined by T-test) between the rates of PAH degradation in liquid and soil experiments were observed for three- and four-ring PAHs. The greatest differences in rates were observed between the axenic *P. janthinellum* inoculated liquid and soil microcosms, particularly for phenanthrene (soil rate was five times that of liquid culture), fluoranthene and pyrene (soil rate was seven times that of liquid culture). The contribution of indigenous PAH degrading organisms in the soil based experiments would have contributed to this improved rate and extent of degradation. There was a lesser difference in rates between VUN 10,010 and *P. janthinellum* and axenic VUN 10,010 inoculated liquid and soil microcosm experiments.

5.2.6 Radiolabelled Spiked Soil Microcosms

Radiolabelled experiments using ^{14}C radiolabelled benzo[*a*]pyrene were conducted to replicate all microcosm experiments to determine if the observed benzo[*a*]pyrene degradation was resulting in mineralisation, demonstrated through the generation of $^{14}CO_2$. Axenic controls of each bacterial isolate and fungal isolate were used as well as uninoculated and killed controls. Percentages of $^{14}CO_2$ generated in the ^{14}C benzo[*a*]pyrene experiments are shown in Table 5.5.

No significant amount of $^{14}CO_2$ was generated in either microcosm, indicating that any degradation of benzo[*a*]pyrene was partial and did not result in mineralisation. In experiments using the VUN 10,010 isolate, only a small amount of $^{14}CO_2$ was evolved. The greatest amount was evolved in the sequentially inoculated microcosm without additional N added. Interestingly, the BSMY amended sequentially inoculated microcosm that was observed to

Table 5.5: Amount of $^{14}CO_2$ evolved from ^{14}C radiolabelled Benzo[*a*]pyrene in PAH spiked soil microcosm experiments incubated for 100 days.

Inocula	**% ^{14}CO2 Evolved**
VUN 10,010	2.5
VUN 10,010 + *P. janthinellum*	1.8
P. janthinellum then VUN 10,010	1.8
P. janthinellum then VUN 10,010 (distilled water)	4.2
P. janthinellum then VUN 10,010 (1% glucose)	2.1
Uninoculated Control	3
$HgCl_2$ Killed Control	1.9

substantially reduce the concentration of benzo[*a*]pyrene (Section 5.2.4.2) in non labelled experiments did not evolve $^{14}CO_2$ to any great extent.

An attempt was made to conduct a mass balance on the soil used in these experiments and was discontinued due to the silty and highly coloured nature of the aqueous extracts (despite centrifugation and filtering) impeding the ability to utilise the scintillation counter. As a consequence, no mass balance for these experiments was determined.

5.3 Discussion

The use of spiked soil allows for the assessment of coculture degradation in a more environmentally relevant medium without the influence of additional variables such as ageing of the contaminants which can affect PAH bioavailability. The experiments conducted in this Chapter were intended to assess the capability of the *P. janthinellum* and VUN 10,010 coculture to degrade PAHs in soil using a variety of inoculation protocols and the provision of various amendments. Following the limited success of the experiments using a liquid medium, the results of the experiments using spiked soil were somewhat encouraging. It appeared that some removal of benzo[*a*]pyrene was achieved, although this was not achieved consistently and mineralisation was not found to occur. The results presented in this Chapter demonstrate the difficulties of the transition from liquid to soil media when undertaking the development of an inoculum for soil bioaugmentation. The influence of indigenous microbial populations growing in the autoclaved soil contributed to PAH removal in many experiments and was a cause of variation in the experiments.

5.3.1 Natural Attenuation

Despite autoclaving and the addition of $HgCl_2$ killed inocula, a viable PAH degrading population grew in uninoculated soil microcosm controls. Autoclaving was considered an effective method for sterilising soils compared to other methods such as chemical treatment (e.g. sodium azide) or γ-irradiation (Kale and Raghu, 1982). This method of sterilisation was selected as although it affects the physical structure of the soil, it does not leave toxic residues that may detrimentally affect the inoculum. Previously, attempts to sterilise agricultural soil for PAH degradation experiments using autoclaving did not kill all organisms present (Horinouchi *et al*., 2000).

The uninoculated control microcosm amended with BSMY was the second best performing microcosm in terms of PAH degradation of all the microcosms tested, achieving a 46.4% reduction in benzo[*a*]pyrene over the 100 day incubation period. The contribution of an indigenous PAH degrading community was also shown in the sequentially inoculated microcosms. These observations were not surprising as a competent PAH degrader, *Mycobacterium* sp. Strain KA5, was isolated following enrichment from the Kanmantoo soil.

A similar phenomenon was observed in an investigation of the PAH degradative capabilities of four white rot fungi in contaminated soil. Canet *et al*. (2001) observed the greatest PAH losses occurred in the biotic control (64.3% total PAH remaining after 32 weeks), compared to 71.8% for the best performing fungi and 76.1% for all four fungal species inoculated together. Soil cultures prepared at the end of the experiment found that the inoculated fungi could be recovered, whereas both fungi and bacteria grew in the cultures from the biotic control. From this observation, the authors proposed that the introduced fungi did not die during the experiments, but were in a metabolically inactive form.

McFarland and Qiu (1995) reported a similar observation in experiments inoculating *Ph. chrysosporium* into PAH contaminated soil. The reduction in benzo[*a*]pyrene concentration was marginally higher in the non-sterile uninoculated microcosms (65%) than that of the inoculated microcosm (62.8%). It was found that after the 95 day incubation period, a large number of PAH degrading fungi had grown in the uninoculated microcosms, thereby increasing degradation.

It is findings such as these that emphasise the importance of determining the contribution of indigenous microflora to the degradation of PAHs in experiments using inoculants. The contribution of indigenous bacteria to degradation in fungi inoculated soils led to the development of the coculture process, as it would appear that the bacterial component of the indigenous microflora contributes to a greater extent of overall PAH degradation. Interactions between fungi and bacteria (e.g. competition), have significant implications for the degradation of PAHs in soil whether the bacteria were inoculated or are part of the indigenous microflora (in der Wiesche *et al*., 1996).

The suitability of the microorganism for the particular soil environment is important to the success of bioaugmentation. Microorganisms introduced to soil are at a disadvantage compared to the indigenous microflora that have established a niche in the soil community. The potential for indigenous organisms to contribute to the degradation of PAHs is an important consideration in experiments assessing bioaugmentation using soils sourced from the environment (Grosser *et al*., 1991).

The findings of these experiments assessing natural attenuation emphasise that analysis of the PAH degradative capacity of the indigenous microbial population is an important step in assessing whether bioaugmentation or biostimulation is required for remediating soils contaminated with PAHs. Where biostimulation is a preferable option, consideration of the type of amendment is vital. These

experiments have shown that in the Kanmantoo soil, the provision of BSMY enhanced natural attenuation whereas the use of 1% glucose inhibited natural attenuation.

5.3.2 Previous Coculture Observations

There are few studies that have attempted to use defined fungal-bacterial cocultures for the degradation of high molecular weight PAHs although some studies have used an undefined coculture composition (augmenting indigenous PAH degrading microorganisms with a PAH degrading fungal strain). In the study upon which much of this work is based, a defined fungal-bacterial coculture composed of *P. janthinellum* and VUN 10,010 was used. In soil microcosm experiments conducted using this coculture combination inoculated concurrently by Boonchan (1998), up to 48% of the ^{14}C benzo[*a*]pyrene added to spiked soil was recovered as $^{14}CO_2$ over the 100 day incubation period. No $^{14}CO2$ was evolved from benzo[*a*]pyrene in the initial 14 days, but was generated over the remaining 86 days. Lower recoveries of $^{14}CO_2$ were observed in axenic bacterial and fungal microcosms. The degradation achieved by the coculture inoculated microcosm was observed to occur in both spiked and contaminated soils (Boonchan *et al*., 2000).

Although the same organisms were used in the experiments presented here, they did not display the effectiveness reported by Boonchan. The extensive mineralisation of ^{14}C benzo[*a*]pyrene (37-48%) achieved by concurrently inoculated *P. janthinellum* and VUN 10,010 was not observed in this work, where a similar experimental protocol yielded only 1.8% $^{14}CO_2$. Whilst it is conceded that the experiment conditions varied in terms of the soil used, it is reasonable to expect that a potential inoculum for bioaugmentation should be able to perform consistently in a variety of conditions and circumstances. Effects such as a reduction in the bioavailability of benzo[*a*]pyrene due to sorption to soil

components over the experimental period affecting the ability to achieve degradation were discounted due to the low organic content of the Kanmantoo soil. As such, the variability of the coculture's performance may be a product of other factors and not simply a change in experimental conditions.

The interaction between fungus and bacteria in soils was also observed in studies in which only the fungal component was inoculated forming an "undefined" coculture. As demonstrated in these experiments, the indigenous microflora can contribute to degradation despite microcosms being initially autoclaved. It is obvious that autoclaving resulted in only a temporary reduction in bacterial numbers. The establishment of inoculated fungi has been shown previously to be enhanced by an initial autoclaving. In experiments where white rot fungi were inoculated into soil, only one fungus (*Hypholoma fasciculare*) was able to establish in non-autoclaved soil, whereas *H. fasciculare*, *Ph. chrysosporium* and *Pleurotus ostreatus* were able to establish in autoclaved soil. This suggests that the indigenous microflora may impair establishment of introduced species, but the authors also proposed that the success in establishing may have also been a product of increased concentrations of soluble nutrients or organic material. In these studies, fungi were also observed to establish in both autoclaved and non-autoclaved soils, but no PAH degradation was observed (Anderson *et al.*, 2000). This demonstrates that establishment of the inoculant is a necessary, but not sufficient, factor in degradation.

The experiments further investigating the potential to use the *P. janthinellum* and VUN 10,010 coculture in spiked soils were developed in recognition of the need for the respective coculture components to establish in soil without the pressure of competition of the other coculture component. They also addressed the need for appropriate nutritional amendments to achieve PAH degradation.

5.3.3 Sequential Inoculation

Sequential inoculation may overcome problems related to the provision of nutrients and establishment of the inoculum by allowing the fungal population to initially establish in a less competitive and nutrient limited environment resulting in the release of extracellular degradative enzymes. Fungal extracellular enzymes convert high molecular weight PAHs to hydroxylated water-soluble intermediates that are more easily transported across the bacterial cell membrane, allowing for intracellular degradation by the bacterial component. This process overcomes the metabolic barrier to bacterial degradation and achieves degradation of the high molecular weight PAHs.

The success of sequential over concurrent inoculation may be a result of a variety of fungal and bacterial interactions. Whilst the overall co-culture relationship may provide a beneficial reduction in high molecular weight PAHs, the degradation pathways (both pathway used and carbon flux in the pathway) of each organism are influenced by the activities of the other organism. A previous study observed a negative correlation between fungal colonisation and PAH degradation and soil microflora levels in contaminated soils (Eggen and Majcherczyk, 1998). Soil bacteria have also been shown to inhibit the growth of *Ph. chrysosporium* under laboratory conditions, whilst in other studies the addition of white rot fungi has been shown to inhibit indigenous soil bacteria (Pointing, 2001). The limited success observed in concurrently inoculated microcosms may result from competition for available degradable substrates and freely colonisable space. It has been demonstrated previously that when introduced organisms are given a selective advantage in soil systems (e.g. nutritionally or spatially protective), only a minimal number of active cells are initially necessary for the application to be effective (van Veen *et al*., 1997).

It has been proposed that fungal degradation of high molecular weight PAHs is a response to nutrient limitation, in particular nitrogen (Kotterman *et al*., 1995). During concurrent inoculation, the bacterial component of the co-culture may rapidly degrade the lower molecular weight PAHs that are not degraded by the fungal component. This may provide an alternative carbon source and result in nutrient cycling in the microcosm, sustaining the fungi and delaying induction of enzymes required for the degradation of high molecular weight PAHs. This may also result in a depletion of nitrogen. At later stages of incubation, bacterial activity may be reduced due to depletion of more readily degradable low molecular weight PAHs. The combination of nitrogen depletion and low bacterial activity may permit fungal activity that could explain the subsequent reduction in benzo[*a*]pyrene during later stages of incubation.

The inoculation of bacteria and fungi concurrently may also have an impact as a result of PAH degradation intermediates formed. The formation of PAH degradation intermediates by bacteria has been shown to impair transformation of other PAHs in soils. This occurs in instances where the intermediates are accumulated and other organisms present in the soil are incapable of further degradation (Kazunga and Aitken, 2000).

Fungi's inhibition of PAH degradation by indigenous microflora has also been reported in experiments using ^{14}C labelled pyrene. Two species of fungi, *Pleurotus ostreatus* and *Dichomitus squalens*, were assessed for their ability to degrade pyrene in solid state fermentation. *D. squalens* mineralisation of the ^{14}C pyrene was found to be less in coculture (12.9%) than that carried out by soil microflora alone (25.8%). It was proposed that *D. squalens* consumed most of the easily available substances in the soil (particularly straw added as a growth substrate and amendment) during axenic pre-incubations with out mineralising ^{14}C pyrene as soil microorganisms would have done. Pyrene was potentially modified by the fungus in this early phase of incubation in a way that made it less

available for the soil microflora (e.g. through oxidation and polymerisation of phenolic compounds generated) (In der Wiesche *et al*., 1996).

Bilateral antagonism between bacteria and fungi has been observed previously (Mille-Lindblom and Tranvik, 2003). In a study of bacterial and fungal decomposers of aquatic plant litter, bacterial biomass was found to be twice as high in the absence of fungi and fungal biomass was twelve times as high in the absence of bacteria. Interestingly, carbon metabolism was similar regardless of whether the fungi and/or bacteria were present in conjunction or independently. A number of factors were considered for these observed variations in biomass including resource (carbon) competition, competition for colonisable space or the production of extracellular compounds by bacteria or fungi (Mille-Linblom and Tranvik, 2003).

The reduction in bacterial numbers following inoculation in liquid culture experiments conducted as part of this investigation was not observed in the soil experiments. In all experiments, the number of PAH degrading bacteria was observed to increase, although the contribution of an indigenous population to the bioaugmented bacterial PAH degraders creating a higher total population cannot be eliminated. It must be recognised that the soil used in these experiments has been substantially disturbed through the autoclaving process and the growth of the introduced bacterial PAH degrading population is considered unlikely in natural soils due to the resistance of the indigenous microbial population (van Veen *et al*., 1997).

5.3.4 Amendments

Amendments are often used to enhance degradation of xenobiotic compounds in the environment. Fungi such as *Ph. chrysosporium* and others often require the provision of a large amount of cosubstrate for bioaugmentation and

biodegradation to succeed (Forsyth *et al*., 1995). Due to the large amount of cosubstrate required for site remediation projects, cost and availability usually dictates that simple sugars (e.g. glucose) or cellulose be used.

The provision of an easily metabolised carbon source (glucose) was attempted to produce large numbers of fungal cells in the initial stages of the experiments. It was considered that this carbon source would be consumed in the initial stages of the experiment increasing the number of fungal cells, which would starve following the consumption of the glucose and induce the production of lignolytic enzymes. Although not reflected in the fungal MPNs conducted on soil samples, fungal growth was evident in many inoculated microcosms on the surface of the soil. This was not observed in the uninoculated glucose amended microcosms.

As observed in this study, the inoculation of fungi in the glucose amended soils did not achieve a reduction in concentration of the PAHs and also was observed to prevent degradation by the indigenous microflora. Carmichael and Pfaender (1997) reported a similar phenomenon where amendments were found to either produce no effect or inhibit degradation. This was considered to be a product of either the organism utilising the amendment as a preferred substrate or the growth of other non-PAH utilising microorganisms. Whilst the potential for this to occur was considered when the experiment was devised, it was considered that the provision of glucose and the immediate inoculation of *P. janthinellum* during sequential inoculation studies would provide a competitive advantage to the inoculum due to the availability of a readily consumable substrate.

The growth of fungi also has the potential to have a detrimental impact on indigenous soil bacteria. This phenomenon has been observed previously, where the mycelia of *P. ostreatus* were observed to kill indigenous microflora, inhibiting degradation (Gramss *et al*., 1999). In the experiments presented here, microbial PAH degraders were routinely found to be present in lower numbers in glucose amended microcosms when compared to non-amended ones. A

bacteriocidal effect from fungal mycelial growth is not indicated as increasing fungal MPNs did not correspond with a reduction in microbial PAH degraders.

The removal of PAHs in microcosms amended with distilled water was not surprising as *Mycobacterium* sp. have previously been shown to achieve the degradation of PAHs in soils with low concentrations of N and P. Leys *et al*. (2005) found that *Mycobacterium* sp. and S*phingomonas* sp. could achieve the degradation of PAHs in soil slurries amended with N and P supplied at the C/N/P ratio of 100/10/1, unbalanced N and P levels and at N and P levels ten times less than the ratio. The provision of N and P salts was found to increase the salinity of the soil slurry solutions causing inhibition of PAH degradation (Leys *et al*., 2005)

As demonstrated in experiments assessing the degradative capacity of the indigenous microorganisms in the Kanmantoo soil, there was an inherent capacity for degradation in uninoculated microcosms. Bioaugmented microcosms were also observed to achieve degradation, suggesting little competition in the microcosms, most likely due to the reduced numbers of indigenous microorganisms from autoclaving and the high numbers of introduced microorganisms. It is interesting to note that while the introduction of BSMY with the inoculated strains and as a soil amendment for uninoculated microcosms did not seem to influence PAH degradation, it has previously been observed to impact on degradation. Kastner *et al*. (1998) found that the introduction of a similar minimal medium inhibited PAH degradation by both autochthonous organisms and the strains introduced as part of the experiment, prompting the use of water to alleviate this inhibitory effect. In the experiments conducted in this Chapter using distilled water to make up water holding capacity, PAH degradation was observed to occur when inoculated, but no degradation was observed in the uninoculated microcosms. This would indicate that the salinity of the solution had little impact on degradation and the addition of the minimal nutrients contained in BSMY allowed indigenous degradation to occur.

5.3.5 Radiolabelled Experiments

Despite the removal of benzo[*a*]pyrene being found in a number of the experiments conducted using unlabelled benzo[*a*]pyrene, experiments using ^{14}C benzo[*a*]pyrene did not result in the production of significant quantities of $^{14}CO_2$. Although mineralisation did not occur, partial degradation of benzo[*a*]pyrene may occur without the production of $^{14}CO_2$. This was observed previously by Eggen and Majcherczyk (1998) in experiments where *Pleurotus ostreatus* was inoculated into aged creosote contaminated soil. Following the addition of ^{14}C-benzo[*a*]pyrene, a 40% reduction in benzo[*a*]pyrene concentration was observed over the month long incubation period, although only 1% of the radiolabel was recovered as $^{14}CO_2$.

The site of ring cleavage has important implications for the evolution of $^{14}CO_2$ in benzo[*a*]pyrene degradation experiments. Juhasz (1998) reported that ring cleavage in the 7, 8-position in liquid media experiments would result in the evolution of $^{14}CO_2$, whereas cleavage in the 4, 5-position would not unless the ^{7}C-benzo[*a*]pyrene was extensively degraded. The radiolabelled benzo[*a*]pyrene used in the experiments presented here was labelled at the 7, 8 position, indicating that an alternative explanation for the lack of $^{14}CO_2$ generation may apply.

McFarland and Qiu (1995) observed a similar lack of $^{14}CO_2$ evolution despite the apparent removal of benzo[*a*]pyrene in *Ph. chrysosporium* inoculated soil composting systems. A half-life for benzo[*a*]pyrene of 11.5 days in inoculated microcosms and 8.6 days in uninoculated microcosms was observed, but it was found that in the *P chrysosporium* inoculated microcosms the removal of benzo[*a*]pyrene was actually the product of bound residue formation rather than mineralisation, hence no $^{14}CO_2$ was formed. The inoculation of *P. Chrysosporium* was considered to increase bound residue formation as the uninoculated and killed controls did not achieve substantial generation of bound

residues. It is difficult to determine if this was occurring in the experiments presented here, although it is worthy of further exploration as it is a potential detoxification mechanism.

5.4 Conclusions

Whilst a reduction in concentration of benzo[*a*]pyrene was achieved in a number of the coculture inoculated experiments in spiked soil microcosms, it was highly variable and did not result in benzo[*a*]pyrene mineralisation. Natural attenuation and the growth of indigenous microbial PAH degraders occurred in most experiments and had an influence on both the rate and extent of PAH degradation. The use of spiked soil, whilst more in keeping with the relevant soil conditions, does not truly reflect the potential impact of other factors such as contaminant aging and the effect on bioavailability. The varying establishment and nutritional requirements of the coculture emphasise the difficulties in developing a suitable inoculum for bioaugmentation.

The results of this work presented here demonstrated that:

- Inoculation with a *P. janthinellum* and VUN 10,010 coculture, in cojunction with indigenous PAH degraders, can achieve the degradation of benzo[*a*]pyrene in soils;
- Sequential inoculation is a key method for the delivery of the coculture inoculum into soil and assists in overcoming issues related to competitive and nutritional pressures; and
- The provision of nutrient amendments must be carefully considered particularly as the BSMY amendment enhanced PAH degradation whereas the 1% glucose amendment inhibited it.

Although the results presented in this Chapter varied in terms of the effectiveness of the coculture process, they do indicate a potential for this method of

bioaugmentation to achieve the degradation of high molecular weight PAHs in soil. Further investigation into the fate and activity of the organisms once introduced into soil, their relationship to indigenous microflora, and process optimisation through the provision of various amendments is required.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Thesis Synopsis

This thesis describes the development of fungal-bacterial cocultures as a bioaugementation inoculum for the remediation of high molecular weight PAH contaminated soils.

Chapter 3 described the process of enrichment of bacteria from various contaminated and uncontaminated soils on PAH mixtures and isolation using pyrene as a growth substrate. Each of the bacterial isolates was able to utilise a range of substrates and one isolate (*Mycobacterium* sp. Strain BS5) produced zones of clearing on BSMY plates with benzo[*a*]pyrene (50 mg/l). The bacterial isolates were identified as species previously found to possess PAH degrading abilities through FAME (*Ralstonia pickettii* and *Stenotrophomonas maltophilia*) and 16S rRNA analysis (*Mycobacterium* sp. Strain BS5, *Mycobacterium* sp. Strain KA5 and *Mycobacterium* sp. Strain KF4). Following isolation and identification of these organisms, they were subjected to a series of experiments in liquid medium to determine their PAH degradation ability.

In Chapter 4, all of the above mentioned bacterial isolates and two additional isolates (VUN 10,010 and *Mycobacterium* 1B) were assessed for their ability to degrade both single and multiple PAHs in liquid medium. This process was to determine the best performing bacterial isolates for combination with the two fungal components in cocultures. All isolates were found to be competent phenanthrene, fluoranthene and pyrene degraders. All isolates were found to extensively mineralise pyrene in radiolabelled experiments. No isolates were able to extensively degrade fluorene and benzo[*a*]pyrene when these compounds were present in isolation. The two fungal isolates (*P. janthinellum* and *Ph. chrysosporium*) were assessed for their ability to degrade benzo[*a*]pyrene in both MYPD and BSM liquid media. Both *P. janthinellum* and *Ph. chrysosporium* was found to be able to transform benzo[*a*]pyrene in MYPD media, but could not achieve removal in BSM. Of the seven bacterial isolates, three were eliminated

from the investigation on the basis of their poor PAH degrading abilities and the remaining four were assessed for their ability to degrade a five PAH mixture in axenic and coculture inoculated cultures with the two fungal components.

All coculture combinations were found to produce a significantly better extent of PAH removal than the axenic bacterial and fungal isolates for the higher molecular weight PAHs in the PAH mixture. Only the VUN 10,010 and *P. janthinellum* coculture achieved a small amount of benzo[*a*]pyrene removal in liquid culture. This coculture was also the only combination to achieve the partial transformation of benzo[*a*]pyrene into water soluble metabolites in experiments using [7-^{14}C] benzo[*a*]pyrene. This prompted its assessment in experiments using PAH spiked soil as the medium.

Chapter 5 described the ability of the *P. janthinellum* and VUN 10,010 coculture to degrade PAHs in PAH spiked soil using a variety of inoculation protocols and amendments (BSMY, distilled water and 1% glucose). The soil was initially assessed for the ability of indigenous microorganisms to achieve the degradation of the five PAH mixture. Despite attempts at sterilising the soil, it was found that the indigenous organisms could achieve degradation of the PAH mixture after a lag period of approximately 40 days in microcosms amended with BSMY and supplements. In uninoculated microcosms amended with distilled water and 1% glucose, extensive degradation was not found to occur. A coculture of VUN 10,010 and *P. janthinellum* was selected for soil based experiments and similar to the liquid culture experiments. The inoculation with the coculture enhanced the rate of PAH degradation; although a similar extent of benzo[*a*]pyrene degradation was observed for coculture (29.4%) and axenic *P. janthinellum* (31.6%) inoculated microcosms.

Whilst the sequential inoculation of VUN 10,010 followed by *P. janthinellum* fifty days later was found to remove three- and four-ring PAHs, only 14.8% of benzo[*a*]pyrene was removed by the end of the incubation period. Although

inoculating *P. janthinellum* followed by VUN 10,010 resulted in a slower initial rate of removal of three- and four-ring PAHs, this inoculation protocol resulted in benzo[*a*]pyrene being degraded below detection limits in two of three microcosms.

This result prompted the assessment of the sequential inoculation protocol using *P. janthinellum* and VUN 10,010 provided with different amendements to stimulate fungal growth and/or enzyme production. Microcosms were amended with BSMY as used in initial experiments or amendments intended to provide nutrient limitation to enhance fungal enzyme production (distilled water) or increase initial fungal numbers to increase fungal degradation (1% glucose). Whilst benzo[*a*]pyrene removal was observed in both BSMY (22.4 mg/kg) and distilled water microcosms (8.1 mg/kg), it was not observed in micrcosms with 1% glucose, where only 1.1 mg/kg benzo[a]pyrene was degraded and reduced the number of microbial PAH degraders was reduced.

6.2 CONCLUSIONS

The studies described in this thesis explored the innovative and yet to be extensively investigated process of using defined fungal-bacterial cocultures to enhance high molecular weight PAH degradation. The purpose of this investigation to undertake an assessment of the capability of the defined coculture process to achieve degradation of PAHs (in particular high molecular weight PAHs) in liquid media and spiked soil. The various cocultures used in this work did enhance the removal of PAHs from both soil and liquid media when compared to axenic microbial inoculants and in some instances achieved substantial removal of benzo[*a*]pyrene.

The key outcomes of the work presented in this thesis were:

- Enrichment and isolation using pyrene of competent PAH degrading organisms from contaminated and uncontaminated environments;
- Assessment of the ability of a variety of coculture combinations to achieve the degradation of a PAH mixture in liquid culture;
- Further assessment of the *P. janthinellum* and VUN 10,010 coculture combination's ability to degrade PAHs in spiked soils;
- The importance of sequential delivery of the coculture components to achieve degradation of benzo[a]pyrene in spiked soil; and
- The influence of BSMY, distilled water and 1% glucose amendments on the degradation of PAHs in spiked soil.

These results demonstrate the potential of bacterial-fungal co-cultures for use in bioremediation of soil contaminated with high molecular weight PAHs and illustrate the importance of the manner of inoculum delivery to achieve maximum bioremediation efficacy. As a consequence, the defined fungal-bacterial coculture process does have merit and is worthy of further exploration.

6.3 RECOMMENDATIONS FOR FUTURE WORK

The defined bacterial-fungal coculture process showed some promise for the degradation of high molecular weight PAHs. The work presented in this thesis investigated the potential for the use of these cocultures to achieve high molecular weight PAH degradation in liquid media and soils. Future work to better elucidate the interactions between the components of defined bacterial cocultures is required if this process is to be exploited for bioaugmentation purposes.

6.3.1 Assessment of Fungal-Bacterial Interactions

As the coculture process is reliant on the effective interaction between the fungal and bacterial components, a more comprehensive assessment of the antagonistic

and synergistic relationships between these organisms is required. This investigation demonstrated, in a number of instances, that the differing nutritional requirements of the fungal and bacterial components can have a deleterious impact on the overall degradative capability of the coculture. Both fungal components were shown to be able to achieve benzo[*a*]pyrene removal in MYPD liquid media, but the addition of glucose to spiked soil experiments prevented PAH degradation. Further assessment of the circumstances that enhance fungal enzyme production (e.g. nutrient deficiency or the provision of Mn and other trace metals) in soils that do not have a deleterious effect on the ability of the bacterial component to establish and colonise soil is necessary. In addition, assessment of the production of the various enzymes (MnP, LiP and laccases) in conjunction with fungal growth, nutrient utilisation and PAH degradation must be examined.

A better characterisation of the metabolites generated by the fungal and bacterial components of the coculture may also assist in determining whether a synergistic or antagonistic relationship has been established. As has been demonstrated previously, some metabolites inhibit further degradation of the compound, preventing mineralisation.

6.3.2 In-Situ Chemical Oxidation and Bioremediation

As the coculture process is based on the principle of an initial extracellular partial oxidative transformation of the high molecular weight PAH increasing water solubility to allow intracellular bacterial degradation, a similar process may be able to be achieved using chemical oxidants. In-situ chemical oxidation using compounds such as Fenton's Reagent are increasingly being exploited to the removal of recalcitrant compounds from contaminated soils. There is the potential for a two phase process to be developed (following careful consideration of soil conditions and the chemical oxidant) for an initial oxidation by the

addition of chemical oxidants followed by the inoculation of competent bacterial PAH degrading organisms.

6.3.3 Development of Effective Tags, Biomarker or Molecular Probes for Monitoring Inoculants

A common finding in the assessment of microbial inoculants for the purposes of bioaugmentation is the inability to directly monitor inoculants once released into soil. As was shown in this investigation, PAH degrading organisms can be isolated from a number of sources both contaminated and uncontaminated and despite attempts to sterilise soils, residual microbial populations can recover to levels to achieve degradation.

The development of "tags" for bacteria (e.g. visual markers such as *gfp* transposon vectors producing a specific colouration or *lux* transposon vectors producing light) to monitor the survival of inoculants in soils would allow the assessment of the survival and viability of the inoculant. Hybridising probes for PAH catabolic genes would allow the assessment of indigenous and inoculant microbial PAH catabolic potential and has been investigated to a limited extent. Biomarkers such as ergosterol could also be used to determine fungal levels and viability (Rossner, 1996). These tags, probes or biomarkers would assist in demonstrating the effectiveness of inoculants if the coculture process was commercialised to differentiate between the contribution of indigenous organisms and that of the inoculated organisms.

APPENDICES

APPENDIX 1

Degradation tables for experiments using the PAH mixture in BSMY medium inoculated with fungal, bacterial and cocultures.

Table A2.1: Combined Abiotic Controls (*n=6*) 5 PAH BSMY Broth Experiments

	PAH Concentration (mg/l)					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[a]	% PAH Decrease[b]
Fluorene	97.71 ± 7.17	97.22 ± 10.11	88.31 ± 5.15	46.87 ± 7.47	44.46 ± 4.42	-
Phenanthrene	261.48 ± 15.83	248.15 ± 12.1	242.66 ± 12.38	224.89 ± 16.26	219.89 ± 19.46	-
Fluoranthene	51.24 ± 3.9	47.73 ± 0.82	49.62 ± 2.81	46.85 ± 1.16	48.47 ± 2.92	3.35
Pyrene	242.66 ± 5.47	242.76 ± 5.15	253.62 ± 14.96	235.23 ± 11.28	249.24 ± 13.37	5.62
Benzo[a]pyrene	44.89 ± 1.43	44.4 ± 1.36	44.58 ± 0.82	43.68 ± 2.27	51.81 ± 3.82	2.7
Total PAH	697.98	680.26	678.79	597.52	613.87	2.67

[a] Combination of killed controls results (*n= 8*) at Day 90

[b] The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.2: Degradation of PAH Mixture in BSMY inoculated with Axenic *P janthinellum*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	102.43 ± 1.24	48.72 ± 4.06	33.91 ± 1.44	19.84 ± 2.17	44.46 ± 4.42	55.38
Phenanthrene	252.72 ± 0.91	246.19 ± 3.79	220.4 ± 12.72	187.43 ± 13.22	219.89 ± 19.46	14.77
Fluoranthene	51.67 ± 0.32	48.36 ± 2.78	43.79 ± 0.92	44.74 ± 2.74	48.47 ± 2.92	7.70
Pyrene	261.77 ± 12.83	247.51 ± 12.68	222.06 ± 5.52	227.65 ± 0.72	249.24 ± 13.37	8.66
Benzo[a]pyrene	43.92 ± 0.39	42.16 ± 0.81	37.33 ± 3.71	44.76 ± 1.44	51.81 ± 3.82	-
Total PAH	721.51	632.94	557.49	524.42	613.87	14.58

Table A2.3: Degradation of PAH Mixture in BSMY inoculated with Axenic *P chrysosporium*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	100.68 ± 5.38	39.17 ± 1.85	30.93 ± 1.39	19.41 ± 1.21	44.46 ± 4.42	56.34
Phenanthrene	259.91 ± 2.8	226.97 ± 5.49	221.98 ± 11.48	188.18 ± 2.9	219.89 ± 19.46	14.42
Fluoranthene	48.43 ± 2.97	50.77 ± 2.95	45.67 ± 2.4	43.3 ± 0.39	48.47 ± 2.92	10.67
Pyrene	249.97 ± 8.96	237.57 ± 15.12	231.1 ± 9.96	231.56 ± 9.9	249.24 ± 13.37	7.09
Benzo[a]pyrene	41.19	39.61 ± 4.02	35.48 ± 1.81	39.11 ± 1.68	51.81 ± 3.82	5.05
Total PAH	700.18	594.09	565.16	521.56	613.87	15.04

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days (n=8)
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.4: Degradation of PAH Mixture in BSMY inoculated with Axenic VUN 10,010

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	110.68 ± 2.41	35.46 ± 4.81	18.18 ± 4.71	4.91 ± 0.08	44.46 ± 4.42	88.96
Phenanthrene	265.48 ± 4.89	94.03 ± 9.91	57.66 ± 11.56	20.24 ± 1.2	219.89 ± 19.46	90.8
Fluoranthene	48.27 ± 1.39	21.96 ± 1.01	18.17 ± 2.35	8.39 ± 1.09	48.47 ± 2.92	82.69
Pyrene	241.8 ± 5.93	141.62 ± 12.18	126.22 ± 3.68	136.88 ± 17.96	249.24 ± 13.37	45.08
Benzo[a]pyrene	43.84 ± 1.77	44.47 ± 0.97	45.89 ± 0.46	47.87 ± 3.58	51.81 ± 3.82	-
Total PAH	710.07	337.54	266.12	218.29	613.87	64.44

Table A2.5: Degradation of PAH Mixture in BSMY inoculated with Axenic *Mycobacterium* 1B

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	116.56 ± 5.97	56.39 ± 0.77	33.21 ± 3.51	10.22 ± 13.09	44.46 ± 4.42	77.01
Phenanthrene	261.57 ± 11.01	143.67 ± 4.29	98.52 ± 11.84	59.98 ± 4.27	219.89 ± 19.46	72.9
Fluoranthene	50.97 ± 1.07	28.48 ± 1.26	23.98 ± 2.34	19.43 ± 1.35	48.47 ± 2.92	59.91
Pyrene	254.54 ± 5.56	166.86 ± 3.88	157.65 ± 2.49	143.53 ± 4.68	249.24 ± 13.37	42.41
Benzo[a]pyrene	46.91 ± 0.93	44.39 ± 1.21	46.69 ± 0.48	49.46 ± 1.12	51.81 ± 3.82	-
Total PAH	730.55	439.79	360.05	282.62	613.87	53.96

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days (n=8)
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.6: Degradation of PAH Mixture in BSMY inoculated with Axenic *Mycobacterium* sp. Strain BS5

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	115.25 ± 1.84	32.57 ± 1.73	19.74 ± 4.37	8.1 ± 0.62	44.46 ± 4.42	81.78
Phenanthrene	261.67 ± 7.24	83.8 ± 3.11	65.37 ± 3.46	37.75 ± 1.48	219.89 ± 19.46	82.83
Fluoranthene	50 ± 0.61	20.17 ± 1.69	18.29 ± 0.24	19.51 ± 0.79	48.47 ± 2.92	59.75
Pyrene	251.19 ± 3.89	152.4 ± 12.64	154.23 ± 6.03	150.38 ± 10.83	249.24 ± 13.37	39.66
Benzo[a]pyrene	43.87 ± 0.78	44.36 ± 0.54	36.83 ± 8.19	43.96 ± 3.35	51.81 ± 3.82	-
Total PAH	721.98	333.3	294.46	259.7	613.87	57.69

Table A2.7: Degradation of PAH Mixture in BSMY inoculated with Axenic *Mycobacterium* sp. Strain KA5

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	108 ± 3.82	44.43 ± 1.12	28.92 ± 4.32	22.96 ± 0.2	44.46 ± 4.42	48.36
Phenanthrene	262.42 ± 7.22	111.76 ± 1.95	78.11 ± 12.56	73.52 ± 0.23	219.89 ± 19.46	66.57
Fluoranthene	49.06 ± 0.2	37.67 ± 2.59	30.13 ± 5.67	34.85 ± 2.29	48.47 ± 2.92	28.1
Pyrene	245.13 ± 0.34	233.94 ± 4.33	204.59 ± 13.84	209.06 ± 9.99	249.24 ± 13.37	16.12
Benzo[a]pyrene	45.96 ± 0.89	45.81 ± 1.5	43.7 ± 0.96	47.94 ± 0.78	51.81 ± 3.82	-
Total PAH	710.57	473.81	385.45	388.33	613.87	36.66

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days (n=8)
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.8: Degradation of PAH Mixture in BSMY inoculated with VUN 10,010 and *P janthinellum*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	97.44 ± 2.54	24.81 ± 1.69	6.13 ± 0.26	0	44.46 ± 4.42	100
Phenanthrene	254.1 ± 1.76	83.19 ± 4.04	19.01 ± 0.5	0	219.89 ± 19.46	100
Fluoranthene	53.54 ± 4.03	2.86 ± 0.13	2.22 ± 0.15	0	48.47 ± 2.92	100
Pyrene	248.57 ± 3.62	125.9 ± 3.66	97.49 ± 0.44	0	249.24 ± 13.37	100
Benzo[a]pyrene	42.15 ± 1.18	44.12 ± 0.08	36.78 ± 0.92	41.21 ± 0.08	51.81 ± 3.82	2.24
Total PAH	695.8	280.88	161.63	41.21	613.87	90.7

Table A2.9: Degradation of PAH Mixture in BSMY inoculated with VUN 10,010 and *P chrysosporium*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	108.81 ± 13.81	23.99 ± 1.02	10.09 ± 0.65	3.47 ± 0.49	44.46 ± 4.42	92.2
Phenanthrene	253.12 ± 9.21	109.21 ± 3.34	36.65 ± 2.95	26.76 ± 1.64	219.89 ± 19.46	87.83
Fluoranthene	53.93 ± 4.51	18.15 ± 2.9	5.26 ± 1.2	11.7 ± 4.68	48.47 ± 2.92	75.86
Pyrene	254.78 ± 22.37	153.05 ± 13.23	115.08 ± 2.22	139.65 ± 14	249.24 ± 13.37	43.97
Benzo[a]pyrene	41.75 ± 0.83	39.46 ± 1.83	33.59 ± 1.71	39.27 ± 0.03	51.81 ± 3.82	5.95
Total PAH	712.39	343.86	200.67	220.85	613.87	64.02

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.10: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* 1B and *P janthinellum*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	118.96 ± 2.34	70.39 ± 4.11	28.56 ± 11.78	0	44.46 ± 4.42	100
Phenanthrene	257.32 ± 3.24	183.82 ± 10.07	73.09 ± 28.59	0	219.89 ± 19.46	100
Fluoranthene	51.61 ± 0.29	37.63 ± 1.65	15.84 ± 5.57	0	48.47 ± 2.92	100
Pyrene	254.53 ± 1.05	209.81 ± 8.26	136.15 ± 23.86	21.53 ± 8.05	249.24 ± 13.37	91.36
Benzo[a]pyrene	44.43 ± 0.65	45.09 ± 0.91	45.94 ± 1.47	46.75 ± 3.26	51.81 ± 3.82	-
Total PAH	726.85	546.74	299.58	68.28	613.87	88.88

Table A2.11: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* 1B and *P chrysosporium*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	106.37 ± 2.33	61.04 ± 3.07	52.39 ± 5.57	0	44.46 ± 4.42	100
Phenanthrene	261.3 ± 5.2	159.81 ± 7.6	136.35 ± 14.07	5.38 ± 3.27	219.89 ± 19.46	97.55
Fluoranthene	47.45 ± 0.75	32.36 ± 1.79	27.96 ± 3.95	0.49 ± 1.72	48.47 ± 2.92	98.99
Pyrene	240.53 ± 3.28	183.45 ± 7.96	182.09 ± 10.32	58.49 ± 22.94	249.24 ± 13.37	76.53
Benzo[a]pyrene	43.57 ± 2.02	42.62 ± 0.88	35.5 ± 1.7	51.19 ± 1.49	51.81 ± 3.82	-
Total PAH	699.22	479.28	434.29	115.55	613.87	81.18

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.12: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* sp. Strain BS5 and *P janthinellum*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	113.71 ± 2.49	64.8 ± 7.99	20.63 ± 8.19	2.64 ± 4.58	44.46 ± 4.42	94.06
Phenanthrene	247.08 ± 3.55	169.56 ± 23.45	56.46 ± 2.07	10.56 ± 1.83	219.89 ± 19.46	95.2
Fluoranthene	49.28 ± 0.53	35.84 ± 4.71	13.01 ± 3.45	3.95 ± 0.84	48.47 ± 2.92	91.85
Pyrene	244.78 ± 1.38	207.53 ± 15.27	140.05 ± 3.28	56.86 ± 7.3	249.24 ± 13.37	77.19
Benzo[a]pyrene	42.98 ± 0.45	45.98 ± 4.77	45.35 ± 0.34	48.43 ± 5.6	51.81 ± 3.82	-
Total PAH	697.83	523.71	275.5	122.44	613.87	80.05

Table A2.13: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* sp. Strain BS5 and *P chrysosporium*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	108.55 ± 4.75	64.35 ± 7.53	45.82 ± 2.15	2.91 ± 5.03	44.46 ± 4.42	93.45
Phenanthrene	258.15 ± 13.11	167.95 ± 2.36	117.01 ± 5.35	10.11 ± 17.5	219.89 ± 19.46	95.4
Fluoranthene	49.09 ± 0.77	35.37 ± 3.68	20.07 ± 6.92	3.31 ± 5.73	48.47 ± 2.92	93.17
Pyrene	248.83 ± 4.65	196.78 ± 2.17	163.57 ± 2.89	76.29 ± 38.84	249.24 ± 13.37	69.39
Benzo[a]pyrene	44.22 ± 1.32	43.12 ± 2.09	33.77 ± 15.34	52.02 ± 4.23	51.81 ± 3.82	-
Total PAH	708.84	507.57	380.24	144.64	613.87	76.44

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

Table A2.14: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* sp. Strain KA5and *P janthinellum*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	106.89 ± 8.84	63.39 ± 0.52	14.39 ± 5.37	0	44.46 ± 4.42	100
Phenanthrene	253.08 ± 1.99	162.72 ± 1.68	35.2 ± 1.52	2.29 ± 0.21	219.89 ± 19.46	98.96
Fluoranthene	48.81 ± 2.72	44.27 ± 1.07	15.74 ± 4.45	2.31 ± 0.22	48.47 ± 2.92	95.23
Pyrene	242.43 ± 1.64	243.2 ± 1.64	180.41 ± 12.76	131.43 ± 32.28	249.24 ± 13.37	47.27
Benzo[a]pyrene	43.55 ± 0.89	43.94 ± 0.59	43.18 ± 1.87	47.56 ± 1.61	51.81 ± 3.82	-
Total PAH	694.76	557.52	288.92	183.59	613.87	70.09

Table A2.15: Degradation of PAH Mixture in BSMY inoculated with *Mycobacterium* sp. Strain KA5 and *P chrysosporium*

	PAH Concentration (mg/l)[a]					
PAH	Initial	30 Days	60 Days	90 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	107.04 ± 5.38	39.01 ± 1.4	24.82 ± 8.55	0	44.46 ± 4.42	100
Phenanthrene	261.14 ± 10.57	102.03 ± 4.83	62.78 ± 2.14	1.82 ± 1.61	219.89 ± 19.46	99.17
Fluoranthene	47.7 ± 1.49	32.58 ± 2.09	23.89 ± 4.04	1.83 ±1.65	48.47 ± 2.92	96.22
Pyrene	239.64 ± 6.99	224.29 ± 12.08	220.84 ± 3.55	113.67 ± 40.7	249.24 ± 13.37	54.39
Benzo[a]pyrene	43.62 ± 0.5	35.82 ± 1.41	43.36 ± 1.85	55.16 ± 0.5	51.81 ± 3.82	-
Total PAH	699.14	433.73	375.69	172.48	613.87	71.9

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed liquid cultures after 90 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo[a]pyrene over the incubation period was calculated with reference to the initial concentration

APPENDIX 2

Degradation tables for experiments using spiked soil inoculated with axenic *P. janthinellum*, VUN 10,010 and the *P. janthinellum* and VUN 10,010 coculture. Also includes degradation tables for experiments using spiked soil sequentially inoculated with the *P. janthinellum* and VUN 10,010 coculture and provided with amendments.

Table A3.1: Natural Attenuation in PAH spiked soil amended with BSMY.

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	77.4 ± 9.3	52.8 ± 3.2	36.5 ± 11.7	0	0	0	38.6 ± 7.3	100
Phenanthrene	212.5 ± 14.4	167.3 ± 11.3	133.1 ± 34.4	0	0	0	149 ± 18	100
Fluoranthene	42 ± 2	39.6 ±2.1	30.7 ± 10.1	0	0	0	51.2 ± 2.5	100
Pyrene	215.9 ± 7.5	214.9 ± 10.2	184.3 ± 28.3	48.4 ± 11.3	10.7 ± 9.5	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	40.1 ± 3.8	38.2 ± 2.1	39.3 ± 9.9	31.8 ± 1.4	29.1 ± 8.3	21.5 ± 6.3	44.5 ± 2.2	46.4
Total PAH	587.9	512.8	423.9	80.2	39.8	21.5	464.6	95.4

Table A3.2: Natural Attenuation in PAH spiked soil amended with distilled water.

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	69.5 ± 1.7	59.6 ± 1.1	65.4 ± 2.3	63.4 ± 6.1	52.1 ± 1.5	49.4 ± 4.6	38.6 ± 7.3	-
Phenanthrene	198.6 ± 3.6	176.2 ± 26.7	198.2 ± 0.4	218.5 ± 19.1	199.9 ± 39.9	196.1 ± 12.4	149 ± 18	-
Fluoranthene	42.4 ± 8.7	36.9 ± 6.2	40.3 ± 3.1	46.5 ± 2.8	43.7 ± 5.4	42.1 ± 2.7	51.2 ± 2.5	17.8
Pyrene	204.1 ± 28.3	202.7 ± 22.7	216.1 ± 18	239 ± 9.3	229 ± 28.8	225.3 ± 6.7	181.3 ± 11.2	-
Benzo(*a*)pyrene	43.8 ± 3.3	39.8 ± 1.2	42.5 ± 6	38.6 ± 2	36.4 ± 9	32.9 ± 3.6	44.5 ± 2.2	24.9[d]
Total PAH	558.4	515.2	562.5	606	561.1	545.8	464.6	-

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed soil cultures after 100 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo(*a*)pyrene over the incubation period was calculated with reference to the initial concentration

Table A3.3: Natural Attenuation in PAH spiked soil amended with 1% glucose.

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	HgCl2[b]	% PAH Decrease[c]
Fluorene	70.88 ± 1.97	64.19 ± 3.24	58.98 ± 0.73	62.22 ± 1.07	56.99 ± 7.04	44.89 ± 0.38	38.63 ± 2.16	-
Phenanthrene	200.66 ± 4.79	208.74 ± 12.45	198.82 ± 1.63	232.66 ± 10.78	237.74 ± 27.27	198.51 ± 7.71	137.73 ± 8.67	-
Fluoranthene	42.61 ± 1.68	44.93 ± 2.94	43.09 ± 1.71	47.69 ± 2.34	50.33 ± 5.01	41.89 ± 0.14	51.21 ± 3.94	18.2
Pyrene	223.51 ± 8.84	232.88 ± 8.88	231.03 ± 10.5	247.85 ± 18.45	259.15 ± 21.22	221.17 ± 3.27	181.32 ± 11.23	-
Benzo(a)pyrene	49.13 ± 6.94	40.64 ± 8.31	43.24 ± 0.38	36.53 ± 1.38	38.08 ± 7.03	45.85 ± 2.33	44.48 ± 1.59	6.68
Total PAH	586.79	591.38	575.16	626.95	642.29	552.31	453.37	-

Table A3.4: Degradation in PAH spiked soil inoculated with VUN 10,010

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	85.4 ± 5	0	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	219.6 ± 14.2	7.8 ± 20.9	0	0	0	0	149 ± 18	100
Fluoranthene	41.7 ± 2.6	0	0	0	0	0	51.2 ± 2.5	100
Pyrene	210.3 ± 10.9	26.3 ± 14.4	7.2 ± 12.4	0	0	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	35.5 ± 1.8	33.6 ± 2.9	34.9 ± 4.1	33.5 ± 2.9	30.8 ± 5.5	30.8 ± 4	44.5 ± 2.2	13.2[d]
Total PAH	592.5	67.6	42.1	33.5	30.8	30.8	464.6	93.4

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed soil cultures after 100 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo(*a*)pyrene over the incubation period was calculated with reference to the initial concentration

Table A3.5: Degradation in PAH spiked soil inoculated with *P janthinellum*

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	73.5 ± 11. 6	42.2 ± 12.6	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	214 ± 13.3	139.7 ± 17.2	31.3 ± 11.1	0	0	0	149 ± 18	100
Fluoranthene	42.4 ± 1	27.3 ± 4.6	13.9 ± 12.4	0	0	0	51.2 ± 2.5	100
Pyrene	218.6 ± 4.2	166.7 ± 11.2	102.4 ± 23.3	28.5 ± 5.2	0	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	40.9 ± 3	39.7 ± 4.5	37.3 ± 3	35.3 ± 2.1	31.9 ± 2.5	28 ± 0.1	44.5 ± 2.2	31.6[d]
Total PAH	589.4	415.6	184.9	63.8	31.9	28	464.6	94

Table A3.6: Degradation in PAH spiked soil inoculated with VUN 10,010 and *P janthinellum*

	PAH Concentration (mg/kg)[a]							
PAH	Initial	20 Days	40 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	93.5 ± 7.3	0	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	237.7 ± 21.3	9.8 ± 16.9	0	0	0	0	149 ± 18	100
Fluoranthene	45.4 ± 2.7	0	0	0	0	0	51.2 ± 2.5	100
Pyrene	227.8 ± 14.2	33.6 ± 11.6	5.9 ± 10.3	0	0	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	37.5 ± 0.3	33.2 ± 4.2	36.4 ± 1.6	32.9 ± 0.9	29.7 ± 3	26.5 ± 1.2	44.5 ± 2.2	29.4[d]
Total PAH	641.9	76.6	42.3	32.9	29.7	26.5	464.6	94.3

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed soil cultures after 100 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo(*a*)pyrene over the incubation period was calculated with reference to the initial concentration

Table A3.7: Degradation in PAH spiked soil sequentially inoculated with VUN 10, 010 followed by *P. janthinellum*.

	PAH Concentration (mg/kg)[a]								
PAH	Initial	20 Days	40 Days	50 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	90.7 ± 7.3	0	0	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	243.7 ± 15.9	0	0	0	0	0	0	149 ± 18	100
Fluoranthene	46.1 ± 2.6	0	0	0	0	0	0	51.2 ± 2.5	100
Pyrene	231.8 ± 11.4	15.8 ± 15.7	0	7 ± 12.2	0	0	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	33.8 ± 4.1	30.6 ± 0.5	30.1 ± 3.4	38.3 ± 3.4	22.3 ± 4.1	20.8 ± 1.7	28.8 ± 4.5	44.5 ± 2.2	14.8[d]
Total PAH	646.1	46.4	30.1	45.3	22.3	20.8	28.8	464.6	93.8

Table A3.8 Degradation in PAH spiked soil sequentially inoculated with *P. janthinellum* followed by VUN 10, 010.

	PAH Concentration (mg/kg)[a]								
PAH	Initial	20 Days	40 Days	50 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	86.5 ± 4.8	62.2 ± 7.1	19.6 ± 17.4	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	224.6 ± 10.4	207.2 ± 22.4	118.7 ± 68.3	17.3 ± 15.3	0	0	0	149 ± 18	100
Fluoranthene	41.2 ± 1.9	39.5 ± 2.1	29.3 ± 9.5	0	0	0	0	51.2 ± 2.5	100
Pyrene	209.7 ± 5.5	210.4 ± 11.7	168 ± 36.8	41.1 ± 23.1	0	0	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	32.1 ± 2.7	29.7 ± 2.7	25.2 ± 4.8	24.6 ± 2.3	12 ± 11.8	7.7 ± 13.3	9.7 ± 16.9	44.5 ± 2.2	69.8[d]
Total PAH	594.1	549	360.8	83	12	7.7	9.7	464.6	93.8

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed soil cultures after 100 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo(*a*)pyrene over the incubation period was calculated with reference to the initial concentration

Table A3.9: Degradation in PAH spiked soil sequentially inoculated with *P. janthinellum* followed by VUN 10, 010 amended with distilled water.

	PAH Concentration (mg/kg)[a]								
PAH	Initial	20 Days	40 Days	50 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	92.6 ± 15.6	68 ± 0.9	30 ± 12.8	0	0	0	0	38.6 ± 7.3	100
Phenanthrene	249.2 ± 20.1	191.8 ± 1.5	88.2 ± 7.1	9.4 ± 1.3	0	0	0	149 ± 18	100
Fluoranthene	50.8 ± 9.41	37.7 ± 0.2	30 ± 11.5	0	0	0	0	51.2 ± 2.5	100
Pyrene	240.5 ± 20.1	197.4 ± 4.4	177.3 ± 4.6	60.6 ± 11.2	7.9 ± 1.1	5.7 ± 0.8	0	181.3 ± 11.2	100
Benzo(*a*)pyrene	50.9	35.5 ± 4.5	35.9 ± 8.11	40.3	34.7 ± 4.4	31.7 ± 2.3	42.1 ± 1.2	44.5 ± 2.2	17.3[d]
Total PAH	684	530.4	361.4	110.3	42.6	37.4	42.1	464.6	90.9

Table A3.10: Degradation in PAH spiked soil sequentially inoculated with *P. janthinellum* followed by VUN 10, 010 amended with 1% glucose.

	PAH Concentration (mg/kg)[a]								
PAH	Initial	20 Days	40 Days	50 Days	60 Days	80 Days	100 Days	$HgCl_2$[b]	% PAH Decrease[c]
Fluorene	80.1 ± 5.2	60.2 ± 4.11	53 ± 1.5	48.8 ± 3.9	45.2 ± 0.7	35.6 ± 1.7	36.1 ± 2.7	38.6 ± 7.3	6.5
Phenanthrene	221.4 ± 6.4	179.8 ± 15.4	167.9 ± 0.5	160.9 ± 7.6	156.5 ± 3.1	157.2 ± 10.5	168 ± 13.7	149 ± 18	-
Fluoranthene	42.7 ± 2.3	36.3 ± 3.2	34.1 ± 0.9	33 ± 1.5	31.5 ± 0.9	37.8 ± 3.5	36.7 ± 3.7	51.2 ± 2.5	28.3
Pyrene	213.3 ± 6.4	189.9 ± 18.6	185.9 ± 0.7	182.9 ± 9.1	180 ± 1.4	203.2 ± 22.6	210.2 ± 19.7	181.3 ± 11.2	-
Benzo(*a*)pyrene	40.1 ± 3.4	36 ± 4	37.9 ± 4.6	37.2	33.9 ± 2.2	35.8 ± 11.6	39 ± 2.3	44.5 ± 2.2	2.8[d]
Total PAH	597.6	502.6	478.8	462.8	447.1	469.6	490	464.6	-

[a]Data reported is average of triplicate samples
[b]PAH concentration in mercuric chloride killed soil cultures after 100 days
[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control
[d]The percentage decrease in benzo(*a*)pyrene over the incubation period was calculated with reference to the initial concentration

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