MICROBIAL DEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS

Submitted by

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree in any university and that to the best of my knowledge, contains no copy or paraphrase material published or written by any other person, except where due reference is made in the text of this thesis.

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Juhasz, A.L, M.L. Britz and G.A. Stanley. Evaluation of Bioaugmentation for the Remediation of Polycyclic Aromatic Hydrocarbon-Contaminated soil. *10th International Biotechnology Symposium*, Sydney, Australia, August (1996), 158.

Juhasz, A.L, M.L. Britz and G.A. Stanley. Acclimation of PAH-Degrading Microorganisms: The Search of Cheap Substrate. *Australian and New Zealand Societies for Microbiology: A Spectrum of Microbiology*, Christchurch, New Zealand, September (1996), A94.

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ABSTRACT

The bacterial degradation of high molecular weight PAHs was investigated by isolating communities and individual strains from a PAH-contaminated site. Microbiological analysis of soils from Port Melbourne, Australia, resulted in the enrichment of five microbial communities capable of degrading pyrene as a sole carbon and energy source. Communities four and five degraded a number of PAH compounds including fluorene, phenanthrene, pyrene and dibenz[*a,h*]anthracene. Three pure cultures were isolated from community five using a spray plate method with pyrene as the sole carbon source. The cultures were identified as strains of *Stenotrophomonas maltophilia* on the basis of multiple sequence alignment analysis of 16SrRNA gene sequences. Differentiation of the three strains was possible by pulse field gel electrophoresis and DNA:DNA hybridisation methods. The *St. maltophilia* strains had similar degradative profiles to community five.

When inoculated at high initial cell densities, community five and the three *St. maltophilia* strains degraded significant concentrations of fluoranthene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene as sole carbon and energy sources. The pyrene-grown microorganisms were also able to degrade all PAHs in a PAH mixture containing three-, four-, five- and seven-ring compounds. Furthermore, improved degradation rates of the five- and seven-ring PAHs was observed when the low molecular weight PAHs were present. Stimulation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was also achieved by the addition of phenanthrene or pyrene to cultures inoculated with low cell numbers. Pyrene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene degradation was not observed by PYEG-grown cells suggesting that the induction of PAH degrading ability appears to be involved in the catabolism of PAHs by community five and the *St. maltophilia* strains.

A characteristic of the kinetics of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolism by cultures containing high initial cell densities of *St. maltophilia* strains was the cessation of five-ring degradation after approximately 10-15 mg/l of the compounds had been degraded. The amount of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degraded by *St maltophilia* strain VUN 10,003 was restricted by the accumulation of their catabolic by-products in the medium, as evidenced by the lack of five-ring PAH degradation after inoculation of fresh cells of VUN 10,003 into benzo[*a*]pyrene or dibenz[*a,h*]anthracene "spent" medium.

The degradation of high molecular weight PAHs by community five and the *St. maltophilia* strains corresponded to a decrease in the mutagenic potential of organic extracts of PAH cultures. In addition, no mutagenic response was observed for PAH culture supernatants although RE intermediate concentrations were detected at

concentration up to 16 mg/l. An increase in the toxicity of benz[*a*]anthracene culture supernatants was observed after incubation with the *St. maltophilia* strains. The increase in benz[*a*]anthracene supernatant toxicity corresponded to the observed increase in RE intermediate concentration.

A number of substrates were evaluated as carbon sources for their use in preparing community five inocula for PAH degradation. As a sole carbon and energy source, creosote was a poor growth substrate. Peptone, yeast extract and glucose were good growth substrates, however, they were unable to induce pyrene degradation. The combination of creosote and yeast extract in BSM provided a medium which supported growth and maintained the pyrene-degrading capacity of the community. This provided an opportunity of using inexpensive sources of PAHs (creosote) and fermentation waste (yeast extract) (CYEM) for cheap, large scale inoculum development for future bioremediation.

Inoculation of pyrene-grown community five into clean soil spiked with PAHs demonstrated the ability of the community to degrade three-, four- and five-ring PAH compounds in a soil matrix. When inoculated into PAH-contaminated soil, CYEM-grown community five degraded all PAH compounds significantly and maintained a high microbial population. Incubation of PAH-contaminated soil with community five resulted in a 43% decrease in the mutagenic potential of organic extracts of soil and a 170-fold decrease in the toxicity of aqueous extracts of soil.

Community five and *St. maltophilia* strain VUN 10,003 rapidly mineralised ^{14}C-pyrene after an initial lag period of 10 hours. Between 65 and 70% of the ^{14}C label was detected as $^{14}CO_2$ after 120 hours. CYEM-grown community five also mineralised pyrene in PAH-contaminated soil: 42% of the ^{14}C label was detected as $^{14}CO_2$ after 48 days. Mineralisation of ^{14}C-benzo[*a*]pyrene was minimal: less than 0.25% of the total radioactivity was detected as $^{14}CO_2$ after 70 days, although ^{14}C label was detected in the aqueous phase (5-7%) and cellular material (12%).

Analysis of samples taken from pyrene cultures inoculated with the *St. maltophilia* strains resulted in the identification of four pyrene metabolites. Pyrenedihydrodiol and pyrenol were identified as ring oxidation products while 4-hydroxyperinaphthenone and 4-phenanthroic acid were identified as ring fission products. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites were isolated by preparative TLC, however, the compounds could not be identified due to the low yield of the metabolites and problems associated with volatilising, ionising and derivatising of the compounds.

SYMBOLS AND ABBREVIATIONS

%	per cent
B[a]P	benzo[*a*]pyrene
BSA	bovine serum albumin
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
BTX	benzene, toluene and xylene
BYP	basal salts yeast extract peptone agar
CBFT	Centre for Bioprocessing and Food Technology
COR	coronene
CFU	colony forming units
°C	degrees Celsius
CHEF	contour-clamped homogeneous electric field
CYEM	creosote yeast extract medium
DBA	dibenz[*a,h*]anthracene
DCM	dichloromethane
DMF	dimethylformamide
EC_{50}	Effective concentration of a test compounds at which a 50% decrease in the light output of *Photobacterium phosphoreum* is observed
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno sorbant assay
FA	fluoranthene
FC	fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene
FLU	fluorene
g	gram
GC	gas chromatography
GC-FID	gas chromatography equipped with flame ionisation detection
GC-MS	gas chromatography equipped with mass spectrometry
h	hour
HBAMGN	histidine/biotin/ampicillin minimal glucose medium
HBMGM	histidine/biotin minimal glucose medium
HPLC	high performance liquid chromatography
kg	kilogram
K_{ow}	octanol:water partitioning coefficient
l	litre
M^+	molecular ion
µg	microgram
mg	milligram

MGM	minimal glucose medium
ml	millilitre
min	minute
MM	minimal medium
mV	milli volts
NA	nutrient agar
NB	nutrient broth
NB II	nutrient broth II
ND	not determined
nm	nanometre
NMR	nuclear magnetic resonance
opm	oscillations per minute
PAH	polycyclic aromatic hydrocarbon
PCP	pentachlorophenol
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
pH	hydrogen ion concentration (minus log of)
PHEN	phenanthrene
PP	*Photobacterium phosphoreum* medium
PPDB	phenanthrene, pyrene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene
PYEG	peptone yeast extract glucose medium
PYR	pyrene
RE	resorcinol equivalent
R*f*	chromatographic mobility
RLU	relative light units
rpm	revolutions per minute
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
uv	ultraviolet
VUN	Victoria University Gram negative bacterium
VUT	Victoria University of Technology
v/v	volume per volume
w/v	weight per volume

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LITERATURE REVIEW

1.1 INTRODUCTION

Over the past 150 years, industrial, agricultural and medical activities have generated large quantities of hazardous chemicals that have caused environmental pollution. Governments have adopted treatment and recycling programs to dispose of toxic chemicals, minimise waste and recycle reusable materials to overcome increasing pollution problems and to decrease use of limited energy resources. In addition, the clean up of contaminated land has become an increasing issue due to the risk that exposure to pollutants pose to human health (Menzie *et al.*, 1992). Currently, a number of physical and chemical approaches have been used for the remediation of contaminated soil (Anderson *et al.*, 1993). Soil washing and soil flushing may be used as an *ex situ* process for the removal of organic, inorganic and radioactive contaminants from soil. Solidification is a process that encapsulates contaminants in a solid of high integrity, while stabilisation converts the waste into a less toxic, soluble or mobile phase. A number of thermal techniques have been developed for treating contaminated soil, which can involve either desorbing the contaminant from a matrix or thermally destroying organic compounds. Chemical treatments include technologies that use reagents to destroy or chemically modify contaminants by means other than pyrolysis or combustion. In addition, solvent and chemical extractions may be used to remove contaminants from soil, sediment or water (Anderson *et al.*, 1993). These methods are based on equilibrium separation techniques commonly used in the chemical industry. Some of the physical and chemical processes may be used as stand-alone techniques or in combination for cleaning up contaminated soils. Unfortunately, the above approaches are often expensive, inefficient and may lead to additional problems such as the collection of concentrated organic waste after solvent extraction or air borne pollution after incineration. Bioremediation, the biological degradation of organic hazardous wastes, has emerged over the past decade as a viable alternative to physical and chemical remediation processes. Bioremediation exploits the metabolic diversity of microorganisms, in particular those organisms displaying an ability to catabolise xenobiotic substances. The advantage of microbial degradation of pollutants over other processes lies in its effectiveness to degrade some compounds to innocuous by-products (Cerniglia, 1992). Furthermore, the process may occur *in situ*, thereby eliminating excavation of the site and reducing operating costs of treatment. Unfortunately,

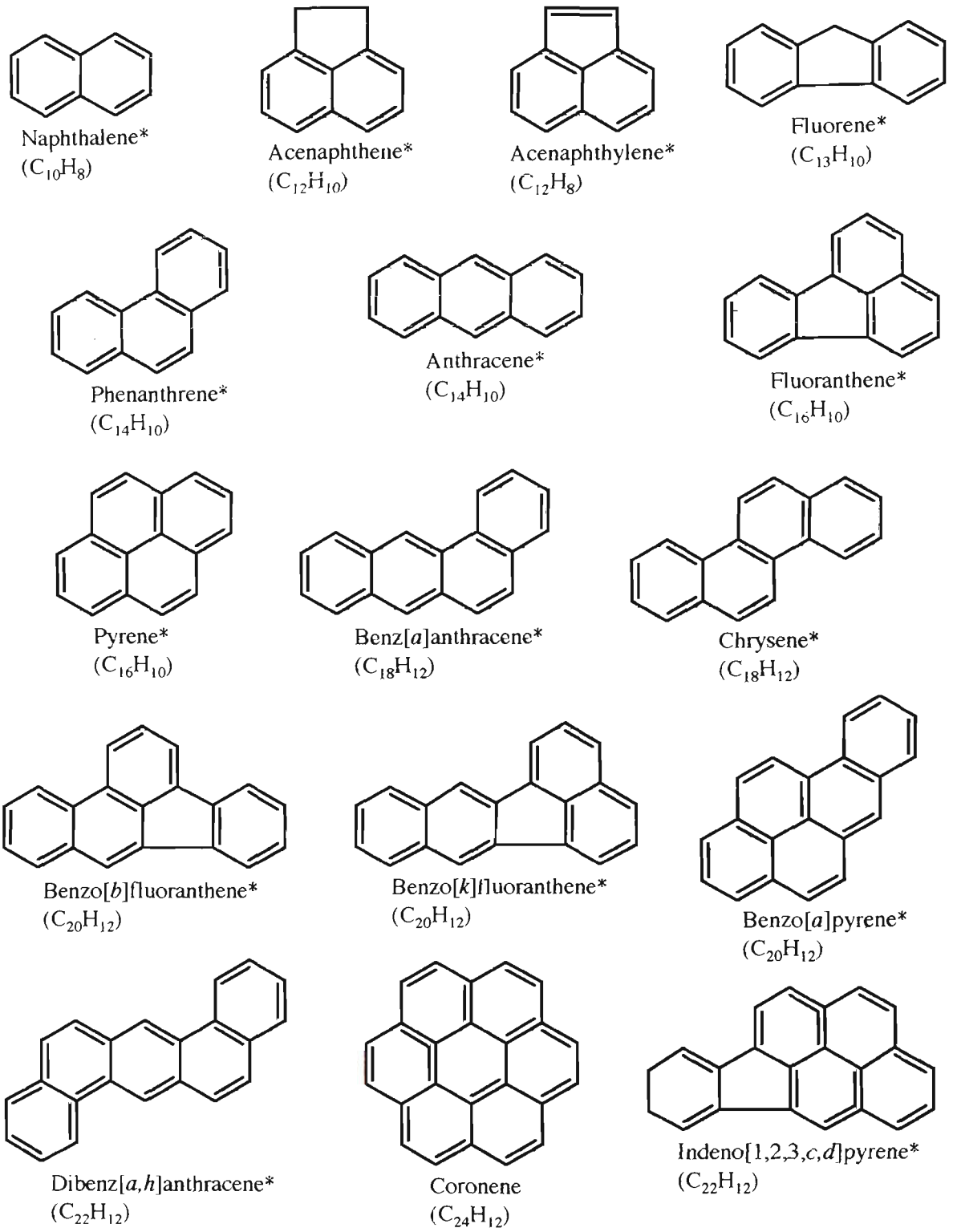

Figure 1.1. Chemical structures of some common polycyclic aromatic hydrocarbons (adapted from Wilson and Jones, 1993).

bioremediation may not be effective for some pollutant types or at some sites due to the physical and chemical characteristics of the soil. The success of bioremediation depends on a number of factors including the presence of microorganisms that are capable of degrading an array of compounds, the availability of pollutants for bioremediation (which may be governed by several factors) and the capacity to scale-up laboratory systems for field application.

Polycyclic aromatic hydrocarbons (PAHs) are one class of environmental pollutants that have accumulated in the environment due to a variety of anthropogenic activities. Presently, bioremediation has been shown to be effective in remediating soils contaminated with low molecular weight PAHs (Mueller *et al.*, 1991a; Kastner and Mahro, 1996; Banerjee *et al.*, 1995), however, the high molecular weight PAHs are generally recalcitrant to microbial attack (Park *et al.*, 1990; Erickson *et al.*, 1993; Cerniglia, 1992). This may be attributed to the scarcity of organisms capable of degrading high molecular weight PAHs together with environmental factors governed by the site. For bioremediation to be an effective tool for the clean up of PAH-contaminated soils, a greater understanding of the processes involved in the degradation of high molecular weight PAHs is required. The subject of this thesis was the isolation of bacteria from PAH-contaminated soil samples and the evaluation of these strains for bioremediation of high molecular weight PAHs. The remainder of this chapter reviews the literature on many of the aforementioned factors important in this evaluation.

1.2 PHYSICAL PROPERTIES OF PAHs AND THEIR OCCURRENCE IN THE ENVIRONMENT

1.2.1 Chemical Structure

The term PAH generally refers to hydrocarbons containing three or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983). PAHs may also contain five member rings, such as fluorene and fluoranthene. Figure 1.1 shows the structures and names of some commonly occurring PAHs. In addition, alkyl groups may be attached to various positions of the unsubstituted parent PAH. Many PAHs contain heteroatoms, where a carbon in the aromatic ring is substituted with nitrogen, sulphur, oxygen or other elements (Blumer, 1976). These heteroaromatic compounds have properties and behaviours that resemble the unsubstituted parent PAHs in analysis. Table 1.1 lists structures and physical-chemical properties of some common PAHs (adapted from Sims and Overcash, 1983).

Table 1.1. Structure and physico-chemical properties of some PAHs (adapted from Sims and Overcash, 1983).

PAH	N° of Rings	mp[a] °C	bp[b] °C	Sol[c] (mg/l)	log K_p[d]	Vapour Pressure[e]
Naphthalene	2	80	218	30	3.37	4.92×10^{-2}
Acenaphthalene	3	96	279	3.47	4.33	2.0×10^{-2}
Acenaphthylene	3	92	265	3.93	4.07	2.9×10^{-2}
Anthracene	3	216	340	0.07	4.45	1.96×10^{-4}
Phenanthrene	3	101	340	1.29	4.46	6.8×10^{-4}
Fluorene	3	116	293	1.98	4.18	1.3×10^{-2}
Fluoranthene	4	111	250	0.26	5.33	6.0×10^{-6}
Benz[*a*]anthracene	4	158	400	0.014	5.61	5.0×10^{-9}
Chyrsene	4	255	488	0.002	5.61	6.3×10^{-7}
Pyrene	4	149	360	0.14	5.32	6.85×10^{-7}
Benzo[*a*]pyrene	5	179	496	0.0038	6.04	5.0×10^{-7}
Benzo[*b*]fluoranthene	5	167		0.0012	6.57	5.0×10^{-7}
Benzo[*k*]fluoranthene	5	217	480	0.0005	6.84	5.0×10^{-7}
Dibenz[*a,h*]anthracene	5	262	524	0.0005	5.97	1.0×10^{-10}
Benzo[*g,h,i*]perylene	6	222		0.0003	7.23	1.0×10^{-10}
Indeno[*1,2,3-c,d*]pyrene	6	163	536	0.062	7.66	1.0×10^{-10}

[a]mp=melting point

[b]bp=boiling point

[c]Sol=aqueous solubility

[d]Log K_p=logarithm of the octanol:water partition coefficient

[e]Vapour pressure=torr at 20°C

The stability of PAHs is related to the arrangement of the benzene rings in the structure. PAHs with an angular arrangement are most stable while PAHs with a linear arrangement are least stable (Blumer, 1976). PAHs are hydrophobic compounds and their persistence in the environment is due chiefly to their low water solubility (Cerniglia, 1992). Generally, PAH solubility decreases with an increase in number of fused benzene rings. Volatility also decreases with an increasing number of fused rings (Wilson and Jones, 1993). Theoretical and experimental evidence has shown that the association of a contaminant with organic material of soil (expressed as the organic carbon normalised partition coefficient K_{OC}) is a function of the hydrophobicity of the compound (expressed as the octanol water partition coefficient K_{OW}) (Dzombak and Luthy, 1984). As PAHs are characterised by extremely high K_{OW} and low vapour pressures, naturally occurring organic material is an excellent sorbent for these compounds (Means *et al.*, 1980; McCarthy and Jimenez, 1985; Weissenfels *et al.*, 1992).

1.2.2 Production of PAHs

PAHs are ubiquitous environment contaminants which have been detected in a wide variety of environmental samples, including air (Freeman and Cattell, 1990; Sexton *et al.*, 1985; Greenberg *et al.*, 1985), soil (Jones *et al.*, 1989a, 1989b, 1989c; Wilson and Jones, 1993), sediments (Youngblood and Blumer, 1975; Laflamme and Hites, 1978; Shiaris, 1986), water (Cerniglia and Heitkamp, 1989), oils, tars (Nishioka *et al.*, 1986) and foodstuffs (Lijinsky, 1991; Dipple and Bigger, 1991). The major source of PAHs is from the combustion of organic material (Cerniglia and Yang, 1984; Guerin and Jones, 1988a). PAHs are formed naturally during thermal geologic production and during burning of vegetation in forest and bush fires (Blumer, 1976; Bjorseth *et al.*, 1979). PAHs and their alkyl homologs may also be derived from biogenic precursors during early diagenesis (Wakeham *et al.*, 1980b, Laflamme and Hites, 1978, 1979). However, anthropogenic sources, particularly from fuel combustion, pyrolytic processes and spillage of petroleum products (Freeman and Cattell, 1990; Wakeham *et al.*, 1980a), are significant sources of PAHs in the environment. In industrial countries, anthropogenic combustion activities are a principal source of PAHs in soils where they arise from atmospheric deposition. This had lead to an increase in soil PAH concentration over the last 100-150 years (Jones *et al.*, 1989a, 1989b).

Anthropogenic sources of PAHs pollution of the environment include coal combustion, industrial operations using fossil fuels, waste incinerators, domestic heaters and vehicles powered by gasoline or diesel fuels (Table 1.2). Industrial activities, such as processing,

Table 1.2. Industrial activities associated with the production, processing, use and disposal of PAH-containing material (Wilson and Jones, 1993).

Gasification/liquefacation of fossil fuels
Heat and power generation by using fossil fuels
Coke production
Catalytic cracking
Carbon-black production and use
Asphalt production and use
Coal-tar/coal-tat-pitch production and use
Refining/distillation of crude oil and crude oil derived products
Wood treatment processes
Wood preservation (eg creosote/anthracene oil)
Fuel/oil storage, transportation, use and disposal
Landfill/waste dumps
Open burning (tyres/refuse/coal etc.)
Incineration

combustion and disposal of fossil fuels, are usually associated with the presence of PAHs at highly contaminated sites. For example, at an oil refinery, PAH concentrations may range up to 1.79 x 10^6 ng PAH/g of soil (Johnson *et al.*, 1985). Thermal PAH formation can occur over a wide range of temperatures and with many source materials. High temperatures (>700°C), which occur in association with coking of coal, automotive engines and domestic heating, favour the formation of unsubstituted aromatics (Youngblood and Blumer, 1975). Lower temperatures (<700°C) produce a greater degree of alkylation. PAH contamination on industrial sites is commonly associated with spills and leaks from storage tanks and with the conveyance, processing, use and disposal of these fuel/oil products (Wilson and Jones, 1993). Harbours, rivers and waterways are endpoints for municipal and industrial sewage, which deposit large quantities of decaying organic matter and oil residues into the sediment. In Boston, USA, it has been estimated that hydrocarbon discharge into the harbour by only two sewage treatment plants is approximately 5 x 10^5 kg dry weight per year (Shiaris, 1986). Motor vehicles, including spark emission and diesel automobiles, trucks and buses, contribute to PAH pollution through exhaust condensate and particulates, tyre particles and lubricating oils and greases (Wakeham *et al.*, 1980a). Harkov *et al.* (1984) proposed that benzo[*a*]pyrene derived from automobiles accounted for 98% of New Jersey's state-wide, non-heating produced benzo[*a*]pyrene emissions. PAHs are also a major constituent of creosote (approximately 85% PAH by weight) and anthracene oil, which are commonly used pesticides for wood treatment (Bumpus, 1989; Bos *et al.*, 1984; Walter *et al.*, 1991). As such, PAH contamination is frequently associated with wood treatment activities (Mueller *et al.*, 1991a, 1993; Vanneck *et al.*, 1995; Sims and Overcash, 1983).

Forest fires, bush fires and general residential wood burning can generate high concentrations of PAHs, including the higher molecular weight PAHs, benzo[*a*]pyrene and coronene. PAH concentrations of 3,000 $\mu g/m^3$, and concentrations of 60 $\mu g/m^3$ for benzo[*a*]pyrene have been measured in flue emissions from small residential stoves (Freeman and Cattell, 1990). Ambient PAH concentrations are usually in the order of a few nanograms per cubic metre of air (Ramadahl *et al.*, 1982). Bonfire smoke may contain up to 70 parts per million of benzopyrenes, which is approximately 350-times the amount found in cigarette smoke (Gamlin and Price, 1988).

Diagenesis is another source of PAH contamination in recent sediments and *in situ* generation of some PAHs in several different depositional environments is more significant than previously thought (Wakeham *et al.*, 1980b; Laflamme and Hites, 1978). Wakeham *et al.* (1980b) found that perylene concentrations generally increased

Table 1.3. Mean, median and range values for PAHs in Welsh surface soil samples (μg/kg soil dry weight) (Jones *et al.*, 1989c).

Compound	Mean	Median	Range	Standard Deviation
Naphthalene	35	2.8	<1.0-1000	147
Acenaphthylene	4.8	<1.0	<1.0-130	19
Acenaphthalene/ Fluorene	217	38	12.4-5500	815
Phenanthrene	273	29	7.7-6700	991
Anthracene	50	2.7	0.6-1500	217
Fluoranthene	514	54	16.8-11600	1710
Pyrene	225	31	9.7-5650	816
Benz[*a*]anthracene/ Chrysene	406	46	12.2-12000	1740
Benzo[*b*]fluoranthene	207	26	7.0-4600	678
Benzo[*a*]pyrene	138	16	3.5-3700	534
Dibenz[*a,h*]anthracene	65	11	<1.0-666	130
Benzo[*g,h,i*]perylene	137	43	<1.0-1600	274
Sum PAH	2325	301	108-54500	7940

with increasing depth in the sedimentary column. In sediments greater than one metre, perylene accounted for 70-90% of the total PAHs. The abundance of perylene in sediments was interpreted as *in situ* generation of the compound by the transformation of an unknown precursor. Laflamme and Hites (1978) suggested that the presence of perylene was due to the reduction of various extended quinone pigments, such as erythroaphin and 4,9-dihydroxyperylene-3,10-quinone, which are found in insects (Cameron *et al.*, 1964) and fungi (Allport and Bu'Lock, 1960).

Both Laflamme and Hites (1978) and Wakeham *et al.* (1980b) also observed retene, an alkylated phenanthrene, in sediments from remote areas. Retene, a naturally occurring compound in pine tar and in high boiling tall oils, arises as a result of the dehydrogenation of abietic acid, which is a major component of conifer resins (Laflamme and Hites, 1978; Wakeham *et al.*, 1980b).

1.2.3 Occurrence of PAHs in the Environment

Temporal studies on PAH concentrations in soils from industrialised countries have revealed an increasing PAH burden since the mid 1800s, with a peak in the 1950/1960s (Jones *et al.*, 1989b). A qualitative PAH pattern for most locations has appeared, with an increase in PAH abundance near urban centres. Anthropogenic combustion of fossil fuels and long range atmospheric transport of PAHs has contributed to the dispersal of PAHs throughout the environment (Bjorseth *et al.*, 1979; Greenberg *et al.*, 1985). A study by Jones *et al.* (1989c) on Welsh soil revealed total PAH levels ranged over three orders of magnitude (100 to 55,000 µg of PAH/kg) in normal soils with no industrial contamination (Table 1.3). The concentration of PAHs in contaminated soils can vary depending on the industrial activity associated with the site (Table 1.4). PAH concentrations at these sites ranged from 451 to 18,704 mg/kg soil. However, it must be appreciated that sampling methods and locations can vary at each site and the values in Table 1.4 are given as an indication of the concentration of PAHs at a few contaminated sites only.

PAHs are also distributed in sediments throughout the world. The input of PAH material into sediments has resulted from the deposition of aeolian transported fossil fuel combustion products (Lake *et al.*, 1979), as well as PAHs generated by forest or grass fires and volcanic activities (Blumer, 1976). PAHs may also be deposited to sediments following transportation by runoff from contaminated soils and by discharge of municipal and industrial waste material into rivers and bays.

Table 1.4. Concentration of selected PAHs in contaminated soils and sediment.

PAH	PAH concentration (mg/kg soil) in PAH-contaminated soils/sediment:				
	Wood preserving site[a]	Creosote production site[b]	Gas works site[c]	Manufacturing gas plant site[d]	Dredged sediment[e]
Naphthalene	3,925	1,313		97	497
Acenaphthalene	49	33		28	
Acenaphthene	1,368		2	49	
Fluorene	1,792	650	225	14	321
Phenanthrene	4,434	1,595	379	26	318
Anthracene	3,307	334	156	11	778
Fluoranthene	1,629	682	2,174	73	512
Pyrene	1,303	642	491	47	237
Chrysene	481	614	345	15	67
Benz[*a*]anthracene	171		317	16	180
Benzo[*a*]pyrene	82		92	14	78
Benzo[*b*]fluoranthene and Benzo[*k*]fluoranthene	140		498	21	205
Dibenz[*a,h*]anthracene			2,451	33	
Indeno[*1,2,3-c,d*]pyrene	23		207	7	142
Sum PAH	18,704	5,863	7,337	451	3,335

[a]Mueller *et al.* (1991a, 1991b)
[b]Ellis *et al.* (1991)
[c]Bewley *et al.* (1989)
[d]Erickson *et al.* (1993)
[c]Huis in't Veld *et al.* (1995)

1.2.4 PAH Toxicity

Many PAHs are carcinogenic and they are therefore of significant concern as environmental contaminants (Sims and Overcash, 1983). Numerous studies have indicated that one-, two- and three-ring compounds are acutely toxic (Sims and Overcash, 1983), while higher molecular weight PAHs are considered to be genotoxic (Lijinsky, 1991; Mersch-Sundermann *et al.*, 1992; Nylund *et al.*, 1992; Phillips, 1983). More than 30 parent PAH compounds and several hundred alkyl derivatives of PAHs were reported to have some carcinogenic effect (Dipple, 1976).

Three-ring and higher PAHs can be active as tumour initiators, complete carcinogens or as cocarcinogens (IARC, 1983). PAHs, such as dibenz[*a,c*]anthracene and benzo[*e*]pyrene, which are inactive as complete carcinogens, may act as tumour initiators. Tumour initiators lead to tumour development only when the treated tissue is subsequently exposed to a promoter and/or when they are applied together with a cocarcinogen over a long time period (IARC, 1983). Generally, neither promoters nor cocarcinogens induce tumours by themselves. To be considered a complete carcinogen, a PAH must induce benign and malignant tumours in epithelial cells or connective tissue after single or repeated applications.

1.2.4.1 *Phenanthrene and Anthracene*

Phenanthrene and anthracene have not been reported to be carcinogenic (Lijinsky, 1991). However, the presence of methyl groups on the respective parent compound (*i.e.* 1,2,3,4-tetramethylphenanthrene and 9,10-dimethylanthracene) produces carcinogens of moderate potency. Anthracene has been tested for its carcinogenicity in mice and rats by a number of methods including skin application (Wynder and Hoffmann, 1959), subcutaneous and/or intramuscular administration (Boyland and Burrows, 1935; Pollia 1941), intraperitoneal administration and pulmonary injection (Stanton *et al.*, 1972). The results obtained were not indicative of a carcinogenic effect or of initiating activity. Phenanthrene has been tested extensively for its carcinogenic ability in animal studies (Huggins and Yang, 1962; LaVoie *et al.*, 1981; Wood *et al.*, 1979; Buening *et al.*, 1979). When phenanthrene was administered orally to female rats, using a single dose of 200 mg phenanthrene dissolved in sesame oil, no mammary tumours were produced within sixty days (Huggins and Yang, 1962). LaVoie *et al.* (1981) reported administering 100 µl of a 0.1% solution of phenanthrene in acetone 10 times on alternate days to Swiss mice. After a 10 day period, 2.5 µg TPA (12-O-tetradecanoylphorbol-13-acetate) in 100 µl acetone was applied three times a week for

20 weeks. No skin tumours were found after this time period, suggesting that phenanthrene is not carcinogenic.

1.2.4.2 *Pyrene*

Studies on the carcinogenic nature of four-ring PAHs, such as pyrene and benz[*a*]anthracene have shown that some of these four-ring PAHs exhibit weak carcinogenic activity, or that the data available is inadequate to permit an evaluation of the carcinogenicity in experimental animals. Pyrene has been tested for its carcinogenicity in several experiments by skin application to mice (Horton and Christian, 1974; Van Duuren and Goldschmidt, 1976). In these experiments, no skin tumours were observed. Pyrene was, however, found to enhance the carcinogenic effects of benzo[*a*]pyrene. Van Duuren and Goldschmidt (1976) treated mice with 0.1 ml of acetone containing 4, 12 or 40 μg of pyrene. The pyrene solution was applied to the skin of the test mice over a period of 368 or 440 days, with three, weekly applications. In addition, 5 μg of benzo[*a*]pyrene was applied to the skin with these various concentrations of pyrene. The results showed that there was an increase in the incidence of papilloma and carcinoma formation with an increase in pyrene concentration. At the lowest pyrene concentration (4 μg pyrene plus 5 μg benzo[*a*]pyrene), 12 mice displayed papilloma and six had squamous-cell carcinomas. However, when pyrene was applied at a concentration of 40 μg (with 5 μg benzo[*a*]pyrene), 35 mice exhibited papillomas and 26 contained squamous-cell carcinomas out of a total of 52 mice. No skin tumours occurred in the solvent-treated or untreated control mice.

Goldschmidt *et al.* (1973) reported that pyrene enhanced benzo[*a*]pyrene's carcinogenic effect: the simultaneous application of pyrene and benzo[*a*]pyrene had a greater effect on papilloma and carcinoma induction than benzo[*a*]pyrene when applied as a single test compound. The application of pyrene alone did not induce tumour formation. Tests with pyrene in both *in vitro* and *in vivo* experiments produced limited evidence that pyrene was active. Pyrene was mutagenic in some assays with *Salmonella typhimurium* in the presence of the rat liver homogenate S9 fraction (Kaden *et al.*, 1979), however, it was not mutagenic to fungi (*Saccharomyces cerevisiae*) (de Serres and Hoffman, 1981). Damage to DNA was not reported in assays performed on *Escherichia coli* or *Bacillus subtilis* (Ashby and Kilby, 1981). Pyrene did induce mutations and unscheduled DNA synthesis in some *in vitro* assays in mammalian cells (Jotz and Mitchell, 1981; Robinson and Mitchell, 1981), but in *in vivo* mammalian tests, sister chromatid exchange or micronuclei were not induced (Paika *et al.*, 1981; Salamone *et al.*, 1981). Experimental

data on the activity of pyrene in animal tests is extensive. From this data, the International Agency for Research on Cancer has given the evaluation that there is no evidence that pyrene is carcinogenic to experimental animals (IARC, 1983).

1.2.4.3 *Benz[a]anthracene*

Sufficient evidence has been gathered to classify benz[*a*]anthracene as carcinogenic to experimental animals. Kennaway and Hieger (1930) were among the initial group of researchers examining the carcinogenic nature of the constituents of tars. Benz[*a*]anthracene was the first pure compound shown to induce tumours in mice following skin application. These experiments were the first to recognise the carcinogenic activity of a chemical of defined structure. Since then, benz[*a*]anthracene has proven to be carcinogenic to mice when administered by several routes. Klein (1963) administered benz[*a*]anthracene to mice by oral application, using 1.5 mg benz[*a*]anthracene, given as 15 treatments over a five-week period. Klein found that repeated oral administration of benz[*a*]anthracene produced hepatomas and lung adenomas. The application of benz[*a*]anthracene in a number of solvents to the skin of mice has shown that the four-ring PAH is a complete carcinogen to mouse skin (Van Duuren *et al.*, 1970; Hadler, 1959). The incidence of tumour formation was greater when benz[*a*]anthracene was applied in a solution of dodecane than toluene. Dodecane has been shown to have the ability to act as a cocarcinogen when tested simultaneously with several PAHs (Bingham and Falk, 1969). Benz[*a*]anthracene produced tumours in mice following subcutaneous injections. Boyland and Sims (1967) tested benz[*a*]anthracene at a range of concentrations. Even at the lowest benz[*a*]anthracene concentration (50 μg), the compound was effective in tumour formation in newborn and adult mice. Tests have demonstrated that benz[*a*]anthracene is mutagenic to *S. typhimurium* (Kaden *et al.*, 1979; Bartsch *et al.*, 1980; Coombs *et al.*, 1976), *D. melanogaster* (Fahmy and Fahmy, 1973) and *in vitro* to cell culture in the presence of the rat liver homogenate S9 fraction (Slaga *et al.*, 1978; Amacher *et al.*, 1980; Amacher and Turner, 1980). Benz[*a*]anthracene also induced unscheduled DNA synthesis in cultured mammalian cells and morphological transformations (Pal, 1981; Pienta *et al.*, 1977), which are indicative of carcinogenic activity.

Methyl substitution of some four-ring compounds gives rise to PAHs of great carcinogenic potency, however, the carcinogenicity depends on the position of the substitution in the molecule. Derivatives of benz[*a*]anthracene are the most extensively studied series of PAH compounds in terms of structure-activity relationships (Dipple *et al.*, 1984). Methyl substitutions at the 1-, 2-, 3- or 4- position on the angular benzene

ring result in products that lack carcinogenic activity whereas substitutions at the 6-, 7-, 8- or 12- position result in compounds of great carcinogenic potential. For example, 7,12-dimethylbenz[*a*]anthracene is one of the most potent PAH carcinogens known (Dipple and Bigger, 1991). Methylchrysenes also vary greatly in their carcinogenic potency: most are weak carcinogens, but 5-methylchrysene is a very potent carcinogen.

1.2.4.4 *Benzo[a]pyrene*

Compounds based on five-ring PAHs vary greatly in their carcinogenic activity. Picene, pentacene, pentaphene, perylene and benzo[*e*]pyrene are not carcinogenic. Dibenz[*a,c*]anthracene and cyclopenta[*c,d*]phenanthrene are considered to have weak carcinogenic potential, whereas benzo[*a*]pyrene and dibenz[*a,h*]anthracene are regarded as potent carcinogens.

Benzo[*a*]pyrene has been shown to be carcinogenic by producing tumours in experimental animals when administered orally, by skin application, inhalation and/or intratracheal administration, subcutaneous and/or intramuscular administration, intraperitoneal administration, intrabronchial implantation and transplacental routes. Benzo[*a*]pyrene was shown to be active in assays for bacterial DNA repair, bacteriophage induction and bacterial and *D. melanogaster* mutations (Fujikawa *et al.*, 1993; Bos *et al.*, 1984; Mersch-Sundermann *et al.*, 1992; Wood *et al.*, 1976). In mammalian cells in culture, benzo[*a*]pyrene can induce DNA binding, sister chromatid exchange, chromosomal aberrations, point mutations and transformations. Tests in mammals *in vivo* have shown that benzo[*a*]pyrene is active in assays for DNA binding, sister chromatid exchange, chromosomal aberrations, sperm abnormality and in the somatic specific locus (spot) test (Hollstein *et al.*, 1979; de Serres and Ashby, 1981).

1.2.4.5 *Dibenz[a,h]anthracene*

Dibenz[*a,h*]anthracene has produced tumours by different routes of administration in a number of experimental animals. It has been shown that dibenz[*a,h*]anthracene produces both local and systematic carcinogenic effects. Snell and Stewart (1962) administered dibenz[*a,h*]anthracene to mice orally at a concentration of 0.76-0.85 mg/day. After 200 days, 100% of the surviving mice (27/27) had developed pulmonary adenomatosis, 24 had alveologenic carcinoma and 16 had haemangio-endotheliomas. In addition, 12/13 female mice had developed mammary carcinomas. In the 35 control mice, no mammary tumours were observed and only two pulmonary adenomatoses were seen. In skin application experiments with mice, dibenz[*a,h*]anthracene was shown to

initiate skin carcinogenesis at doses of as little as 0.02 μg given as a single dose (Klein 1960). The Ames test has revealed that dibenz[*a,h*]anthracene was mutagenic to *S. typhimurium* (Kaden *et al.*, 1979; Baker *et al.*, 1980) in the presence of the rat liver homogenate S9 fraction. *In vitro* mammalian cell assays have illustrated that dibenz[*a,h*]anthracene was mutagenic, causing unscheduled DNA synthesis (Lake *et al.*, 1978; Martin *et al.*, 1978), induced sister chromatid exchange (Pal, 1981) and was positive for morphological transformations (Pienta *et al.*, 1977; Casto *et al.*, 1977).

1.2.4.6 *Coronene*

Coronene, a seven-ring compound, has generally been accepted to be non-carcinogenic (Lijinsky, 1991). Skin applications to mice did not induce tumour formation, however, coronene was active as an initiator in mouse skin initiation promotion assays (Van Duuren *et al.*, 1968). Table 1.5 details the carcinogenic activity of a range of PAHs.

1.3 BIODEGRADABILITY OF PAHs

Microorganisms play an important role in the environment as they serve as biogeochemical agents for the conversion of organic compounds to simple inorganic compounds or their constituent elements. The conversion of organic compounds to carbon dioxide with the concomitant reduction in molecular oxygen is facilitated by a wide variety of bacteria, fungi and algae. The ability of microorganisms to degrade environmental pollutants, such as petroleum hydrocarbons, PAHs, polychlorinated biphenyls (PCBs), pentachlorophenol (PCP) and pesticides, has generated considerable interest in the use of microorganisms for waste minimisation and bioremediation of contaminated soils and waste streams.

Table 1.6 lists a variety of environmental microorganisms with broad metabolic capabilities in PAH degradation. *Pseudomonas*, *Mycobacterium*, *Rhodococcus*, *Beijernickia* and *Alcaligenes* species have been shown to degrade numerous organic compounds including *n*-alkanes, aromatics, polyaromatics and halogenated derivatives. The versatility of these microorganisms may be as a consequence of the structural diversity of substrates in the environment which have led to the evolution of an extremely wide range of degradative activities. Many catabolic pathways have evolved on conjugative plasmids and by mutational change in enzyme components and regulators of gene expression. The transmission of new genetic material on broad host range plasmids provides a huge gene pool which allows the acquisition of degradative pathways for organic compounds.

Table 1.5. Evaluation of the Carcinogenic Activity of Selected PAHs (IARC, 1983).

Compound	Carcinogenicity in Experimental Animals§	Activity in Short Term Tests†	Mutagenicity to *S. typhimurium* (Ames test)¥
Fluorene	I	I	-
Phenanthrene	I	L	+
Anthracene	No	No	-
Fluoranthene	No	L	+
Pyrene	No	L	+
Benzo[*a*]fluorene	I	I	No
Benzo[*b*]fluorene	I	I	No
Benzo[*c*]fluorene	I	I	No
Benzo[*g,h,i*]fluoranthene	I	I	+
Benzo[*c*]phenanthrene	I	I	+
Cyclopenta[*c,d*]pyrene	L	S	+
Benz[*a*]anthracene	S	S	+
Chrysene	L	L	+
Triphenylene	I	I	+
Benzo[*b*]fluoranthene	S	I	+
Benzo[*j*]fluoranthene	S	I	+
Benzo[*k*]fluoranthene	S	I	+
Benzo[*e*]pyrene	I	L	+
Benzo[*a*]pyrene	S	S	+
Perylene	I	I	+
Indeno[*1,2,3-c,d*]pyrene	S	I	+
Dibenz[*a,c*]anthracene	L	S	+
Dibenz[*a,h*]anthracene	S	S	+
Dibenz[*a,j*]anthracene	L	I	+
Benzo[*g,h,i*]perylene	I	I	+
Anthanthrene	L	I	+
Coronene	I	I	+
Dibenzo[*a,e*]fluoranthene	L	No	-
Dibenzo[*a,e*]pyrene	S	I	+
Dibenzo[*a,h*]pyrene	S	I	+
Dibenzo[*a,i*]pyrene	S	I	+
Dibenzo[*a,l*]pyrene	S	No	-

§: Overall evidence of carcinogenicity in experimental animals.

S=*Sufficient evidence*: there is an increased incidence of malignant tumours (a) in multiple species or strains; (b) in multiple experiments; or (c) to an unusual degree with regard to incidence, site, or type of tumour or age at onset.

L=*Limited evidence:* the data suggests a carcinogenic effect but are limited because (a) the studies involve a single species, strain or experiment; (b) the experiments are restricted by inadequate dosage levels, inadequate duration of exposure to the agent, inadequate period of follow up, poor survival, too few animals, or inadequate reporting; or (c) the neoplasms produced often occur spontaneously and, in the past, have been difficult to classify as malignant by histological criteria alone.

I=*Inadequate evidence:* the studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations.

No=*No evidence:* this applies when several adequate studies show, within the limits of the tests used, that the chemical is not carcinogenic.

†: Overall evidence of activity in short term tests.

S=*Sufficient evidence:* there was a total of at least three positive results in at least two of the three test systems measuring DNA damage, mutagenicity or chromosomal anomalies.

L=*Limited evidence:* there was at least two positive results, either from different endpoints or in systems representing two levels of biological complexity.

I=*Inadequate evidence:* there were too few data for an adequate evaluation, or there were contradicting data.

No=*No evidence:* there were many negative results from a variety of short-term tests with different endpoints, and at different levels of biological complexity. If certain biological endpoints are not adequately covered, this is indicated.

¥: Neg=No mutagenic activity was observed in the presence or absence of an exogenous metabolic system.

Pos=The compound was mutagenic in the presence of an exogenous metabolic system.

No=No data available.

Table 1.6. Polycyclic aromatic hydrocarbons oxidised by difference species of bacteria (adapted from Cerniglia, 1992).

Compound	Organism	Reference
Naphthalene	*Acinetobacter calcoaceticus, Alcaligenes denitrificans, Mycobacterium sp., Pseudomonas sp., P. putida, P. fluorescens, Sp.paucimobilis, Brevundimonas vesicularis, Burkholderia cepacia, Comamonas testosteroni, Rhodococcus sp., Corynebacterium renale, Moraxella sp., Streptomyces sp., B. cereus, P. marginalis, P. stutzeri, P. saccharophila*	Ryu *et al.* (1989), Weissenfels *et al.* (1990a, 1991), Kelly *et al.* (1991), Dunn and Gunsalus (1973), Davies and Evans (1964), Foght and Westlake (1988), Jeffrey *et al.* (1975), Mueller *et al.* (1990b), Kuhm *et al.* (1991), Walter *et al.* (1991), Dua and Meera (1981), Tagger *et al.* (1990), Garcia-Valdes *et al.* (1988), Trower *et al.* (1988), Grund *et al.* (1992), Barnsley (1983a), Yang *et al.* (1994), Burd and Ward (1996), Allen *et al.* (1997), Stringfellow and Aitken (1995)
Acenaphthene	*Beijernickia sp., P. putida, P. fluorescens, Bu. cepacia, Pseudomonas sp.*	Chapman (1979), Schocken and Gibson (1984), Ellis *et al.* (1991)
Anthracene	*Beijernickia sp., Mycobacterium sp., P. putida, Sp. paucimobilis, Bu. cepacia, Rhodococcus sp., Flavobacterium sp., Arthrobacter sp., P. marginalis*	Colla *et al.* (1959), Akhtar *et al.* (1975), Jerina *et al.* (1976), Evans *et al.* (1965), Ellis *et al.* (1991), Weissenfels *et al.* (1991), Foght and Westlake (1988), Walter *et al.* (1991), Mueller *et al.* (1990b), Savino and Lollini (1977), Tongpim and Pickard (1996), Burd and Ward (1996)
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*	Kiyohara *et al.* (1976, 1982b, 1990), Weissenfels *et al.* (1990a, 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 (1988, 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 (1983), Yang *et al.* (1994), Kohler *et al.* (1994), Stringfellow and Aitken (1995)
Fluoranthene	*A. denitrificans, Mycobacterium sp., P. putida, Sp. paucimobilis, Bu. cepacia, Rhodococcus sp., Pseudomonas sp.*	Kelly and Cerniglia (1991), Walter *et al.* (1991), Weissenfels *et al.* (1991), Foght and Westlake (1988), Mueller *et al.* (1989b, 1990b), Ye *et al.* (1996), Kelly *et al.* (1993)
Pyrene	*A. denitrificans, Mycobacterium sp., Rhodococcus sp. , Sp. paucimobilis*	Heitkamp *et al.* (1988a), Walter *et al.* (1991), Weissenfels *et al.* (1991), Grosser *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996)
Chrysene	*Rhodococcus sp., P. marginalis, Sp. paucimobilis*	Walter *et al.* (1991), Burd and Ward (1996), Ye *et al.* (1996)
Benz[*a*]anthracene	*A. denitrificans, Beijernickia sp., P. putida, Sp. paucimobilis*	Gibson *et al.* (1975), Mahaffey *et al.* (1988), Weissenfels *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996)
Benzo[*a*]pyrene	*Beijernickia sp., Mycobacterium sp., Sp. paucimobilis*	Gibson *et al.* (1975), Heitkamp and Cerniglia (1988), Grosser *et al.* (1991), Schneider *et al.* (1996), Ye *et al.* (1996)
Dibenz[*a,h*]anthracene	*Sp. paucimobilis*	Ye *et al.* (1996)

Numerous genera of microorganisms have been observed to oxidise PAHs (Table 1.6). While there is a great diversity of organisms capable of degrading the low molecular weight PAH, such as naphthalene, acenaphthene and phenanthrene, relatively few genera have been observed to degrade the high molecular weight PAHs, such as the four- and five-ring compounds. Kastner *et al.* (1994) suggested that nocardioform bacteria (*e.g. Rhodococcus*, *Nocardia*, *Mycobacterium* and *Gordona*) may play a crucial role in the degradation of high molecular weight PAHs in soils. This suggestion was based on results reported by the authors for screening pyrene-degrading bacteria (from soil samples) and on the observation that there are very few reports of bacteria outside the nocardioform actinomycetes group capable of growing on high molecular weight PAHs. However, some *Pseudomonas* species have been observed to degrade some four- and five-ring PAHs (Table 1.6).

Fungi, in particular the white rot fungi, play an important environmental role in recycling wood and related materials, which is due primarily to the relatively non-specific processes used to initiate the degradation of the lignin fraction (Fiechter, 1993). Lignin degradation is carried out by mechanisms related to the production of highly reactive intermediates by enzymes such as lignin peroxidase and manganese dependent peroxidase (Barbosa *et al.*, 1996). The reactivity of these non-specific enzymes has led to the application of these organisms for degradation of a range of organic compounds. Fungal metabolism of low molecular weight PAHs has been studied extensively by a number of researchers (Table 1.7). Numerous genera of fungi with the ability to oxidise naphthalene have been identified. Most degradative mechanisms reported are cometabolic, where an alternate carbon source is utilised for energy and growth while the PAH is transformed as a consequence of this growth. However, the white rot fungus, *Phanerochaete chrysosporium*, has been reported to mineralise phenanthrene, fluorene, fluoranthene, anthracene and pyrene in nutrient-limited cultures (Bumpus, 1989). Degradation of benzo[*a*]pyrene to carbon dioxide and water has also been reported (Sanglard *et al.*, 1986). Fungi metabolise PAHs in a manner similar to mammalian enzyme systems. Enzymes from both fungal and mammalian systems oxidise PAHs to arene oxides by the cytochrome P450 enzyme system. The oxides can isomerise to yield phenols or undergo enzymatic hydration to yield *trans*-dihydrodiols (Gibson and Subramanian, 1984).

Algae and cyanobacteria have also been shown to oxidise PAHs (Table 1.8). While the oxidation of naphthalene by a number of algae and cyanobacteria has been reported, relatively few studies have demonstrated the degradation of high molecular weight

Table 1.7. Polycyclic aromatic hydrocarbons oxidised by difference species of fungi (adapted from Cerniglia, 1992).

Compound	Organism	Reference
Naphthalene	*Absida glauca, Aspergillus niger, Basidiobolus ranarum, Candida utilis, Choanephora campincta, Circinella sp., Claviceps paspali, Cokeromyces poitrassi, Conidiobolus gonimodes, C. bainieri, C. elegans, C. japonica, Emericellopsis sp., Epicoccum nigrum, Gilbertella persicaria, Gliocladium sp., Helicostylum piriforme, Hyphochytrium catenoides, Linderina pennispora, Mucor hiemalis, Neurospora crassa, Panaeolus cambodginensis, Panaeolus subbalteatus, Penicillium chrysogenum, Pestalotia sp., Phlyctochytrium reinboldtae, Phycomyes blakesleeanus, Phytophthora cinnamomi, Psilocybe cubensis, Psilocybe strictipes, Psilocybe stuntzii, Psilocybe subaeruginascens, Rhizophlyctis harderi, Rhizophlyctis rosea, Rhizopus oryzae, Rhizopus stolonifer, S. cervisiae, Saprolegnia parasitica, Smittium culicis, Smittium culisetae, Smittium simulii, Sordaria fimicola, Syncephalastrum racemosum, Thamnidium anomalum, Zygorhynchus moelleri*	Cerniglia and Gibson (1977), Cerniglia *et al.* (1978, 1982b), Smith and Rosazza (1974), Cerniglia and Crow (1981), Ferris *et al.* (1973)
Acenaphthene	*C. elegans*	Pothuluri *et al.* (1992b)
Anthracene	*Bjerkandera sp., C. elegans, P. chrysosporium, Ramaria sp., R. solani, Trametes versicolor*	Cerniglia (1982), Cerniglia and Yang (1984), Hammel *et al.* (1991), Sutherland *et al.* (1992), Field *et al.* (1992), Collins *et al.* (1996)
Phenanthrene	*C. elegans, P. chrysosporium, Pleurotus ostreatus, T. versicolor*	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)
Fluoranthene	*C. elegans*	Pothuluri *et al.* (1990, 1992a)
Pyrene	*C. elegans, P. chrysosporium, Penicillium sp., P. janthinellum, P. ostreatus, Syncephalastrum racemosum*	Cerniglia *et al.* (1986), Hammel *et al.* (1986), Launen *et al.* (1995), Bezelel *et al.* (1996)
Benz[*a*]anthracene	*C. elegans,*	Cerniglia *et al.* (1980d)
Benzo[*a*]pyrene	*A. ochraceus, B. adusta, Bjerkandera sp., C. maltosa, C. tropicalis, Chrysosporium pannorum, C. elegans, Mortierella verrucosa, N. crassa, Penicillium sp., P. janthinellum, P. chrysosporium, P. ostreatus, Ramaria sp., S. cerevisiae, S. racemosum, T. versicolor, Trichoderma viride*	Cerniglia and Gibson (1979, 1980), Ghosh *et al.* (1983), Cerniglia and Crow (1981), Lin and Kapoor (1979), Bumpus *et al.* (1985), Wiseman and Woods (1979) Field *et al.* (1992), Cerniglia *et al.* (1980a), Sanglard *et al.* (1985), Haemmerli *et al.* (1986), Launen *et al.* (1995), Bezelel *et al.* (1996)

Table 1.8. Polycyclic aromatic hydrocarbons oxidised by different species of cyanobacteria and algae (adapted from Cerniglia, 1992).

Compound	Organism	Reference
Naphthalene	*Oscillatoria sp., Microcoleus chthonoplastes, Nostoc sp., Anabaena sp., Agmenellum quadruplicatum, Coccochloris elabens, Aphanocapsa sp., Chlorella sorokiniana, Chlorella autotrophica, Dunaliella tertiolecta, Chlamydomonas angulosa, Ulva fasciata, Cylindrotheca sp., Amphora sp., Nitzschia sp., Navicula sp., Porphyridium cruentum*	Cerniglia *et al.* (1979, 1980b, 1980c, 1982), Narro *et al.* (1992a)
Phenanthrene	*Oscillatoria sp., Agmenellum quadruplicatum*	Narro *et al.* (1992b)
Benzo[*a*]pyrene	*Selenastrum capricornutum*	Warshawsky *et al.* (1988, 1990), Lindquist and Warshawsky (1985)

compounds by these organisms. Warshawsky *et al.* (1988) demonstrated the oxidation of benzo[*a*]pyrene by the green algae *Selanastum capricornutum*. These results indicated that *S. capricornutum* produced *cis* vicinal dihydrodiols via a dioxygenase enzyme pathway. The dioxygenase enzymes are characteristic of bacterial metabolic pathways and are unlike those of eukaryotic organisms which involve monooxygenase enzymes (Warshawsky *et al.*, 1988) .

1.4 ADAPTATION OF MICROORGANISMS TO PAH DEGRADATION

Prolonged exposure to chemical toxicants can cause adaptations in microbial populations which result in greater resistance to toxicity or enhanced ability to utilise toxicants as substrates for metabolism or cometabolism (Cerniglia and Heitkamp, 1989; Thomas *et al.*,1989; Bauer and Capone, 1985, 1988; Heitkamp and Cerniglia, 1987; Heitkamp *et al.*, 1987). Prior exposure of a microbial community to PAHs is important in determining how rapidly hydrocarbons can be degraded. This phenomenon, which results from increases in the PAH-oxidising potential of the community, is known as adaptation (Spain *et al.*, 1980).

An adaptation lag period can be described as the length of time during the initial exposure of microorganisms to a chemical in which the transformation rates of the chemical are too slow to be measurable or are significantly less than subsequent rates (Lewis *et al.*, 1986). The adaptation/acclimation period results in changes in the microbial community that bring about an increase in the rate of transformation of the subject compounds. Previous work has shown that adaptation of microorganisms can play a major role in determining biodegradation rates of environmental pollutants (Spain and Van Veld, 1983; Lewis *et al.*, 1986; Cerniglia and Heitkamp, 1989; Wilson and Jones, 1993).

1.4.1 Prior Exposure to PAHs

The major mechanism which accounts for the adaptation period is the need for populations to become sufficiently large to bring about a detectable loss of the compound (Spain *et al.*, 1980; Ventullo and Larson, 1986) or for the growth of a specific sub-population of a microbial community (van deer Meer *et al.*, 1992). Exposure to PAHs may result in the selection of populations which contain microorganisms that possess constitutively-synthesised PAH-degrading enzymes or, indeed, it may induce PAH-degrading enzymes in some indigenous microorganisms. A number of other mechanisms may account for the acclimation period. These include:

(i) the initial concentration of the compound (Spain and Van Veld, 1983);
(ii) time needed for microorganisms to adapt to toxins, or for toxins to be inactivated or to disappear (Wiggins *et al.*, 1987);
(iii) insufficient supply of inorganic nutrients (Lewis *et al.*, 1986; Vashon *et al.*, 1982);
(iv) preferential utilisation of other organic compounds before the chemical of interest is degraded (Lewis *et al.*, 1986); and
(v) time needed for enzymes to be induced (Torstensson *et al.*, 1975; Spain and Van Veld, 1983) or for mutation or genetic exchange to occur (Schmidt *et al.*, 1983; Spain *et al.*, 1980).

The concentration of the PAH may be a significant factor affecting its susceptibility to microbial attack. Some compounds may persist in the environment as a result of their low concentrations or low solubility in water. A phenomenon may exist where there is a concentration threshold for the compounds below which adaptation will not occur (Boethling and Alexander, 1979a; Spain *et al.*, 1980). Spain and Van Veld (1983) tested the adaptation period of microorganisms in eco-cores on the Escambia River near Pensacola, Florida, USA, to *p*-nitrophenol. Results obtained with different concentrations of *p*-nitrophenol indicated that there was a threshold concentration of *p*-nitrophenol (10 ng/ml) below which there was no detectable adaptation of the community. Communities adapted to *p*-nitrophenol degradation were obtained by pre-exposing the microbial community to concentrations of *p*-nitrophenol above the threshold concentration. Rates of *p*-nitrophenol degradation were not proportional to the pre-exposure concentration above the threshold concentration. Only a slight increase in biodegradation rates was seen in communities pre-exposed to higher concentrations. Degradation rates were influenced more by the test concentration than by the pre-exposure concentrations.

In naturally occurring environments, the lag period may be influenced by the presence and concentrations of vitamins, cofactors, nitrogen, phosphorous and additional carbon sources. The pH of the environment and the presence of inhibitory compounds may govern the metabolic activities of the microorganisms present. Toxins may contribute to the acclimation period. These compounds may inhibit the growth of the degrading species until the microorganisms adapt to the inhibitory substrates, or the toxins are inactivated or disappear (Wiggins *et al.*, 1987). Long lag times may indicate that the compound is toxic at that particular chemical-to-biomass ratio (Larson, 1979), or that the initial population capable of metabolising the chemical is small. It has been

suggested that part of the acclimation period for the biodegradation of oil is the time needed for the loss by volatilisation of the toxic, low molecular weight constituents of the oil (Atlas and Bartha, 1972).

The availability of essential elemental nutrients, such as nitrogen and phosphorus, can affect the microbial adaptation period to environmental pollutants. Lewis *et al.* (1986) illustrated this with microorganisms capable of degrading *p*-cresol. They found that microbial samples collected from sites that contained low concentrations of dissolved nitrogen and phosphorus exhibited longer adaptation periods to *p*-cresol than other samples which had higher concentrations of nitrogen and phosphorus. The effect of limiting phosphorus on the lag period was shown by adding various concentrations of phosphorus to batch cultures. Below a phosphorus concentration of 0.01 mg/l, the lag times were greatly lengthened by even small decreases in the initial phosphorus concentration. Above a phosphorus concentration of approximately 0.15 mg/l, the lag times were unaffected by increases in the initial phosphorus concentration (Lewis *et al.*, 1986). Although Lewis *et al.* (1986) illustrated that lag periods may be related to limiting nutrients, they could not determine the specific mechanism responsible for the lag period. Limiting nutrients may:

(i) limit the production of enzymes capable of transforming the particular compound;
(ii) limit the mRNA synthesis which is required to induce the enzymes capable of transforming the particular compound(s);
(iii) limit the production of enzymes that are capable of removing the compounds that repress the synthesis of enzymes capable of transforming the particular compound;
(iv) limit the synthesis of plasmids that are involved in transforming the particular compound; or
(v) cause any combination of the above (Lewis *et al.*, 1986).

Little is known about the molecular events that lead to the adaptation of microorganisms to PAHs. Increased knowledge about these events may provide a better insight into the metabolic capabilities of microorganisms to utilise these compounds as growth substrates (van deer Meer *et al.*, 1992). A number of mechanisms may be responsible for genetic adaptation of microorganisms to environmental pollutants. These mechanisms include:

Table 1.9. Catabolic plasmids from environmental microorganisms (Sayler, 1991).

Reference Plasmid	Compounds Degraded[a]	Original Bacterial Host	Environmental Source
pSS50	4-CB	*Alcaligenes, Acinetobacter*	sediment
pJP4	2,4-D, 3-CBA, MCPA	*Alcaligenes*	soil and water
OCT	Octane, Hexane, Decane	*P. putida*	soil
CAM	Camphor	*P. putida*	soil
pJP2	2,4-D, MCPA	*Alcaligenes*	soil and water
Naph	Naphthalene	*Pseudomonas*	soil
pWW0	Toluene, *p*- and *m*-Xylene	*P. putida*	soil
NAH7	Naphthalene	*P. putida*	soil
Creosol	Creosol	*P. putida*	soil

[a]Abbreviations: 4-CD, 4-chlorobiphenyl; 2,4-D, 2,4-dichlorophenoxyacetic acid; 3-CBA, 3-chlorobenzoic acid; MCPA, 4-chloro-2-methylphenoxyacetic acid.

(i) gene transfer;
(ii) mutational drift; and
(iii) genetic recombination and transposition.

Some of these mechanisms are difficult to prove experimentally, since the final results can only be observed (van deer Meer *et al.*, 1992).

1.4.2 Gene Transfer

Gene transfer among indigenous microorganisms in microbial communities may be facilitated by the mechanisms of transformation, transduction and conjugation via information residing on plasmids. Table 1.9 outlines a number of catabolic plasmids associated with environmental microorganisms. Two plasmids that carry genes for the degradation of aromatic compounds are the TOL and NAH plasmids. At present the best understood catabolic plasmid is the TOL plasmid, which encodes enzymes that degrade toluene. The catabolic genes of the TOL plasmid, pWW0, are organised into two operons: the lower and upper (or *meta*) pathways. The upper pathway, *xylCAB*, encodes the degradation of toluene and xylene to benzoates and toluates (Harayama *et al.*, 1987), while the lower pathway, *xylDLEGFJKIH*, encodes the degradation of benzoates and toluates to acetaldehyde and pyruvate (Harayama *et al.*, 1984). In the plasmid NAH7, the naphthalene catabolic genes are organised on two operons, *nah* and *sal*. Both operons are controlled by *nah*R, a positive regulator gene, that is located upstream from the *nah*G gene. Induction of the catabolic operons is controlled by salicylate, a metabolite of naphthalene degradation. The upper pathway for naphthalene degradation (*nah*) mediates the oxidation of naphthalene to salicylate, while the lower pathway (*sal*) mediates salicylate oxidation. The *sal* operon contains similar *meta* cleavage pathway genes to those present on the pWW0 plasmid (van deer Meer *et al.*, 1992). NAH7 and NAH7-like plasmids have been implicated in the oxidation of higher molecular weight PAHs. Menn *et al.* (1993) demonstrated NAH-plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acids by *Pseudomonas fluorescens* strain 5R and a mutant, *P. fluorescens* 5RL.

The ramification of gene transfer includes conferring the ability to degrade compounds previously not able to be degraded, the expansion of degradative pathways by replacing narrow-specificity enzymes by broader-specificity ones (horizontal expansion) or by providing peripheral enzymes which can direct substrates into existing degradative pathways (vertical expansion) (Ramos and Timmis, 1987). Jeense *et al.* (1982) illustrated the ability to expand the degradative capabilities of *Pseudomonas* strain B13

horizontally by the transfer of the TOL plasmid pWW0 from *P. putida* strain mt-2. Previously, strain B13 was able to degrade 3-chlorobenzoate, but with the transfer of the TOL plasmid, its degradative ability was expanded to include 4-chlorobenzoate and 3,5-dichlorobenzoate. This transfer provided strain B13 with the toluate dioxygenase encoded by *xylXYZ* of the TOL plasmid, an enzyme with a broader substrate range than chlorobenzoate dioxygenase of strain B13 (van deer Meer *et al.*, 1992).

Yang *et al.* (1994) expressed PAH transformation activity in *E. coli* using a large DNA insertion (20-30 kb) from *P. putida* strain NCIB 9,816. Clones containing 20 kb, 28 kb and 30 kb DNA insertions, with each of the two possible orientations, were produced in strain NCIB 9,816. Additional subclones were constructed containing 16 kb, 12 kb and 8.5 kb DNA fragments after digestion of DNA with *EcoRI*, *ScaI* and *XhoI*. The identification of degradation products from the NCIB 9,816 clones following incubation with naphthalene, fluorene and phenanthrene demonstrated that multiple PAH degradations can be encoded by a single gene cluster. All clones produced the same metabolites for the three PAH substrates, with the exception of the clone containing the 16 kb DNA fragment inserted in the reverse orientation without an additional promoter. Salicylic acid was identified as a metabolite of naphthalene while 1-hydroxy-2-naphthoic acid was identified from phenanthrene degradation. Fluorene was transformed to 9-hydroxyfluorene, 9-fluorenone and two unidentified metabolites.

The genes encoding naphthalene dioxygenase of *P. putida* strain NCIB 9,816 were cloned into *E. coli* strain HB101 by Kurkela *et al.* (1988). Naphthalene dioxygenase activity was recognised, based on the ability of the organism to oxidise indole to indigo. DNA fragments (10-20 kb) were prepared from total *P. putida* DNA after partial digestion with *Sau3A*. The fragments were ligated into *BamHI*-digested pBR322 and the library was transformed into strain HB101. Transformed colonies were selected and screened for enzyme activity towards indole. The genes responsible for naphthalene dioxygenase activity from *P. putida* strain NCIB 9,816 were successfully cloned into *E. coli*. Plating transformed cells into media containing indole resulted in the production of blue colonies as a consequence of indigo production. However, naphthalene dioxygenase expression levels were 10- to 20-fold lower than in *P. putida* (Ensley *et al.*, 1990).

1.4.3 Mutational Drift

A number of researchers have demonstrated that single-site mutations can alter substrate specificity of enzymes or effector specificities. Ramos *et al.* (1987) extended the

substrate range of catechol 2,3-dioxygenase (encoded by TOL plasmid pWW0) by single substitutions of amino acids. The specificity of the *xylS* regulatory protein was modified by mutagenesis, resulting in other compounds, such as 4-ethyl-benzoate, salicylate and 3,5-, 2,5- and 2,6-dichlorobenzoate as effector molecules (Ramos *et al.*, 1986). Single site mutations are believed to arise continuously and randomly as a result of errors in DNA replication or repair (van deer Meer *et al.*, 1992). However, other mechanisms, such as selective pressure, have been proposed for point mutations. Stress factors, including chemical pollutants, may stimulate error prone DNA replication and hence accelerate DNA evolution (Blom *et al.*, 1992).

1.4.4 Genetic Recombination and Transposition

DNA rearrangements may be responsible for genetic adaptation of microorganisms to environmental pollutants. For example, the gene order of part of the *meta* cleavage operons of the TOL- and NAH-encoded catabolic genes (*xylTEGFJ* and *nahTHJNL*) are identical (Harayama *et al.*, 1987). There are, however, differences in downstream and upstream genes. As yet there are no clear indications of what mechanisms may direct these rearrangements.

Gene duplications have been considered to be an important mechanism for the evolution of microorganisms (Beacham, 1987). Once the genes have been duplicated, the extra gene copy is virtually free of selective constraints. This may lead to faster changes in the gene by the accumulation of mutations. These mutations could eventually lead to full inactivation, rendering this gene copy silent. Reactivation of the silent gene copy could then occur through the action of insertion elements (van deer Meer, 1992). Researchers have shown that the upper and lower pathway operons, as well as some regulatory genes of the TOL-type plasmid, have sometimes switched position, inverted or increased their copy number (Chatfield and Williams, 1986; Osborne *et al.*, 1988; Williams *et al.*, 1990).

Insertion elements have been shown to play an important role in rearrangement of DNA fragments, in gene transfer and in activation or inactivation of silent genes (van deer Meer *et al.*, 1992). A number of insertion elements are known for catabolic pathways. The adaptability and catabolic potential of *P. cepacia* strain 249 is thought to be due to insertion elements. Strain 249 was shown to carry at least nine different insertion elements, which were present in one to thirteen copies in its genome (Lessie *et al.*, 1990). Insertion elements may also activate or inactivate genes (Parker *et al.*, 1988; Aronson *et al.*, 1989). Insertion elements often contain promoter-like sequences which

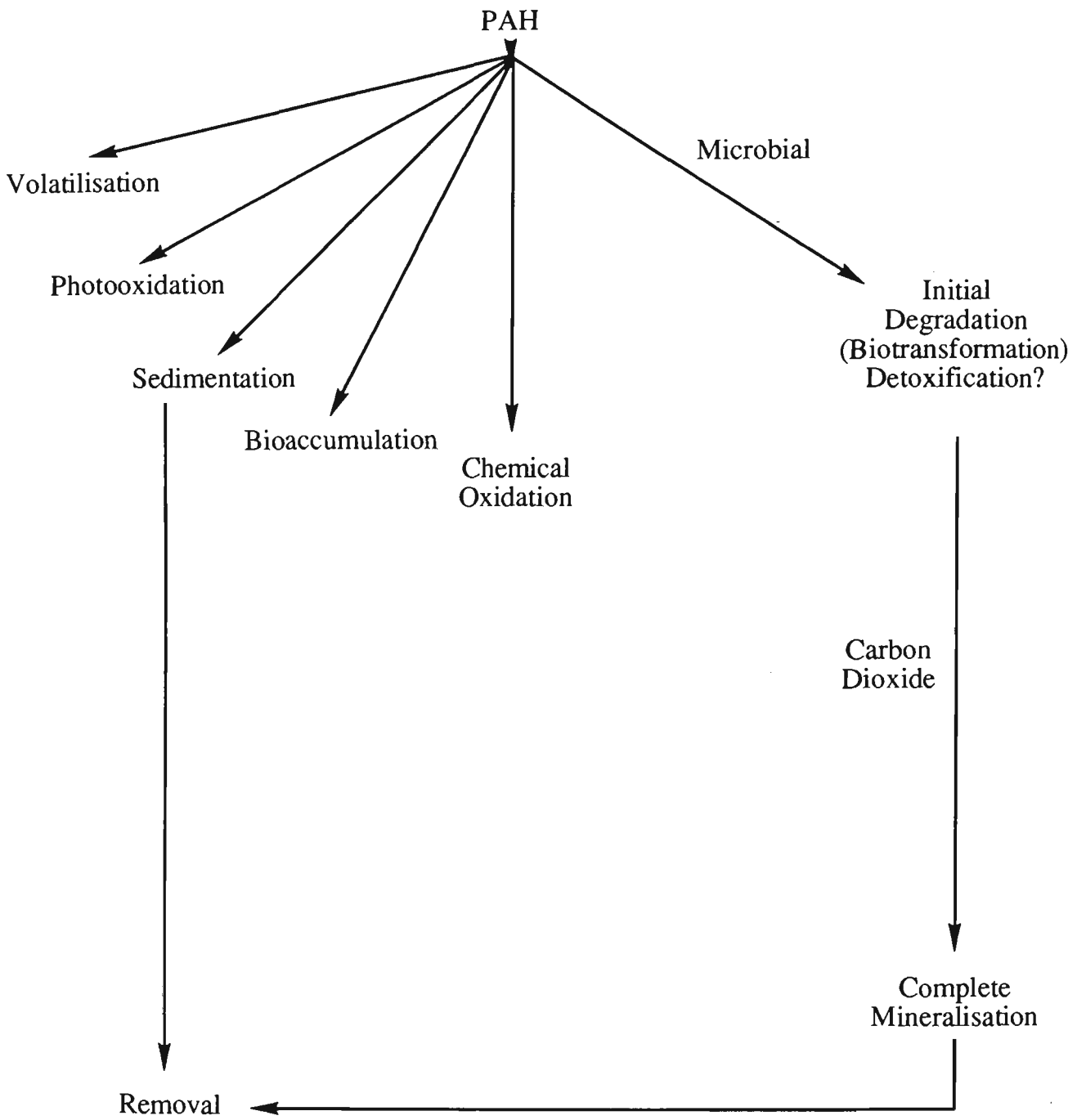

Figure 1.2. Schematic representation of the environmenal fate of polycyclic aromatic hydrocarbons (Cerniglia, 1992).

may activate the expression of a gene. Wood *et al.* (1990) and Scordilis *et al.* (1987) found that the *lacZ* gene in *P. cepacia* could be activated by the insertion of two elements, *IS406* and *IS407*. Adjacent genes could also be activated be the insertion elements *IS931* and *IS932*.

Adaptation of microbial communities to PAHs (*i.e.*, increased rates of transformation of PAHs associated with contaminated environments) have been reported in several studies (Cerniglia, 1984a; Atlas, 1981). Reports of turnover times of 10- to 400-times longer in sediments of pristine streams compared to contaminated sediment are not uncommon. Bauer and Capone (1988) provided evidence for acclimation of sediment microbial communities to PAHs. Prior exposure to a PAHs effected an increase in metabolism rates of compounds with similar structure. The occurrence of this phenomenon was attributed to the broad specificity of the selected microbial populations for PAHs and/or the existence of common pathways for PAH catabolism (Bauer and Capone, 1988).

1.5 MICROBIAL METABOLISM OF PAHs

1.5.1 General Aspects of PAH Degradation

The persistence of PAHs in the environment depends on the physical and chemical characteristics of the PAH. PAHs are degraded by photo-oxidation and chemical oxidation (Shiaris, 1989a), but biological transformation is probably the prevailing route of PAH loss (Mueller *et al.*, 1990a) (Figure 1.2). The recalcitrance of PAHs to microbial degradation increases directly with the molecular weight and the octanol:water partition coefficient (log K_{OW}) (Cerniglia, 1992) (Figure 1.3). The microbial metabolism of PAHs containing up to three rings (naphthalene, phenanthrene, anthracene, fluorene) has been studied extensively. The rates, metabolic pathways, enzymatic regulation and genetic regulation have been well documented (Evans *et al.*, 1965; Kiyohara and Nagao, 1978; Kiyohara *et al.*, 1976, 1982b, 1990; Barnsley, 1975a, 1983a, 1983b; Ghosh and Mishra, 1983; Guerin and Jones, 1988a, 1988b; Sutherland *et al.*, 1990; Keuth and Rehm, 1991; Narro *et al.*, 1992b; Foght and Westlake, 1988; Mueller *et al.*, 1990b; Weissenfels *et al.*, 1990a, 1991, Jerina *et al.*, 1976; Heitkamp and Cerniglia, 1988; Kelly *et al.*, 1990; DiGrazia *et al.*, 1991; Aranha and Brown, 1981; Cox and Williams, 1980; Heitkamp *et al.*, 1987; Guerin and Boyd, 1992; Wodzinski and Bertolini, 1972; Trenz *et al.*, 1994; Mihelcic and Luthy, 1988; Monna *et al.*, 1993; Grifoll *et al.*, 1992, 1994, 1995). However, until recently there has been a paucity of information on the bacterial degradation of the larger, more recalcitrant, high molecular weight PAHs containing four or more fused benzene rings.

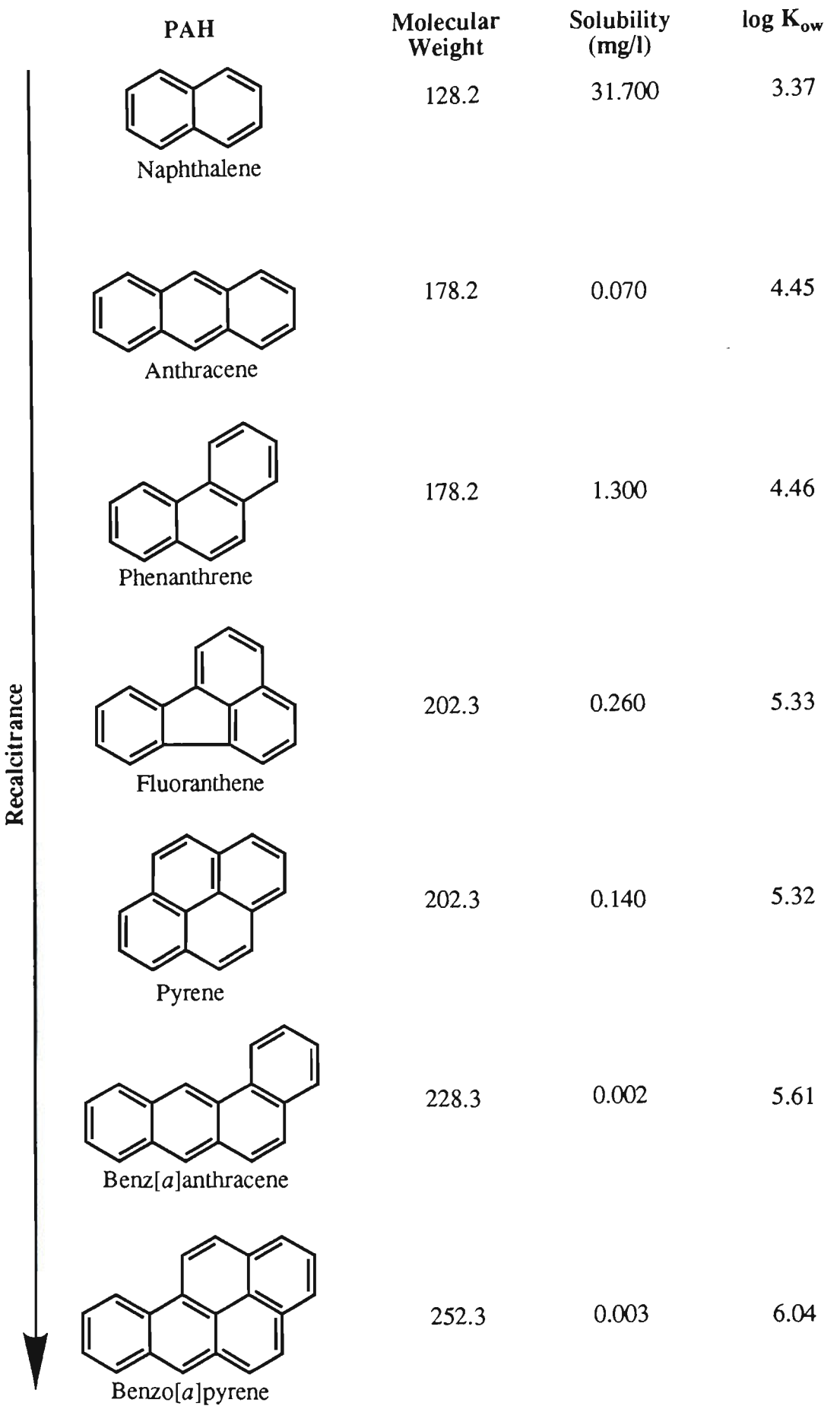

Figure 1.3. Resistance of PAHs to microbial degradation (adapted from Cerniglia, 1992).

Bacteria initially oxidise aromatic hydrocarbons to *cis*-dihydrodiols (Cerniglia, 1984a; Heitkamp *et al.*, 1988b; Kelly *et al.*, 1991; Koreeda, 1978; Jerina *et al.*, 1976; Evans *et al.*, 1965). The oxidation of these compounds involves the enzymatic incorporation of atmospheric oxygen into the substrate. Characteristically, bacteria produce dioxygenases, which incorporate two oxygen atoms into the aromatic nucleus (Albaiges *et al.*, 1983). The dioxygenase that catalyses these initial reactions is a multicomponent enzyme system. The initial ring oxidation is usually the rate-limiting step in the biodegradation reaction of PAHs (Cerniglia, 1992). *cis*-Dihydrodiols are re-aromatised through a *cis*-dihydrodiol dehydrogenase to yield a dihydroxylated derivative (Cerniglia, 1984a). Further oxidation of the *cis*-dihydrodiols leads to the formation of catechols (Atlas, 1981; Gibson and Subramanian, 1984) that are substrates for other dioxygenases that bring about enzymatic cleavage of the aromatic ring. Catechol can be oxidised via two pathways. The *ortho* pathway involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield *cis*,*cis*-muconic acid. On the other hand, the *meta* pathway involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group. Ring cleavage results in the production of succinic, fumaric, pyruvic and acetic acids and aldehydes, all of which are utilised by the microorganism for the synthesis of cellular constituents and energy (Wilson and Jones, 1993). A by-product of these reactions is the production of carbon dioxide and water. Figure 1.4 illustrates the *ortho* and *meta* cleavage pathways of catechol.

Once the initial hydroxylated aromatic ring of the PAH is degraded (to pyruvic acid and carbon dioxide), the second ring is then attacked in the same manner (Atlas and Bartha, 1981). However, many high molecular weight PAHs, such as benzo[*a*]pyrene are only degraded with difficulty or not at all, due to their low water solubilities, high resonance energies and toxicities (Cerniglia, 1992; Wilson and Jones, 1993).

1.5.2 Cooxidation and Cometabolism of PAHs

Despite the widespread occurrence of many PAHs in the environment, few micro-organisms are capable of utilising the high molecular weight compounds as a sole source of carbon. Detailed experiments have established this (Mahaffey *et al.*, 1988; Gibson *et al.*, 1975; Schneider *et al.*, 1996; Wu and Wong, 1981; Barnsley, 1975b; Shiaris, 1989b; Herbes and Schwall 1978), however, the results suggested that PAHs might be biodegraded via a cooxidation or cometabolism mechanism through less recalcitrant compounds (Beam and Perry, 1973).

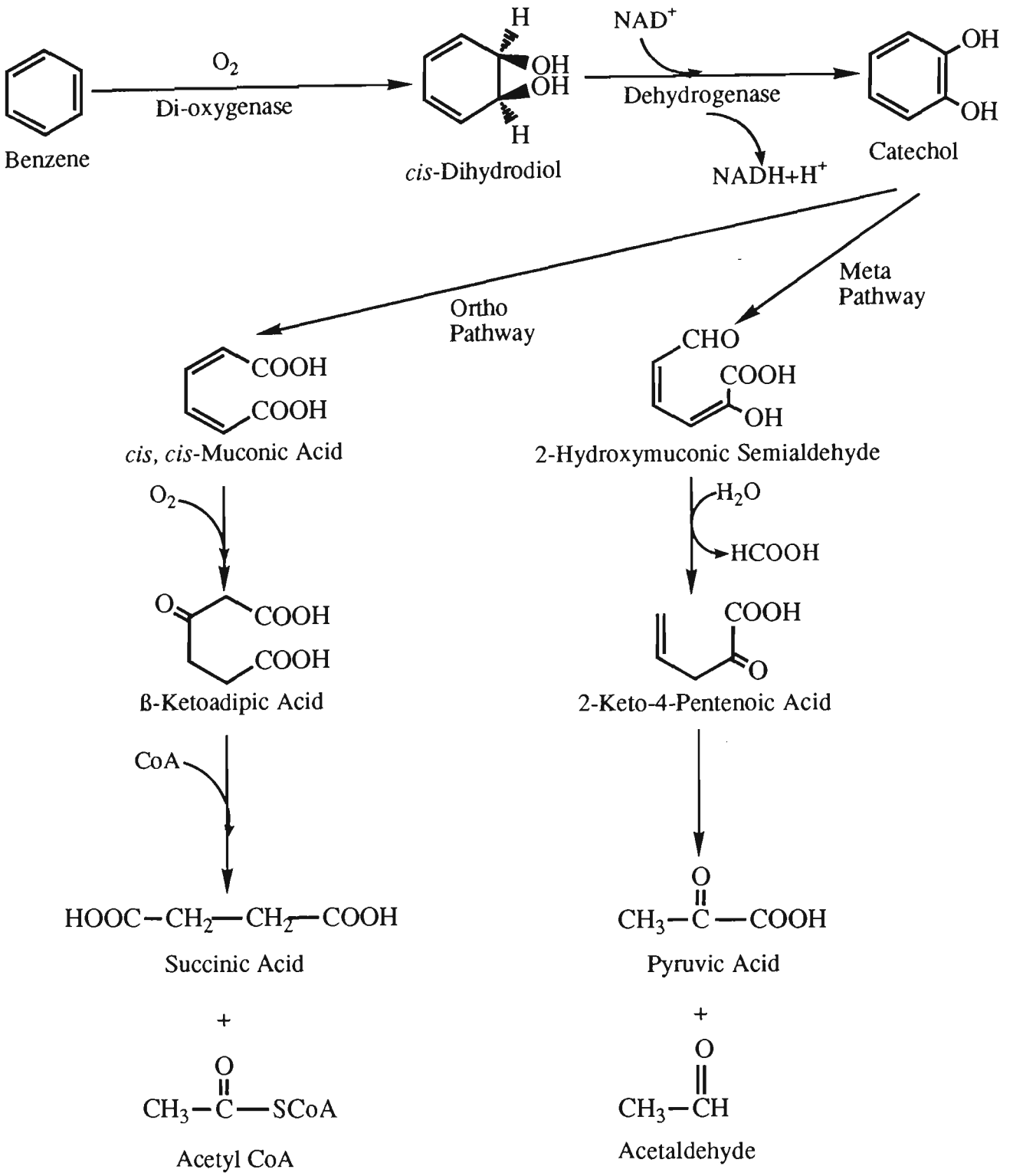

Figure 1.4. Microbial metabolism of the aromatic ring by ortho or meta cleavage (adapted from Cerniglia, 1984a and Rochkind-Dubinsky *et al.*, 1987).

Foster (1962) defined cooxidation as the condition where "non-growth hydrocarbons are oxidised when present as cosubstrates in a medium in which one or more hydrocarbons are furnished for growth". This definition was based on the observation that *P. methanica* could cooxidise various gases when growing on methane (Leadbetter and Foster, 1959). Cometabolism is defined as the simultaneous oxidation of an organic "cosubstrate" which is neither essential nor sufficient to support the replication of a microorganism (Hulbert and Krawiec, 1977). Some characteristics of cometabolism include:

(i) the cosubstrate does not support the growth of chemoheterotrophs;
(ii) the production of waste products is stoichiometrically related to the disappearance of the cosubstrate;
(iii) utilisation of the cosubstrate is associated with increased oxygen consumption; and
(iv) cosubstrate transformation involves adventitious utilisation of existing enzyme systems (Hulbert and Krawiec, 1977).

Horvath (1972) differentiated cooxidation from cometabolism by using cometabolism when referring to the oxidation of any non-growth substrate, regardless of whether a growth substrate was present.

The initial attack via cooxidation of a recalcitrant molecule in an environmental niche is a coincidental attack on the compound that is probably of little significance to the microorganism involved in the oxidative reaction (Perry, 1979). Neither energy nor carbon for biosynthesis results from this oxidation. The inability of an organism to grow at the expense of a hydrocarbon does not preclude its ability to oxidise the compound. The lack of growth on the hydrocarbon may be due to its inability to assimilate the oxidation products (Foster, 1962).

The incorporation of oxygen into diverse substrates is often a prerequisite to the mineralisation of those molecules. Oxygenases play an important role in the environment (Colby *et al.*, 1977) serving as an indispensable indicator of biodegradative attack (Perry, 1979). These enzymes are inducible in microorganisms by a wide variety of inducers and their activities can yield partially oxidised compounds that might be subjected to mineralisation by bacteria in the community or the environment. The induction of enzymes in microorganisms for the degradation of

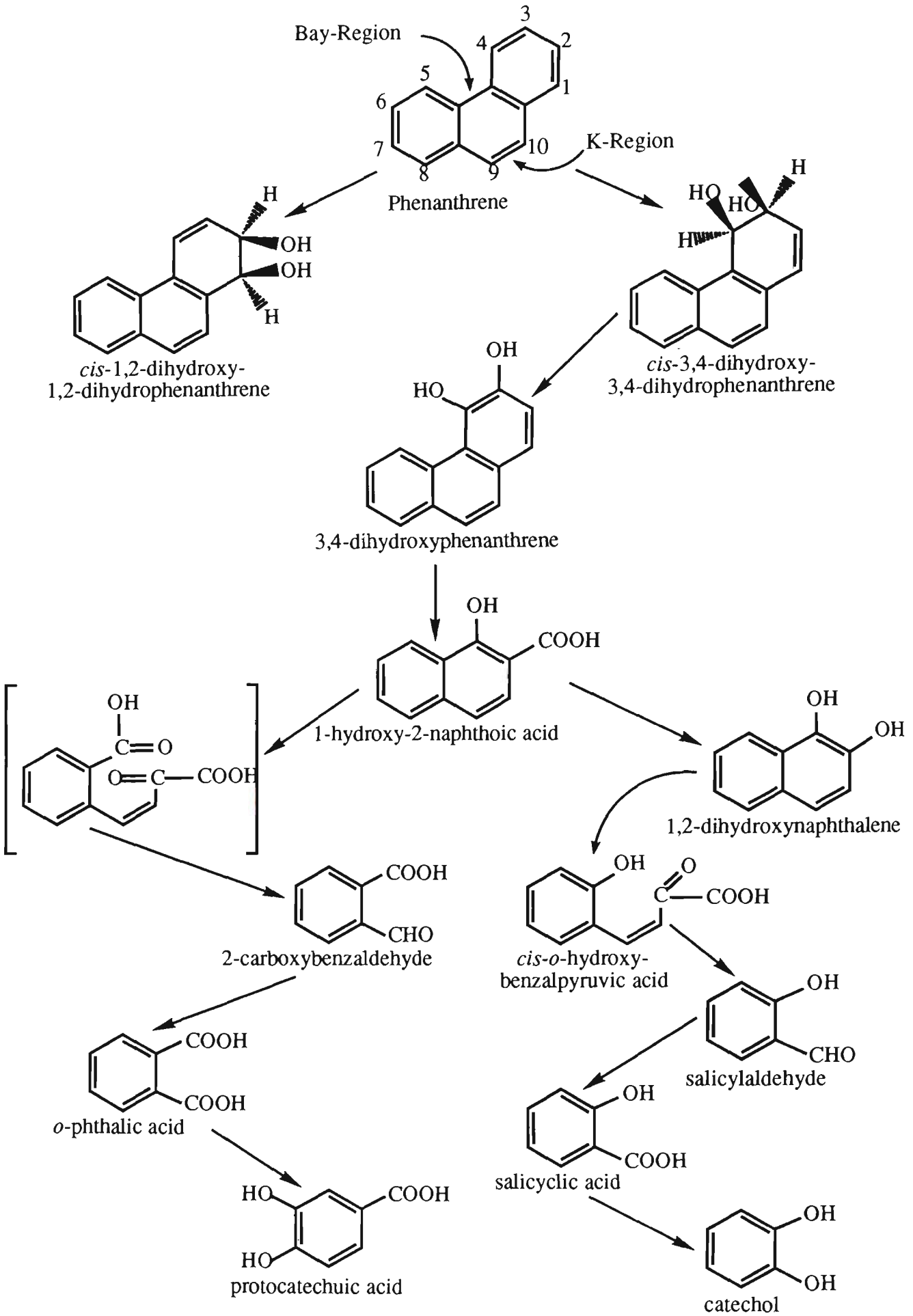

Figure 1.5. The different pathways for the bacterial oxidation of phenanthrene (Cerniglia, 1984b).

particular compounds may account for the long lag phases often observed before the degradation of recalcitrant compounds.

1.5.3 Phenanthrene Metabolism

A considerable amount of information exists on the microbial metabolism of phenanthrene. Phenanthrene, a tricyclic aromatic hydrocarbon, is widely distributed throughout the environment as a result of pyrolytic processes and occurs as minor contaminants in waste water effluents from coal gasification and liquefication processes (Blumer, 1976). Although phenanthrene has not been found to be mutagenic or carcinogenic, it has been used as a model substrate for studies on the environmental degradation of PAHs. Phenanthrene is the simplest aromatic hydrocarbon that contains a "bay-region" and a "*K*-region" (Figure 1.5) (Cerniglia, 1984a) and its structure is found in the carcinogenic PAHs benzo[*a*]pyrene and benz[*a*]anthracene.

A number of studies have shown the ability of pure and mixed cultures to metabolise phenanthrene as a sole carbon and energy source (Evans *et al.*, 1965; Kiyohara and Nagao, 1978; Kiyohara *et al.*, 1976, 1982a, 1982b, 1990; Barnsley, 1983a, 1983b; Ghosh and Mishra, 1983; Guerin and Jones, 1988a, 1988b; Sutherland *et al.*, 1991; Keuth and Rehm, 1991; Narro *et al.*, 1992a; Foght and Westlake, 1988; Mueller *et al.*, 1990a; Weissenfels *et al.*, 1990a, 1991; Jerina *et al.*, 1976; Heitkamp and Cerniglia, 1988). Bacteria initially oxidise phenanthrene at the 1,2- and 3,4- positions to form (+)-*cis*-1R,2S-dihydroxy-1,2-dihydrophenanthrene and (+)-*cis*-3S,4R-dihydroxy-3,4-dihydrophenanthrene, with the latter being the predominant isomer (Jerina *et al.*, 1976; Koreeda, 1978). Evans *et al.* (1965) showed that phenanthrene was oxidatively metabolised by soil pseudomonads through *trans*-3,4-dihydro-3,4-dihydroxyphenanthrene. The dihydroxylated derivative was further oxidised to produce *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid. The ring cleavage product was then metabolised to 1-hydroxy-2-naphthoic acid. This intermediate product may then be further catabolised via a naphthalene catabolising pathway or through a protocatechuate pathway. Evans *et al.* (1965) proposed that 1-hydroxy-2-naphthoic acid, after oxidative decarboxylation to 1,2-dihydroxynaphthalene was metabolised further through the naphthalene pathway. 1,2-Dihydroxynaphthalene is enzymatically cleaved by a dioxygenase to yield *cis*-2'-hydroxybenzalpyruvate. An aldose catalyses the cleavage of *cis*-2'-hydroxybenzalpyruvate to pyruvate and salicylaldehyde, the latter of which is subsequently oxidised to salicylate by a dehydrogenase (Cerniglia and Heitkamp, 1989). Salicylate may be further oxidised by salicylate hydroxylase to yield catechol (Heitkamp *et al.*, 1987; Kelly *et al.*, 1990) (Figure 1.5), which can then undergo *ortho* or *meta*

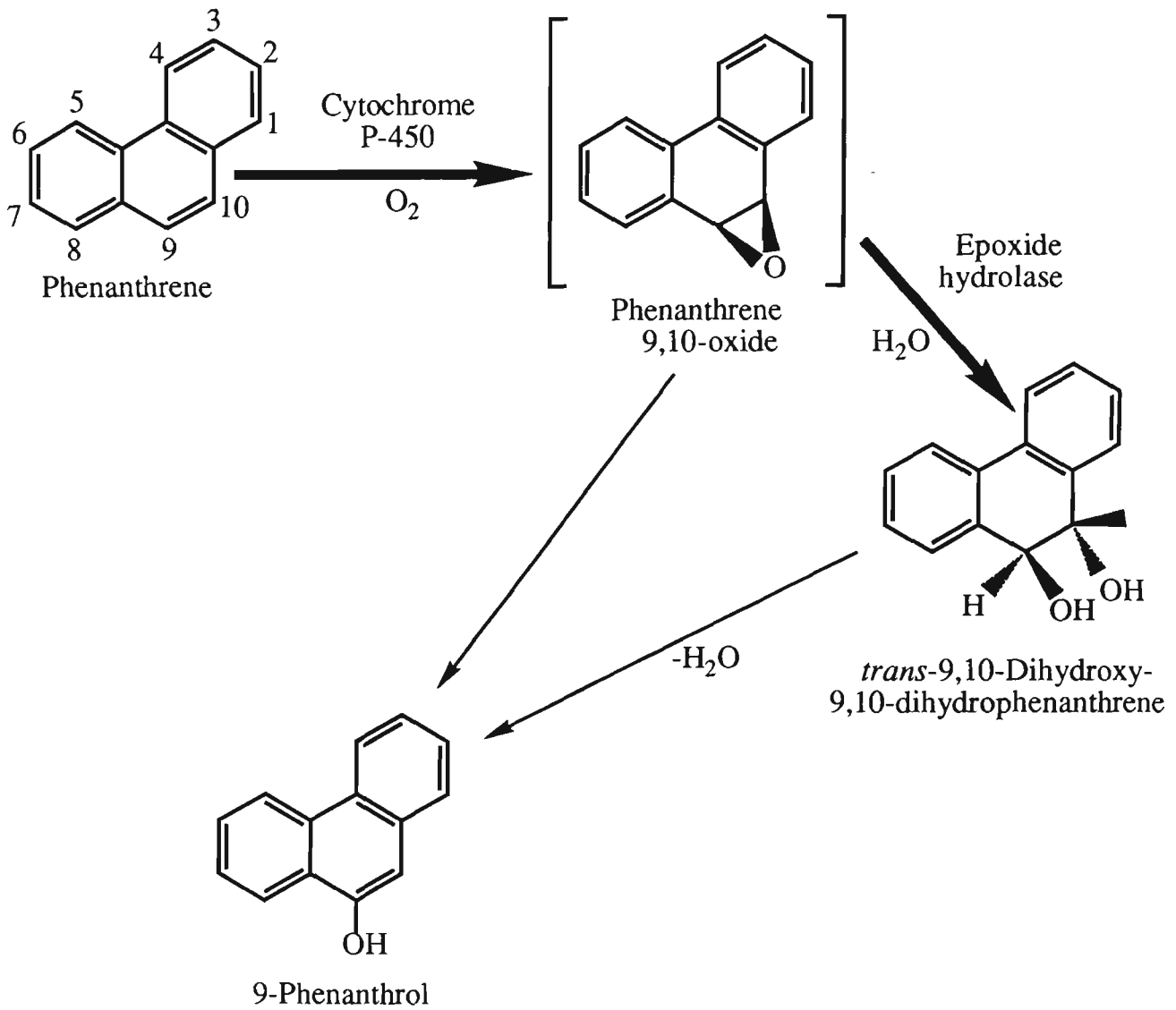

Figure 1.6. Proposed pathway for phenanthrene metabolism by *S. flavovirens*. The bold arrows indicate the predominant pathway (Sutherland *et al.*, 1990).

fission depending on the bacterial species. Kiyohara and Nagao (1978) found that various bacteria, such as *Aeromonas* strain S45P1, fluorescent and non-fluorescent pseudomonads, vibrios as well as some unidentified bacteria, oxidised 1-hydroxy-2-naphthoate, 2-carboxybenzaldehyde, *o*-phthalate and protocatechuate, but showed little oxygen uptake with salicylaldehyde, salicylate and catechol. These results suggested that the strains utilised the protocatechuate pathway for phenanthrene metabolism. Kiyohara and Nagao (1978) proposed that strain S45P1 converted 1-hydroxy-2-naphthoic acid via a intradiol cleavage to form *o*-phthalic acid, which was then hydroxylated and decarboxylated to protocatechuate. Protocatechuate was then further metabolised via *ortho* or *meta* cleavage. Ghosh and Mishra (1983) isolated a strain of *Micrococcus* from petroliferous soil that degraded phenanthrene through the same (protocatechuate) pathway.

Sutherland *et al.* (1990) described the metabolism of phenanthrene by *S. flavovirens*. Unlike the initial oxidation of phenanthrene at the 1,2- or 3,4- positions as decribed by Jerina *et al.* (1976), Koreeda (1978) and Evans *et al.* (1965), the actinomycete oxidised the hydrocarbon at the *K*-region to form *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene. Trace amounts of 9-phenanthrol were also detected (Figure 1.6). A cytochrome P450 oxygenase detected in *S. flavovirens* was responsible for the initial oxidation step, formation of the arene oxide. Degradation experiments in an atmosphere of $^{18}O_2$, followed by gas chromatography-mass spectrometry of metabolites, revealed that only one atom of oxygen was incorporated into each molecule of the phenanthrene t*rans*-9,10-dihydrodiol, while the other atom was obtained from water. 9-Phenanthrol could be produced by either the arrangement of the arene oxide (Bruice *et al.*, 1976; Chaturapit and Holder, 1978) or by the dehydration of the dihydrodiol (Jerina *et al.*, 1976). The 9,10- dihydrodiol produced by *S. flavovirens* was similar to the principal dihydrodiol produced from phenanthrene by mammals (Balani *et al.*, 1986). The results which showed the absolute configuration of the dihydrodiol metabolite suggest that *S. flavovirens* metabolised phenanthrene in a stereospecific manner to produce a compound that is also typical of mammalian phenanthrene metabolism (Sutherland *et al.*, 1990).

Narro *et al.* (1992a) reported the oxidation of phenanthrene by the marine cyanobacterium *A. quadruplicatum* strain PR-6. The initial oxidation of phenanthrene was catalysed by a monooxygenase reaction to yield 9,10- and 1,2- oxides. The 9,10-oxide underwent enzymatic hydration to yield *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene whereas the 1,2- oxide was unstable in solution and underwent rapid isomerisation to form 1-phenanthrol. This compound may serve as a substrate for a methyl transferase to yield 1-methoxyphenanthrene. The metabolism of phenanthrene

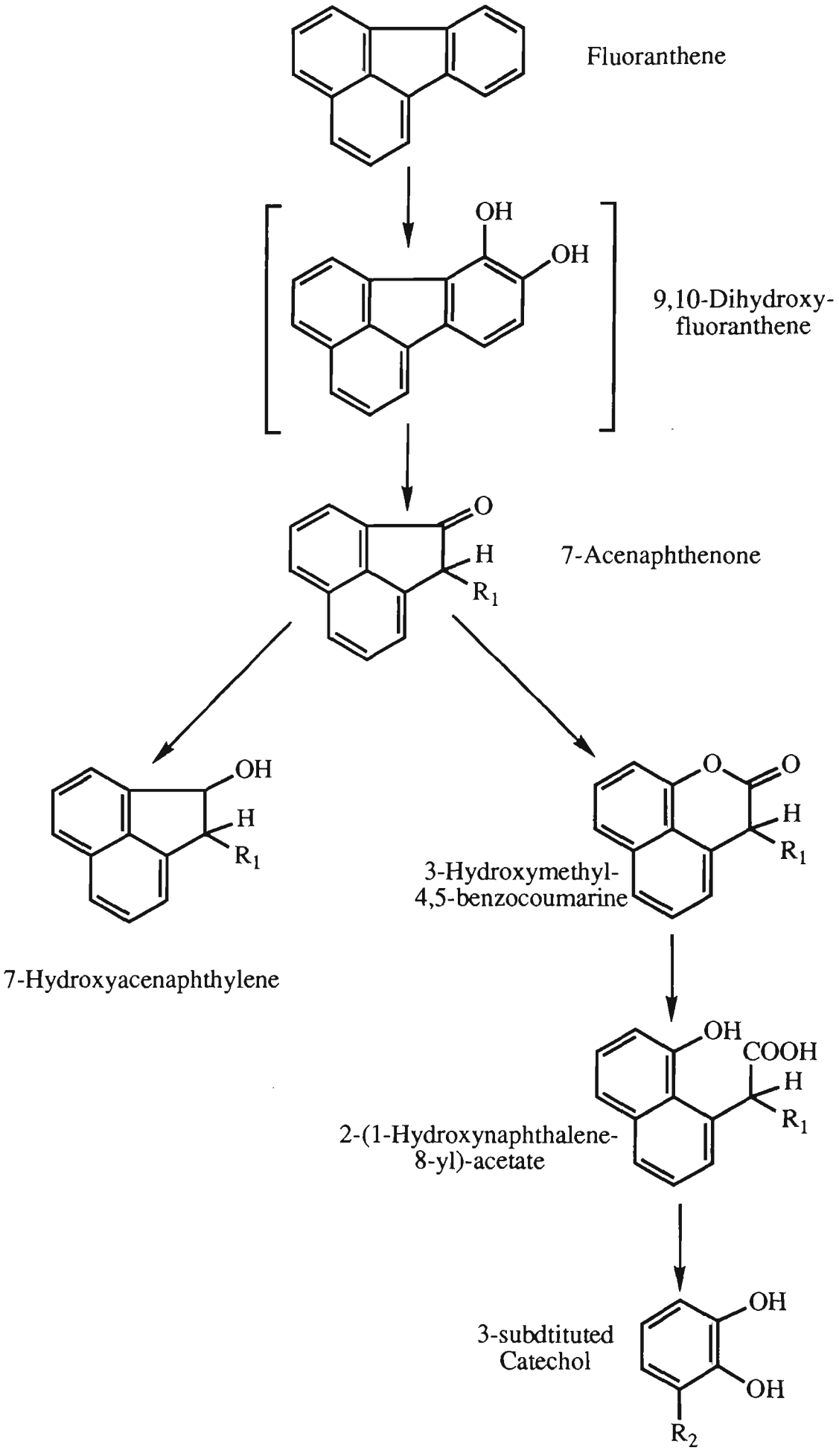

Figure 1.7. Proposed pathway for the degradation of fluoranthrene by *A. denitrificans* strain WW1 (Weissenfels *et al.*, 1991).

by *A. quadruplicatum* strain PR-6 shows similarities to the detoxification reactions catalysed by mammalian liver microsomes (Boyland and Wolf, 1950; Pelkonen and Nebert, 1982).

1.5.4 Fluoranthene Metabolism

Fluoranthene has been shown to be degraded microbially as a sole carbon and energy source by mixed microbial cultures (Mueller *et al.*, 1989b, 1990a) as well as by pure cultures (Mueller *et al.*, 1990b; Weissenfels *et al.*, 1991; Kelly *et al.*, 1991; Trzesicka-Mlynaez and Ward, 1996; Tiehm and Fritzsche, 1995; Bouchez *et al.*, 1995; Kastner *et al.*, 1994). Mueller *et al.* (1990b) isolated a strain of *Sp. paucimobilis* (strain EPA 505) from a seven-member microbial community previously isolated from creosote-contaminated waste. Although resting cells of EPA 505 could transform a range of PAHs (naphthalene, 2,3-dimethylnaphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*b*]fluorene and chrysene) this *Sp. paucimobilis* could only grow on naphthalene, 2,3-dimethylnaphthalene, phenanthrene, anthracene, fluoranthene and benzo[*b*]fluorene. Although EPA 505 could efficiently utilise fluoranthene as a sole carbon and energy source, no attempt to ascertain the metabolic pathway was made.

The degradative pathway of fluoranthene by *A. denitrificans* strain WWI was elucidated by Weissenfels *et al.* (1991) (Figure 1.7). In addition to fluoranthene, strain WW1 could utilise naphthalene, 1- and 2-methylnaphthalene, phenanthrene and anthracene as sole carbon and energy sources. The metabolites identified from fluoranthene metabolism showed that a dioxygenase mechanism initiated the degradation of the PAH. Metabolite F1, identified as acenaphthenone, was obtained by the complete degradation of the exposed, terminal aromatic ring of fluoranthene. From the identification of this metabolite, it seemed likely that the initial attack was on the exposed terminal ring in the 9, 10- position, which resulted in the formation of the corresponding dihydrodiol. 7-hydroxyacenaphthylene was identified as a dead-end metabolite of acenaphthenone degradation. The mechanism of formation for this metabolite was unclear, however, its formation required a 1-carbon excision from the aromatic aldehyde. Acenaphthenone was also degraded to 3-hydroxymethyl-4,5-benzocoumarine (metabolite FL2). The formation of FL2 from acenaphthenone required the insertion of one atom of oxygen into the carbon ring system. This type of reaction has not been reported for the degradation of other PAHs (Weissenfels *et al.*, 1991). Weissenfels *et al.* (1991) proposed that the degradation of 4,5-benzocoumarine would lead to the formation of 1-hydroxynaphthalene, substituted with a carbon unit in the 8- position. The transformation of this compound, be it on either aromatic ring, would produce a catechol

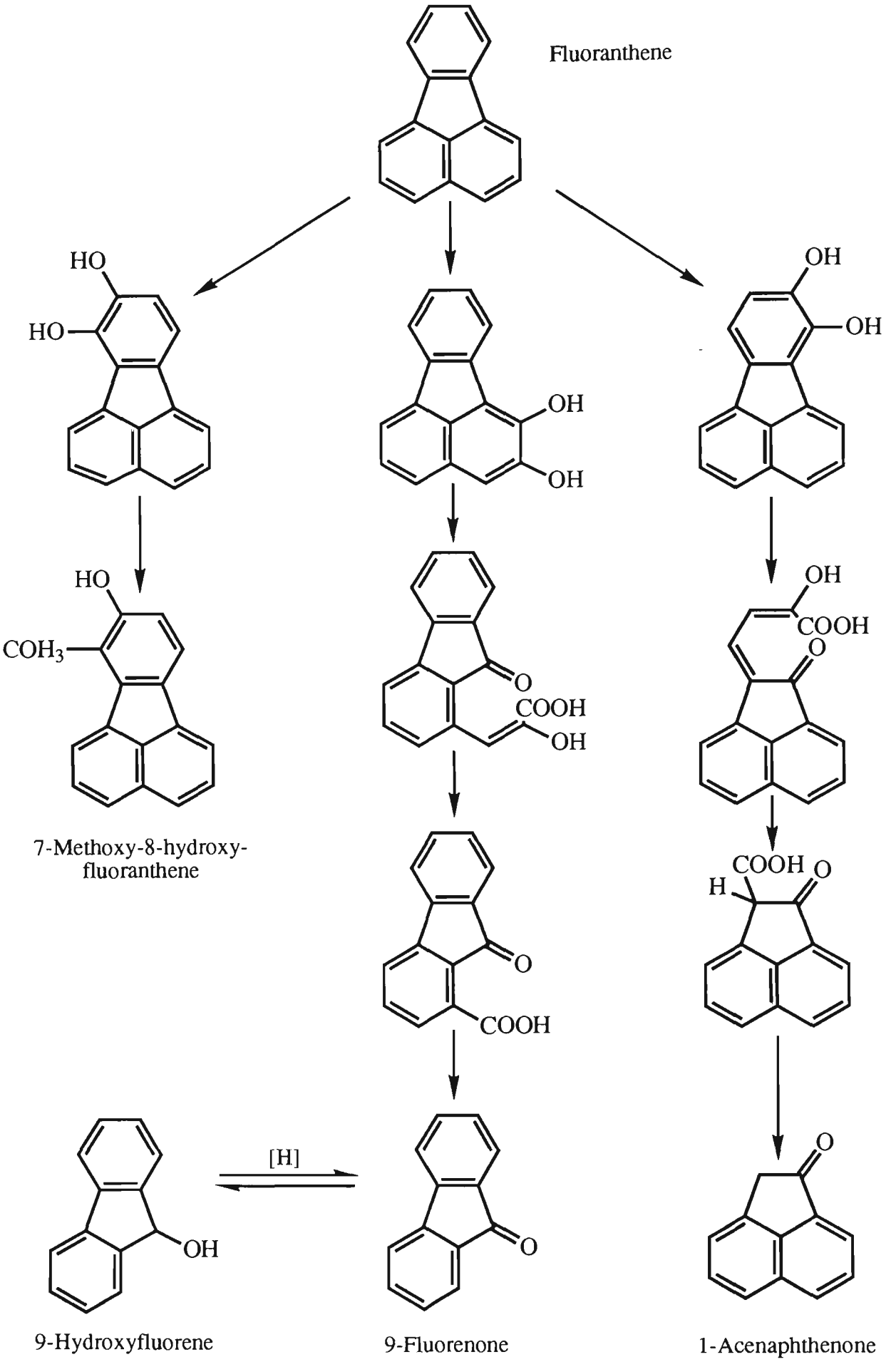

Figure 1.8. Pathways proposed for the metabolism of fluoranthene by *Mycobacterium* strain PYR-1 (adapted from Cerniglia, 1992).

substituted in the 3- position. Alternatively the substituted naphthalene metabolite may be degraded by a sequence analogous to the naphthalene pathway.

Kelly *et al.* (1991) demonstrated a different degradative pathway for fluoranthene by a *Mycobacterium* species previously isolated from an oil-contaminated estuarine sediment (Heitkamp and Cerniglia, 1989). Although a number of fluoranthene metabolites were detected during HPLC analysis of culture extracts, one major metabolite was isolated, purified and identified. ^{1}H-NMR, ^{13}C-NMR, GC-MS analysis and comparison with authentic standards confirmed that the metabolite was 9-fluorenone-1-carboxylate. Presumably, the formation of 9-fluorenone-1-carboxylate resulted from the initial attack of fluoranthene at the 1,2- or 2,3- position. The dihydroxylated intermediate may have been further oxidatively cleaved at the 1,2- and 2,3- positions resulting in the formation of the isolated metabolite. It was not possible to identify further ring fission products. Further fluoranthene metabolite studies with the *Mycobacterium* species led to the proposed pathway shown in Figure 1.8.

1.5.5 Pyrene Metabolism

The microbial degradation of pyrene has been reported by Heitkamp *et al.* (1988a, 1988b), Walter *et al.* (1991), Schneider *et al.* (1996), Thibault *et al.* (1996), Tiehm and Frtizsche (1995), Kastner *et al.* (1994), Bouchez *et al.* (1995), Ye *et al.* (1996) and Trzesicka-Mlynars and Ward (1995), although only a few researchers have identified metabolites from pyrene degradation. Heitkamp *et al.* (1988b) were the first to propose a chemical pathway for the microbial catabolism of pyrene (Figure 1.9). Degradation of pyrene was achieved by a pure culture of a *Mycobacterium* species isolated from sediment located near a point source for petrogenic chemicals. Over 60% of [^{14}C]pyrene was mineralised to CO_2 after 96 hours incubation. One major metabolite and six minor metabolites were identified from the ^{14}C-labelled residue by UV and mass spectrometry and gas chromatography. Pyrene *cis*- and *trans*-4,5-dihydrodiols and pyrenol were identified as initial microbial ring oxidation products of pyrene. 4-Phenanthroic acid, 4-hydroxyperinaphthenone, cinnamic acid and phthalic acid were identified as ring fission products, with 4-phenanthroic acid being the major metabolite. Similar findings were reported by Schneider *et al.* (1996). The degradation of pyrene by *Mycobacterium* strain RJGII-135 resulted in the formation of 4,5-pyrenedihydrodiol and 4-phenanthrene-carboxylic acid, as well as 4,5-phenanthrene dicarboxylic acid, the latter which was proposed by Heitkamp *et al.* (1988b) but not previously isolated.

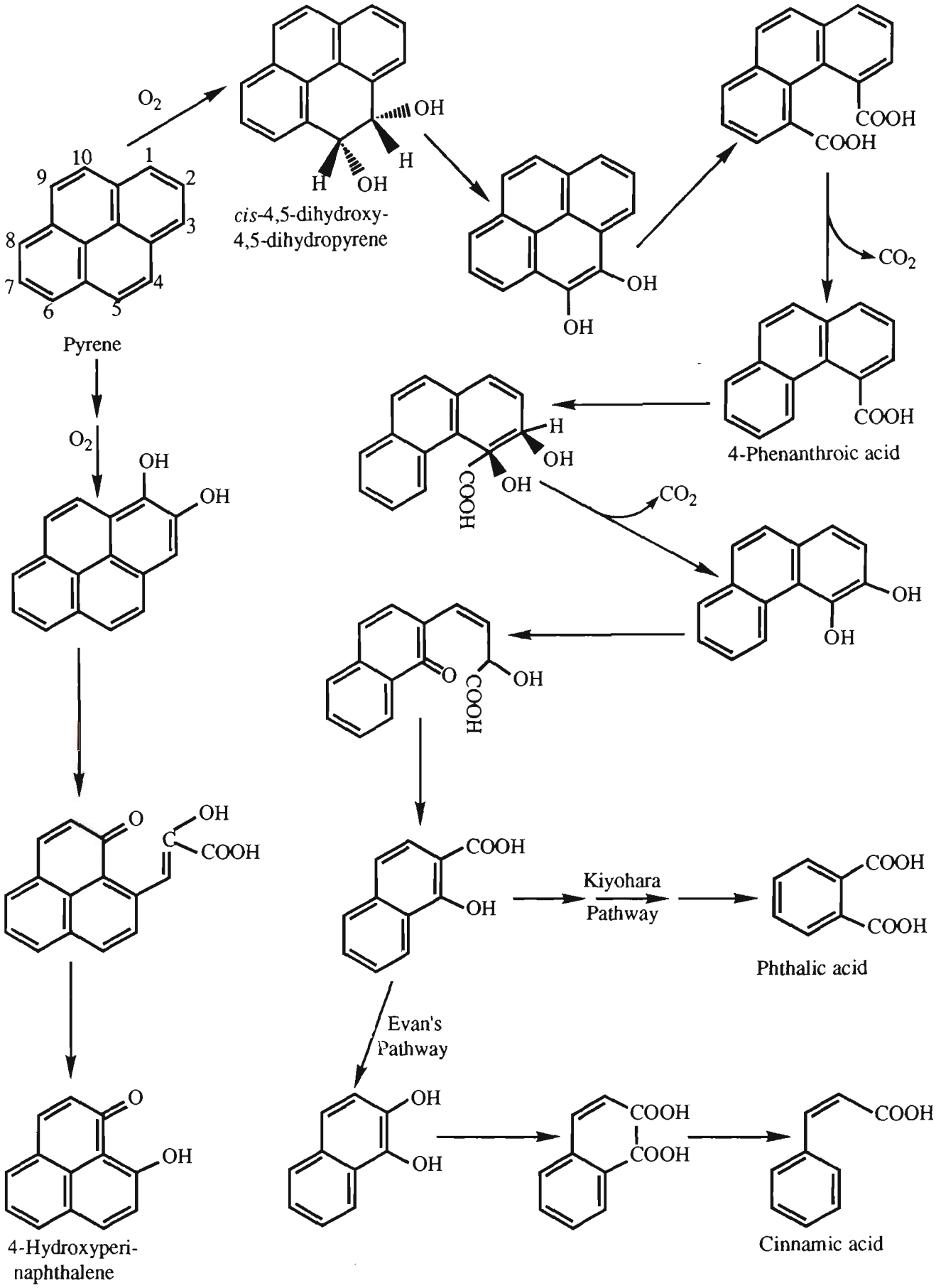

Figure 1.9. Pathways proposed for the metabolism of pyrene by *Mycobacterium* strain PYR-1 (Cerniglia, 1992).

Walter *et al.* (1991) demonstrated the ability of *Rhodococcus* strain UW1, which was isolated from contaminated soil, to degrade pyrene as a sole source of carbon and energy. When pyrene was supplied in cultures at a concentration of 500 mg/l, 72% of the pyrene was mineralised within two weeks. During the growth period, one metabolite accumulated in the culture supernatant which was characterised by spectrophotometry. Although the identity of the metabolite was not achieved, the fragment pattern of the mass spectrum obtained led to the molecular formula of $C_{16}H_{10}O_4$. Walter *et al.* (1991) proposed that the metabolite resulted from the recrystalisation of the *meta*-ring-fission product of pyrene. The proposed pathway for the initial oxidation and ring fission of pyrene by strain UW1 involves the initial attack on pyrene by ring hydroxylation at either the 1,2- or 4,5- positions. This leads to the fission of the first pyrene ring, resulting in the formation of either *cis*-2-hydroxy-3-(perinaphthenone-9-yl)-propenic acid or 2-hydroxy-2-(phenanthren-5-one-4-enyl)-acetic acid. Recrystalisation of either of these products resulted in the formation of the metabolite isolated.

1.5.6 Benz[*a*]anthracene Metabolism

Benz[*a*]anthracene has been shown to be degraded via a cometabolic process. Mahaffey *et al.* (1988) demonstrated that a *Beijernickia* strain, designated B1, was able to oxidise benz[*a*]anthracene. Strain B1 was unable to utilise benz[*a*]anthracene as a source of carbon and energy, however, after induction with biphenyl, *m*-xylene or salicylate, intact cells oxidised benz[*a*]anthracene to CO_2 and a mixture of *o*-hydroxypolyaromatic acids. The initial hydroxylation of benz[*a*]anthracene occurred at the 1,2-, 8,9- and 10,11-positions, with oxidation at the 1,2- position being the most prevalent. 1-Hydroxy-2-anthranoic acid was the major product formed by the oxidation of benz[*a*]anthracene at the 1,2- position, while 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid were minor metabolites produced by the oxidative cleavage of the catechol formed at the 10,11- and 8,9- positions of benz[*a*]anthracene respectively (Figure 1.10). Benz[*a*]anthracene degradation occurred due to the ability of biphenyl, *m*-xylene and salicylate to induce the enzymes systems required for the oxidation of benz[*a*]anthracene.

Gibson *et al.* (1975) also illustrated that a mutant strain B-836 of *Beijernickia* species could oxidise benz[*a*]anthracene to dihydrodiols. Strain B-836 was unable to utilise benz[*a*]anthracene as a sole carbon and energy source, however, after growth with succinate, in the presence of biphenyl, strain B-836 oxidised benz[*a*]anthracene to polar

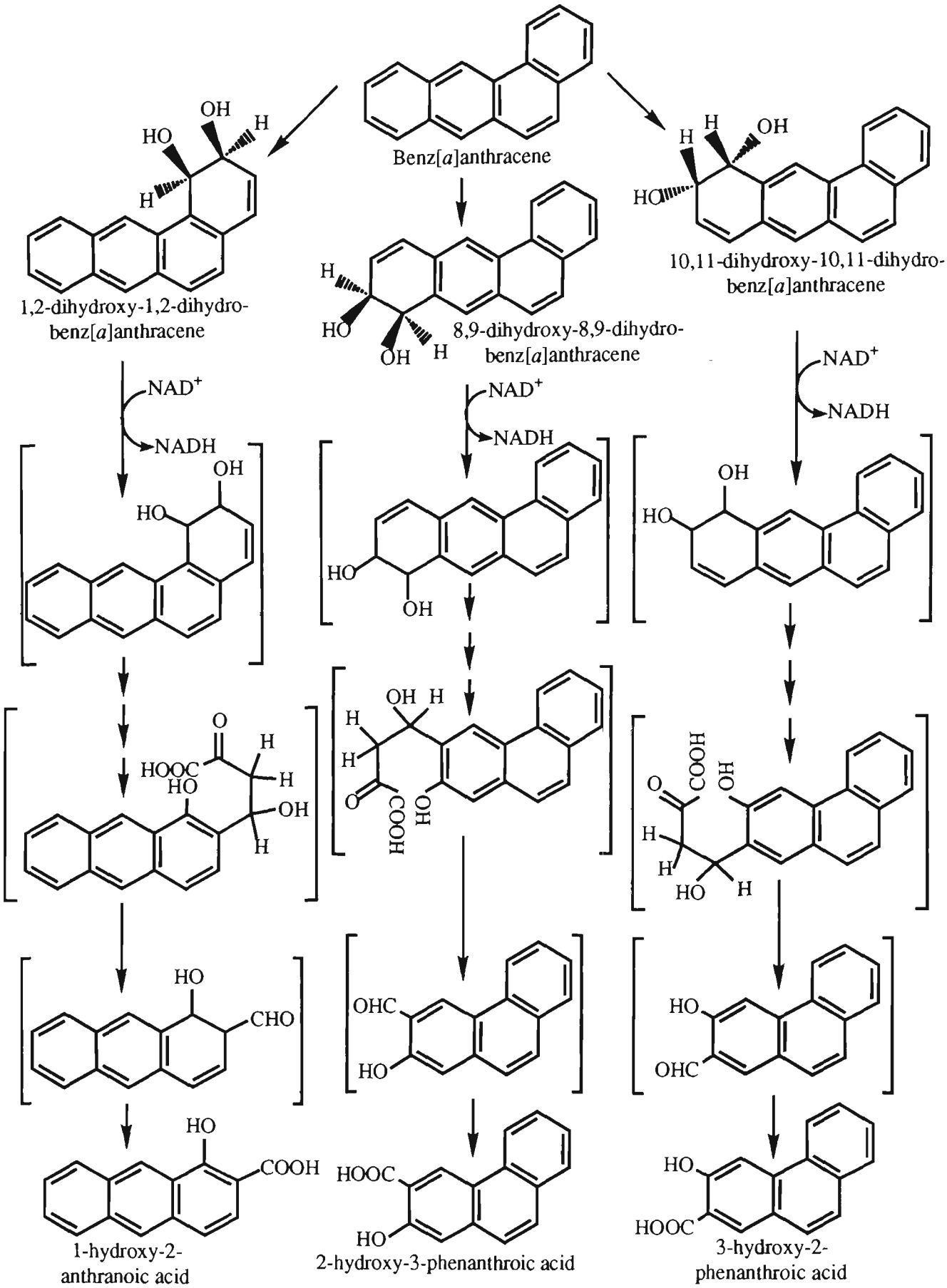

Figure 1.10. Proposed pathway for the metabolism of benz[*a*]anthracene by *Beijerinckia* strain B1. The structures shown in brackets are proposed intermediates (Mahaffey *et al.*, 1988).

products. The major metabolite formed from the oxidation of benz[*a*]anthracene was *cis*-1,2-dihydroxy-1,2-dihydro-benz[*a*]anthracene.

Schneider *et al.* (1996) reported the initial degradation products of benz[*a*]anthracene by *Mycobacterium* strain RJGII-135 grown in the presence of trace amounts of yeast extract, peptone and soluble starch (250 mg/l each) (Figure 1.11). The initial degradation of benz[*a*]anthracene by strain RJGII-135 resulted in the formation of three benz[*a*]anthracene dihydrodiols. Hydroxylation occurred at the 5,6-, 8,9- and 10,11-positions, with the 5,6-dihydrodiol being the dominant metabolite. The minor benz[*a*]anthracene dihydrodiols (8,9- and 10,11-) are the same as reported by Mahaffey *et al.* (1988) for the initial benz[*a*]anthracene hydroxylation products produced by *Beijernickia* strain B1. In addition, strain RJGII-135 produced a metabolite with a chemical formula of $C_{17}H_{10}O_2$. Although a structure could not be assigned to the metabolite, its formula is representative of a ring cleavage intermediate.

1.5.7 Benzo[*a*]pyrene Metabolism

Little is known about the bacterial oxidation of PAHs containing five or more aromatic rings. To date, no bacteria have been isolated that have the metabolic capabilities of utilising these PAHs as growth substrates. However, microorganisms can oxidise some of these high molecular weight PAHs when grown on an alternative carbon source (Barnsley, 1975b; Shiaris, 1989b; Gibson *et al.*, 1975; Herbes and Schwall, 1978). Gibson *et al.* (1975) showed that *Beijernickia* strain B-836 could oxidise benzo[*a*]pyrene to dihydrodiols. Strain B-836 was unable to utilise benzo[*a*]pyrene as a sole carbon and energy source but after growth with succinate, in the presence of biphenyl, oxidised benzo[*a*]pyrene to a mixture of vicinal dihydrodiols. The major dihydrodiol formed was identified as *cis*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene (Figure 1.12). These results indicate that although strain B-836 was unable to utilise benzo[*a*]pyrene as a carbon source, growth on an alternate substrate induced the synthesis of enzymes capable of transforming the five-ring PAH.

Mycobacterium strain RJGII-135 also formed benzo[*a*]pyrene dihydrodiols which was the same as seen for the initial degradation of benzo[*a*]pyrene by *Beijernickia* strain B-836. The further formation of *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid resulted from the *meta* fission of the hydroxylated compound. This product may be transformed to 7,8-dihydro-pyrene-8-carboxylic acid through several subsequent steps which have not yet been characterised fully. Furthermore, Schneider *et al.* (1996) isolated 4,5-chrysene-dicarboxylic acid, which they proposed was a *ortho* fission

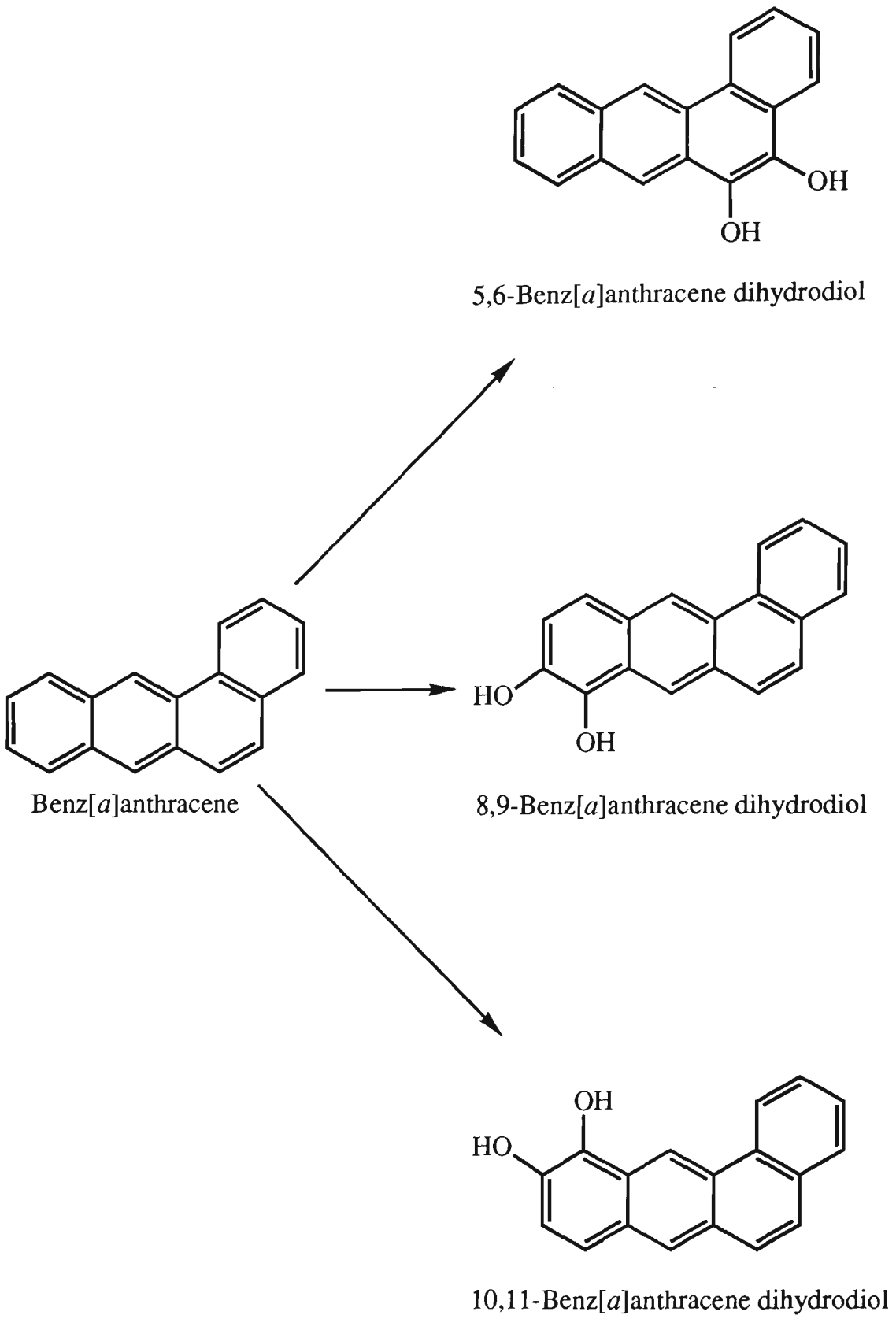

Figure 1.11. Proposed pathway for the initial hydroxylation of benz[*a*]anthracene by *Mycobacterium* strain RJGII-135 (Schneider *et al.*, 1996).

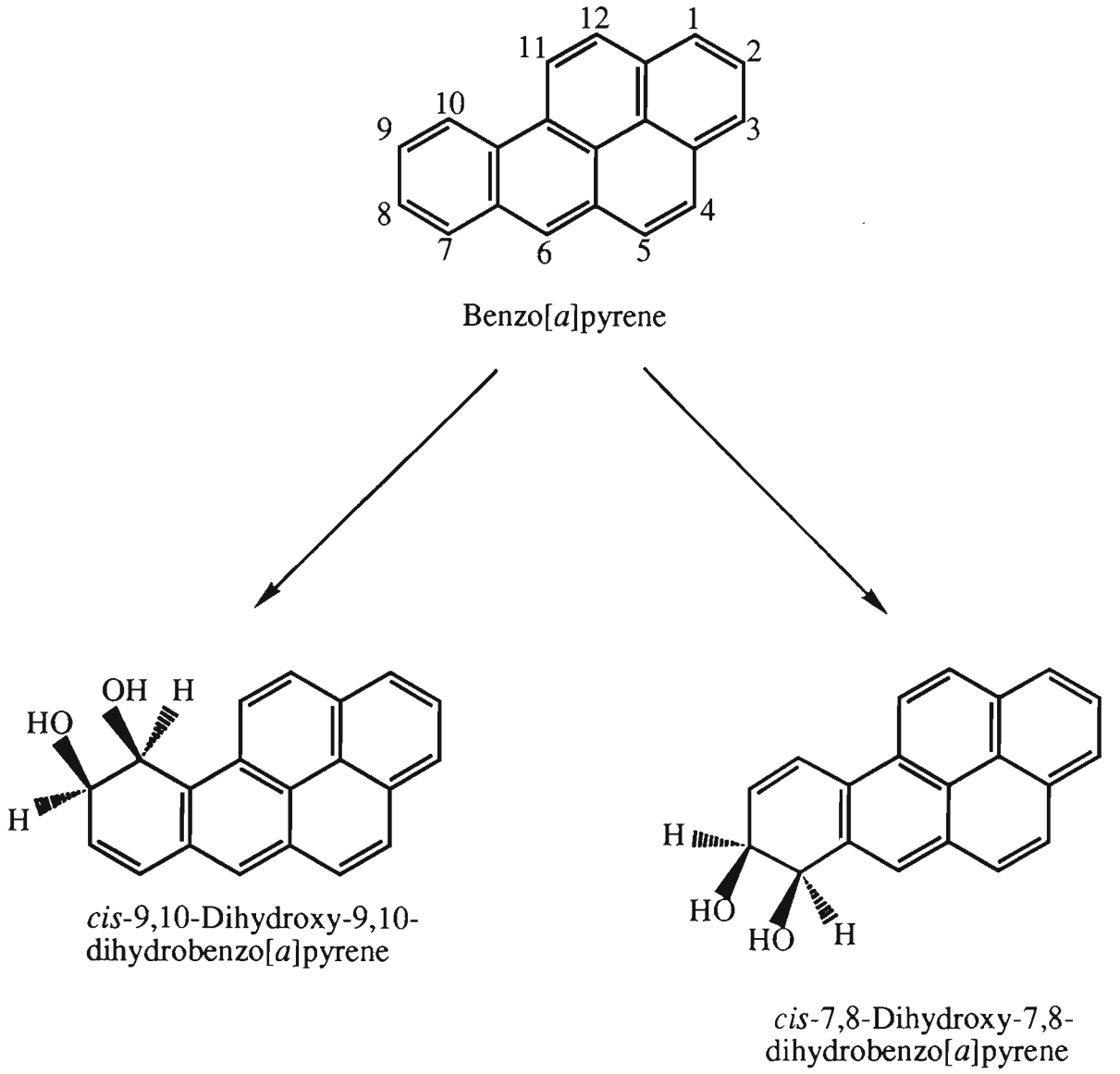

Figure 1.12. Initial reactions in the oxidation of benzo[*a*]pyrene by *Beijernickia* strain B-836 (Gibson *et al.*, 1975).

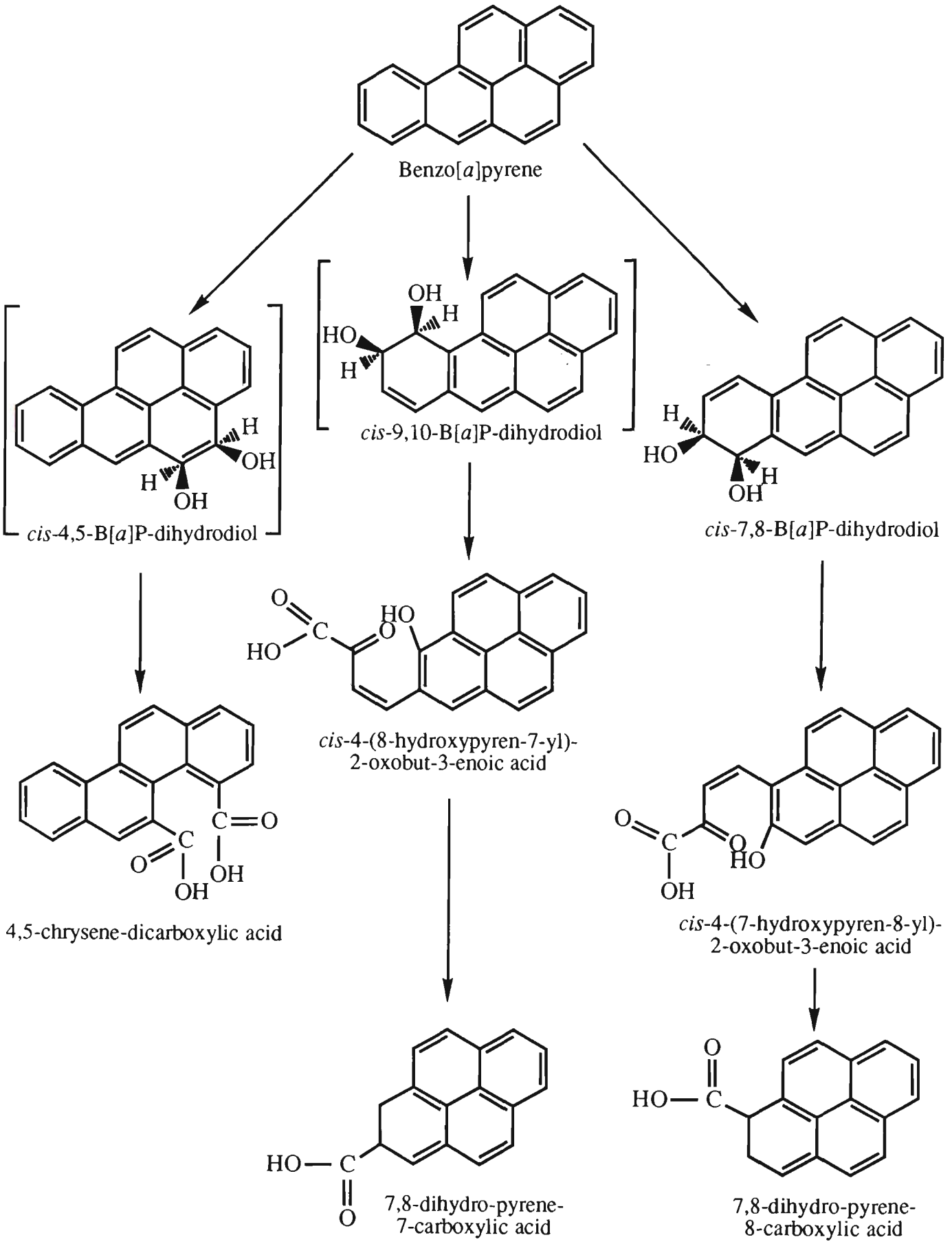

Figure 1.13. Proposed pathway for the degradation of benzo[*a*]pyrene by *Mycobacterium* strain RJGII-135 (Schneider *et al.*, 1996).

product of *cis*-4,5-benzo[*a*]pyrene dihydrodiol, an alternate initial hydroxylation product (Figure 1.13).

Ye *et al.* (1996) observed a significant evolution of $^{14}CO_2$ from [7-^{14}C] benzo[*a*]pyrene cultures inoculated with *Sp. paucimobilis* strain EPA 505, indicating hydroxylation and ring cleavage of the 7,8,9,10- benzo ring. Since no metabolites from this degradation of benzo[*a*]pyrene were characterised and benzo[*a*]pyrene was labelled only at the C-7 position, ring cleavage on other aromatic rings could not be excluded.

1.5.8 Dibenz[*a,h*]anthracene Metabolism

To date, a pathway has not been proposed for the microbial degradation of dibenz[*a,h*]anthracene. However, the degradation of dibenz[*a,h*]anthracene by resting cells of *Sp. paucimobilis* strain EPA 505 has been demonstrated by Ye *et al.* (1996). Over the incubation period (16 hours), there was a 7.8% decrease in the concentration of dibenz[*a,h*]anthracene which was added at an initial concentration of 10 mg/l to a resting cell suspension of 1 mg protein/ml. Metabolites were not identified. Metabolites produced from degradation of high molecular weight PAHs (four- and five-ring PAHs) are shown in Table 1.10.

1.6 MICROBIAL DEGRADATION OF PAHs IN SOIL

The possible fate of PAHs in the environment includes volatilisation, photooxidation, chemical oxidation, bioaccumulation and adsorption to soil particles (Figure 1.2). However, the principal processes for the removal of PAHs from the environment are thought to be microbial transformation and degradation (Gibson *et al.*, 1975). A number of techniques, such as *in situ* remediation, on site landfarming and bioreactors have been developed to enhance the degradation rate of PAHs in contaminated soil.

1.6.1 Natural Attenuation of PAHs

Natural attenuation, the degradation of contaminants by indigenous microorganisms, has been observed to reduce the mobility, mass and associated risk of soil contaminants. In some situations, where contaminants do not pose a direct risk to human health or where there is low priority for land reclamation, it may be more economical to allow bioremediation to proceed naturally rather than hastening removal rates by the addition of electron acceptors and nutrients. Gin *et al.* (1995) observed natural attenuation of PAHs from a manufacturing gas plant waste site in New York, USA. The concentration

Table 1.10. Metabolites produced from the microbial degradation of high molecular weight PAHs.

PAH	Species	Strain	Metabolites Isolated	Reference
Fluoranthene	*A. denitrificans*	WW1	acenaphthenone, 7-hydroxy-acenaphthylene, 3-hydroxymethyl-4,5-benzocoumarine	Weissenfels *et al.* (1991)
Fluoranthene	*Mycobacterium* species		9-fluorenone-1-carboxylate	Kelly *et al.* (1991)
Pyrene	*Mycobacterium* species		*cis*-4,5-pyrene dihydrodiol, *trans*-4,5-pyrene dihydrodiol, pyrenol, 4-phenanthroic acid, 4-hydroxy-perinaphthenone, cinnamic acid, phthalic acid	Heitkamp *et al.* (1988b)
Pyrene	*Mycobacterium*	RJGII-135	4,5-pyrene dihydrodiol 4-phenanthroic acid 4,5-phenanthrene dicarboxylic acid	Scneider *et al.* (1996)
Pyrene	*Rhodococcus*	UW1	$C_{16}H_{10}O_4$	Walter *et al.* (1991)
Benz[*a*]anthracene	*Beijernickia*	B1	1,2-, 8,9- and 10,11-benz[*a*]anthracene dihydrodiols 1-hydroxy-2-anthroic acid 2-hydroxy-3-phenanthroic acid 3-hydroxy-2-phenanthroic acid	Mahaffey *et al.* (1988)
Benz[*a*]anthracene	*Beijernickia*	B-836	*cis*-1,2-benz[*a*]anthracene dihydrodiol	Gibson *et al.* (1975)
Benz[*a*]anthracene	*Mycobacterium*	RJGII-135	5,6-, 8,9- and 10,11-benz[*a*]anthracene dihydrodiols $C_{17}H_{10}O_2$	Schneider *et al.* (1996)
Benzo[*a*]pyrene	*Beijernickia*	B-836	*cis*-7,8- and *cis*-9,10- benzo[*a*]pyrene dihydrodiols	Gibson *et al.* (1975)
Benzo[*a*]pyrene	*Mycobacterium*	RJGII-135	7,8-benzo[*a*]pyrene dihydrodiol 4,5-chrysene dicarboxylic acid *cis*-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid 7,8-dihydropyrene-7-carboxylic acid or 7,8-dihydropyrene-8-carboxylic acid	Schneider *et al.* (1996)

of dissolved naphthalene in the groundwater plume was observed to dissipate in the direction of the groundwater flow. In addition, the concentration of oxygen was found to increase away from the centre of the plume. Biotransformation studies indicated that the indigenous soil microorganisms were capable of degrading naphthalene and phenanthrene. 1-Hydroxy-2-naphthoic acid, a metabolite of phenanthrene, was detected in contaminated soil from the site, indicating that *in situ* bioremediation may play an important role in natural attenuation of PAHs at the site.

Natural attenuation of coal tar organics in groundwater was also observed by King *et al.* (1995). Sand, containing residual coal tar, was placed under the water table to assess the migration of selected creosote compounds (*m*-xylene and naphthalene). The plumes were monitored over a 1,008-day period to assess whether contaminant migration would reach steady state. The *m*-xylene plume migrated approximately 46 metres from the source after 640 days. After 1,008 days, the plume was observed to be receding (approximately 33 metres) indicating that the compound was being transformed. The rate of *m*-xylene mass flux into the plume had decreased to below the rate of transformation within the plume. The naphthalene plume continued to migrate from the point source, indicating that the rate of input from the source was still in excess of microbial transformations for this compound.

1.6.2 Bioaugmentation of PAH-Polluted Soil

Bioaugmentation, the introduction of non-indigenous microorganisms for bioremediation, is one technology which can potentially increase degradation rates compared to natural attenuation. The addition of microorganisms with specific degradative capabilities is believed to overcome the catabolic limitations of the indigenous microorganisms towards target compounds. Natural degradation rates may be low due to the lack of appropriate enzymes, low population densities or contaminant toxicity which impedes growth of suitable microbes. Bioaugmentation is especially important for sites containing high PAH concentrations, sites which contain a significant proportion of high molecular weight PAHs and for recently polluted soils which do not have an adapted microbial population (Mueller *et al.*, 1989a; Trzesicka-Mlynarz and Ward, 1996). At such sites, the indigenous microorganisms are often ineffective at removing high molecular weight PAH compounds (Aprill *et al.*, 1990; Grosser *et al.*, 1991; Kastner *et al.*, 1995) due to the aforementioned reasons. Other site characteristics may also influence the need to bioaugment. Microorganisms with PAH-catabolic capabilities and the ability to grow at low or high temperatures, with minimal nutrient requirements, with heavy metal tolerance, the ability to produce surfactants or the ability

to survive in a wide range of conditions may be necessary at particular sites depending on their particular characteristics. If the speed of decontamination is a prime factor, the addition of a microbial community with known degradative capabilities can be used to start the remediation process with little or no lag period (Forsyth *et al.*, 1995).

The majority of research conducted into microbial PAH degradation has involved liquid cultivations to determine the degradative potential of isolated microorganisms and to elucidate pathways for the degradation of these compounds. Promising isolates from these studies could be used for the *in situ* bioaugmentation of contaminated soils (inoculating soil with selected PAH-degrading organisms) and for *ex situ* soil decontamination practices such as landfarming, biopiles and solid-phase bioreactors (Wilson and Jones, 1993). *In situ* bioremediation processes have less practical application for the remediation of PAH-contaminated soils, because the limiting factor is usually oxygen delivery, which can be overcome by landfarming, biopiles and solid-phase bioreactors.

Previous examples of bioaugmentation of contaminated sites have met with limited success for reasons including die-off of laboratory-adapted strains, limited substrate availability and the inability of inocula to compete with indigenous microflora, exemplified by the results from a creosote-contaminated site as reported by Mueller *et al.* (1989a). Of the few bioaugmentation studies conducted in soil matrices, most have been concerned with, or limited to, the bacterial degradation of low molecular weight PAH compounds (Aamand *et al.*, 1995; Venosa *et al.*, 1995; Erickson *et al.*, 1993) and four-ring PAHs such as fluoranthene and pyrene (Trzesicka-Mlynarz and Ward, 1996; Grosser *et al.*, 1991).

The degradation of phenanthrene in coal tar polluted soil was assesses by Aamand *et al.* (1995) with and without the inoculation of a PAH-degrading microbial community isolated from an industrial sewage treatment plant. Two polluted soils, varying in coal tar contamination (2,902 and 240 mg/kg soil), were supplemented with [9-^{14}C] phenanthrene. One set of soils were inoculated with the mixed culture suspended in a mineral salts medium while the other soils were supplemented with the mineral salts medium only. After 22 days, 35% of added ^{14}C-phenanthrene was mineralised to $^{14}CO_2$ in the heavily contaminated soil inoculated with the microbial community; only 5% of ^{14}C-phenanthrene was mineralised in uninoculated soils. In contrast, inoculation of the less contaminated soil with the mixed culture did not significant increase phenanthrene mineralisation. After 14 days, 55% of ^{14}C-phenanthrene was mineralised to $^{14}CO_2$ in inoculated soils compared to 52% in uninoculated soils. Although

microbial numbers in soil cultures were not determined, Aamand *et al.* (1995) accounted for the indigenous mineralisation of ^{14}C-phenanthrene in the low coal tar contaminated soil by the presence of a considerable microbial population which was already adapted to PAH degradation. The inhibition of ^{14}C-phenanthrene mineralisation by the indigenous microbial population in the heavily contaminated soil may have arose due to the toxic effects of high concentrations of the coal tar components. The increase in microbial numbers and the resistance of the inoculated microorganisms to the high concentrations of the coal tar components may account for the increased mineralisation of ^{14}C-phenanthrene in the inoculated heavily contaminated soil.

Bioaugmentation of crude oil-contaminated plots with indigenous microorganisms by Venosa *et al.* (1995) did not result in additional enhancement of *n*-alkane or PAH degradation. *n*-Alkane degradation rates were greater at plots where nutrients or an inoculum plus nutrients were added compared to no treatment. However, no significant difference was observed between nutrient-amended plots and nutrient plus inoculum-amended plots. Analysis of the PAH fraction of the extracted crude oil revealed that small decreases in the concentration of the two- and three-ring PAHs and the lower alkyl-substituted homologs occurred. The four-ring PAHs were unaffected over the six week period. However, no significant difference in PAH degradation rates was observed among any of the treatments at any sampling time.

Erickson *et al.* (1993) investigated the bioremediation of PAHs from a manufacturing gas plant soil using indigenous microorganisms as well as an inoculum containing PAH-degrading microorganisms. A number of different strategies were applied to the soils to effect PAH degradation, including nutrient addition (manure), inoculum addition (using uncontaminated soil or activated sludge), pH adjustment and incubation at different temperatures (7, 15, 20 and 35°C). The test soil contained PAHs ranging in size from two- to six-rings (naphthalene to benzo[*g,h,i*]perylene) and varying in concentration from 6 to 55 mg/kg soil for each compound. Baseline studies (no treatment) indicated that the indigenous microorganisms were not capable of degrading any of the PAHs present in the manufacturing gas plant soil after a three month period. In fact, varying the temperature, soil moisture or nutrient conditions did not stimulate the degradation of PAHs. Augmentation of soils with organisms known to be capable of degrading PAHs also did not increase PAH loss in the manufacturing gas plant soil. Erickson *et al.* (1993) concluded that the failure to observed PAH loss in any of the soils may be due to the toxicity of the soil or the poor availability of the PAHs to the soil microorganisms. To test whether soil toxicity was responsible for the lack of microbial activity towards PAHs, Erickson *et al.* (1993) evaluated the degradation of added naphthalene or

phenanthrene in manufacturing gas plant soil. The objective of the study was to determine whether a component in the soil inhibited the microbial degradation of naphthalene or phenanthrene. The added PAHs were rapidly degraded in augmented and non-augmented soils; between 60 and 100 mg/kg soil of naphthalene and phenanthrene were degraded after 28 days. It also appeared that background naphthalene and phenanthrene from the contaminated soil remained undegraded at the conclusion of the four week incubation period. These results led to the conclusion that the soils were not toxic to the indigenous or augmented microorganisms, however, the PAHs contained with in the soil were bound in such a way that made them unavailable for degradation.

Trzesicka-Mlynarz and Ward (1996) evaluated the use of a fluoranthene-degrading microbial community to degrade fluoranthene in soil. The effects of bioaugmentation on the degradation of fluoranthene in sterile and unsterile soil were assessed as well as the degradative potential of the indigenous soil microorganisms without bioaugmentation. Over a nine week period, fluoranthene concentrations were found to decrease from 14.4% in sterile uninoculated soils to 52.1% in unsterile inoculated soils. Degradation of fluoranthene by the indigenous microflora accounted for a 38.2% decrease in PAH concentration. A good correlation was found between PAH degradation rates and microbial counts. In inoculated soils, initial microbial populations were approximately 10-fold greater than the indigenous soil count (4×10^5 cfu/g soil). However, over the nine week period, the total viable counts in inoculated unsterile soil gradually declined to the level observed for the original indigenous microbial population. Viable counts in inoculated sterile soil were even smaller, dropping to levels significantly lower than that of the indigenous population, which remained fairly constant over the incubation period. Trzesicka-Mlynarz and Ward (1996) attributed the drop in microbial numbers to strain starvation due to limited availability of essential nutrients.

Grosser *et al.* (1991) demonstrated that PAH mineralisation in soil can be enhanced by the reintroduction of PAH-degrading microorganisms isolated from the same site. Studies were conducted in soils with different histories of past exposure to hydrocarbons, different amounts of total organic carbon (2-39% of dry weight), microbial biomass (1×10^5-6.4×10^7) and microbial activity. The degradation of ^{14}C-labelled pyrene, carbazole and benzo[*a*]pyrene by the indigenous microflora was assessed over a 180 day period. The levels of mineralisation of pyrene, carbazole and benzo[*a*]pyrene varied depending on the soil type. Pyrene and carbazole were degraded with short or no lag periods, however, benzo[*a*]pyrene mineralisation occurred only after

a 28 day lag period. PAH mineralisation varied from 10 to 48% for pyrene, 0 to 46% for carbazole and from undetectable to 25% for benzo[*a*]pyrene. PAH degraders were isolated from soils by enrichment using selected PAHs (naphthalene, phenanthrene, anthracene) as the sole carbon and energy source. The isolated microorganisms, identified as a *Mycobacterium* species and a *Xanthamonas* species, were capable of mineralising pyrene (55%) and carbazole (60%) respectively in a minimal basal salts medium after two days. Attempts to isolate a benzo[*a*]pyrene degrading microorganism were unsuccessful. Reintroduction of the pyrene degrading *Mycobacterium* species (2 x 10^9 cfu/g soil) into soil containing ^{14}C-pyrene resulted in the enhanced mineralisation of the PAH. Within one week of reintroduction, 58% of pyrene was mineralised, compared to approximately 14% in soils without inoculation. The addition of different concentrations of pyrene-degrading microorganisms to the soil had little effect on the total amount of pyrene mineralised. At cell concentrations of 10^7 cfu/g soil, 5 x 10^7 cfu/g soil and 10^8 cfu/g soil, the amounts of pyrene mineralised were 55%, 62% and 58% respectively. When the carbazole degrading microorganism was reintroduced to ^{14}C-carbazole containing soil (4 x 10^7 cfu/g soil), approximately 48% of the ^{14}C was recovered as $^{14}CO_2$ after 14 days. Little carbazole mineralisation (<10%) was measured in soils without inoculation. Reintroduction of different numbers of carbazole degraders into soil showed that carbazole mineralisation was dependent on the concentration of introduced cells. The level of mineralisation for three cell concentrations were 29% for 10^6 cfu/g soil, 38% for 5 x 10^6 cfu/g soil and 42% for 10^7 cfu/g soil after 14 days.

These studies have a practical limitation in that they do not address the degradation of the more recalcitrant high molecular weight PAH compounds containing five benzene rings, such as benzo[*a*]pyrene or dibenz[*a,h*]anthracene, which can be major components of PAH-contaminated soil (Erickson *et al.*, 1993; Wilson and Jones, 1993). This is probably attributable to the nature of the microbes used and the rarity of reported isolated microorganisms which degrade four- and five-ring PAHs efficiently, plus the loss of high molecular weight PAH-degrading ability by the inocula when introduced to a soil matrix. Furthermore, previous experimental work on bioaugmentation of contaminated soils has mainly been concerned with the degradation of single PAH substrates, which does not represent the mixed PAH profile typical of PAH-contaminated sites (Erickson *et al*, 1993).

1.7 ENVIRONMENTAL FACTORS AFFECTING PAH DEGRADATION

The microbial degradation of PAHs in the environment is strongly influenced by a wide variety of abiotic and biotic factors (Cerniglia, 1992). Environmental factors such as

Table 1.11. Environmental conditions affecting the degradation of PAHs in soil (adapted from Wilson and Jones, 1993).

Parameter	Conditions required for microbial activity	Optimum values for PAH degradation	Reference
Soil moisture	25-85% of water holding capacity	30-90%	Dibble and Bartha (1979)
Soil pH	5.5-8.5	7.0-7.8	Dibble and Bartha (1979), Weissenfels *et al.*(1990a)
Redox potential	Aerobes and facultative anaerobes >50 mV; Anaerobes <50 mV	Aerobic, +500 mV	Mihelcic and Luthy (1988), Delaune *et al.* (1981), Atlas (1981)
Oxygen content	Aerobic, minimum air-filled pore space of 10% Anaerobic <1% by volume	10-40% O_2	Bauer and Capone (1985)
Nutrient content	Nitrogen (N) and Phosphorus (P) for microbial growth	Carbon:N 10-60:1 Carbon:P 100-800:1	Manilal and Alexander (1991), Dibble and Bartha (1979), Atlas and Bartha (1973)
Temp. (°C)	15-45	20-30	Dibble and Bartha (1979), Bauer and Capone (1985), Weissenfels *et al.* (1990a), Heitkamp *et al.* (1988a), Walter *et al.* (1991)

temperature, pH, oxygen availability, water availability and nutrient availability can directly affect the rate at which PAHs are degraded by microorganisms (Table 1.11).

1.7.1 Soil Texture and Structure

The significance of soil texture and structure in the microbial degradation of PAHs lies in its overriding control of the above factors (Pollard *et al.*, 1994). Soil texture and structure can influence oxygen diffusion rates, nutrient availability, contaminant bioavailability, tilth, water infiltration, retention and yield. In soils with a clay content of approximately 12% w/w, aggregation can result in the formation of micro-environments. Entrapment of microorganisms and substrate within a pore space may provide a suitable environmental niche for localised enzymatic activity. However, soils containing high clay content may form aggregates which have anaerobic centres due to their fine mesoporous structure and water absorbent properties.

Mott *et al.* (1990) examined the influence of soil aggregate size on the degradation of a heavy gas oil in an uncontaminated silty clay loam. Soil aggregates tested ranged in diameter from 19-25 mm (coarse), 5-10 mm (medium) to 1-2 mm (fine). After 14 days, the authors observed that aggregate size greatly affected biotransformation rates. Heavy gas oil degradation rates decreased with increasing aggregate size. Mott *et al.* (1990) proposed that the differences in biotransformation rates arose due to differences in soil surface areas which directly affected the bioavailability of the gas oil.

1.7.2 Bioavailability

The bioavailability of the PAHs plays a critical role in the microbial degradation of these compounds. Many PAHs are readily degraded *in situ* (*e.g.* naphthalene and phenanthrene), however, their persistence in soil can be attributed to the compound being inaccessible to the microorganism. PAHs are hydrophobic compounds and tend to adsorb onto soil material (Bouwer *et al.*, 1994; Blackburn and Hafker, 1993). Over a long contact time, adsorbing compounds may diffuse into the inorganic and organic matrix and may also form bound residues (Bouwer *et al.*, 1994). As most evidence indicates that biotransformation of PAHs occurs in the liquid phase (Wodzinski and Bertolini, 1972), adsorption of compounds to humic material or the clay fraction of the soil reduces the concentration of the compound available to the liquid phase (Bouwer *et al.*, 1994; Wilson and Jones, 1993) and ultimately reduces the degradation rate. In addition, the accumulation of contaminants in fissures and cavities renders them inaccessible to microorganisms and their enzymes.

1.7.3 Temperature

Seasonal factors can influence the rate and extent of PAH degradation (Cerniglia, 1992; Wilson and Jones, 1993; Dibble and Bartha, 1979; Bauer and Capone, 1985; Weissenfels *et al.*, 1990a; Heitkamp *et al.*, 1988a). Microbial activity decreases below optimum temperatures due to membrane gelling which decreases the transport of nutrients and waste products across the cell membrane. At temperatures above optimum conditions, deterioration of the cell membrane occurs, resulting in protein denaturation, protein dysfunction and ultimately thermal death.

Temperature has a marked influence on kinetic or rate constants as described by Arrhenius equations. Atlas (1981) reported petroleum degradation rates an order of magnitude lower at 5°C compared to 25°C. By decreasing the incubation temperature from 30°C to 20°C, Rosenberg *et al.* (1992) observed a decrease in the amount of pentane-extractable hydrocarbons degraded (55% to 45%) by a hydrocarbon degrading microbial community. When the incubation temperature was increased to 60°C, 85% of the hydrocarbons were still present, indicating decreased catabolic activities at elevated temperatures. The seasonal biotransformation of naphthalene, phenanthrene and benzo[*a*]pyrene in surficial estuarine sediments was observed by Shiaris (1989b). The author observed PAH mineralisation maxima to occur in the warmer months of the year (spring to early summer). Low transformation rates during the winter and early spring were probably the result of low sediment temperatures and the resulting suppression of bacterial activity.

1.7.4 pH

The pH of the medium or environment can greatly affect the rate of PAH degradation. Enzyme function is influenced by pH and extreme variations in pH denature enzymes. A study by Delaune *et al.* (1981) conducted using an estuarine sediment under controlled pH demonstrated the effect of pH on the degradation of benzo[a]pyrene. Under aerobic conditions (500 mV) and a pH of 8.0, approximately 6.4% of added ^{14}C-benzo[*a*]pyrene was mineralised to $^{14}CO_2$ after 37 days. Benzo[*a*]pyrene mineralisation was six-fold less at pH 9.0 and 40-fold less at pH 5.0. Shiaris (1989b) did not observe any change in the biotransformation rates of PAHs due to high pH. Although the high pH of marine sediments may result in lower PAH adsorption to particles, leading to a more bioavailable form of the PAH, the sites sampled did not vary enough in pH to affect PAH transformation rates.

1.7.5 Redox Potential and Oxygen Availability

Oxygen is a vital factor in the degradation of PAHs as indicated by the observation that the major degradative pathways involve oxygenases and molecular oxygen. This is not to say that PAH degradation cannot occur at reduced redox potential, however, hydrocarbons which enter anaerobic environments such as anoxic sediments are well preserved and may persist indefinitely (Atlas, 1981). Hambrick *et al.* (1980) observed that the mineralisation of hydrocarbons was highly dependent on oxygen availability. Rates of hydrocarbon degradation decreased with decreasing redox potential. Under anaerobic conditions, naphthalene mineralisation was insignificant (0.4%) after 35 days. However, when oxygen was introduced into the system and the redox potential increased from -220 mV to +130 mV, naphthalene mineralisation increased from 0.4% to 22.6% after 35 days. Mihelcic and Luthy (1988) observed the degradation of PAHs under various redox conditions in soil-water systems. The degradation of naphthol, naphthalene and acenaphthene by indigenous soil microorganisms was assessed under aerobic, anaerobic and denitrification conditions. Naphthol could be degraded under all incubation conditions, however, the rate of naphthol degradation decreased with decreasing oxygen concentration. Under aerobic conditions, naphthol (9 mg/l) was degraded to undetectable levels after three days incubation, compared to 15 and 16 days under anaerobic and denitrification conditions respectively. Naphthalene (7 mg/l) and acenaphthene (1 mg/l), under aerobic conditions, were degraded to undetectable levels after 10 days compared to 40-45 days under denitrification conditions. In contrast, these compounds showed no microbial degradation under anaerobic conditions for test durations of up to 10 weeks. Benzo[*a*]pyrene mineralisation was shown to be dependent on the redox potential in estuarine sediment (Delaune *et al.*, 1981). Under reduced conditions (-250 and 0 mV), mineralisation of benzo[*a*]pyrene was insignificant (0.09% and 0.15% respectively). However, as the redox potential was increased to +250 mV and +500 mV, benzo[*a*]pyrene mineralisation increased to 1.89% and 6.37% respectively.

1.7.6 Nutrient Availability

Nutrient availability, in particular nitrogen and phosphorus, plays and important role in the rate and extent of hydrocarbon degradation. Since microorganisms require nitrogen and phosphorus for incorporation into biomass, the availability of these nutrients within the same area as the hydrocarbon is critical (Atlas, 1981). Nutrient limitation may also inhibit the production of catabolic enzymes, inhibit mRNA synthesis required to induce

catabolic enzymes or limit the synthesis of plasmids that are involved in the degradative process (Lewis *et al.*, 1986). Researchers have estimated the optimal ratios of carbon to nitrogen and carbon to phosphorus for maximum hydrocarbon degradation rates. Optimal ratios ranged from 10:1 (Atlas and Bartha, 1973) to 60:1 (Dibble and Bartha, 1979) for carbon to nitrogen and 100:1 (Atlas and Bartha, 1973) to 800:1 (Dibble and Bartha, 1979) for carbon to phosphorus. These values varied depending on whether the fertiliser was applied to stimulate hydrocarbon degradation in seawater or in oily sludges.

The effect of nutrient amendments on the microbial utilisation of oil in soil has been assessed by a number of researchers (Jobson *et al.*, 1974; Odi, 1978). These studies have conclusively found that nitrogen and phosphorus amendments stimulate microbial growth and the rate of hydrocarbon utilisation. In addition, nutrient limitation may affect adaptation lag periods for microbial transformations. Lewis *et al.* (1986) observed that *p*-cresol degradation lag periods were up to 10-times longer for periphyton samples taken from field sites that were low in dissolved nitrogen and phosphorus (approximately 0.004 mg/l nitrogen, 0.009 mg/l phosphorus) compared to those with higher nutritional levels (approximately 0.4 mg/l nitrogen, 0.03 mg/l phosphorus). In addition, lag periods decreased in samples amended with nitrogen or phosphorus.

1.7.7 Heavy Metals

The presence of heavy metals can affect the rate of PAH degradation. Heavy metals such as lead, cadmium, mercury, zinc, copper, arsenic, chromium and nickel are often present in wood preserving and refinery wastes. Bowen (1996) demonstrated the inhibition of microbial activity with mercury (II), nickel (II), lead (II) and chromium (VI) at concentrations below 1 mg/l. Heavy metal toxicity can occur via enzyme inhibition, chelation with essential metals inhibiting their bioavailability, catalysis of essential metabolites or through impairing cell function or acting as antimetabolites (Gadd, 1991).

1.8 MEASUREMENT OF MICROBIAL DETOXIFICATION OF PAHs: TESTS AND THEIR APPLICATION

The biodegradation of a number of PAHs may be incomplete depending on the environmental conditions and the microbial population present. This is of concern as relatively little is known about whether biotransformations reduce the toxicity of the parent compound (Pothuluri *et al.*, 1992a). Measuring the loss of PAHs initially present

does not ensure that complete detoxification and immobilisation has occurred. Intermediate degradation products, which may be more mobile and toxic than the parent compound, may be generated as the initial compounds degrade (Dasappa and Loehr, 1991; Cerniglia and Heitkamp, 1989; Pothuluri *et al.*, 1992a) and as such pose a greater threat to human health. Numerous assays have been developed to determine the toxicity or mutagenicity of chemicals used in industrial applications and the food and pharmaceutical industries (Ames test, germ cell mutation test, somatic mutation and recombination tests, Tradescantia-micronucleus test, SOS chromotest and Microtox™ assay). In fact, since the 1940's about 30 different bioassays including animal, plant and bacterial systems have been used for the detection of mutagens (Ma *et al.*, 1984). Some of these assays have been adopted as monitoring tools in bioremediation to determine the efficacy of degradation and detoxification of hazardous wastes (Baud-Grasset *et al.*, 1993; Symons and Sims, 1988; Dasappa and Loehr, 1991; Aprill *et al.*, 1990). When combined with information from waste, site and soil characterisation, toxicity and mutagenicity data may be used in predictive mathematical models to:

(i) evaluate the effectiveness of on site bioremediation for the treatment of wastes in solid systems;
(ii) develop appropriate containment structures to prevent waste transport from the treatment zone; and
(ii) design performance monitoring systems (Aprill *et al.*, 1990).

Assays employing microorganisms are often used for testing toxicity due to their speed, simplicity, ease of handling, cost effectiveness and use of statistically significant numbers of test organisms that is required to detect the effect of potentially toxic materials in the environment (Sims, 1990). A number of assays have been developed to test the acute toxicity of aqueous samples. Conventional toxicity tests with fish, such as rainbow trout, guppy, flag fish and zebra fish, are expensive and time consuming. Assays normally require 48 to 96 hours exposure time plus an additional two to four weeks for acclimatisation and other preparations (Ribo and Kaiser, 1987). Microbial tests, based on the measurement of different indicators such as enzymatic activity, growth inhibition, reproduction rate, oxygen demand, metabolic light and heat release, have been used to assess the toxicity of potentially hazardous materials (Ribo and Kaiser, 1987; Aprill *et al.*, 1990; Symons and Sims, 1988; Elnabarawy *et al.*, 1988).

1.8.1 Tradescantia-Micronucleus (Trad-MCN) Test

The tradescantia-micronucleus test is a simple short term bioassay for gases, liquids and physical agents (Ma *et al.*, 1984). The test assesses the mutagenic potential of test compounds towards *Tradescantia paludosa* by its response in the production of micronuclei. Chromosome breaks, as a result of chemical treatment, can be observed as micronuclei in the synchronised tetrads. Micronuclei serve as the indicator of mutagenicity. *T. paludosa* has six pairs of large metacentric chromosomes in its pollen mother cells (Ma, 1982a, 1982b). Because chromosome fragments in a meiotic process usually result in micronuclei in the tetrad stage, the mutagen test was developed using micronuclei as the indicator of genetic damage (Ma *et al.*, 1978). The advantages of this system are that the chromosomes are in haploid numbers (n=6) and that the generative nucleus of the mature pollen is usually synchronised in the G_2 stage of interphase. Aberrations in the mature pollen are usually of the chromatid types (Ma, 1982a, 1982b). Plant cuttings of young inflorescences, in which the pollen mother cells undergo various stages of meiosis (pachytene and diplotene division), are exposed to the test chemicals. Chemicals are applied to the plant cuttings by either absorption of the soluble agents through the stem, peduncle or pedicle, by diffusion of gaseous agents through the leaves and buds or by *in situ* exposure to air pollutants. After chemical exposure, a recovery (meiotic) period is needed (24-30 hours) which allows the damaged chromosomes of early prophase I to proceed to the tetrad stage. Micronuclei are counted and the number serve as the indicator of mutagenicity.

The Trad-MCN test has been used to test the mutagenicity of common chemicals, beverages, drugs, pesticides, household chemicals, radiation and isotopes (Ma *et al.*, 1984). In addition, the test has been applied to the *in situ* monitoring of exhaust fumes and assessing the mutagenic potential of complex environmental mixtures. The environmental mixtures tested included aqueous forms found in lakes and wells (drinking water, well water with radium), gaseous or particulate forms from the air (air pollutant and diesel exhaust condensates, diesel exhaust fumes) and extracts from soil (water extract from lead smelter ground). Out of the 39 samples tested, 26 gave the same response for both the Trad-MCN test and the Ames test (Ma *et al.*, 1984). Examples of the use of this test in bioremediation are described in the following.

Reduction of the genotoxicity of a creosote-contaminated soil after fungal treatment was reported by Baud-Grasset *et al.* (1993). Creosote contaminated soil was inoculated with *P. chrysosporium* and incubated over an eight week period. Genotoxicity tests were conducted with *Tradescantia* clone 4430 cuttings, which were exposed for 30 hours to

different dilutions of soil extracts from the PAH contaminated soil before and after fungal treatment. Aqueous soil extracts were prepared by agitating soil samples in deionised water. Soils used for the genotoxicity assays included untreated soil, uninoculated, incubated soil and inoculated (*P. chrysosporium*), incubated soil. The inoculated soil showed a reduction in the PAH loading of up to 86%, with up to 79% removal of the four-ring compounds. A reduction in the control incubated soil also seen (49% PAH removal, with a 22% decrease in the four-ring compounds), which was accounted for by the presence of indigenous microflora capable of degrading PAHs. All of the soils gave a positive dose-related increase in micronucleus frequencies. After eight weeks incubation, the fungal-treated soil was two-fold less genotoxic at an extract concentration of 2%. Overt toxicity also appeared lower for the fungal treated soil, based on signs of overdose at an extract concentration of 4% before treatment but only at the 8% concentration after treatment (Baud-Grasset *et al.*, 1993). There was, however, an increase in the genotoxicity of the soil without fungal inoculation after the eight week incubation period. Baud-Grasset *et al.* (1993) accounted for this by the generation of water-soluble metabolic intermediates by indigenous microflora. It appeared that *P. chrysosporium* was capable of degrading the water soluble genotoxic metabolites.

1.8.2 *Escherichia coli* SOS Chromotest

The SOS chromotest is a quantitative bacteria colorimetric assay for genotoxins (Quillardet and Hofnung, 1985). It is based on the *E. coli* SOS response to DNA damage (Walker, 1984) as well as damage inducible (*din*) genes (Kenyon and Walker, 1980). The *E. coli* PQ37 tester strain used in the SOS chromotest carries a *sfiA::lacZ* fusion and has a deletion for the normal *lac* region (Quillardet *et al.*, 1982). *LacZ*, the gene which controls ß-galactosidase activity, is placed under the control of the *sfiA* gene (Huisman and d'Ari, 1981), an SOS function involved in cell division inhibition. As a consequence, ß-galactosidase activity is strictly dependent on *sfiA* expression. Two other mutations allow for increased responses to test compounds. The *uvrA* mutation renders the strain deficient in excision repair and therefore increases the response to certain DNA damaging agents. The *rfa* mutation allows for greater diffusion of chemicals into the cell by rendering the strain lipopolysaccharide deficient.

The assay consists of the tester strain incubated with increasing concentration of the test chemical. After two hours (time for protein synthesis), ß-galactosidase activity is assayed. Under extreme conditions, high concentrations of the test compound may inhibit protein synthesis which would lead to an underestimation of ß-galactosidase

Table 1.12. Genotypes of *S. typhimurium* strains used for mutagenesis testing (Maron and Ames, 1983).

Histidine Mutation[a]				LPS[b]	Repair[c]	R-factor[d]
hisD6610 his01242 =TA88	hisD3052	hisG46	hisG428 (pAQ1)			
TA90	TA1538	TA1535	-	*rfa*	Δ*uvrB*	-R
[TA97]	[TA98]	[TA100]	-	*rfa*	Δ*uvrB*	+R
-	TA1978	TA1975	-	*rfa*	+	-R
TA110	TA94	TA92	-	+	+	+R
-	TA1534	TA1950	-	+	Δ*uvrB*	-R
-	-	TA2410	-	+	Δ*uvrB*	+R
TA89	TA1964	TA1530	-	Δ*gal*	Δ*uvrB*	-R
-	TA2641	TA2631	-	Δ*gal*	Δ*uvrB*	+R
-	-	-	[TA102]	*rfa*	+	+R

[a]All strains were originally derived from *S. typhimurium* LT2. Wildtype genes are indicated by a +. Tester strains in brackets are recommended for general mutagenesis testing.

[b]LPS mutations cause an increase in cell wall permeability. The *rfa* mutation causes partial loss of the lipopolysaccharide barrier of the cell wall. The Δ*gal* strains and the *rfa/uvrB* strains have a single deletion through *gal, chl, bio, uvrB*. The *rfa* repair$^+$ strains have a mutation *galE*.

[c]The *uvrB* mutation is a deletion of a gene coding for the DNA excision repair system. The deletion (Δ) through *uvrB* also includes the nitrate reductase (*chl*) and biotin (*bio*) genes.

[d]The R-factor plasmid, pKM101, carries an ampicillin resistance gene.

activity. To correct for this, protein synthesis is estimated during the incubation period by assaying alkaline phosphatase in parallel with ß-galactosidase. The assay may also be performed with the inclusion of a microsomal activation preparation (Maron and Ames, 1983) in the incubation mixture. The assay is quantitative and dose-response curves present a linear region. The slope of the linear region, named the SOS-inducing potency (SOSIP), reflects the inducing ability of the test compound.

The SOS chromotest has been used to assess the genotoxicity of a number of test compounds. PAHs have been shown to exhibit high genotoxicity when incubated in the presence of an exogenous metabolic activation system (Mersch-Sundermann *et al.*, 1992). In particular, the high molecular weight PAHs, such as fluoranthene, chrysene, benzo[*a*]pyrene, benzo[*j*]fluoranthene and benzo[*g,h,i*]fluoranthene exhibited high genotoxic activities. Anthracene, benzo[*a*]fluorene, coronene, phenanthrene, fluorene, naphthalene, pyrene and perylene showed little or no response.

1.8.3 Ames Test

The Ames test has been extensively used for testing the mutagenicity of individual compounds as well as complex environmental and biological mixtures (Maron and Ames, 1983). The test is a mutational reversion assay employing several strains of *S. typhimurium* (Table 1.12), each of which has a different mutation in the histidine biosynthesis operon. The strains also contain other mutations which increase their ability to detect mutagens.

(i) *rfa* mutation: this mutation increases the permeability of the cell wall by eliminating the lipopolysaccharide barrier. This allows large molecules to penetrate the cell.

(ii) *uvB* mutation: the *uvB mutation* results in increased sensitivity for detecting mutagens. These organisms have the gene encoding for the DNA excision repair system deleted. The deletion of the *uvB* genes extends into the *bio* gene and as a consequence these strains require biotin for growth.

(iii) *his*46 mutation: Strains TA100 and TA1535 contain the *his*46 mutation which codes for the first enzyme of histidine biosynthesis. This mutation substitutes proline for leucine in the wild type organism and as such detect mutagens that cause base pair substitutions.

(iv) *his*D3052 mutation: strains TA98 and TA1538 contain the *his*D3052 mutation which codes for histidinol dehydrogenase. These strains detect various frameshift mutations (Maron and Ames, 1983).

Strains TA98 and TA100 also contain the plasmid *pkm*101. This plasmid codes for ampicillin resistance and a highly error-proned DNA repair system.

In the Ames test (overlay method) the *S. typhimurium* strains are exposed to suspected mutagens in molten agar and overlayed on a minimal medium containing glucose. To ensure that DNA replication takes place in the presence of the mutagen, trace amounts of histidine and biotin are added to the molten agar. Initially, the histidine autotrophs will grow in the presence of the test compound until the histidine is depleted. Once the histidine supply is exhausted, only revertants that have mutationally regained the ability to synthesis histidine will grow (Ames, 1972). The visible colonies are counted and are compared to controls in order to estimate the relative mutagenicity of the compound; more colonies, indicate higher mutagenicity.

Often a mammalian liver extract is added to the molten top layer before plating. The extract converts the potential mutagens into electrophilic derivatives that will readily react with DNA (Ames, 1972). The conversion or activation of PAHs by the mammalian system is catalysed by at least two enzyme systems. The initial step, catalysed by the cytochrome P450-dependent, mixed-function oxidase, transforms the parent PAH compound to phenols and arene oxides. Arene oxides are then hydrolysed by epoxide hydrolases to form dihydrodiols, which then may undergo further oxidation to form diol epoxides. These compounds are highly mutagenic and act as the ultimate carcinogen (Dipple and Bigger, 1991). Since bacteria do not contain this activation system, the liver extract is added to promote the transformations that occur in mammals. The addition of the liver extract illustrates which compounds have intrinsic mutagenicity and which ones require activation after uptake.

Numerous studies incorporating the Ames test have demonstrated that PAHs are compounds with mutagenic potential. Positive mutagenic responses have been found with fluoranthene (Bos *et al.*, 1987; Pothuluri *et al.*, 1992b; Mercsh-Sundermann *et al.*, 1992), pyrene (Mercsh-Sundermann *et al.*, 1992), benz[*a*]anthracene (Bos *et al.*, 1984; Mercsh-Sundermann *et al.*, 1992), chrysene (Mercsh-Sundermann *et al.*, 1992), benzo[*a*]pyrene (Zeiger *et al.*, 1979; Wood *et al.*, 1976; Bos *et al.*, 1984 Maron and Ames, 1983; Mercsh-Sundermann *et al.*, 1992; Andrews *et al.*, 1978; Phillips, 1983; Dipple and Bigger, 1991), dibenz[*a,h*]anthracene (Mercsh-Sundermann *et al.*, 1992; Andrews *et al.*, 1978; Dipple and Bigger, 1991) and coronene (Mercsh-Sundermann *et al.*, 1992) in the presence of the mammalian liver extract. Mutagenic assays of mixtures with PAHs are often performed with the assumption that the mutagenic potential of the

mixture approximates the sum of the mutagenicities of the individual components (Haugen and Peak, 1983). A number of studies have demonstrated synergistic and antagonistic effect of PAH mixtures on bacterial mutagenicity (Nylund *et al.*, 1992; Hermann, 1981; Haugen and Peak, 1983; Shahin and Fourier, 1978; Petrilli *et al.*, 1980). Several non-mutagenic unsubstituted PAHs have been shown to enhance the mutagenicity of high molecular weight PAHs, while some mutagenic PAHs produce a large decrease in the mutagenic potential of benzo[*a*]pyrene when combined (Hermann, 1981). Studies on coal-derived oil (Haugen and Peak, 1983), tar-sand fractions (Shahin and Fourier, 1978) and shale oil fractions (Petrilli *et al.*, 1980) have shown that these complex PAH mixtures have the ability to decrease the mutagenicity of indirect mutagens like benzo[*a*]pyrene. The inhibition of mutagenicity with complex mixtures may be due to interactions of P450 metabolism, assuming that structural analogues inhibit epoxide formation. The enhancement of benzo[*a*]pyrene mutagenicity in the presence of unsubstituted low molecular weight PAHs may be due to enhancement of epoxide hydrolysis by P450 metabolism (Hermann *et al.*, 1980).

While these studies give us an understanding of the potential mutagenicity of single PAHs, individual PAHs within a PAH mixture and PAH mixtures, relatively little is known about the decrease or increase in mutagenic potential of PAHs degraded by microorganisms. Pothuluri *et al.* (1992b) demonstrated the fungal detoxification of fluoranthene (FA) by *C. elegans*. The metabolism of FA by *C. elegans* produced five metabolites; 3-FA-ß-glucopyranoside, 3-(8-hydroxy-FA)-ß-glucopyranoside, FA-*trans*-2,3-dihydrodiol, 8-hydroxy-FA-*trans*-2,3-dihydrodiol and 9-hydroxy-FA-*trans*-2,3-dihydrodiol. These compounds were tested for their mutagenic activities towards *S. typhimurium* strains TA100 and TA104 in the presence of a rat liver homogenate fraction. The parent compound displayed a positive dose-related mutagenic response in both of the *S. typhimurium* test strains. 9-Hydroxy-FA-*trans*-2,3-dihydrodiol showed a weak positive response to *S. typhimurium* TA100, where as the other metabolites were negative in both strains. A time course mutagenicity assay performed with extracted samples (24 hour intervals for 120 hours) indicated that mutagenic activity decreased with time.

Aprill *et al.* (1990) also observed a reduction in the mutagenicity of soils contaminated with wood preserving and petroleum waste. The dose-response curve for the creosote sludge/soil mixture showed a decrease in the number of revertants as the dose increased beyond 100 mg soil/plate in the presence of the mammalian liver extract. This indicated that the waste mixture was toxic to the Ames strain (TA98) at these concentrations. A positive dose-response curve for the "slop oil" emulsion solids was observed with and

without the presence of the mammalian liver extract, indicating that components other than PAHs were also responsible for the mutagenic potential of the waste. After 350 days incubation, significant decreases in the concentration of non-carcinogenic and carcinogenic PAHs were observed. In addition, neither of the contaminated-waste soils exhibited a positive mutagenic response. The decrease in mutagenic potential of the slop oil emulsion solids and the creosote sludge amended soil correlated with the decrease in the concentration of PAHs in the waste soils.

Although the Ames test has been used to screen a wide variety of potential carcinogens by a number of government, industrial and academic laboratories, the test has some technical and theoretical limitations. The Ames test reveal mutants by a restoration of enzymatic activity caused by the mutagenic activity of the test compound (indirect mutagenesis). However, any mutation that does not reconstruct the precise DNA sequence that codes for the histidine-synthesising enzyme is not observed (Devoret, 1979). For example, a large number of antitumour drugs, that work by damaging the DNA of tumour cells, exhibit a false-negative response in the Ames test such that they fail to induce the histidine mutation (Devoret, 1979).

Some chemicals exhibit a false-positive response where they exhibit mutagenic activity in bacterial and mammalian cells without being carcinogenic. These results may raise some doubts about the validity of the Ames test in identifying potential carcinogens. The incorporation of an analogue of one of the nitrogenous bases and the methylation of certain sites on the bases causes negligible alteration in DNA structure that are not sensed in the cell as DNA damage. DNA replication continues, however, the new DNA carries a different coded message (Devoret, 1979). This form of mutagenesis is termed direct mutagenesis. In the Ames test, the mutagenic activity of a test compound is correlated with carcinogenesis in mammals through indirect mutagenesis which results from DNA damage (Devoret, 1979).

1.8.4 Microtox™ Test

The Microtox™ system is a standardised acute toxicity test which uses a suspension of bioluminescent bacteria (*Photobacterium phosphoreum*) as the biological indicator of toxicity. The assay is based on the measurement of light emission from the bacteria (metabolic activity); in the presence of a toxic aqueous sample, *P. phosphoreum* will become "challenged" and a decrease in light output will result. A dose-response curve for each sample can be determined. The EC_{50}, the concentration of the sample required to reduce the light output by 50% over a specified time period, can be calculated which

indicates the relative toxicity of a set of samples (Symons and Sims, 1988). In addition, the bacterial response to chemicals tested may be dependent on the exposure time. Some chemicals need longer to react with the test organism while others react within a few seconds.

Light emission by *P. phosphoreum* results from the interaction of the enzyme luciferase, reduced flavin and a long chain aldehyde (Ribo and Kaiser, 1987). It occurs in the presence of oxygen and constitutes part of the cells electron transport system. The emission of light depends on the flow of electrons and therefore the light output is an indicator of the metabolic activity of the general health of the organism.

The Microtox™ system has been used as a monitoring tool in demonstrating the detoxification of hazardous wastes in environmental samples. Huling *et al.* (1995) demonstrated the detoxification of soil contaminated with wood preserving waste using this system. The concentrations of indicator compounds (PCP, pyrene and total carcinogenic PAHs [benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene and indeno[*1,2,3-c,d*]pyrene]) were also monitored in the soil by taking both composited and discrete samples. Over the incubation period (53-60 days), significant decreases in the concentration of the indicator compounds were observed. PCP concentration in the discrete samples decreased by 58% after 53 days, while a 60% decrease in concentration was observed in the composited after 60 days. Pyrene concentration decreased by 49% and 52% respectively, while the total carcinogenic PAH concentration decreased by 50% and 67%. The decrease in the concentration of the indicator compounds correlated with the decrease in toxicity of aqueous soil extracts. The toxicity of soil extracts decreased from day 1 (EC_{50}: 6.6) to day 53 when seven of the 10 samples had no response at any dose.

The Microtox™ bioassay was applied to a land treatment system to assess the detoxification of a complex hazardous waste (Symons and Sims, 1988). The waste contained a cocktail of heavy metals (arsenic, barium, cadmium, chromium, cobalt, lead, mercury, nickel and vanadium), monoaromatics (benzene, ethyl benzene, toluene, xylene, cresol and phenol) as well as PAHs (two- to five-rings). The waste material was applied to soils at three concentrations (two, four and eight percent) and the concentration of individual compounds, as well as the toxicity of aqueous soil extracts were determined over a 180 day period. EC_{50}'s were determined for each soil loading and the detoxification rate was determined by plotting the $\ln[EC_{50}(t)/EC_{50}(t=0)]$ vs time. The degradation of individual PAHs correlated with the decrease in Microtox™

toxicity for soils containing two and four percent loadings. No correlation was observed for the high loading (eight percent) in batch reactors even though significant decreases in PAH concentrations were observed. Presumably other constituents of the waste mixture contributed to the toxic response in the Microtox™ system or intermediates produced from the degradation of the PAHs accumulated to toxic concentrations.

An increase in the toxicity of the water soluble fraction of an API separator sludge-amended soil was observed during its treatment by Aprill *et al.* (1990). Although a 71% decrease in the concentration of non-carcinogenic PAHs and a 24% decrease in the concentration of carcinogenic PAHs was observed over the treatment period, this did not correlate to a decrease in Microtox™ toxicity. The EC_{50} of soil extracts decreased from 82.1±19.35% at day zero to 18.3±3.2% at day 340. The degradation of PAHs is known to result in the formation of more polar intermediate metabolites, which are more water soluble than the parent compound and are more likely to leach from the soil solid phase. Presumably, the degradation of PAHs by the indigenous microflora lead to the formation and accumulation of intermediate metabolic products which resulted in the increase in toxicity of the soil leachate.

Although the Microtox™ system is a standardised acute toxicity assay, the test has limitations with respect to compounds that have low water solubilities. PAHs are characterised by their low water solubilities and as such aqueous extracts of PAH contaminated soil will not reflect the degree of contamination and toxicity of the sample. Even though metabolites produced from the degradation of PAHs are more water soluble than the parent compound, the intermediate products may still have limited water solubilities. Data from Microtox™ tests of aqueous extracts from soil samples will reflect the degree of toxicity of water soluble compounds, however, little information will be gained on the total toxicity of the soils.

In an attempt to gain a better understanding of the total toxicity/genotoxicity of contaminated soils being biologically treated, Aprill *et al.* (1990) performed Microtox™ and Ames tests, in combination with chemical tests, to evaluate the extent of microbial degradation and detoxification of PAH-containing wastes. Although the decrease in mutagenic potential of the PAH-containing wastes correlated with the decrease in concentration of total PAHs, an increase in Microtox™ toxicity was observed over the test period. Aprill *et al.* (1990) attributed the increase in toxicity to the production of water soluble metabolites from the degradation of PAHs. To aid in predicting the fate of soil detoxification by microbial processes, the metabolic pathways of PAH degrading microorganisms need to be elucidated. This will allow researchers to predict whether

microorganisms are suitable for the decontamination of particular compounds. In addition, microorganisms that can mineralise PAHs would be advantageous as the accumulation of possibly toxic metabolites would not occur.

1.9 SCOPE AND OBJECTIVES OF THE THESIS

The work presented in this thesis examines the microbial degradation of high molecular weight PAHs. The objectives for the study were to:

(i) Enrich, isolate and identify microorganisms capable of degrading high molecular weight PAHs;
(ii) Determine the PAH degradative capabilities of the microorganisms in liquid and soil matrices; and
(iii) Take some preliminary steps in identifing catabolic pathways for the degradation of high molecular weight PAHs.

CHAPTER 2

MATERIALS AND METHODS

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2.6.2.1 *Degradation of PAHs in PAH-Spiked Soil*

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2.7 **ANALYTICAL METHODS**

2.7.1 Determination of Cellular Protein Levels

2.7.2 Phenolic (PAH Intermediate) Assay

2.7.3 Extraction of PAHs from Culture Fluids and Soil

2.7.3.1 *Extraction of PAHs or Creosote from Culture Fluid*

2.7.3.2 *Extraction of Pyrene, Benzo[a]pyrene and Dibenz[a,h]anthracene Metabolites from Culture Supernatants*

Table 2.1. Source of bacterial strains used in this study.

Microorganism[a]	Culture Collection Number	Source	Medium[b]
Stenotrophomonas maltophilia	VUN 10,001	PAH-contaminated soil	BSM+pyrene
Stenotrophomonas maltophilia	VUN 10,002	PAH-contaminated soil	BSM+pyrene
Stenotrophomonas maltophilia	VUN 10,003	PAH-contaminated soil	BSM+pyrene
Stenotrophomonas maltophilia	VUN 10,075	VUT Culture Collection	NA
Pseudomonas aeruginosa	VUN 0030	VUT Culture Collection	NA
Escherichia coli	VUN 0100	VUT Culture Collection	NA
Proteus mirabilis	-	VUT Culture Collection	NA
Bacillus cereus	VUP 0001	VUT Culture Collection	NA
Photobacterium phosphoreum	VUN 2050	VUT Culture Collection	PP
Salmonella typhimurium TA98	VUN 0040	VUT Culture Collection	HBAMGM
Salmonella typhimurium TA100	VUN 0041	VUT Culture Collection	HBAMGM

[a]Stock cultures of microorganisms were stored in glycerol at -80°C.

[b]Microorganisms were revived from glycerol stocks by plating the organisms onto an appropriate medium: BSM+pyrene, basal salts medium sprayed with an etheral solution of pyrene (2% w/v); NA, Nutrient Agar; PP, PP medium; HBAMGM, histidine/biotin/ampicillin minimal glucose medium.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

All strains used in this work were stored in the Centre for Bioprocessing and Food Technology (CBFT) culture collection and given Victoria University strain numbers. Their origin and properties are shown in Table 2.1. The research focused on the three strains that were isolated from PAH-contaminated soil (VUN 10,001, VUN 10,002 and VUN 10,003). Strains were stored at -80°C in glycerol stocks. Cultures were revived from storage by inoculating a loopful of each strain into an appropriate medium.

2.2 GENERAL METHODS

Fine chemicals were weighed using a Mettler AE200 analytical balance (FSE); large quantities of media and chemical were weighed using an AC-4K balance (Denver Instrument Company). All media and stock solutions were prepared using ultra pure water produced using a Millipore Milli Q ultra pure water system (Millipore). The pH of media and stock solutions was measured using a HI 8418 pH meter (Hanna Instruments). Optical densities were measured using a Ultrospec III UV/Vis spectrophotometer (Pharmacia). Centrifugation was performed with a Bechman J2-HS centrifuge with a JA 14 rotor. Media and stock solutions were sterilised by autoclaving at 121°C for 20 minutes, with the exception of media used in the 10 litre fermenter, which was autoclaved at 121°C for 40 minutes. Unless otherwise stated, solutions were filter sterilised using 0.20 μm disposable filters (Sartorius Minisart). Soil isolates were incubated on an orbital shaker (Paton Scientific OI3422) in the dark at 30°C/175 opm. PAH-exposed glassware was cleaned by rinsing with dichloromethane, followed by soaking in Extran 300 (BDH) for 24 hours. Glassware was rinsed with tap water followed by distilled water. All other glassware was washed with Extran 300 and rinsed appropriately.

2.3 MATERIALS

2.3.1 Procurement of Materials

Chemicals, solvents, reagents, enzymes and microbiological media were purchased from Sigma, Aldrich, BDH, Ajax, EM Science, Cambridge Isotope Laboratory, Pierce, Boehringer, Biolabs, Promega, Oxoid, Difco and Gibco. All chemicals, solvents and reagents were of analytical grade of higher. Where the source of the reagent is important for the experimental outcome, sources are specified.

2.3.2 Stock Solutions

Ames Salts Solution: (Maron and Ames, 1983) KCl (123.0 g) and $MgCl_2.6H_2O$ (81.4 g) were dissolved in one litre of H_2O. The salts solution was sterilised by autoclaving and stored at room temperature.

Ampicillin Solution: Ampicillin was dissolved in sterile H_2O at a concentration of 25 mg/ml. The stock solution was stored at 4°C and discarded after three months.

1 M Ammonium Acetate: Ammonium acetate (77.1 g) was dissolved in one litre of H_2O. The stock solution was sterilised by autoclaving and stored at room temperature.

Bovine Serum Albumin (BSA): The stock solution of bovine serum albumin (5% w/v) was diluted in 0.45M NaH_2PO_4 to achieve a concentration of 100 µg/ml BSA. The BSA solutions were stored at 4°C.

Creosote Solutions: Creosote (Sparko, Australia) stock solutions were prepared with dimethylformamide (DMF) at ratios of 1:1, 1:10, 1:100, 1:1,000 and 1:10,000 (creosote:DMF). Solutions were stored in the dark at 4°C.

Denatured Salmon Sperm DNA: Salmon sperm DNA was prepared in sterile H_2O at a concentration of 1 mg/ml and denature at 95°C for 10 minutes prior to use. After denaturation, the salmon sperm DNA was place on ice before use.

50X Denhardt's Reagent: (Sambrook *et al.*, 1989) Ficoll (5 g), polyvinylpyrrolidone (5 g) and bovine serum albumin (5 g) were dissolved in 500 ml H_2O and stored at 4°C.

2% Deoxycholate (v/v): Deoxycholate (20 ml) was dissolved in 980 ml of H_2O. The stock solution was stored at room temperature.

EC Lysis Solution: (Cantor *et al.*, 1988) The composition of EC lysis solution was 6 mM Tris Cl (pH 7.6), 100 mM EDTA (pH 7.5), 1 M NaCl, 20 µg/ml DNase free RNase, 10 mg/ml Lysozyme, 0.5% Sarkosyl and 0.2% Deoxycholate. The EC lysis solution was prepared prior to use by combining stock solutions of the respective constituents and diluting with the appropriate volume of distilled H_2O.

0.5 M EDTA (pH 7.5, 8.0 and 9.0): Disodium ethylenediaminetetra-acetate.$2H_2O$ (186.1 g) was dissolved in 800 ml of H_2O. The pH was adjusted to the appropriate pH with the addition of NaOH pellets. The stock solutions were sterilised by autoclaving and stored at room temperature.

ESP: (Cantor *et al.*, 1988) The composition of ESP was 0.5 M EDTA (pH 9.0), 1 mg/ml Proteinase K and 1.0% Sarkosyl. ESP was prepared prior to use by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O.

Ethidium Bromide: Ethidium bromide (1 g) was dissolved in 100 ml of H_2O. The solution was stored in the dark at 4°C.

Folin-Ciocalteau Reagent: Dilute Folin reagent was prepared by diluting Folin-Ciocalteau reagent with distilled water (2:3). The reagent was prepared immediately before protein assays.

Glucose Solution: Glucose was dissolved in H_2O at a concentration of 50 mg/ml and sterilised by filtration. The stock solution was stored at room temperature.

40% Glucose Solution (w/v): Glucose (400 g) was dissolved in one litre of H_2O. The stock solution was sterilised by filtration and stored at room temperature.

1 M Glucose-6-phosphate Solution: Glucose-6-phosphate was dissolved in distilled H_2O at a concentration of 0.282 g/ml. The solution was stored at -20°C.

0.5 mM Histidine/Biotin Solution: Biotin (123.6 mg) and Histidine (96.0 mg) were dissolved in one litre of H_2O. To assist the dissolution of biotin, the solution was

heated. The histidine/biotin solution was sterilised by autoclaving and stored at room temperature.

Hybridisation Wash (First): (Sambrook *et al.*, 1989) The washing solution consisted of SSC (2X) and SDS (0.1%). The hybridisation wash solution was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at room temperature and heated to 65°C prior to use.

Hybridisation Wash (Second): (Sambrook *et al.*, 1989) The washing solution consisted of SSC (0.2X) and SDS (0.1%). The hybridisation wash solution was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at room temperature and heated to 65°C prior to use.

Lowry Reagent A: (Lowry *et al.*, 1951) Reagent A consisted of 2% Na_2CO_3 in 0.1M NaOH. The solution was stored at room temperature.

Lowry Reagent B: (Lowry *et al.*, 1951) Reagent B consisted of 0.5% $CuSO_4.5H_2O$ in 1% Na-K-tartrate. The pH was adjusted to 9.0 and the solution was stored at 4°C.

Lowry Reagent C: (Lowry *et al.*, 1951) Reagent C was prepared by mixing Lowry reagent A with Lowry reagent B at a ratio of 50:1. Lowry reagent C was prepared immediately before protein assays and discarded after one day.

Lysozyme Solution: Lysosyme was dissolved in TE (pH 8.0) to achieve a final concentration of 5 mg/ml. Lysosyme solution was prepared immediately prior to use.

Magnesium/Calcium Solution: The magnesium/calcium solution consisted of $MgSO_4.7H_2O$ (400 mg) and $CaCl_2.2H_2O$ (400 mg) per litre of H_2O. The solution was filter sterilised and stored at 4°C.

5 M NaCl: Sodium chloride (292.2 g) was dissolved in one litre of H_2O. The stock solution was sterilised by autoclaving and stored at room temperature.

0.1 M NADP Solution: NADP was dissolved in distilled H_2O at a concentration of 77.6 mg/ml. The solution was stored in the dark at -20°C.

5 M NaOH: Sodium hydroxide (200 g) was dissolved in one litre of H_2O. The stock solution was stored at room temperature.

PAH stock solutions: Stock solutions of each PAH were prepared in dimethylformamide (DMF) (Cerniglia and Yang, 1984) at the following concentrations: 100 mg/ml, phenanthrene and pyrene; 25 mg/ml, fluorene, phenanthrene and pyrene, 10 mg/ml, fluorene, phenanthrene, fluoranthene, pyrene and benz[*a*]anthracene; 5 mg/ml, dibenz[*a,h*]anthracene and benzo[*a*]pyrene and 1 mg/ml coronene. Stock mixtures of selected PAHs (fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene or phenanthrene, pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene) were also prepared at a concentration of 5 mg/ml for each PAH. PAH stock solutions were stored in the dark at 4°C.

Peptone Solution: Peptone was dissolved in H_2O at a concentration of 50 mg/ml and sterilised by autoclaving. The stock solution was stored at room temperature.

0.45 M Phosphate Solution: Phosphate solution was prepared by dissolving $NaH_2PO_4.2H_2O$ (70.2 g) in one litre of H_2O. The solution was sterilised by autoclaving and stored at room temperature.

PIV: (Cantor *et al.*, 1988) The composition of PIV was 10 mM Tris Cl (pH 7.6) and 1 M NaCl. PIV was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was sterilised by autoclaving and stored at 4°C.

Prehybridisation/hybridisation Solution: (Sambrook *et al.*, 1989) Hybridisation solutions consisted of Denhardt's solution (5X), SSC (6X), SDS (0.5%) and denatured salmon sperm DNA (1 mg/ml). Solutions were prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. Hybridisation solutions were stored at 4°C.

Proteinase K Solution: Proteinase K was dissolved in TE (pH 8.0) to achieve a final concentration of 20 mg/ml. Proteinase K solution was prepared immediately prior to use.

Resorcinol Solution: Resorcinol was dissolved in H_2O at a concentration of 20 mg/l. The stock solution was filter sterilised and stored at 4°C.

Ringer's Solution (1/4 Strength): Ringer's solution was prepared by dissolving one Ringer's solution tablet in 500 ml of H_2O. The solution was sterilised by autoclaving and stored at room temperature.

RNase Solution: DNase free RNase was dissolved in sterile H_2O to achieve a final concentration of 1 mg/ml. RNase solution was prepared immediately prior to use.

S9 Mixture (4%): (Maron and Ames, 1983) The S9 mixture (4%, Moltox) contained Rat Liver S9 (2 ml), Salts Solution (1.0 ml), 1 M Glucose-6-phosphate (0.25 ml), 0.1 M NADP (2.0 ml), 0.2 M Sodium Phosphate Buffer (pH 7.4) (25.0 ml) and 19.75 ml sterile H_2O. The mixture was prepared prior to use and stored on ice.

Salicylic Acid Solution: Salicylic acid was dissolved in H_2O at a concentration of 50 mg/ml and sterilised by autoclaving. The stock solution was stored at room temperature.

10% Sarkosyl (v/v): Sarkosyl (100 ml) was dissolved in 900 ml of H_2O. The stock solution was stored at room temperature.

20% SDS: Electrophoresis grade SDS (200 g) was dissolved in 800 ml of H_2O. The solution was heated to 68°C to assist dissolution. The volume was adjusted to one litre with H_2O and the pH to 7.2 by the addition of HCl. The stock solution was stored at room temperature.

Sodium Acetate (pH 4.8): NaC_2H_3O (164 g) was dissolved in one litre of H_2O. The pH was adjusted to 4.8 by the addition of HCl. The solution was sterilised by autoclaving and stored at room temperature.

0.2 M Sodium Phosphate Buffer (pH 7.4): Sodium phosphate buffer consisted of $NaH_2PO4.H_2O$ (27.6 g/l) (120 ml) and Na_2HPO_4 (28.4 g/l) (880 ml). The buffer was sterilised by autoclaving and stored at room temperature.

Southern Blot Denaturing Solution: (Sambrook *et al.*, 1989) The composition of the southern blot denaturing solution was 1.5 M NaCl and 0.5 M NaOH. Denaturing solution was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The solution was stored at room temperature.

Southern Blot Neutralising Solution: (Sambrook *et al.*, 1989) The composition of the southern blot neutralising solution was 1M ammonium acetate and 0.02 M NaOH. Neutralising solution was prepared prior to use by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O.

20X SSC: (Sambrook *et al.*, 1989) NaCl (175.3 g) and sodium citrate (88.2 g) were dissolved in one litre of H_2O. The pH was adjusted to 7.0 with the addition of NaOH. The stock solution was stored at room temperature.

STE: (Sambrook *et al.*, 1989) The composition of STE was 0.1 M NaCl, 10 mM Tris. Cl (pH 8.0) and 1 mM EDTA (pH 8.0). STE was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at room temperature.

Succinate Solution: Succinate was dissolved in H_2O at a concentration of 50 mg/ml and sterilised by autoclaving. The stock solution was stored at room temperature.

25% Sucrose Solution (w/v): Sucrose (250 g) was dissolved in one litre of H_2O. The stock solution was sterilised by filtration and stored at room temperature.

10x TAE Buffer: (Sambrook *et al.*, 1989) Trizma base (48.5 g), 0.25 M EDTA (pH 8) (50 ml) and glacial acetic acid (11.4 ml) were added to 938.6 ml H_2O. The stock solution was stored at room temperature.

50X TAE: (Sambrook *et al.*, 1989) Tris base (242 g), glacial acetic acid (57.1 ml) and 0.5 M EDTA (pH 8.0) (100 ml) were added to 842.9 ml of H_2O. The stock solution was stored at room temperature.

TBE: (Sambrook *et al.*, 1989) The composition of TBE was 0.09 M Tris Borate and 0.002 M EDTA (pH 8.0). TBE was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at 4°C.

TE Buffer (pH 8): (Sambrook *et al.*, 1989) The composition of TE Buffer was 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0). TE buffer was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at 4°C.

TES Buffer (pH 8): (Sambrook *et al.*, 1989) The composition of TES Buffer was 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0) and 25% sucrose. TES buffer was prepared by combining stock solutions of the respective constituents and diluting with the appropriate volume of H_2O. The stock solution was stored at room temperature.

Trace Element Solution: (Bogardt and Hemmingsen, 1992) The trace element solution consisted of $FeSO_4.7H_2O$ (200 mg), $ZnSO_4.7H_2O$ (10 mg), $MnCl_2.4H_2O$ (3 mg), $CoCl_2.6H_2O$ (20 mg), $CuCl_2.2H_2O$ (1 mg), $NiCl_2.6H_2O$ (2 mg), $Na_2MoO_4.2H_2O$ (500 mg) and H_3BO_4 (30 mg) per litre of H_2O. The solution was filter sterilised and stored at 4°C.

2 M Tris (pH 8): Trisma Base (106 g) and Trisma HCl (177.6 g) were dissolved in one litre H_2O and sterilised by autoclaving. The stock solution was stored at room temperature.

2 M Tris.Cl (pH 7.6 and 8.0): Tris (hydroxymethyl) methylammonium chloride (315.2 g) was dissolved in one litre of H_2O. The pH was adjusted to the appropriate value by the addition of NaOH. The stock solutions were sterilised by autoclaving and stored at room temperature.

Vitamin Solution: (Bogardt and Hemmingsen, 1992) The vitamin solution consisted of biotin (20 mg), folic acid (20 mg), thiamine HCl (50 mg), D-calcium pantothenate (50 mg), vitamin B_{12} (50 mg), riboflavin (50 mg), niacin (200 mg), pyridoxal HCl (30 mg) and *p*-aminobenzoic acid (20 mg) per litre of H_2O. The solution was filter sterilised and stored at 4°C.

Yeast Extract Solution: Yeast extract was dissolved in H_2O at a concentration of 50 mg/ml and sterilised by autoclaving. The stock solution was stored at room temperature.

2.3.3 Media Composition

All media was prepared with ultra high purity water. Where solid media was used, plates were poured once the agar had cooled to 50°C.

Basal Salts Medium (BSM) (this thesis)

BSM was composed of (per litre); K_2HPO_4 (0.4 g), KH_2PO_4 (0.4 g), $(NH_4)_2SO_4$ (0.4 g) and NaCl (0.3 g) and was sterilised by autoclaving. Sterile vitamin, trace element and magnesium/calcium solutions (5 ml/l each) were added to autoclaved BSM. Growth substrates and PAH stock solutions were added to the BSM prior to inoculation. When solid media was required, BSM was supplemented with 12.0 g/l of agar.

Basal Salts Yeast Extract Peptone Agar (BYP) (Foght *et al.* 1990)

BYP was composed of (per litre); K_2HPO_4 (0.5 g), NH_4Cl (1.0 g), Na_2SO_4 (2.0 g), KNO_3 (2.0 g), $MgSO_4.7H_20$ (0.2 g) Yeast Extract (Difco) (1.0 g) Proteose Peptone No. 3 (Difco) (1.0 g) and Agar (15.0 g). BYP was sterilised by autoclaving.

Creosote Yeast Extract Medium (CYEM) (this thesis)

CYEM was composed of (per litre); K_2HPO_4 (0.4 g), KH_2PO_4 (0.4 g), $(NH_4)_2SO_4$ (0.4 g), NaCl (0.3 g), yeast extract (1.0 g/l) and creosote (Sparko) (2.0 ml/l). Creosote was added to the medium after autoclaving.

Histidine/Biotin/Ampicillin Minimal Glucose Medium (HBAMGM) (Maron and Ames, 1983)

Histidine biotin ampicillin plates consisted of Minimal Media (933 ml), 40% Glucose Solution (50 ml), 0.5 mM Histidine Solution (10 ml), 0.5 mM Biotin Solution (6 ml), Ampicillin Solution (1 ml) and agar (12 g). Glucose and ampicillin solutions were added to the medium after autoclaving and once the agar had cooled to 50°C. Plates were poured after these solutions had been added to the medium.

Histidine/Biotin Minimal Glucose Medium (HBMGM) (Maron and Ames, 1983)

Histidine biotin plates consisted of Minimal Media (934 ml), 40% Glucose Solution (50 ml), 0.5 mM Histidine Solution (10 ml), 0.5 mM Biotin Solution (6 ml) and agar (12 g). Histidine/biotin medium was sterilised by autoclaving. Glucose solution was added to the medium after autoclaving and once the agar had cooled to 50°C. Plates were poured after the glucose had been added to the medium.

Minimal Glucose Medium (MGM) (Maron and Ames, 1983)

Minimal glucose medium consisted of Minimal Medium (950 ml), 40% Glucose Solution (50 ml) and Agar (12 g). Minimal glucose medium was sterilised by autoclaving. Glucose solution was added to the medium after autoclaving and once the agar had cooled to 50°C. Plates were poured after the glucose had been added to the medium.

Minimal Medium (MM) (Maron and Ames, 1983)

Minimal medium was composed of (per litre); KH_2PO_4 (3.0 g), Na_2HPO_4 (6.0 g), NH_4Cl (2.0 g), NaCl (5.0 g) and $MgSO_4.7H_2O$ (0.1 g). The medium was sterilised by autoclaving.

Nutrient Agar (NA)

The medium was prepared by adding of Nutrient Agar (28.0 g, Oxoid) to one litre of H_2O. Nutrient agar was sterilised by autoclaving.

Nutrient Broth (NB)

The medium was prepared by dissolving Nutrient Broth (13.0 g, Oxoid) in one litre of H_2O. Nutrient broth was sterilised by autoclaving.

Nutrient Broth II (NBII)

The medium was prepared by dissolving Nutrient Broth II (25.0 g, Oxoid) in one litre of H_2O. Nutrient broth II was sterilised by autoclaving.

PP Medium (Simonov, personal communications)

PP medium consisted of Nutrient Broth II (12.5 g), Yeast Extract (5.0 g), NaCl (27.5 g) and glycerol (3.0 ml) per litre of H_2O. The medium was sterilised by autoclaving.

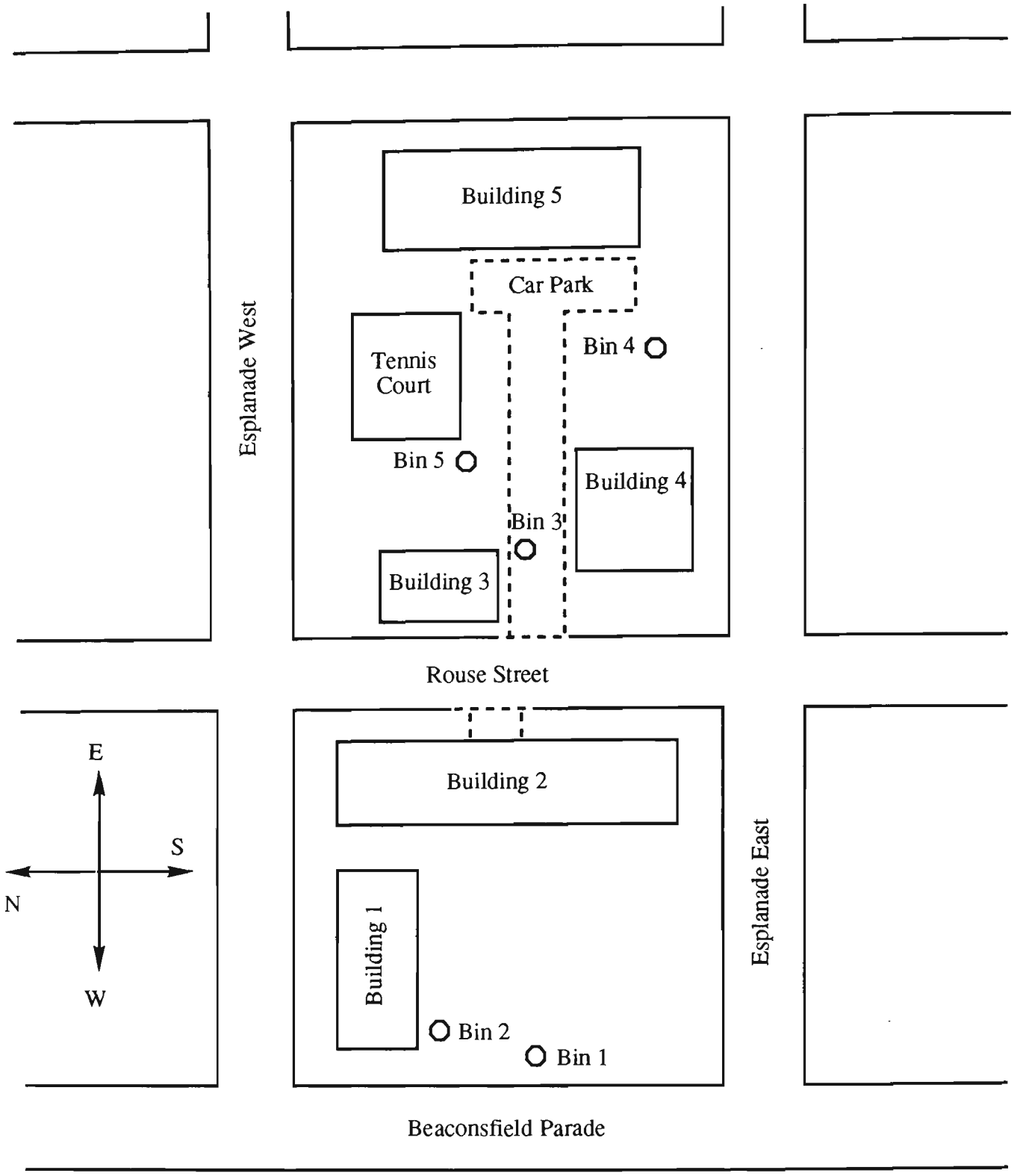


Figure 2.1. Schematic representation of the Port Lonsdale site from where contaminated soil samples were obtained. Soils were sampled from five 280 litre sealed bins (bins 1-5) located at various positions on the site. Soils were collected (0.5-1.0 kg) in 1.5 litre plastic screw capped jars and were stored at 4°C until used.

Peptone, Yeast Extract, Glucose Medium (PYEG)

PYEG was composed of (per litre); K_2HPO_4 (0.4 g), KH_2PO_4 (0.4 g), $(NH_4)_2SO_4$ (0.4 g), NaCl (0.3 g), Yeast Extract (Difco) (1.0 g) Proteose Peptone No. 3 (Difco) (1.0 g) and glucose (1.0 g) and was sterilised by autoclaving.

Top Agar (Maron and Ames, 1983)

Top agar consisted of NaCl (5.0 g) and Agar (6.0 g) per litre of H_2O. Top agar was sterilised by autoclaving and maintained in a molten state (45°C) prior to use.

2.4 MICROBIOLOGICAL METHODS

2.4.1 Enrichment of PAH-Degrading Microorganisms from PAH-Contaminated Soil

Soils for enrichment studies were obtained from an abandoned factory site located near Port Melbourne, Victoria, Australia. The site previously housed a manufacturing gas plant and recently was used as a defence facility site (for more information see page 159). Previously cored soils from various positions on the site were housed in 280 litre sealed bins (Figure 2.1). Samples (0.5-1.0 kg) were collected from five sample sites (designated bins one to five) from the top 10 cm of the cores. Soils were collected in 1.5 litre plastic screw capped jars and were stored at 4°C until used.

Inocula for PAH-enrichment cultures were obtained by shaking 20 g (wet weight) of contaminated soil overnight in 100 ml of 1/4-Strength Ringer's Solution at 30°C/175 opm. After shaking, soil suspensions were left to stand for 1 hour to allow for soil particles to settle out. Suspension supernatants (5 ml) were used to inoculate BSM (45 ml) containing individual PAHs (phenanthrene, pyrene, benz[*a*]anthracene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene) with final PAH concentrations of 50 mg/l for the five-ring compounds and 100 mg/l for the three- and four-ring compounds. Enrichment cultures were incubated for up to 10 weeks. When visible growth had occurred, as determined by observations of cultures and microscopy (Olympus CH2, Selby Anax), 5 ml of cultures were transferred to fresh BSM containing PAH (45 ml). Enrichments were subcultured through three successive transfers in the respective PAH medium. Subsequently, pyrene-degrading pure cultures were then isolated from selected microbial communities.

2.4.2 Enrichment of PAH-Degrading Microorganisms on Aromatic and Non-Aromatic Substrates

The enrichment of a pyrene-degrading microbial community (community five) on aromatic and non-aromatic carbon sources was performed using a number of substrates in BSM. The inocula for enrichments were grown in BSM (400 ml) containing pyrene (250 mg/l) as the sole carbon and energy source. Pyrene-enriched microorganisms (5 ml) were inoculated into BSM (45 ml) containing peptone (1 g/l), yeast extract (1 g/l), glucose (1 g/l), succinate (1 g/l), salicyclic acid (1 g/l) or creosote (2 ml/l). Enrichments were subcultured after three days incubation for three successive subcultures. The growth of enrichment cultures was established by the increase in protein concentration (see Section 2.7.1). Enrichments were also performed in BSM containing creosote (2.0, 0.2. 0.02, 0.002 or 0.0002 ml/l) with the addition of peptone, yeast extract or glucose (0.1, 0.5, 1.0 or 2.0 g/l).

2.4.3 Isolation of PAH-Degrading Microorganisms

The microbial community from bin five, enriched on pyrene, was used for the isolation of PAH-degrading pure cultures using a spray plate technique (Kiyohara *et al.*, 1982a) with pyrene as the sole carbon source. Ten-fold serial dilutions of community five were prepared in 1/4-Strength Ringers Solution to a dilution factor of 10^{-6}. Dilutions (0.1 ml) were transferred onto BSM agar and plated using a sterile glass plate spreader. Immediately thereafter, an etheral solution of pyrene (2% w/v) was uniformly sprayed over the surface of the agar plates using a Preval Power Spray Unit (Precision Valve Corporation). The ether instantaneously volatilised leaving a thin white layer of pyrene on the agar surface. The pyrene spray plates were incubated at 30°C (Thermoline model I00FA) for up to 21 days. Petri dishes were sealed with parafilm to retain moisture. Pyrene-degrading cultures were visualised by a distinct pyrene free zone surrounding individual colonies. Individual pyrene-degrading pure colonies were streaked onto BSM agar, sprayed with pyrene and incubated. Once the purity of the isolated colonies was established, colonies were removed from agar plates and transferred to BSM containing pyrene (100 mg/l). Microorganisms were routinely subcultured in BSM containing pyrene. Pure cultures were given Victoria University of Technology culture collection numbers for Gram negatives (VUN).

2.4.4 Phenotypic Identification of PAH-Degrading Microorganisms

For identification, microorganisms were inoculated into standard diagnostic biochemical substrates and reactants (MacFadden, 1980; Palleroni, 1984) (see Appendix 1) after growth on BYP agar plates (Foght *et al.*, 1990); Gram staining properties were also noted.

2.4.5 Inoculum Preparation for Evaluating PAH-Degradation Profiles

Inocula (mixed and pure cultures) for PAH degradation and growth experiments (see Section 2.6.1.1 and 2.6.1.3) were prepared in 100 ml serum bottles containing BSM (45 ml) and pyrene (100 mg/l) as the sole carbon and energy source. Serum bottles were stoppered with neoprene septa (Alltech) and sealed with aluminium crimp caps. The medium was inoculated with pyrene-grown cultures (5 ml) and incubated for up to 10 days. Filter sterilised air (0.20 µm Sartorius Minisart) (100 ml) was introduced into the bottles every second day.

Some experimental protocols (Sections 2.6.1.4, 2.6.1.5, 2.6.1.6 and 2.6.2.1) required high initial cell numbers of pyrene-degrading microorganisms as the inoculum. These were prepared as follows: community five, VUN 10,002 and VUN 10,003 were grown in a fermenter (Applikon) containing BSM (10 litres) with pyrene (250 mg/l) as the sole carbon and energy source. The fermenter was incubated at 30°C with air supplied at a rate of 10 litres/min and agitation maintained at 250 rpm. Following the complete degradation of pyrene (5-7 days), cells were harvested by centrifugation (JA 14 rotor) at 5,000 rpm for 10 minutes at 4°C. Cell pellets were washed twice in 1/4-strength Ringer's solution and resuspended in BSM to achieve a 10-fold concentration in cell biomass.

Inocula used to determine degradation of PAHs in contaminated soil (see Section 2.6.2.2) were prepared in two, 1.5 litre volumes of CYEM. Cultures were incubated for three days on a rotary shaker at 30°C/175 opm. Cells were harvested by centrifugation (JA 14 rotor) at 5,000 rpm for 10 minutes at 4°C. Cell pellets were washed twice in 1/4-strength Ringer's solution and resuspended in BSM to achieve a 10-fold concentration in biomass.

Inocula used for large-scale degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene (see Section 2.6.1.6) were prepared in 60 litre bioreactors containing BSM (40 litres) and pyrene (250 mg/l). Bioreactors consisted of galvanised rubbish bins containing

coiled aeration tubes. The air supply was obtained from a compressor pump (Dynavac Engineering Pty Ltd, Model OD1) which also provided mixing. Cells were collected by centrifugation as mentioned above. Washed cell pellets were resuspended in 500 ml BSM.

2.4.6 Substrate Range of PAH-Degrading Microorganisms

Pyrene-enriched microorganisms were tested for their ability to grow on a variety of carbon sources: succinate, pyruvate, salicylic acid, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, Tween 80, catechol, benzene, 4-chlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, pentachlorophenol, hexane, nitrobenzene, cinnamic acid, gentisic acid, phthalic acid, toluene and octane. Test compounds were added to BSM (20 ml) as the sole carbon and energy source (50 mg/l) in 100 ml flasks sealed with neoprene stoppers. Volatile compounds were supplied to the microorganisms in the vapour phase: the liquid form of the volatile compounds (0.5 ml) were added to test tubes (75 x 10 mm) which were placed into the inoculated medium. Flasks were inoculated with 0.1 ml (1-3 μg protein per ml) of the respective pyrene-enriched microorganisms (community four, community five, VUN 10,001, VUN 10,002 and VUN 10,003) and incubations were performed in duplicate for each set of culture conditions. The evaluation of growth was carried out by visual monitoring and scored relative turbidity after seven days incubation.

2.4.7 Microtox™ Test (Ribo and Kaiser, 1987)

The toxicity of PAH containing culture supernatants and aqueous soil extracts were evaluated using *P. phosphoreum* in a modified Microtox™ assay. The modification to the assay allowed for the monitoring of light output over an extended period of time. Culture fluids (2 x 1.5 ml) were routinely removed from PAH containing media incubated with community five, VUN 10,002 or VUN 10,003. Controls consisted of uninoculated PAH media or media containing autoclaved cells. Cellular material and undegraded PAHs were removed from the fluid by centrifugation in a bench top microfuge at 14,000 rpm for one minute (Eppendorf 5415C Centrifuge). NaCl (30 mg) was added to the supernatants (1.0 ml) to achieve a final concentration of 3% (w/v). Soil samples (5 g) were diluted in deionised water (20 ml) and shaken for 2 hours at 22°C. The soil was allowed to settle out and the liquid was centrifuged for 15 minutes at 3,000 rpm (Symons and Sims, 1988). The salinity of the supernatant was adjusted to 3% (w/v) by adding solid NaCl. An overnight culture of *P. phosphoreum* was prepared in PP medium at 24°C. Toxicity assays were performed by the addition of *P.*

phosphoreum (10 µl) to dilutions (1.0 ml) of PAH culture supernatants or soil extracts. The light output of *P. phosphoreum* (RLU) was monitored for up to 108 minutes using a Liquid Scintillation Counter (Wallac 1410, Pharmacia) (see section 2.7.9).

2.4.8 **Ames Test** (Maron and Ames, 1983)

2.4.8.1 *Confirmation of Genotypes*

The genotypes of the Ames strains (*S. typhimurium* TA98 and TA100) were tested immediately after reviving the cultures from -20°C glycerol stocks. The histidine auxotrophic phenotype of the strains was confirmed by demonstrating that histidine was required for growth on minimal glucose agar. Biotin is also required by the strains because of the *uvrB* deletion which extends through the *bio* genes. The strains were streaked onto minimal glucose agar plates containing histidine or biotin or histidine and biotin. Cultures were also plated onto minimal glucose plates lacking the growth requirements. Plates were incubated at 37°C for 24-48 hours. Histidine and biotin auxotrophs resulting from *uvrB* deletion (Ames and Maron, 1983) were indicated by growth on plates containing both histidine and biotin.

Ampicillin resistance (R-factor) of strains TA98 and TA100 was determined by streaking onto minimal glucose plates containing 25 µg/ml ampicillin. After incubation at 37°C (Thermoline Incubator Model I100FA) for 24-48 hours, plates were observed for growth. Strains TA98 and TA100 contain an R-factor which encodes ampicillin resistance and which enables growth in the presence of 25 µg/ml ampicillin.

The *rfa* mutation was demonstrated by testing the sensitivity of the strains to crystal violet. An overnight culture of each strain (0.1 ml) was added to 2.0 ml of molten top agar (45°C). After mixing, the agar was evenly distributed over a nutrient agar plate and allowed to solidify on a level surface. A sterile filter paper disc (10 mm) impregnated with crystal violet (10 µl of a 1 mg/ml solution) was placed onto the centre of the overlayed nutrient agar plate. Plates were incubated at 37°C (Thermoline Incubator Model I100FA) for 24-48 hours. A clear zone of inhibition (approximately 14 mm) is observed around the crystal violet impregnated disc for *S. typhimurium* strains with the *rfa* mutation. The *rfa* mutation permits large molecules, such as crystal violet to enter the cell, resulting in cell death. Wild-type strains or strains containing the *gal* deletion are not inhibited because the crystal violet cannot penetrate the cell.

2.4.8.2 *PAH-Dose-Related Mutagenic Response Curves*

The toxic and mutagenic effects of three-, four-, five- and seven-ring PAHs, PAH mixtures and extracts from PAH-contaminated soils were tested over a range of concentrations to determine the dose-related mutagenic response curve for each compounds and *S. typhimurium* strains. Individual PAHs were tested at concentrations of 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 µg/plate while soil extracts from PAH-contaminated soils were tested at a number of dilutions. The PAH mixtures were tested at the above concentrations for each individual PAH in the mixture. Stock solutions of the PAHs as well as soil extracts were prepared in dimethylformamide (DMF). After soils (1 g) were extracted with DCM (see Section 2.7.3.5), aliquots of the DCM extract (0.1 to 2.0 ml) were dried under a gentle stream of nitrogen and resuspended in DMF (0.1 ml). Top agar (2.0 ml) was distributed into test tubes (75 x 10 mm) held in a 45°C heating block (Thermoline Scientific Equipment). Fresh overnight cultures of strains TA98 or TA100 (0.1 ml) and the test chemicals (0.1 ml) were added to the molten top agar and mixed by vortexing (setting 3, MT19 Chiltern) for 2-3 seconds. When activation of PAHs by rat liver microsomal preparations (S9 fraction) was evaluated, 0.5 ml of the S9 fraction was added to the tester strain/chemical/top agar mixture. The top agar was evenly distributed onto minimal glucose plates and allowed to solidify on a level surface. Five replicate plates were poured for each test chemical and strain combination. Negative controls containing the tester strain, S9 and DMF without test chemical were prepared to estimate the number of revertants that arise from spontaneous mutations. The plates were incubated at 37°C for 48 hours. The number of revertant colonies were counted using a colony counter (Stuart Scientific). Dose-response curves were prepared by plotting the net revertants against the concentration of test chemical (µg/plate) or dilution. To obtain net revertants, the average number of spontaneous revertants was subtracted from the average of counts for tests.

2.4.8.3 *Mutagenic Potential of PAH Culture Extracts*

The dose-response curves for the individual PAHs, PAH mixtures and soil extracts demonstrated the concentration range at which these compounds could be tested before they reached toxic concentrations. Extracts of PAH containing cultures were diluted appropriately in DMF so that PAH concentrations were below toxic levels for *S. typhimurium* strains. The appropriate dilutions were maintained throughout the mutagenicity experiments. Mutagenicity assays for the liquid culture and soil extracts were performed as described above using *S. typhimurium* strains TA98 and TA100 with and without exogenous (S9) activation. In addition, culture supernatants and aqueous

soil extracts were assayed for mutagenicity. Culture fluids (2 x 1.5 ml) were centrifuged at 14,000 rpm for one minute (Eppendorf benchtop centrifuge 5415C) to remove undegraded PAHs and cellular material. Aqueous soil extracts were prepared as described for the Microtox™ test (see Section 2.4.7). Supernatants (0.1 and 1.0 ml), S9 (0.5 ml) and the *Salmonella* strains (0.1 ml) were added to top agar (2.0 ml) and plated onto minimal glucose agar. After incubation (37°C for 48 hours) (Thermoline Incubator Model I100FA) the number of revertant colonies were counted and the number of net revertants calculated.

2.4.9 Determination of Most Probable Numbers (MPNs)

Microbial numbers in liquid and soil cultures were determined by the most probable number technique. 10-Fold serial dilutions of soil and culture fluids were made in 1/4-strength Ringer's solution to a dilution of 10^{-10}. Sterile Nutrient Broth (225 µl) was dispensed into 96-well disposable ELISA trays (Disposable Products). Triplicate wells were inoculated with 25 µl of the respective dilutions. ELISA trays were incubated at 30°C for 48 hours. After incubation, growth was scored by observing the presence or absence of turbidity. The viable count was estimated from the results using statistical tables (Taylor, 1962).

2.5 MOLECULAR BIOLOGY METHODS

2.5.1 Isolation of Total Genomic DNA (Scott *et al.*, 1981)

Strains VUN 10,001, VUN 10,002 and VUN 10,003 were grown in 100 ml of NB overnight. Cells were harvested in 50 ml polycarbonate centrifuge tubes at 7,000 rpm for 10 minutes at 4°C (JA 21 rotor). The cell pellets were washed with TES buffer (10 ml) and centrifuged as above. The pellets were resuspended in TES buffer (5.0 ml) and 0.25 M EDTA (0.4 ml, pH 8.0) and lysozyme solution (0.2 ml) were added to the cell suspensions then the tubes were incubated at 37°C (Ratex Instruments waterbath) for 10-15 minutes. Warm SDS (0.1 ml of 20% solution at 65°C), RNase A solution (0.5 ml) and sterile water (2.0 ml) were added to the suspended cells and incubated at 37°C for 30 minutes. After incubation, proteinase K (82 µl of 20 mg/ml in TE), which had been autodigested at 37°C for 60 minutes, was added to the samples to achieve a final concentration of 0.2 mg/ml. Samples were further incubated at 37°C for 60 minutes then TE (8.0 ml) and phenol (16 ml) were added. Samples were shaken vigorously for 10 seconds and incubated in a horizontal position on an orbital shaker (SS60, Chiltern)

at room temperature with gentle agitation for 60 minutes. Once incubation was complete, the samples were centrifuged at 15,000 rpm for 20 minutes at 4°C (JA 21 rotor). A sterile bent sterile pasteur pipette was used to remove the top aqueous layer without disturbing the white protein precipitate at the interface between the aqueous and organic phases. The top aqueous layer was transferred to sterile polyallomer tubes, while the precipitates and the bottom aqueous layer were discarded. Phenol (8.0 ml) and chloroform:isoamylalcohol (8.0 ml of 24:1 v/v solution) were added to each tube. After mixing, the tubes were incubated on an orbital shaker at room temperature for 60 minutes. Samples were centrifuged at 15,000 rpm for 20 minutes at 4°C (JA 21 rotor) then the phenol:chloroform:isoamylalcohol extraction of the aqueous phase was repeated. The accumulated DNA solutions obtained from the phenol:chloroform:isoamylalcohol extractions were divided into precooled (-20°C) Corox glass tubes in 2.5 ml aliquots. Sodium acetate (250 µl, pH 4.8) and ice cold absolute ethanol (5.0 ml) were added to DNA aliquots. The tubes were sealed with parafilm, mixed and incubated on ice overnight. A white stringy DNA precipitate formed upon mixing. The DNA precipitate was collected by centrifugation at 10,000 rpm for 30 minutes at 4°C (JA 21 rotor) and by carefully decanting the ethanol. Ice cold ethanol (70%, 10 ml) was added to the DNA pellets and further centrifuged for 25 minutes. The ethanol was decanted and the pellets left to dry for one to two hours by inverting them on a clean dry paper towel at room temperature. When dry, the DNA pellets were resuspended in sterile TE (100-300 µl) and stored at -20°C.

2.5.2 Determination of Quality and Quantity of DNA

2.5.2.1 Spectrophotometric Method (Sambrook *et al.*, 1989)

Genomic DNA extracts (10 µl) were added to sterile distilled water (990 µl). The optical density of the diluted DNA extracts were measured at 260 and 280 nm using an Ultrospec III UV/Vis spectrophotometer (Pharmacia). Sterile distilled water was used as the zero. The reading at 260 nm allowed for calculation of nucleic acid in the sample: an optical density of one corresponded to approximately 50µg/ml double stranded DNA. The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid. Pure preparations of DNA had an OD_{260}/OD_{280} value of 1.8. If there was contamination with protein or phenol, the OD_{260}/OD_{280} value was less.

2.5.2.2 **Agarose Plate Method** (Sambrook *et al.*, 1989)

A 1.0% agarose solution containing ethidium bromide (2 µl) was prepared in 1 x TAE buffer (50 ml). DNA samples were prepared in sterile distilled water (5 µl) containing loading buffer (2 µl) and DNA (3 µl). The samples (10 µl) were loaded into the gel and run for one hour at 100 V with 1 x TAE as the running buffer. DNA concentration standards (varying from 1-20 µg/ml) were run in parallel with the DNA samples. The quantity of DNA was estimated after photography of the gel by comparing the intensity of the sample DNA bands with the DNA markers.

2.5.3 Construction and Design of Oligonucleotide Primers for 16SrRNA Gene Amplification

Because the strains were biochemically most similar to *Bu. cepacia*, the design of oligonucleotide primers for 16SrRNA gene amplification was based on the initial phenotypic identification of the three strains. Primers were designed using a multiple sequence alignment of *Bu. cepacia* strains. A set of primers, designated as MAS2F and MAS2R, corresponding to positions 18-1495 of the DNA sequence of *Bu. cepacia* strain DSM 50181 (Genbank excession number X87275) was used for amplification. Primers were prepared by a service provided at CBFT.

3.5.4 **PCR Amplification of 16SrRNA Gene Regions** (Perkin Elmer, 1992)

PCR reactions were prepared in a total volume of 100 µl. The reactions contained 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each of the dNTPs (dATP, dCTP, dGTP, dTTP), 2.5 units Ampli Taq DNA polymerase, 1 µM of each of the primers and 500-700 ng of total genomic DNA. The PCR cycle consisted of an initial denaturation step at 97°C for seven minutes followed by 35 cycles of 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing) and 72°C for 2 minutes (extension time). At the end of the cycling, a final extension time of 5 minutes was used. DNA concentration was determined using the agarose plate method (see Section 2.5.2.2).

2.5.5 **Purification of PCR Products: Wizard PCR Preparations** (Promega, 1993)

PCR products were purified from contaminants, primers-dimers and amplification primers by Wizard preparations (Promega). Mini columns were prepared by removing the plunger from a 3 ml disposable syringe (Talus Manufacturing, Sydney) and

attaching a mini column to the syringe barrel. DNA purification resin (1.0 ml, Promega) was added to the PCR products and vortexed for 20 seconds. The resin/DNA were mixed for 20 seconds and the vortexing/inversion procedure repeated a further two times. The resin/DNA mixtures were pipetted into the syringe barrels and carefully pushed into the mini columns with the plunger. Isopropanol (80%, 2 ml) was used to wash the columns. The columns were removed from the syringes, placed in 1.5 ml Eppendorf tubes and centrifuged for 20 seconds at 12,000 g to dry the resins. The columns were transferred to new Eppendorf tubes and 50 µl of sterile water was applied to the columns. After two minutes, the columns were centrifuged (20 seconds at 12,000 g) to elute the bound DNA fragments. The purified DNA was stored at -20°C until use.

2.5.6 Sequencing of PCR Products

Preparation of templates for automated sequencing was performed using the Dye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Samples were submitted for automated sequencing to the Department of Microbiology, Monash University (joint VUT-Monash University facility).

2.5.7 Analysis of DNA Sequences

DNA sequences were analysed using the database similarity search program (BLASTn) with the assistance of Dr Maria Serafica (Centre for Bioprocessing and Food Technology, Victoria University of Technology). BLASTn was accessed through ANGIS or through the Internet at *www.angis.su.org.au*.

2.5.8 Genomic DNA Digests (Sambrook *et al.*, 1989)

Genomic DNA digests were prepared with DNA isolated from VUN 10,001, VUN 10,002 and VUN 10,003. DNA (500 and 1,000 ng) was digested with *Sma I* (Biolabs) and *Eco 01091* (Biolabs) endonucleases (20 units) with the addition of NEB4 restriction buffer (3 µl, Biolabs) and BSA (3 µl, Biolabs) where appropriate. Sterile water was added to DNA digests to achieve a final digestion volume of 50 µl. *Sma I* and *Eco 01091* digests were incubated at 25°C and 37°C respectively for 24 hours. Digested samples were loaded onto agarose gels (0.8% in 1 x TAE) containing ethidium bromide (75 µl), with 1 x TAE as the running buffer. Samples were heat inactivated at 65°C for 20 minutes prior to the addition of loading buffer (1 µl loading buffer per 5 µl digested sample). Digested DNA samples (10-20 µl) were loaded into

Table 2.2. Enzymes used for restriction digestion of purified *St. maltophilia* 16SrRNA gene PCR amplification products. DNA was digested with 10 units of the respective enzymes, restriction buffer (3 µl) and BSA (3 µl) where appropriate. The reaction time for DNA digests was 3.5 hours at the indicated temperatures.

Enzyme	Restriction Buffer (10 x)	BSA Required	Reaction[a] Temperature (°C)
BssH II	NEB3	Yes	60
Eco 01091	NEB4	No	37
Hinf I	NEB2	No	37
Hpa II	NEB1	No	37
Rsa I	NEB1	No	37
Sma I	NEB4	No	37

[a]Manufacturers instructions.

wells (GNA 200 gel tank, Pharmacia) and gels were run at 30 volts for 16 hours using an EPS 500/400 electrophoresis power supply (Pharmacia). A lambda DNA, cut with *Hind III* and *EcoR1,* was used as the molecular marker (size range 125-21,226 bp). Bands were observed by placing the gel on a transilluminator (LKB 2011 Macrovue, Bromma, 302 nm) and sizes determined by comparison with the lambda marker.

2.5.9 **Restriction Digest of Purified 16SrRNA Gene PCR Amplification Products** (Sambrook *et al.*, 1989)

Restriction digests of purified 16SrRNA gene products were performed with the enzymes outlined in Table 2.2. DNA (1 µg) was digested with 10 units of the respective enzymes (Biolabs), restriction buffer (3 µl, Biolabs) and BSA (3 µl, Biolabs) where appropriate. Sterile water was added to DNA digests to achieve a final digest volume of 30 µl. The reaction time for DNA digests was 3.5 hours. Samples were heat inactivated after digestion at 65°C for 20 minutes, with the exception of *BssH II* digests. Loading buffer (1 µl) was added to aliquots of digested DNA (5 µl) and samples were applied to agarose gels (1.0% in 1 x TAE) containing ethidium bromide (15 µl). 1 x TAE was used as the running buffer. Probase 50TM (Progen) was used as the molecular marker (size range 50-3,147 bp). Separation of restriction fragments was obtained by running the gel at 80 volts for 60 minutes using an EPS 500/400 electrophoresis power supply (Pharmacia). Bands were observed by placing the gel on a transilluminator (LKB 2011 Macrovue, Bromma, 302 nm) and sizes determined by comparison with the Probase 50TM marker.

2.5.10 **Southern Blotting** (Sambrook *et al.*, 1989)

Genomic DNA was restriction digested and run on gels in preparation for southern blotting. Gels were placed in denaturing solution for 45 minutes at room temperature with gentle agitation. Gels were transferred to neutralising solution and agitated for 20 minutes. Excess solution was removed by blotting the gels on filter paper. The gels were transferred to perspex boards (DNA side up) and precut nitrocellulose membranes (Hybons, Amersham) were placed on top. Air bubbles were removed by rolling the membranes with a 10 ml pipette. Three layers of filter paper (Whatman 3 mm Chromatographic paper) were applied to the membranes plus approximately 4 cm of absorbent paper towelling. A second perspex board was applied on top of the paper towel and a 1 kg weight placed on top. Gels were blotted overnight. After blotting the membranes were washed in 2 x SSC for 5 minutes, blotted on filter paper to remove excess solution and wrapped in glad wrap. Membranes were exposed to UV light

(LKB 2011 Macrovue transilluminator, Bromma) for 5 minutes to fix the DNA to the membrane then stored at room temperature.

2.5.11 Hybridisation and Probing of Southern Blots (Sambrook *et al.*, 1989)

Southern blot membranes were placed in hybridisation bags and preheated prehybridisation solution (65°C) was added (30 ml). Air bubbles were carefully removed and the plastic bags were double sealed using a heat sealer (Venus Packaging Machines Pty, Ltd, model UHIB 300). Membranes were incubated at 65°C overnight (Extron HI2001, Bartelt Instruments Pty Ltd). Labelling of the probe was performed using High Prime Labelling mixture (Boehringer). The PCR probe was denatured at 95°C for 10 minutes in a single dry block heater (Ratex Instruments). Denatured DNA (25 ng) was added to High Prime Labelling mix (4 µl), ^{32}P (20 µCi, 3,000 Ci/mmol) and the volume was adjusted to 25 µl with sterile water. Samples were centrifuged for 5 seconds at 14,000 rpm (Eppendorf benchtop centrifuge, 5415C) and incubated at 37°C for 10 minutes. The labelling reaction was stopped by the addition of 0.2 M EDTA (pH 8.0, 2 µl). Transfer RNA (2 µl of tRNA stock solution, 5 µg/ml) and TE buffer (75 µl) were added to the labelled samples and centrifuged for 5 seconds at 14,000 g. Samples were placed on ice while sephadex G50 columns were prepared.

G50 columns consisted of syringe barrels (1 ml, Terumo Medical Corporation, USA) plugged with sterile glass wool. G50 in TE buffer was added to the syringe barrels up to the 1 ml mark. Columns were placed in disposable centrifuge tubes (10 ml) and centrifuged for 30 seconds (Spinette centrifuge, International Equipment Company, USA). STE buffer (100 µl) was added to the columns and the columns were centrifuged for 30 seconds (Spinette centrifuge, International Equipment Company, USA). TE buffer (100 µl) was applied to the columns and the columns were centrifuged as above. Capless Eppendorf tubes were placed inside new centrifuge tubes (10 ml). The G50 columns were placed inside the centrifuge tubes, the labelled probes were added to the columns and the columns were centrifuged for 30 seconds. The syringe barrel was discarded and the contents of the Eppendorf tubes were transferred to capped Eppendorf tubes. TE buffer (200 µl) was added to the probes and the samples were denatured at 95°C for 10 minutes in a single dry heating block (Ratek Instruments). Labelled probes were placed on ice until needed.

Probes were added to hybridisation bags containing southern blot membranes by cutting one end of the bag. Probes were added, air bubbles removed and the bags were resealed and incubated at 65°C overnight (Extron HI2001, Bartelt Instruments Pty Ltd).

After incubation, the membranes were removed and placed into the first hybridisation wash solution. Membranes were washed at room temperature for 10 minutes. Wash solutions were replaced until the radiolabel being removed had decreased to negligible levels (less than 2 counts/second). The membranes were placed in the second hybridisation wash solution and incubated at 65°C for 5 minutes. Excess solution was removed from the membranes by blotting them on filter paper (Whatman 3 mm Chromatography paper) and membranes were wrapped in cling wrap. Membranes were placed in an X-ray cassette with an intensifying screen and X-ray film (Amersham Hyperfilm-MP) was added to the cassettes in the dark. Cassettes were incubated at -70°C for 12-24 hours after which the films were developed.

2.5.12 Pulse Field Gel Electrophoresis (PFGE) (Cantor *et al.*, 1988)

2.5.12.1 *Preparation of Agarose Blocks*

VUN 10,001, VUN 10,002 and VUN 10,003 were grown overnight in 100 ml of NB. Cells of VUN 10,001, VUN 10,002 and VUN 10,003 were collected by centrifugation at 4,000 rpm for five minutes (Hetlich Universal bench top centrifuge). The pellets were resuspended in PIV (5 ml), centrifuged for five minutes (as above) and washed similarly a further four times. After washing, the pellets were resuspended in PIV (10 ml final volume) and incubated on ice for 10 minutes. Suspensions were transferred to a 37°C shaking waterbath (Ratex Instruments) and incubated for a further 10 minutes. After incubation, the cells suspensions were divided into aliquots (500 µl) in Eppendorf tubes and warm 1% agarose in PIV (500 µl) added to each tube. After mixing thoroughly, suspensions were placed in agarose block molds (Pharmacia). Molds were incubated on ice for 1-2 hours.

Agarose blocks were removed from molds and placed in freshly prepared EC Lysis solution (20 ml). Blocks were incubated at 37°C for 4-5 hours. After incubation, the blocks were transferred into ESP solution and incubated at 45°C overnight. Blocks were washed twice in TE buffer containing phenyl methyl sulphyl fluoride (0.00175 mg/ml) for one hour at 45°C . Thereafter, the blocks were washed in TE buffer for 30 minutes at 45°C. The TE washing procedure was repeated a further three times. After washing, the blocks were stored in TE buffer at 4°C until further use.

2.5.12.2 *Restriction Digestion of Agarose Blocks*

Half an agarose block was used for each restriction endonuclease digest. Blocks were cut in half using a sterile scalpel blade. The blocks were washed twice in TE buffer, at 45°C for 15 minutes. Each half block was digested overnight at 37°C in sterile deionised water (115 µl) containing 40 units of the enzyme *SpeI* (Biolabs), NEB2 restriction buffer (15 µl, Biolabs) and BSA (1 mg/ml, 15 µl, Biolabs). Digested blocks were washed four times in TE buffer at 45°C before being transferred into the loading wells of a 1% agarose gel in 0.1 x TBE.

2.5.12.3 *PFGE of Agarose Blocks*

Pulse field gel electrophoresis was performed using a Clamped Homogenous Electric Field (CHEF) Apparatus (Pharmacia) with HEX electrode. The electrical parameters used were: voltage- 170 V, temperature- 12°C and field strength- 10 V/cm. The running buffer was 0.1 x TBE and the pulse and running times used were:

(i) 5 seconds for 24 hours.
(ii) 10 seconds for 12 hours followed by 15 seconds for 18 hours.
(iii) 20 seconds for 30 hours.
(iv) 25 seconds for 12 hours followed by 30 seconds for 18 hours.
(v) 40 seconds for 40 hours.
(vi) 1 second for 12 hours followed by 5 seconds for 15 hours.
(vii) 5 seconds for 10 hours, 25 seconds for 10 hours followed by 30 seconds for 15 hours.

After PFGE, gels were stained in ethidium bromide (0.5 µg/ml) for 30 minutes to two hours. Gels were viewed under UV light (302 nm) and fragment sizes estimated by comparison with Lambda *Hind*III fragments (size range 0.1-200 kbp, Biolabs), Lambda *Xho*I fragments (size range 15-300 kbp, Biolabs), Lambda ladder (48.5 kbp concatamers, Biolabs) and yeast chromosome marker (size range 225-1,900 kbp, Biolabs). The level of similarity between between different PFGE patterns were calculated using the Dice coefficient (Dice, 1945).

2.5.13 Photography of Agarose Gels and Photo Development

Photographs of agarose gels were taken with a Polaroid MP4 Land Camera with black and white positive (Polaroid Polapan 667) or positive/negative (Polaroid Polapan 665)

film. Agarose gels were placed on a transilluminator (302 nm) (LKB 2001 Macrovue) and photos were taken with an orange filter in the dark. The camera shutter speed was 1 - 1/8 second and the f-stop was 5.6 for black and white positive film (667). Photos of pulse field gels were taken with positive/negative film using a shutter speed of 45 seconds and an f-stop of 5.6.

Negatives were immediately placed in water to remove the film lining. Once the black lining was removed, the negatives were washed in 18% Na_2SO_3. Negatives were fixed in Kodak Photoflow 600 solution (1/600 dilution in distilled H_2O) for 10 seconds and hung up to dry. X-ray films were developed in AGFA Gevaert G150 manual X-ray developer (1/5 dilution in distilled H_2O) for 3-5 minutes. Films were rinsed in H_2O for one minute followed by fixing in Kodak Photoflow 600 solution (1/600 dilution in distilled H_2O) for 1-5 minutes. Films were rinsed in running water for five minutes and hung up to dry. The size of restriction or hybridisation bands were determined from the positive and X-ray films by comparing the bands with the molecular size markers.

2.6 MICROBIAL DEGRADATION OF PAHs

PAH degradation due to microbial activity was recognised as a greater decrease in the PAH concentration in inoculated cultures compared to controls treated similarly. Cultures without a carbon source, uninoculated PAH medium and PAH media inoculated with killed cells served as the controls. Cells were killed by autoclaving (15 minutes at 121°C/15 psi) or by the addition of mercuric chloride (2% w/v) to PAH containing media. PAH concentration in liquid and soil cultures were determined in triplicate by gas chromatography-flame ionisation detection (see Section 2.7.5) after extraction with DCM. Microbial growth was established by an increase in protein concentration (see Section 2.7.1) or an increase in microbial numbers as determined by the most probable number technique (see Section 2.4.9). The production of metabolic intermediates in culture fluids was monitored by the Folin-Ciocalteau reaction (see Section 2.7.2).

2.6.1 Degradation of PAHs in Liquid Culture

2.6.1.1 *PAH Degradation by PAH-Enriched Microorganisms*

All media used in the degradation experiments contained the respective PAH as the sole carbon source. Replicate serum bottles containing BSM (9.9 ml) were inoculated

with 0.1 ml (1-3 μg protein per ml) of the respective pyrene-enriched microorganisms (community four, community five, VUN 10,001, VUN 10,002 and VUN 10,003). PAH stock solutions (0.1 ml) were added to achieve final PAH concentrations of 50 or 100 mg/l. Bottles were incubated for up to 56 days and samples removed routinely for analysis over the incubation period. Degradation experiments with pyrene were also performed at higher pyrene concentrations (250, 500 and 1,000 mg/l) for VUN 10,001, VUN 10,002 and VUN 10,003.

2.6.1.2 *PAH or Creosote Degradation by Microorganisms Enriched on Aromatic or Non-Aromatic Substrates*

Following the enrichment of community five on aromatic and non-aromatic substrates (see Section 2.4.2), the community was tested for its ability to grow on and degrade pyrene. The media used in the degradation experiments contained pyrene as the sole carbon and energy sources. Replicate serum bottles containing BSM (9.0 ml) were inoculated with cultures grown on different substrates (1.0 ml). Pyrene was added to BSM to achieve a final concentration of 250 mg/l. Bottles were incubated and samples removed for analysis at 3, 7, 10, 14 and 21 days.

The degradation of creosote (1.0 ml/l) or a PAH mixture (fluorene, phenanthrene, fluoranthene, pyrene benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene, 50 mg/l each) by community five enriched on creosote (2 ml/l) and yeast extract (1 g/l) (CYEM) was evaluated with and without the addition of yeast extract (1.0 g/l). Media were inoculated with 1.0 ml of the CYEM-grown culture. Bottles were incubated and samples removed for analysis at 3, 7, 10, 14 , 21, 28 and 42 days.

2.6.1.3 *Cometabolism of High Molecular Weight PAHs*

Due to the poor degradation and growth of pyrene-enriched isolates on the high molecular weight PAHs, community five, VUN 10,001, VUN 10,002 and VUN 10,003 were tested for their ability to degrade benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the presence of a growth supporting PAH compounds. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to BSM to achieve final PAH concentrations of 50 mg/l. Phenanthrene or pyrene (100 mg/l) were added to the respective serum bottles as the cosubstrate. PAH containing media were inoculated with 0.1 ml (1-3 μg protein per ml) of the respective pyrene-enriched cultures. Bottles were incubated and samples removed for analysis at 28, 42 and 56 days.

2.6.1.4 *PAH Degradation Using High Initial Cell Densities*

High cell density suspensions were prepared by concentrating cells from pyrene cultures 10-fold and adding 5 or 10 ml of VUN 10,001, VUN 10,002 and VUN 10,003 into serum bottles, to evaluate the degradation of individual PAHs or a PAH mixture. PAHs were added to cell suspensions at individual concentrations of 250 mg/l (fluorene, phenanthrene and pyrene), 100 mg/l (fluoranthene and benz[*a*]anthracene) and 20 mg/l (coronene). Benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to cell suspensions to achieve final concentrations of 25, 50 or 100 mg/l. When the PAH mixture was added to BSM, each component was at a concentration of 50 mg/l with the exception of coronene which was supplied at 20 mg/l. Incubations were performed in triplicate for each set of culture conditions and samples removed for analysis at 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 35, 42, 49, 56 and 63 days.

High initial cell density experiments were also prepared to determine the effect of pyrene (250 mg/l) on the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene (50 mg/l) by and VUN 10,003. After 63 days, pyrene (250 mg/l) was readded to the cultures and incubated further. Cultures were incubated and samples were removed for analysis at weekly or fortnightly intervals over the following 126 day incubation period.

The influence of pyrene metabolites on the degradation of benzo[*a*]pyrene or dibenz[*a,h*]anthracene was determined by inoculating high initial cell densities of VUN 10,003 into media containing pyrene metabolites and one of the five-ring compounds. BSM was prepared by first using it as a medium for the degradation of pyrene (250 mg/l). After the complete degradation of pyrene (7-10 days), the cells were removed by centrifugation (10,000 rpm for 10 minutes at 4°C, JA 14 rotor). The spent BSM was supplemented with pyrene (250 mg/l), benzo[*a*]pyrene or dibenz[*a,h*]anthracene (50 mg/l), nitrate and phosphate (see recipe for BSM, Section 2.3.3) (designated "pyrene-spent medium") before inoculation. A high cell density inoculum of VUN 10,003, freshly grown in BSM containing pyrene (see Section 2.4.5), was added to the pyrene spent medium. Cultures were incubated and samples were removed for analysis at weekly intervals over the 63 day incubation period.

The influence of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites on the degradation of five-ring PAHs was evaluated by inoculating VUN 10,003 into media containing the respective PAH metabolites. The influence of benzo[*a*]pyrene or dibenz[*a,h*]anthracene polar metabolites on the degradation of five-ring PAHs was

determined by collecting culture supernatants from benzo[*a*]pyrene or dibenz[*a,h*]anthracene cultures (5,000 rpm for 10 minutes at 4°C, JA 14 rotor) after 63 days incubation. The supernatants were transferred to sterile serum bottles, benzo[*a*]pyrene or dibenz[*a,h*]anthracene were readded to the supernatants to achieve the same PAH concentration prior to centrifugation and freshly grown cells from BSM containing pyrene of VUN 10,003 (high initial cell density) were added to the medium. Pyrene (250 mg/l) was also readded to the medium. Cultures were incubated for a further 63 days and samples were removed at weekly or fortnightly intervals.

The effect of polar and non-polar metabolites on the degradation of benzo[*a*]pyrene or dibenz[*a,h*]anthracene was also determined after DCM extraction of 63 day cultures containing the five-ring PAHs (see Section 2.7.3.3). DCM extracts were solvent exchanged with DMF (100µl). The extracts were added to fresh BSM containing pyrene (250 mg/l) and benzo[*a*]pyrene or dibenz[*a,h*]anthracene and the media was inoculated with fresh pyrene-grown VUN 10,003 (high initial cell density). Cultures were incubated for a further 63 days and samples were removed at weekly or fortnightly intervals.

2.6.1.5 *Mineralisation of Pyrene and Benzo[a]pyrene*

Pyrene and benzo[*a*]pyrene mineralisation ($^{14}CO_2$ evolution) by community five and VUN 10,003 was monitored in three replicate biometer flasks (Bellco Glass). Aliquots (20 ml) of high cell density cell suspensions were inoculated into flasks. Each flask was supplemented with 1.0 µCi of [4, 5, 9, 10- ^{14}C] pyrene (58.7 mCi/mmol) or 7-^{14}C benzo[*a*]pyrene (26.6 mCi/mmol). Unlabelled PAH was added to the respective cultures at a concentration of 250 mg/l for pyrene and 50 mg/l for benzo[*a*]pyrene. Pyrene cultures were incubated at 30°C/150 opm for 120 hours and benzo[*a*]pyrene cultures were incubated for 70 days: samples were taken routinely over this period. Mineralisation of pyrene and benzo[*a*]pyrene was determined by monitoring the distribution of ^{14}C in the culture medium, cell pellet and gaseous phase (see Section 2.7.8). Uninoculated PAH containing media and mercuric chloride killed cells served as the controls.

2.6.1.6 Degradation of *Pyrene, Benzo[a]pyrene and Dibenz[a,h]anthracene: Formation of Metabolites*

High initial cell density degradation experiments were prepared to monitor the production of metabolites produced from the degradation of pyrene, benzo[*a*]pyrene

and dibenz[*a,h*]anthracene by VUN 10,001, VUN 10,002 and VUN 10,003. Washed cell pellets of VUN 10,001, VUN 10,002 and VUN 10,003 were inoculated into 5 litre Schott bottles containing 3.5 litres of BSM. Pyrene was added at a concentration of 500 mg/l while benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added at a concentration of 50 mg/l. Pyrene cultures were incubated at 30°C/125 opm and samples (100 ml) removed at four hour intervals for 72 hours then at 96 and 120 hours. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene cultures were incubated at 30°C without shaking. Aeration was supplied by stirring the cultures with 60 x 10 mm magnetic stirrers (Industrial equipment and Control Pty Ltd) at 75% maximum speed. Samples (500 ml) were removed after 2, 4, 6, 8, 10 and 12 weeks incubation. Samples were frozen at -20°C until extractions (see Section 2.7.3.2) were performed for TLC, GC and HPLC.

2.6.2 Degradation of PAHs in Soil Culture

2.6.2.1 *Degradation of PAHs in PAH-Spiked Soil*

Soil for this study was obtained from St Albans, Melbourne, Australia. The soil was air dried for 2-3 days, followed by oven drying (Memmert Incubators, Model 500) at 150°C overnight. Soils were sieved through a 2 mm mesh and the pH was adjusted from 4.7 to 6.7 by the addition of garden lime (Nurserymens Products Pty Ltd, Melbourne) (T. G. Juhasz, personal communications). The physical and chemical characteristics of the soil were analysed by National Analytical Laboratories Pty Ltd, Melbourne, Australia. Soils were distributed in 200 g amounts into sealable, 1.5 litre glass jars and sterilised by autoclaving for one hour at 121°C and 15 psi. PAHs were spiked into soils by dissolving appropriate volumes of PAH stock solutions in dichloromethane (50 ml) (DCM) and adding them to the soils. The soil-DCM slurry was stirred vigorously to effect even distribution of the PAHs. Dichloromethane was removed by evaporation at room temperature. PAHs were added at a concentration of 100 mg/kg for the three- and four-ring compounds, 50 mg/kg for the five-ring compounds and 20 mg/kg for the seven-ring compound. When PAH mixtures were used, each component was at a concentration of 50 mg/kg, with the exception of coronene, which was supplied at 20 mg/l.

Cell suspensions (30 ml, 5 x 10^7 cells/ml) of pyrene-grown community five were added to 200 g dry weight of PAH-spiked soil. During the addition of microorganisms, the soils were stirred with sterile glass rods to distribute the inoculum evenly throughout the soil. Sterile double strength BSM (30 ml) was added to the uninoculated control

cultures. Soils were incubated at room temperature and samples removed at regular intervals over a 70 day period. PAH concentration was determined by GC-FID (see Section 2.7.5) after extraction of soils with DCM (see Section 2.7.3.4). Microbial numbers were monitored using the most probable number method (see Section 2.4.9).

2.6.2.2 *Degradation of PAHs in PAH-Contaminated Soil*

Soil for this study was obtained from Sydney, Australia. The soil was dried at room temperature for four days. Preparation of the soil was performed as described above (Section 2.6.2.1) without the addition of spiked PAHs.

Cell suspensions (30 ml, 5 x 10^8 cells/ml) of CYEM-grown community five were added to 200 g dry weight of PAH-contaminated soil. During the addition of the inoculum, the soils were stirred with sterile glass rods to evenly distribute the inoculum throughout the soil. Sterile double strength BSM was added to the uninoculated soil to evaluate PAH degradation by the indigenous microflora. Degradation of PAHs by the indigenous microflora and the inoculum was also assessed with the addition of yeast extract (1.0 g/kg). Control soil cultures consisted of uninoculated soil and soils inoculated with mercuric chloride-killed cells. The inoculum was resuspended in a saturated solution (30 ml) of mercuric chloride and uninoculated soils were wetted with the mercuric chloride solution only. Soils were incubated at room temperature for 91 days. PAH concentration was determined by GC-FID (see Section 2.7.5) after extraction of soils with DCM (see Section 2.7.3.5). Microbial numbers were monitored using the most probable number method (see Section 2.4.9). Toxicological analysis of soil extracts (aqueous and organic) were performed using the modified Microtox™ assay (see Section 2.4.7) and the Ames test (see Section 2.4.8).

2.6.2.3 *Mineralisation of Pyrene in PAH-Contaminated Soil*

Mineralisation of pyrene by community five in PAH-contaminated soil was monitored in three replicate biometer flasks (Bellco Glass). Contaminated soils (20 g dry weight) were supplemented with 1.0 µCi of [4,5,9,10-^{14}C] pyrene (58.7 mCi/mmol). Aliquots (1.5 ml) of high cell density suspensions of CYEM-grown community five (5 x 10^8 cells/ml) were inoculated into flasks to evaluate the mineralisation of pyrene. Control flasks consisted of ^{14}C-pyrene supplemented soil with BSM (1.5 ml) or soils inoculated with mercuric chloride killed community five (1.5 ml). Soils were incubated at room temperature for 49 days. The evolution of $^{14}CO_2$ was monitored routinely over the

incubation period. Mineralisation of pyrene was determined by monitoring the distribution of ^{14}C in the gaseous phase and the soil matrix (see Section 2.7.8).

2.7 ANALYTICAL METHODS

2.7.1 Determination of Cellular Protein Levels (Lowry *et al.*, 1951)

Cells for protein assays (10 ml) were collected by centrifugation at 5,000 rpm for 10 minutes (Hettich Universal, HD Scientific, Melbourne) and washed twice in 1/4-strenght Ringer's solution. Cell pellets were resuspended in 4.6 M NaOH (1.0 ml) and boiled for 10 minutes to lyse the cells. Protein concentrations were measured by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) in 0.45 M NaH_2PO_4 was used as the reference standard at concentrations ranging from 10-200 µg/ml. Lysed cells (50 µl) were diluted 10-fold in 0.45 M NaH_2PO_4. Lowry C (2.5 ml) was added to the respective diluted protein samples and BSA standards and vortexed. After exactly 10 minutes, 250 µl of dilute Folin-Ciocalteau reagent was added and the solutions mixed. Samples were incubated at room temperature for 30 minutes before the absorbance was measured at a wavelength of 750 nm.

2.7.2 Phenolic (PAH Intermediate) Assay (Box, 1983)

Analysis of PAH metabolic intermediates was carried out by the Folin-Ciocalteau reaction (Box, 1983). Since many of the known PAH metabolites are hydroxylated aromatic compounds (Guerin and Jones, 1988a), culture supernatants were assayed for the presence of phenolic compounds. Resorcinol (2.5-20 µg/ml) was used as the standard. Culture fluids for phenolic assays (10 ml) were centrifuged at 5,000 rpm for 10 minutes (Hettich Universal, HD Scientific, Melbourne) to remove undegraded PAHs and cellular material. Supernatants (1.0 ml) were distributed into 5 ml test tubes to which sodium carbonate (150 µl of 200 g/l) and Folin-Ciocalteau phenol reagent (50 µl) were added. Reagents were vortexed (Chiltern, Model M19) and incubated at room temperature for 60 minutes. After incubation, the absorbance was measured at a wavelength of 750 nm. Intermediate values were expressed as milligram resorcinol equivalents per litre.

2.7.3 Extraction of PAHs from Culture Fluids and Soil

2.7.3.1 *Extraction of PAHs or Creosote from Culture Fluid* (Ryan, personal communications)

PAHs or creosote were extracted from bacterial culture fluids (10 ml) with dichloromethane (DCM) (1.0 ml). Benzo[*b*]fluorene (1,000 µg/ml) (100 µl) was used as an internal standard which was added prior to extraction. After DCM and the internal standard were injected into the serum bottles, cultures were shaken vigorously for 20 seconds. Cultures and controls were stored in the dark at -20°C overnight to facilitate separation of the water/solvent emulsion. After thawing at room temperature, DCM extracts (400 µl) were removed and transferred to brown glass sample bottles (2.0 ml). Extracts were stored at -20°C until analysed by GC-FID.

2.7.3.2 *Extraction of Pyrene, Benzo[a]pyrene and Dibenz[a,h]anthracene Metabolites from Culture Supernatants*

Culture fluids were centrifuged (15,000 rpm/10 minutes, Beckman JA21) to remove undegraded PAHs and cellular material. Supernatants were extracted with two equal volumes of DCM. To enhance the recovery of acidic metabolites, supernatants were acidified to pH 2.5 (Heitkamp *et al.*, 1988b) with 10 M HCl and extracted with two additional volumes of DCM. The extracts were pooled, dried with anhydrous Na_2SO_4 and evaporated *in vacuo* at 35°C (Eyela Rotary Evaporator with SB-650 waterbath) to approximately 1.5 ml. The extracts were transferred to brown glass sample vials and further concentrated under a gentle stream of nitrogen (Teche Sample Concentrator). Extracts were stored at -20°C until further use.

2.7.3.3 *Extraction of PAHs and Metabolites from Culture Fluid (for Ames Tests)*

Extraction of PAHs and metabolites from culture fluid (for Ames Test) were performed as outline above (2.7.3.2) without prior centrifugation. After sample concentration, total culture extracts were solvent exchanged with DMF (1.0 ml). Extracts were stored at -20°C until mutagenicity assays were performed.

2.7.3.4 *Extraction of PAHs from PAH-Spiked Soil* (EPA, 1992)

Recovery of PAHs from soils was according to methods described in EPA Method 3550A, Ultrasonic extraction of nonvolatile and semivolatile organics from solids

(EPA, 1992). Anhydrous sodium sulphate (0.5 g) was added to soil samples and mixed thoroughly. Internal standard (2,3-benzo[*b*]fluorene; 100 µl of a 1,000 µg/ml in DCM) and DCM (2.0 ml) were added to samples and controls. Sample bottles were placed on ice and extracted ultrasonically using a 1/8" tapered microtip attached to a 1/2" horn (Branson Sonifier 450). Samples were sonicated for 30 seconds with an output control of seven and a duty cycle of 50%. After 30 seconds, the sonication procedure was repeated. Samples were extracted for a total sonication time of two minutes (4 x 30 seconds). Extracts were cleaned by passing through mini-columns containing glass wool and anhydrous sodium sulphate. Samples were stored at -20°C until analysis by GC-FID.

2.7.3.5 *Extraction of PAHs from PAH-Contaminated Soil*

Recovery of PAHs from PAH-contaminated soil was achieved using a modified ultrasonic extraction method. DCM (9 ml) and internal standard (2,3-benzo[*b*]fluorene, 1 ml of a 1,000 µg/ml in DCM) were added to soil samples. After 30 seconds sonication (3 x 10 seconds with 10 seconds separating each sonication), the DCM was removed and replaced with fresh DCM (10 ml) and the sonication process repeated. This process was repeated a further four times. DCM extracts were pooled and centrifuged at 14,000 rpm for 20 seconds at 4°C (JA 14 rotor) to remove soil particles. Soil extracts were cleaned by passing through a Sep-Pak Florisil cartridge (Waters) and stored at -20°C until GC-FID analysis.

2.7.4 **Separation and Isolation of PAH Metabolites Using Thin Layer Chromatography (TLC)** (Guerin and Jones, 1988b)

Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites were isolated and purified by TLC which was performed with silica gel 60 plates (Merck) using a three phase solvent system (Guerin and Jones, 1988b). Separation was achieved with benzene:hexane (1:1, v/v), hexane:acetone (8:2, v/v) and benzene:acetone:acetic acid (85:15:5 v/v/v). The first solvent system separated the undegraded PAHs and other non-polar compounds from more polar PAH metabolites. The hexane:acetone solvent system was used to elute ring oxidation products and the benzene:acetone:acetic acid system was used to separated the highly polar metabolites. Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene DCM extracts (5-10 µl) were applied to the TLC plates. Plates were developed until the solvent front was 1-2 cm from the top of the plate. Plates were removed from the solvent tank, air dried for two minutes and replaced in the tank containing the new solvent system. After solvent development, PAH metabolites were

visualised by observing the plates on a UV light box (302 nm) (LKB 2011 Macrovue transilluminator, Bromma). The chromatographic mobility (R_f values) of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites were recorded by measuring the distance the metabolites migrated from the origin compared to the solvent front.

Silica gel containing the PAH metabolite bands were removed from the plates and extracted twice (2 ml) with methanol. The methanol extracts were centrifuged (14,000 rpm for 10 seconds) to remove residual silica gel. Samples were concentrated by evaporation under a gentle stream of nitrogen. Dried samples were resuspended in 50-100 µl methanol and reapplied to silica gel plates to check the purity of the isolated metabolites. Purified metabolites were extracted from the silica gel and stored in 100 µl methanol at -20°C. Metabolites that were not pure were extracted from the silica gel and reapplied to TLC plates until single bands were observed.

2.7.5 Determination of PAH and Creosote Concentration by Gas Chromatography (GC)-Flame ionisation Detection (FID)

Gas chromatographic analysis of DCM extracts and of PAH standards was performed on a Varian Star 3400 gas chromatogram equipped with a flame ionisation detector (GC-FID). The following conditions were standard for all analyses:

Column	BPX-5 capillary column (25 m x 0.22 mm, SGE, Melbourne, Australia)
Carrier Gas	Nitrogen
Injector Temperature	300°C
Detector Temperature	300°C

For the analysis of PAH extracts from liquid media and PAH-spiked soil, the oven temperature was programmed at 200°C for one minute, followed by a linear increase of 10°C/min to 320°C, holding at 320°C for 10 minutes. Due to the presence of more volatile hydrocarbon components in creosote, a lower initial column temperature was used. The oven temperature was programmed at 50°C for one minute, followed by a linear increase of 10°C/min to 320°C, holding at 320°C for 5 minutes.

The concentration of PAHs was calculated using benzo[*b*]fluorene (1,000 ng/µl) as the internal standard. Standard solutions (1 ml) were prepared with benzo[*b*]fluorene (100 µg) and PAHs ranging in concentration from 25 to 500 µg/ml. Standards were analysed by GC-FID and the peak area ratio of PAHs to internal standard was calculated. The

concentration of PAHs verses the peak ratio was plotted and a line of best fit was obtained. If the line of best fit was less than R^2=0.95, fresh standards were prepared and analysed. The concentration of PAHs in extracted samples was calculated using the ratio between the PAH and internal standard peaks and the respective PAH line of best fit.

2.7.6 Derivatisation of PAH Metabolites (Pierce, 1995)

PAH metabolites were derivatised using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce). PAH metabolites were dried under a gentle stream of nitrogen. BSTFA+1% TMCS (300 µl) was added to the dried samples and mixed to effect dissolution. Samples were also derivatised using heat. Samples dissolved in BSTFA+1% TMCS (300 µl) were incubated at 60°C for 15 to 60 minutes. Derivatised samples were analysed by GC-MS (see Section 2.7.7.3).

2.7.7 Analysis of PAH Metabolites

Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites, purified by preparative TLC, were identified and characterised by a number of analytical methods.

2.7.7.1 *High Performance Liquid Chromatography (HPLC)*

HPLC analyses of purified PAH metabolites were performed using a Varian liquid chromatographic system containing a solvent delivery system (Varian 9012), automated injection system (Varian 9100), variable wavelength UV/Vis detector (Varian 9050), diode array detector (Varian 9065 Polychrom), controlled by Star chromatography software. PAH metabolites were separated using a Spherex 5 µm C18 column (250 x 4.6 mm, Phenomenex) and a linear methanol-water gradient (50 to 100% methanol in 30 minutes, maintaining at 100% methanol for 20 minutes) with a flow rate of 1 ml/min (Heitkamp *et al.*, 1988b). Fixed wavelength UV detection was at 254 nm and the diode array detector scanned between 190 and 367 nm.

2.7.7.2 *Gas Chromatography (GC)-Flame Ionisation Detection (FID)*

GC analyses of PAH metabolites were performed on a Varian Star 3400 gas chromatograph equipped with a FID using BPX-5 (25 m x 0.22 mm, SGE, Melbourne, Australia), BP-21 (25 m x 0.54 mm, SGE, Melbourne, Australia) and BP-70 (25 m x 0.22 mm, SGE, Melbourne, Australia) capillary columns. The oven temperature for the

BPX-5 column was programmed at 100°C for one minute, followed by a linear increase of 10°C/min to 300°C, holding at 300°C for 9 minutes. For BP-21 and BP-70 columns, the oven temperature was programmed at 100°C for one minute, followed by a linear increase of 10°C/min to 250°C, holding at 300°C for 14 minutes. Injector and detector temperatures were maintained at 300°C. The column back pressure was 20 psi, the carrier gas flow rate was 0.8 ml/minute and the spilt ratio was 90:1.

2.7.7.3 *Gas Chromatography-Mass Spectroscopy (GC-MS)*

Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites were analysed using a Varian Star 3400 gas chromatograph equipped with a Varian Saturn II mass spectrometer (MS) and a BPX-5 (25 m x 0.22 mm, SGE, Melbourne, Australia) capillary column. The MS was operated in electron impact mode with an electron energy of 70 eV over a scan range of 45-400 Da. The column temperature was programmed at 100°C for one minute, followed by a linear increase of 10°C/min to 300°C, holding at 300°C for 9 minutes. The injector and transfer line temperatures were maintained at 250°C and 300°C respectively. Spectra were analysed using Star Chromatography software (Varian).

2.7.7.4 *Nuclear Magnetic Resonance (NMR)*

^{1}H NMR spectra of PAH metabolites were recorded on a DPX 300 recording ^{1}H NMR (Bruker) at 300 MHz. PAH metabolites were recorded as deuteriodichloromethane solutions (DCM-d_2, Cambridge Isotope laboratory) under the following conditions (Smallridge, personal communications):

Data points:	16,384
Number of scans:	256
Number of dummy scans:	2
Sweep width:	4,006.4 Hz
Temperature:	305.2 K
Power level of pulse:	-6.00 dB
Frequency of pulse:	300.131 MHz
Length of pulse:	9.5 s

2.7.8 Detection of Radioactivity (Fedorak *et al.*, 1982)

$^{14}CO^2$ from [4, 5, 9, 10-^{14}C] pyrene and [7-^{14}C] benzo[*a*]pyrene degradation experiments was collected in 0.1 M NaOH (5.0 ml). At various time intervals the NaOH was removed from the flask side arm and replaced with fresh NaOH. At the final sample point, 10 M HCl (0.5 ml) was added to the culture medium to release dissolved CO_2. Aliquots (2 x 1.0 ml) of the NaOH were assayed for radioactivity. The NaOH was added to Cytoscint scintillation cocktail (9.0 ml; ICN) and beta emissions were measured over a 10 minute period using a Liquid Scintillation counter (Wallac 1410, Pharmacia) (see Section 2.7.9).

To determine the distribution of residual ^{14}C-label, culture fluid was centrifuged at 15,000 rpm for 10 minutes at 4°C (JA 21 rotor). The culture supernatants were assayed for radioactivity by combining 1.0 ml aliquots (duplicate) of the supernatants with 9.0 ml of Cytoscint scintillation cocktail. The PAH and cell pellets were extracted with DCM and aliquots of the DCM extracts (2 x 1.0 ml) were added to Insta Gel scintillation cocktail (Packard) (9.0 ml) and the beta emissions measured. To determine the amount of ^{14}C incorporated into cellular material, the cell debris, after extraction with DCM, was suspended in 5.0 ml of water. Aliquots of the cell debris (2 x 1.0 ml) were combined with Cytoscint scintillation cocktail (9.0 ml) and the radioactivity assayed.

Residual ^{14}C-pyrene in soil was determined by extracting the soil with DCM. Soils were extracted as previously described in Section 2.7.3.5. Aliquots of the DCM extracts (2 x 1.0 ml) were added to Insta Gel scintillation cocktail (Packard) (9.0 ml) and the beta emissions measured. The distribution of ^{14}C-residues was calculated with reference to the total radioactivity recovered from the respective control samples which lacked living cells.

2.7.9 Parameters for Bioluminescence and Radioactivity Measurement

Bioluminescence (RLU) and ^{14}C radioactivity were measured using a liquid scintillation counter (Wallac 1410, Pharmacia). Bioluminescence was determined under the following conditions (Park, personal communications):

Counting Mode:	CPM
Isotope:	H^3
Counting Time:	10 seconds

Repeats:	1
Cycles:	1
Replicates:	3

RLU was calculated using the following formula:

RLU = (left count+right count-12,500)/10,000

^{14}C radioactivity was determined under the following conditions:

Measuring Parametres:	^{14}C
Counting Time:	600 seconds
Repeats:	1
Cycles:	1
Replicates:	3

CHAPTER 3

ENRICHMENT, ISOLATION AND IDENTIFICATION OF PAH-DEGRADING MICROORGANISMS

CHAPTER 3

ENRICHMENT, ISOLATION AND IDENTIFICATION OF PAH-DEGRADING MICROORGANISMS

3.1 INTRODUCTION

Bioremediation is a technology that exploits the natural metabolic capabilities of bacteria and fungi. Many microorganisms have been isolated from contaminated soils on the basis of their ability to metabolise various carbon sources, including aliphatic and aromatic compounds and their chlorinated derivatives (Pritchard *et al.*, 1992; Heitkamp *et al.*, 1988a; Stanlake and Finn, 1982). While microorganisms can degrade most naturally occurring compounds, they often lack the appropriate enzymes to degrade many synthetic compounds. Prolonged exposure of microorganisms to these chemicals may cause adaptations in the microbial population, resulting in greater resistance to toxicity or the production of enzymes to degrade some of the compounds (Spain *et al.*, 1980).

The isolation of microorganisms from contaminated environments is the first step in screening microorganisms with the metabolic capabilities to degrade target compounds. Selective enrichment and isolation procedures may be used for the isolation of microorganisms with specific metabolic capabilities and not for microorganisms that are more representative of the autochthonous population. This type of selection procedure has been used for the isolation of PAH-degrading microorganisms (Kiyohara *et al.*, 1982a; Kastner *et al.*, 1994).

Kiyohara *et al.* (1982a) developed a rapid screening method for the detection of bacteria capable of degrading water insoluble solid hydrocarbons on agar plates. Bacteria that were screened for their phenanthrene degrading abilities (*A. faecalis*, *Beijernickia* strain Bwt and *Pseudomonas* strain SPM64) were inoculated onto a minimal salts agar plate. An etheral solution of phenanthrene (10% w/v) was sprayed onto the surface of the agar plate as the sole carbon and energy source. The ether volatilised almost instantaneously leaving a thin white layer of phenanthrene covering the plate. Microorganisms showing phenanthrene degrading abilities grew at the expense of the phenanthrene and colonies were surrounded by clear zones on the opaque plate.

Once microorganisms with specific metabolic capabilities have been isolated, identification of the strains is required. The traditional microbial classification system

is based largely on phenotypic properties such as morphology, physiological and biochemical properties. However, phenotypic resemblance is often an unreliable guide to genealogy (Shleifer and Ludwig, 1989). Often organisms sharing similar properties are not necessarily genealogically related to one another and characteristics arising from adaptation are often acquired independently by unrelated taxa. Present day classification is more reliably based on genotypic methods which analyse information from nucleic acid (DNA and RNA) structures present in the cell (Vandamme *et al.*, 1996). It has now been accepted that rRNA is the best target for studying phylogenetic relationships. It offers several advantages over other genotypic methods: rRNA is present in all bacteria, it is functionally constant and is composed of highly conserved as well as more variable domains (Schleifer and Ludwig, 1989; Stackerbrandt and Goebel, 1994; Yamamoto *et al.,* 1983; Vandamme *et al.*, 1996). Pulse field gel electrophoresis (PFGE) is also another valuable tool for microbial classification or differentiation. PFGE is considered to be the most discriminatory DNA-based typing method (Gordillo *et al.*, 1993; Maslow *et al.*, 1993; Tenover *et al.*, 1995). While genotyping data may be used to allocate taxa on a phylogenetic tree, phenotyping consistency is still required to generate useful classification systems (Wayne *et al.*, 1987; Vandamme *et al.*, 1996). For many new isolates, especially organisms isolated from new sources, several phenotypic and genotypic methods are often required for the identification, classification and differentiation of the organism.

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

1. enrich microorganisms from PAH-contaminated soil with the ability to grow on PAH compounds;

2. isolate pure cultures from PAH-enriched microbial communities with the ability to grow on PAHs;

3. determine the substrate utilisation range of the PAH-degrading microorganisms;

4. identify the PAH-degrading microorganisms at the genus and species levels using the 16SrRNA gene sequencing approaches; and

5. differentiate the PAH-degrading strains by restriction digestion profiles of their 16SrRNA genes, ribotyping and pulsed field gel electrophoresis.

Table 3.1. Location and description of soils samples collected from the HMAS Port Lonsdale site for PAH-enrichment studies (see Figure 2.1 for bin locations). Soils were sampled from five 280 litre sealed bins located at various positions on the site. Soils (0.5-1.0 kg) were collected in 1.5 litre plastic screw capped jars and stored at 4°C until used.

Bin Number	Soil Description
1	Bin 1 was located at the western area of the site. The bin was approximately 10 metres from the fence bordering on Beaconsfield Parade and midway between Esplanade East and Esplanade West. The bin contained approximately 30 cm of soil which was covered with plastic bags and approximately 10 cm of water. The soil was composed of dark brown to black uniformly sized fine particles, with a strong tar-like smell.
2	Bin 2 was located at the western area of the site next to the southern wall of building 1. The bin contained approximately 70 cm of soil which was covered with plastic bags and approximately 10 cm of water. The soil was composed of fine black particles with some grey clay areas, with a very strong tar-like smell.
3	Bin 3 was located 7-8 metres east of the Rouse St entrance at the south eastern corner of building 3. The bin contained approximately 70 cm of soil which was covered with plastic bags and approximately 10 cm of water. The soil was composed of very fine orange, brown clay particles and some larger (approximately 0.5 mm in diametre) black particles. A very weak tar-like smell was present.
4	Bin 4 was located in the eastern area of the site midway between buildings 4 and 5. The bin contained approximately 80 cm of soil. Soil located near the periphery of the bin was covered with approximately 5-10 cm of water, however, the soil in the centre of the bin was above the water line. The soil was sampled from the exposed section of the bin. The soil was composed of a variety of soil types and sizes, including brown soil particles up to 5 mm, small rocks and pebbles and tar balls up to 20 mm in diametre. A weak tar-like smell was present.
5	Bin 5 was located on the grassed area between building 3 and the tennis court. The bin contained approximately 50 cm of soil and was partly submerged under 10 cm of water, similar to bin 4. Samples were taken from the exposed section of the bin. The soil had a similar composition to bin 4.

3.2 SAMPLING OF PAH-CONTAMINATED SOIL

Soils for enrichment studies were collected from the HMAS Port Lonsdale site located in Port Melbourne on the 24th of May 1994. Port Melbourne is located approximately three km from the central business district of Melbourne. Land within the vicinity of the site is used for light industry, business, recreation and housing. The site was previously used as a manufacturing gas plant for over 100 years and more recently was a defence facility site. The majority of the site was covered with concrete or asphalt; only a small grassed section was present near the tennis court in the eastern area of the site (Figure 2.1). Previously cored soils were housed on-site in 280 litre sealed bins. Five bins were sampled for PAH enrichment studies from various positions on the site. The soils collected from the site showed great heterogeneity. The samples ranged from fine sediment-like material to heavy clay soils containing large tar balls. Table 3.1 describes the location and the physical properties of the sampled soils.

3.3 ENRICHMENT OF PAH-DEGRADING MICROORGANISMS

Soil samples collected from the HMAS Port Lonsdale site were used to prepare selective enrichment cultures for the isolation of PAH-degrading microorganisms. BSM was supplemented with individual PAH compounds (phenanthrene, pyrene, benz[*a*]anthracene [100 mg/l], dibenz[*a,h*]anthracene and benzo[*a*]pyrene [50 mg/l]) to select for microorganisms with the ability to degrade these PAHs as sole carbon and energy sources. The inoculum for enrichment cultures was prepared by shaking 20 g of soil in 100 ml of 1/4-strenght Ringer's solution overnight. After shaking, the soil particles were allowed to settle and the supernatant was used as the inoculum. A 10% inoculum was added to BSM containing PAHs (see Section 2.4.1). PAH utilisation in the enrichment cultures was evidenced by a visual decrease in the amount of PAH crystals, by a colour change in the medium and by a visual increase in bacterial biomass.

Microbial growth was observed in all primary enrichments containing the three-, four- and five-ring PAHs. Subsequent serial transfers using BSM containing PAHs demonstrated that the growth observed on the high molecular weight PAHs (benzo[*a*]pyrene and dibenz[*a,h*]anthracene) in the primary enrichments was a consequence of organic carbon carry over from the initial inoculum. Growth by microbial communities from all soil samples was observed on phenanthrene and pyrene, while communities from bins one and five were able to grow on benz[*a*]anthracene (Table 3.2). A colour change in the medium, from colourless to

Table 3.2 Growth of secondary enrichment cultures on a variety of PAHs after 28 days. Enrichment of microbial communities was performed with phenanthrene (PHEN), pyrene (PYR), benz[*a*]anthracene (BA), dibenz[*a,h*]anthracene (DBA) and benzo[*a*]pyrene (B[a]P) as sole carbon and energy sources. A 10% inoculum from primary enrichments was added to fresh BSM containing the respective PAH. The evaluation of growth was carried out by visual monitoring and turbidity scored relatively.

	Growth on:				
Site Code	PHEN[a]	PYR[a]	BA[a]	DBA[b]	B[a]P[b]
bin 1	+++[c]	+	+	-	-
bin 2	+++[c]	++	-	-	-
bin 3	+++	+	-	-	-
bin 4	+++[c]	+++[c,d]	-	-	-
bin 5	+++[c]	+++[d]	++	-	-

[a]PAH concentration was 100 mg/l.

[b]PAH concentration was 50 mg/l.

[c]Culture supernatants turned a yellow colour.

[d]Enrichment cultures used for degradation experiments.

bright yellow, was observed in several cultures grown on phenanthrene (communities from bins one, two, four and five) and by the community from bin four grown on pyrene. This colouration persisted even after PAH crystals were no longer visible. After the primary enrichment, no growth was observed on benzo[*a*]pyrene or dibenz[*a,h*]anthracene by any of the microbial communities. Community five was selected for the isolation of pure cultures due its rapid growth on pyrene compared to the other communities.

3.4 ISOLATION OF PAH-DEGRADING MICROORGANISMS

After five successive transfers in BSM containing pyrene, community five was used for the isolation of pure cultures. Isolation of PAH-degrading pure cultures was performed using a spray plate technique (Kiyohara *et al.,* 1982a) with pyrene as the sole carbon and energy source on BSM agar plates (see Section 2.4.3). Pyrene-degrading pure cultures were visualised on spray plates by colonies surrounded by a zone of clearing, where the pyrene had been utilised (Figure 3.1). Distinct colonies were removed, restreaked onto BSM agar and sprayed with pyrene. Once the purity of the colonies was established, colonies were inoculated into BSM containing pyrene (100 mg/l). Three colony types (designated VUN 10,001, VUN 10,002 and VUN 10,003) of pyrene-degrading bacteria were observed. The isolated microorganisms were similar in cellular morphology: colonies were cream in colour, circular and convex in shape, varying only slightly in size (Table 3.3).

3.5 IDENTIFICATION OF PAH-DEGRADING MICROORGANISMS

The identification of the pyrene-enriched microorganisms, VUN 10,001, VUN 10,002 and VUN 10,003, was performed using both phenotypic and genotypic methods. Initially, biochemical tests were performed to identify the microorganisms to genus level and tentatively to species level. The initial identification from the biochemical tests were then used for the design and construction of olignucleotide primers for amplifying and sequencing the 16SrRNA genes of the three strains.

3.5.1 Biochemical and Substrate Utilisation Tests

VUN 10,001, VUN 10,002 and VUN 10,003, as well as communities four and five, were tested for their ability to utilise a variety of substrates as sole carbon and energy sources. Compounds tested included *n*-alkanes, mono-, chlorinated-, nitro-aromatic compounds and potential breakdown products of PAHs. Cinnamic acid, phthalic acid,

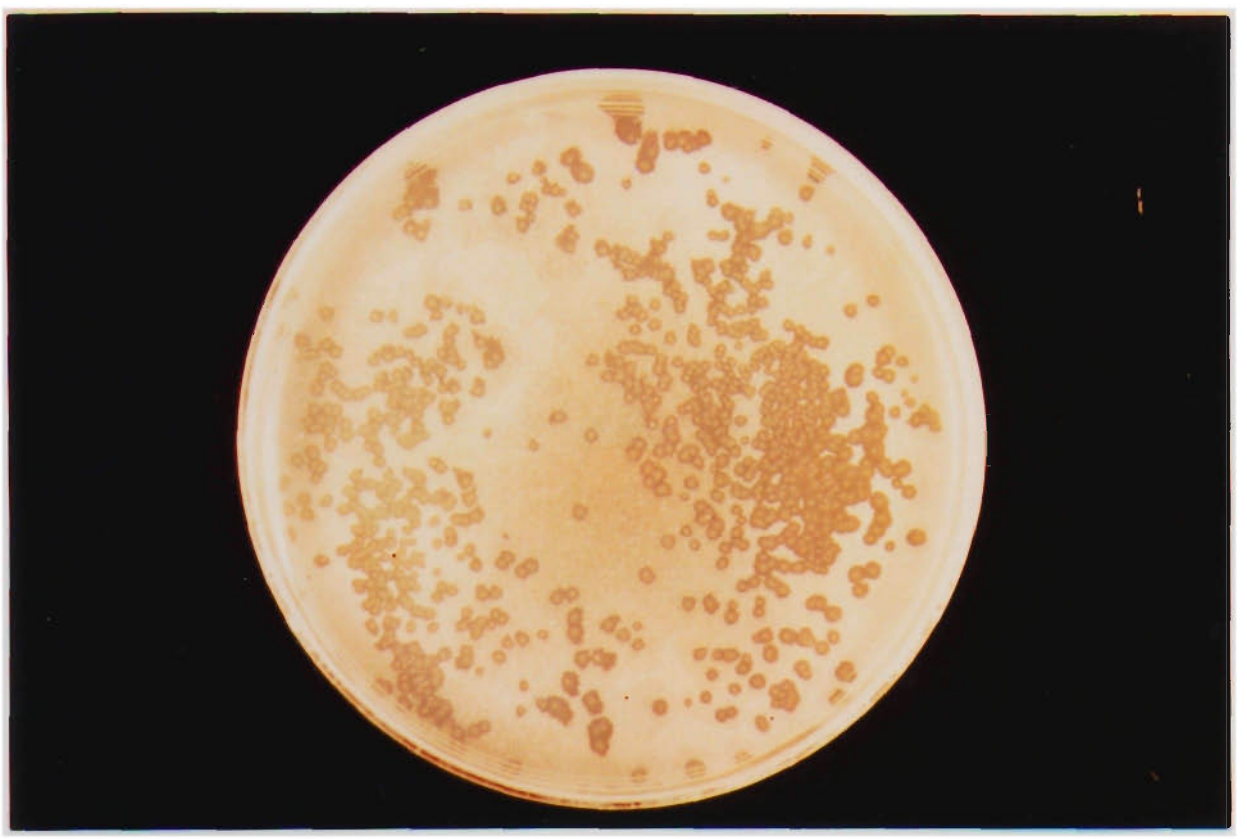


Figure 3.1. Utilisation of pyrene by community five on a BSM agar plate sprayed with a 2% etheral solution of pyrene. Individual pyrene-degrading microorganisms are visualised by colonies surrounded by a zone of clearing where the pyrene was utilised.

Table 3.3. Colony morphology of pyrene-degrading isolates, VUN 10,001, VUN 10,002 and VUN 10,003, after growth on Basal Salts Yeast Extract Peptone Agar (BYP) for 48 hours.

Characteristic	VUN 10,001	VUN 10,002	VUN 10,003
Size (mm)	3.0	2.5	2.0
Shape	circular	circular	circular
Elevation	convex	convex	convex
Surface	Smooth/glistening	Smooth/glistening	Smooth/glistening
Edge	entire	entire	entire
Pigment	cream	cream	cream
Opacity	opaque	opaque	opaque

Table 3.4. Substrate utilisation patterns of microbial communities and bacterial isolates. Test compounds were added to BSM as the sole carbon and energy source at a concentration of 50 mg/l. Volatile compounds were supplied to the microorganisms in the vapour phase. Media were inoculated with 1-3 µg/ml protein of the respective pyrene-grown microorganisms. The evaluation of growth on various substrates by the mixed and pure PAH-degrading microorganisms was carried out by visual monitoring and scored relatively.

Substrate[a]	Growth pattern observed by:				
	Community four	Community five	VUN 10,001	VUN 10,002	VUN 10,003
Cinnamic Acid	+	+	-	-	-
Gentisic Acid	+	+	-	+	-
Phthalic Acid	-	+	+	+	+
Toluene	+	+	+	-	+
Octane	+	+	-	+	+
4-Nitrophenol	-	-	-	-	-

[a]All cultures showed positive growth on succinate, pyruvate, salicylic acid, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, Tween 80, catechol, benzene, 4-chlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, pentachlorophenol, hexane and nitrobenzene.

salicylic acid, protocatechuic acid, succinate and pyruvate are known intermediates of phenanthrene metabolism (Evans *et al.*, 1965; Kastner *et al.*, 1994) as well as *ortho*- and *meta*-pathway endproducts. The compounds were added to BSM at a concentration of 50 mg/l; volatile compounds were supplied in the vapour phase.

All pyrene-enriched microorganisms were able to grow on a broad range of substrates supplied as sole carbon and energy sources including succinate, pyruvate, salicylic acid, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, Tween 80, catechol, benzene, hexane, nitrobenzene, 4-chlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol and pentachlorophenol (Table 3.4). VUN 10,001, VUN 10,002 and VUN 10,003 were unable to utilise cinnamic acid and 4-nitrophenol as carbon sources. VUN 10,002 could be differentiated from VUN 10,001 and VUN 10,003 by its ability to grow on gentisic acid and its inability to utilise toluene. VUN 10,001 was the only isolate that could not utilise octane as a carbon source. Community five could be differentiated from community four by its ability to grow on phthalic acid (Table 3.4).

Biochemical, bacteriological and growth tests were performed for the initial identification of the three isolates. VUN 10,001, VUN 10,002 and VUN 10,003 produced similar results in 20 biochemical tests, with the only difference being that VUN 10,002 could grow at 42°C (Table 3.5). The three isolates were oxidase positive, Gram negative motile rods, which utilised glucose oxidatively and cleaved protocatechuate via ortho cleavage. The isolates did not produce fluorescent pigments, but accumulated poly-ß-hydroxybutyrate in the cell. VUN 10,001, VUN 10,002 and VUN 10,003 were able to grow on a variety of sugars including lactose, sucrose, arabinose, mannose, maltose, sorbitol, glucose and cellibriose (Table 3.6). VUN 10,003 could be differentiated from VUN 10,001 and VUN 10,002 by its inability to grow on adonitol and rhamnose. Minimal growth by VUN 10,002 and VUN 10,003 was observed on mannitol, while neither of the microorganisms were capable of utilising raffinose as a growth source. VUN 10,001, VUN 10,002 and VUN 10,003 were classified as belonging to the genus *Burkholderia* (formally *Pseudomonas*) and tentatively identified as *cepacia* strains.

3.5.2 Determination of 16SrRNA Gene Sequences

16SrRNA gene analysis of VUN 10,001, VUN 10,002 and VUN 10,003 was performed for the further identification of the PAH-degrading microorganisms. Since the strains were identified as *Bu. cepacia* based on the results of the biochemical tests, a pair of oligonucleotide primers were designed based on the 16SrRNA gene sequence of *Bu.*

Table 3.5. Biochemical and physiological characteristics of pyrene-degrading isolates VUN 10,001, VUN 10,002 and VUN 10,003 and test organisms *P. aeruginosa*, *E. coli*, *Proteus mirabilis* and *Bacillus cereus*. Organisms were inoculated into standard biochemical substrates and reactants after growth on Basal Salts Yeast Extract Peptone Agar (BYP) for 48 hours. Results are shown for a 48 hour incubation period except for gelatin liquefaction which was incubated for up to two weeks. Microorganisms were incubated at 30°C with the exception of *E. coli* which was incubated at 37°C.

Test	VUN 10,001	VUN 10,002	VUN 10,003	*P. aeruginosa*	*E. coli*	*Proteus mirabilis*	*Bacillus cereus*
Citrate	+[a]	+	+	+	-	+	+
Lysine Decarboxylase	+	+	+	+	+	-	-
Arginine Decarboxylase	-[b]	-	-	-	-	-	+
Gelatin Liquefaction	-	-	-	+	-	+	+
Indole	-	-	-	-	+	-	+
Methyl Red	-	-	-	-	+	+	-
O-F Glucose	O[c]	O	O	O	O/F[d]	O	O
Nitrate Reduction	NO_2[e]	NO_2	NO_2	N_2[f]	NO_2	NO_2	NO_2
H_2S Production	-	-	-	-	-	+	-
Urease	-	-	-	-	-	+	+
Voges-Proskauer	-	-	-	-	-	+	+
m-Hydroxybenzoate utilisation	+	+	+	-	-	ND[g]	ND
Poly-ß-hydroxybutyrate accumulation	+	+	+	-	ND	ND	ND
Phenylalanine Deaminase	-	-	-	-	-	+	-
Starch Hydrolysis	-	-	-	-	-	-	+
Litmus Milk	-	-	-	-	ND	ND	+
MacConkey Agar	+	+	+	+	+	+	+
DNase Agar	-	-	-	-	-	+	ND
Growth at 42°C	-	+	-	+	+	+	+
Growth with 6.5% NaCl	-	-	-	+	+	ND	+
ONPG	+	+	+	-	+	-	ND
Cleavage Mechanism	ortho[h]	ortho	ortho	ortho	ortho	NR[i]	NR
Tween 80 Hydrolysis	+	+	+	+	-	ND	+
Catalase	+	+	+	+	-	ND	+
Oxidase	+	+	+	+	-	-	-
Fluorescent Pigments	-	-	-	+	-	-	-
Motility	+	+	+	+	+	+	-
Gram Stain	-	-	-	-	-	-	+

[a]+: The test gave a positive result.
[b]-: The test gave a negative result.
[c]O: Utilised glucose oxidatively.
[d]O/F: Utilised glucose oxidatively and fermentatively.
[e]O_2: Nitrate was reduced to nitrite.
[f]N_2: Nitrate was reduced to N_2.
[g]ND: Not determined.
[h]ortho: Cleaved *p*-hydroxybenzoate via the ortho mechanism.
[i]NR: No reaction.

Table 3.6. Growth of pyrene-degrading microorganisms, VUN 10,001, VUN 10,002 and VUN 10,003, and test strains, *E. coli* and *P. aeruginosa,* on various sugars. Sugars were added to BSM as the sole carbon and energy source at a concentration of 1 g/l. Media were inoculated with single colonies from Basal Salts Yeast Extract Peptone Agar (BYP). Microorganisms were incubated for 48 hours at 30°C with the exception of *E. coli* which was incubated at 37°C. The evaluation of growth on various sugars by the microorganisms was carried out by visual monitoring and scored relatively.

Sugar	Growth on Sugars by:				
	VUN 10,001	VUN 10,002	VUN 10,003	*E. coli*	*P. aeruginosa*
Lactose	+	+	+	+	-
Mannitol	-	-/+	-/+	+	+
Adonitol	+	+	-	-	-
Sucrose	+	+	+	+	-
Arabinose	+	+	+	+	-
Raffinose	-	-	-	+	-
Maltose	+	+	+	+	+
Mannose	+	+	+	+	-
Sorbitol	+	+	+	+	-
Glucose	+	+	+	+	+
Cellobiose	+	+	+	-	-
Rhamnose	+	+	-	+	-

Table 3.7. Similarity of VUN 10,001, VUN 10,002 and VUN 10,003 16SrRNA gene sequences generated from mas2f and mas1r primers with known gene sequences from the Australian National Genomic Information Service (ANGIS). The Blastn similarity search was run by accessing ANGIS from the World Wide Web.

Primer	Organisms	Accession Number	Similarity to: VUN 10,001	VUN 10,002	VUN 10,003
mas2f	*St. maltophilia*	X95923 emb	95	95	97
	St. maltophilia	X95924 emb	96	96	92
	St. maltophilia	X95925 emb	91	92	94
	X. campestris	X95917 emb	94	94	94
	X. oryzae	X95921 emb	94	94	94
	X. axonopodis	X95919 emb	94	94	94
	Xanthomonas sp.	U64004 gb	94	96	96
	Hydrothermal vent bacterium	U15111 gb	88	89	87
	St. africae	U62646 gb	92	94	93
mas1r	*St. maltophilia*	X95923 emb	96	94	99
	St. maltophilia	X95924 emb	96	94	99
	St. maltophilia	X95925 emb	95	94	98
	X. campestris	X95917 emb	95	93	97
	X. oryzae	X95921 emb	95	93	97
	X. axonopodis	X95919 emb	93	91	99
	Xanthomonas sp.	U64004 gb	-[a]	-	-
	Hydrothermal vent bacterium	U15111 gb	95	93	98
	St. africae	U62646 gb	95	94	98

[a]No match was given for *Xanthomonas* sp. (U64004 gb) at the mas1r end due to the sequence being only 500 bp.

cepacia strain DSM50181 (X87275, GenBank accession number). This PCR primer set was called mas2f-mas1r and corresponded to position 18-38 (for mas2f) and position 1495-1475 (for mas1r) of the 16SrRNA gene of *Bu. cepacia* (M. D. E. Serafica, personal communications). A PCR product of about 1.5 kb was amplified from each strain using Taq polymerase (ABI-PE) as well as from the Expand Long Template PCR system (Boehringer). Each of the PCR products was used as a template for automated DNA sequencing using the two primers, mas2f and mas1r as sequencing primers. About 400 bp of sequence was generated from each primer. The identity of the sequences was determined by a BLASTn similarity search which was run by accessing ANGIS (Australian National Genomic Information Service). Results of the identity search revealed that the DNA sequences obtained were 91% to 99% similar to *Stenotrophomonas maltophilia* strain LMG 958-T (X95924), *St. maltophilia* strain LMG 11114 (X95925) and strain LMG 11087 (X95923) (Table 3.7).

To determine the sequence of the internal region, a pair of internal PCR and sequencing primers, designated as PD31f-PD31r, were constructed based on the initial sequence data obtained. Assembly of the DNA fragments obtained from mas2f-mas1r and from PD31f-PD31r into one continuous sequence was not achieved with these two primer sets alone. In order to join these fragments and to unambiguously determine the 16SrRNA gene sequences from these strains, the MAZE set of primers was constructed (M. D. E. Serafica, personal communications) in the following manner.

The top eight high scoring pairs of 16SrRNA DNA sequences obtained from the BLASTn search results were aligned using the program Clustal W and the output consensus sequence was used for constructing internal primers. This enabled the sequence determination of the unsequenced region of the 16SrRNA gene from the three strains. The DNA sequences of the 16SrRNA genes from VUN 10,001, VUN 10,002 and VUN 10,003 are shown in Figures 3.2, 3.3 and 3.4. The length of the nearly completed 16SrRNA gene sequences for the three strains were 1,393 bp for VUN 10,001, 1,391 bp for VUN 10,002 and 1,399 bp for VUN 10,003. In both VUN 10,001 and VUN 10,002, the strand that was sequenced was the reverse complement whereas in VUN 10,003, the sequenced strand was the positive strand. The assembled DNA sequences are 93% complete and only approximately 110 bp need to be sequenced to obtain a full length 16SrRNA gene. The DNA sequencs of VUN 10,001, VUN 10,002 and VUN 10,003 were deposited to GenBank. The similarity of VUN 10,001, VUN 10,002 and VUN 10,003 gene sequences was determined using a balstn similarity search (Figure 3.5). The high degree of similarity is shown by the black highlighted section throughout the sequence output.

1	gttaagctac	ctgcttctgg	tgcaacaaac	tcccatggtg	tgacgggcgg
51	tgtgtacaag	gcccgggaac	gtattcaccg	cagcaatgct	gatctgcgat
101	tactagcgat	tccgacttca	tggagtcgag	ttgcagactc	caatccggac
151	tgagataggg	tttctgggat	tggcttaccg	tcgccggctt	gcagccctct
201	gtccctacca	ttgtagtacg	tgtgtagccc	tggccgtaag	ggccatgatg
251	acttgacgtc	atccccacct	tcctccggtt	tgtcaccggc	ggtctcctta
301	gagttcccac	cattacgtgc	tggcaactaa	ggacaagggt	tgcgctcgtt
351	gcgggactta	acccaacatc	tcacgacacg	agctgacgac	agccatgcag
401	cacctgtgtt	cgagttcccg	aaggcaccaa	tccatctctg	gaaagttctc
451	gacatgtcaa	ggccaggtaa	ggttcttcgc	gttgcatcga	attaaaccac
501	atactccacc	gcttgtgcgg	gcccccgtca	attcctttga	gtttcagtct
551	tgcgaccgta	ctccccaggc	ggcgaactta	acgcgttagc	ttcgatactg
601	cgtgccaaat	tgcacccaac	atccagttcg	catcgtttag	ggcgttgtga
651	ctaccagggt	atctaatcct	gtttgctccc	cacgctttcg	tgcctcagtg
701	tcagtgttgg	tccaggtagc	tgccttcgcc	atggatgttc	ctcctgatct
751	ctacgcattt	cactgctaca	cccaggaaat	tccgcttacc	ctctaaccac
801	aactctagtc	gcccagtatc	cactgcagtt	cccaggttga	gcccagggct
851	ttcacaacgg	acttaaacga	ccacctacgc	acgctttacg	cccagtaatt
901	ccgagtaacg	cttgcaccct	tcgtattacc	gcggctgctg	gcacgaagtt
951	agccggtgct	tattctttgg	gtaccgtcat	cccaaccggg	tattagccag
1001	ctggatttct	ttcccaacaa	aagggcttta	caacccgaag	gccttcttca
1051	cccacgcggt	atggctggat	caggcttgcg	cccattgtcc	aatattcccc
1101	actgctgcct	cccgtaggag	tctggaccgt	gtctcacttc	cagtgtggct
1151	gatcatcctc	tcagaccagc	tacggatcgt	cgccttggtg	ggcctttacc
1201	ccgccaacta	gctaatccga	catcggctca	ttcaatcgcg	caaggtccga
1251	agatcccctg	ctttcacccg	taggtcgtag	gtcgtatgcg	gtattagcgt
1301	aagtttccct	acgttatccc	ccacgaaaaa	gtagattccg	atgtattcct
1351	cacccgtccg	ccactggcca	cccagngcgc	aggctntgcc	tgt

Figure 3.2. 16SrDNA gene sequence strand of strain VUN 10,001. A PCR product of approximately 1.5 kb was amplified from strain VUN 10,001 and the primers mas2f and mas1r were used as sequencing primers. The sequence of the internal region was determined using the primers PD31f and PD31r while the sequence fragments were joined using the MAZE set of primers (Length: 1393 bp).

1 ctcccgtaag gttaagctac ctgcttctgg tgcaacaaac tcccatggtg

51 tgacgggcgg tgtgtacaag gcccgggaac gtattcaccg cagcaatgct

101 gatctgcgat tactagcgat tccgacttca tggagtcgag ttgcagactc

151 caatccggac tgagataggg tttctgggat tggcttaccg tcgccggctt

201 gcagccctct gtccctacca ttgtagtacg tgtgtagccc tggccgtaag

251 ggccatgatg acttgacgtc atccccacct tcctccggtt tgtcaccggc

301 ggtctcctta gagttcccac cattacgtgc tggcaactaa ggacaagggt

351 tgcgctcgtt gcgggactta acccaacatc tcacgacacg agctgacgac

401 agccatgcag cacctgtgtt cgagttcccg aaggcaccaa tccatctctg

451 gaaagttctc gacatgttca aggccaggta aggttcttcg cgttgcatcg

501 aattaaacca catactccac cgcttgtgcg ggcccccgtc aattcctttg

551 agtttcagtc ttgcgaccgt actccccagg cggcgaactt aacgcgttag

601 cttcgatact gcgtgccaaa ttgcacccaa catccagttc gcatcgttta

651 gggcgtggac taccagggta tctaatcctg tttgctcccc acgctttcgt

701 gcctcagtgt cagtgttggt ccaggtagct gccttcgcca tggatgttcc

751 tcctgatctc tacgcatttc actgctacac caggaattcc gctaccctct

801 accacactct agtcgcccag tatccactgc agttcccagg ttgagcccag

851 ggctttcaca acggacttaa acgaccacct acgcacgctt tacgcccagt

901 aattccgagt aacgcttgca cccttcgtat taccgcggct gctggcacga

951 agttagccgg tgcttattct ttgggtaccg tcatcccaac cgggtattag

1001 ccagctggat ttctttccca acaaaagggc tttacaaccg cgaaggcttt

1051 cttcacccac gcggtatggc tggatcaggc ttgcgcccat tgtccaaata

1101 ttccccaatg ctgcctcccg taggagtctg gaccgtgtct cagttccagt

1151 gtggctgatc atcctctcag accacctacg gatcgtcgcc ttggtgggcc

1201 tttaccccgc caactagcta atccgacatc ggctcattca atcgcgcaag

1251 gtccgaagat cccctgcttt cacccgtagg tggtatgcgg tattagcgta

1301 agtttcccta cgttatcccc cacgaaaaag tagattccga tgtattcctc

1351 acccgtccgc cactcgccac ccagagagca tgctctncnt g

Figure 3.3. 16SrDNA gene sequence strand of strain VUN 10,002. A PCR product of approximately 1.5 kb was amplified from strain VUN 10,002 and the primers mas2f and mas1r were used as sequencing primers. The sequence of the internal region was determined using the primers PD31f and PD31r while the sequence fragments were joined using the MAZE set of primers (Length: 1391 bp).

1 caaacggcag cacccgagac cttgctctct gggtggcgag tggcggacgg

51 gtgaggaata catcggaatc tacttttteg tgggggataa cgtagggaaa

101 cttacgctaa taccgcatac gacctacggg tgaaagcagg ggatcttcgg

151 accttgcgcg attgaatgag ccgatgtcgg attagctagt tggcggggta

201 aaggcccacc aaggcgacga tccgtagctg gtctgagagg atgatcagcc

251 acactggaac tgagacacgg tccagactcc tacgggaggc agcagtgggg

301 aatattggac aatgggcgca agcctgatcc agccataccg cgtgggtgaa

351 gaaggccttc gggttgtaaa gccctttgt tgggaaagaa atccagctgg

401 ctaatacccg gttgggatga cggtacccaa agaataagca ccggctaact

451 tcgtgccagc agccgcggta atacgaaggg tgcaagcgtt actcggaatt

501 actgggcgta aagcgtgcgt aggtggtcgt ttaagtccgt tgtgaaagcc

551 ctgggctcaa cctgggaact gcagtggata ctgggcgact agagtgtggt

601 agagggtagc ggaattcctg gtgtagcagt gaaatgcgta gagatcagga

651 ggaacatcca tggcgaaggc agctacctgg accaacactg acactgaggc

701 acgaaagcgt ggggagcaaa caggattaga taccctggta gtccacgccc

751 taaacgatgc gaactggatg ttgggtgcaa tttggcacgc agtatcgaag

801 ctaacgcgtt aagttcgccg cctggggagt acggtcgcaa gactgaaact

851 caaaggaatt gacgggggcc cgcacaagcg gtggagtatg tggtttaatt

901 cgatgcaacg cgaagaacct tacctggcct tgacatgtcg agaactttcc

951 agagatggat tggtgccttc gggaactcga acacaggtgc tgcatggctg

1001 tcgtcagctc gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa

1051 cccttgtcct tagttgccag cacgtaatgg tgggaactct aaggagaccg

1101 ccggtgacaa accggaggaa ggtggggatg acgtcaagtc atcatggccc

1151 ttacggccag ggctacacac gtactacaat ggtagggaca gagggctgca

1201 agccggcgac ggtaagccaa tcccagaaac cctatctcag tccggattgg

1251 agtctgcaac tcgactccat gaagtcggaa tcgctagtaa tcgcagatca

1301 gcattgctgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac

1351 accatgggag tttgttgcac cagaagcagg tagcttaacc ttcggaggg

Figure 3.4. 16SrDNA gene sequence strand of strain VUN 10,003. A PCR product of approximately 1.5 kb was amplified from strain VUN 10,003 and the primers mas2f and mas1r were used as sequencing primers. The sequence of the internal region was determined using the primers PD31f and PD31r while the sequence fragments were joined using the MAZE set of primers (Length: 1399 bp).

The BLASTn search results of these nearly completed sequences are shown in Figure 3.6. Since October 1997, three new 16SrRNA sequences of *Stenotrophomonas* species have been submitted to GenBank (Moore *et al.*, 1997) with the following accession numbers: AJ002814 (isolate S3), AJ002807 (isolate R3) and AJ002806 (isolate R2). These sequences are reported in the BLASTn results (Figure 3.6). VUN 10,001 is 98.75% similar to *Stenotrophomonas sp.* AJ002814, 98.25% similar to *St. maltophilia* strain LMG 11087, 97.75% similar to *St. maltophilia* strain LMG 958-T and 94.75% similar to *St. maltophilia* strain AJ002807 and AJ002806. VUN 10,002 is 98% similar to *St. maltophilia* LMG 958-T and *Stenotrophomonas sp.* AJ002814. VUN 10,003 is 99% similar to *St. maltophilia* LMG 958-T and *Stenotrophomonas sp.* AJ002814 (Tables 3.8, 3.9 and 3.10).

Based on 16SrRNA gene sequence determination, the three VUN strains were identified as belonging to *St. maltophilia.* The restriction maps generated, based on the sequence data, are shown in Figure 3.7. VUN 10,001, VUN 10,002 and VUN 10,003 restriction maps are similar, with the exception of the absence of an *EcoRI* restriction site in VUN 10,001. The restriction enzyme maps of *St. maltophilia* strains LMG 958-T, LMG 11087 and *Stenotrophomonas sp.* AJ002814 are similar to VUN 10,001, VUN 10,002 and VUN 10,003. In addition to having restriction enzyme map patterns similar to VUN 10,002 and VUN 10,003, strains AJ002807 and AJ002806 have an extra *DraII* site at about position 176 of the gene.

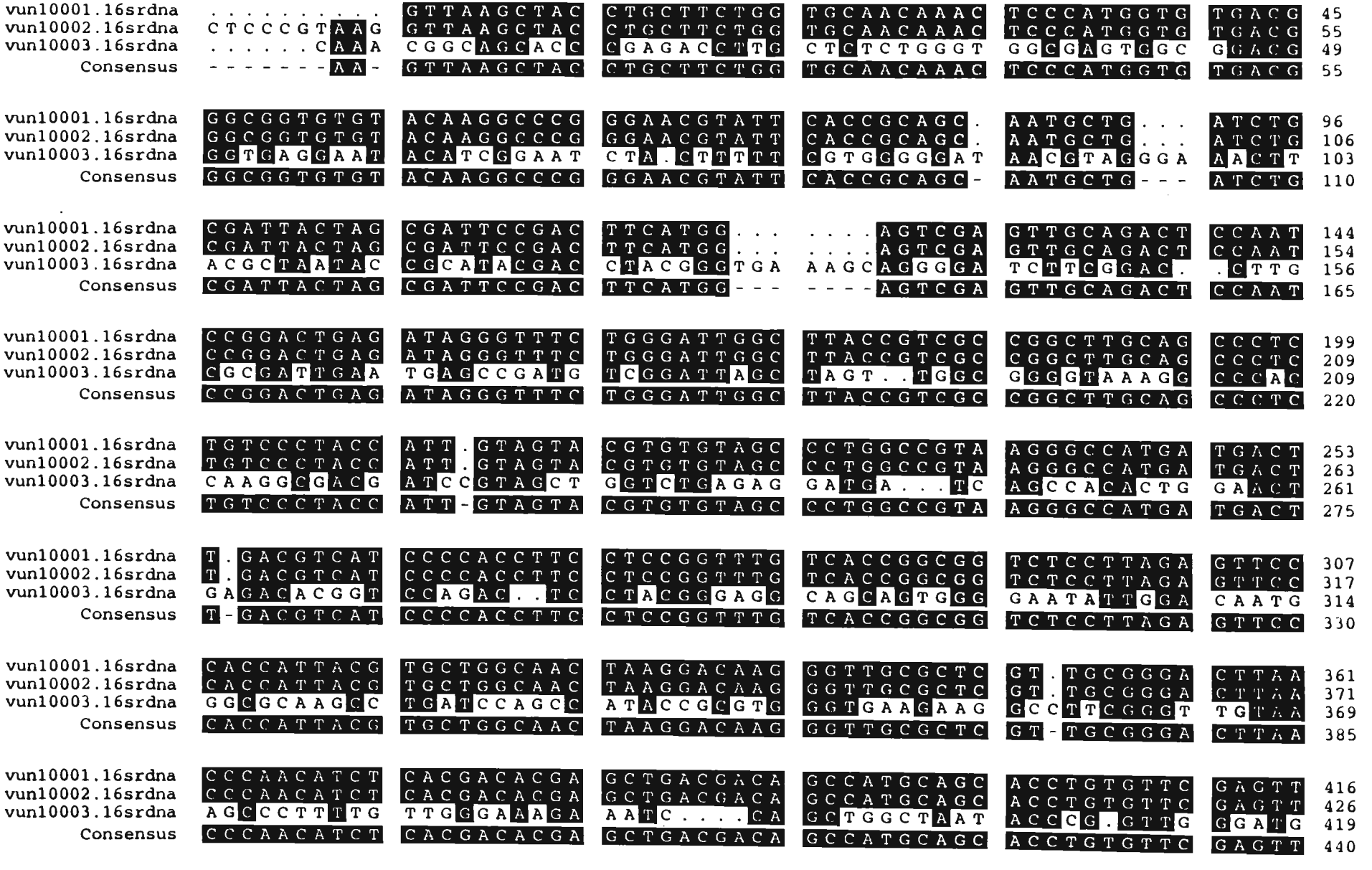
3.6 DIFFERENTIATION OF THE *ST. MALTOPHILIA* STRAINS

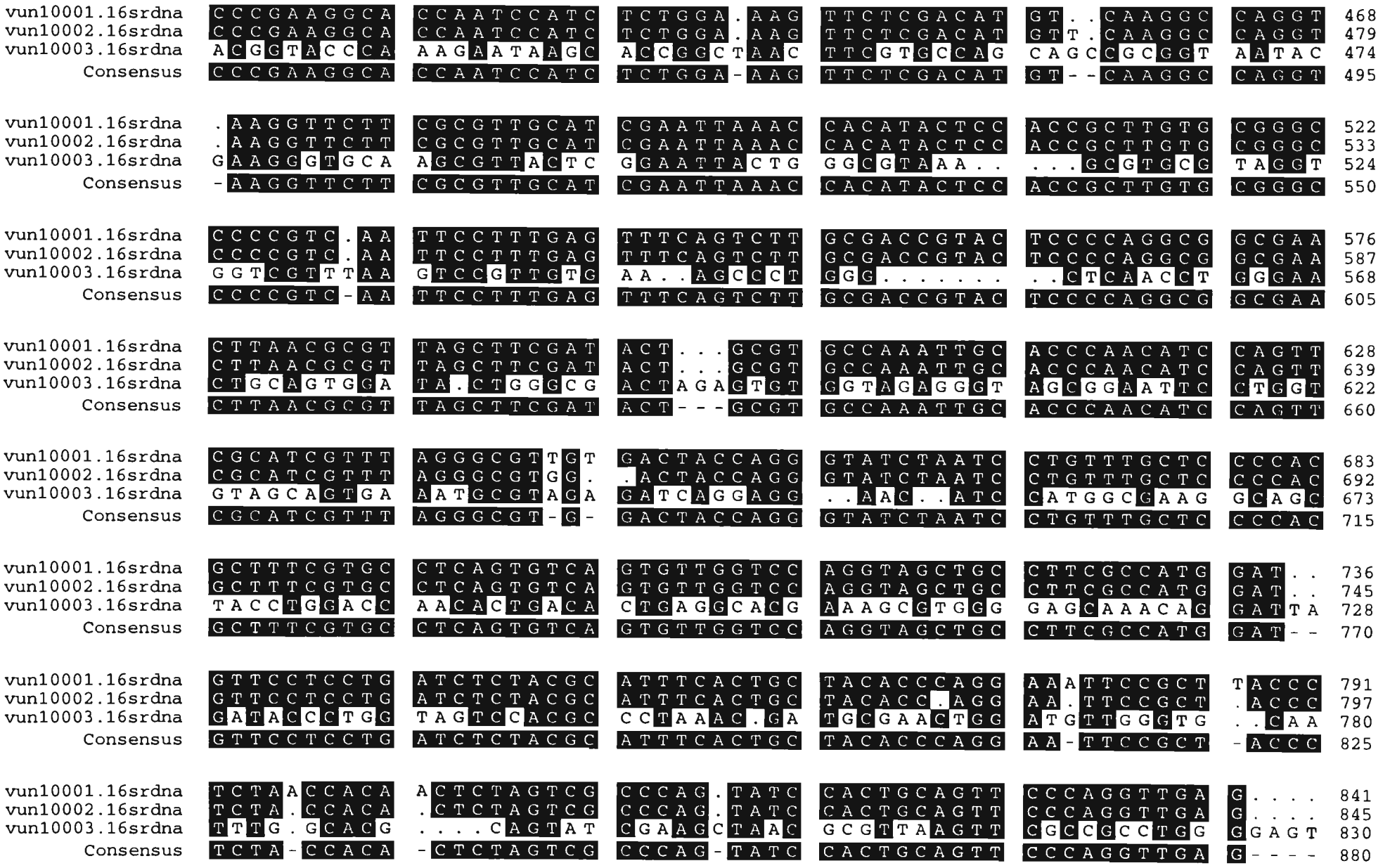
The 16SrRNA gene PCR products amplified from each pyrene-enriched microorganism was then used for strain differentiation in two ways: firstly by digesting each 16SrRNA gene with four-base cutting enzymes and comparing the digestion profiles and secondly, by ribotyping whereby the 16SrRNA gene was used as a probe to total genomic DNA which had been digested with six-base cutting enzymes. Finally, pulse field gel electrophoresis was used to obtain the macrorestriction fragment profile of each isolate by using an enzyme that cuts most prokaryotic genomes infrequently.

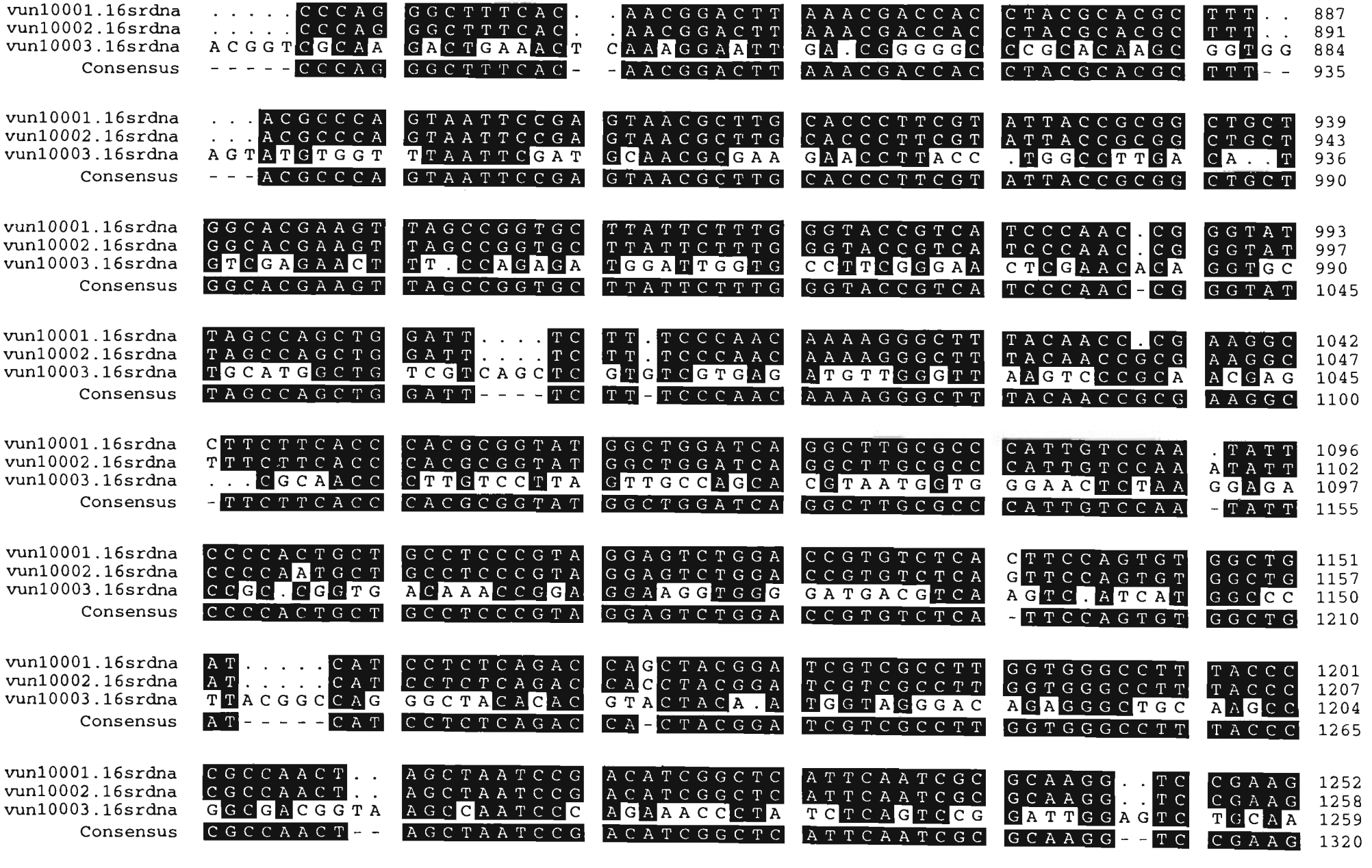
3.6.1 Restriction Digestion of the 16SrRNA Gene

A number of four-base cutting enzymes were used for digesting the 16SrRNA gene of the three strains. Differentiation of the *St. maltophilia* strains using restriction digestion profiles of the 16SrRNA gene may be possible since a four-base cutting enzyme will cut

Figure 3.5. Similarity of VUN 10,001, VUN 10,002 and VUN 10,003 16SrDNA gene sequences using a BLASTn similarity search. The degree of similarity is shown by the black highlighted section throughout the sequence output. The BLASTn similarity search was accessed from the Australian National Genomic Information Service (ANGIS) through the World Wide Web.




vun10001.16srdna CCCGAAGGCA CCAATCCATC TCTGGA.AAG TTCTCGACAT GT..CAAGGC CAGGT 468
vun10002.16srdna CCCGAAGGCA CCAATCCATC TCTGGA.AAG TTCTCGACAT GTT.CAAGGC CAGGT 479
vun10003.16srdna ACGGTACCCA AAGAATAAGC ACCGGCTAAC TTCGTGCCAG CAGCCGCGGT AATAC 474
Consensus CCCGAAGGCA CCAATCCATC TCTGGA-AAG TTCTCGACAT GT--CAAGGC CAGGT 495
vun10001.16srdna .AAGGTTCTT CGCGTTGCAT CGAATTAAAC CACATACTCC ACCGCTTGTG CGGGC 522
vun10002.16srdna .AAGGTTCTT CGCGTTGCAT CGAATTAAAC CACATACTCC ACCGCTTGTG CGGGC 533
vun10003.16srdna GAAGGGTGCA AGCGTTACTC GGAATTACTG GGCGTAAA.. ...GCGTGCG TAGGT 524
Consensus -AAGGTTCTT CGCGTTGCAT CGAATTAAAC CACATACTCC ACCGCTTGTG CGGGC 550
vun10001.16srdna CCCCGTC.AA TTCCTTTGAG TTTCAGTCTT GCGACCGTAC TCCCCAGGCG GCGAA 576
vun10002.16srdna CCCCGTC.AA TTCCTTTGAG TTTCAGTCTT GCGACCGTAC TCCCCAGGCG GCGAA 587
vun10003.16srdna GGTCGTTTAA GTCCGTTGTG AA..AGCCCT GGG....... ..CTCAACCT GGGAA 568
Consensus CCCCGTC-AA TTCCTTTGAG TTTCAGTCTT GCGACCGTAC TCCCCAGGCG GCGAA 605
vun10001.16srdna CTTAACGCGT TAGCTTCGAT ACT...GCGT GCCAAATTGC ACCCAACATC CAGTT 628
vun10002.16srdna CTTAACGCGT TAGCTTCGAT ACT...GCGT GCCAAATTGC ACCCAACATC CAGTT 639
vun10003.16srdna CTGCAGTGGA TA.CTGGGCG ACTAGAGTGT GGTAGAGGGT AGCGGAATTC CTGGT 622
Consensus CTTAACGCGT TAGCTTCGAT ACT---GCGT GCCAAATTGC ACCCAACATC CAGTT 660
vun10001.16srdna CGCATCGTTT AGGGCGTTGT GACTACCAGG GTATCTAATC CTGTTTGCTC CCCAC 683
vun10002.16srdna CGCATCGTTT AGGGCGTGG. .ACTACCAGG GTATCTAATC CTGTTTGCTC CCCAC 692
vun10003.16srdna GTAGCAGTGA AATGCGTAGA GATCAGGAGG ..AAC..ATC CATGGCGAAG GCAGC 673
Consensus CGCATCGTTT AGGGCGT-G- GACTACCAGG GTATCTAATC CTGTTTGCTC CCCAC 715
vun10001.16srdna GCTTTCGTGC CTCAGTGTCA GTGTTGGTCC AGGTAGCTGC CTTCGCCATG GAT.. 736
vun10002.16srdna GCTTTCGTGC CTCAGTGTCA GTGTTGGTCC AGGTAGCTGC CTTCGCCATG GAT.. 745
vun10003.16srdna TACCTGGACC AACACTGACA CTGAGGCACG AAAGCGTGGG GAGCAAACAG GATTA 728
Consensus GCTTTCGTGC CTCAGTGTCA GTGTTGGTCC AGGTAGCTGC CTTCGCCATG GAT-- 770
vun10001.16srdna GTTCCTCCTG ATCTCTACGC ATTTCACTGC TACACCCAGG AAATTCCGCT TACCC 791
vun10002.16srdna GTTCCTCCTG ATCTCTACGC ATTTCACTGC TACACC.AGG AA.TTCCGCT .ACCC 797
vun10003.16srdna GATACCCTGG TAGTCCACGC CCTAAAC.GA TGCGAACTGG ATGTTGGGTG ..CAA 780
Consensus GTTCCTCCTG ATCTCTACGC ATTTCACTGC TACACCCAGG AA-TTCCGCT -ACCC 825
vun10001.16srdna TCTAACCACA ACTCTAGTCG CCCAG.TATC CACTGCAGTT CCCAGGTTGA G.... 841
vun10002.16srdna TCTA.CCACA .CTCTAGTCG CCCAG.TATC CACTGCAGTT CCCAGGTTGA G.... 845
vun10003.16srdna TTTG.GCACG ....CAGTAT CGAAGCTAAC GCGTTAAGTT CGCCGCCTGG GGAGT 830
Consensus TCTA-CCACA -CTCTAGTCG CCCAG-TATC CACTGCAGTT CCCAGGTTGA G---- 880


vun10001.16srdna . . . . . CCCAG GGCTTTCAC . . AACGGACTT AAACGACCAC CTACGCACGC TTT . . 887
vun10002.16srdna . . . . . CCCAG GGCTTTCAC . . AACGGACTT AAACGACCAC CTACGCACGC TTT . . 891
vun10003.16srdna ACGGTCGCAA GACTGAAACT CAAAGGAATT GA . CGGGGGC CCGCACAAGC GGTGG 884
Consensus - - - - - CCCAG GGCTTTCAC - - AACGGACTT AAACGACCAC CTACGCACGC TTT - - 935
vun10001.16srdna . . . ACGCCCA GTAATTCCGA GTAACGCTTG CACCCTTCGT ATTACCGCGG CTGCT 939
vun10002.16srdna . . . ACGCCCA GTAATTCCGA GTAACGCTTG CACCCTTCGT ATTACCGCGG CTGCT 943
vun10003.16srdna AGTATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC . TGGCCTTGA CA . . T 936
Consensus - - - ACGCCCA GTAATTCCGA GTAACGCTTG CACCCTTCGT ATTACCGCGG CTGCT 990
vun10001.16srdna GGCACGAAGT TAGCCGGTGC TTATTCTTTG GGTACCGTCA TCCCAAC . CG GGTAT 993
vun10002.16srdna GGCACGAAGT TAGCCGGTGC TTATTCTTTG GGTACCGTCA TCCCAAC . CG GGTAT 997
vun10003.16srdna GTCGAGAACT TT . CCAGAGA TGGATTGGTG CCTTCGGGAA CTCGAACACA GGTGC 990
Consensus GGCACGAAGT TAGCCGGTGC TTATTCTTTG GGTACCGTCA TCCCAAC - CG GGTAT 1045
vun10001.16srdna TAGCCAGCTG GATT . . . . TC TT . TCCCAAC AAAAGGGCTT TACAACC . CG AAGGC 1042
vun10002.16srdna TAGCCAGCTG GATT . . . . TC TT . TCCCAAC AAAAGGGCTT TACAACCGCG AAGGC 1047
vun10003.16srdna TGCATGGCTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAG 1045
Consensus TAGCCAGCTG GATT - - - - TC TT - TCCCAAC AAAAGGGCTT TACAACCGCG AAGGC 1100
vun10001.16srdna CTTCTTCACC CACGCGGTAT GGCTGGATCA GGCTTGCGCC CATTGTCCAA . TATT 1096
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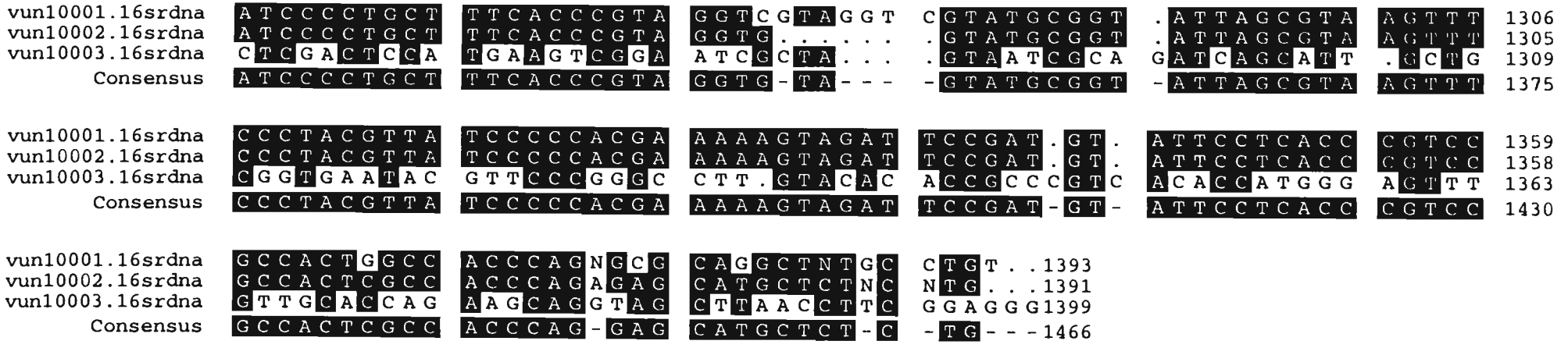
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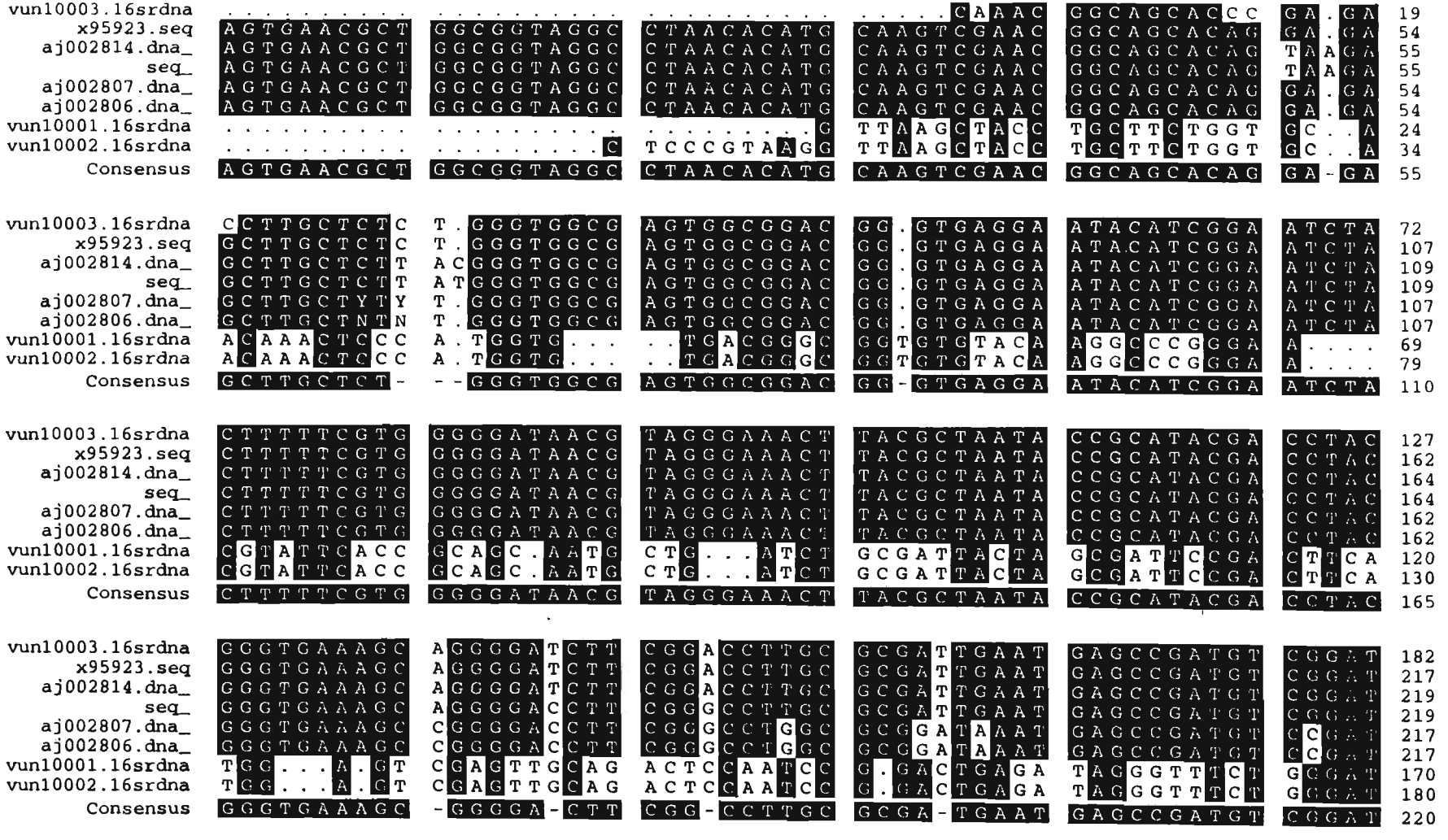
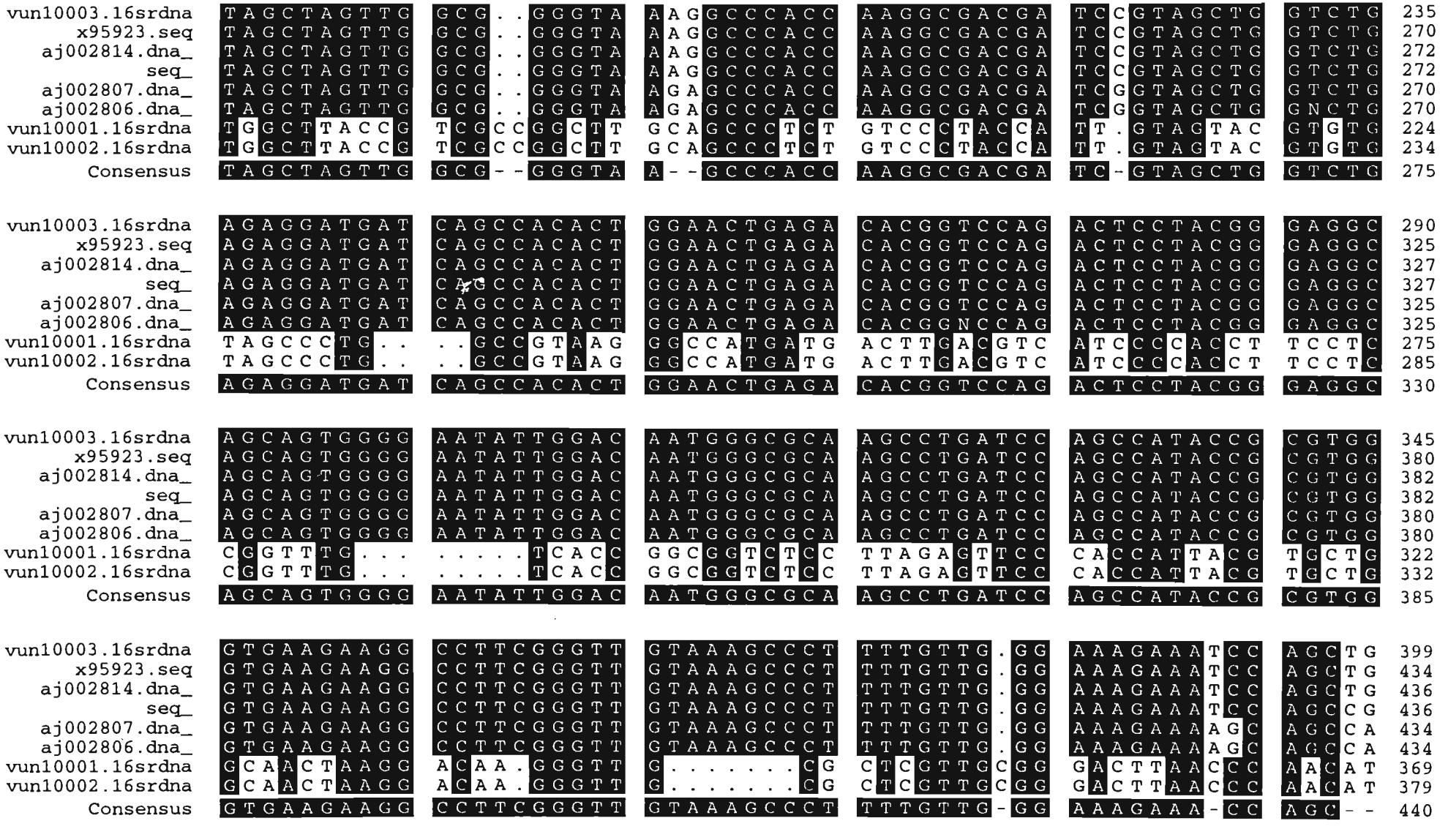
Figure 3.6. Comparison of 16SrDNA gene sequences of VUN 10,001, VUN 10,002 and VUN 10,003 to *St. maltophilia* strain LMG 958-T (X95923), *Stenotrophomonas* species, isolate S3 (AJ002814), *Stenotrophomonas* species, isolate R3 (AJ002807) and *Stenotrophomonas* species, isolate R2 (AJ002806). The degree of similarity is shown by the black highlighted section throughout the sequence output. The BLASTn similarity search was accessed from the Australian National Genomic Information Service (ANGIS) through the World Wide Web.

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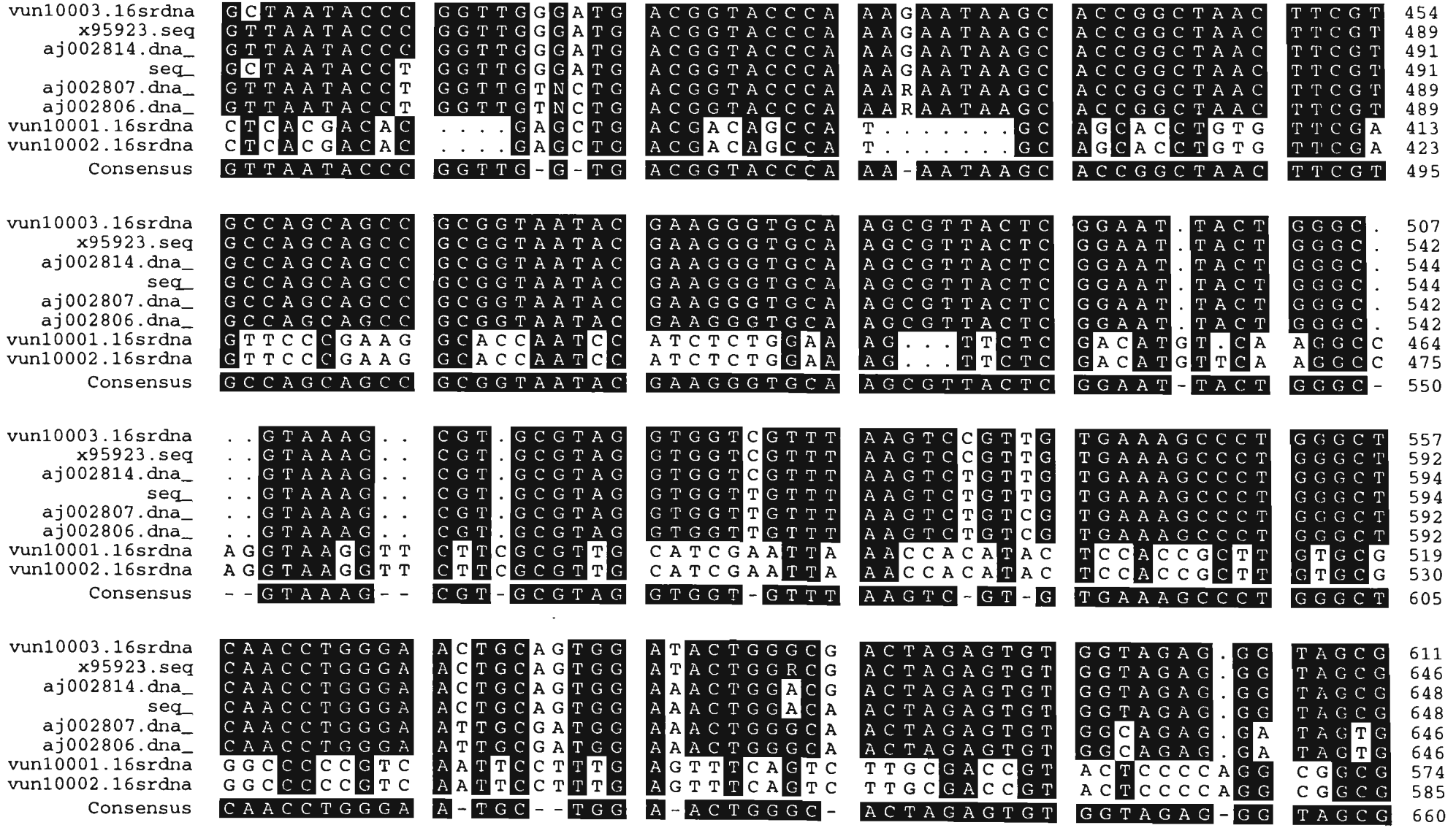
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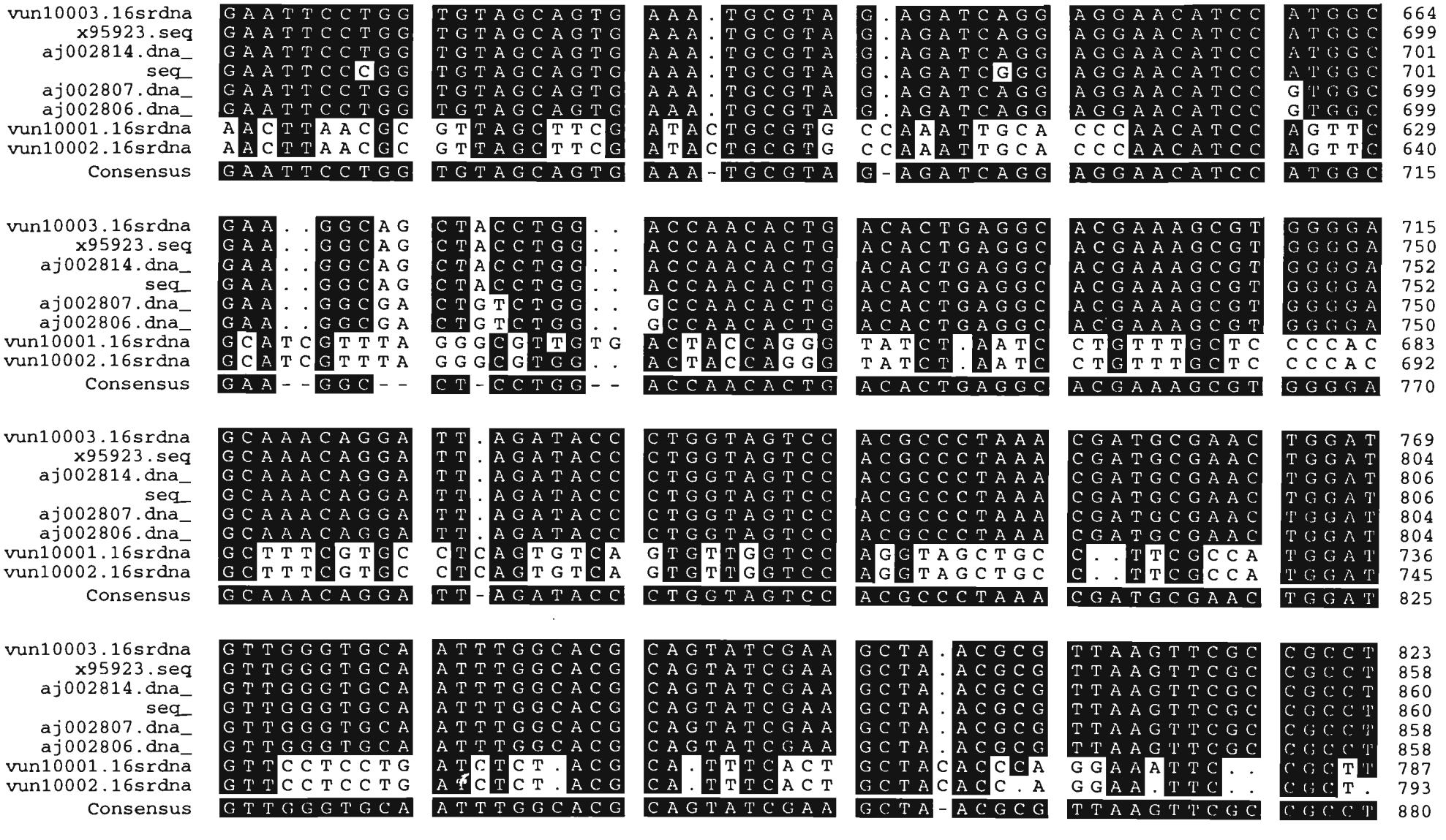
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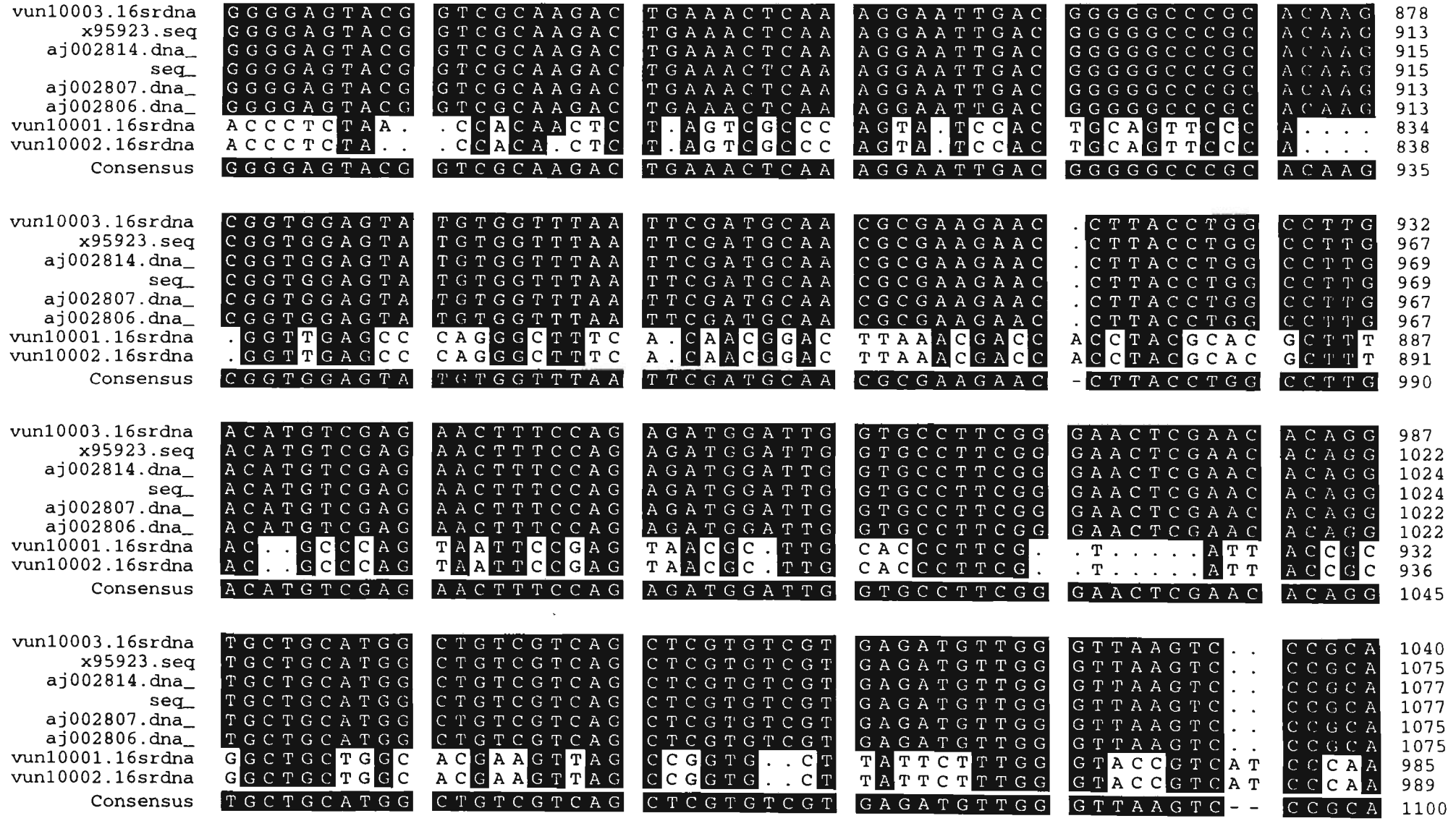
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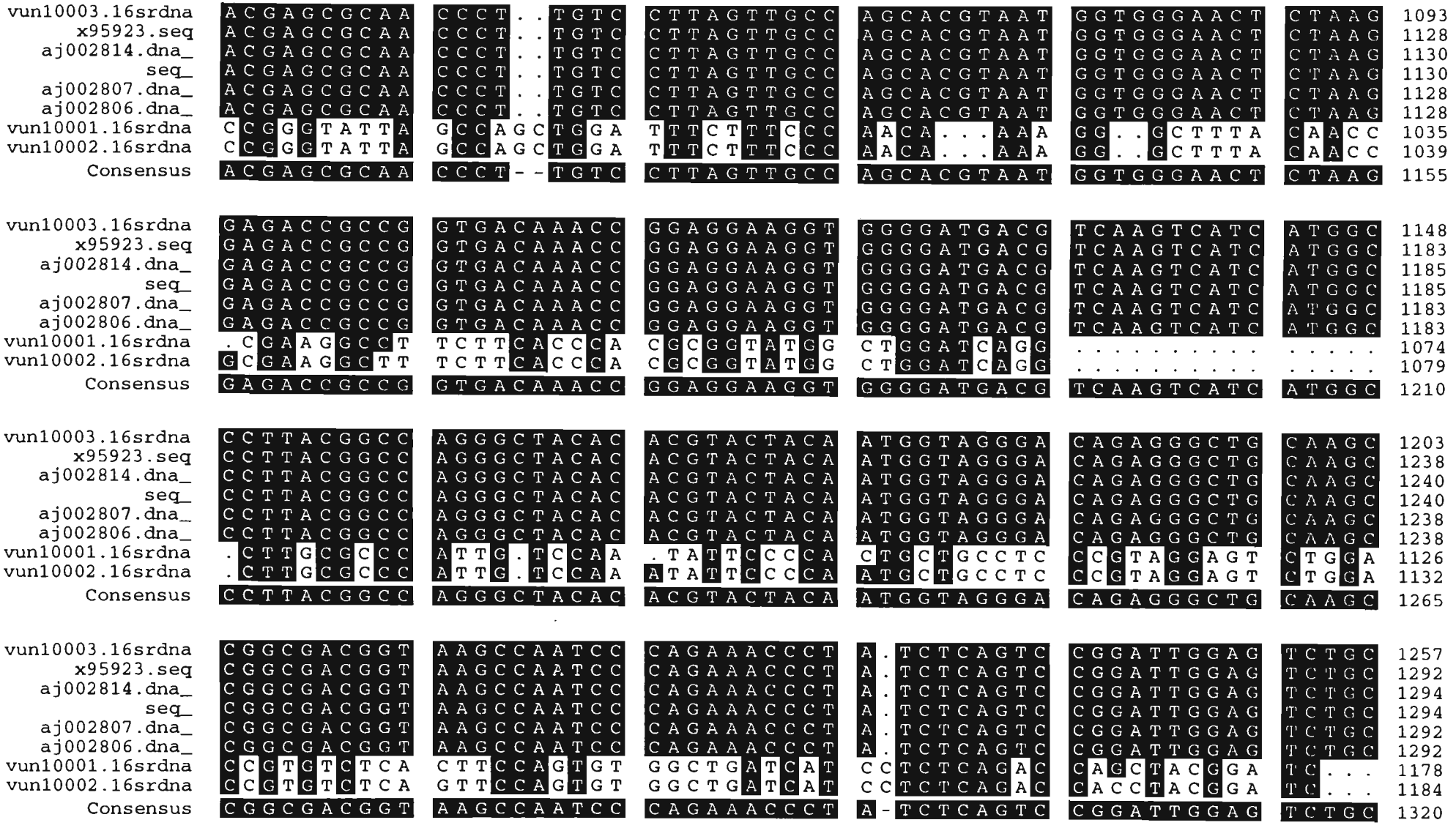
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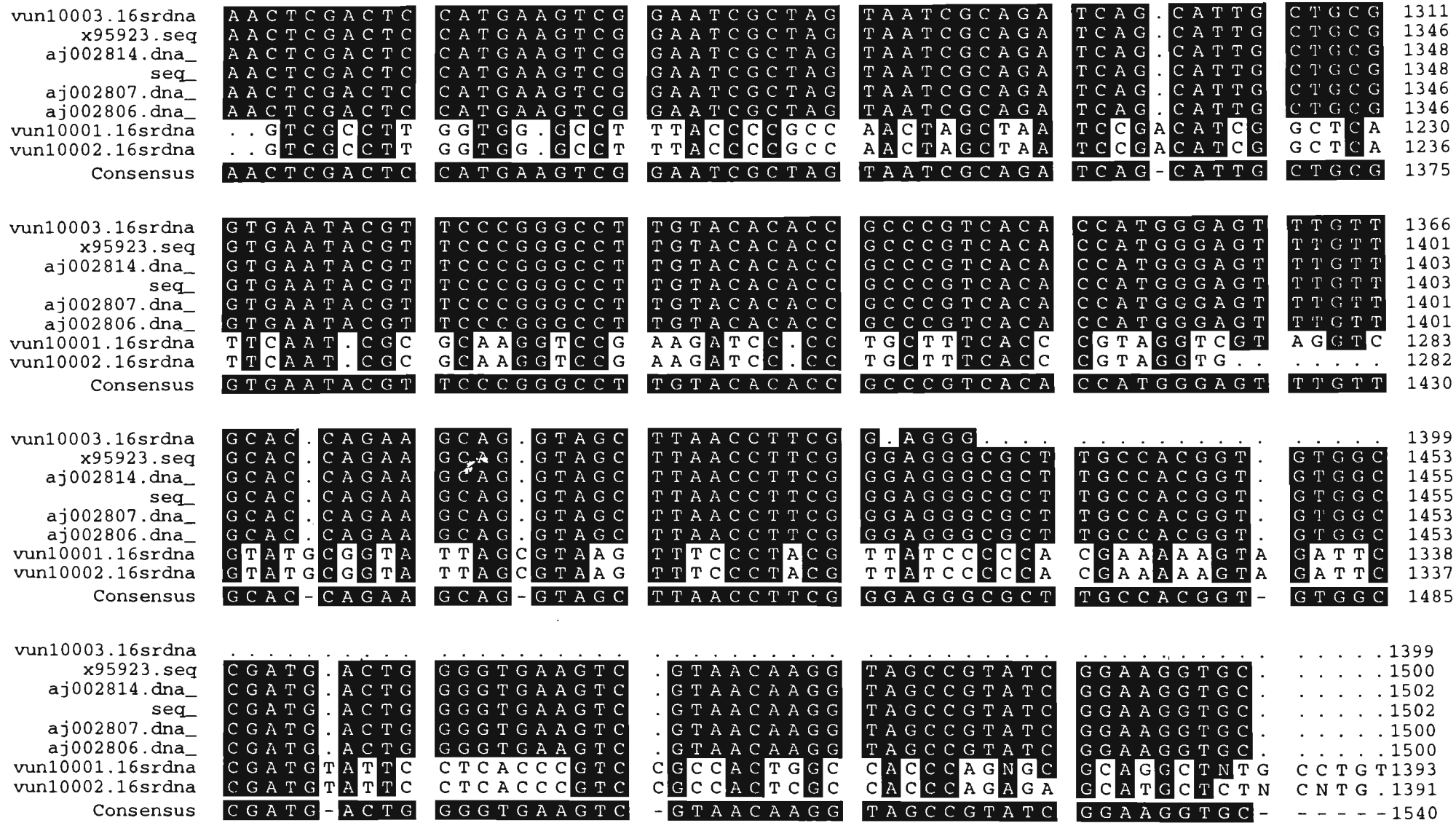
x95923.seq GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1183
aj002814.dna_ GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1185
seq_ GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1185
aj002807.dna_ GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1183
aj002806.dna_ GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1183
vun10001.16srdna .CGAAGGCCT TCTTCACCCA CGCGGTATGG CTGGATCAGG 1074
vun10002.16srdna GCGAAGGCTT TCTTCACCCA CGCGGTATGG CTGGATCAGG 1079
Consensus GAGACCGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGC 1210
vun10003.16srdna CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1203
x95923.seq CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1238
aj002814.dna_ CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1240
seq_ CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1240
aj002807.dna_ CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1238
aj002806.dna_ CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1238
vun10001.16srdna .CTTGCGCCC ATTG.TCCAA .TATTCCCCA CTGCTGCCTC CCGTAGGAGT CTGGA 1126
vun10002.16srdna .CTTGCGCCC ATTG.TCCAA ATATTCCCCA ATGCTGCCTC CCGTAGGAGT CTGGA 1132
Consensus CCTTACGGCC AGGGCTACAC ACGTACTACA ATGGTAGGGA CAGAGGGCTG CAAGC 1265
vun10003.16srdna CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1257
x95923.seq CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1292
aj002814.dna_ CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1294
seq_ CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1294
aj002807.dna_ CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1292
aj002806.dna_ CGGCGACGGT AAGCCAATCC CAGAAACCCT A.TCTCAGTC CGGATTGGAG TCTGC 1292
vun10001.16srdna CCGTGTCTCA CTTCCAGTGT GGCTGATCAT CCTCTCAGAC CAGCTACGGA TC... 1178
vun10002.16srdna CCGTGTCTCA GTTCCAGTGT GGCTGATCAT CCTCTCAGAC CACCTACGGA TC... 1184
Consensus CGGCGACGGT AAGCCAATCC CAGAAACCCT A-TCTCAGTC CGGATTGGAG TCTGC 1320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aj002814.dna_ AACTCGACTC CATGAAGTCG GAATCGCTAG TAATCGCAGA TCAG.CATTG CTGCG 1348
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aj002807.dna_ AACTCGACTC CATGAAGTCG GAATCGCTAG TAATCGCAGA TCAG.CATTG CTGCG 1346
aj002806.dna_ AACTCGACTC CATGAAGTCG GAATCGCTAG TAATCGCAGA TCAG.CATTG CTGCG 1346
vun10001.16srdna ..GTCGCCTT GGTGG.GCCT TTACCCCGCC AACTAGCTAA TCCGACATCG GCTCA 1230
vun10002.16srdna ..GTCGCCTT GGTGG.GCCT TTACCCCGCC AACTAGCTAA TCCGACATCG GCTCA 1236
Consensus AACTCGACTC CATGAAGTCG GAATCGCTAG TAATCGCAGA TCAG-CATTG CTGCG 1375
vun10003.16srdna GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT TTGTT 1366
x95923.seq GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT TTGTT 1401
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aj002806.dna_ GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT TTGTT 1401
vun10001.16srdna TTCAAT.CGC GCAAGGTCCG AAGATCC.CC TGCTTTCACC CGTAGGTCGT AGGTC 1283
vun10002.16srdna TTCAAT.CGC GCAAGGTCCG AAGATCC.CC TGCTTTCACC CGTAGGTG.. 1282
Consensus GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT TTGTT 1430
vun10003.16srdna GCAC.CAGAA GCAG.GTAGC TTAACCTTCG G.AGGG.... 1399
x95923.seq GCAC.CAGAA GCAG.GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT. GTGGC 1453
aj002814.dna_ GCAC.CAGAA GCAG.GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT. GTGGC 1455
seq_ GCAC.CAGAA GCAG.GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT. GTGGC 1455
aj002807.dna_ GCAC.CAGAA GCAG.GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT. GTGGC 1453
aj002806.dna_ GCAC.CAGAA GCAG.GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT. GTGGC 1453
vun10001.16srdna GTATGCGGTA TTAGCGTAAG TTTCCCTACG TTATCCCCCA CGAAAAAAGTA GATTC 1338
vun10002.16srdna GTATGCGGTA TTAGCGTAAG TTTCCCTACG TTATCCCCCA CGAAAAAAGTA GATTC 1337
Consensus GCAC-CAGAA GCAG-GTAGC TTAACCTTCG GGAGGGCGCT TGCCACGGT- GTGGC 1485
vun10003.16srdna 1399
x95923.seq CGATG.ACTG GGGTGAAGTC .GTAACAAGG TAGCCGTATC GGAAGGTGC. 1500
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aj002807.dna_ CGATG.ACTG GGGTGAAGTC .GTAACAAGG TAGCCGTATC GGAAGGTGC. 1500
aj002806.dna_ CGATG.ACTG GGGTGAAGTC .GTAACAAGG TAGCCGTATC GGAAGGTGC. 1500
vun10001.16srdna CGATGTATTC CTCACCCGTC CGCCACTGGC CACCCAGNGC GCAGGCTNTG CCTGT1393
vun10002.16srdna CGATGTATTC CTCACCCGTC CGCCACTCGC CACCCAGAGA GCATGCTCTN CNTG.1391
Consensus CGATG-ACTG GGGTGAAGTC -GTAACAAGG TAGCCGTATC GGAAGGTGC- -----1540

Table 3.8. Similarity of VUN 10,001 16SrRNA gene sequence with known gene sequences from the Australian National Genomic Information Service (ANGIS). The Blastn similarity search was run by accessing ANGIS from the World Wide Web.

Organism	Accession Number	16SrRNA Region	% Similarity to VUN 10,001
Stenotrophomonas species isolate S3	AJ002814	1373-1276	98
		1287-802	98
		772-649	100
		647-1	99
Stenotrophomonas species isolate R3	AJ002807	1373-1276	92
		1287-802	93
		772-649	95
		647-1	99
Stenotrophomonas species isolate R2	AJ002806	1373-1276	92
		1287-802	92
		772-649	95
		647-1	99
St. maltophilia strain LMG958-T	X95923	1373-1276	94
		1287-802	98
		772-649	100
		647-1	99

Table 3.9. Similarity of VUN 10,002 16SrRNA gene sequence with known gene sequences from the Australian National Genomic Information Service (ANGIS). The Blastn similarity search was run by accessing ANGIS from the World Wide Web.

Organism	Accession Number	16SrRNA Region	% Similarity to VUN 10,002
St. maltophilia strain LMG 958-T	X95923	1388-1095	97
		1049-468	98
		467-4	99
		1105-1011	80
Stenotrophomonas species isolate S3	AJ002814	1372-1095	98
		1049-468	98
		467-4	99
		1105-1011	80
St. maltophilia	AF017749	1388-1095	96
		1061-487	96
Azoancus strain BSI-14	AF011348	1368-468	83
		473-318	86
		321-96	85
		95-1	88

Table 3.10. Similarity of VUN 10,003 16SrRNA gene sequence with known gene sequences from the Australian National Genomic Information Service (ANGIS). The Blastn similarity search was run by accessing ANGIS from the World Wide Web.

Organism	Accession Number	16SrRNA Region	% Similarity to VUN 10,003
St. maltophilia strain LMG 958-T	X95923	1-1399	99
Stenotrophomonas species isolate S3	AJ002814	31-1399	99
St. maltophilia strain LMG 11087	X95924	28-1399	98
Stenotrophomonas species isolate R3	AJ002807	1-1399	96
Stenotrophomonas species isolate R2	AJ002806	1-1399	96
St. maltophilia strain LMG 11114	X95925	1-1399	96

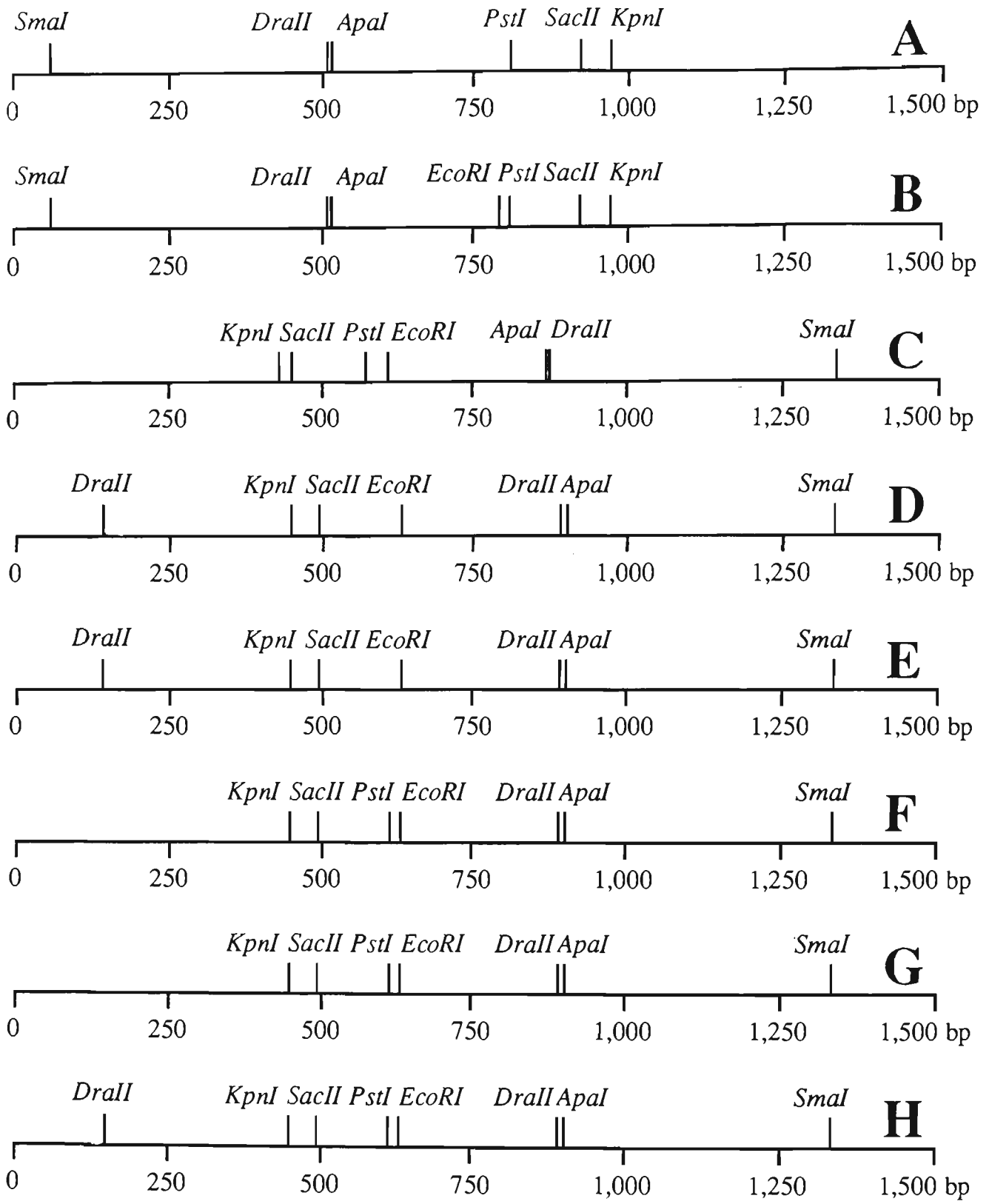


Figure 3.7. 16SrDNA restriction maps of *St. maltophilia* strains VUN 10,001 (A), VUN 10,002 (B), VUN 10,003 (C), *Stenotrophomonas* species isolate R2 [AJ002806] (D), *Stenotrophomonas* species isolate R3 [AJ002807] (E), *Stenotrophomonas* species isolate S3 [AJ002814] (F), *St. maltophilia* strain LMG 958-T [x95923] (G) and *St. maltophilia* strain LMG 11087 [x95924] (H). Restriction sites for the enzymes *ApaI*, *DraII*, *EcoRI*, *KpnI*, *PstI*, *SacII* and *SmaI* were determined from the sequences shown in Figure 3.6. Note that in strains VUN 10,001 and VUN 10,002, the strand that was sequenced was the reverse complement, where as in strain VUN 10,003, the sequence strand was the positive strand.

Table 3.11. Number and size of restriction fragments from VUN 10,001, VUN 10,002 and VUN 10,003 16SrRNA gene products after digestion with a number of endonucleases. No differences in number and size of restriction fragments were observed for the three strains.

Enzyme	Restriction Site	Nº of Restriction Fragments	Size of Restriction Fragments (bp)[a]
BssH II	5' GCG↓CGC 3'	4	70, 230, 510, 710
Eco 01091	5' PuG↓GNCCPy 3'	2	515, 980
Hinf I	5' G↓ANTC 3'	4	50, 160, 200, 1080
Hpa II	5' C↓CGG 3'	4	60, 125, 560, 800
Rsa I	5' GT↓AC 3'	4	100, 375, 450, 550
Sma I	5' CCC↓GGG 3'	2	50, 1410

[a]The size of restriction fragments was determined by comparison with a Promega PCR marker (100-1,500 bp, 100 bp increments up to 1,000 bp).

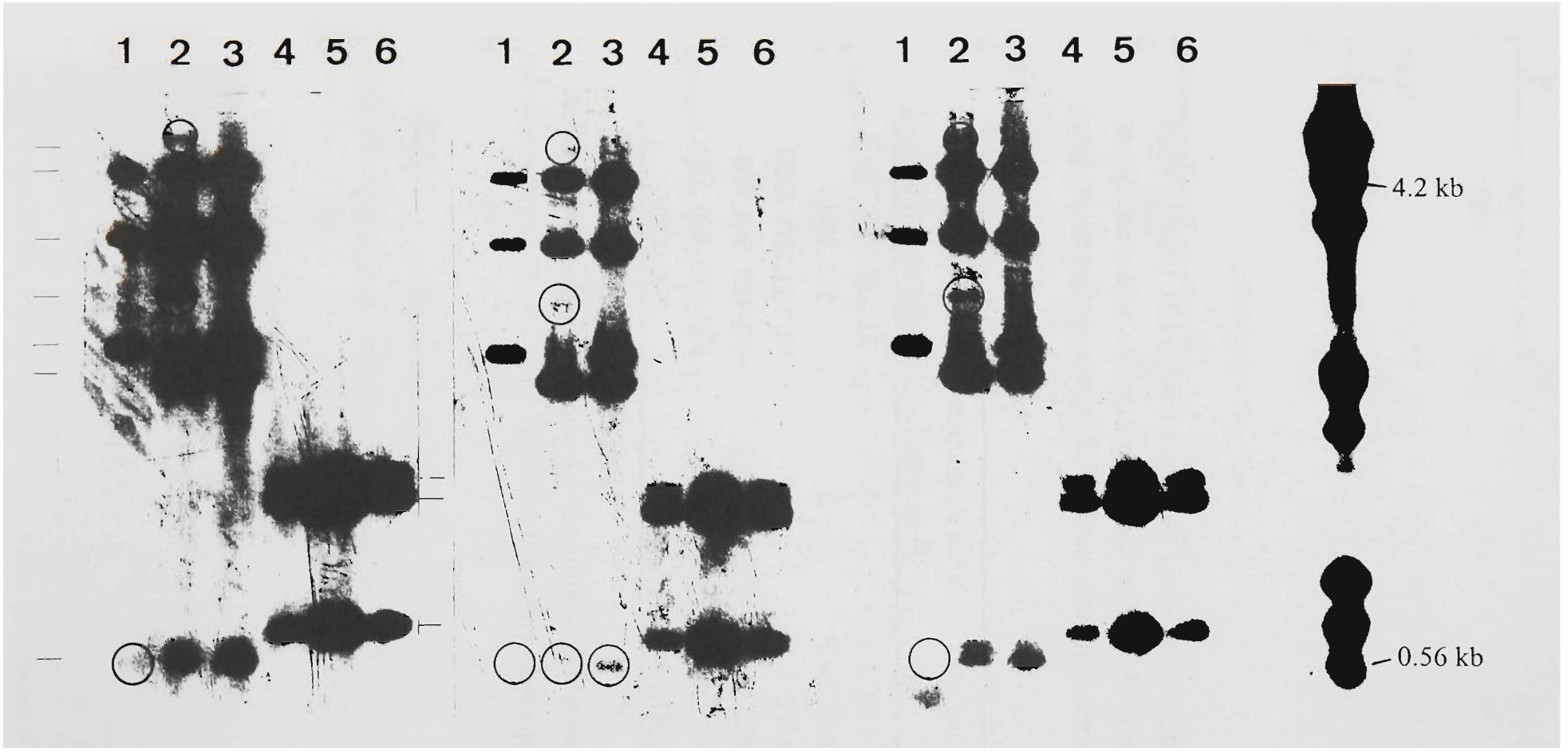


Figure 3.8. Hybridisation patterns of the three *St. maltophilia* strains using VUN 10,001 (A), VUN 10,002 (B) and VUN 10,003 (C) 16SrRNA gene probes. Total genomic DNA was digested with either *SmaI* (lanes 1-3) or *DraII* (lanes 4-6) and run on an agarose gel overnight. After denaturation, the DNA was transferred to a nylon membrane using a dry capillary blotting method and probed with each of the 16SrRNA genes. X-ray films were exposed to the labelled blots for 24 hours at -70°C. After photo development, the size of hybridisation bands were estimated by comparison to the *EcoRI/HindIII* marker (D). Lane 1, VUN 10,003; lane 2, VUN 10,002; lane 3, VUN 10,001 (*SmaI* digests), lane 4, VUN 10,003; lane 5, VUN 10,002; lane 6, VUN 10,001 (*DraII* digests).

a 1.5 kb DNA fragment every 256 bp. A useful enzyme for strain differentiation will be one that will show differences in the number of fragments as well as in the sizes of restriction fragments. No differences in the restriction digestion profiles of 16SrRNA genes of the *St. maltophilia* strains were observed with any of the enzymes used (Table 3.11): the number and size of restriction fragments were the same for VUN 10,001, VUN 10,002 and VUN 10,003.

3.6.2 Ribotyping

DNA:rDNA hybridisation patterns can be used for determining the relatedness of bacterial species. Isolated chromosomal DNA from the test microorganisms is cut by restriction endonucleases and separated by gel electrophoresis. The fractionated DNA is transferred to a nylon membrane and the membrane is probed with a labelled 16SrRNA probe. Taxonomic relatedness is determined by the similarity in the restriction digest length polymorphism (Priest and Austin, 1993). Total genomic DNA from the three strains were individually digested with the restriction enzymes *DraII* (5'PuG$^{\downarrow}$GNCCPy 3') and *SmaI* (5' CCC$^{\downarrow}$GGG 3'), run on an agarose gel overnight and the denatured DNA was transferred to a nylon membrane by a dry capillary blotting method. Each of the 16SrRNA genes were used as probes against these restricted genomic digests.

Hybridisation patterns of VUN 10,001, VUN 10,002 and VUN 10,003, using each of the 16SrRNA gene probes are shown in Figure 3.8. The hybridisation patterns of the three strains digested individually with either *DraII* and *SmaI* were similar when each of the whole 16SrRNA genes were used as probes. Based on the DNA sequences determined for the three strains, there was a *DraII* site at position 870 both for VUN 10,001 and VUN 10,003 and at position 857 for VUN 10,002. The *DraII* hybridisation patterns in terms of number and size were similar for VUN 10,001 and VUN 10,003 (1.23 kb, 1.14 kb and 0.66 kb) but slightly different in intensity and length for VUN 10,002. Based on the hybridisation patterns obtained, there were at least three copies of the gene in all three strains.

There was a *SmaI* site at position 1,329 for VUN 10,001, position 1,317 for VUN 10,002 and position 1,325 for VUN 10,003. Using the 16SrRNA gene from VUN 10,003 as a probe against *SmaI* total genomic digests, five hybridising bands were obtained for VUN 10,001, six bands for VUN 10,002 and four bands were detected in VUN 10,003. The same result was obtained when the 16SrRNA gene of VUN 10,001 and VUN 10,002 were used as probes (Figure 3.8). All strains had three bands in

common (4.15 kb, 3.32 kb and 0.59 kb). Furthermore, VUN 10,001 shared a common band with VUN 10,003, unique to the two strains only (1.95 kb) and VUN 10,001 also shared a common band with VUN 10,002, unique to these two strains only (1.61 kb). VUN 10,002 was the only strain which exhibited 5.31 kb and 2.57 kb bands. The results from the ribotyping, using VUN 10,001, VUN 10,002 and VUN 10,003 16SrRNA genes as probes, demonstrated that the three strains could be differentiated in terms of their *SmaI* digestion and hybridisation patterns.

3.6.3 Pulse Field Gel Electrophoresis

Agarose cell inserts of the three strains were prepared, digested with the enzyme *SpeI* (5'ACT↓AGT 3') and run on a CHEF pulsed field gel apparatus. A number of pulse times and ramping programs were tested in order to separate and resolve the small, medium and large *SpeI* fragments. A 40-second pulse time and a 25/30 second pulse ramp were used for the separation of the largest fragments (250-600 kb), a single pulse time of 20 seconds and a 10/15 second pulse ramp were used for the medium-sized fragments (100-350 kb) and a 5 second pulse time was used to resolve the small fragments (50-100 kb). In addition, a 35 hour gel was run using 5 seconds for 10 hours, 25 seconds for 10 hours and 30 seconds for 15 hours and a 27 hour gel was run using 1 second for 12 hours and 5 seconds for 15 hours. The choice of the *SpeI* enzyme was based on the following:

1. the strains were initially identified as pseudomonads which are known to have high G+C content (57-71 mol%);
2. this enzyme cuts at the tetranucleotide CTAG, which has been shown to be rare in bacteria with high G+C content (McClelland *et al.*, 1987; Huber and Selenska-Pobel, 1994); and
3. *SpeI* has been shown to work well with most known pseudomonads (Escuadra, 1992; Holloway *et al.*, 1992).

Figure 3.9 show the *SpeI* restriction patterns of the three VUN strains while Table 3.12 shows the estimated sizes of the *SpeI* fragments. The PFGE-*SpeI* patterns of the three strains were similar in the number of bands resolved, however, there were some marked differences seen (Figure 3.9). The presence of the same or corresponding doublet bands were observed in the three strains, namely fragment 6 (250 kb) and fragment 8 (175 kb). Also, the three strains (based on the table 3.12) had 14 other similar *SpeI* fragments in terms of size *(SpeI* fragments 1, 3, 4, 5, 7, 9, 11, 13, 14, 15, 16, 17, 18 and 19). For VUN 10,001, fragment 1 was slightly larger than 475 kb, furthermore, fragment 3b

Table 3.12. Genome size (kb) of *St. maltophilia* strains VUN 10,001, VUN 10,002 and VUN 10,003 estimated by summation of the *SpeI* digests shown in Figure 3.9.

SpeI Fragment Number	Size of *SpeI* Fragment (kb)		
	VUN 10,001	VUN 10,002	VUN 10,003
1	~650	~650	~650
2	~475	-	475
3	400	400	400
3b	-	~375	-
4	350	350	350
5	325	325	325
6	250 D[a]	250 D	250 D
7	225	225	225
8	175 D	175 D	175 D
9	125	125	125
10	-	~115	~115
11	~100	~100	~100
12	~85	-	-
13	75	75	75
14	37	37	37
15	34	34	34
16	12	12	12
17	8	8	8
18	7.5	7.5	7.5
19	5.5	5.5	5.5
Total Size	3764	3694	3794

[a]Doublet bands are labelled with a D after the band size. For all three strains, bands 6 and 8 were doublets due to the intensity of the ethidium bromide fluorescence and the thickness of the bands.

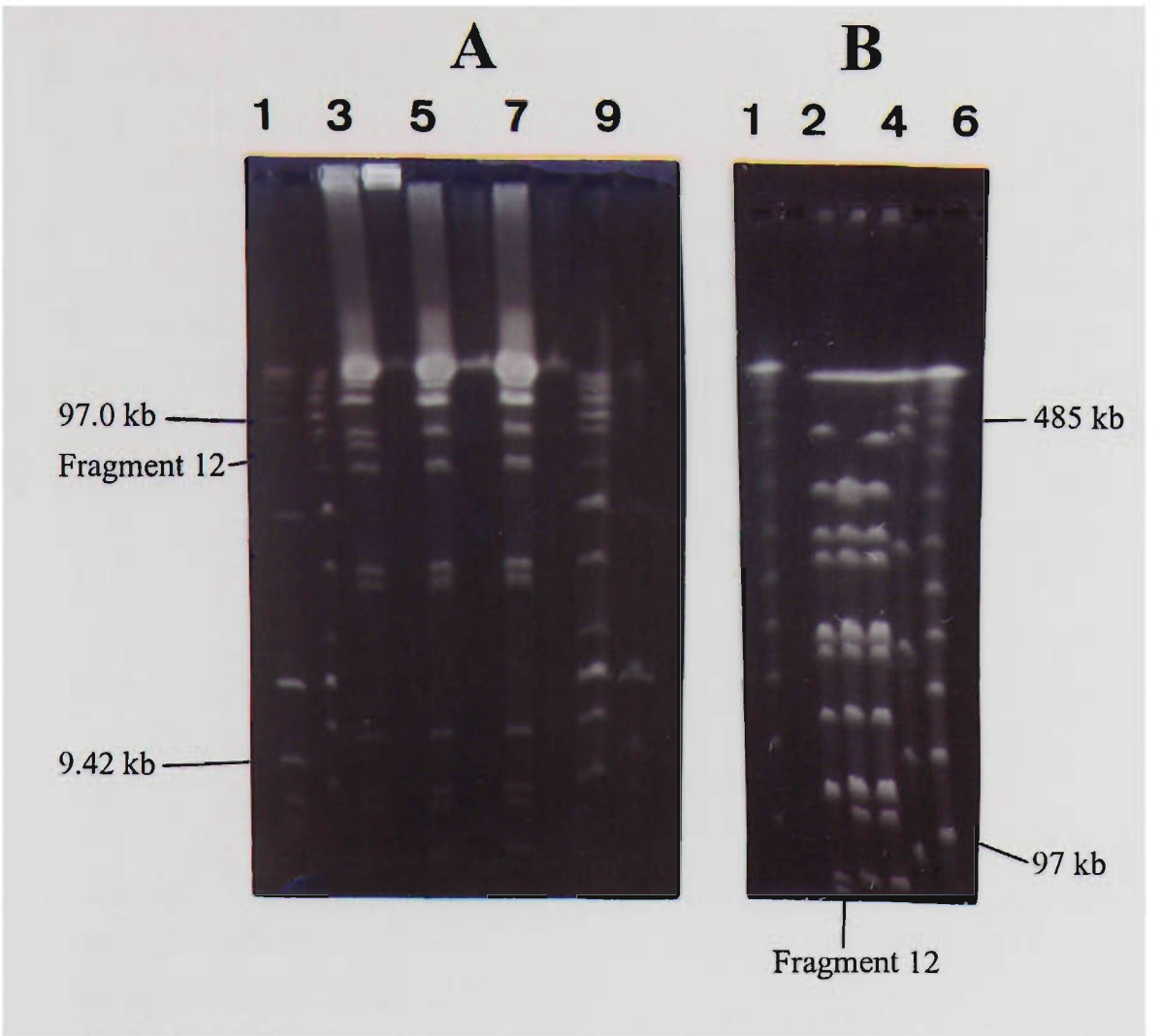


Figure 3.9. Pulse field gel electrophoresis patterns of *SpeI* digests of genomic DNA from VUN 10,001, VUN 10,002 and VUN 10,003. Electrophoresis was performed with a pulse time of 5 seconds for 24 hours (A) or 5 seconds for 10 hours, 25 seconds for 10 hours followed by 30 seconds for 15 hours (B).

Photograph A: lane 1, low range marker; lane 2, mid range marker; lane 3, VUN 10,001; lane 4, undigested VUN 10,001; lane 5, VUN 10,002; lane 6, undigested VUN 10,002; lane 7, VUN 10,003; lane 8, undigested VUN 10,003; lane 9, mid range marker; lane 10, low range marker.

Photograph B: lane 1, lambda ladder marker; lane 2, VUN 10,001; lane 3, VUN 10,002, lane 4, VUN 10,003; lane 5, *Stenotrophomonas maltophilia* strain VUN 10,075; lane 6, lambda ladder marker.

(approximately 375 kb) and fragment 10 (125 kb) were absent. The absence of fragment 10 was unique to VUN 10,001. For VUN 10,002, a large fragment (fragment 2) was absent but a slightly smaller, diffuse band (designated 3b) (approximately 375 kb) was observed. The disappearance of fragment 2 and the presence of fragment 3b was unique to this strain. An 85 kb *SpeI* fragment (fragment 12) was missing in both VUN 10,002 and VUN 10,003, while the corresponding fragment was present in VUN 10,001. When pairwise comparisons of the restriction fragments were made for each organism and the similarity coefficients calculated (given as Dice coefficient [Dice, 1945]), the similarity coefficient for VUN 10,001 and VUN 10,002 was 91%; for VUN 10,001 and VUN 10,003 was 93% and for VUN 10,002 and VUN 10,003 was 98%.

3.7 DISCUSSION

3.7.1 Enrichment of Microorganisms on PAHs

In this study, microbial communities from PAH-contaminated soil were enriched on three-, four- and five-ring PAH compounds. The Port Lonsdale site has had a history of PAH contamination. In the late 1800's, the site contained a manufacturing gas plant, which as a consequence of its operation contaminated the surrounding land with high concentrations of PAHs. Even though the operation of the plant ceased decades ago, up to 5,000 ppm of PAHs (R. B. Davey personal communication) were detected in recently sampled soils.

Enrichment of phenanthrene-degrading microbial communities from the soil was not surprising given the long exposure time of indigenous microorganisms to the PAHs and the relative ease of phenanthrene utilisation as a carbon source (compared to other PAH compounds). Heitkamp *et al.* (1987) suggested that chronic exposure to petrogenic chemicals may not increase the total number of heterotrophic microorganisms, but it may selectively increase the hydrocarbon-degrading microbial population. The enrichment of five microbial communities on pyrene is quite noteworthy as until recently four-ring compounds have been considered as rather recalcitrant against microbial degradation (Cerniglia and Heitkamp, 1989). The long exposure time to the four-ring PAHs may have allowed the selection of specific enzymes or new metabolic capabilities for the degradation of these compounds.

Kastner *et al.* (1994) screened the microflora from five different oil or PAH-contaminated sites in Germany for PAH-degrading ability. Enumeration and

characterisation of the soil microflora resulted in the isolation of 25 different pure cultures with the ability to degrade two- to four-ring PAHs. Although a large number of microorganisms could be enriched on the lower molecular weight PAHs, no isolates were found that could grow on perylene, triphenylene, chrysene or benzo[*a*]pyrene as sole carbon sources. Similar finding to Kastner *et al.* (1994) were observed in this study. The enrichment of microbial communities on phenanthrene and pyrene was achieved within three weeks, however, neither of the communities were able to grow on benzo[*a*]pyrene or dibenz[*a,h*]anthracene. Although, *in situ* microbial adaptation to high molecular weight PAHs may occur, degradation of these compounds may not be possible even after prolonged exposure. When a complex mixture of compounds is present in soil, water or a waste stream, the more readily degradable compounds will be the first to be utilised. Low molecular weight PAHs, such as naphthalenes and phenanthrenes are more likely to be utilised as growth substrates than benzo[*a*]pyrene and dibenz[*a,h*]anthracene. Enrichment of microorganisms on a particular compound will only proceed if the substrate can support good microbial growth. High molecular weight PAHs may not support microbial growth due to their chemical properties, toxicity and structural complexity.

3.7.2 Identification of PAH-Degrading Microorganisms

Prior to the use of PCR and its variations, automated sequencing and PFGE, delineation to the geno-species level was based on DNA hybridisation (Stackebrandt and Goebel, 1994). The reason for using DNA reassociation was based on the high degree of correlation between DNA similarity and chemotaxonomic, genomic and serological similarity. Organisms with 70% or greater DNA similarity were used as the benchmark for assignation to the species level. Since the primary structure of the 16SrRNA gene is highly conserved, species having 70% or greater DNA similarity usually have more than 97% sequence identity (Woese, 1987; Stackebtandt and Goebel, 1994).

By sequencing the 16SrRNA genes of the three strains, identification at the genus and species levels were easily obtained. The reliability and accuracy of this method can be seen from the fact that the initial PCR primers used were based on the presumptive identification of the strains as *Bu. cepacia.* However, the resulting DNA fragments flanked by these primers (namely the first 400 bp from the 5' end and the last 400 bp from the 3' end) and that of the internal region showed up to 99% similarity to *St. maltophilia.* Assembly of nearly 1,400 bp for each strain showed the same result; 98-99% similarity to *St. maltophilia.* This illustrated the usefulness, reliability, rapidity as well as the ease at which a bacterial isolate can be identified.

There was little similarity between 16SrRNA genes of *Bu. cepacia* and VUN 10,001, VUN 10,002 and VUN 10,003, despite phenotypic similarities. However, the BLASTn results showed a 94-96% 16SrRNA gene sequence similarity with members of the genus *Xanthomonas*. This finding was just recently reported by the group of Moore *et al.* (1997). Historically, *St. maltophilia* was classified under the *Xanthomonas* group; its former name being *X. maltophilia* and prior to this, *P. maltophilia* (Hugh and Ryschenkow, 1961; Swings *et al.*, 1990). This classification was based on DNA-RNA hybridisation, quinone type, enzyme characterisation and cellular fatty acid composition. This grouping was not widely accepted because of specific features which distinguished *Stenotrophomonas* as a separate taxon, namely:

1. *Stenotrophomonas* species are not pathogenic to plants (while most *Xanthomonas* species are);
2. absence of yellow pigments called xanthomonadins;
3. presence of a tuft of flagella; and
4. growth is not inhibited by the presence of 0.1% triphenyltetrazolium chloride (van Zyl and Stein, 1992; Palleroni and Bradbury, 1993).

Phylogenetic analyses done by Moore *et al.* (1997) also showed that *St. maltophilia* formed a separate cluster from the xanthomonas group. The very high degree of similarity of the 16SrRNA gene sequence amongst the three strains was underscored in Figure 3.5. The degree of similarity is shown by the black highlight throughout the output file.

There are few reports of the degradation of aromatic compounds by *St. maltophilia* strains. The organism has previously been shown to degrade toluene and xylene (Mallakin and Ward, 1996; Su and Kafkewitz, 1994, 1996), 1-naphthoic acid (Phale *et al.*, 1995) as well as the herbicide 2,4-dichlorophenoxyacetic acid (McGhee and Burns, 1995; Clarkson *et al.*, 1995). The bacterial cultures isolated in this study grew on a diverse range of organic compounds suggesting that these microorganisms possess enzymes with broad substrate specificities. *St. maltophilia* strains VUN 10,001, VUN 10,002 and VUN 10,003, were able to utilise a wide range of organics as sole carbon and energy sources, including alkanes, chlorinated phenols, nitroaromatics as well as possible PAH degradation products. This is a desirable feature of PAH-degrading bacteria since many bioremediation sites are contaminated with a wide variety of PAHs, as well as other organics such as phenolic and heterocyclic compounds (Mueller *et al.*, 1989a; Cerniglia, 1992; Wilson and Jones, 1993).

Although there have been a number of reports in recent years of the degradation of pyrene by *Mycobacterium* species (Heitkamp *et al.*, 1988a, 1988b; Heitkamp and Cerniglia, 1988, 1989; Kastner *et al.*, 1994; Schneider *et al.*, 1996) and other Gram positive microorganisms (Walter *et al.*, 1991; Kastner *et al.*, 1994), there has been no reports of a Gram negative microorganism capable of growing on pyrene. Ye *et al.* (1996) and Weissenfels *et al.* (1991) isolated a *Sp. paucimobilis* strain and an *A. denitrificans* strain respectively capable of degrading pyrene, however, degradation of pyrene was via cometabolism. The ability of the *St. maltophilia* strains isolated in this work to grow on pyrene is the first report of a Gram negative microorganism capable of utilising this compound as a growth substrate.

3.7.3 Differentiation of the *St. maltophilia* Isolates

The resolving power of the 16SrRNA gene sequencing approach is somewhat limited when it comes to discrimination between strains of a species or between genospecies (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). For instance, two species of the genus *Aeromonas* had a low level of relatedness based on DNA-DNA hybridisation (30%) yet their 16SrRNA genes showed only a single base difference (Martinez-Murcia *et al.*, 1992). A number of different molecular methods were tested in an attempt to differentiate the *St. maltophilia* strains. Restriction digestion of 16SrRNA genes with different endonucleases gave identical fragment profiles for the three strains. This was not unexpected, since the 16SrRNA gene sequences of the three strains were nearly identical.

Differentiation of the three strains was possible by ribotyping, after digestion of total genomic DNA with *SmaI* and probing with the 16SrRNA gene products. No difference in polymorphism was observed when *SmaI* digests were probed with VUN 10,001, VUN 10,002 or VUN 10,003 16SrRNA genes. All three strains showed different ribotyping patterns, indicating different copy numbers of the 16SrRNA gene. This may have occurred through lateral gene transfer between the strains resulting in the aquisition of extra copies of the gene in strains VUN 10,002 and VUN 10,003.

PFGE provides an alternative method for the study of the relationship among strains by facilitating the study of their genomic structures (Pavon and Gaju, 1997). Restriction fragments can reveal degrees of relatedness among bacterial strains (MacDonald and Kalmakoff, 1995) and genome sizes can be estimated from the sum of these fragments. Pairwise comparisons of *SpeI* restriction fragment numbers from the three *St.*

maltophilia strains using Dice similarity coefficient showed intra-strain similarities of between 91 and 98%. Although the number and size of *SpeI* restiction fragments were similar for VUN 10,001, VUN 10,002 and VUN 10,003, differences were observed. Most notably was the absence of a 475 kb fragment in VUN 10,002 and the appearance of a 375 kb fragment. Restriction fragment differences may be due to DNA rearrangements such as inversions or translocations or point mutations at restriction sites (Le Blond *et al.*, 1990). Although the 16SrRNA gene sequences (approximately 1,400 bp) of VUN 10,001, VUN 10,002 and VUN 10,003 were almost identical, PFGE of *SpeI* digested chromosomal DNA provided a method for the differentiation of the three strains.

3.7.4 Conclusion

Research reported in this chapter demonstrated the enrichment and growth of five microbial communities on phenanthrene and pyrene as sole carbon and energy sources. No microbial communities were able to grow on the five-ring compounds benzo[*a*]pyrene or dibenz[*a,h*]anthracene. Three pure cultures were isolated from pyrene-enriched community five with the ability to utilise pyrene as a growth substrate. The isolates were identified as strains of *St. maltophilia* based on 16SrRNA gene sequence determination. Differentiation of the three strains was possible by analysis of ribosomal DNA restriction fragment length polymorphism (ribotyping) with *SmaI*, probed with VUN 10,001, VUN 10,002 or VUN 10,003 16SrRNA genes or by PFGE after digestion of genomic DNA with *SpeI*. The following chapter reports the PAH degradative potential of the *St. maltophilia* strains, community four and community five in liquid medium.

CHAPTER 4

MICROBIAL DEGRADATION OF PAHs IN LIQUID CULTURE

CHAPTER 4

MICROBIAL DEGRADATION OF PAHs IN LIQUID CULTURE

4.1 INTRODUCTION

The bioremediation of PAH-contaminated sites has been effective in removing low molecular weight PAH compounds, but PAH-bioremediation continues to be unreliable in removing higher molecular weight PAHs. The major reasons for the recalcitrance of high molecular weight PAHs to microbial degradation include the lack of indigenous or inoculated microflora in the soil that contain the appropriate catabolic pathways, the low bioavailability of the compounds and a deficit of essential nutrients in the soil. Other factors may also play a role depending on the microbial, chemical and physical environment of the site. For example, high molecular weight PAH biodegradation may be inhibited by the presence of low molecular weight PAHs or other more easily degraded organic compounds.

In an effort to overcome some of these shortfalls in PAH bioremediation, a major focus of research in this area has been on the isolation of high molecular weight PAH-degrading microorganisms. Isolated microorganisms which have demonstrated ability to degrade high molecular weight PAHs could be useful for *in situ* or *ex situ* remediation of soils containing these compounds. The inoculation of such isolates into contaminated soils or soil slurries could improve the overall PAH degradation rate or, in particular, degradation of the high molecular weight PAHs which would otherwise remain undegraded.

The search for such isolates has so far met with limited success. Weissenfels *et al.* (1991) isolated *A. denitrificans* strain WW1 from PAH-contaminated soil. Although strain WW1 was able to degrade naphthalene, phenanthrene, anthracene and fluoranthene, degradation of pyrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene was not observed when the high molecular weight PAHs were supplied as sole carbon and energy sources. Similar observations were reported by Walter *et al.* (1991). *Rhodococcus* strain UW1 could grow on phenanthrene, anthracene, fluoranthene as well as pyrene and chrysene, however, no growth was observed on benz[*a*]anthracene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene.

Table 4.1. PAH degradation by microbial communities and bacterial isolates.

PAH	Initial PAH Concentration (mg/l)	% PAH degraded[a]					Time (days)
		Community four	Community five	VUN 10,001	VUN 10,002	VUN 10,003	
FLU	100	90.0 ± 6.8	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	10
PHEN	100	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	7
PYR	100	89.3 ± 8.1	94.0 ± 1.6	100.0 ± 0.0	99.7 ± 7.1	95.3 ± 4.3	7
FA	100	2.2 ± 4.0	12.3 ± 0.4	9.9 ± 5.5	3.1 ± 2.0	8.2 ± 2.1	21
BA	100	10.7 ± 0.8	1.5 ± 0.9	6.0 ± 0.8	6.8 ± 5.1	9.5 ± 5.2	21
DBA	50	13.8 ± 1.1[b]	10.2 ± 1.6[b]	4.4 ± 6.3	9.4 ± 6.8	23.1 ± 4.1	56
B[*a*]P	50	4.6 ± 2.5[b]	2.8 ± 3.2[b]	1.4 ± 6.3	5.5 ± 1.8	6.2 ± 1.3	56

[a] Values are mean ± standard deviation percentages of the recovery of three separate incubations for each PAH relative to the amount of PAHs recovered from uninoculated media. Greater than 95% of added PAHs were recovered from the respective controls. Media containing three- and four-ring PAHs was inoculated with 1% unwashed pyrene-grown cells while benzo[*a*]pyrene and dibenz[*a,h*]anthracene containing media was inoculated with a 10% unwashed pyrene-grown inoculum.

[b] Degradation experiments performed for 28 days.

The PAH degraders described in the previous chapter, communities four and five and *St. maltophilia* strains VUN 10,001, VUN 10,002 and VUN 10,003, were enriched in BSM containing pyrene as the sole carbon and energy source. Their ability to degrade and grow on PAHs is a necessary requirement for the decontamination of PAH-polluted soils, but to do this these isolates must also be able to degrade a spectrum of high and low molecular weight PAHs if they are to be effective in PAH bioremediation.

The general aim of research reported this chapter was to examine the potential of bacteria described in Chapter 3 for use in the bioremediation of PAH-contaminated sites. In particular, the specific aims were to:

1. determine the PAH-substrate range in liquid media of community four and five, and *St. maltophilia* strains VUN 10,001, VUN 10,002 and VUN 10,003;

2. investigate whether the degradation of PAHs by VUN 10,001, VUN 10,002 and VUN 10,003 results in the detoxification of the compounds; and

3. evaluate if a cheap and readily available substrate and nutrient source could be used to condition community five inocula for optimum PAH degradation subsequently.

4.2 METABOLISM OF PAHs BY MICROBIAL COMMUNITIES AND *ST. MALTOPHILIA* ISOLATES

To determine the substrate range of the microorganisms described in Chapter 3, PAHs were added to BSM as sole carbon and energy sources and then inoculated with pyrene-grown starter cultures. PAH degradation was demonstrated by a decrease in PAH concentration compared to uninoculated and killed cell control cultures and growth on test substrates was determined by an increase in microbial numbers or bacterial protein concentration.

4.2.1 Microbial Communities Four and Five

Pyrene-enriched microbial communities from bins four and five were capable of degrading various PAHs of different molecular weight (Table 4.1): both communities were able to degrade fluorene, phenanthrene and pyrene (100 mg/l) to undetectable levels within 10 to 14 days. Community four also degraded small amounts of

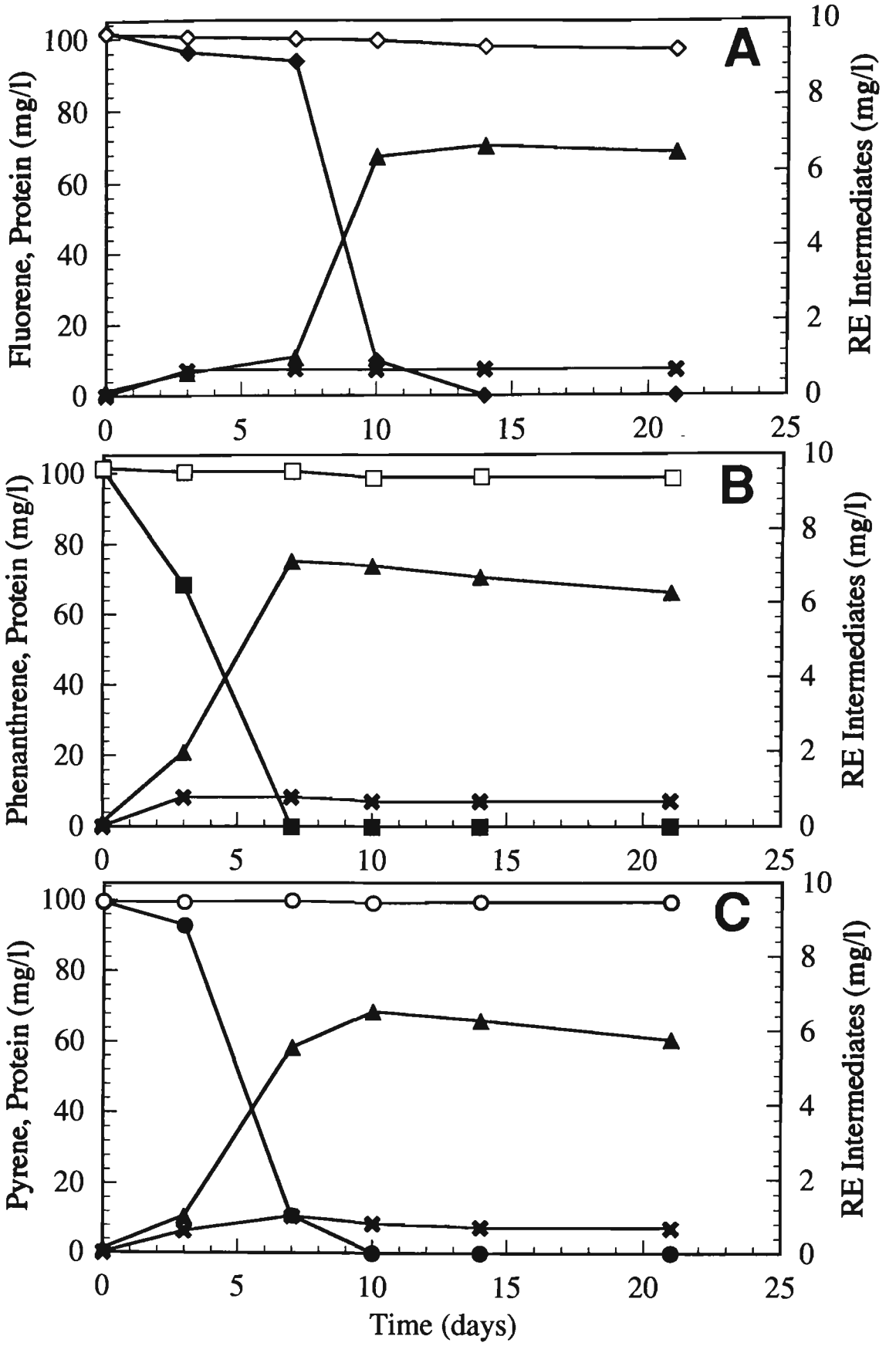


Figure 4.1. Time course for fluorene (A), phenanthrene (B) and pyrene (C) degradation by community four. PAH containing media was inoculated with 1% unwashed pyrene-grown cells. Fluorene (◆), phenanthrene (■), pyrene (●), protein (▲) and resorcinol equivalent (RE) intermediates (✖) were determined as described in the materials and methods. The fluorene (◇), phenanthrene (□) and pyrene (○) concentration in uninoculated control cultures is also shown.

benz[*a*]anthracene and dibenz[*a,h*]anthracene and community five degraded fluoranthene and dibenz[*a,h*]anthracene. Neither of the microbial communities had the ability to degrade significant amounts of benzo[*a*]pyrene (Table 4.1).

Cultures in which significant PAH removal was observed were further examined by measuring changes in the concentration of protein, PAH and resorcinol equivalent (RE) intermediates over a time period, where the latter can indicate the degree of biotransformation of the PAH into polar compounds. As many known PAH metabolites are hydroxylated compounds, the concentration of these compounds in the culture medium can be determined by an assay which detects phenolic compounds (see Section 2.7.2).

Fluorene, phenanthrene, pyrene and benz[*a*]anthracene degradation by community four was accompanied by an increase in protein concentration (Figures 4.1 and 4.2). RE intermediates were detected at low concentrations in the PAH culture media over the incubation period, reaching maximum concentrations of 1 mg/l. Dibenz[*a,h*]anthracene degradation by community four resulted in a 13.8% decrease in the concentration of the compounds after 28 days (Figure 4.2), however, only a small increase in protein concentration was observed over the incubation period (10.1 to 15.2 mg/l) (Table 4.2). The RE intermediate concentration over the incubation period was less than 1 mg/l. These data suggest that communuty four grew poorly on dibenz[*a,h*]anthracene as a sole carbon and energy source.

Fluorene, phenanthrene, fluoranthene and pyrene degradation by community five was accompanied by an increase in protein concentration (Figures 4.3 and 4.4). RE intermediates were detected at low concentrations in fluorene, fluoranthene and pyrene culture media over the incubation period, reaching maximum concentrations of 1 mg/l. In phenanthrene cultures, however, the concentration of RE intermediates reached a maximum of 4.5 mg/l after seven days, and subsequently decreased to 2.0 mg/l after 21 days. The accumulation of RE intermediates coincided with an increase in yellow colouration of the culture medium. Dibenz[*a,h*]anthracene degradation by community five (10.2%) was similar to that of community four (Figure 4.4). Only a small increase in protein concentration was observed over the incubation period (9.1 to 13.8 mg/l) (Table 4.2) while RE intermediate concentration was less than 1 mg/l.

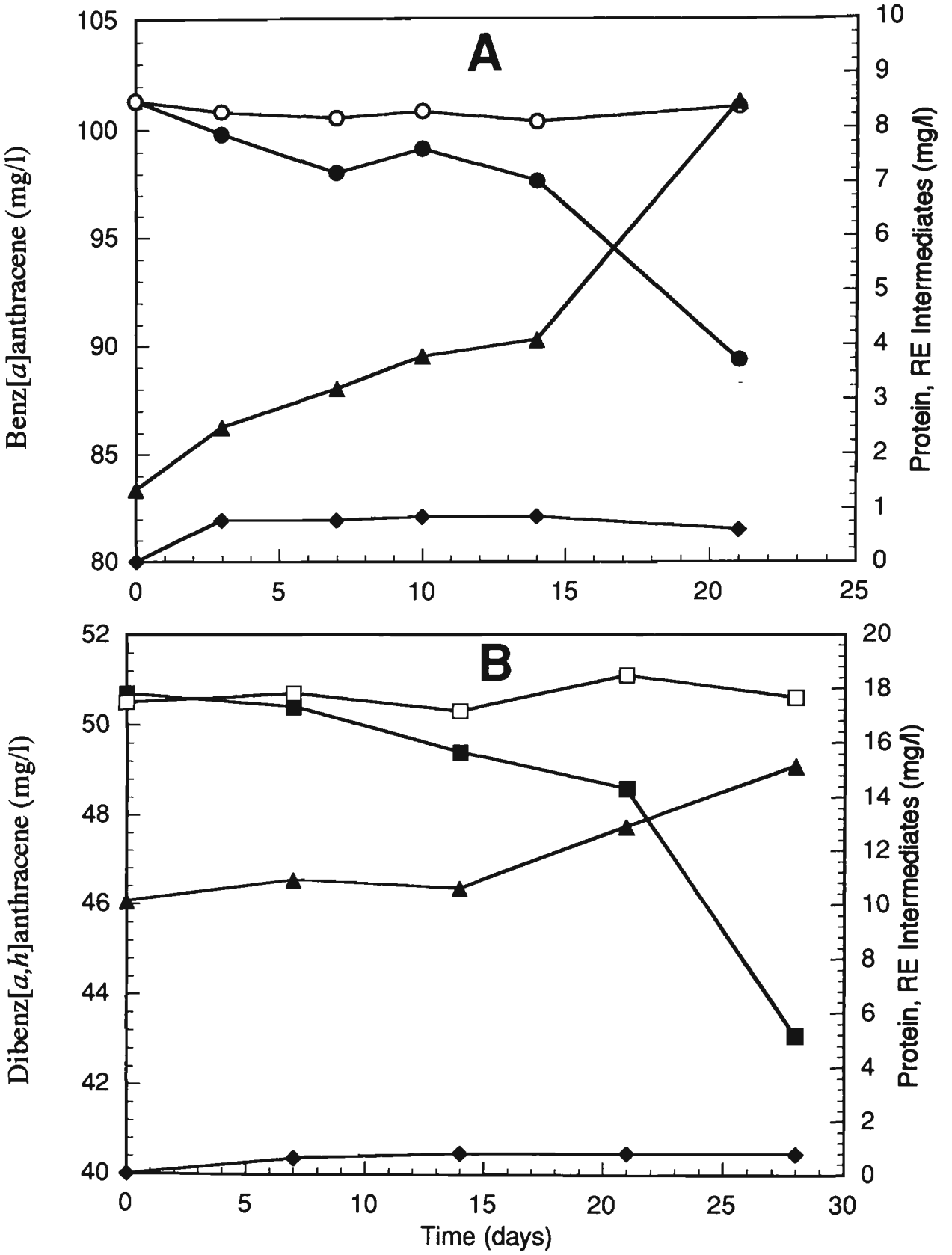


Figure 4.2. Time course for benz[*a*]anthracene (A) and dibenz[*a,h*]anthracene (B) degradation by community four. Benz[*a*]anthracene containing media was inoculated with 1% unwashed pyrene-grown cells while dibenz[*a,h*]anthracene containing media was inoculated with a 10% unwahed pyrene-grown inoculum. Benz[*a*]anthracene (●), dibenz[*a,h*]anthracene (■), protein (▲) and resorcinol equivalent (RE) intermediates (◆) were determined as described in the materials and methods. The benz[*a*]anthracene (□) and dibenz[*a,h*]anthracene (○) concentration in uninoculated control cultures is also shown.

Table 4.2. Protein concentrations of microbial communities and bacterial isolates before and after incubation on PAHs.

	Protein Concentration (mg/l)									
PAH	Community four		Community five		VUN 10,001		VUN 10,002		VUN 10,003	
FLU	1.4[a]	70.1[b]	1.4	73.9	2.3	44.9	2.5	43.2	2.3	41.2
PHEN	1.1	75.1	0.9	90.5	1.1	68.1	1.4	68.1	1.3	68.6
PYR	1.1	68.5	1.0	83.7	2.4	60.2	3.8	59.6	2.9	59.1
FA	1.3	3.1	1.4	11.7	1.8	7.1	2.3	2.9	2.1	8.3
BA	1.4	8.5	1.5	3.8	1.9	5.9	1.8	5.4	1.6	8.1
DBA	10.1	15.2	9.1	13.8	3.0	3.8	3.2	6.0	3.2	9.7
B[*a*]P	11.4	11.8	9.0	9.4	2.8	3.1	5.9	6.4	2.6	3.8

[a] Protein concentration after inoculation.

[b] Maximum protein concentration measured after incubation on the respective PAH as the sole carbon and energy source (see Table 4.1). Maximum protein concentrations were measured at the times indicated in Table 4.1.

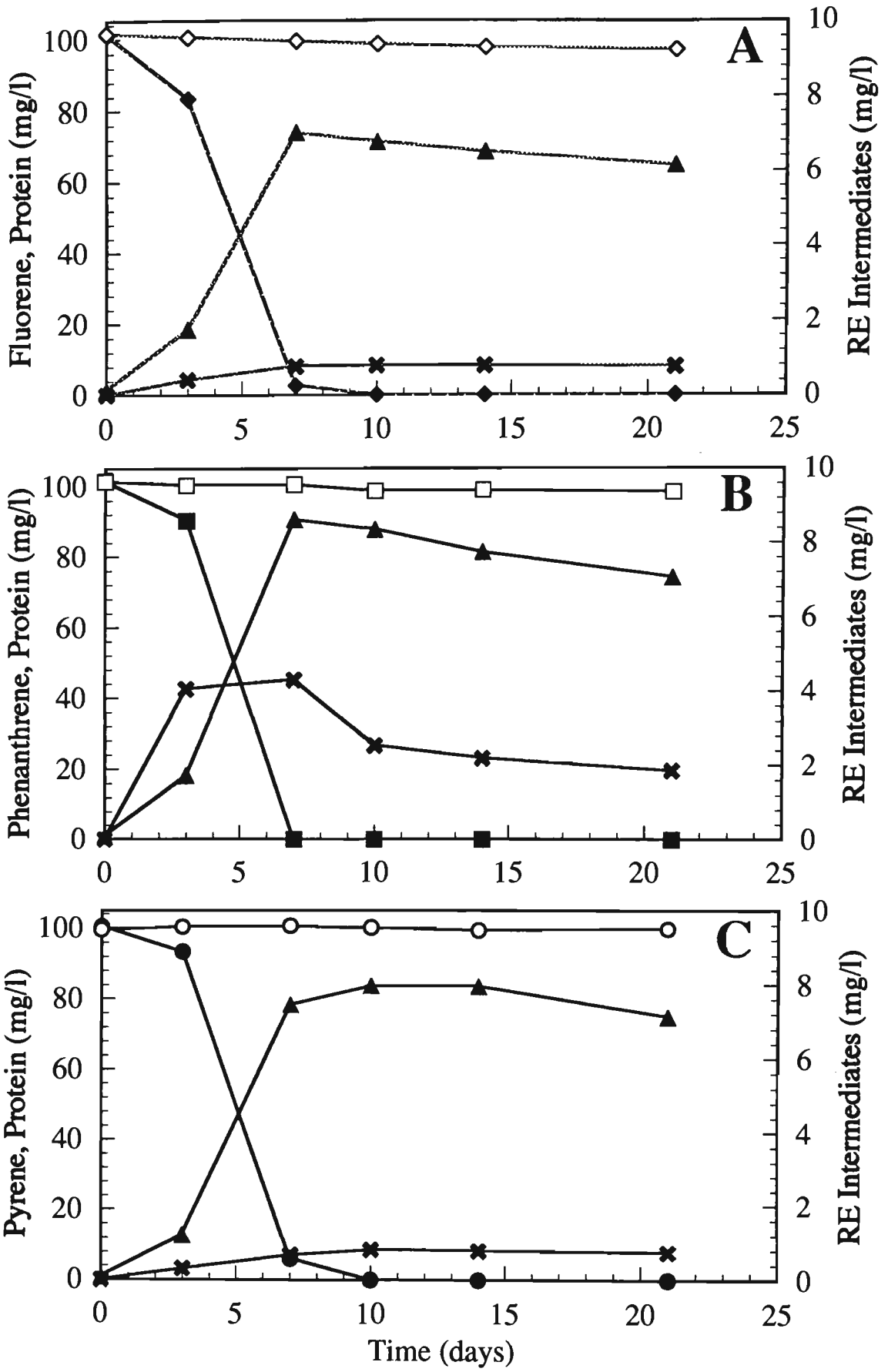


Figure 4.3. Time course for fluorene (A), phenanthrene (B) and pyrene (C) degradation by community five. PAH containing media was inoculated with 1% unwashed pyrene-grown cells. Fluorene (◆), phenanthrene (■), pyrene (●), protein (▲) and resorcinol equivalent (RE) intermediates (✖) were determined as described in the materials and methods. The fluorene (◇), phenanthrene (□) and pyrene (○) concentration in uninoculated control cultures is also shown.

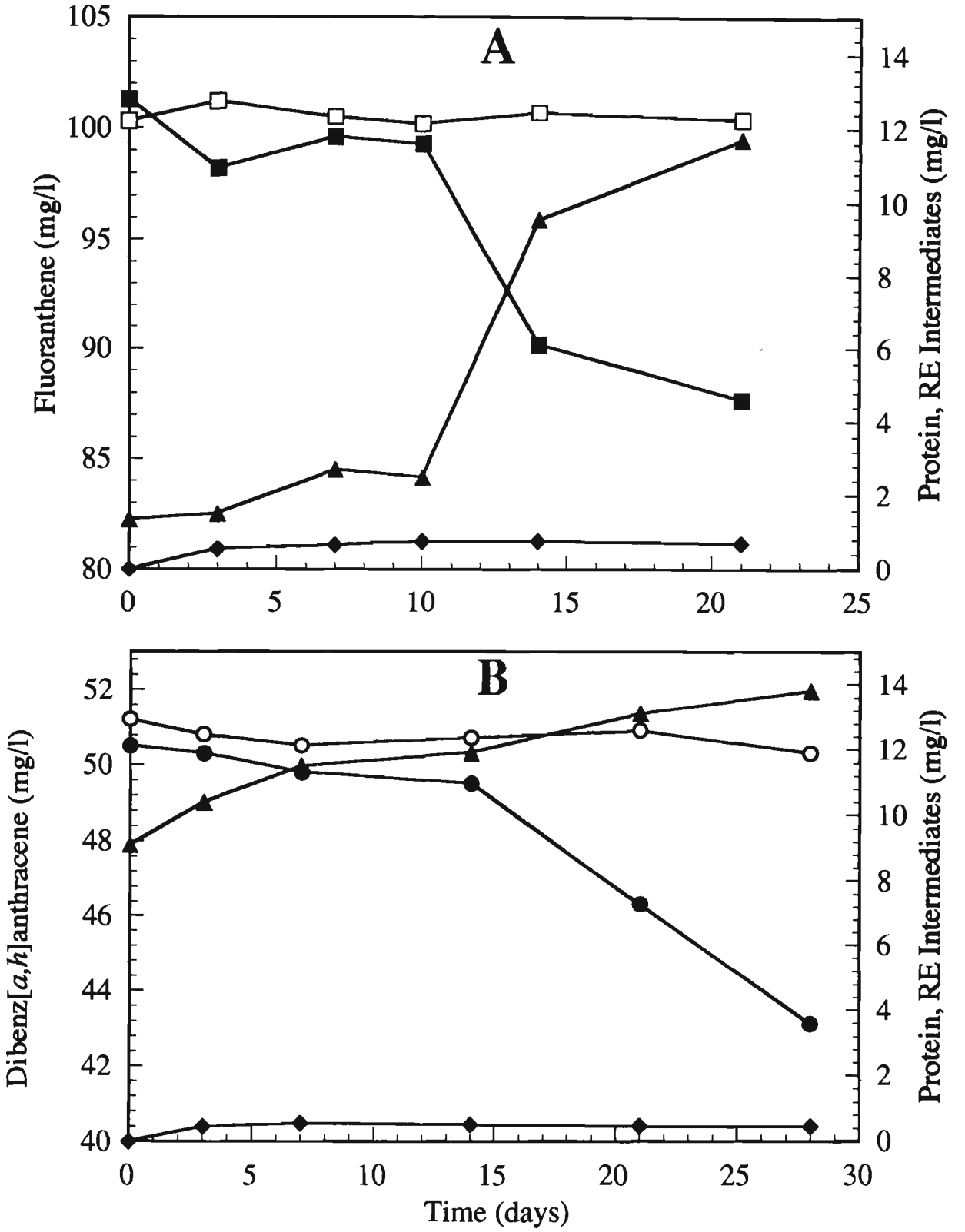


Figure 4.4. Time course for fluoranthene (A) and dibenz[*a*,*h*]anthracene (B) degradation by community five. Fluoranthene containing media was inoculated with 1% unwashed pyrene-grown cells while dibenz[*a*,*h*]anthracene containing media was inoculated with a 10% unwashed pyrene-grown inoculum. Fluoranthene (■), dibenz[*a*,*h*]anthracene (●), protein (▲) and resorcinol equivalent (RE) intermediates (◆) were determined as described in the materials and methods. The fluoranthene (□) and dibenz[*a*,*h*]anthracene (○) concentration in uninoculated control cultures is also shown.

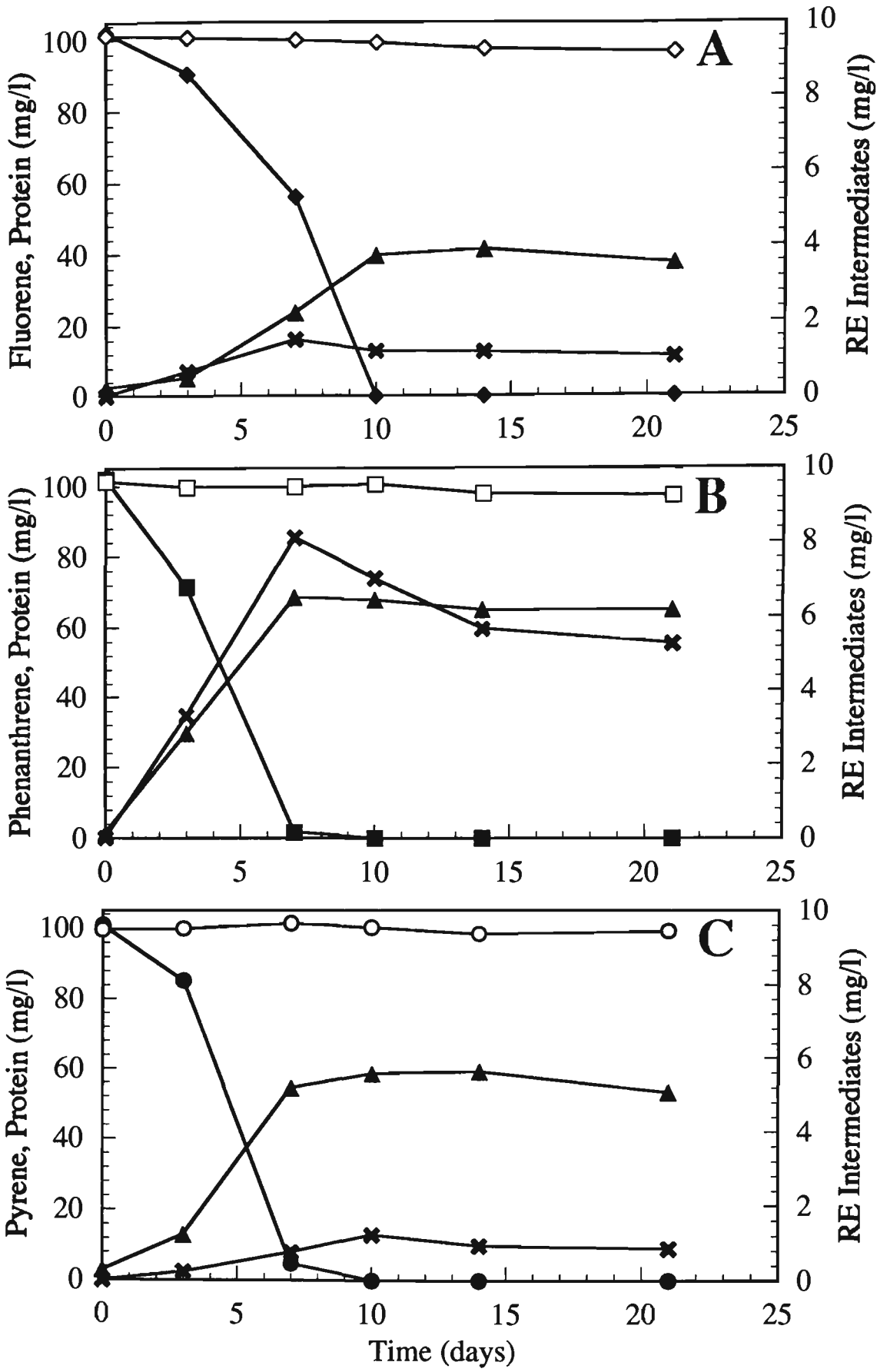


Figure 4.5. Time course for fluorene (A), phenanthrene (B) and pyrene (C) degradation by VUN 10,003. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoclum of VUN 10,003. Fluorene (◆), phenanthrene (■), pyrene (●), protein (▲) and resorcinol equivalent (RE) intermediates (✖) were determined as described in the materials and methods. The fluorene (◇), phenanthrene (□) and pyrene (○) concentration in uninoculated control cultures is also shown.

4.2.2 *St. maltophilia* Isolates

The above experiments were repeated using pure cultures of the three *St. maltophilia* isolates. The inocula were also grown in BSM containing pyrene as the sole carbon source. Degradation experiments were performed with the pure cultures after three successive transfers through BSM containing pyrene.

The pure cultures had degradative profiles which were similar to that of community five (from which they were isolated). All three isolates degraded 100 mg/l of fluorene, phenanthrene and pyrene to undetectable levels within 7 to 10 days (Table 4.1). Fluorene, phenanthrene and pyrene degradation was accompanied by an increase in protein concentration (Table 4.2). High concentrations of RE intermediates accumulated in VUN 10,002 and VUN 10,003 cultures containing phenanthrene, reaching maximum concentrations of 12.5 mg/l and 8.2 mg/l respectively after 7 days (data for VUN 10,003 shown in Figure 4.5). RE intermediate concentrations decreased to 6.0 mg/l and 5.0 mg/l after 21 days incubation. Only low concentrations of RE intermediates (2.0 mg/l) were measured in VUN 10,001 cultures, culminating in a final concentration of 1.5 mg/l after 21 days. When grown on fluorene and pyrene, VUN 10,001, VUN 10,002 and VUN 10,003 produced only low concentrations of RE intermediates (<1.0 mg/l) over the 21 day time course period (data for VUN 10,003 shown in Figure 4.5).

VUN 10,001, VUN 10,002 and VUN 10,003 were also able to degrade high concentrations (250, 500 and 1,000 mg/l) of pyrene (data for VUN 10,003 shown in Figure 4.6). The *St. maltophilia* isolates degraded 95-100% of the pyrene at an initial concentration of 250 mg/l within 10 to 14 days. This was accompanied by an increase in protein concentration from 1.4-1.9 mg/l to 62.4-65.0 mg/l. All three pure cultures grew at high pyrene concentrations (500 and 1,000 mg/l), however, pyrene degradation ceased after approximately 400 mg/l of the initial pyrene in the culture had been degraded (results for VUN 10,003 shown in Figure 4.6). Protein concentrations in these cultures increased from an initial amount of 1.4-3.5 mg/l to 66.1-73.5 mg/l after 28 days.

The three *St. maltophilia* strains grew poorly, if at all, on some of the high molecular weight PAHs when a 10% inoculum from pyrene-grown cultures was used (Table 4.2). Despite this, small decreases in PAH concentration were observed for dibenz[*a,h*]anthracene (VUN 10,002 and VUN 10,003) and fluoranthene (VUN 10,001 and VUN 10,003) (Table 4.1). In particular, VUN 10,003 degraded 11.65 mg/l (23.1%)

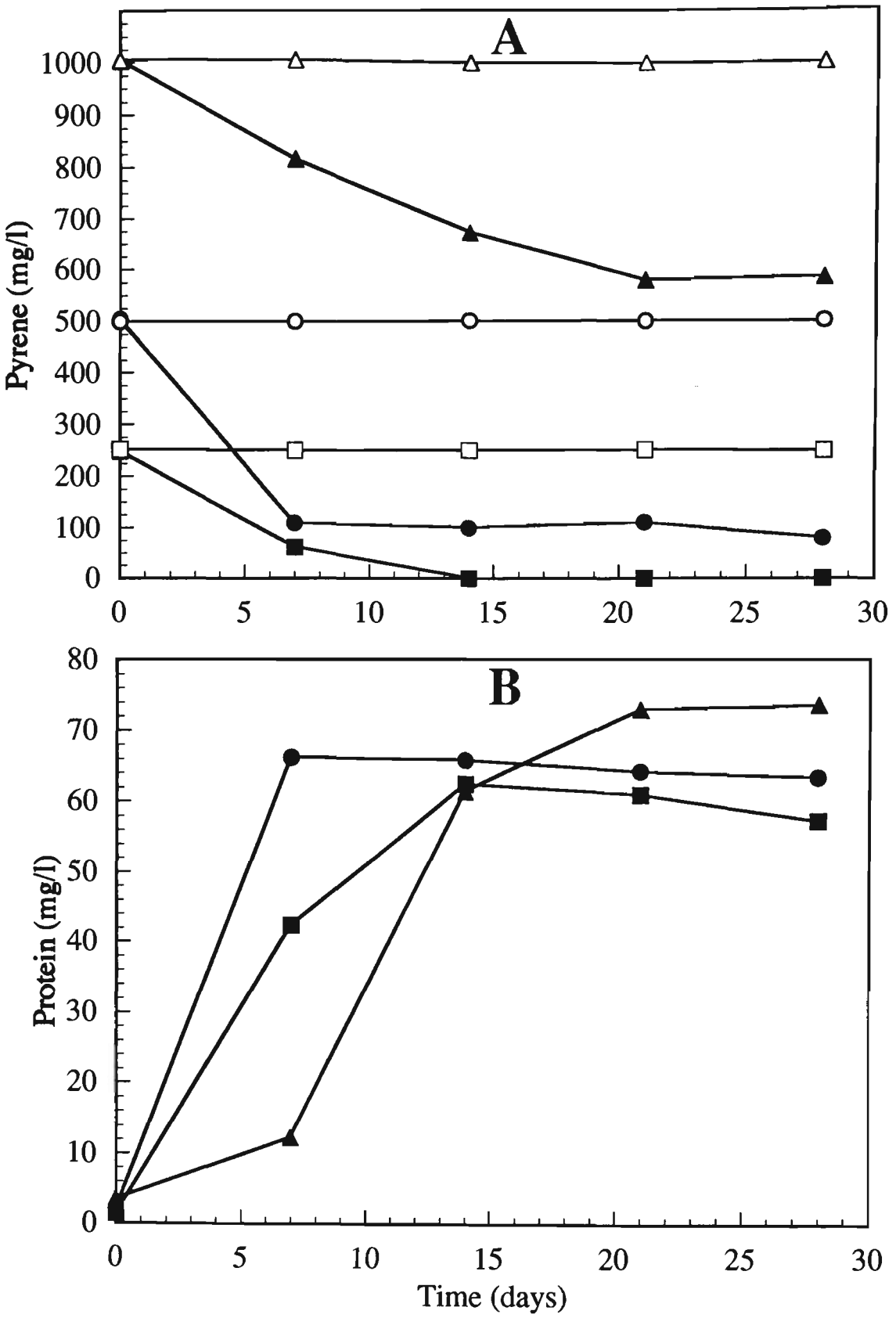


Figure 4.6. Time course for pyrene degradation (A) and growth (B) by VUN 10,003. Pyrene was used as the sole carbon and energy source at the following concentrations; 250 mg/l (■), 500 mg/l (●) and 1000 mg/l (▲). Pyrene containing media was inoculated with 1% unwashed pyrene-grown cells. The concentration of pyrene in uninoculated control cultures [250 mg/l (□), 500 mg/l (○) and 1,000 mg/l (△)] is also shown.

of dibenz[*a,h*]anthracene after 56 days, with only a small increase (3.2 to 9.7 µg/ml) in bacterial protein concentration. VUN 10,003 was also the only culture that could utilise benz[*a*]anthracene as a sole carbon and energy source (Table 4.1).

4.2.3 Microbial Degradation of High Molecular Weight PAHs

Results described in Section 4.2.2 indicated that the *St. maltophilia* isolates could not initiate growth on high molecular weight compounds so that only small amounts of the substrates were used. To investigate whether the small decreases in concentration resulted from biodegradation, inocula containing high cell numbers of pyrene-grown cells were used in BSM medium containing high molecular weight PAHs. Alternatively, other approaches involved adding a low molecular weight PAH as an alternative carbon and energy source (to test for cometabolism) and inoculating media with high cell numbers of microorganisms which had been grown on an alternative substrate.

4.2.3.1 *PAH Degradation Using High Initial Cell Populations*

All starter cultures used in this section (community five, VUN 10,001, VUN 10,002 and VUN 10,003) were grown on pyrene as the sole carbon and energy source (see Section 2.6.1.4). Cells were collected by centrifugation, washed and resuspended in BSM to achieve a ten-fold concentration in cell biomass. Aliquots of these cell suspensions were added to serum bottles and PAHs (benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene) added as sole carbon and energy sources.

Autoclaved and mercuric chloride killed cell controls were prepared to assess the extent of PAH adsorption to cellular material. The killed cells were added to PAH media at a concentration of 0.85 mg/l protein. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to the BSM at a concentration of 50 mg/l while coronene was added at a concentration of 20 mg/l. No significant difference in the PAH extraction yield was observed between controls containing no cells, autoclaved cells or mercuric chloride killed cell controls. Greater than 97% of the added PAHs were recovered from control flasks after 63 days. Figure 4.7 shows the amount of benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene recovered from control cultures containing no cells, autoclaved cells or mercuric chloride killed cell controls over the incubation period. Due to the PAH extraction yield from the three controls being similar, only the results for the uninoculated controls will be shown in the following figures.

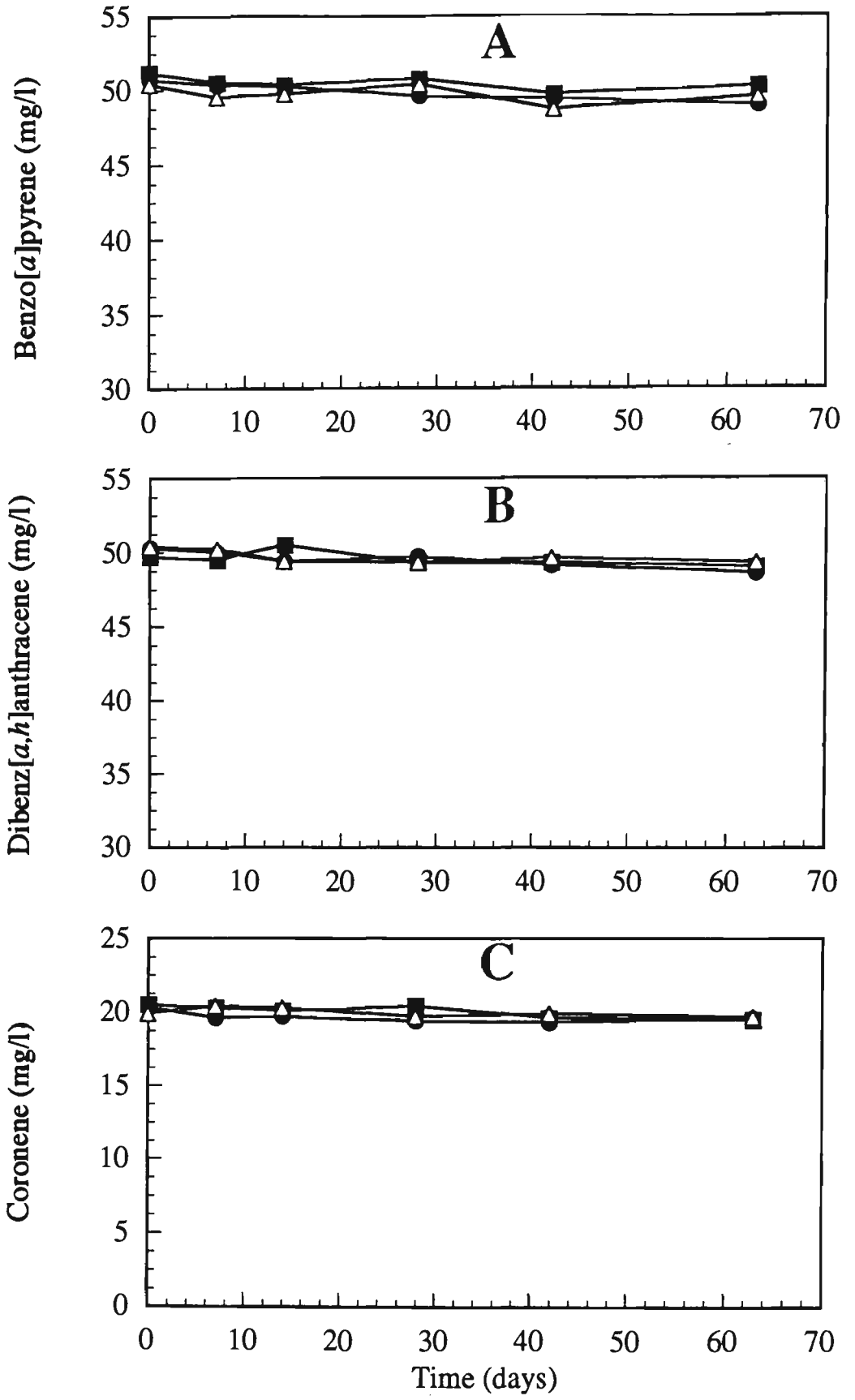


Figure 4.7. Recovery of benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) from controls flasks containing no cells (■), autoclaved VUN 10,003 cells (●) and mercuric chloride killed VUN 10,003 cells (Δ). Benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to BSM at a concentration of 50.2-51.2 mg/l, while coronene was added at a concentration of 19.9-20.5 mg/l. Autoclaved or mercuric chloride killed cells were added to BSM at a concentration of 0.85 mg protein/l.

All three *St. maltophilia* isolates were able to degrade five- and seven-ring PAHs (Figures 4.8, 4.9 and 4.10); similar results were obtained for the degradation of these compounds by community five (Figure 4.11). Although the degradation of benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene was slow, significant decreases of 20-30% (10-14 mg/l) in benzo[*a*]pyrene and dibenz[*a,h*]anthracene were observed after 63 days; community five degraded 18-21% (9.0-10.5 mg/l) of benzo[*a*]pyrene and dibenz[*a,h*]anthracene after 42 days. Lag periods of up to 21 days were observed for the three strains and community five before the onset of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. Protein concentrations decreased over the incubation period (1.31-1.47 g/l to 1.12-1.24 g/l) indicating that little cell growth occurred and, indeed, some cell lysis occurred. Coronene degradation by the *St. maltophilia* strains was extensive, with 65-70% (13-14 mg/l) being degraded after 63 days; community five degraded 53% (10.5 mg/l) of the coronene supplied after 42 days. A lag period of approximately 14 days occurred before the onset of coronene degradation. Similarly, protein concentrations decreased over time (1.31-1.47 g/l to 1.12-1.24 g/l).

Most PAH-contaminated sites contain a variety of PAH compounds, ranging in size from two- to seven-rings. Under these conditions, it is possible that the high molecular weight PAH-degrading ability of the isolates may be affected by the presence of the low molecular weight PAH compounds. This aspect was investigated by performing experiments using a mixture of substrates containing different PAHs (three- to seven-ring PAH compounds) and inocula containing high numbers of cells.

The results demonstrated that all three *St. maltophilia* strains (Figures 4.12, 4.13 and 4.14) and community five (Figure 4.15) were capable of degrading high and low molecular weight PAHs concurrently. Degradation of the lower molecular weight PAHs (three-ring compounds) was fast and extensive; in the case of VUN 10,002, over 90% of fluorene and phenanthrene was degraded in seven days. Fluoranthene and pyrene were degraded to similar extents, with a 57-75% decrease in the concentration of these compounds after 42 days. The lag period in the degradation of five- and seven-ring compounds by all the isolates was approximately 7-10 days, a significant decrease when compared to a lag period of 14-21 days prior to degradation in the single substrate experiments. Degradation of benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene by community five, VUN 10,001 and VUN 10,003 resulted in decreases in the concentration of the compounds of 29-42% after 42 days. Five-ring PAH degradation by VUN 10,002 was less extensive compared to the other isolates, however, significant decreases of 19% and 24% were observed for

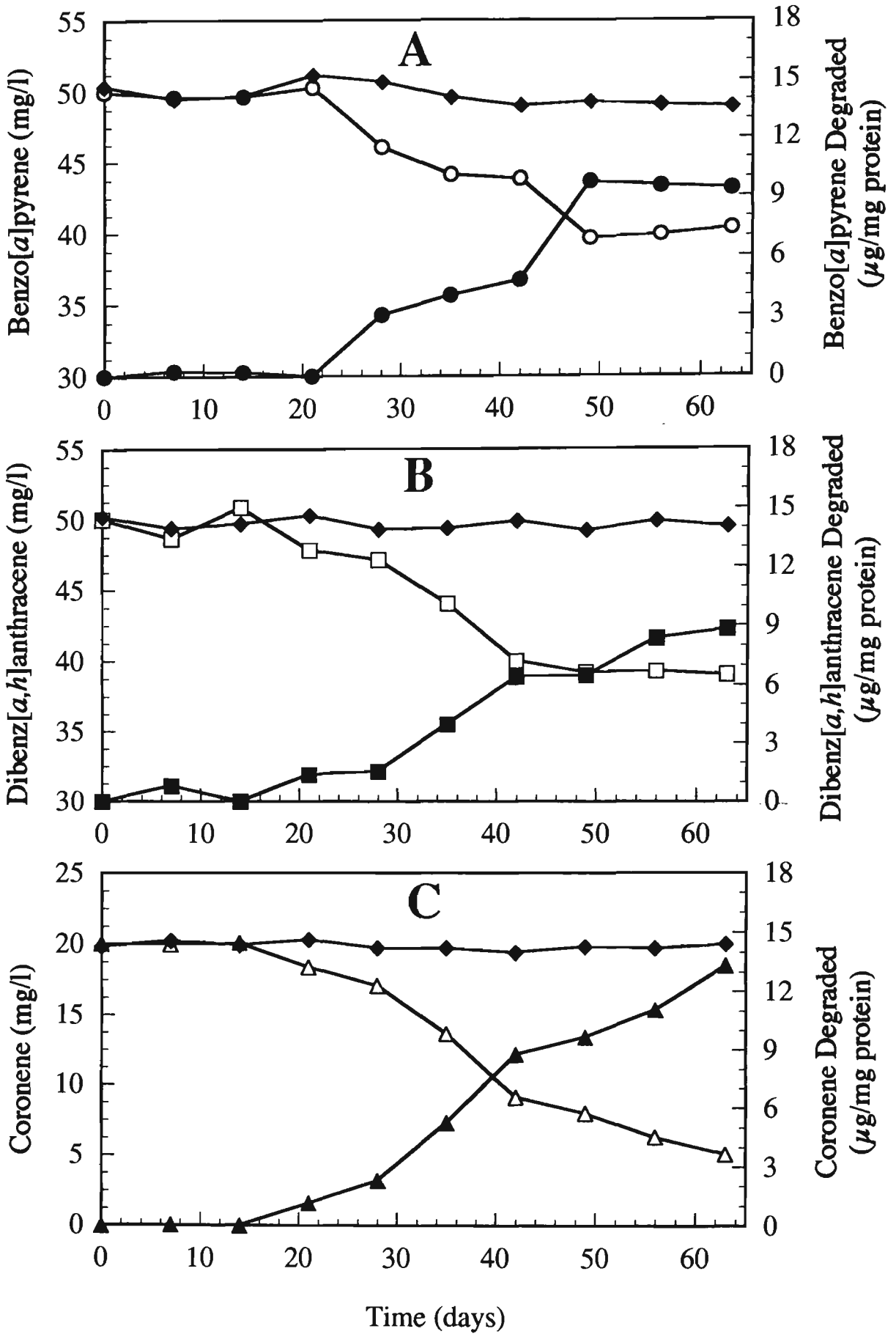


Figure 4.8. Degradation of benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) by VUN 10,001 when PAHs supplied as single substrates. Benzo[*a*]pyrene (O), dibenz[*a,h*]anthracene (□) and coronene (Δ) were added to BSM inoculated with high initial cell densties of pyrene-grown VUN 10,001. Degradation rates per mg of protein are shown for benzo[*a*]pyrene (●), dibenz[*a,h*]anthracene (■) and coronene (▲). Controls (◆) contained uninoculated BSM and the respective PAH.

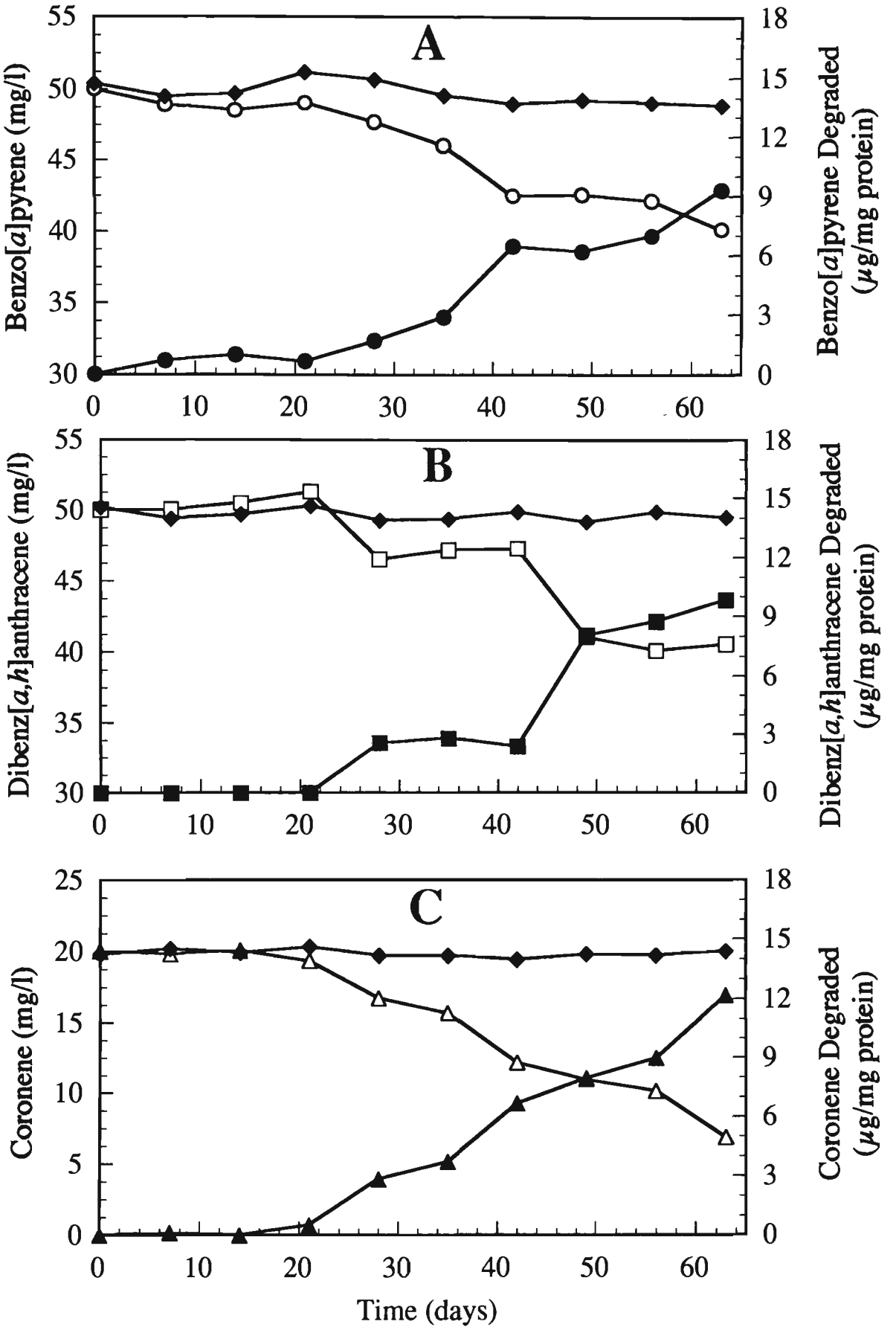


Figure 4.9. Degradation of benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) by VUN 10,002 when PAHs were supplied as single substrates. Benzo[*a*]pyrene (O), dibenz[*a,h*]anthracene (□) and coronene (Δ)were added to BSM inoculated with high cell numbers of pyrene-grown VUN 10,002. Degradation rates per mg of protein are shown for benzo[*a*]pyrene (●), dibenz[*a,h*]anthracene (■) and coronene(▲). Controls (◆) contained uninoculatedBSM and the respectivePAH.

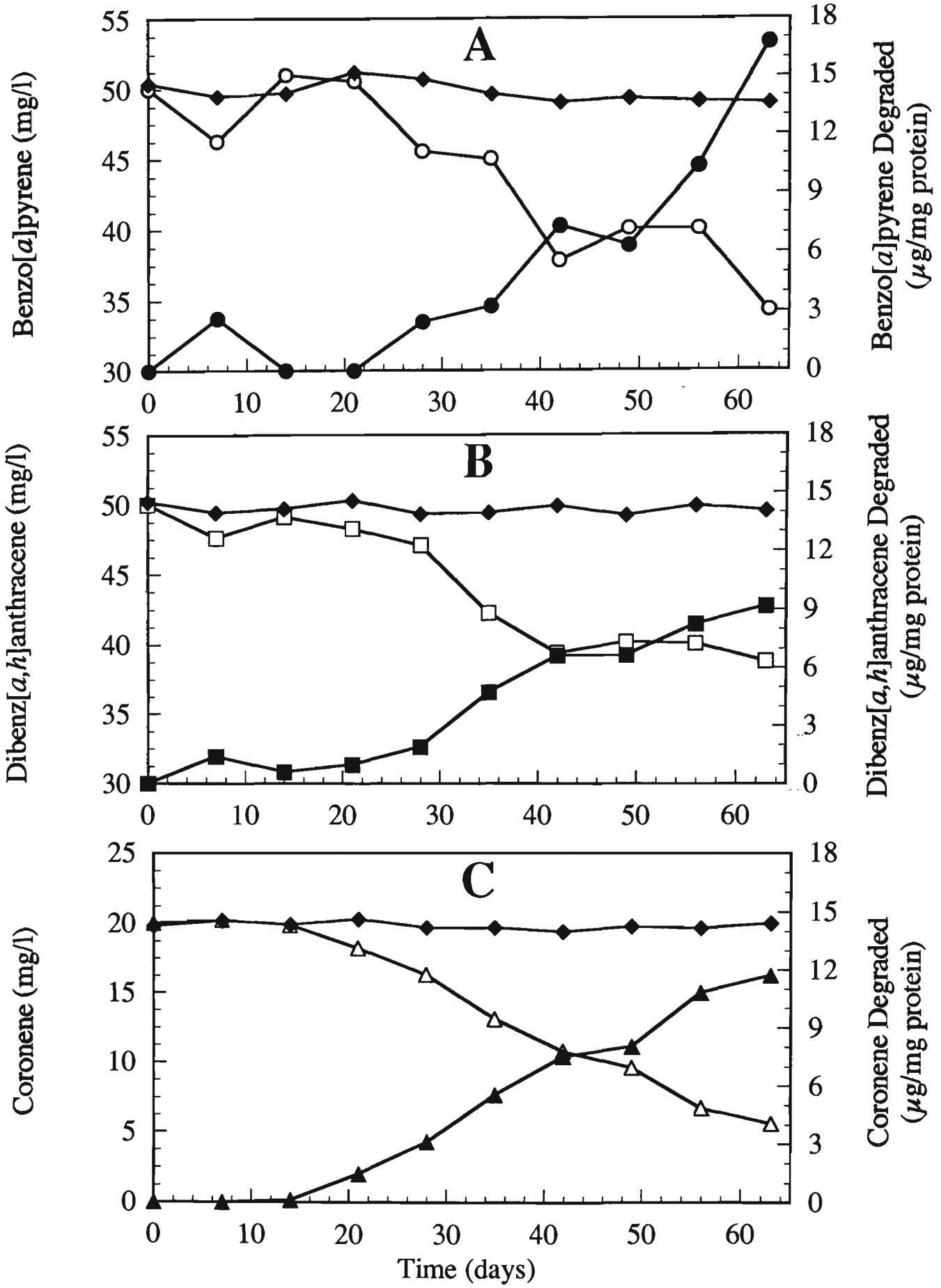


Figure 4.10. Degradation of benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) by VUN 10,003 when PAHs supplied as single substrates. Benzo[*a*]pyrene (O), dibenz[*a,h*]anthracene (□) and coronene (Δ) were added to BSM inoculated with high initial cell densties of pyrene-grown VUN 10,003. Degradation rates per mg of protein are shown for benzo[*a*]pyrene (●), dibenz[*a,h*]anthracene (■) and coronene (▲). Controls (◆) contained uninoculated BSM and the respective PAH.

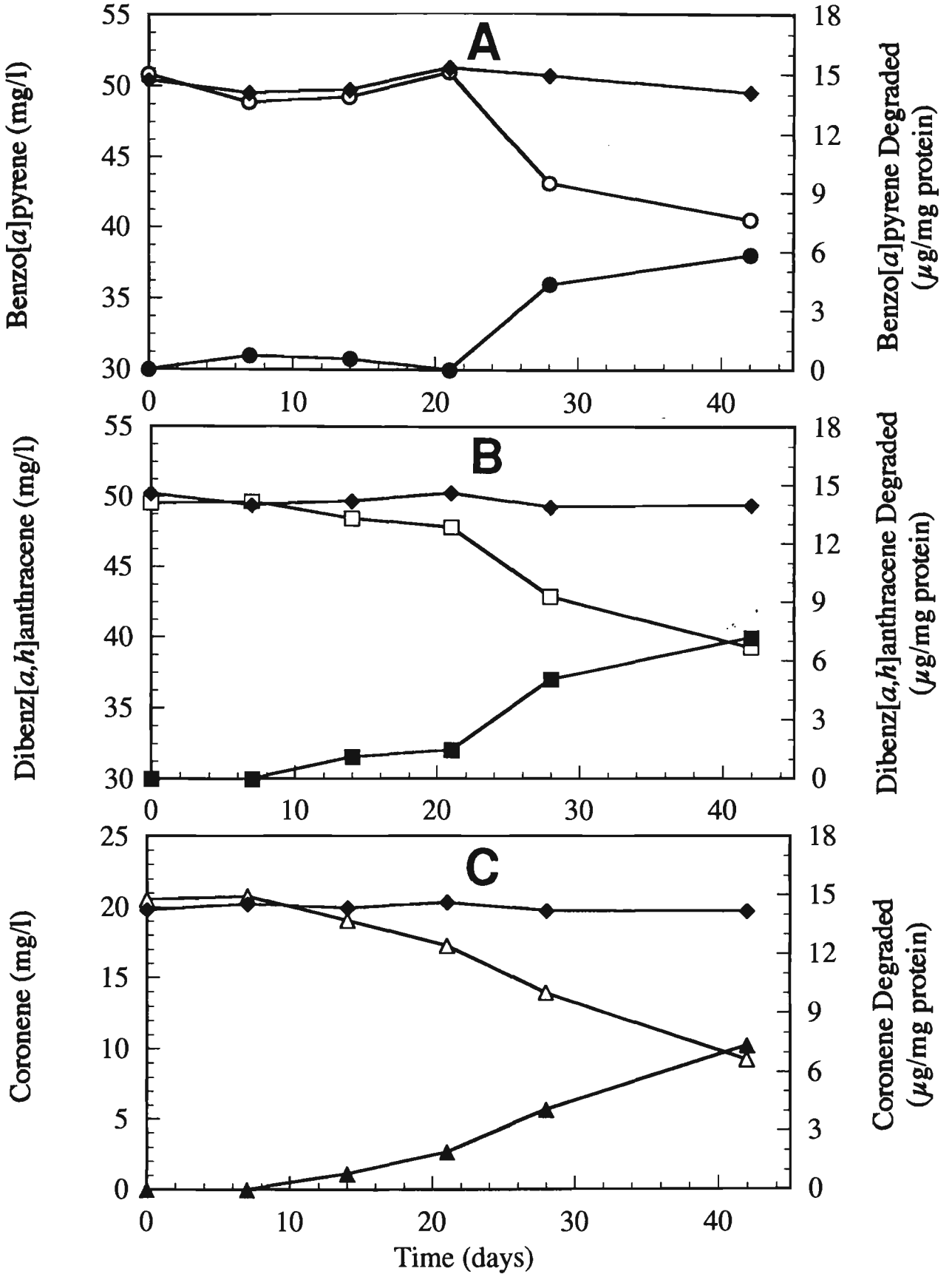


Figure 4.11. Time course for benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) degradation by community five when PAHs were supplied as single substrates. Benzo[*a*]pyrene (O), dibenz[*a,h*]anthracene (□) and coronene (Δ) were added to BSM inoculated with high initial cell densities of pyrene-grown community five. Degradation rates per mg of protein are shown for benzo[*a*]pyrene (●), dibenz[*a,h*]anthracene (■) and coronene (▲). Controls (◆) contained uninoculated BSM and the respective PAH.

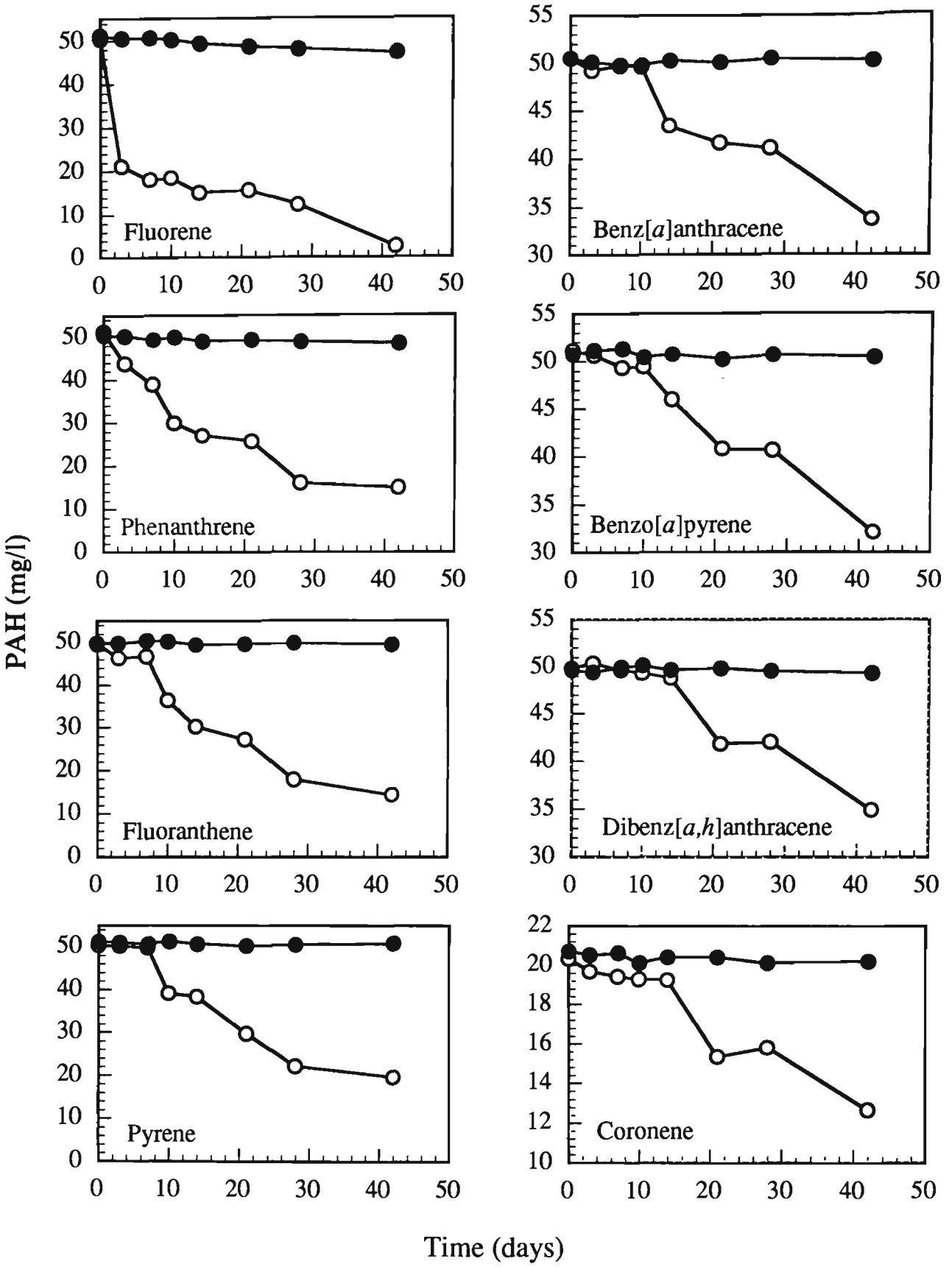


Figure 4.12. PAH concentration in BSM containing a PAH mixture inoculated with high initial cell densities of pyrene-grown VUN 10,001. The panels represent the concentration profile of each PAH in cultures containing all of the above PAHs (O). Control cultures containing autoclaved cells of VUN 10,001 and PAHs are also shown (●).

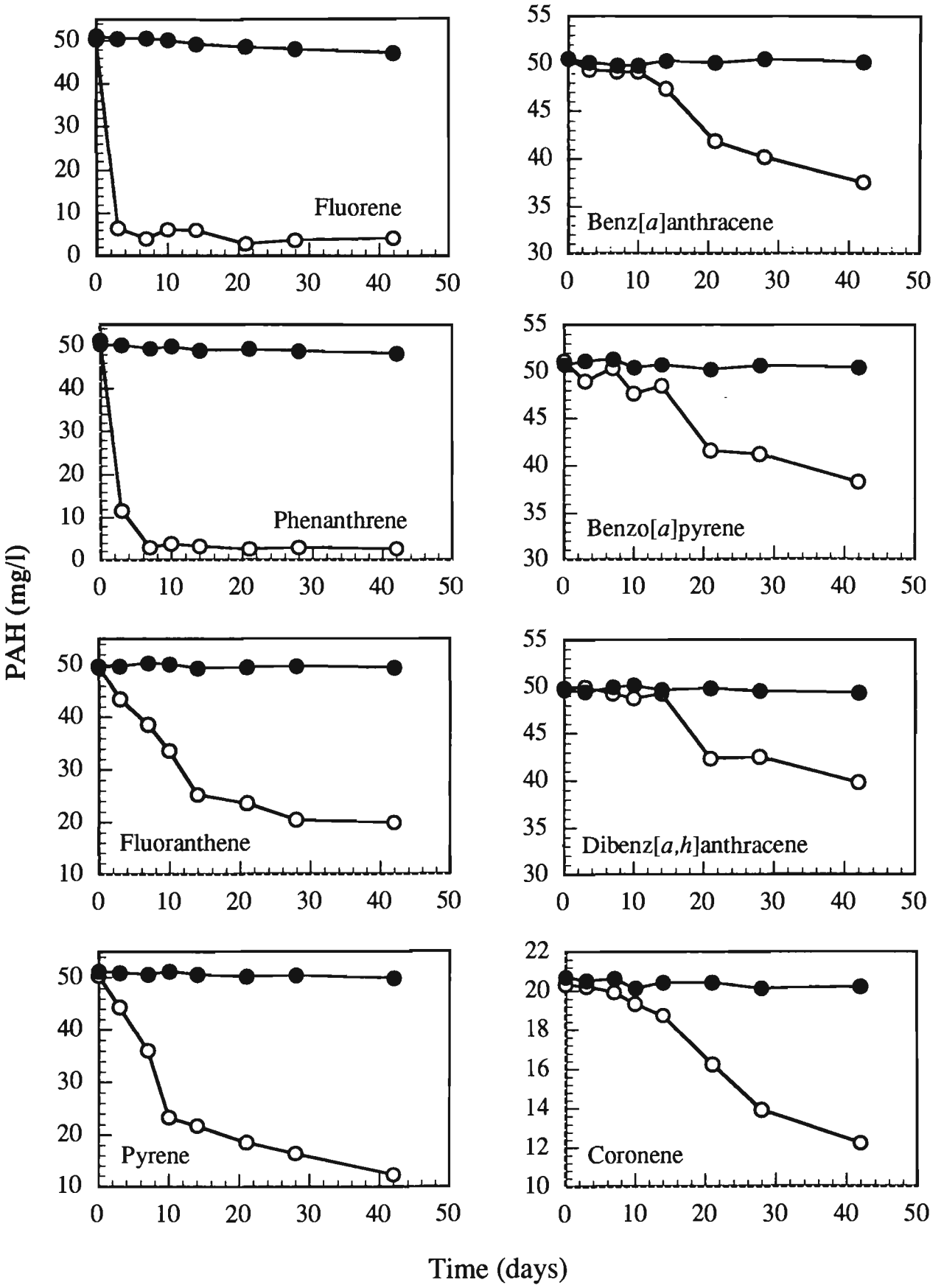


Figure 4.13. PAH concentration in BSM containing a PAH mixture inoculated with high initial cell densities of pyrene-grown VUN 10,002. The panels represent the concentration profile of each PAH in cultures containing all of the above PAHs (O). Control cultures containing autoclaved cells of VUN 10,002 and PAHs are also shown (●).

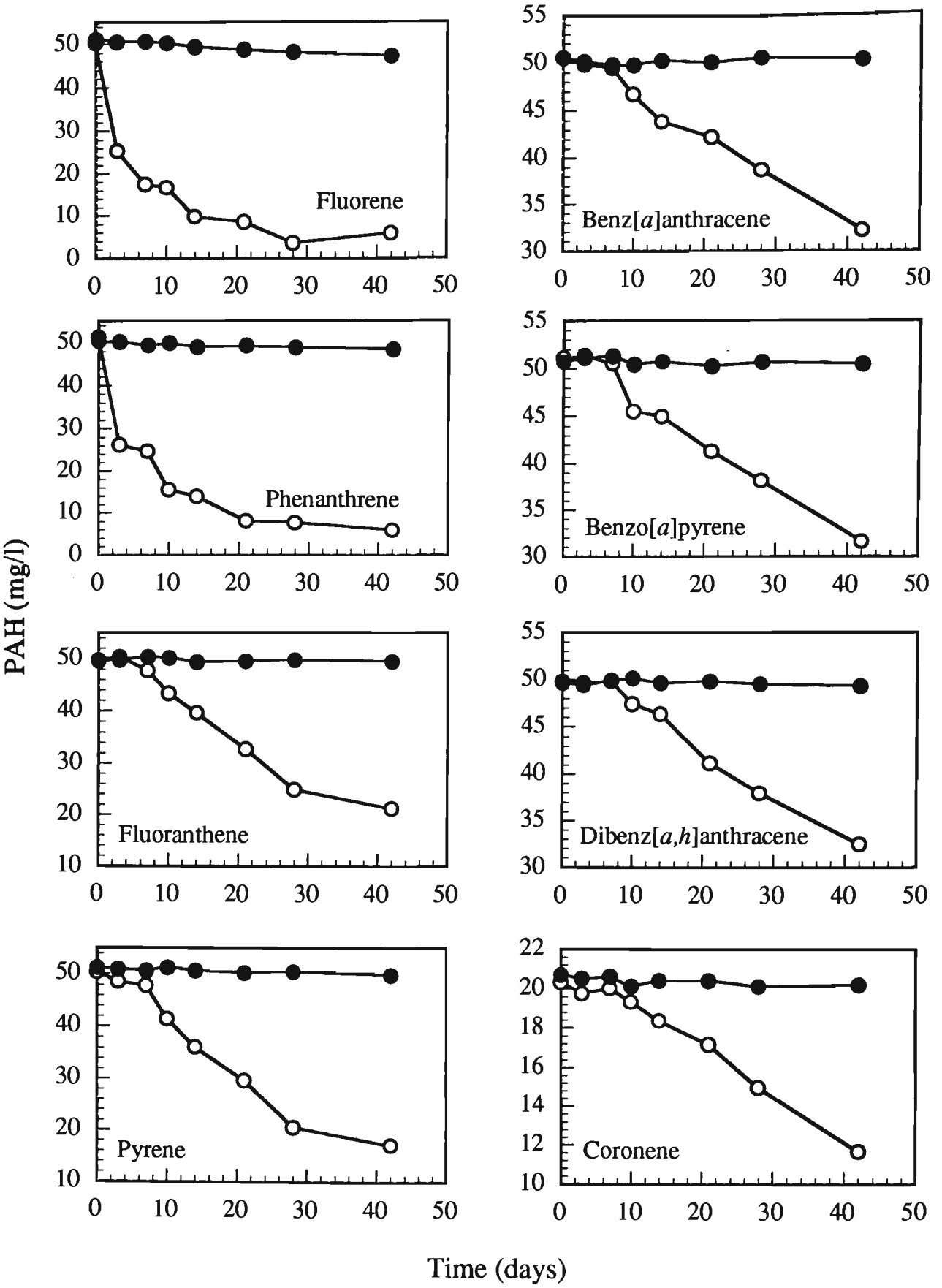


Figure 4.14. PAH concentration in BSM containing a PAH mixture inoculated with high initial cell densities of pyrene-grown VUN 10,003. The panels represent the concentration profile of each PAH in cultures containing all of the above PAHs (O). Control cultures containing autoclaved cells of VUN 10,003 and PAHs are also shown (●).

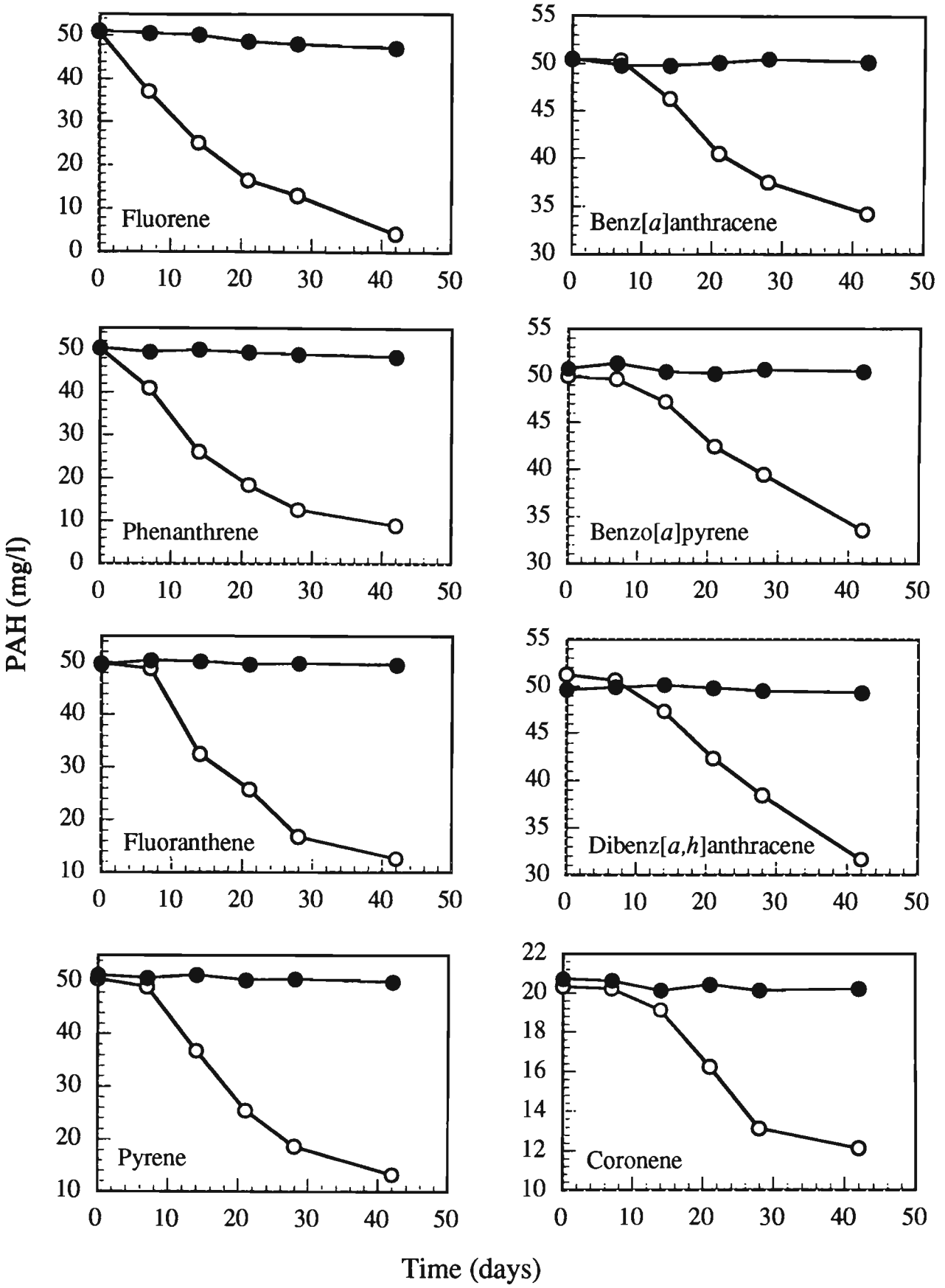


Figure 4.15. PAH concentration in BSM containing a PAH mixture inoculated with high initial cell densities of pyrene-grown community five. The panels represent the concentration profile of each PAH in cultures containing all of the above PAHs (O). Control cultures containing autoclaved cells of community five and PAHs are also shown (●).

Table 4.3. Five- and seven-ring PAH degradation rates by VUN 10,001. PAHs were added to BSM containing high initial cell numbers of pyrene-grown VUN 10,001 as individual compounds or as part of a PAH mixture.

PAH Supplied as:	PAH	Incubation Time (days)	Lag Period (days)	Biomass (g/l)	PAH degraded (mg/l)	µg PAH degraded/mg protein	Degradation Rate (µg PAH degraded/mg protein/day)
Single	B[a]P	42	21	1.16	6.2	5.3	0.252
Substrate	DBA		21	1.14	10.0	8.8	0.419
	COR		14	1.18	10.9	9.3	0.332
Mixed	B[a]P	42	7	0.64	17.9	28.0	0.800
Substrate	DBA		7	0.64	15.1	23.6	0.674
	COR		7	0.64	7.3	11.5	0.329

benzo[*a*]pyrene and dibenz[*a,h*]anthracene respectively. Protein concentrations decreased over the incubation period from initial concentrations of 0.72-0.78 g/l to 0.64-0.69 g/l after 42 days.

To compare degradation rates of *St. maltophilia* strains for five- and seven-ring PAH in single and mixed PAH cultures, the specific degradation rates of these compounds were calculated by determining the amount of each PAH degraded during the period of microbial degradation and dividing it by the average protein concentration and the time lapsed during that period (mg PAH degraded/mg protein/day). It should be made clear that the specific degradation rates were calculated from data collected only during the periods when degradation was occurring; no data was used from the degradation lag period.

The high molecular weight PAHs in the mixed PAH cultures were degraded to a greater extent per mg protein and at a faster rate compared to single PAH, high cell density cultures; the exception was coronene degradation by VUN 10,001, where the specific PAH degradation rate was comparable in single and mixed substrate experiments (Tables 4.3, 4.4 and 4.5). After 42 days, the amount of each five-ring PAH degraded per mg of protein in the mixed PAH incubations was 1.7- to 8.8-fold greater than the amount degraded when the PAHs were supplied as single substrates. In addition, the specific degradation rates of benzo[*a*]pyrene and dibenz[*a,h*]anthracene were 0.6- to 4.9-fold greater when the PAH was degraded in the PAH mixture. The amount of coronene degraded per mg of protein was also greater (24-84%) when the compound was supplied as part of the PAH mixture compared to its degradation as a single substrate. Small increases in the specific degradation rates of coronene were observed for VUN 10,002 (47%) and VUN 10,003 (29%), however, the specific degradation rate of VUN 10,001 was similar in both single and mixed substrate experiments.

To compare the rate at which each individual PAH was degraded in the PAH mixture, the cumulative degradation rate for each PAH was determined at each sampling point and plotted over the time course period. The degradation values in Figure 4.16 represents the total amount of PAH degraded by VUN 10,003 (from time zero), divided by the average protein concentration during the time period. The rate at which each PAH was degraded correlated to the size and the number of benzene ring of the compound. The lower molecular weight compounds were degraded at the fastest rate; after 42 days, 62 μg of fluorene and phenanthrene were degraded per mg protein. The degradation rate of the four-ring compounds, fluoranthene and pyrene, was 26-35% less than for fluorene and phenanthrene. Benz[*a*]anthracene, benzo[*a*]pyrene and

Table 4.4. Five- and seven-ring PAH degradation rates by VUN 10,002. PAHs were added to BSM containing high initial cell numbers of pyrene-grown VUN 10,002 as individual compounds or as part of a PAH mixture.

PAH Supplied as:	PAH	Incubation Time (days)	Lag Period (days)	Biomass (g/l)	PAH degraded (mg/l)	µg PAH degraded/mg protein	Degradation Rate µg PAH degraded/mg protein/day)
Single	B[a]P	42	21	1.29	7.5	5.8	0.276
Substrate	DBA		21	1.56	2.7	1.7	0.081
	COR		14	1.25	7.9	6.3	0.225
Mixed	B[a]P	42	7	0.67	11.8	17.6	0.503
Substrate	DBA		7	0.67	11.2	16.7	0.447
	COR		7	0.67	7.8	11.6	0.331

Table 4.5. Five- and seven-ring PAH degradation rates by VUN 10,003. PAHs were added to BSM containing high initial cell numbers of pyrene-grown VUN 10,003 as individual compounds or as part of a PAH mixture.

PAH Supplied as:	PAH	Incubation Time (days)	Lag Period (days)	Biomass (g/l)	PAH degraded (mg/l)	µg PAH degraded/mg protein	Degradation Rate µg PAH degraded/mg protein/day
Single	B[a]P	42	21	1.67	12.3	7.4	0.352
Substrate	DBA		21	1.60	10.6	6.6	0.314
	COR		14	1.23	9.2	7.5	0.268
Mixed	B[a]P	42	7	0.69	18.4	26.7	0.763
Substrate	DBA		7	0.69	17.5	25.4	0.726
	COR		7	0.69	8.3	12.1	0.346

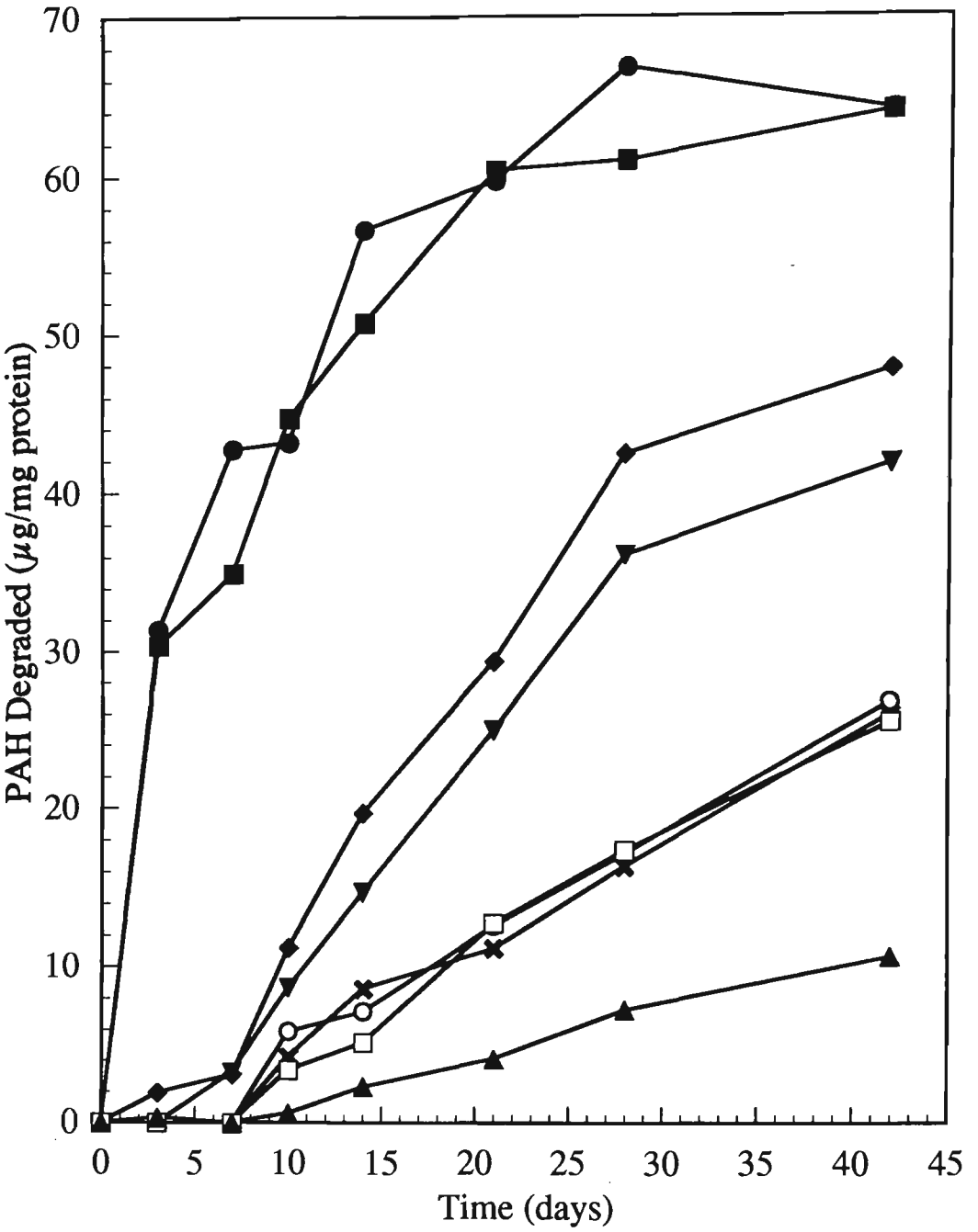


Figure 4.16. Time course degradation experiment using high initial cell numbers of VUN 10,003 and a mixture of PAHs. PAHs were added to flasks at a concentration of 50 mg/l each, the exception being coronene which was supplied at a concentration of 20 mg/l. The degradation rates of fluorene (●), phenanthrene (■), fluoranthene (▼), pyrene (◆), benz[*a*]anthracene (✖), benzo[*a*]pyrene (O), dibenz[*a*,*h*]anthracene (□), and coronene (▲) are expressed as mg PAH degraded per mg protein.

dibenz[*a,h*]anthracene were all degraded at similar rates; after 42 days, between 25.5 and 26.8 μg of the PAHs supplied were degraded per mg protein. Coronene was degraded at the slowest rate, which was 83% lower than seen for the three-ring compounds.

4.2.3.2 *Cometabolism of PAHs*

In Section 4.2.1 and 4.2.2, it was shown that community five and strains VUN 10,001, VUN 10,002 and VUN 10,003 could use phenanthrene and pyrene as sole carbon and energy sources and produce cultures with relatively high microbial populations (approximately 5 x 10^6-1 x 10^7 cells/ml). Section 4.2.3.1 demonstrated that community five and the individual strains could degrade high molecular weight PAHs if cell numbers in the inoculum were high and that the rate of degradation of high molecular weight PAHs increased in mixed-substrate cultures. This section tested whether high molecular weight PAHs could be degraded in the presence of low molecular weight substrates which would have allowed good growth to occur from low initial cell numbers in the inoculum. This was achieved by adding a 1% pyrene-grown inoculum of community five and the three *St. maltophilia* strains into BSM containing either phenanthrene or pyrene and a five-ring PAH. The purpose of the lower molecular weight PAH was to stimulate growth and support a cell population of sufficient magnitude to degrade significant quantities of the five-ring compound.

Community five, VUN 10,001, VUN 10,002 and VUN 10,003 were able to degrade significant amounts of benzo[*a*]pyrene and dibenz[*a,h*]anthracene (50 mg/l) when these cultures were supplemented with phenanthrene as the only other source of carbon and energy. In these cultures, greater than 97% of the phenanthrene (100 mg/l) was degraded after 28 days and there was a corresponding increase in protein concentration from 3 to 52-56 mg/l, indicating that growth had occurred. Degradation of the five-ring compounds by VUN 10,001 proceeded slowly over the 56 day experimental period, resulting in a 52% decrease in dibenz[*a,h*]anthracene concentration or a 41% decrease in benzo[*a*]pyrene concentration (Figure 4.17). A lesser amount of these five-ring compounds was degraded by community five, VUN 10,002 and VUN 10,003 (data shown in Appendix 2) but there was still a significant decrease of between 30% to 49% in the concentration of these compounds. Protein concentrations decreased slightly after day 28, when the five-ring PAHs were the only source of carbon in the cultures.

Similar results to those described above were observed for all isolates when pyrene was substituted for phenanthrene as the cosubstrate. A decrease in the pyrene concentration

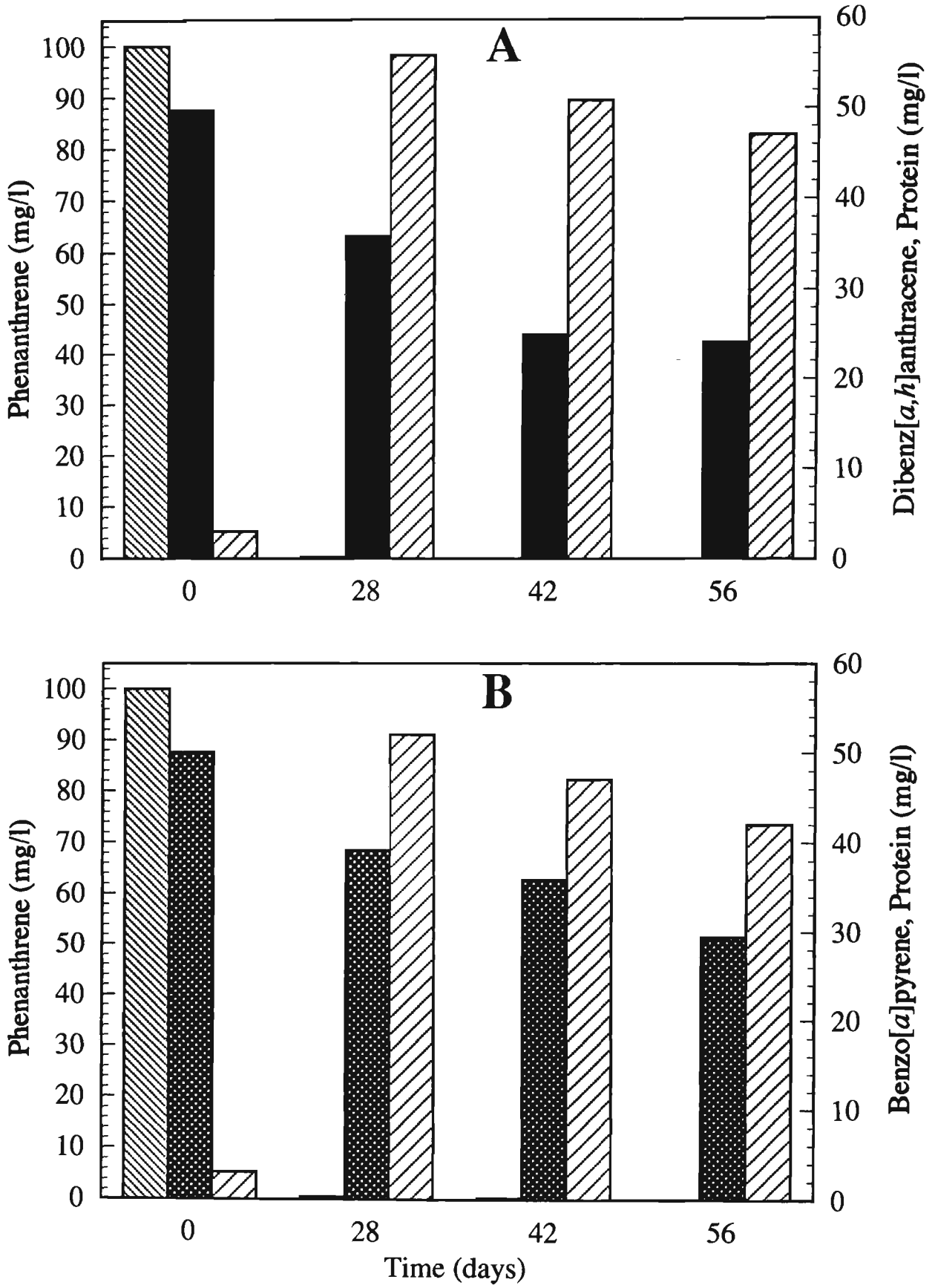


Figure 4.17. Effect of phenanthrene (▨) on the cometabolism of dibenz[*a*,*h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,001. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,001. Protein concentrations (▨) were determined as described in the material and methods.

of greater than 90% was observed during the first 28 days of incubation. A concurrent increase in protein concentration from 3.4 to 43.9-48.3 mg/l was also observed during this period. Degradation of the five-ring compounds by community five resulted in a 41% decrease in dibenz[*a,h*]anthracene concentration and a 37% decrease in benzo[*a*]pyrene concentration (Figure 4.18). A lesser amount of the five ring compounds was degraded by VUN 10,001, VUN 10,002 and VUN 10,003 (data shown in Appendix 2) but there was still a significant decrease of between 30.6 to 36.4% in the concentration of these compounds. As observed with the phenanthrene cometabolism experiments, protein concentrations decreased slightly when the five-ring PAHs were the only source of carbon in the cultures.

4.2.3.3 *Degradation of PAHs after Growth on Peptone, Yeast Extract and Glucose (PYEG)*

The previous sections demonstrated that significant amounts of benzo[*a*]pyrene and dibenz[*a,h*]anthracene could be degraded by community five and the *St. maltophilia* strains if the density of cells in the inoculum was high or if a growth supporting PAH was supplied in the medium. Another approach for testing the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by community five and VUN 10,001, VUN 10,002 and VUN 10,003 was to grow the microorganisms on a substrate which produced higher microbial numbers than pyrene and subsequently inoculating these microorganisms into BSM containing PAH.

The inoculum for degradation experiments was prepared by growing community five and the *St. maltophilia* strains in a basal salts medium containing peptone, yeast extract and glucose (PYEG). Microorganisms were incubated for 48 hours and then tested for their ability to degrade pyrene, pyrene and benzo[*a*]pyrene or pyrene and dibenz[*a,h*]anthracene. Mercuric chloride killed cells were prepared to assess the extent of PAH adsorption to cellular material.

Growth of community five, VUN 10,001, VUN 10,002 and VUN 10,003 on PYEG resulted in high microbial numbers. After 48 hours growth on PYEG, microbial numbers were approximately 67 times greater (5×10^8 cells/ml) compared to microbial numbers obtained after seven days growth on pyrene (7.5×10^6 cells/ml) (Figure 4.19). The ability of the PYEG-grown microorganisms to degrade PAHs was assessed by adding a 10% unwashed inoculum into BSM containing 250 mg/l of the four-ring compound or 50 mg/l of the five ring compounds. The microorganisms grown on PYEG were unable to degrade pyrene in BSM over a 21 day period (data for VUN

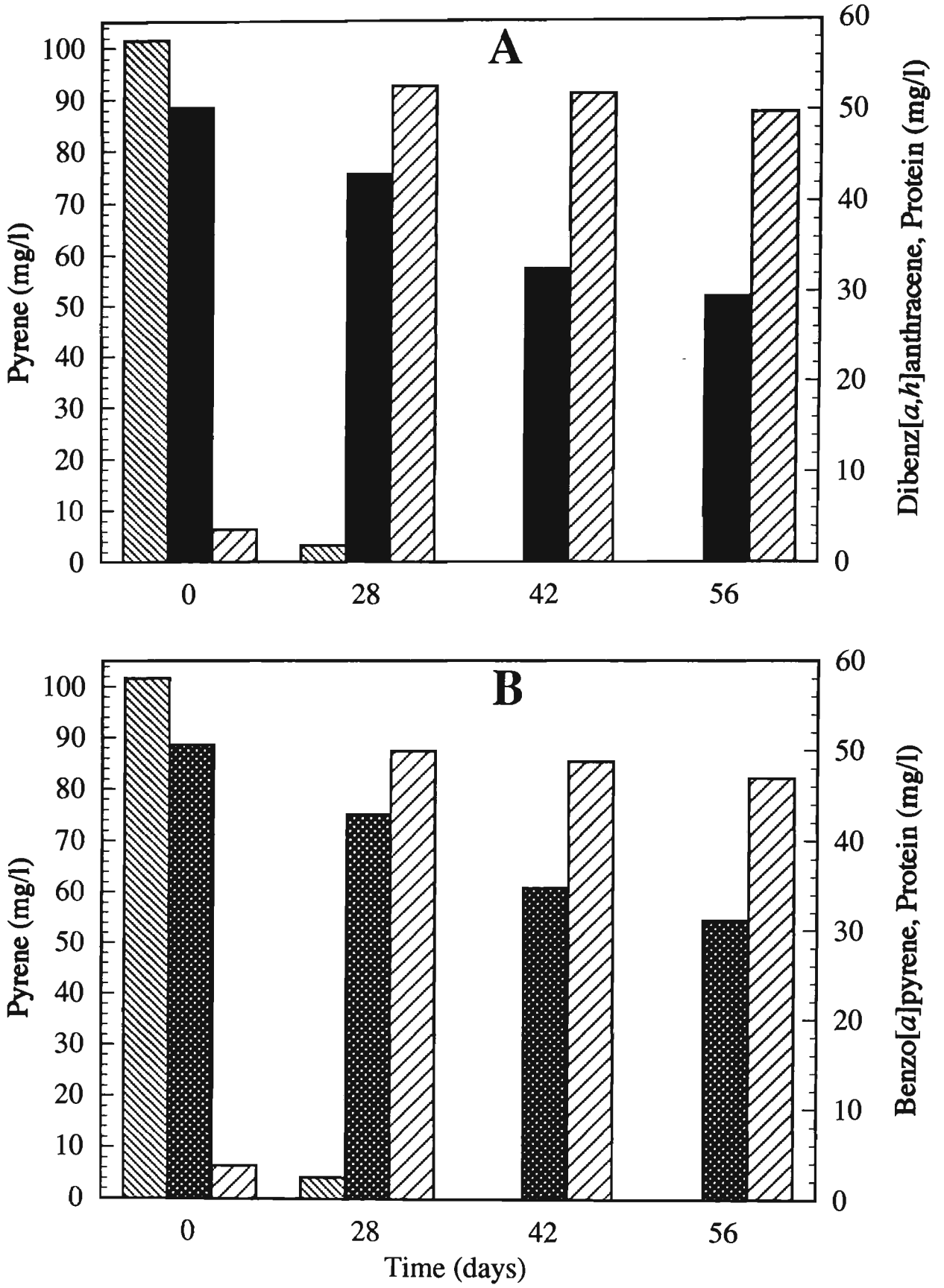


Figure 4.18. Effect of pyrene ( ) on the cometabolism of dibenz[*a*,*h*]anthracene ( ) (A) or benzo[*a*]pyrene ( ) (B) by community five. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of community five. Protein concentrations ( ) were determined as described in the material and methods.

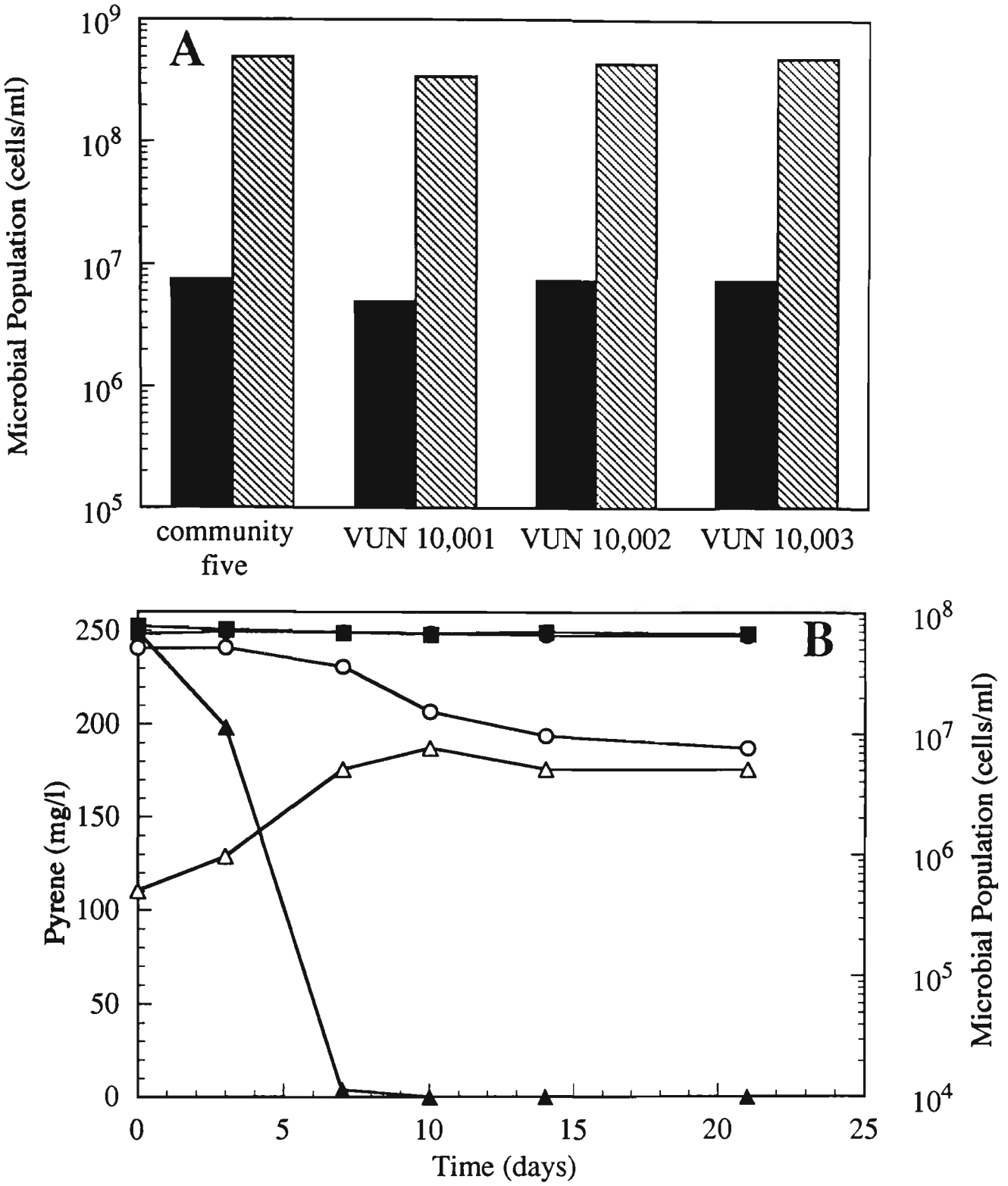


Figure 4.19. Microbial numbers (A) of community five, VUN 10,001, VUN 10,002 and VUN 10,003 after growth on peptone, yeast extract and glucose (PYEG) (▨) or pyrene (250 mg/l) (■). A 10% unwashed pyrene-grown inoculum was added to PYEG and BSM containing pyrene and incubated for 48 hours and seven days respectively. The ability of VUN 10,003, grown on PYEG (●) or pyrene (▲), to degrade pyrene (B) was assessed by adding a 10% unwashed inoculum into BSM containing pyrene as the sole carbon and energy source. The growth of PYEG-grown (O) and pyrene-grown (Δ) VUN 10,003 on pyrene and the concentration of pyrene in mercuric chloride killed control cultures (■) is also shown.

10,003 shown in Figure 4.19). Pyrene grown inocula completely degraded pyrene in BSM over a seven day period. Furthermore, neither of the PYEG grown microorganisms were able to degrade benzo[*a*]pyrene or dibenz[*a,h*]anthracene in the presence of pyrene after 56 days. Greater than 97% of the added PAHs were recovered from the $HgCl_2$ killed control cultures at the end of the incubation period. These results demonstrated that although growth of community five and the *St. maltophilia* strains on PYEG resulted in higher microbial numbers, subsequent degradation of PAHs did not occur. This indicates that induction of PAH degrading ability appears to be involved in the catabolism of PAHs by community five, VUN 10,001, VUN 10,002 and VUN 10,003.

4.2.4 Benzo[*a*]pyrene and Dibenz[*a,h*]anthracene Degradation By Community Five and *St. maltophilia* Strain VUN 10,003: Effect of Pyrene-spiking.

The *St. maltophilia* isolates were shown in section 4.2.3 to be capable of degrading significant amounts of benzo[*a*]pyrene and dibenz[*a,h*]anthracene if the microbial population was sufficiently high in the inocula (7.5×10^7 cells/ml starting numbers in culture). However, a characteristic of the kinetics of degradation was an initial lag period before degradation started and a rapid decline in the degradation rate of the five-ring PAHs after approximately 56 days (Figures 4.8 to 4.15). This is an undesirable characteristic of the isolates if they are to be used for the remediation of sites contaminated with high molecular weight PAHs. Cessation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation after 56 days was not due to a low cell density as cellular protein concentrations remained high. A possible explanation was that, in the absence of a readily metabolised carbon and energy source such as pyrene, the metabolic state of the cells was depleted so that further cometabolism of benzo[*a*]pyrene and dibenz[*a,h*]anthracene could not occur. A way of testing this was to provide a fresh spike of a readily metabolised substrate to the 56 day old culture to see whether this stimulated further metabolism of high molecular weight PAHs. Pyrene was the obvious choice to use because it supported the growth of the isolates and it appeared to stimulate benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation (Sections 4.2.3.1 and 4.2.3.2). In this section, high initial cell density experiments were conducted by inoculating pyrene-grown cells into BSM containing both pyrene and either benzo[*a*]pyrene or dibenz[*a,h*]anthracene, and after the degradation rate of the five-ring compounds had substantially decreased, the cultures were spiked with pyrene.

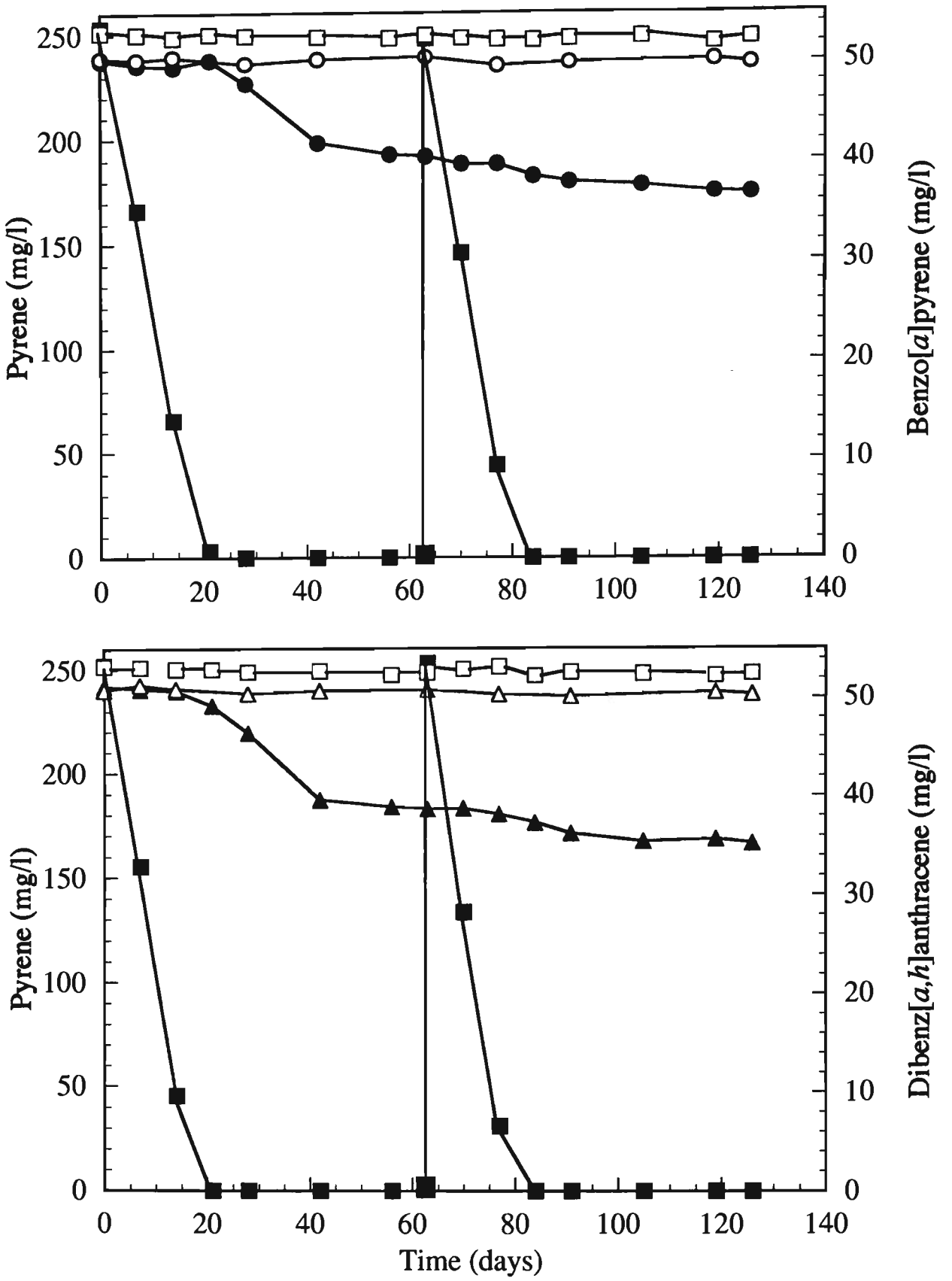


Figure 4.20. Degradation of benzo[*a*]pyrene (●) or dibenz[*a,h*]anthracene (▲) in the presence of pyrene (■) using high initial cell densities of pyrene-grown VUN 10,003. Pyrene was resupplied to the cultures after 63 days. Mercuric chloride killed controls for pyrene (□) benzo[*a*]pyrene (○) and dibenz[*a,h*]anthracene (△) are also shown.

When inoculated into BSM containing pyrene (250 mg/l) and benzo[*a*]pyrene or dibenz[*a,h*]anthracene (50 mg/l), VUN 10,003 rapidly degraded the pyrene in the presence of benzo[*a*]pyrene or dibenz[*a,h*]anthracene (Figure 4.20). Over 95% of the initial pyrene was degraded after 21 days. A lag period of 21 days was observed before the commencement of benzo[*a*]pyrene and dibenz[*a,h*]anthracene which was similar to the degradation lag periods observed in the single PAH degradation experiments using benzo[*a*]pyrene and dibenz[*a,h*]anthracene (see Section 4.2.3.1). Degradation of the five-ring compounds was slow relative to pyrene, however, significant decreases in the concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene of 19-22.6% (9.5-11.6 mg/l) were observed after 63 days. After 63 days, the degradation rate of the five-ring compounds had decreased substantially. At this stage, more pyrene (250 mg/l) was added to the benzo[*a*]pyrene and dibenz[*a,h*]anthracene cultures. Pyrene was again rapidly degraded by VUN 10,003; pyrene was undetectable in all cultures on day 84 (21 days after spiking). Small decreases in the concentration of benzo[*a*]pyrene (3.8 mg/l) and dibenz[*a,h*]anthracene (3.5 mg/l) were observed in the cultures 63 days after pyrene-spiking. Microbial numbers decreased over the incubation period for all benzo[*a*]pyrene and dibenz[*a,h*]anthracene incubations (2.5×10^8 cells/ml to $5.0\text{-}7.5 \times 10^7$ cells/ml) (Figure 4.20).

4.2.5 Evaluation of Factors Affecting the Decrease in Benzo[*a*]pyrene and Dibenz[*a,h*]anthracene Degradation Rates During Long Term Incubations.

The above results indicated that cells remained metabolically active after 63 days incubation, as they were capable of degrading pyrene without further growth occurring. However, addition of fresh pyrene failed to stimulate further significant degradation of the high molecular weight PAHs. Furthermore, most probable number estimates taken during the experiments suggested that the cultures still contained a high viable cell population after 126 days. In addition, after 63 days, VUN 10,003 was able to degrade pyrene rapidly even though the degradation rate of the five-ring PAHs had deteriorated significantly and could not be restored. This suggests that the failure of the cells to perpetuate a significant decrease in the concentration of the five-ring compounds was specifically associated with the benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism. The most likely reason for the loss in five-ring PAH-degrading activity by the viable cell population are:

1. A decrease in benzo[*a*]pyrene and dibenz[*a,h*]anthracene to a threshold concentration below which their degradation rate drops significantly. This may

be due to a fall in the concentration-dependent transportation rate of the compound to or into the cell or a lack of sufficient stimulation of the benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism by these PAHs at the threshold concentration.

2. The accumulation of polar or non-polar by-products of the pyrene catabolism which inhibit benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation.

3. A loss of ability by the cells to catabolise benzo[*a*]pyrene and dibenz[*a,h*]anthracene resulting from irrepairable damage to the catabolic system responsible for degradation, possibly via loss of essential plasmids or enzyme damage due to low endogenous respiration.

4. The accumulation of polar or non-polar by-products of the benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism which inhibit their further degradation.

Without further experimentation it is difficult to determine which one or more of the above mechanisms are responsible for the failure of the cells to perpetuate benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation over an extended period of time. Therefore, each of the above proposed mechanisms were tested experimentally in the next four subsections of this chapter.

4.2.5.1 *The Effect of Benzo[a]pyrene and Dibenz[a,h]anthracene Concentration*

The pyrene-spiking experiment (Section 4.2.4) demonstrated that the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene virtually ceased when the concentration of these compounds had decreased from 50 mg/l to around 36-38 mg/l. The purpose of the work in this section was to determine whether the degradation of the five-ring compounds failed to continue because benzo[*a*]pyrene and dibenz[*a,h*]anthracene had reached a minimum threshold concentration below which the degradation of these compounds will no longer occur. To test this hypothesis, a high initial cell density experiment was performed by inoculating pyrene-grown cells into BSM containing benzo[*a*]pyrene or dibenz[*a,h*]anthracene at one of three different concentrations (25, 50 and 100 mg/l). These concentrations lie below, at and above the concentration of the compounds in the pyrene-spiking experiment (Section 4.2.4). Pyrene was not present in the experimental cultures because of the possible inhibitory effects of its catabolic by-products. Viable cell numbers were monitored by the most probable number estimations.

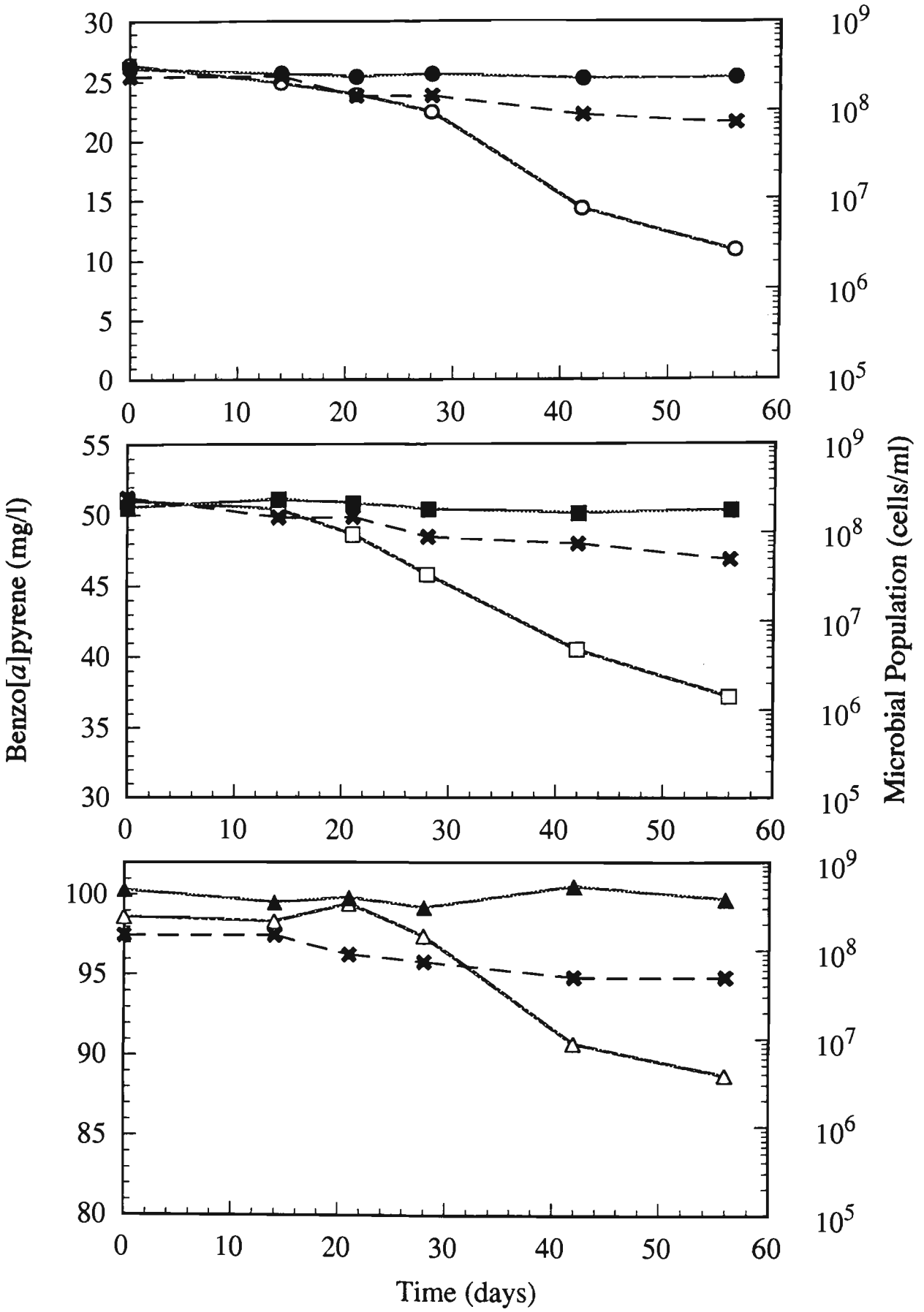


Figure 4.21. Relationship between initial concentration of benzo[*a*]pyrene and the kinetics of degradation by VUN 10,003. Benzo[*a*]pyrene was added to BSM containing high initial cell densities of pyrene-grown VUN 10,003 at the following concentrations: 25 mg/l (O), 50 mg/l (□) and 100 mg/l (Δ). Microbial numbers (✖) and benzo[*a*]pyrene concentrations in mercuric chloride killed cell controls [25 mg/l (●), 50 mg/l (■) and 100 mg/l (▲)] are also shown.

The degradation of benzo[*a*]pyrene (Figure 4.21) and dibenz[*a,h*]anthracene (Figure 4.22) at all concentrations was proceeded by lag period of 21 days. Although the percentage of the five-ring compounds degraded over 56 days decreased with increasing initial concentration (57%, 25% and 11% for initial concentrations of 25, 50 and 100 mg/l respectively), the amounts degraded were similar. Degradation of benzo[*a*]pyrene by VUN 10,003 resulted in decreases of 14.3, 12.8 and 11.3 mg/l from cultures with initial concentrations of 25, 50 and 100 mg/l respectively (Figure 4.21). A similar phenomenon was observed for dibenz[*a,h*]anthracene (Figure 4.22). Degradation by VUN 10,003 resulted in decreases of 16.3, 15.8 and 14.3 mg/l for cultures with initial dibenz[*a,h*]anthracene concentrations of 25, 50 and 100 mg/l respectively. Microbial numbers decreased slightly over 56 days for all benzo[*a*]pyrene and dibenz[*a,h*]anthracene incubations (2.5 x 10^8 cells/ml to 5.0-7.5 x 10^7 cells/ml). The results of these experiments clearly demonstrate that benzo[*a*]pyrene and dibenz[*a,h*]anthracene can be degraded by VUN 10,003 when the initial concentration of these PAHs is as low as 25 mg/l. The degradation rate of these PAHs was independent of their initial concentration. The concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the pyrene-spiking experiment (Section 4.2.4) does not appear to be the inhibitory mechanism responsible for the termination of their degradation during the incubation period.

4.2.5.2 *Inhibition by Pyrene Metabolites*

The pyrene-spiking experiments in section 4.2.4 demonstrated that pyrene could be degraded after its readdition at 63 days, however, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were not significantly degraded after this time period. Conceivably, the inhibition of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation after 63 days may be due to the accumulation of pyrene metabolites in the culture medium. To test this hypothesis, high initial cell density experiments were conducted using spent BSM which had been supplied initially for the degradation of pyrene (250 mg/l) and which therefore contained presumptive pyrene catabolism by-products. After all the pyrene was degraded, the cells were removed and the spent BSM was supplemented with pyrene (250 mg/l), benzo[*a*]pyrene or dibenz[*a,h*]anthracene (50 mg/l) and nitrate and phosphate (400 mg/l each of $(NH_4)_2SO_4$, NaH_2PO_4 and Na_2HPO_4; designated "pyrene-spent medium"). This was then inoculated with high cell numbers of VUN 10,003 from BSM containing pyrene.

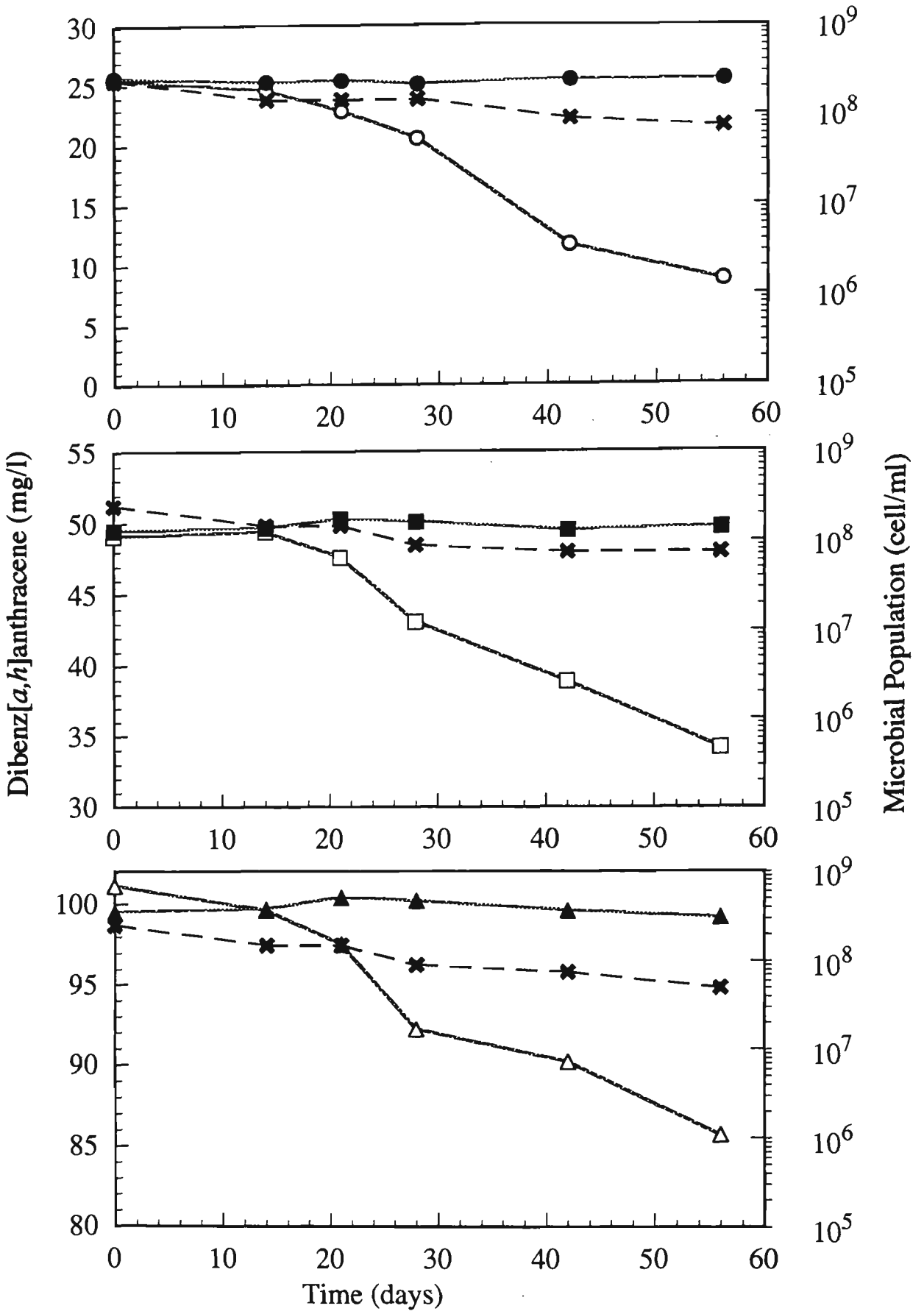


Figure 4.22. Relationship between initial concentration of dibenz[*a*,*h*]anthracene and kinetics of degradation by VUN 10,003. Dibenz[*a*,*h*]anthracene was added to BSM containing high initial cell densities of VUN 10,003 at the following concentrations: 25 mg/l (○), 50 mg/l (□) and 100 mg/l (△). Microbial numbers (✖) and dibenz[*a*,*h*]anthracene concentrations in mercuric chloride killed controls [25 mg/l (●), 50 mg/l (■) and 100 mg/l (▲)] are also shown.

When inoculated into pyrene-spent medium, VUN 10,003 degraded greater than 97% of the initial pyrene concentration after 21 days (Figure 4.23). Benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was proceeded by a lag period of approximately 21 days, after which 27-28% (14.2 and 13.6 mg/l) of the five-ring compounds were degraded after 56 days respectively (Figure 4.23). Microbial numbers decreased over the incubation period for all benzo[*a*]pyrene and dibenz[*a,h*]anthracene incubations (2.5 x 10^8 cells/ml to 5.0-7.5 x 10^7 cells/ml). These results demonstrated that the presence in the medium of by-products from pyrene catabolism did not inhibit the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by VUN 10,003. Hence, pyrene catabolic by-products do not appear to be responsible for the cessation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation during the pyrene-spiking experiments (Section 4.2.4).

4.2.5.3 *Loss of Catabolic Activity by Nutrient-Depleted Cells*

In this section, experiments were prepared to determine whether cells exposed for extended periods of time to benzo[*a*]pyrene or dibenz[*a,h*]anthracene are unable to further degrade the five-ring compounds because the cells have lost their catabolic ability. To investigate this hypothesis, VUN 10,003 cells were "preconditioned" in a high initial cell density incubation in the presence of pyrene and either benzo[*a*]pyrene or dibenz[*a,h*]anthracene. After 63 days, when degradation of the five-ring compounds had ceased, the cells were removed and inoculated at a low initial cell density into BSM containing either pyrene and benzo[*a*]pyrene or dibenz[*a,h*]anthracene or to BSM containing pyrene only.

VUN 10,003, after exposure to benzo[*a*]pyrene or dibenz[*a,h*]anthracene was capable of degrading pyrene when supplied alone or in combinations of pyrene and benzo[*a*]pyrene or pyrene and dibenz[*a,h*]anthracene. Pyrene (250 mg/l) was degraded to undetectable levels after 10-14 days and accompanied by an increase in protein concentration (6.8 to 55.5-58.9 mg/l) (Figure 4.24). When benzo[*a*]pyrene or dibenz[*a,h*]anthracene was supplemented with pyrene, greater than 90% of the pyrene was degraded after 28 days (Figures 4.25 and 4.26). A concurrent increase in protein concentration from 6.7 mg/l to 49.6-53.4 mg/l was observed during this period. Degradation of the five-ring compounds resulted in a 20-22.5% decrease in benzo[*a*]pyrene and dibenz[*a,h*]anthracene concentration by benzo[*a*]pyrene exposed cells after 56 days (Figure 4.25). Degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by dibenz[*a,h*]anthracene exposed cells resulted in similar decreases (23-25%) in the concentration of the five-ring compounds (Figure 4.26).

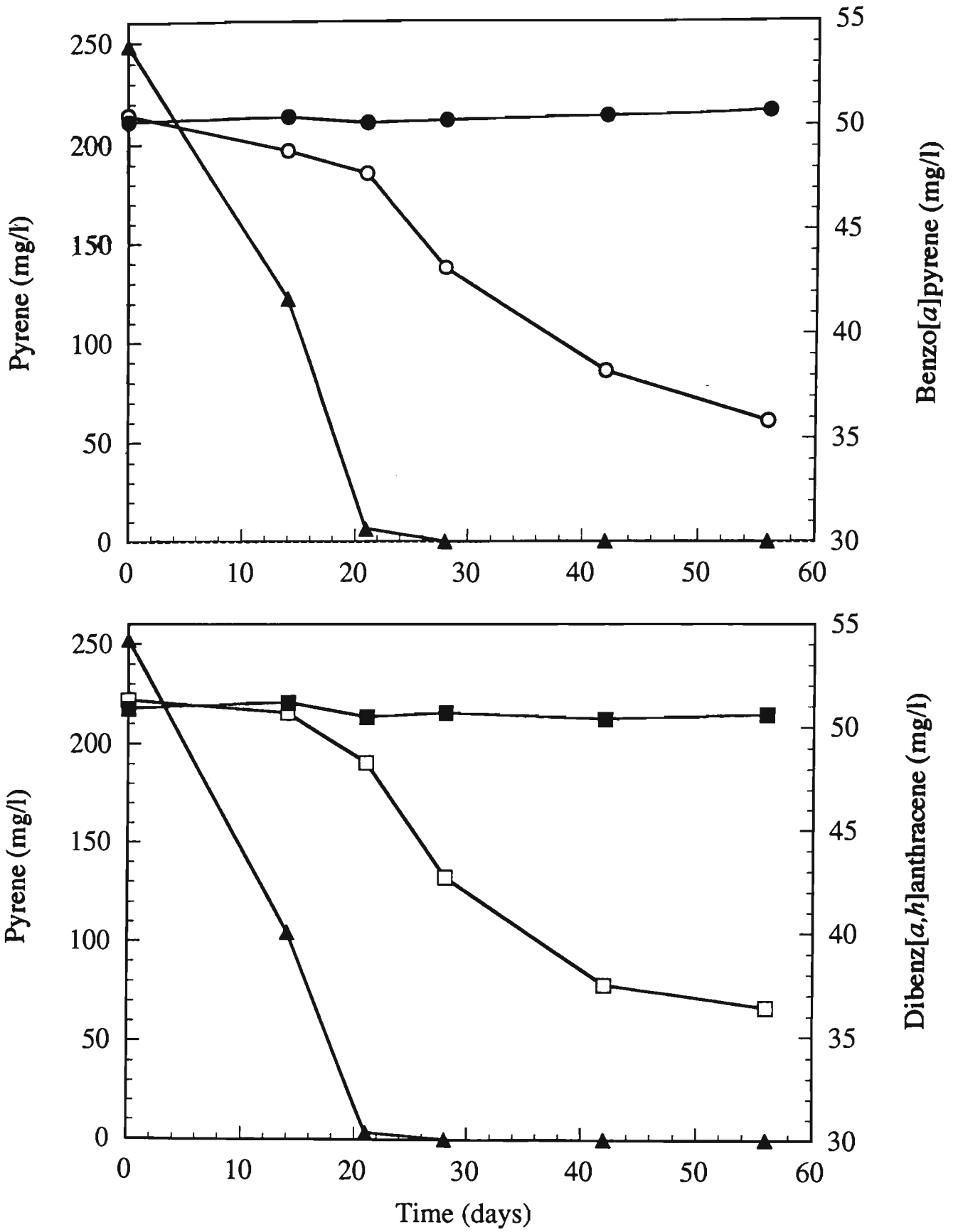


Figure 4.23. Effect of presumptive pyrene catabolism by-products on degradation of benzo[a]pyrene (O) and dibenz[*a*,*h*]anthracene (□) in the presence of pyrene (▲) by VUN 10,003. High initial cell numbers of pyrene-grown VUN 10,003 were inoculated into spent BSM which had previously been supplied initially for the degradation of pyrene and which therefore contained presumptive pyrene catabolism by-products. The benzo[*a*]pyrene (●) and dibenz[*a*,*h*]anthracene (■) concentrations in mercuric chloride killed controls are also shown.

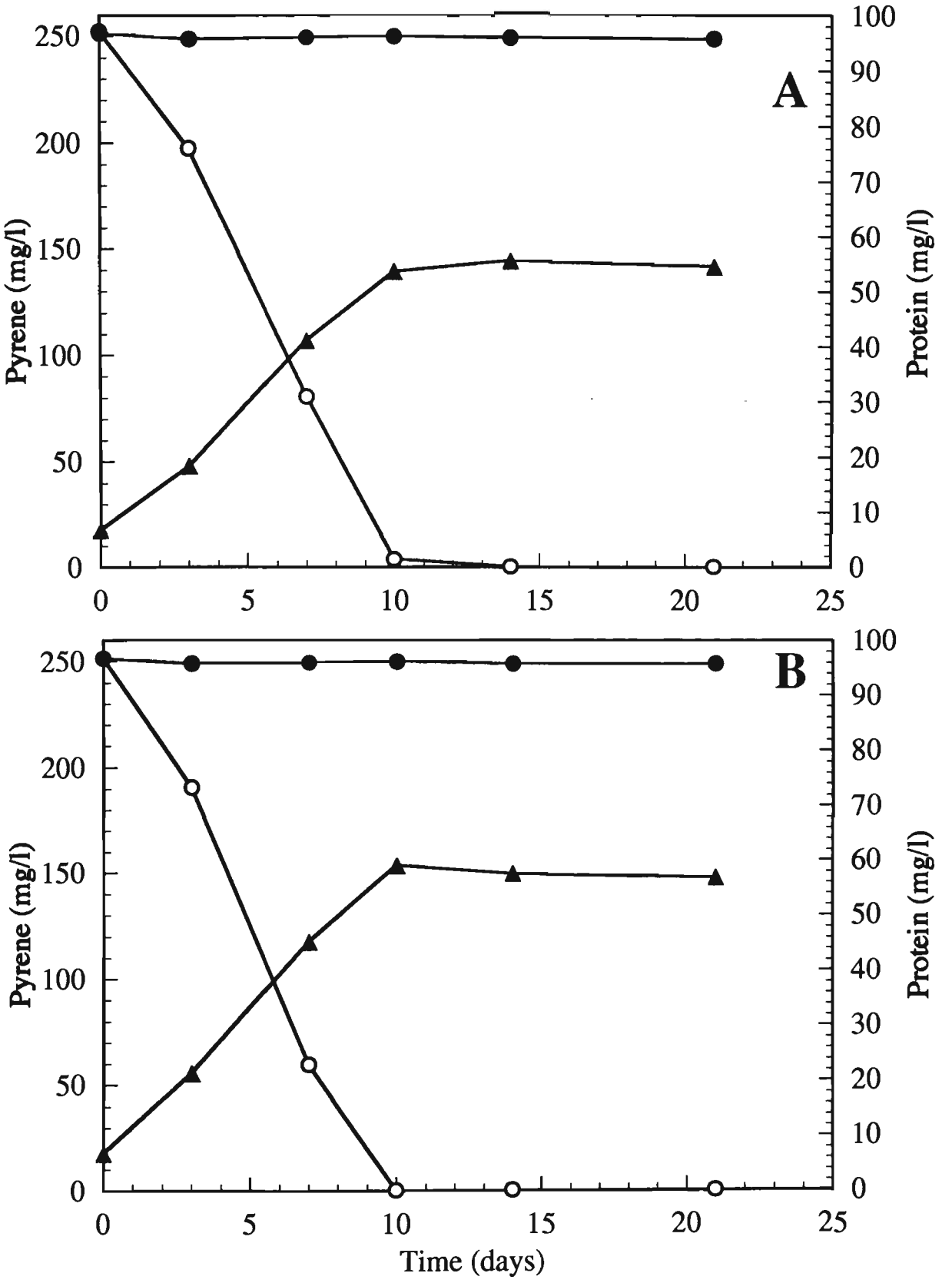


Figure 4.24. Time course for pyrene degradation by VUN 10,003 previously exposed to benzo[*a*]pyrene (A) or dibenz[*a*,*h*]anthracene (B) for 63 days from the high initial cell density degradation experiment shown in Figure 4.27 and 4.28. Pyrene containing medium was inoculated with 10% unwashed benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene exposed cells. Pyrene (O) and protein (▲) concentrations were determined as described in the material and methods. The pyrene (●) concentration in uninoculated control cultures is also shown.

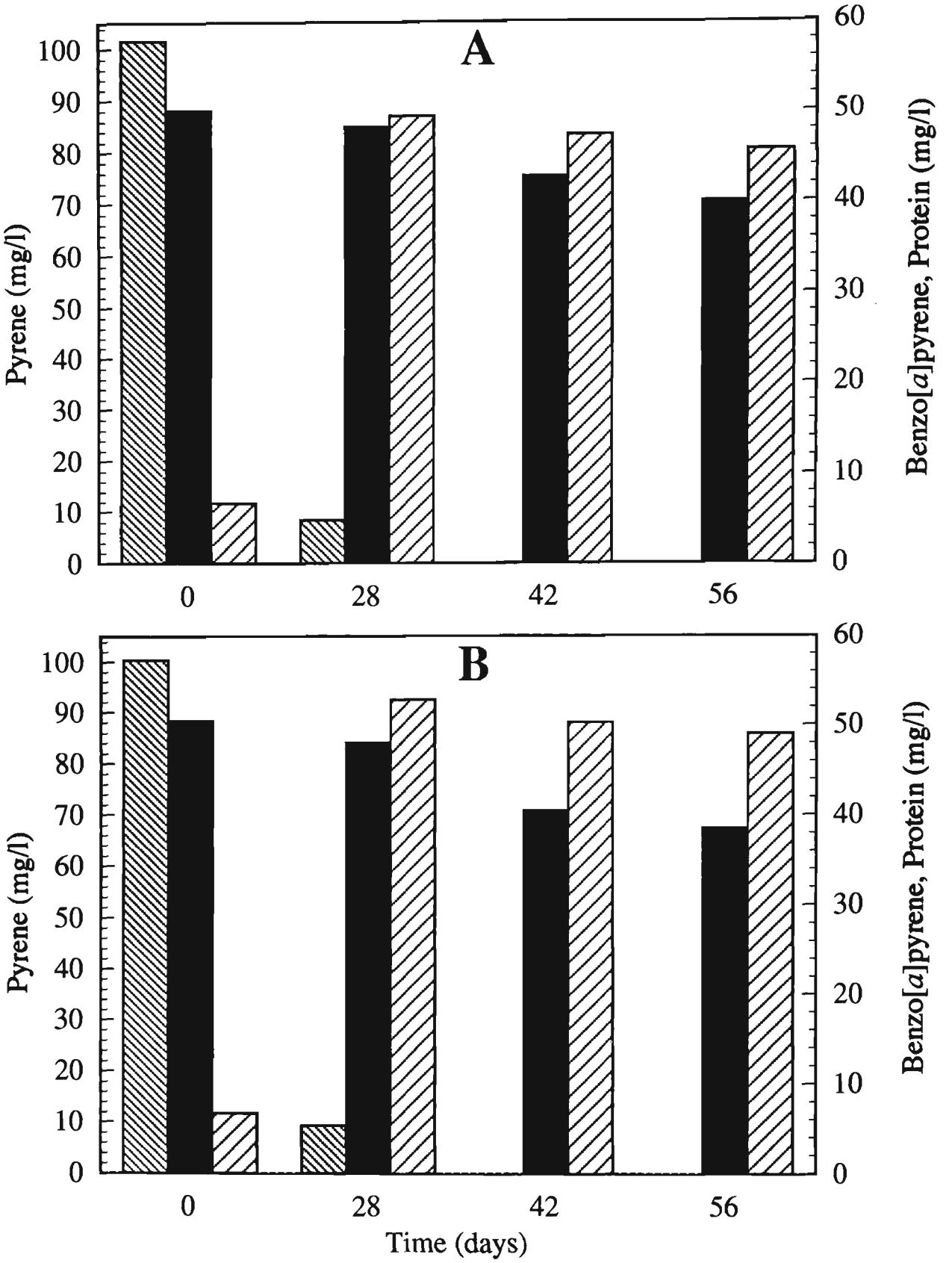


Figure 4.25. Degradation of benzo[*a*]pyrene (■) in the presence of pyrene (▨) by VUN 10,003 previously exposed to benzo[*a*]pyrene (A) or dibenz[*a,h*]anthracene (B) for 63 days from high initial cell density degradation experiments shown in Figure 4.27. PAH containing media was inoculated with 10% unwashed benzo[*a*]pyrene or dibenz[*a,h*]anthracene exposed cells. Protein concentrations (▨) were determined as described in the material and methods.

Protein concentrations decreased slightly after day 28 resulting in final protein concentrations of 45.8-49.2 mg/l. These results demonstrated that exposure of VUN 10,003 to benzo[*a*]pyrene or dibenz[*a,h*]anthracene for extended periods of time did not have an effect on the ability to degrade the five-ring compounds: cells could still grow on pyrene and degrade the five-ring compounds when added to fresh medium, hence the benzo[*a*]pyrene and dibenz[*a,h*]anthracene-catabolic activity of VUN 10,003 still remained. Therefore, the decrease in benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation rates during the pyrene-spiking experiment (Section 4.2.4) does not appear to be due to the loss of PAH-catabolic activity.

4.2.5.4 *Inhibition by Benzo[a]pyrene and Dibenz[a,h]anthracene Metabolites*

A possible mechanism to explain the substantial decrease in the degradation rate of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the pyrene-spiking experiment (Section 4.2.4) is that the by-products of benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism accumulate in the medium to a point where they inhibit their own catabolism. To test this hypothesis, the experimental approach in this work investigated the inhibitory effects of the non-polar and polar by-products.

The experimental medium was first prepared by conducting high initial cell density experiments in BSM using pyrene and benzo[*a*]pyrene or dibenz[*a,h*]anthracene as the carbon sources. When the degradation of the five-ring PAHs ceased (after 63 days as observed previously with the pyrene-spiking experiments in Section 4.2.4), the cells were removed and the culture broth treated in the following manner:

1. Cells and undegraded benzo[*a*]pyrene or dibenz[*a,h*]anthracene were removed from the respective cultures by centrifugation. The culture supernatants (presumably containing mostly polar by-products of five-ring catabolism) were used as the culture medium for the following experiments. Supernatants were distributed into serum bottles and fresh benzo[*a*]pyrene or dibenz[*a,h*]anthracene were added at a concentration equal to that before centrifugation. Pyrene (250 mg/l) was also added to the culture supernatants, which were then inoculated with a high cell population of fresh pyrene-grown VUN 10,003.

2. Benzo[*a*]pyrene or dibenz[*a,h*]anthracene, non-polar and some polar by-products of five-ring catabolism were extracted (see Section 2.7.3.2) from the 63 day pyrene-spiking cultures. The extracts were concentrated, resuspended in DMF

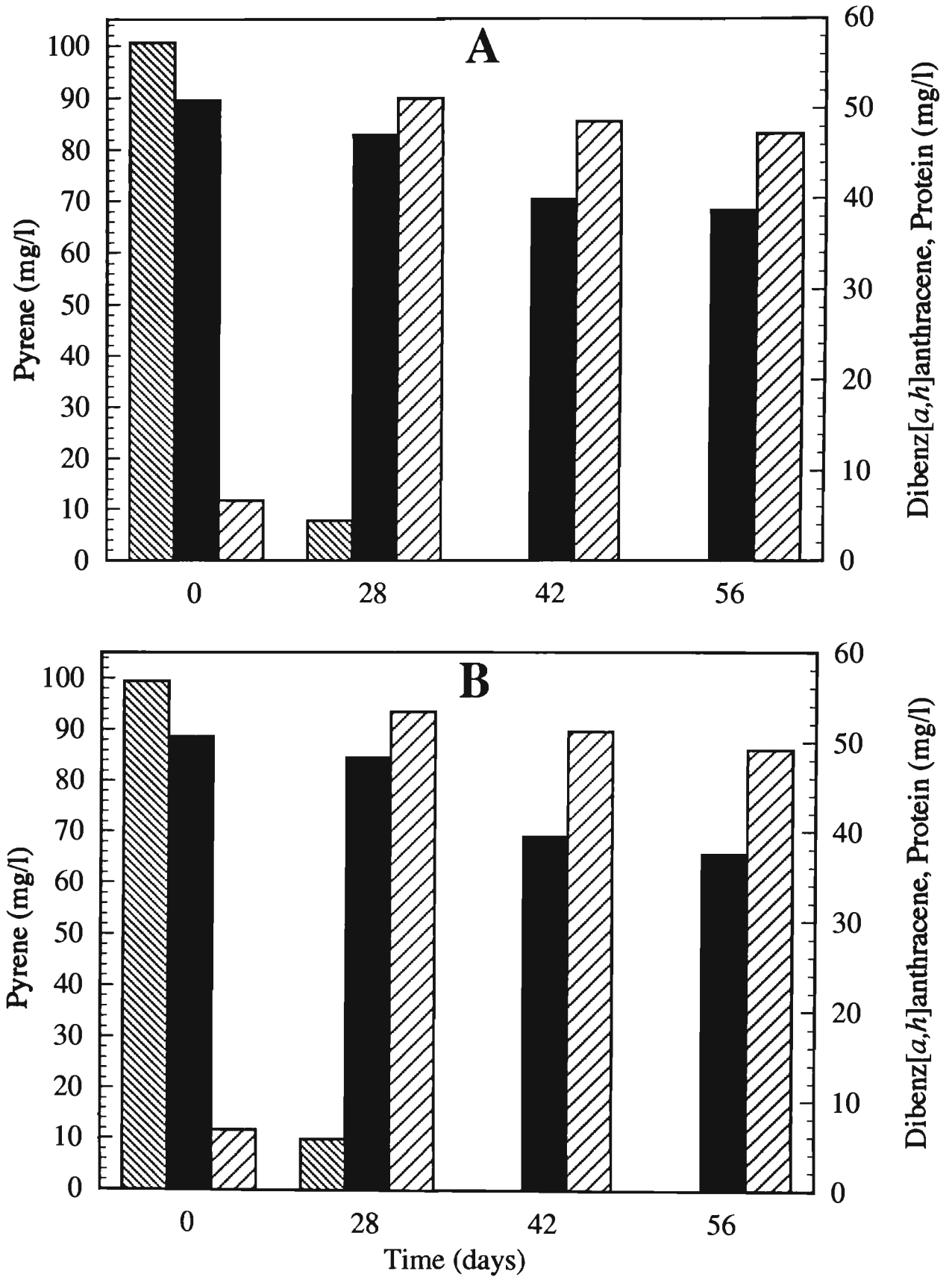


Figure 4.26. Degradation of dibenz[*a*,*h*]anthracene (■) in the presence pyrene (▨) by VUN 10,003 previously exposed to benzo[*a*]pyrene (A) or dibenz[*a*,*h*]anthracene (B) for 63 days from high initial cell density degradation experiments shown in Figure 4.27. PAH containing media was inoculated with 10% unwashed benzo[*a*]pyrene or dibenz[*a*,*h*]anthracene exposed cells. Protein concentrations (▨) were determined as described in the material and methods.

and added to fresh BSM containing pyrene (250 mg/l). This medium was then inoculated with fresh pyrene-grown VUN 10,003.

Experiment 1 was designed to examine the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by fresh VUN 10,003 inocula in the presence of mostly polar by-products of their metabolism. Experiment 2 was designed to examine the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by fresh VUN 10,003 inocula in the presence of mostly non-polar by-products of their catabolism. The addition of fresh cells to the medium, as opposed to the 63 day old cells, should not have an influence on the outcome since in the previous section it was demonstrated that the 63 day old cells can perform like fresh cells when inoculated into fresh medium.

In the first part of the experiment (before day 63), when the experimental medium was being "conditioned", pyrene was rapidly degraded by VUN 10,003 in the presence of benzo[*a*]pyrene and dibenz[*a,h*]anthracene (Figure 4.27 and 4.28). The degradation rates were similar to those observed in the pyrene-spiking experiments conducted earlier (see Section 4.2.4); greater than 95% of the added pyrene was degraded after 21 days. A lag period of 21 days was observed before the commencement of benzo[*a*]pyrene and dibenz[*a,h*]anthracene. Degradation of the five-ring compounds by VUN 10,003 resulted in concentration decreases of 19-24% for benzo[*a*]pyrene and 21-23% for dibenz[*a,h*]anthracene by day 63.

After fresh cells were inoculated into benzo[*a*]pyrene or dibenz[*a,h*]anthracene supernatants for experiment 1, pyrene was again rapidly degraded by VUN 10,003; greater than 95% of added pyrene was degraded at day 84 (21 days after spiking). Small decreases in the concentrations of benzo[*a*]pyrene (4.6 mg/l) and dibenz[*a,h*]anthracene (3.2 mg/l) were observed 63 days after pyrene-spiking (Figure 4.27).

For experiment 2, similar results were observed when fresh cells were inoculated into BSM containing non-polar by-products from benzo[*a*]pyrene or dibenz[*a,h*]anthracene degradation. Greater than 95% of pyrene was degraded at day 84 (21 days after re-spiking). At the end of the incubation period, small decreases in the concentrations of benzo[*a*]pyrene (2.5 mg/l) and dibenz[*a,h*]anthracene (2.7 mg/l) were also observed (Figure 4.28). Microbial numbers decreased over the incubation period for all benzo[*a*]pyrene and dibenz[*a,h*]anthracene incubations (2.5×10^8 to $5.0\text{-}7.5 \times 10^7$). The failure of the fresh inocula to continue the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the 63 day old culture broths indicates that by-products of the

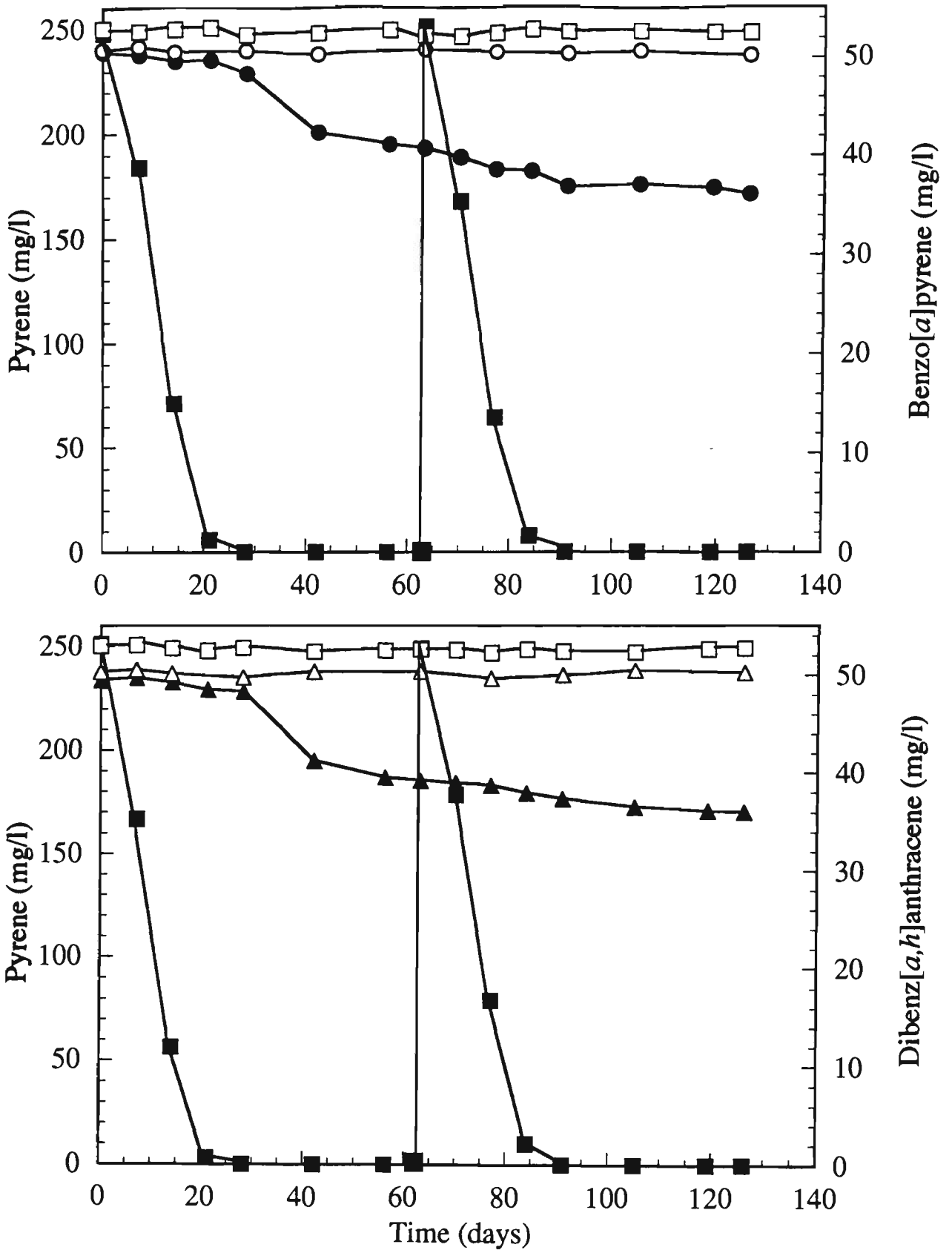


Figure 4.27. Effect of benzo[*a*]pyrene and dibenz[*a,h*]anthracene polar metabolic by-products on the degradation of benzo[*a*]pyrene (●) or dibenz[*a,h*]anthracene (▲) by VUN 10,003 in the presence of pyrene (■). Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to high initial cell densities of pyrene-grown VUN 10,003. After 63 days, cultures were centrifuged and the supernatants, containing presumptive five-ring metabolic by-products, were used as the medium for the remainder of the experiment. Pyrene was readded at a concentration of 250 mg/l, benzo[*a*]pyrene or dibenz[*a,h*]anthracene were added at a concentration equal to that prior to centrifugation and the medium was inoculated with fresh VUN 10,003 cells. Mercuric chloride killed controls for pyrene (□), benzo[*a*]pyrene (○) and dibenz[*a,h*]anthracene (△) are also shown.

five-ring PAH catabolism accumulate in the medium until they reach a concentration that is inhibitory to the further degradation of these compounds. These by-products appear to be specific for the benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism, since the degradation of pyrene is unaffected by their presence. Inhibition of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was observed in both experiments 1 and 2, indicating that the inhibitory product(s) occur in both the polar and non-polar fractions. These results clearly show that the amount of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degraded by VUN 10,003 is restricted by the accumulation in the medium of their catabolic by-products.

4.3 DETOXIFICATION OF PAHs

The microbial degradation of PAHs may lead to a reduction in the concentration of the compounds, however, for bioremediation to be effective, the degradation of the PAHs must also result in the detoxification of the environment. Microbial degradation of PAHs may not result in the complete mineralisation of the compound, *i.e.* conversion to carbon dioxide and water. In some cases, PAH degradation may result in the formation of intermediate compounds or by-products, which if toxic, may pose a greater threat to the environment due to their increased mobility compared to the parent compound. By combining chemical analysis, mutagenicity and toxicity assays, the extent of both degradation and detoxification of hazardous substances can be evaluated (Aprill *et al.*, 1990).

The purpose of research reported in this section was to determine whether degradation of the high molecular weight PAHs by the pyrene-enriched isolates results in a reduction in the mutagenicity and toxicity of culture extracts and supernatants. The change in the concentration of the PAHs was monitored by GC-FID after solvent extraction, while two bioassays (Ames Test and Microtox™ Test) were performed to test the mutagenicity and toxicity of the culture extracts and supernatants. The accumulation of PAH by-products in the culture medium was also assessed using an assay which detects phenolic compounds (see Section 2.7.2).

4.3.1 PAH Dose-Response Curves

The mutagenic effects of single PAHs and PAH mixtures were tested at a number of dose levels to obtain a dose response curve. Dose response curves of single high molecular weight PAHs and PAH mixtures with metabolic activation (S9 fraction) demonstrated the mutagenicity of the PAH compounds towards *S. typhimurium* strain

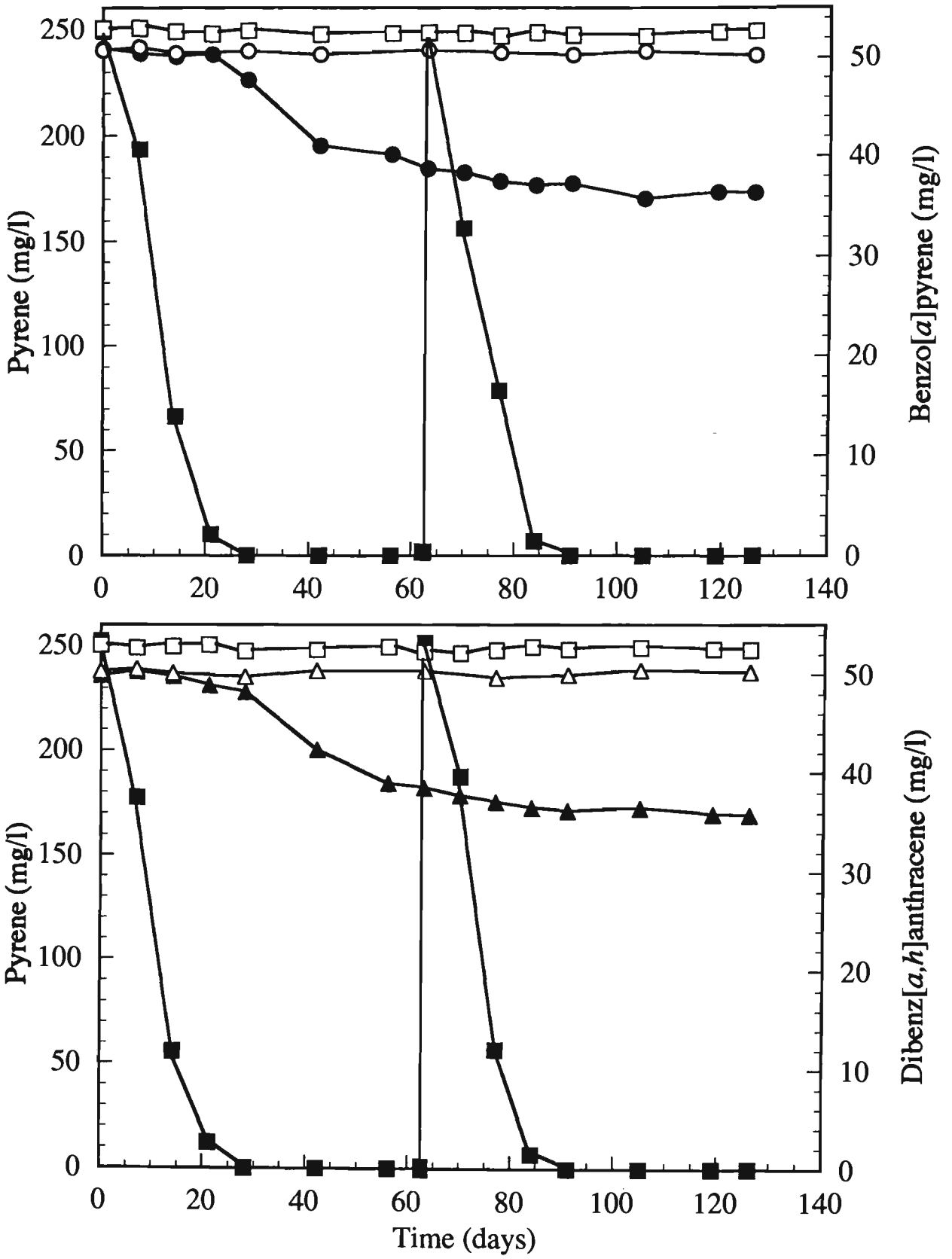


Figure 4.28. Effect of benzo[*a*]pyrene or dibenz[*a,h*]anthracene metabolic by-products on the degradation of benzo[*a*]pyrene (●) or dibenz[*a,h*]anthracene (▲) by VUN 10,003 in the presence of pyrene (■). Pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were added to high initial cell densities of pyrene-grown VUN 10,003. After 63 days, the cultures were extracted and dissolved in dimethylformamide (0.1 ml). Culture extracts were added to sterile BSM, pyrene was added at a concentration of 250 mg/l and the cultures were inoculated with fresh VUN 10,003 cells. Mercuric chlorinde killed controls for pyrene (□), benzo[*a*]pyrene (○) and dibenz[a,*h*]anthracene (△) are also shown.

TA100 at concentrations of 2.5 μg PAH per plate or higher. The PAHs exerted a dose-related mutagenic response (Table 4.6), however, toxic effects, exhibited by decreasing revertant colony numbers and a sparser background lawn of auxotrophic bacteria were noticed when testing at the highest concentrations. With the exception of coronene, an increase in the mutagenicity of the test compounds corresponded to the increase in molecular weight of the compounds tested. The PAH mixtures exerted the greatest mutagenic effect. No dose response was observed in the absence of the mammalian microsomal activation (S9). *S. typhimurium* strain TA98 did not show a dose-related response to the PAHs at the concentrations tested in the presence or absence of metabolic activation (Table 4.6). Further Ames tests were conducted with strain TA100 in the presence or absence of the S9 fraction.

4.3.2 Mutagenicity of Spent Culture Fluids and Extracts Following Growth on PAHs

The *Salmonella* reversion assay was used to determine the mutagenic potential of culture extracts and culture supernatants collected over a time period from cultures containing individual PAHs and PAH mixtures; these cultures were incubated with high initial cell numbers of community five, VUN 10,002 or VUN 10,003. The concentration of residual PAHs was determined over the incubation period after solvent extraction of the medium and GC-FID analysis. In addition, the concentration of phenolic compounds, produced as a consequence of PAH degradation, was determined from culture supernatants. Total culture extracts were assayed to determine the mutagenic activity of undegraded PAHs and any by-products (polar and non-polar) from the degradation of the PAH compounds that accumulated in the culture medium. Culture supernatants were also assayed to determine the mutagenic activity of water soluble by-products.

As observed previously in this chapter (Section 4.2.3.1), incubation of PAH-containing BSM with high initial cell densities of community five, VUN 10,002 and VUN 10,003 resulted in a decrease in the concentration of all PAHs over the incubation period compared to the killed control culture (Tables 1, 2 and 3 in Appendix 2). When assayed with *S. typhimurium* strain TA100 in the presence of the S9 fraction, the decrease in the concentration of the residual PAHs corresponded to a reduction in the mutagenic potential of the total culture extracts (Figures 4.29-4.37). Table 4.7 summarises the results from the mutagenicity tests. These results are described below.

Table 4.6. Reversions induced in *Salmonella typhimurium* TA98 and TA100 by PAHs.

Compound[b]	Conc. (μg/plate)	His$^+$ revertant colonies/plate[a]			
		TA98		TA100	
		-S9[c]	+S9[d]	-S9	+S9
FA	2.5	23.4±4.5	43.5±5.3	38.3±4.5	53.1±2.9
	5.0	33.2±6.3	55.2±6.9	44.3±5.3	129.1±7.2
	10.0	31.5±7.3	66.2±4.1	41.9±6.3	163.1±10.2
	25.0	44.1±5.3	61.4±9.4	36.7±4.1	204.9±7.2
	50.0	37.8±3.9	70.3±5.3	44.2±9.4	198.3±6.9
	100.0	34.5±6.3	74.8±9.3	35.9±6.3	163.1±7.9
PYR	2.5	28.4±5.7	60.3±5.2	44.5±5.3	73.8±7.3
	5.0	30.3±5.6	71.4±7.7	55.2±8.3	138.1±6.7
	10.0	44.2±7.3	72.3±6.3	51.2±4.2	192.3±11.3
	25.0	41.9±4.7	79.3±4.6	42.9±5.7	229.1±9.3
	50.0	30.2±3.4	73.5±5.5	42.8±3.6	258.6±12.9
	100.0	35.6±6.6	84.3±7.1	49.2±7.4	232.1±16.1
BA	2.5	47.9±6.3	77.8±10.3	55.6±3.5	100.3±6.3
	5.0	59.3±7.4	88.2±9.2	63.9±5.3	192.0±12.9
	10.0	51.0±8.4	102.3±12.3	46.8±3.5	263.7±11.5
	25.0	66.8±7.2	110.7±15.3	42.9±9.4	347.2±15.1
	50.0	56.8±9.0	140.2±12.3	55.2±3.6	307.0±11.7
	100.0	62.7±7.5	133.8±9.4	47.6±3.6	274.3±17.9
B[a]P	2.5	58.2±6.7	120.4±12.5	55.3±7.3	197.3±14.8
	5.0	44.6±10.4	133.5±17.8	62.3±7.3	316.0±17.4
	10.0	61.9±5.6	160.3±14.2	69.2±4.7	417.0±20.3
	25.0	77.3±7.8	166.7±20.4	49.6±5.7	402.0±16.3
	50.0	58.3±9.2	159.9±24.6	55.9±6.8	305.1±10.9
	100.0	53.9±7.7	177.4±21.4	61.9±3.9	286.7±16.3

[a]Means±standard deviations from five plates. The number of spontaneous revertants (<20) observed on plates containing no PAHs have been subtracted from the above values.

[b]Compounds tested were: FA, fluoranthene; PYR, pyrene; BA, benz[*a*]anthracene; B[a]P, benzo[*a*]pyrene; DBA, dibenz[*a,h*]anthracene; COR, coronene; PPDB mixture containing: phenanthrene, pyrene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene; FC mixture containing: fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene.

Compound[b]	Conc. (μg/plate)	His$^+$ revertant colonies/plate[a]			
		TA98		TA100	
		-S9[c]	+S9[d]	-S9	+S9
DBA	2.5	43.5±6.7	99.3±12.5	33.6±8.9	146.0±13.9
	5.0	55.3±4.7	110.6±18.3	55.8±5.2	208.3±10.9
	10.0	59.9±10.3	162.4±20.4	46.8±3.9	304.1±12.3
	25.0	43.9±6.3	155.8±18.3	46.8±8.2	379.1±11.6
	50.0	49.4±8.8	170.3±19.3	53.5±3.0	341.5±7.9
	100.0	53.8±12.1	188.3±14.5	50.3±5.3	303.3±11.9
COR	2.5	20.3±5.3	30.9±4.6	23.5±4.2	49.6±5.4
	5.0	24.4±7.8	42.3±5.5	29.5±7.5	68.3±7.9
	10.0	30.4±4.5	49.3±7.2	33.9±7.2	103.4±4.4
	25.0	43.4±9.3	50.6±10.9	40.2±4.0	129.3±7.4
	50.0	39.4±7.7	48.9±8.3	38.4±6.7	139.6±11.6
	100.0	40.2±6.0	44.9±6.3	31.8±4.4	121.3±6.9
PPDB	2.5	89.4±9.2	156.9±21.5	103.4±14.6	198.2±16.3
	5.0	118.3±14.2	153.8±19.3	130.3±15.3	347.6±17.9
	10.0	103.5±20.4	163.9±24.2	123.3±12.4	537.1±18.1
	25.0	124.8±18.3	200.4±23.8	100.2±20.4	673.9±19.8
	50.0	144.9±17.0	193.9±15.2	142.8±21.3	600.1±17.9
	100.0	138.8±24.3	212.8±23.6	152.5±14.9	416.8±17.9
FC	2.5	112.8±12.3	213.2±33.2	123.1±23.5	243.6±23.1
	5.0	132.8±20.3	189.4±24.2	150.2±20.2	483.9±23.9
	10.0	142.6±16.9	243.9±26.3	144.2±15.4	691.3±19.3
	25.0	128.7±15.2	260.3±20.4	130.8±16.7	1003.6±29.7
	50.0	155.2±16.8	244.8±19.3	160.3±30.1	734.6±21.3
	100.0	133.9±24.6	264.9±25.3	140.5±19.9	586.0±23.1

[c]No mammalian microsomal preparation (S9) was added to the assays.
[d]The mammalian microsomal preparation (S9) was added to the assays.

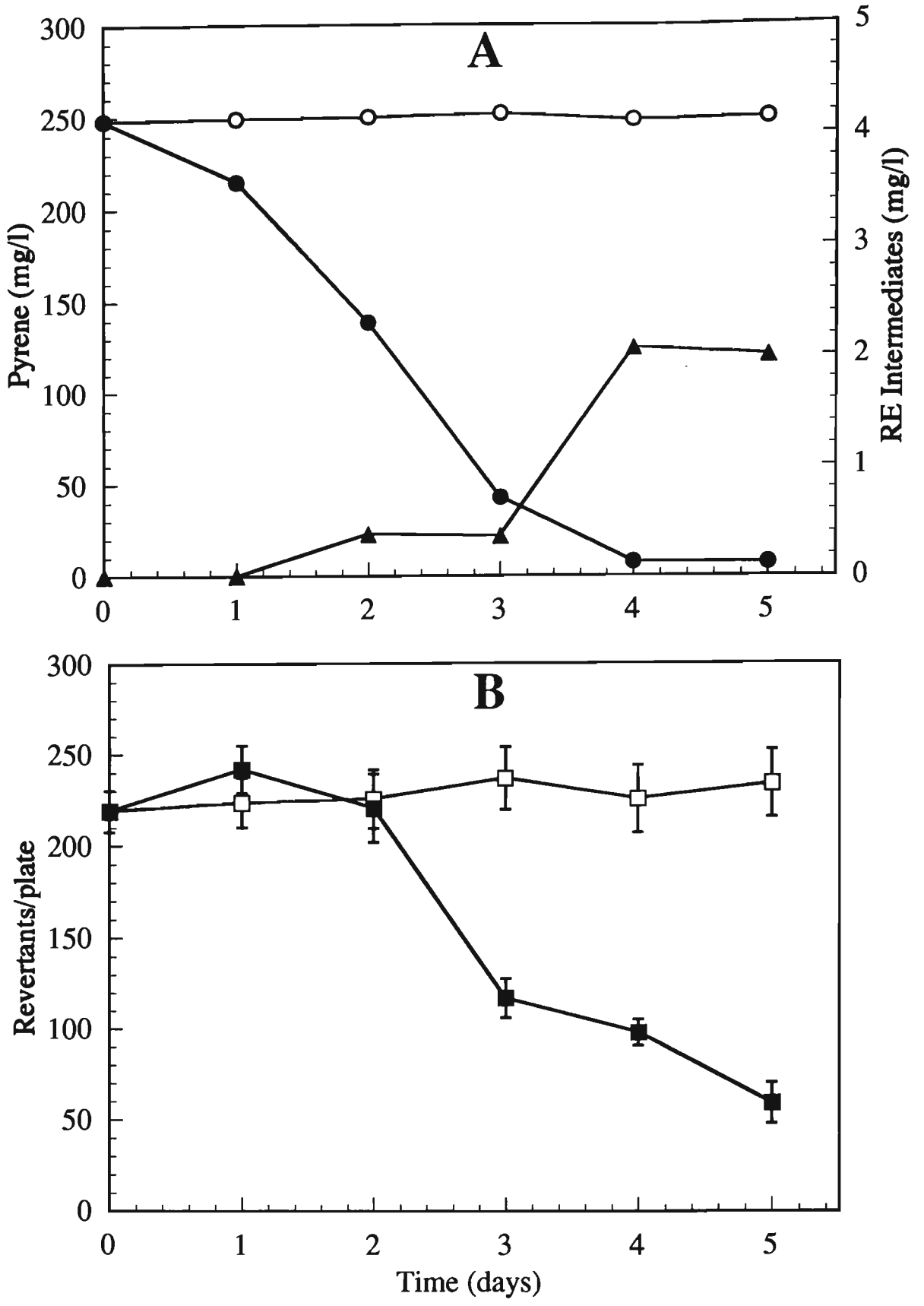


Figure 4.29. Pyrene degradation (A) and change in mutagenic potential (B) of cultures containing high initial cell densities of pyrene grown VUN 10,003. Pyrene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 5 day incubation period. The pyrene concentration (O) and mutagenicity (□) in killed cell cultures is also shown.

4.3.2.1 *Degradation of Single High Molecular Weight PAHs*

The degradation of single high molecular weight PAHs by high initial cell densities of community five, VUN 10,002 and VUN 10,003 resulted in significant decreases in the concentration of all PAH compounds tested. In all experiments, the PAH concentration in the killed cell control cultures did not vary significantly from their initial concentrations over the incubation period. In the inoculated cultures (data shown for VUN 10,003), the concentrations of pyrene (Figure 4.29), fluoranthene (Figure 4.30), benzo[*a*]anthracene (Figure 4.31), benzo[*a*]pyrene (Figure 4.32), dibenz[*a,h*]anthracene (Figure 4.33) and coronene (Figure 4.34) decreased by around 98% (243-248 mg/l), 45-53% (45-53 mg/l), 26-31% (26-31 mg/l), 19-21% (9-11 mg/l), 14-22% (7-11 mg/l) and 45-50% (9-10 mg/l) respectively. RE intermediate concentrations were observed to accumulated in cultures containing fluoranthene (VUN 10,002 and VUN 10,003) and benz[*a*]anthracene (VUN 10,002 and VUN 10,003) (Table 4.8).

Mutagenicity assays with extracts from the $HgCl_2$-killed cell control PAH cultures taken during the incubation period demonstrated little change in mutagenicity over the incubation period (Figures 4.29-4.34). However, inoculation of the PAH-containing medium with community five, VUN 10,002 and VUN 10,003 resulted in significant decreases in the number of revertant colonies per plate at the end of the incubation period. In the inoculated cultures, the number of revertant colonies observed at the end of the incubation period from pyrene (Figure 4.29), fluoranthene (Figure 4.30) and coronene extracts (Figure 4.34) had decreased by 75-77%, 44-50% and 49-57% respectively from initial revertant numbers. A smaller decrease in the number of revertant colonies were observed from cultures containing benz[*a*]anthracene (Figure 4.31), benzo[*a*]pyrene (Figure 4.32) and dibenz[*a,h*]anthracene (Figure 4.33). At the end of the incubation period, the number of revertant colonies observed from benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene extracts had decreased by 26-30%, 17-18% and 13-16% respectively (Table 4.8).

Generally, the reduction in the mutagenic potential of total PAH culture extracts corresponded to the decrease in the concentration of PAHs in the inoculated cultures over the incubation period. Although community five, VUN 10,002 and VUN 10,003 were able to degrade only small amounts of benzo[*a*]pyrene and dibenz[*a,h*]anthracene, a significant reduction in the mutagenic potential of the five-ring culture extracts was observed after 42 days.

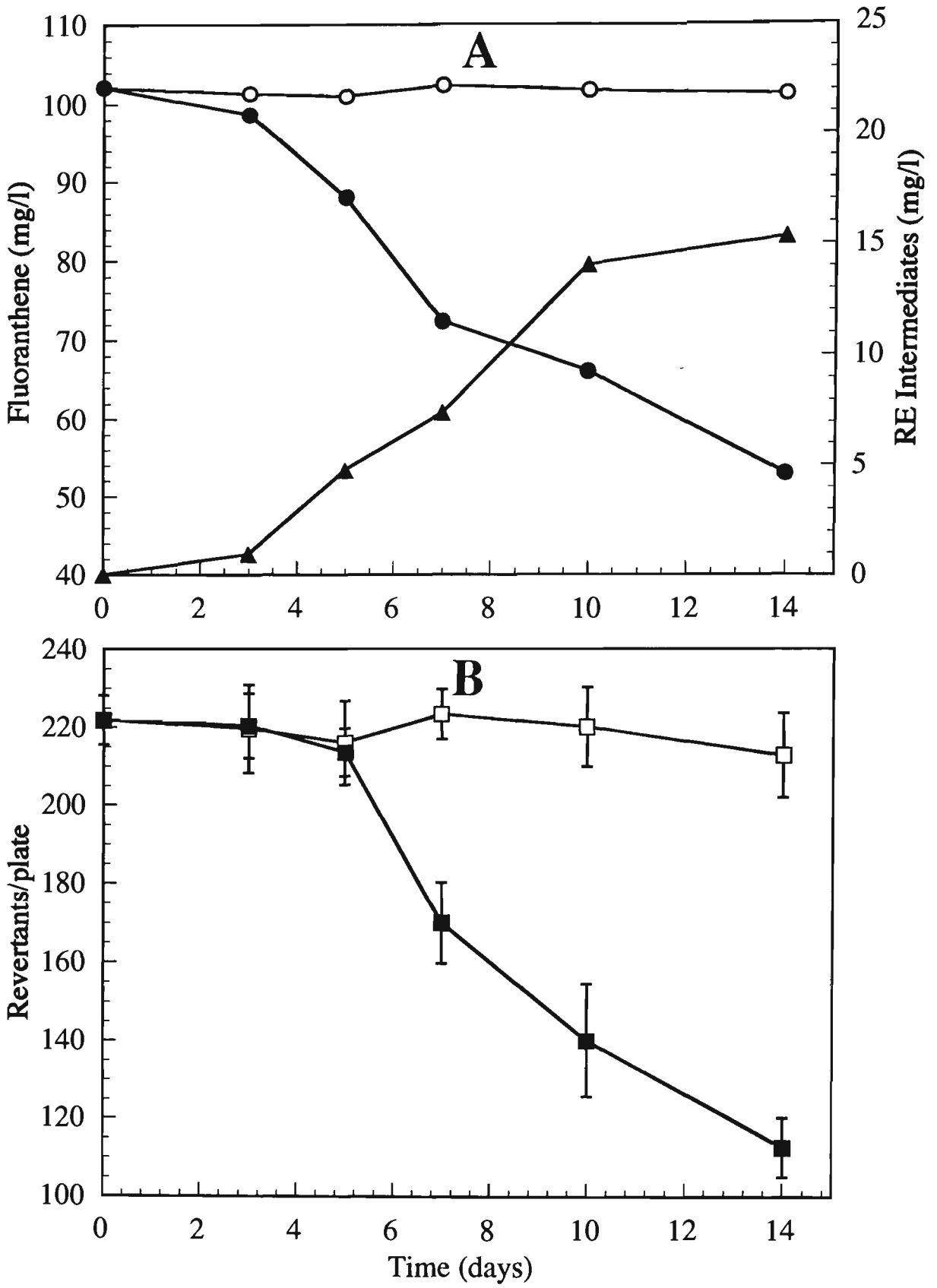


Figure 4.30. Fluoranthene degradation (A) and change in mutagenic potential (B) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. Fluoranthene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 14 day incubation period. The fluoranthene concentration (O) and mutagenicity (□) in killed cell cultures is also shown.

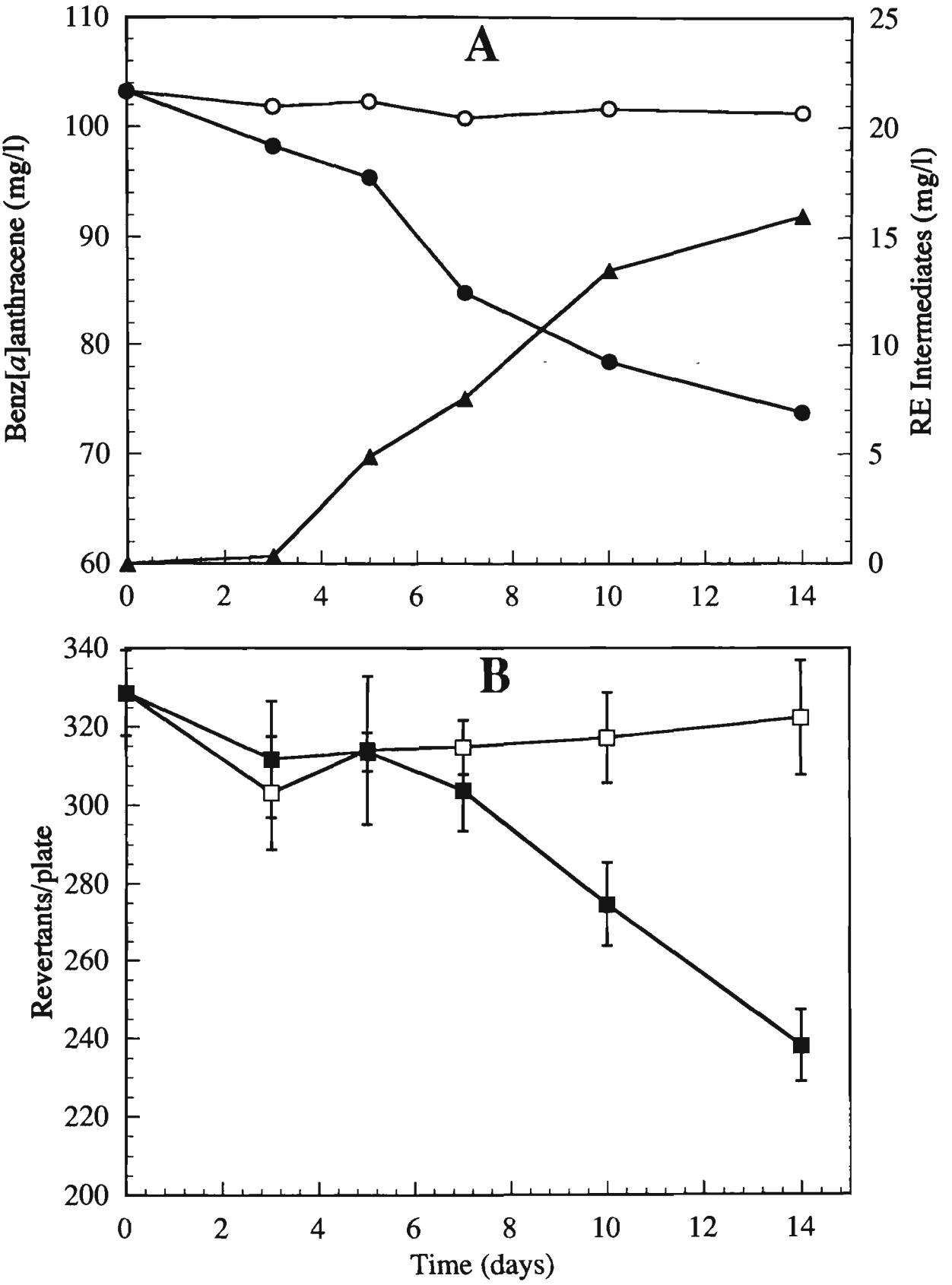


Figure 4.31. Benz[*a*]anthracene degradation (A) and change in mutagenicity (B) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. Benz[*a*]anthracene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 14 day incubation period. The benz[*a*]anthracene concentration (○) and mutagenicity (□) in control cultures is also shown.

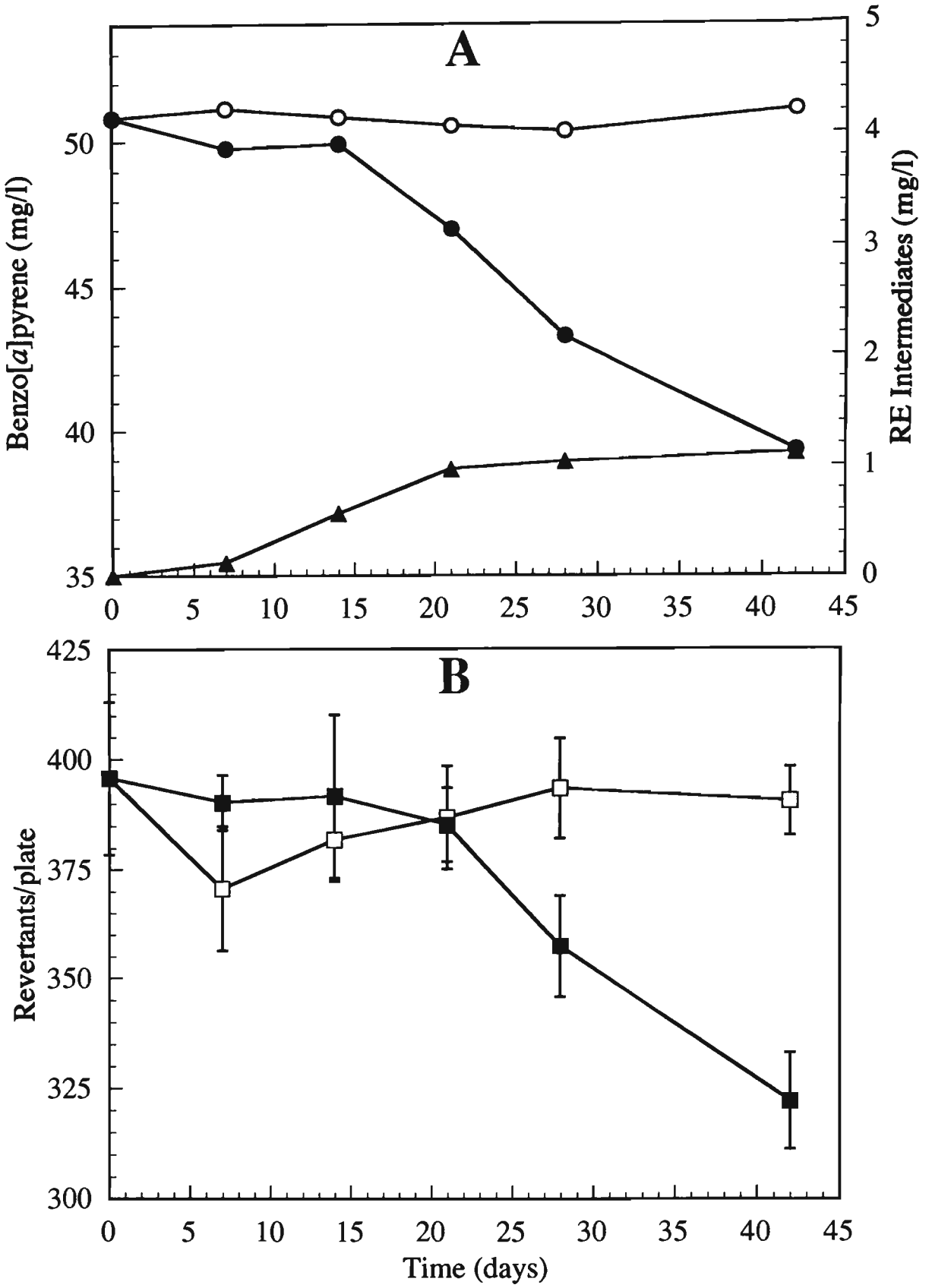


Figure 4.32. Benzo[*a*]pyrene degradation (A) and change in mutagenic potential (B) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. Benzo[*a*]pyrene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 42 day incubation period. The benzo[*a*]pyrene concentration (○) and mutagenicity (□) in killed cell cultures is also shown.

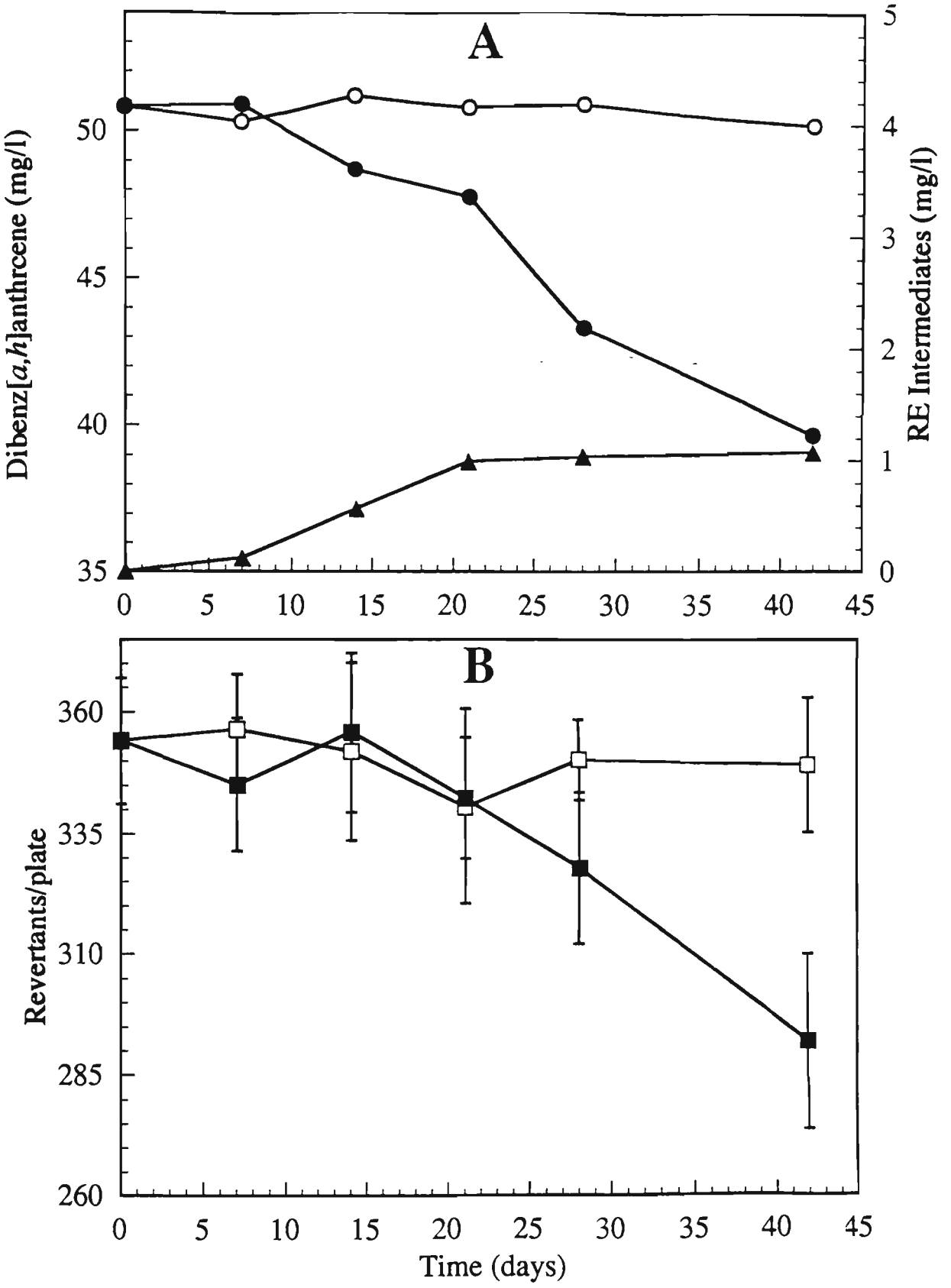


Figure 4.33. Dibenz[*a*,*h*]anthracene degradation (A) and change in mutagenic potential (B) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. Dibenz[*a*,*h*]anthracene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 42 day incubation period. The dibenz[*a*,*h*]anthracene concentration (O) and mutagenicity (□) in killed cell cultures is also shown.

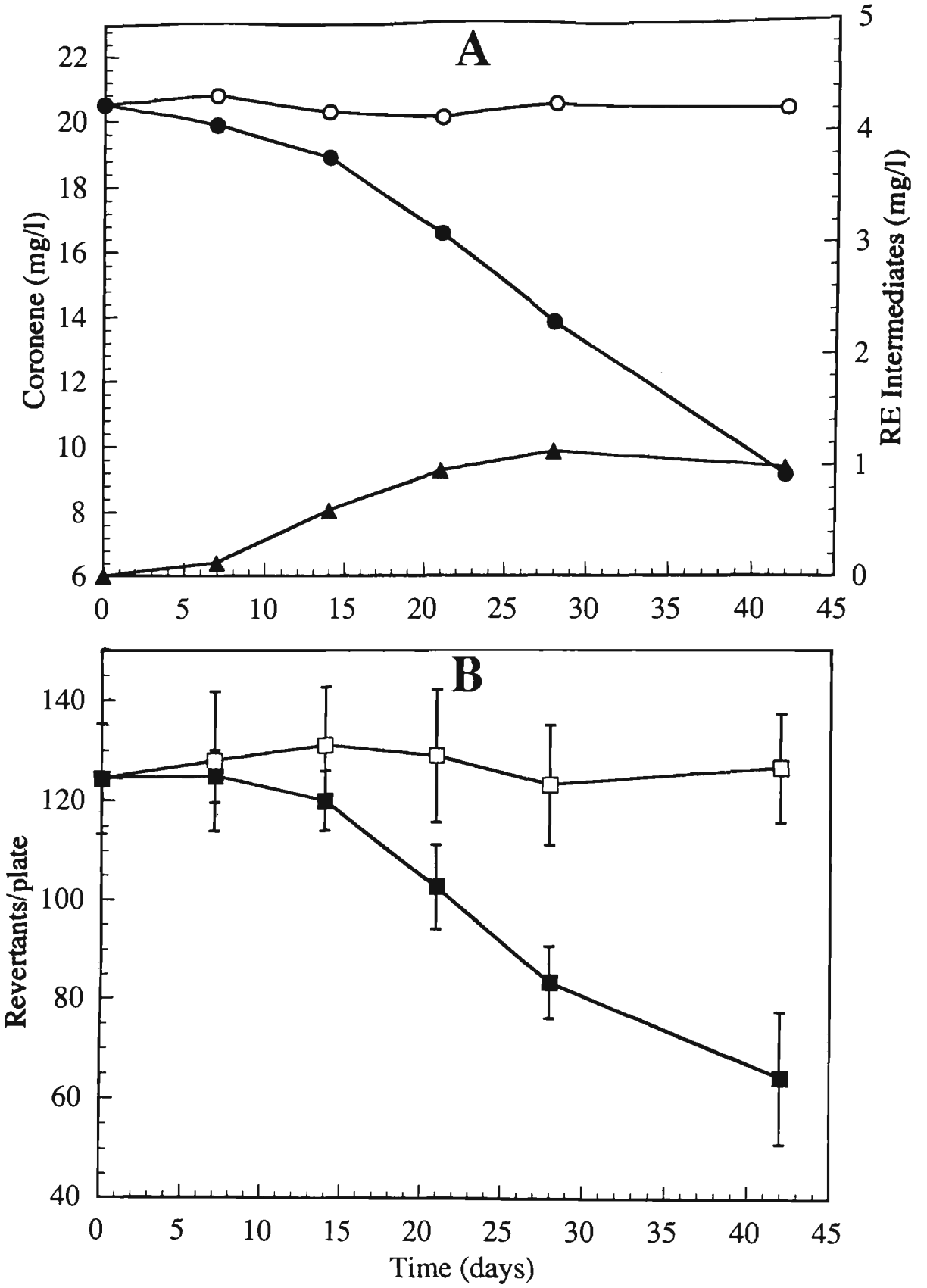


Figure 4.34. Coronene degradation (A) and change in mutagenic potential (B) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. Coronene (●), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (■) were determined in inoculated flasks over the 42 day incubation period. The coronene concentration (O) and mutagenicity (□) in killed cell cultures is also shown.

Table 4.7. Reduction in the mutagenicity of PAH-containing cultures inoculated with high initial cell densities of pyrene-grown community five, VUN 10,002 and VUN 10,003 compared to the respective $HgCl_2$ killed cell control cultures.

Compound	Incubation Time (days)	%Decrease in revertant colonies/plate[a]		
		Community five	VUN 10,002	VUN 10,003
Pyrene	5	76.7	74.7	76.0
Fluoranthene	14	49.9	47.1	43.5
Benz[*a*]anthracene	14	29.7	26.2	26.9
Benzo[*a*]pyrene	42	18.4	17.3	17.5
Dibenz[*a*,*h*]anthracene	42	13.9	13.4	16.4
Coronene	42	57.3	52.5	49.1
PPDB	42	38.1	36.5	37.5
FC	42	45.9	45.6	44.0

[a]The percentage reduction in the number of revertant colonies per plate was calculated with reference to the respective $HgCl_2$ killed cell control cultures in the presence of the S9 fraction.

Table 4.8. Aqueous solubilities of individual PAHs and the maximum resorcinol equivalent intermediate concentration obtained when the compounds were inoculated with high initial cell densities of pyrene-grown community five, VUN 10,002 and VUN 10,003.

PAH	Aqueous Solubility (mg/l)	Maximum RE Intermediate Conc. (mg/l)[a]		
		Community five	VUN 10,002	VUN 10,003
Fluoranthene	0.26	3.39 (14)[b]	10.95 (14)	15.30 (14)
Pyrene	0.14	1.79 (3)	2.25 (4)	2.07 (4)
Benz[*a*]anthracene	0.014	2.52 (14)	11.55 (4)	15.98 (14)
Benzo[*a*]pyrene	0.0038	0.58 (21)	0.99 (21)	1.11 (42)
Dibenz[*a,h*]anthracene	0.0005	0.62 (42)	1.53 (42)	1.08 (42)
Coronene		1.28 (42)	0.91 (21)	1.12 (28)
PPDB	-	5.30 (21)	4.60 (21)	5.90 (21)
FC	-	6.30 (21)	5.70 (21)	7.80 (21)

[a]Resorcinol equivalent (RE) intermediate concentrations were determined as described in the material and methods.

[b]The value in brackets represents the time (in days) when the maximum RE intermediate concentration was reached.

4.3.2.2 *Degradation of the Phenanthrene, Pyrene, Dibenz[a,h]anthracene and Benzo[a]pyrene (PPDB) Mixture*

Degradation of a low and high molecular weight PAH mixture (PPDB) by community five, VUN 10,002 and VUN 10,003 resulted in significant decreases in the concentration of all PAHs over the incubation period (data for VUN 10,003 shown in Figure 4.35). Greater than 95% of added phenanthrene and 85% of added pyrene were degraded after 14 days. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene were degraded at a slower rate, however, 29-38% of the five-ring compounds were degraded after 42 days. Resorcinol equivalent (RE) intermediate concentrations in inoculated cultures reached a maximum after 21 days (4.6-5.9 mg/l), however, RE intermediate concentrations decreased over the remaining incubation period (3.7-4.3 mg/l) (Table 4.8).

Total culture extracts from the PPDB control cultures taken over the incubation period exerted a strong mutagenic effect towards *S. typhimurium* strain TA100; high numbers of revertant colonies per plate (554-603) were observed. However, inoculation of the PPDB containing medium with community five, VUN 10,002 and VUN 10,003 resulted in a 36-38% reduction in the number of revertant colonies per plate after 42 days incubation (data for VUN 10,003 shown in Figure 4.35).

4.3.2.3 *Degradation of the Fluorene, Phenanthrene, Fluoranthene, Pyrene, Benz[a]anthracene, Benz[a]pyrene, Dibenz[a,h]anthracene and Coronene (FC) Mixture*

High initial cell density degradation experiments performed with a PAH mixture (FC) demonstrated that community five, VUN 10,002 and VUN 10,003 were capable of degrading high and low molecular weight PAHs concurrently as observed earlier in Section 4.2.3.1. Degradation of the low molecular weight PAHs (three-ring compounds) resulted in a 90-95% decrease in the concentration of fluorene and a 70-91% decrease in the concentration of phenanthrene after 42 days. Fluoranthene and pyrene were degraded to similar extents by the pyrene-enriched cultures; after 42 days a 61-74% decreases in the concentration of these compounds was observed. Degradation of benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene by community five, VUN 10,002 and VUN 10,003 resulted in decreases in the concentration of the compounds by 23-36% after 42 days (data for VUN 10,003 shown in Figure 4.36). Resorcinol equivalent (RE) intermediate concentrations in inoculated cultures reached a maximum after 21 days (5.7-7.8 mg/l), however, RE intermediate

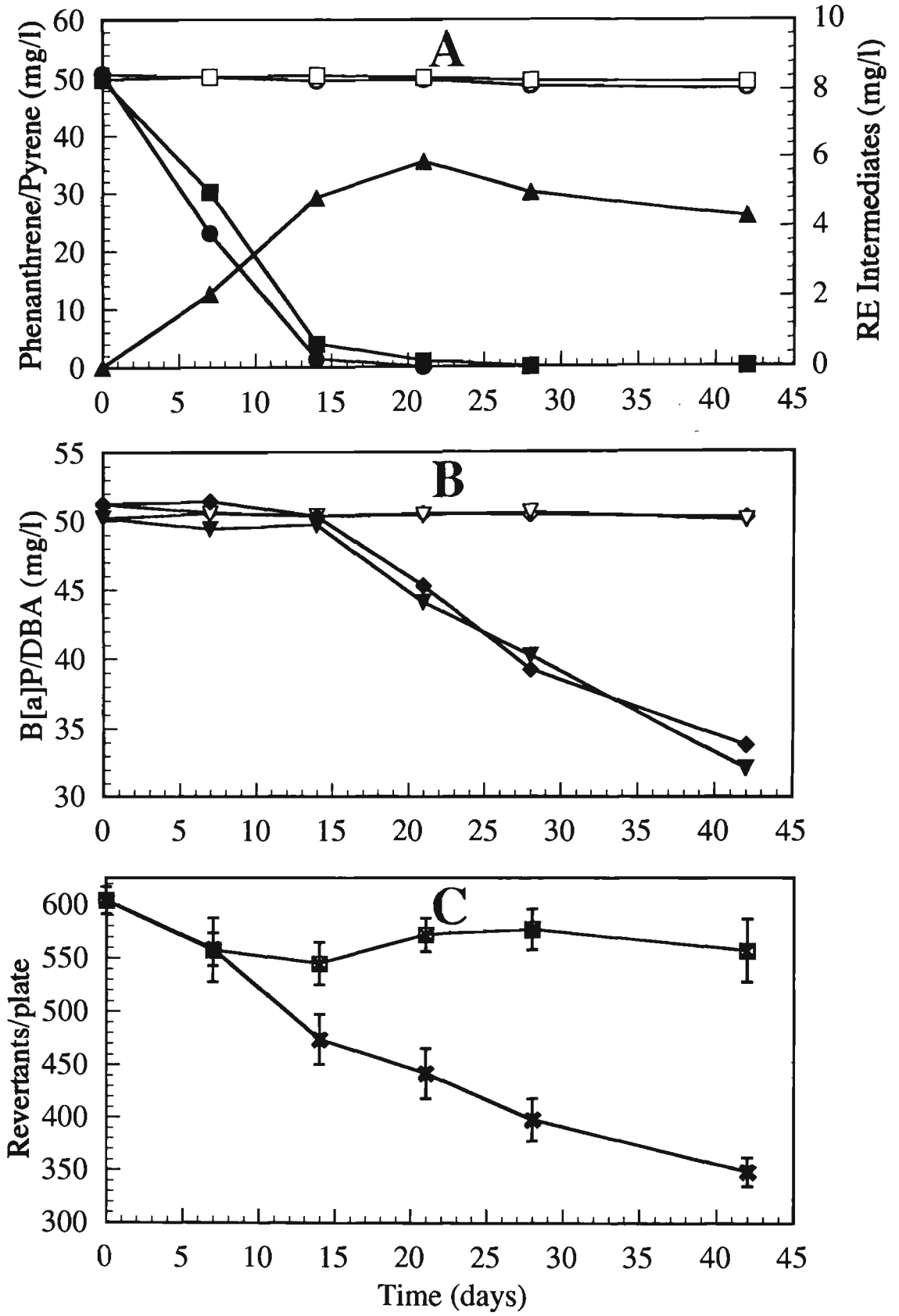


Figure 4.35. Phenanthrene, pyrene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene (PPDB) degradation (A and B) and change in mutagenic potential (C) of cultures containing high initial cell densities of pyrene-grown VUN 10,003. All panels represent data from the same culture. Phenanthrene (●), pyrene (■), benzo[*a*]pyrene (◆), dibenz[*a,h*]anthracene (▼), resorcinol equivalent (RE) intermediates (▲) and the mutagenicity of total culture extracts (✖) were determined in inoculated flasks over the 42 day incubation period. The phenanthrene (○), pyrene (□), benzo[*a*]pyrene (◇), dibenz[*a,h*]anthracene (▼) concentrations and mutagenicity (⊠) in killed cell cultures is also shown.

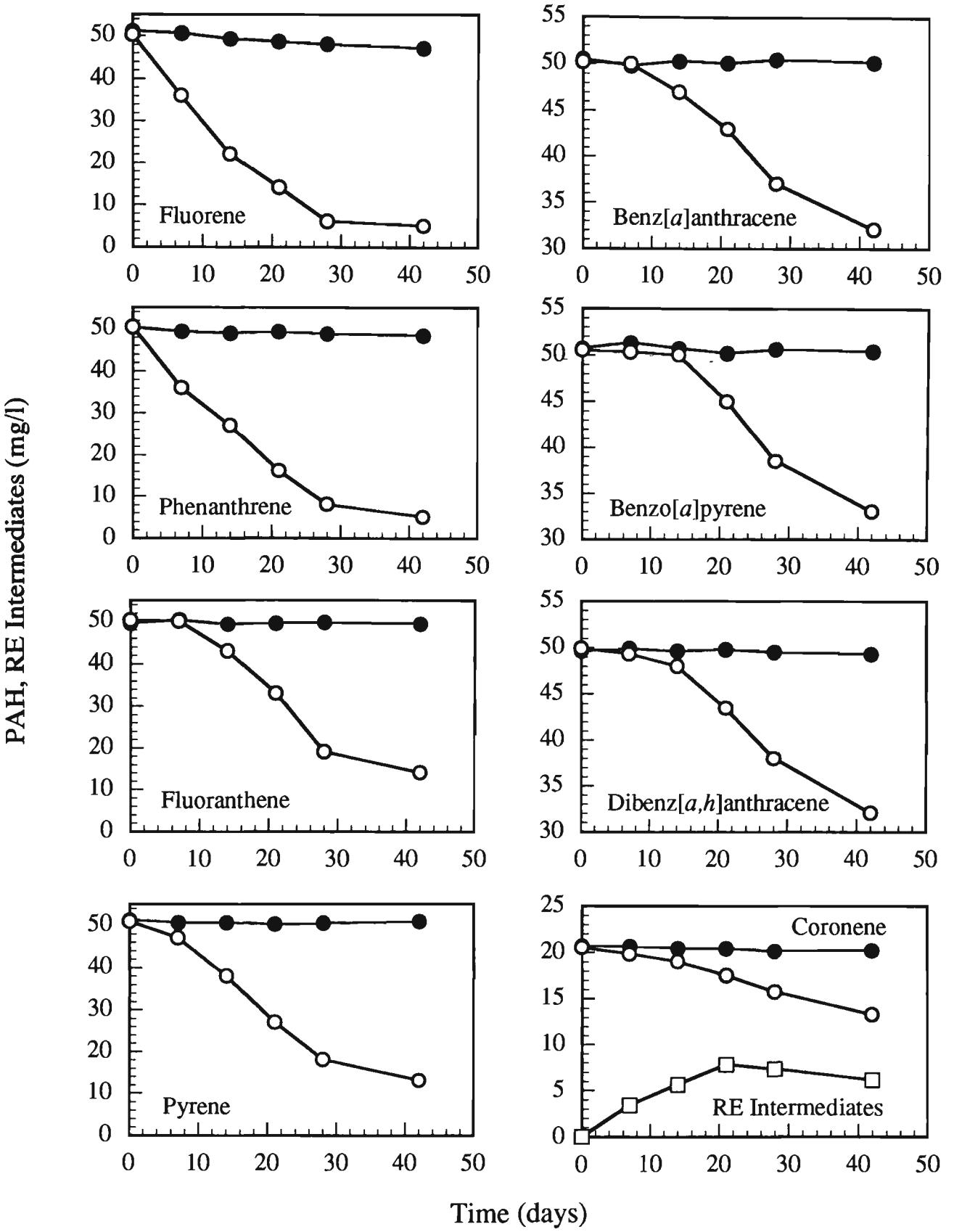


Figure 4.36. Concentration of PAHs and phenolic intermediates (□) in BSM containing a PAH mixture (FC) inoculated with high initial cell densities of pyrene-grown VUN 10,003. The panels represent the concentration profile of each PAH (○) in cultures containing all of the above compounds. The PAH concentration in control cultures (mercuric chloride killed, inoculated similarly) (●) is also shown.

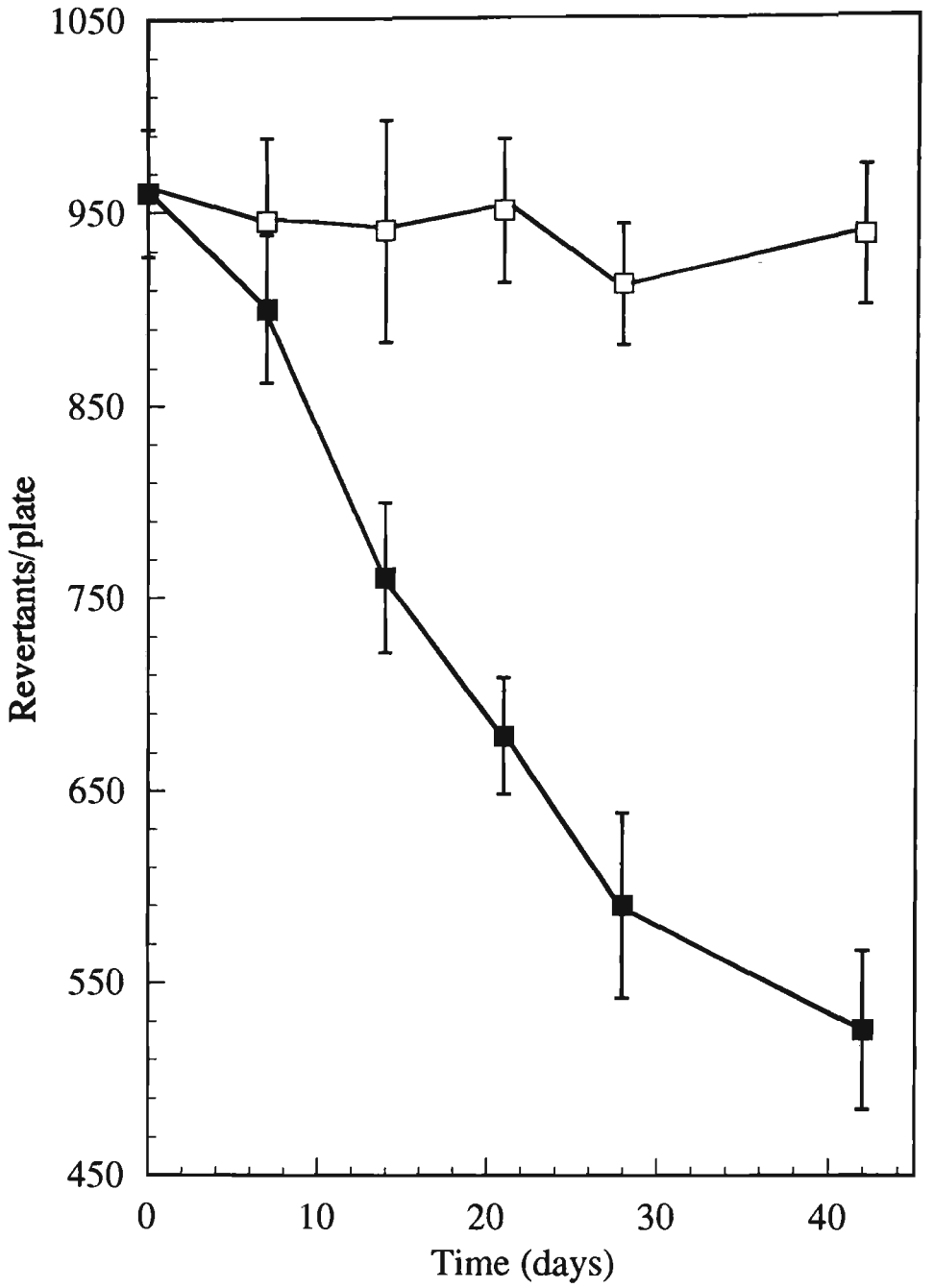


Figure 4.37. Change in the mutagenic potential of cultures containing high initial cell densities of pyrene-grown VUN 10,003 and a PAH mixture (FC) (■). The mutagenicity in mercuric chloride killed cell cultures (□) is also shown.

concentrations decreased over the remaining incubation period (4.5-6.1 mg/l) (Table 4.8).

Total culture extracts from the FC control cultures taken over the incubation period exerted a strong mutagenic effect towards *S. typhimurium* strain TA100; high numbers of revertant colonies per plate (880-987) were observed initially (Figure 4.37). However, inoculation of the FC containing medium with community five, VUN 10,002 and VUN 10,003 resulted in a 44-46% reduction in the number of revertant colonies per plate after 42 days incubation (data for VUN 10,003 shown in Figure 4.37).

4.3.2.4 *Mutagenicity of PAH Culture Supernatants*

The mutagenic activity of PAH culture supernatants was tested to determine whether the degradation of individual PAHs or PAH mixtures by community five, VUN 10,002 or VUN 10,003 resulted in the production of mutagenic by-products. Culture fluids were routinely taken from PAH incubations and cellular material and undegraded PAHs were removed by centrifugation. Ames tests were performed with the culture supernatants and a phenolic assay was used to determine the concentration of RE intermediates.

Significant concentrations of RE intermediates were detected in culture supernatants of VUN 10,002 and VUN 10,003 initially containing fluoranthene and benz[*a*]anthracene (11-16 mg/l) (Table 4.8). Lower concentrations of RE intermediates were observed in cultures containing the PAH mixtures (4.6-7.8 mg/l), while less than 3.4 mg/l RE intermediates were detected in the remaining cultures. Although there was some variation in the concentration of RE intermediates in the culture supernatants from different PAH incubations, mutagenicity assays with culture supernatants (0.1 and 1.0 ml) from all PAH incubations showed no mutagenic activity towards *S. typhimurium* strain TA100 with or without metabolic activation (S9). The number of revertant colonies observed after exposure to the culture supernatants (18-31 revertant colonies per plate) were similar to the number of spontaneous revertants observed when *S. typhimurium* strains were exposed to BSM (15-27 revertant colonies per plate). This indicates that the by-products produced as a result of the degradation of PAHs by community five, VUN 10,002 and VUN 10,003 do not exhibit mutagenic activities and that the degradation of PAHs by the pyrene-enriched isolates reduces the overall mutagenicity of the compounds.

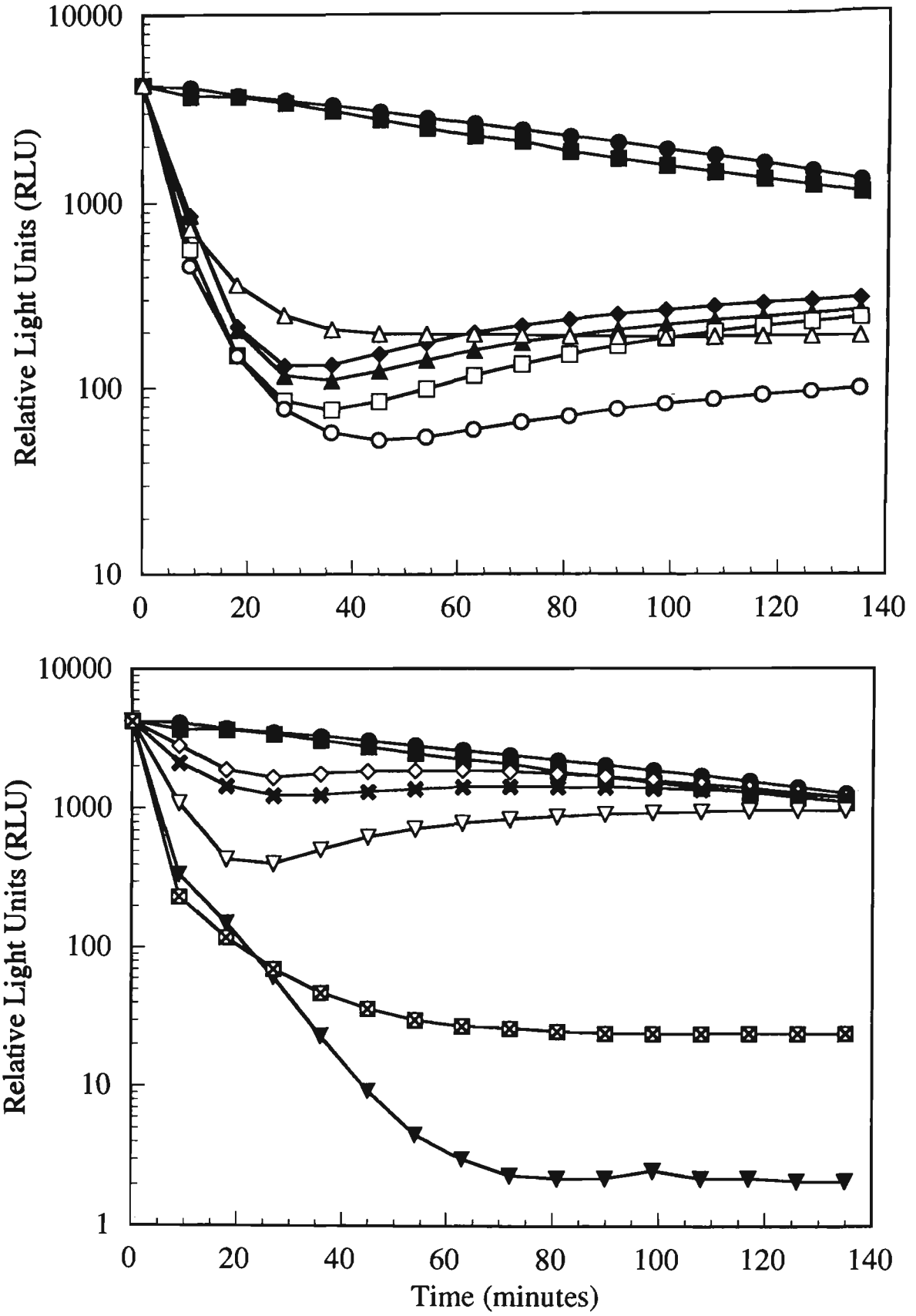


Figure 4.38. Toxicity of PAHs to *P. phosphoreum* as determined by a decrease in light output. *P. phosphoreum* was exposed to aqueous supernatants from BSM-containing fluorene (▲), phenanthrene (◆), fluoranthene (□), pyrene (○), benz[*a*]anthracene (△), benzo[*a*]pyrene (◇), dibenz[*a*,*h*]anthracene (✖), coronene, (▼), PPDB (▼) and FC (⊠) for 135 minutes. The decrease in light output for *P. phosphoreum* exposed to BSM containing DMF (0.1 ml/10 ml) (■) and 3% NaCl without additives (●) is also shown.

4.3.3 Toxicity of PAH Culture Supernatants

PAHs are recognised for their toxic and mutagenic effects on the environment. The previous section specifically examined the mutagenicity of liquid cultures containing PAHs and the pyrene-enriched isolates. In this section, results of testing the toxicity of the aqueous phase of culture fluids initially containing various PAHs using the Microtox™ test are reported. The aqueous phase toxicity of cultures containing various PAHs was assessed using a modified Microtox™ test. A modification to the assay was used in this work which allows for the monitoring of light over an extended period of time (see Section 2.4.7).

4.3.3.1 *Aqueous-Phase Toxicity of PAH Solutions*

The toxicity of aqueous supernatants from uninoculated solutions of PAHs in BSM was measured prior to determining the effect on toxicity of inoculating these solutions with the microbial isolates. PAHs were added to BSM in excess of their aqueous solubilities (50 mg/l in DMF) and allowed to equilibrate for 5 days at room temperature. PAH solutions were centrifuged to remove residual PAHs and *P. phosphoreum* was exposed to the supernatants to assess the toxicity of the PAHs in solution.

When *P. phosphoreum* was exposed to aqueous supernatants from solutions of fluorene, phenanthrene, fluoranthene, pyrene and benz[*a*]anthracene in BSM, large decreases in light output were observed after the initial nine minutes (4,200 to 458-858 RLU) (Figure 4.38). Light output continued to decrease up to 36 minutes following the initial exposure. No further decrease in bioluminescence was observed for *P. phosphoreum* exposed to benz[*a*]anthracene, however, slight increases in light output were observed over the remaining test period for fluorene, phenanthrene, fluoranthene and pyrene.

The high molecular weight PAHs (benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene) had a lesser toxic effect on *P. phosphoreum* compared with the lower molecular weight compounds, which is most likely due to their lower aqueous solubilities. Light emitted by *P. phosphoreum* decreased over the initial 27 minute exposure period (4,211 to 1,639, 1,228 and 401 RLU for benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene respectively), however, an increase in bioluminescence was observed for *P. phosphoreum* exposed to coronene over the remaining test period (965 RLU) (Figure 4.38). This increase was probably the result of adaptive recovery of *P. phosphoreum* on the stress of the initial exposure to the coronene supernatants. The light emitted by *P. phosphoreum* exposed to

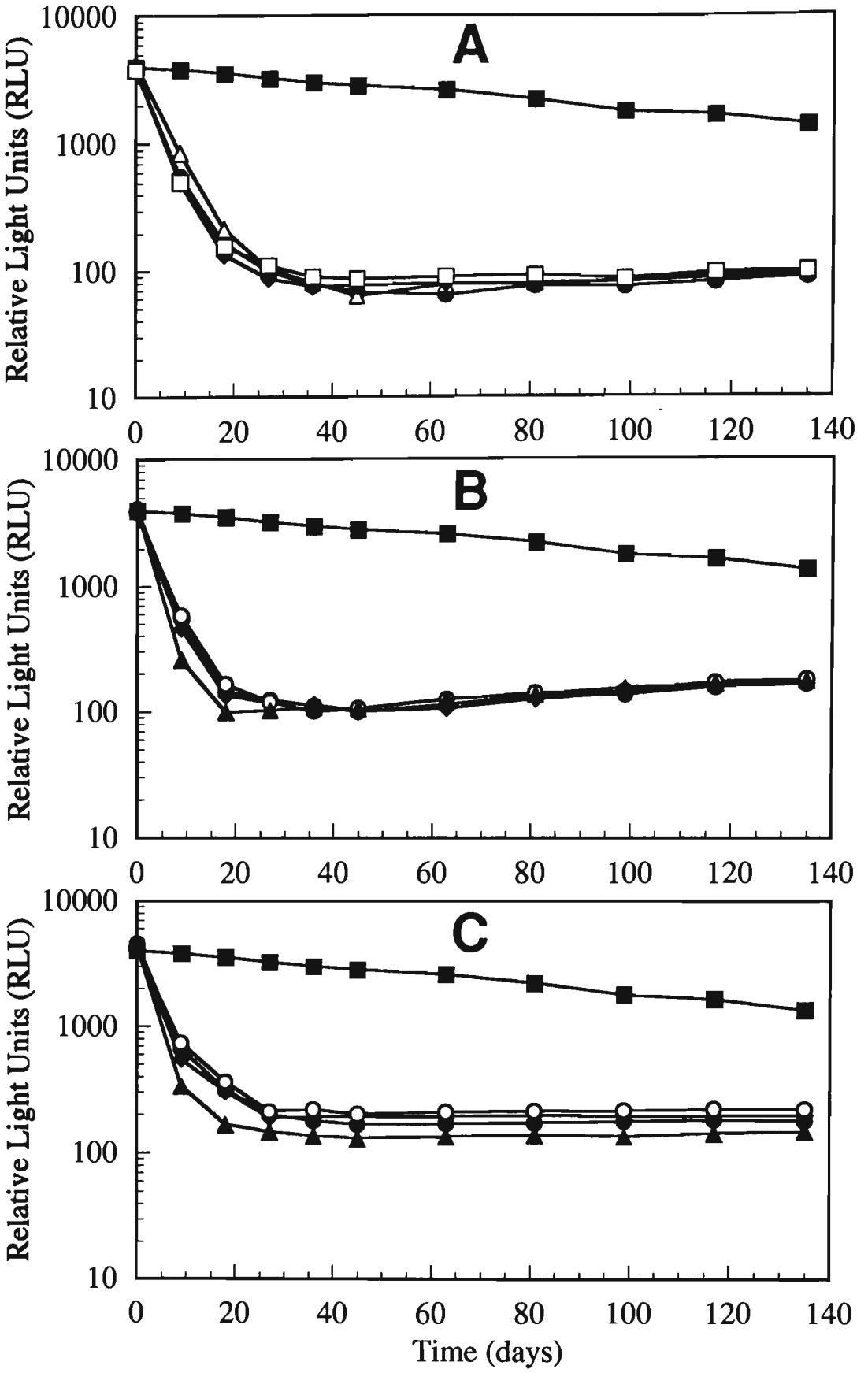


Figure 4.39. Change in the toxicity of culture supernatants containing pyrene (A), fluoranthene (B) and benz[*a*]anthracene (C) after incubation with high initial cell densities of pyrene-grown VUN 10,003. The light output of *P. phosphoreum* is shown after exposure to 1 (Δ), 3 (◆), 5 (□), 7 (O) and 14 day (▲) culture supernatant samples. The decrease in light output for *P. phosphoreum* exposed to BSM (■) and 5 or 14 day uninoculated control culture supernatants (●) is also shown.

benzo[*a*]pyrene and dibenz[*a,h*]anthracene supernatants was relatively stable 27 minutes after the initial exposure. The light output of *P. phosphoreum* exposed to the high molecular weight PAH supernatants at the end of the test period was similar to the test control (BSM) (Figure 4.38). This reconciling of light output between the high molecular weight PAH supernatants and the control probably reflects adaptation of *P. phosphoreum* to the relatively low concentrations of the PAHs in solution.

The exposure of *P. phosphoreum* to the PAH mixtures (phenanthrene, pyrene, dibenz[*a,h*]anthracene, benzo[*a*]pyrene [PPDB] and fluorene, phenanthrene, fluoranthene, pyrene, benzo[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, coronene [FC]) resulted in substantial decreases in light output after the initial nine minutes (4,211 to 338 and 234 RLU). *P. phosphoreum* light output continued to decrease after this time period resulting in final light reading of 2.1 and 24 RLU for the PPDB and FC mixtures respectively (Figure 4.38).

4.3.3.2 *Toxicity of PAH Water Soluble Fractions of Cultures*

Due to the presence of aqueous solubilities of PAHs in all culture supernatants over the incubation period, the toxicity of PAH metabolites was determined as the difference between the light output of *P. phosphoreum* exposed to the aqueous solubilities of PAHs (uninoculated PAH culture medium) and the aqueous solubilities of the PAHs plus their respective water soluble metabolites (inoculated PAH culture medium).

No significant difference (95% confidence limit) between control supernatants and supernatants obtained from cultures containing pyrene, fluoranthene (Figure 4.39), benzo[*a*]pyrene, dibenz[*a,h*]anthracene, coronene (Figure 4.40), PPDB and FC mixtures (Figure 4.41) inoculated with community five, VUN 10,002 and VUN 10,003 were observed over the incubation period. The light output of *P. phosphoreum* exposed to fluoranthene (14 day sample), benzo[*a*]pyrene (42 day sample) and dibenz[*a,h*]anthracene (42 day sample) decreased at a faster initial rate than control supernatants, however, at the end of the assay period (108 minutes) no differences in light output ($p>0.05$) were observed.

No significant difference in the light output of *P. phosphoreum* was observed when the organism was exposed to benz[*a*]anthracene control supernatants and the three and seven day inoculated culture supernatants (Figure 4.39). However, a decrease in light output was observed for the 14 day inoculated culture supernatants compared to the 14 day benz[*a*]anthracene control supernatant. The light output of *P. phosphoreum*,

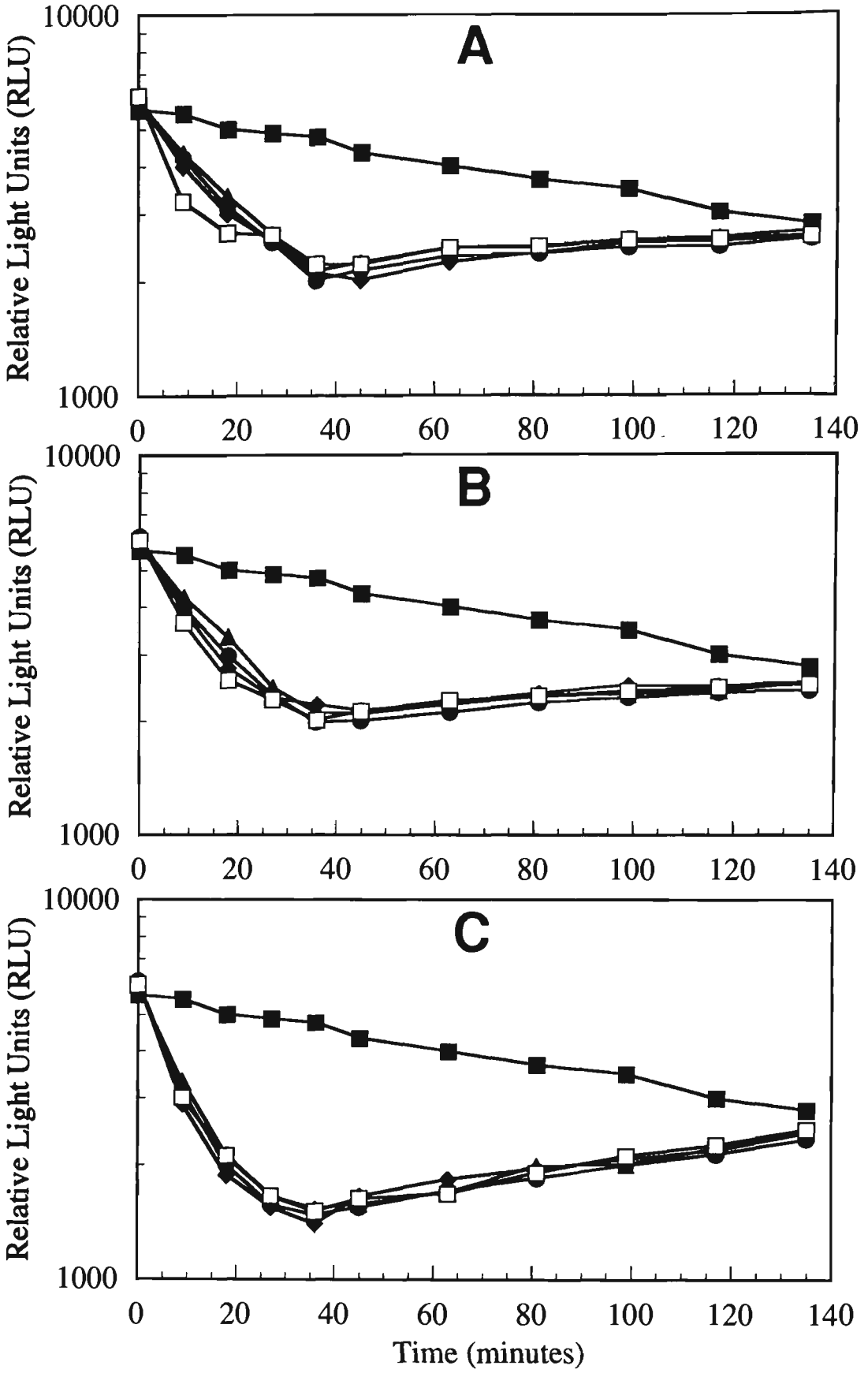


Figure 4.40. Change in the toxicity of culture supernatants containing benzo[*a*]pyrene (A), dibenz[*a,h*]anthracene (B) and coronene (C) after incubation with high initial cell densities of pyrene-grown VUN 10,003. The light output of *P. phosphoreum* is shown after exposure to 7 (▲), 21 (◆) and 42 day (□) culture supernatant samples. The decrease in light output for *P. phosphoreum* exposed to BSM (■) and 42 day uninoculated control culture supernatants (●) is also shown.

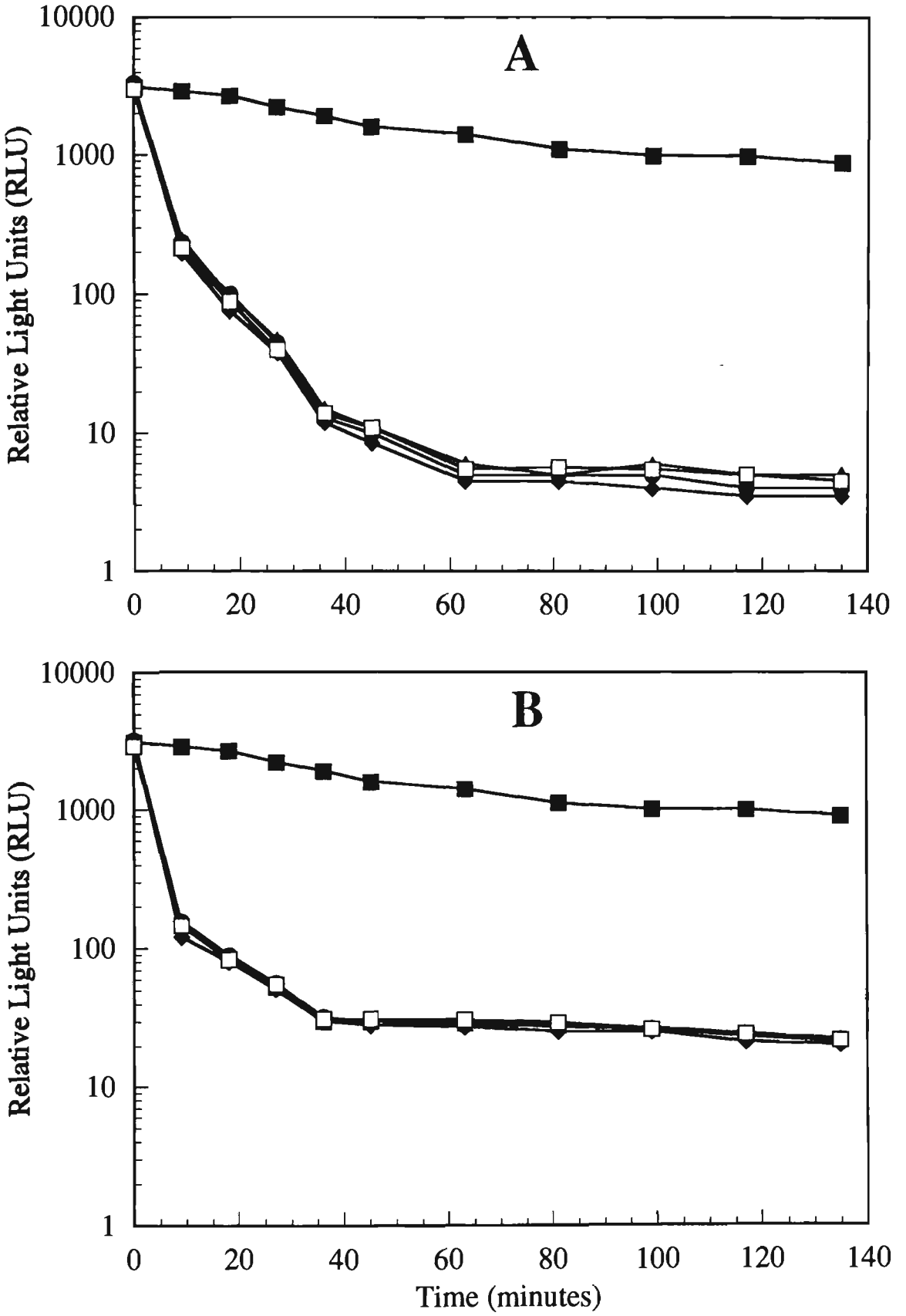


Figure 4.41. Change in the toxicity of culture supernatants containing PPDB (A) and FC (B) PAH mixtures after incubation with high initial cell densities of pyrene-grown VUN 10,003. The light output of *P. phosphoreum* is shown after exposure to 7 (▲), 21 (◆) and 42 day (□) culture supernatant samples. The decrease in light output for *P. phosphoreum* exposed to BSM (■) and 42 day uninoculated control culture supernatants (●) is also shown.

exposed to the inoculated culture supernatants (4,322 to 335 RLU after 9 minutes), decreased at a faster rate than the control supernatants (4,554 to 655 RLU after 9 minutes). At the end of the test period, *P. phosphoreum's* light output after exposure to the inoculated culture supernatants was 18% less than the bioluminescence from the control supernatant. The increase in toxicity of benz[*a*]anthracene culture supernatants corresponded to the increase RE intermediate concentration over the incubation period.

4.4 EVALUATION OF CARBON SOURCES FOR THEIR POTENTIAL USE IN PREPARING INOCULA FOR PAH DEGRADATION

Bioaugmentation of polluted soil may be considered when the indigenous microbial population capable of degrading the target compounds is small. Newly contaminated soil may contain low microbial numbers due to insufficient time for adaptation and growth. Augmentation of complex wastes may be required after physical or chemical pretreatment to remove toxic non-biodegradable materials (*e.g.* heavy metals). Also, when the minimum time period required to remediate a site is of primary importance the addition of adapted microorganisms with little or no lag period can significantly reduce bioremediation times compared to indigenous biodegradation rates. A major factor when considering bioaugmentation is whether the process is cost effective. An inexpensive substrate is necessary for the production of large inocula if bioremediation processes are to be economically viable.

The first section of this chapter reported the ability of community five to degrade a range of PAHs in liquid media. The inocula used in these experiments were prepared and maintained in BSM containing pyrene as the sole carbon and energy source. Pyrene as a growth substrate for inoculum preparation is prohibitively expensive for commercial scale operations. The objective of the study reported in this section was to find an enrichment substrate that:

1. is inexpensive;
2. supports microbial growth;
3. produces substantial biomass yields; and
4. maintains/induces the PAH degradative capabilities of the inocula.

Creosote is a complex mixture of over 200 chemical constituents encompassing diverse chemical structures. PAHs comprise approximately 85% of creosote while phenolic and N-, S-, and O-heterocyclic compounds comprise the remaining constituents. Coal tar creosote is a commonly used wood preserving agent used for treating telephone

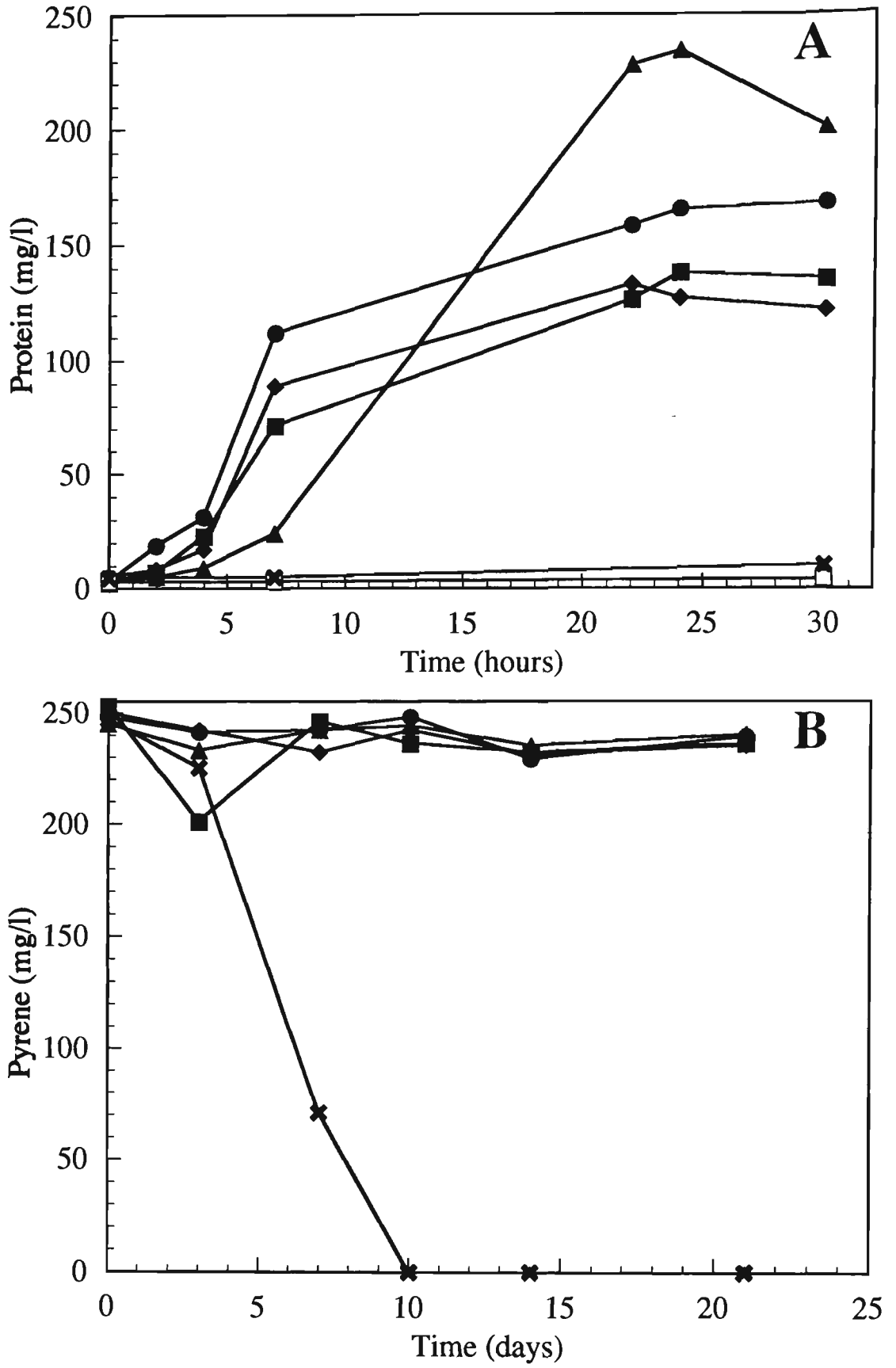


Figure 4.42. Growth of community five (A) in BSM containing 1 g/l peptone (■), yeast extract (●), glucose (▲), succinate (◆), 2 ml/l creosote (□) and 250 mg/l of pyrene (✖). Media was inoculated with 1% unwashed pyrene-grown cells. The ability of the microbial communities, grown on the above substrates, to degrade pyrene was assessed by adding a 10% unwashed inoculum into BSM containing pyrene (250 mg/l) as the sole carbon source (B).

poles, fencing and timbers for farm buildings. It also makes an excellent pesticide, is relatively inexpensive as well as being readily available. Because community five could grow on a variety of substrates (Table 3.1 and 4.1) and on mixtures of PAHs, it was worth testing creosote as a substrate for inoculum preparation. These results are reported in this section.

4.4.1 Effect of Growth Substrate on Subsequent Degradation of PAHs

A number of substrates (creosote, peptone, yeast extract, glucose, succinate, pyrene) were tested for their ability to support the rapid growth of community five. After three successive transfers in the respective medium, each culture was tested for its ability to degrade pyrene in BSM as a sole carbon and energy source. Growth on peptone, yeast extract, glucose and succinate (1 g/l) by community five was rapid compared to its growth on pyrene (Figure 4.42). Protein concentrations were up to five times greater on these substrates and the stationary phase was reached within 24 hours compared to approximately 170 hours for pyrene. The greatest biomass yield was obtained when glucose was used as the substrate: protein concentrations reached a maximum of 230 mg/l after 24 hours. The biomass yield on peptone, yeast extract and succinate was less than glucose, reaching protein concentrations of 120-165 mg/l in 24 hours. Biomass concentrations of community five grown on pyrene was small compared to peptone, yeast extract, glucose and succinate. After 30 hours, protein concentrations increased from 4.7 to 10.2 mg/l and this increased to 53 mg/l after 168 hours. Limited growth was observed by community five on creosote: over a 30 day period, the protein concentration increased from 2.9 μg/ml to 12.9 μg/ml.

The ability of the microbial communities, grown on the above substrates, to degrade pyrene was assessed by adding a 10% unwashed inoculum into BSM containing 250 mg/l of pyrene. The protein concentrations of the inocula varied depending on the extent of growth of community five on the different substrates used. The microorganisms enriched on peptone, yeast extract, glucose and succinate were unable to degrade pyrene (Figure 4.42) in BSM over a 21 day period and were therefore unsuitable for preparing inocula for PAH degradation. Pyrene-grown inocula completely degraded pyrene in BSM over a 10 day period. Pyrene degradation tests for creosote-enriched microorganisms were not performed as the cell population in enrichment cultures was too small.

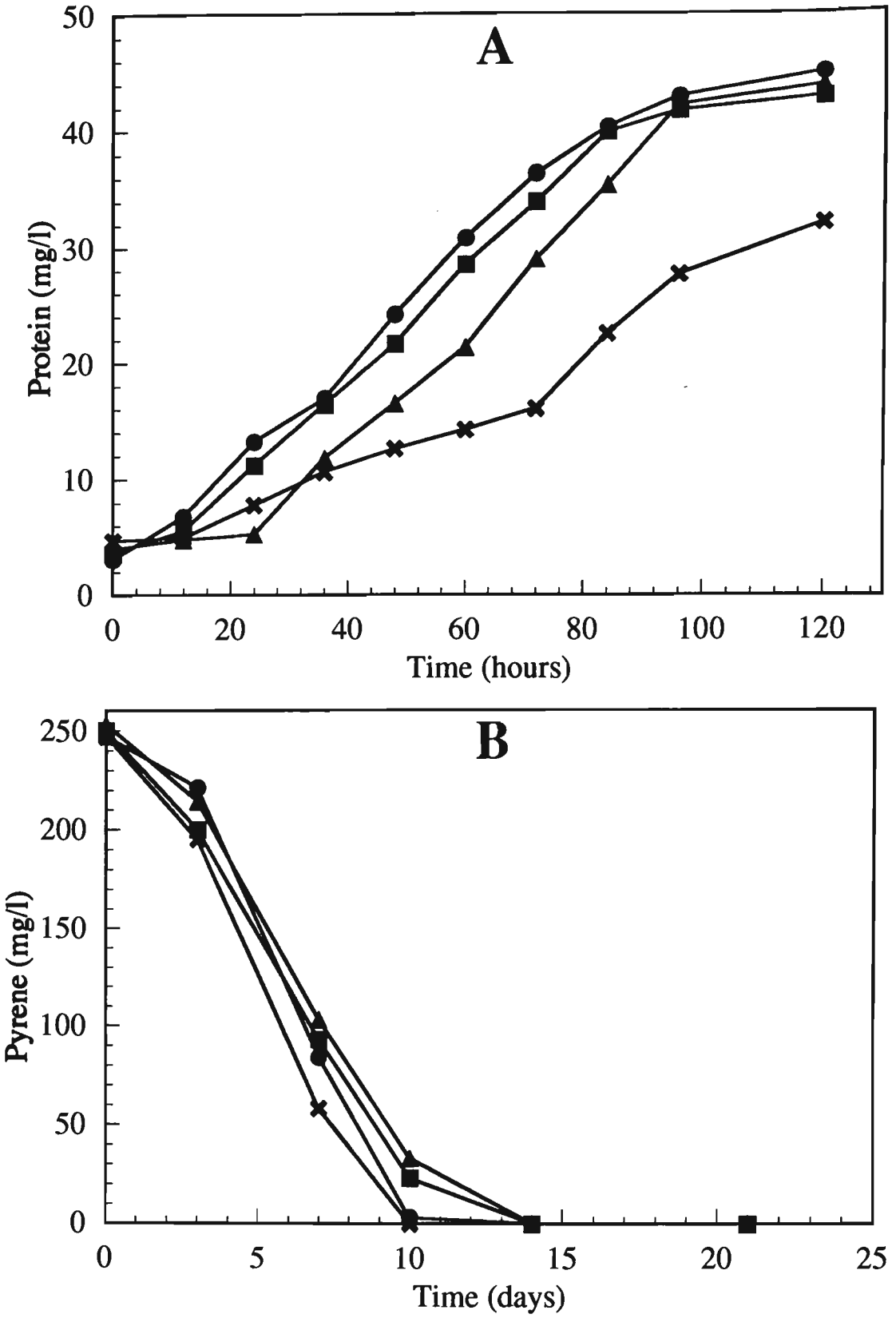


Figure 4.43. Growth of community five (A) in BSM containing creosote (2 ml/l) and one of the following: peptone (1 g/l) (■), yeast extract (1 g/l) (●) and glucose (1 g/l) (▲). Media was inoculated with 1% unwashed pyrene-grown cells. The ability of the microbial communities, grown on the above substrates, to degrade pyrene was assessed by adding a 10% unwashed inoculum into BSM containing pyrene (250 mg/l) as the sole carbon source (B). The growth of community five on pyrene and the degradation of pyrene after pyrene enrichment is also shown (✖).

4.4.2 Evaluation of Creosote Plus Other Nutrients for Preparing Inocula

Although peptone, yeast extract, succinate and glucose promoted rapid growth and high yields of community five, the resulting inocula could not degrade pyrene. Creosote could not support rapid growth of community five, but its chemical make-up would most likely furnish the necessary selective pressure to produce a community five inoculum with PAH-degrading ability. Results in this section report the evaluation of using creosote (2 ml/l) in BSM plus 1 g/l of either peptone, yeast extract or glucose to provide a suitable medium for both the rapid growth of community five inocula plus subsequent PAH-degrading competence.

Growth on these substrate combinations resulted in lower biomass yields compared to that observed on BSM plus peptone, yeast extract or glucose alone (Figure 4.43). However, after 96 hours the protein concentrations obtained were similar to cells grown in BSM containing pyrene but these values were reached in half the incubation time.

When transferred into BSM containing pyrene, the above inocula were able to degrade pyrene as the sole carbon and energy source. The rate of pyrene degradation by community five grown on yeast extract and creosote (Figure 4.43) was comparable to pyrene-grown microorganisms (Figure 4.42). The degradation of pyrene by inocula grown in creosote plus glucose or creosote plus peptone was slightly slower, however, pyrene was degraded to undetectable levels after 14 days.

As a growth substrate, yeast extract is more attractive than glucose as it offers a variety of carbon sources, vitamins, trace elements and amino acids compared to a single carbohydrate source. Yeast extract is also a more attractive substrate compared to peptone, as biomass yields were slightly higher when yeast extract was supplied alone or in combination with creosote. The pyrene degradation rate by creosote- and yeast extract-grown cells was faster than for cells grown in creosote plus peptone and creosote plus glucose. For these reasons, the medium comprising a combination of creosote and yeast extract was selected for further studies.

4.4.3 Optimisation of Creosote Concentration in Inoculum Preparation

The optimum concentration of cresosote required for high biomass yield and subsequent PAH-degradation capacity was evaluated by adding 0.0002 to 2 ml/l of creosote to BSM containing 1 g/l of yeast extract. Growth of community five at the lower concentrations of creosote (0.2, 0.02, 0.002 and 0.0002 ml/l) was rapid and

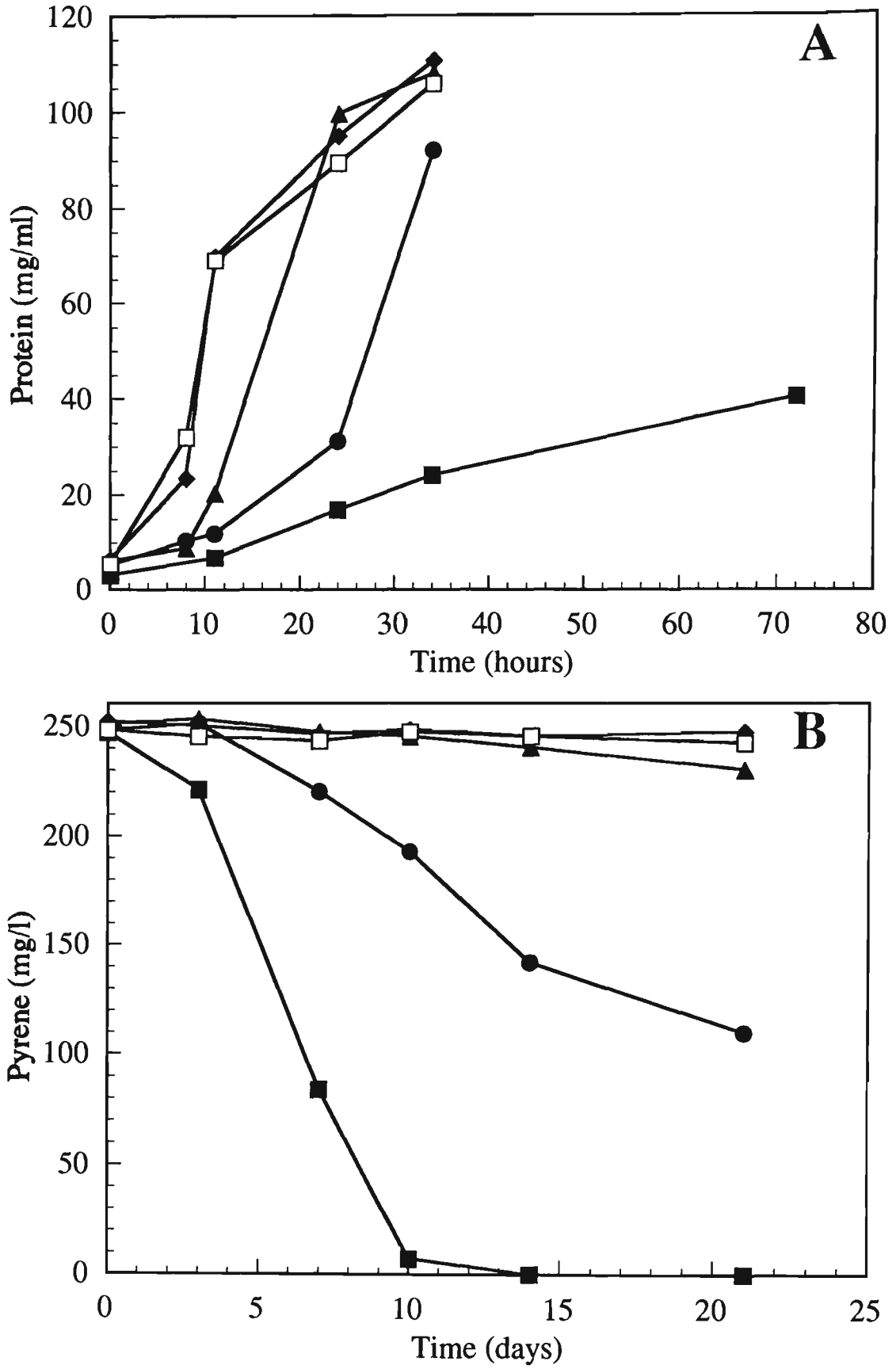


Figure 4.44. Growth of community five (A) in BSM containing yeast extract (1 g/l) and the following concentrations of creosote: 2.0 ml/l (■), 0.2 ml/l (●), 0.02 ml/l (▲), 0.002 ml/l (◆) and 0.0002 ml/l (□). Media was inoculated with 1% unwashed pyrene-grown cells. The ability of the microbial communities, grown on various creosote concentrations, to degrade pyrene (B) was assessed by adding a 10% unwashed inoculum into BSM containing pyrene as the sole source of carbon.

protein concentrations reached 90-110 μg/ml after 36 hours (Figure 4.44). At the highest concentration of creosote (2.0 ml/l), growth was slow, reaching 40 μg/ml protein after 72 hours. The degradation of pyrene by inocula prepared from these cultures demonstrated that high biomass yields did not equate to increased rates of pyrene degradation. Enrichments containing 0.02, 0.002 and 0.0002 ml/l creosote did not significantly degrade pyrene after 21 days. A 56% decrease in pyrene concentration after 21 days was observed by community five grown on BSM containing yeast extract (1.0 g/l) and 0.2 ml/l creosote, while 97% of added pyrene was degraded by microorganisms grown on the highest creosote concentration after 10 days (Figure 4.44). These results indicate that the lower concentrations of creosote failed to induce pyrene-degrading activity in community five in the presence of yeast extract.

4.4.4 Optimisation of Yeast Extract Concentration in Inoculum Preparation

Yeast extract was added at concentrations of 0.1, 0.5, 1.0 and 2.0 g/l to BSM containing 2.0 ml/l creosote, which was then inoculated with community five. Growth by community five at the lower concentrations (0.1 and 0.5 g/l) of yeast extract was typified by lower growth rates and biomass yields: protein concentrations reached 18 μg/ml after 96 hours from initial protein concentrations of 1.75-4.7 mg/l. A higher growth rate occurred using 1.0 and 2.0 g/l yeast extract which, after 29 hours, slowed considerably reaching protein concentrations of 65 μg/ml and 110 μg/ml respectively (Figure 4.45).

The degradation of pyrene by inocula prepared from these cultures demonstrated that the slow growth in media containing the lower concentrations of yeast extract (0.1 and 0.5 g/l) corresponded to a slower pyrene degradation rate (Figure 4.45). There was no significant decrease in pyrene concentration by community five grown on BSM containing creosote (2.0 ml/l) and yeast extract (0.1 g/l). The rate of pyrene degradation by community five grown on BSM containing creosote (2.0 ml/l) and yeast extract (1.0 g/l) was faster than community five grown on BSM containing creosote (2.0 ml/l) and 2.0 g/l yeast extract, even though the protein concentration was 40% greater in this latter medium. The rate of pyrene degradation by microorganisms grown on BSM containing creosote (2.0 ml/l) and yeast extract (1.0 g/l) was comparable to the pyrene degradation rate of pyrene-enriched microorganisms (Figure 4.42). These results indicate that the ratio of non-PAH growth substrate to PAH-containing substrate is important in conditioning the inoculum.

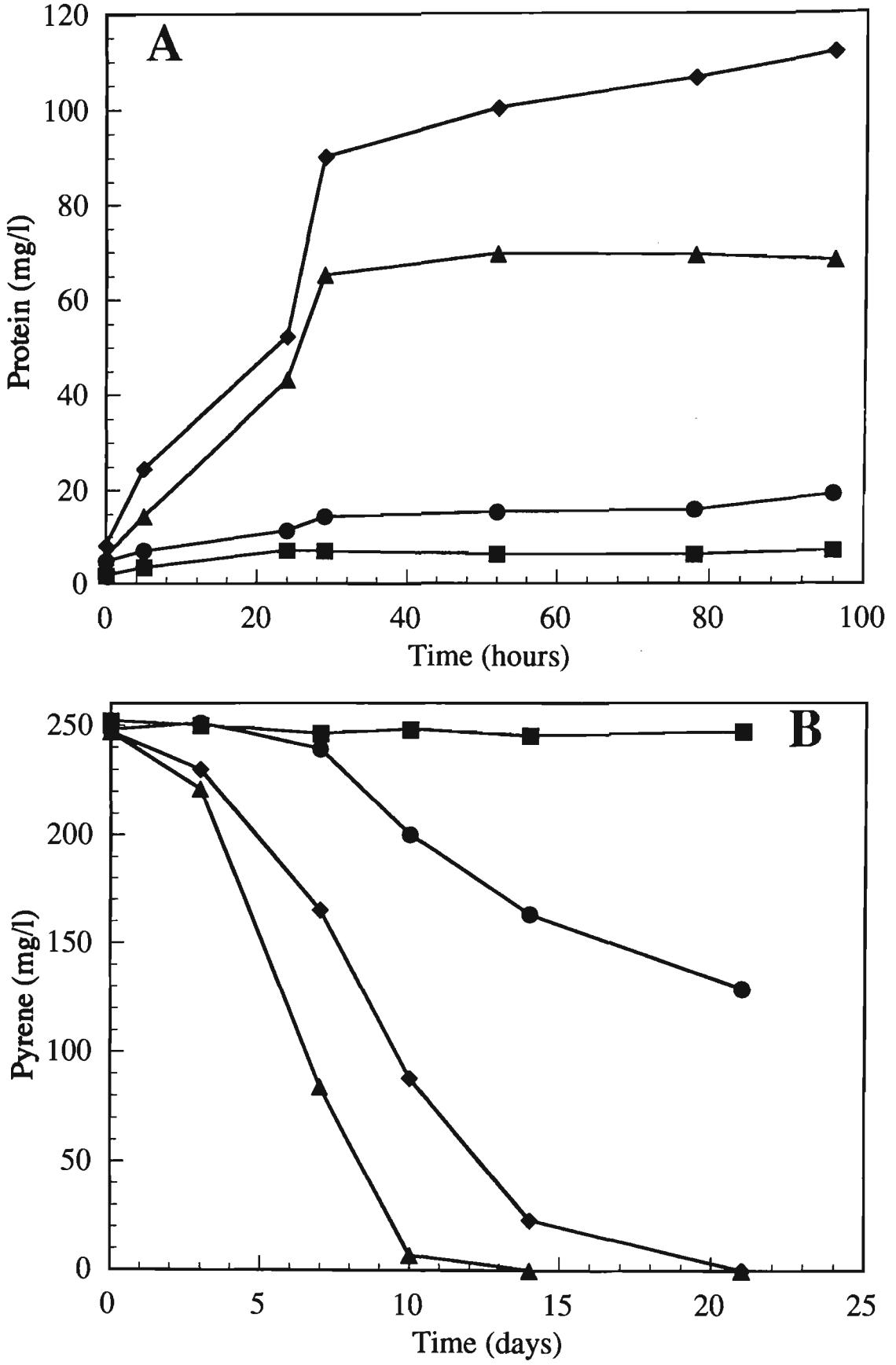


Figure 4.45. Growth of community five (A) in BSM containing creosote (2 ml/l) and the following concentrations of yeast extract: 0.1 g/l (■), 0.5 g/l (●), 1.0 g/l (▲) and 2.0 g/l (◆). Media was inoculated with 1% unwashed pyrene-grown cells. The ability of the microbial communities, grown on various yeast extract concentrations, to degrade pyrene (B) was assessed by adding a 10% unwashed inoculum into BSM containing pyrene as the sole carbon source.

4.4.5 Degradation of Creosote and a PAH Mixture by Inocula Grown in a Creosote Medium

In the previous section, it was shown that community five inocula were best able to degrade pyrene when the inoculum culture was grown on a substrate mix of creosote (2 ml/l) and yeast extract (1 g/l) in BSM (CYEM). The purpose of the experiments reported in this section was to examine the ability of community five inocula grown on CYEM to degrade a synthetic PAH mixture and creosote. Degradation experiments were performed with the PAH mixture and creosote as the sole carbon and energy source as well as in the presence of yeast extract (1.0 g/l).

4.4.5.1 *Degradation of a Synthetic PAH Mixture*

The synthetic PAH mixture was comprised of PAHs identified in the creosote (fluorene, phenanthrene, fluoranthene and pyrene) as well as some higher molecular weight compounds (benz[*a*]anthracene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene). Each component was at a concentration of 50 mg/l which was the approximate concentration of phenanthrene, fluoranthene and pyrene in the creosote.

Long degradation lag periods were observed for the CYEM-grown cells when inoculated into BSM containing the synthetic PAH mixture (Table 4.9). Degradation lag periods were 10-14 days for the three-ring compounds, 14-21 days for the four-ring compounds and 28 days for the five-ring compounds. The degradation of fluorene, phenanthrene, fluoranthene and pyrene by CYEM-grown community five resulted in PAH concentration decreases of 29-43% after 42 days (Table 4.9). However, the amount of benz[*a*]anthracene and the five-ring compounds degraded was not as great; a decrease of around 7.6-10.9% in the concentration of benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were observed over the time period (Figure 4.46). Degradation lag periods in media supplemented with yeast extract were reduced for phenanthrene (7 days), fluorene, fluoranthene, pyrene, benz[*a*]anthracene and benzo[*a*]pyrene (10 days), however, no change was observed for the lag period preceding dibenz[*a,h*]anthracene degradation (Table 4.10). The rate and extent of fluorene (46.0%), phenanthrene (55.2%), fluoranthene (33.5%) and pyrene (53.6%) degradation was increased in the presence of yeast extract compared to when the PAH mixture was added as the sole carbon source (Figure 4.46). Small increases in the amounts of benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene (9.2-11.7%) degraded were also observed in yeast extract supplemented media. The amounts of

Table 4.9. Degradation of a PAH mixture by community five enriched on BSM containing creosote (2 ml/l) and yeast extract (1 g/l) (CYEM). Degradation was assessed by adding a 10% unwashed inoculum into BSM containing the PAH mixture as the sole carbon and energy source.

	Concentration (mg/l)[a]									
	Initial	After incubation for:							Killed cell	
Compound[b]	Conc.	3 days	7 days	10 days	14 days	21 days	28 days	42 days	Control (42 days)[c]	% Decrease
FLU	48.7	48.9	47.4	45.1	42.9	39.9	37.6	31.4	46.1	31.9
PHEN	51.5	51.8	50.2	46.3	41.2	37.4	32.4	28.1	49.3	43.0
FA	52.1	50.3	49.7	48.9	46.1	42.9	40.3	35.2	49.5	28.9
PYR	48.9	49.5	49.2	48.1	45.2	40.3	36.2	30.9	50.6	38.9
BA	51.3	50.9	51.3	50.2	49.3	47.9	46.8	44.1	49.5	10.9
DBA	48.6	49.3	49.1	48.3	48.9	48.3	47.3	45.0	48.7	7.6
B[a]P	49.1	48.1	47.9	48.3	48.6	47.4	47.0	44.5	48.5	8.2

[a]Data reported are averages of triplicate samples.

[b]See Table 4.6 for abbreviations.

[c]Inoculated cultures were killed with 2% $HgCl_2$.

Table 4.10. Degradation of a PAH mixture in the presence of yeast extract (1.0 g/l) by community five enriched on BSM containing creosote (2 ml/l) and yeast extract (1 g/l). Degradation was assessed by adding a 10% inoculum into BSM containing the PAH mixture.

Compound[b]	Concentration (mg/l)[a]									
	Initial Conc.	After incubation for:							Killed cell Control (42 days)[c]	% Decrease
		3 days	7 days	10 days	14 days	21 days	28 days	42 days		
FLU	48.7	48.9	49.3	47.3	41.0	34.1	28.4	24.9	46.1	46.0
PHEN	51.5	51.3	49.8	46.4	42.3	34.9	27.4	22.1	49.3	55.2
FA	52.1	50.8	50.1	51.7	46.1	40.2	36.2	32.9	49.5	33.5
PYR	48.9	49.9	50.4	50.3	44.8	37.2	31.2	23.5	50.6	53.6
BA	51.3	50.7	49.7	49.5	46.9	47.2	45.1	43.7	49.5	11.7
DBA	48.6	49.3	48.9	48.7	47.9	47.4	46.3	44.2	48.7	9.2
B[a]P	49.1	48.9	49.4	49.0	47.3	46.9	45.7	43.9	48.5	9.5

[a]Data reported are averages of triplicate samples.

[b]See Table 4.6 for abbreviations.

[c]Inoculated cultures were killed with 2% $HgCl_2$.

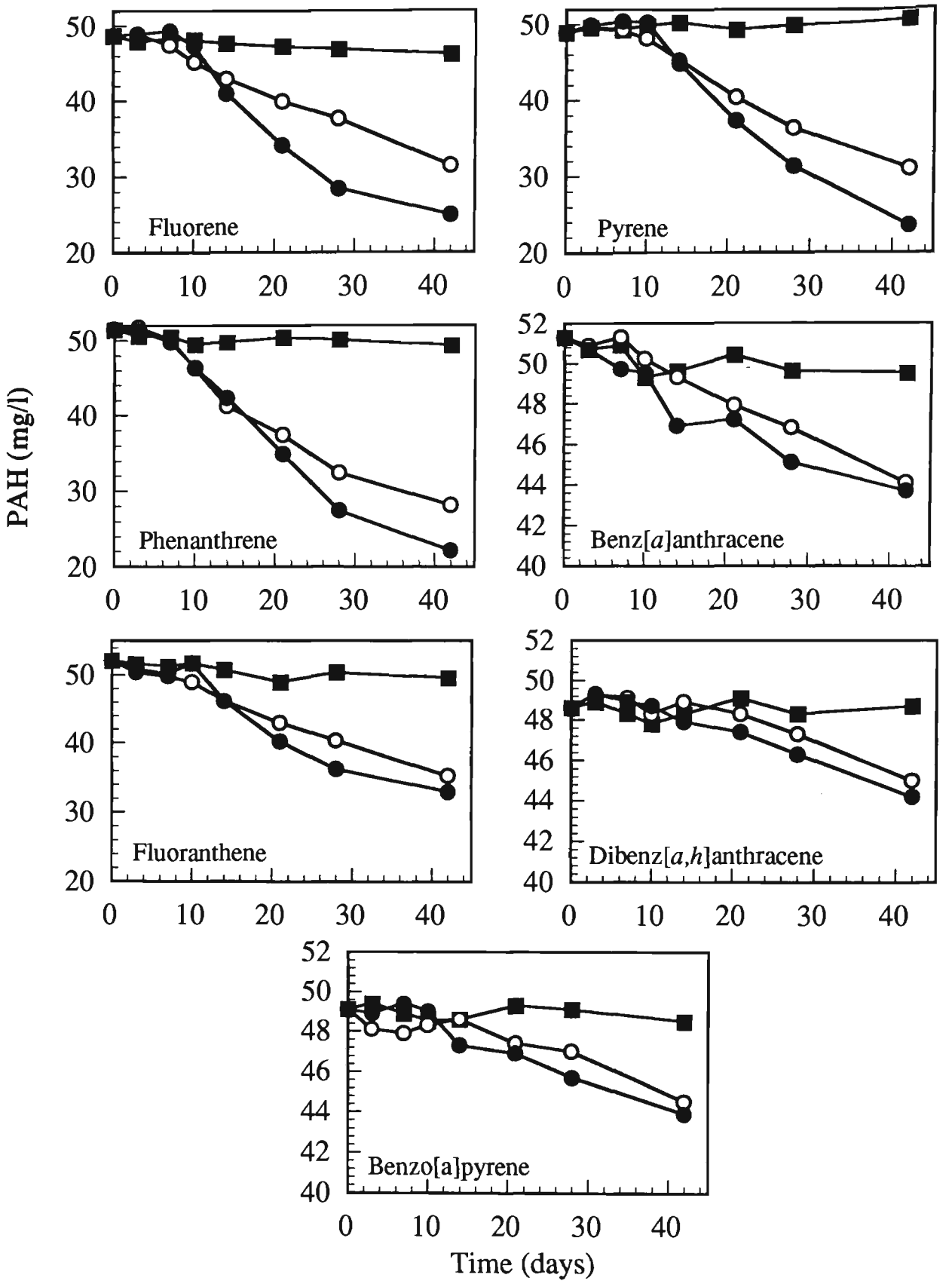


Figure 4.46. Change in PAH concentration following growth of CYEM-grown inoculum of community five in BSM containing an added PAH mixture. The panels represent the concentration profile of each PAH in cultures containing all of the indicated PAHs. CYEM-grown community five was inoculated (10%) into BSM (O) and BSM containing yeast extract (1 g/l) (●). Uninoculated controls are shown for BSM containing yeast extract (■)

individual PAHs degraded by the CYEM-grown microbial community are illustrated in Tables 4.9 and 4.10.

A lag period of 10-14 days was observed before cell biomass increased in BSM containing the PAH mixture (Figure 4.47). Protein concentrations increased from 8 µg/ml to a maximum concentration of 43 µg/ml after 42 days. When yeast extract was included, protein concentrations were approximately 2.3 times greater compared to cultures containing the PAHs as the sole carbon source. Protein concentrations increased rapidly, reaching 94 µg/ml after seven days. Protein concentrations continued to increase at a slower rate until day 21 (102 µg/ml) and remained constant until the end of the incubation period (Figure 4.47).

4.4.5.2 *Degradation of Creosote*

When creosote was added to BSM, there was a 15-20% decrease in the concentration of some creosote components in the sterile controls, presumably due to the effects of volatilisation of the lower molecular weight hydrocarbons (Table 4.11). The degradation of creosote by CYEM-grown community five resulted in a 20% (117.6 mg/l) decrease in the total hydrocarbon concentration over and above the 15-20% decrease observed in the 42 day sterile controls (Table 4.11). After a lag period of 14-21 days, small decreases in the concentration of fluorene, phenanthrene, fluoranthene and pyrene (9.0-12.9%, 1.9-4.6 mg/l)) were observed. In yeast extract supplemented media, the lag period before the degradation of the lower molecular weight hydrocarbons (compounds 1-4, fluorene, phenanthrene) was reduced to 3-7 days; a 7-10 day lag period occurred before the onset of fluoranthene and pyrene degradation. Significant decreases in the concentration of all creosote components were observed over the incubation period (Table 4.12). A 64-85% reduction in the concentration of fluorene, phenanthrene, fluoranthene and pyrene was observed after 42 days compared to the control. The lower molecular weight compounds (compounds 1-4) were degraded to a greater extent, with decreases of 86-94% being observed. A 77% decrease (452 mg/l) in the total hydrocarbon concentration was observed after 42 days.

In BSM containing creosote, protein concentrations increased slowly over the incubation period after a lag period of 10-14 days (Figure 4.47). Protein concentrations reached a maximum of 27 µg/ml after 42 days. In media supplemented with yeast extract, growth was rapid; final protein concentrations were approximately three times greater compared to cultures containing the creosote alone. Protein concentrations

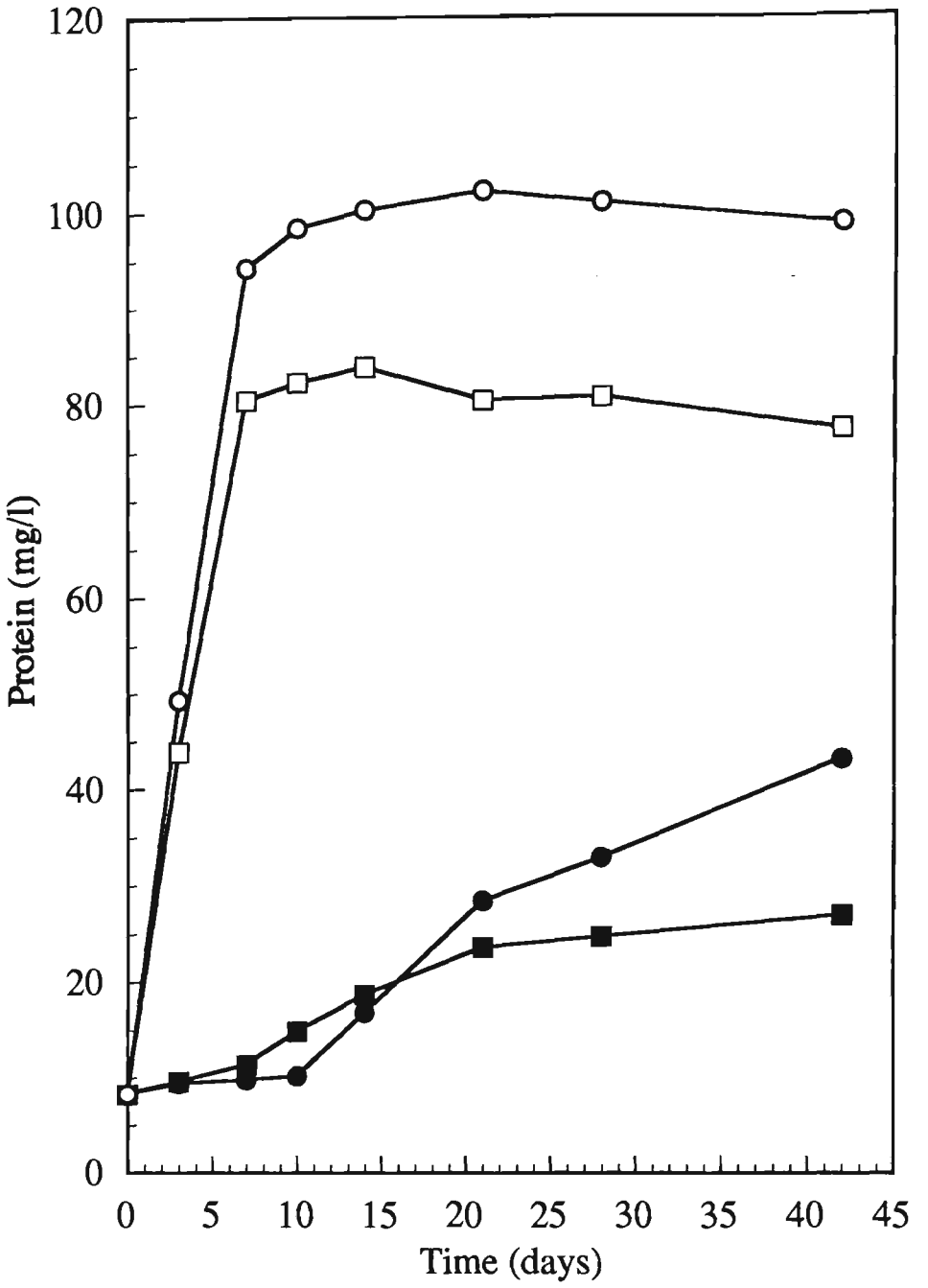


Figure 4.47. Growth of community five in BSM containing a PAH mixture (●), PAH mixture plus yeast extract (○), creosote (■) and creosote plus yeast extract (□). The inoculum for degradation experiments was 10% unwashed cells of community five grown in CYEM.

Table 4.11. Degradation of PAHs and selected creosote constituents in BSM by community five enriched on BSM containing creosote (2 ml/l) and yeast extract (1 g/l). Degradation was assessed by adding a 10% unwashed inoculum into BSM containing creosote as the sole carbon and eneregy source.

Compound	Concentration (mg/l)[a]									
	Initial Conc.	After incubation for:							Killed cell Control (42 days)[b]	% Decrease
		3 days	7 days	10 days	14 days	21 days	28 days	42 days		
1[c]	111.3	106.7	109.2	102	99.1	89.2	82.7	78.7	95.1	17.3
2[c]	23.7	24.6	22.5	22.6	20.3	18.5	17.2	16.3	20.8	21.6
3[c]	26.3	25.4	22.9	24.1	20.3	18.6	17.9	16.9	21	19.5
4[c]	18.7	19.5	19	17.9	17.2	16.3	15	14.3	16.5	13.3
FLU	20.3	20.1	21.1	19.9	19.3	17.2	16	15.3	17.2	11.0
PHEN	45.6	44.5	43.9	44.1	42.6	40.8	38.9	38.1	42.4	10.1
FA	51.3	52.2	51.4	49.9	49.7	46.9	47.1	46.5	51.1	9.0
PYR	59.9	60.2	58.7	58.2	58.9	56.2	53.7	52.7	60.5	12.9
Total HCs	709.6	712.1	688.7	674.5	643.4	572.3	512.2	466.7	584.3	20.1

[a]Data reported are averages of triplicate samples.

[b]Inoculated cultures were killed with 2% Hg Cl_2.

[c]The concentrations of unknown compounds was calculated using the phenanthrene standard curve and are expressed as phenanthrene equivalent concentrations

Table 4.12. Degradation of PAHs and selected creosote constituents in yeast extract (1.0 g/l) supplemented BSM by community five enriched on BSM containing creosote (2 ml/l) and yeast extract (1 g/l) (CYEM). Degradation was assessed by adding a 10% unwashed inoculum into BSM containing creosote.

Compound	Concentration (mg/l)[a]									
	Initial Conc.	After incubation for:							Killed cell Control (42 days)[b]	% Decrease
		3 days	7 days	10 days	14 days	21 days	28 days	42 days		
1[c]	111.3	103.4	90.3	73.4	48.3	31.2	17.9	12.4	95.1	87.0
2[c]	23.7	22.4	21.4	17.9	13.2	6.1	2.3	1.3	20.8	93.7
3[c]	26.3	25.3	22	14.2	7.9	3.2	2.6	3.2	21	84.8
4[c]	18.7	19.2	18.6	16.4	12.3	7.3	4.0	2.3	16.5	86.0
FLU	20.3	17.9	18.4	15.3	11.1	8.9	8.1	6.2	17.2	64.0
PHEN	45.6	42.3	41.6	37.2	28.9	23.5	19.2	13.2	42.4	68.9
FA	51.3	52.4	50.3	46.4	39.4	31.1	23.4	12.1	51.1	76.3
PYR	59.9	57.3	55.3	47.8	40.2	29.5	17.4	9.2	60.5	84.8
Total HCs	709.6	700.2	676.3	581.7	471.4	299.5	189.6	132.34	584.3	77.4

[a]Data reported are averages of triplicate samples.

[b]Inoculated cultures were killed with 2% $HgCl_2$.

[c]The concentrations of unknown compounds was calculated using the phenanthrene standard curve and are expressed as phenanthrene equivalent concentrations.

increased during the initial stage of incubation reaching 80 µg/ml after 7 days. Protein concentrations remained constant until the end of the incubation period (Figure 4.47).

4.5 DISCUSSION

The microbial degradation of PAHs is thought to be the major process involved in effective site bioremediation (Cerniglia, 1992). Numerous microorganisms have been isolated that degrade low molecular weight PAHs, however, the number of microorganisms that can degrade the high molecular weight PAHs, compounds containing four- or more fused benzene rings is limited. PAHs such as pyrene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene are generally resistant to microbial attack due to their low water solubilities and the resonance energy of their structures.

4.5.1 Degradation of High Molecular Weight PAHs Community five and *St. maltophilia* Isolates.

4.5.1.1 *Pyrene, Fluoranthene and Benz[a]anthracene*

All three *Stenotrophomonas* strains isolated in this work were able to degrade, and grow on, pyrene when present at concentrations up to 1,000 mg/l, demonstrating their high tolerance to this compound. However, degradation of all the pyrene present in the 500 mg/l and 1,000 mg/l cultures was not observed. Pyrene utilisation appeared to cease once approximately 400 mg/l pyrene had been catabolised. This may be due to the accumulation of growth inhibitory intermediates or end-products which reach critical concentrations in the medium once 400 mg/l of pyrene has been metabolised. The accumulation of end-products in the medium or in the cell may also inhibit the activity of enzymes involved in the formation of the products through regulation of enzyme activity or synthesis. When the product of a pathway accumulates in the cell, it may combine with a repressor protein to give an active repressor. The latter may bind to the operator region and prevents transcription of the operon (Gottshalk, 1986) and further degradation of the compound.

Gram negative microorganisms have been shown to degrade fluoranthene, benz[*a*]anthracene, chrysene, pyrene and benzo[*a*]pyrene (Mahaffey *et al.*, 1988; Gibson *et al.*, 1975; Ye *et al.*, 1996; Mueller *et al.*, 1989b, 1990b; Weissenfels *et al.*, 1991). Although Ye *et al.* (1996) described the degradation of pyrene by *Sp.*

paucimobilis strain EPA 505, the microorganism was unable to utilise the compound as a growth substrate but could utilise fluoranthene as a sole carbon and energy source. The results of degradation experiments with VUN 10,001, VUN 10,002 and VUN 10,003 are the first to demonstrate the growth of Gram negative bacteria on pyrene as the sole carbon and energy source.

The degradation of fluoranthene and benz[*a*]anthracene by *St. maltophilia* strains VUN 10,001, VUN 10,002, and VUN 10,003 was slow but substantial amounts of these PAHs were degraded in the high initial cell density experiments as the sole carbon and energy source. In previous reports, the microbial degradation of benz[*a*]anthracene has only occurred via co-metabolism (Gibson *et al.*, 1975; Mahaffey *et al.*, 1988; Walter *et al.*, 1991; Weissenfels *et al.*, 1991), despite attempts to isolate microorganisms that can degrade benz[*a*]anthracene as a sole carbon and energy source.

4.5.1.2 *Benzo[a]pyrene, Dibenz[a,h]anthracene and Coronene*

In this work, PAH utilisation in the presence of low cell densities indicated that VUN 10,002 and VUN 10,003 were capable of degrading dibenz[*a,h*]anthracene as a sole carbon and energy source, but only a small PAH concentration change was observed. Protein concentrations did not increase significantly over the incubation period, thus it appeared that the cells could not grow on the five-ring PAHs. However, larger amounts of these high molecular weight PAHs were degraded when a larger population of cells was used in the inoculum. It was also demonstrated that all three strains could degrade benzo[*a*]pyrene and the seven-ring PAH, coronene, when large numbers of pyrene-grown cells were provided in BSM. The ability of VUN 10,001, VUN 10,002 and VUN 10,003 to degrade coronene is unique. There have been no previous reports of the bacterial degradation of this compound.

It is known that some enzymes involved in PAH degradation are inducible (Heitkamp and Cerniglia, 1988), as they are synthesised only when a particular metabolite or substrate is present (Hamzah and Al-Baharna, 1994). Growth of community five and the *St. maltophilia* strains on PYEG would not allow for the consequent degradation of pyrene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene. This indicates that induction of PAH degrading ability appears to be involved with these organisms. Heitkamp *et al.* (1988a) also concluded that inducible enzymes seem responsible for pyrene catabolism by a *Mycobacterium* species. It is possible that pyrene, or metabolites produced during its degradation, induce the synthesis of enzymes necessary for benzo[*a*]pyrene, dibenz[*a,h*]anthracene and coronene catabolism. These enzymes may not be induced,

or may have long induction lag periods, when the bacteria are exposed to the high molecular weight PAH without prior exposure to another PAH.

Strains VUN 10,001, VUN 10,002 and VUN 10,003 were also able to degrade significant concentrations of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the presence of phenanthrene and pyrene when a small initial microbial population was present. Increased degradation rates of these compounds were observed compared to single substrate degradation studies.

Ye *et al.* (1996) proposed that the growth of *Sp. paucimobilis* strain EPA 505 on fluoranthene induced enzymes that were capable of degrading a variety of PAHs and the decrease in the concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene was a result of cometabolism. Growth of community five and the three *St. maltophilia* strains on phenanthrene or pyrene may induce a number of enzymes which can attack a variety of PAHs, however, the efficiency of degradation varies with the substrate, *i.e.* lower molecular weight PAHs are preferred. Because the higher molecular weight PAHs are not fully broken down, their intermediates cannot enter the carbon cycle pathways. As a consequence of this, degradation halts after a period of time. Cometabolism allows greater degradation of the five-ring compounds because phenanthrene and pyrene metabolism drives growth and energy maintenance and it therefore allows more high molecular weight PAHs to be degraded.

4.5.1.3 *By-product Inhibition of Benzo[a]pyrene and Dibenz[a,h]anthracene Degradation*

The transformation of high molecular weight PAHs has been observed by a limited number of researchers (Heitkamp and Cerniglia, 1989; Shiaris, 1989b; Grosser *et al.*, 1991; Schneider *et al.*, 1996), however, none of the work has addressed the reasons why the degradation of the five-ring compounds is limited. In this study, although the *St. maltophilia* strains were capable of degrading benzo[*a*]pyrene and dibenz[*a,h*]anthracene, degradation of these five-ring compounds ceased after approximately 10-15 mg/l of the PAHs were degraded. It was presumed that the cessation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was due to the loss of enzyme activity. Although VUN 10,001, VUN 10,002 and VUN 10,003 were unable to utilise benzo[*a*]pyrene or dibenz[*a,h*]anthracene as a growth source, prior growth of the cells on pyrene appeared to induce the synthesis of a catabolic pathway that could degrade the five-ring compounds at least to some extent. Presumably, the activity of this catabolic pathway was not perpetuated when benzo[*a*]pyrene or

dibenz[*a,h*]anthracene were the only PAHs in the cultures or inhibitory intermediates or products formed which limited further activity.

To test this hypothesis, an experiment was conducted in which pyrene was spiked into cultures containing the five-ring PAHs when their degradation had ceased (after 63 days). The reasoning behind the pyrene addition was that supplementation of the medium with a growth supporting PAH may stimulate the continuation of degradation of the five-ring PAHs. The failure of pyrene-spiking to restart benzo[*a*]pyrene or dibenz[*a,h*]anthracene degradation suggests that other factors apart from enzyme induction may be responsible for the inability of the strains to degrade benzo[*a*]pyrene and dibenz[*a,h*]anthracene significantly when they are supplied as the sole carbon and energy source.

One possibility was that the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by VUN 10,001, VUN 10,002 and VUN 10,003 may be affected by a minimum threshold PAH concentration, below which degradation does not proceed. Spain *et al.* (1980) observed a threshold concentration for *p*-nitrophenol, below which adaptation and subsequent degradation of the compound did not occur. The effect of concentration of organic compounds on their biodegradation by natural microbial communities was also investigated by Boethling and Alexander (1979a). Little mineralisation of 2,4-dichlorophenoxyacetate, 1-naphthyl-*N*-methylcarbamate or 1-naphthol-*N*-methylcarbamate occurred when these compounds were present at initial concentrations of 2-3 µg/l or less. However, when the compounds were supplied at higher concentrations, 60% or more of the compounds were converted to CO_2. This was found not to be the case in cultures containing the five-ring PAHs and VUN 10,003 since the initial concentration of the five-ring compounds in the culture medium (at the concentrations tested) did not affect the total amount of benzo[*a*]pyrene or dibenz[*a,h*]anthracene degraded.

Metabolite or by-product repression was found to be responsible for the inhibition of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. Metabolites or by-products produced from the degradation of the five-ring compounds inhibited their further degradation. Interestingly, benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was not inhibited by the presence of pyrene metabolites present in spent pyrene medium. This indicates that the inhibition of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation stems only from products specific to their degradation. The production and accumulation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites or by-products may be inhibitory to the cells or the metabolites may repress enzyme activity which

prevents further degradation of the compounds. The concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites appears to accumulate to high enough levels to repress enzyme activity after 10-15 mg/l of the five-ring compounds had been degraded. Cells that were previously exposed to benzo[*a*]pyrene or dibenz[*a,h*]anthracene and their metabolites were able to degrade the five-ring compounds in the presence of pyrene when transferred to fresh medium. This data indicates that the cells are still metabolically active and capable of degrading benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the absence of the metabolites and therefore loss of plasmid/genetic ability did not occur. The lack of literature regarding the mechanisms and pathways involved in the degradation of the five-ring compounds makes it difficult to interpret which by-product of their catabolism limits the amount degraded to 10-15 mg/l for VUN 10,001, VUN 10,002 and VUN 10,003.

4.5.1.4 *Degradation of Synthetic PAH Mixtures*

To be effective in site decontamination, microbial populations must possess the ability to degrade all PAH compounds present in complex mixtures. All three *St. maltophilia* strains were able to simultaneously degrade all the PAHs used in this work when present as a substrate mixture. Furthermore, improved degradation of the five- and seven-ring PAH compounds was observed in the presence of lower molecular weight PAHs. This was reflected by a decrease in the degradation lag period of around 50% and an increase in the degradation rate per mg of protein over a 42 day incubation period. The amounts degraded per mg of protein (for VUN 10,003) in the PAH mixture were 280% greater for benzo[*a*]pyrene and dibenz[*a,h*]anthracene, and 33% greater for coronene compared to single substrate experiments. The improved degradation of the high molecular weight PAHs was probably a result of increased metabolic activity due to the presence of the more easily degradable low molecular weight PAHs. In an earlier study using PAH mixtures, the sequential removal of PAHs by a bacterial community was observed during the biotransformation of a PAH mixture (Mueller *et al.*, 1989b). The authors found that the high molecular weight PAHs (fluoranthene and pyrene) were utilised only after degradation of the more labile compounds. It was interesting in the present study that there was concurrent degradation of the PAHs after a short lag period of 10 days. The degradation of coronene and phenanthrene occurred simultaneously, indicating that for the *St. malotphilia* isolates the catabolic pathways for these compounds are not repressed by the presence of more readily metabolised PAH compounds.

It has recently been suggested (Kastner *et al.*, 1994) that nocardioform bacteria (*e.g. Rhodococcus*, *Nocardia*, *Mycobacterium* and *Gordona*) may play a crucial role in the degradation of high molecular weight PAHs in soils. This conclusion was based on results where the authors screened for pyrene-degrading bacteria in soil samples and found nocardioforms dominant. Furthermore, there are few reports of bacteria capable of growing on high molecular weight PAHs which are outside the nocardioform/actinomycetes group. While these results do not challenge the importance of nocardioform bacteria in the degradation of high molecular weight PAHs, the degradation of benz[*a*]anthracene, dibenz[*a,h*]anthracene and rapid degradation of pyrene by *St. maltophilia* in this work indicates that the microbial degradation of high molecular weight PAHs is not restricted to the actinomycetes group.

4.5.2 Detoxification of PAHs in Liquid Culture

Few studies have focussed on the contribution of individual microorganisms in reducing the toxicity of PAH compounds: the majority of research has investigated the microbial detoxification of complex waste mixtures (Aprill *et al.*, 1990; Symons and Sims, 1988). While this provides information on whether natural processes can reduce the toxicity of wastes in soil, it does not provide an understanding of how the waste is detoxified. PAH degradation by organisms with known catabolic pathways could lead to better predictions of the toxicological fate of intermediate metabolites or by-products produced from the degradation of the parent compound. The degradation of PAHs by the pyrene-enriched microorganisms in a defined environment (liquid medium) provided an opportunity to test whether the reduction in the concentration of the PAHs corresponded to a decrease in the toxicity and mutagenicity of culture supernatant and extracts. By testing culture supernatants, the toxicity of polar intermediate compounds produced from the degradation of the PAH was assessed. These assays can provide useful information on whether the isolated microorganisms have the potential to be applied to the detoxification of PAH-contaminated soil.

4.5.2.1 *Toxicity*

The pyrene-enriched microorganisms significantly degraded the high molecular weight PAHs when supplied alone or as part of a PAH mixture when sufficient numbers of cells were supplied. In addition, relatively low quantities of water soluble metabolites were detected in culture supernatants. The formation and accumulation of intermediate products may increase the mutagenicity and toxicity of the PAH water-soluble fraction,

as PAH metabolites may be more toxic than the parent compound. Consequently, PAH intermediate products may pose a greater risk to human health due to the increased mobility of the toxicants.

In many cases the biological degradation of PAHs results in a large percentage of the target compound being converted to polar products. Pothuluri *et al.* (1992a) demonstrated that 76% of the ^{14}C label was found in metabolites during the degradation of fluoranthene by *C. elegans*. After 14 hours incubation, 56% of added benz[*a*]anthracene was converted to a mixture of three isomeric *o*-hydroxypolyaromatic acids by *Beijernickia* strain B1 (Mahaffey *et al.*, 1988). The mammalian degradation of high molecular weight PAHs has been shown to be incomplete, resulting in a variety of phenolic, dihydrodiol and epoxide metabolites. Numerous studies have demonstrated the activation of PAHs to mutagens by mammalian metabolism or degradation. Several known phenolic and dihydrodiol metabolites of benzo[*a*]pyrene are metabolised to products that are mutagenic to the Ames *S. typhimurium* strains. Wood *et al.* (1976) demonstrated that when benzo[*a*]pyrene was replaced by *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene as a substrate, the number of mutations generated per nmol of hemoprotein was three- to four-times higher for the cytochrome P448 dependent monooxygenase system. In addition, photodegradation of PAHs has been shown to produce a number of mutagenic nitro-PAH compounds and nitro-PAH lactones. Sasaki *et al.* (1995) reacted a number of two- to four-ring PAHs under simulated atmospheric conditions in an environmental chamber. The reactant mixtures were collected and the mutagenic activity of the resulting products was shown using a microsuspension modification of the Ames test.

No increase in the supernatant toxicity of pyrene, fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, coronene, PPDB or FC cultures was observed after incubation of these compounds with community five, VUN 10,002 and VUN 10,003 using the Microtox™ test. The formation and accumulation of low quantities of RE intermediates did not increase the toxicity of culture supernatants. Similar findings were observed by Dasappa and Loehr (1991) for degradation of phenolic and chlorinated phenolic compounds: by-products did not increase the toxicity of the water soluble fraction of the contaminated soil. Their research demonstrated the detoxification of soil contaminated with phenolic and chlorinated phenolic compounds (phenol, 2-, 3-, 4-chlorophenol, 2,3-, 2,4-, 2,6-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol) after incubation for 30 days.

Symons and Sims (1988) also observed that the degradation of individual compounds from petroleum refining waste correlated with the decrease in Microtox™ toxicity of soil leachates and aqueous soil extracts. The trend was consistent in soil columns and batch reactors with the exception of the 8% loaded batch reactor where a decrease in the PAH concentration did not correlate with a decrease in Microtox™ toxicity. This indicated that the toxicity of the water soluble fraction remained relatively unchanged.

Microtox™ results from degradation studies with benz[*a*]anthracene demonstrated a slight increase in the toxicity of culture supernatants after 14 days incubation. The change in the toxicity of culture supernatants corresponded to the change in the concentration of phenolic compounds that accumulated in the medium. The accumulation of polar metabolites has been suspected to be responsible for the increase in the toxicity of soil leachates and aqueous soil extract from petroleum waste/creosote contaminated soils (Aprill *et al.*, 1990; Baud-Grasset *et al.*, 1993). Although there was a 29% decrease in the concentration of the petroleum separator sludge waste over the incubation period, an increase in the toxicity of the water soluble fraction of the separator sludge amended soil was observed by Aprill and co-workers (1990). Similar results were observed by Baud-Grasset *et al.* (1993) where a 49% decrease in the concentration of PAHs occurred due to degradation by the indigenous microbial population, however, an increase in the genotoxicity of aqueous soil extracts was observed.

The RE intermediate assay provides a quantitative analysis of the concentration of phenolic compounds in the culture supernatants, however, it does not provide qualitative information regarding the composition of the phenolic compounds. It is reasonable to assume that the concentration of resorcinol equivalent intermediates during the initial stages of an incubation may reflect a different class of compounds compared to those present at the end of the incubation period. Utilisation of PAH intermediate compounds may not result in the mineralisation of the parent compound, however, it may affect the toxicity of the water soluble fraction.

4.5.2.2 *Mutagenicity*

Although an increase in the toxicity of some culture supernatants was observed during the degradation of PAHs with community five, VUN 10,002 and VUN 10,003, the production and accumulation of resorcinol equivalent intermediates did not result in a mutagenic response from the supernatant samples. These findings are contrary to those of Donnelly *et al.* (1987), who observed that the degradation of wood preserving waste

produced a significant increase in both the total and direct acting mutagenicity of soil extracts. Their results indicated that microbial, chemical or photochemical transformations of the waste constituents resulted in intermediate compounds with mutagenic activities greater than the parent compounds.

The *Salmonella* reversion assay demonstrated a decrease in the mutagenicity of total culture extracts of community five, VUN 10,002 and VUN 10,003 with PAHs over the incubation period. The decrease in mutagenicity corresponded with the decrease in the concentration of the PAHs. Assays conducted without the addition of the rat liver microsomal preparation (S9) showed no dose-related response for TA100. However, mammalian metabolic activation of the PAHs with the S9 preparation demonstrated a dose-related response for individual PAHs and PAH mixtures. Ames tests conducted with culture supernatants showed no mutagenic response with or without the addition of the S9 fraction, indicating that the degradation of PAHs by community five, VUN 10,002 and VUN 10,003 resulted in a reduction in the mutagenicity of the culture.

A reduction in the mutagenicity of individual PAH compounds and complex PAH mixtures as a result of biodegradation has also been observed by Pothuluri *et al.* (1992) and Aprill *et al.* (1990). In both cases, the mutagenicity of culture and soil extracts was shown to decrease with time, which corresponded with the decrease in PAH concentration. Although the metabolism of fluoranthene by *C. elegans* resulted in the formation of fluoranthene *trans*-2,3-dihydrodiol, a previously identified rat liver microsome proximal mutagenic metabolite of fluoranthene, the circular dichoism spectrum of the metabolite showed no optical activity. Aprill *et al.* (1990) accounted for the significant decrease in the mutagenic potential of the contaminated soil by the degradation of the carcinogenic and co-carcinogenic PAHs (fluoranthene, pyrene, benz[*a*]anthracene and chrysene). The ability of the pyrene-enriched microorganisms in this study to reduce the mutagenic potential of individual PAHs as well as PAH mixtures indicates the potential of the microorganisms to be used for the bioremediation of PAH-contaminated sites (although such studies were outside the scope of this thesis).

4.5.3 Effect of Enrichment Substrate on PAH Degradation

One of the major factors to be considered when assessing whether bioaugmentation is feasible for the bioremediation of a contaminated site is the economics of providing large amounts of biomass. In the degradation experiments performed in this chapter, pyrene-grown microorganisms were used as the inoculum. Large-scale fermentations of these microorganisms on a pyrene substrate for the production of biomass for

bioaugmentation would not be economical, as pyrene is expensive and does not stimulate high biomass production as a growth substrate.

Community five was selected as the inoculum to study the effect of enrichment substrate on PAH-degrading performance. The microbial consortium was selected in preference to one of the *St. maltophilia* strains as the development of the enrichment medium was geared towards the application of bioaugmentation of PAH-polluted soil. Microbial communities are generally more versatile in their metabolic capabilities, less fastidious and offer greater genetic stability than pure cultures for this application.

The enrichment of community five on peptone, yeast extract, glucose and succinate resulted in rapid growth and high yields of biomass of the community, however, when transferred to pyrene containing medium, the enriched microorganisms were unable to degrade pyrene. Enzyme activity is commonly regulated by the concentration of the reaction product. In the catabolic system of PAH degradation, substrate concentration often determines whether its catabolic enzymes are synthesised. Non-aromatic substrates such as peptone, yeast extract, glucose and succinate were unable to induce the enzymes responsible for PAH degradation of community five or allowed non-PAH degrading microorganisms of the community to outgrow the degraders. Although salicylate has been shown to induce the enzymes for naphthalene oxidation (Barnsley, 1975a) in *P. putida*, it was unable to induce the PAH degradative enzymes of the microbial community. Catabolite repression of the degradation of aromatic compounds by succinate has been reported previously in other bacterial species (Rohm and Werner, 1985). Rotert *et al.* (1995) examined the enhancement of benzene, toluene and xylene (BTX) biodegradation by indigenous microorganism from pristine soil by the addition of aromatic substrates. The addition of benzoate or phenylalanine (1 mg/l) exerted preferential selection for BTX degraders; the concentration of BTX degraders increased two orders of magnitude in supplemented microcosms. However, the addition of a non-aromatic substrate, acetate, did not stimulate an increase in the concentration of BTX degraders compared to the non-supplemented soil. Rotert *et al.* (1995) proposed that the aromatic nature of benzoate and phenylalanine provided a competitive advantage for the proliferation of BTX degraders.

Although community five was shown to be capable of utilising a variety of PAHs as well as mono-, nitro- and chlorinated-aromatic compounds as growth substrates (see Chapter 3), creosote as a sole carbon and energy source did not stimulate good growth even though many of creosote's components are readily degraded by the community. When yeast extract, peptone or glucose were added to the creosote medium, growth was

evident, however, protein concentrations were up to four-times less compared to enrichments when these substrates were supplied as the sole carbon source. Clearly, some components in the creosote mixture exhibit inhibitory or toxic effects towards community five, thus limiting growth. This is not surprising as creosote oils have been used industrially as wood preserving agents for almost 150 years (Bos *et al.*, 1984). Although community five grew poorly on creosote, creosote itself acted as an inducer for the synthesis of PAH-degrading enzymes after growth was achieved on an alternative substrate. Creosote is composed of a number of PAHs and structurally related compounds which in this case have a similar enzyme inducing effect on community five as pyrene. Kuhm *et al.* (1991) demonstrated that structurally related compounds could induce the synthesis of enzymes reponsible for naphthalene catabolism. *P. paucimobilis*, after growth with biphenyl or naphthalene, synthesised the same enzyme for the ring cleavage of 2,3-dihydroxybiphenyl or 2,3-dihydroxynaphthalene.

Enzyme induction is commonly regulated by the concentration of the reaction product. The extent of adaptation achieved by a microbial community may be reflected by the pre-exposure concentration of the substrate. Creosote at low concentrations (0.02-0.0002 ml/l) in the presence of yeast extract (1.0 g/l) had little effect on inducing the enzymes responsible for pyrene oxidation. However, at creosote concentrations of 0.2 and 2.0 ml/l, enzyme induction occurred as demonstrated by the subsequent degradation of pyrene by the enriched microorganisms. The adaptation of community five and its subsequent ability to degrade pyrene was dependent on the concentration of creosote in the enrichment medium. Below a creosote threshold concentration (0.2 ml/l) induction and synthesis of enzymes for PAH degradation did not occur. Spain and Van Veld (1983) also observed a difference in the adaptation of a microbial community to *p*-nitrophenol when different concentrations of the test compound were used for pre-exposure. A threshold concentration existed below which no detectable adaptation of the microbial community was observed. Active *p*-nitrophenol degrading microbial communities were obtained by exposing the microorganisms to *p*-nitrophenol above the threshold concentration. Higher pre-exposure concentration caused only slight increases in the degradation rates of *p*-nitrophenol. Although community five was able to degrade pyrene after enrichment on yeast extract and 0.2 ml/l creosote, the rate and extent of pyrene degradation was significantly less than microorganisms pre-exposed to yeast extract and 2.0 ml/l creosote.

Varying the concentration of yeast extract in the creosote enrichment medium also affected the rate and extent of pyrene degradation by yeast extract plus creosote-grown

community five. Supplying higher concentrations of yeast extract in the medium resulted in higher biomass yields, however, the biomass yields did not result in higher degradation rates. At a yeast extract concentration of 2.0 g/l, biomass yields reached 110 mg/l protein after 96 hours compared to 65 mg/l protein for the 1.0 g/l yeast extract enrichment culture. However, the pyrene degradation rate was substantially greater for microorganisms enriched on creosote and 1.0 g/l yeast extract compared to the higher yeast extract concentration; pyrene was degraded to undetectable levels after 10-14 days compared to 21 days. The decreased pyrene degradation rates by microorganisms enriched on the highest yeast extract concentration may be due to repressed enzyme induction due to the presence of high concentrations of non-inducing growth substrates which were preferentially used for growth.

The aims of the enrichment experiments were to formulate an enrichment medium which was relatively inexpensive, that promoted rapid growth, high biomass production and microorganisms with PAH-degrading capabilities. These aims were achieved with the enrichment of community five in BSM containing creosote (2.0 ml/l) and yeast extract (1.0 g/l) (CYEM). Growth of community five in CYEM resulted in a 40% increase in biomass yield after 120 hours compared to pyrene-containing medium. In addition, the pyrene degradation rate by inocula grown on CYEM was comparable to the pyrene degradation rate of pyrene-enriched microorganisms.

Having optimised the creosote medium used to grow community five inocula for PAH degradation, it was decided to use this medium to grow inocula for the degradation of creosote and a synthetic PAH mixture. The addition of yeast extract to cultures containing CYEM-grown community five and creosote resulted in a three-fold increase in biomass compared to cultures containing the creosote alone. Although community five, enriched on pyrene, was unable to utilise creosote as a growth substrate, the CYEM-enriched community five exhibited some growth on creosote when supplied as the sole carbon source. The prior exposure of community five to creosote in the enrichment medium resulted in the acclimation of the microorganisms to some of the growth inhibitory components of creosote. This is an additional benefit of using creosote as a growth substrate for inocula to be used for PAH degradation. The increase in microbial numbers in cultures containing yeast extract was proportional to the increase in the amount of the creosote degraded; after 42 days incubation a 285% increase in creosote degradation was observed in cultures supplemented with yeast extract. The growth of community five on the yeast extract resulted in an increase in the microbial population which was capable of degrading the creosote components. As

a consequence, the amount of creosote degraded in the presence of the yeast extract increased.

An increase in the amount of PAHs degraded in a synthetic PAH mixture by community five was observed in media supplemented with yeast extract compared to non-supplemented cultures. This was not surprising given that the biomass concentrations in the yeast extract-supplemented cultures were 2.5 times greater than in cultures without yeast extract. However, the increase in PAH degradation was not as great as the observed increase when creosote was supplemented with yeast extract. Although the addition of yeast extract stimulated the growth of CYEM-grown community five in the presence of the synthetic PAH mixture, the utilisation of yeast extract may inhibit the degradation of PAHs due to the preferential utilisation of the yeast extract. However, increased PAH degradation rates were still observed due to the large increase in biomass. On the other hand, the increased PAH degradation rate when yeast extract was supplemented into creosote-containing medium may occur due to the cometabolism of creosote components when the CYEM-grown cells are utilising yeast extract components as carbon sources.

The total amount of PAHs degraded was 29% greater in cultures containing yeast extract compared to cultures containing the PAH mixture alone. However, the increase in the degradation of PAHs was not evenly distributed among the individual components of the PAH mixture. A greater increase in the degradation of the lower molecular weight compounds (fluorene, phenanthrene and pyrene) was observed in the presence of yeast extract compared to fluoranthene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene, illustrating the resistance of the higher molecular weight PAHs to microbial attack.

4.5.4 Conclusion

Research reported in this chapter demonstrated the degradation of high molecular weight PAHs alone and in PAH mixtures by community five and the *St. maltophilia* strains. The decrease in the concentration of these compounds resulted in a reduction in the mutagenic potential of total culture extracts over the incubation period. Degradation also resulted in the production of RE intermediate compounds. Information regarding the regulation of PAH metabolism, *i.e.* induction of PAH-degrading ability, concurrent degradation of PAHs, cometabolism of high molecular compounds and the inhibition of high molecular weight PAH degradation as a result of by-product accumulation was also described. The following chapter reports the PAH

degradative potential of community five in a soil matrix and these results are compared with data reported here in liquid culture.

CHAPTER 5

MICROBIAL DEGRADATION OF PAHs IN SOIL

CHAPTER 5

MICROBIAL DEGRADATION OF PAHs IN SOIL

5.1 INTRODUCTION

A majority of research conducted into microbial PAH degradation has involved liquid cultivations which cannot accurately predict the performance of the microorganisms when inoculated into a soil environment. This is primarily due to the differences between the two medium types in parameters such as nutrient type, availability, the presence of other organics, oxygen diffusion rates, temperature profiles, pH, moisture content, the extent of mixing and the sorption of pollutants to soil particles rendering them less biologically available (Mueller *et al.*, 1991a; Banerjee *et al.*, 1995). This dearth of soil-based research into PAH degradation provides a poor framework for understanding interactions between incoming microbes, soil components and indigenous microflora, where an understanding of such interactions may be crucial in developing successful bioremediation strategies. Previous examples of bioaugmentation of contaminated sites have met with limited success for reasons including die-off of laboratory-adapted strains, limited substrate availability and the inability of inocula to compete with indigenous microflora.

Park *et al.* (1990) studied the transformation of two-, three-, four-, five- and six-ring PAH compounds in soil systems. PAH degradation by the indigenous microflora was observed after the addition of the PAHs to Kidman and McLaurin sandy loam. Degradation of the two- and three-ring compounds was observed, however, no significant loss of PAH compounds containing more than three-rings was observed. Park *et al.* (1990) estimated that the transformation half lives were approximately two days for the two-ring compound (naphthalene and 1-methylnaphthalene), 59 days for the three-ring compounds (anthracene and phenanthrene) and more than 300 days for compounds containing more than three-rings. Aprill *et al.* (1990) also observed the limited degradation of the high molecular weight PAHs from wood preserving and petroleum refining wastes in soil. Degradation of the low molecular weight PAHs (naphthalene, methylnaphthalene, dimethylnaphthalene, trimethylnaphthalene, fluorene, phenanthrene and anthracene) was substantially greater than the high molecular weight PAHs (fluoranthene, pyrene, benz[*a*]anthracene and chrysene), however, there was no detectable degradation of the five-ring PAHs, benzo[*a*]pyrene and dibenz[*a,h*]anthracene.

The bioaugmentation of microorganisms into PAH-contaminated soil has to contend with problems associated with the die off of augmented organisms and competition with the indigenous microflora. Although Trzesicka-Mlynars and Ward (1996) observed a decrease in the concentration of fluoranthene in soil after the addition of fluoranthene-degrading microorganisms, microbial die off over the nine week period resulted in a drop in numbers from 2.1 x 10^7 cfu/g soil to 7.3 x 10^4 cfu/g soil. To sustain PAH degradation, it may be necessary to seed microorganisms routinely into soil or augment adapted microorganisms prepared for site specific bioremediation.

In moving towards the practical application of bioaugmentation for PAH-polluted soil, the purpose of the research reported in this chapter was to assess the performance of community five in degrading high molecular weight PAH compounds in soil. More specifically, this research:

1. determined the PAH-degrading ability of pyrene-enriched community five in soil spiked with PAHs.

2. determined the PAH-degrading ability of CYEM-enriched community five in PAH-contaminated soil.

3. investigated whether bioaugmentation of community five into PAH-contaminated soil results in the detoxification of the soil.

5.2 DEGRADATION OF PAHs IN SPIKED SOIL

The experiments described in Chapter 4 demonstrated the ability of community five and the *St. maltophilia* isolates from this community to degrade a range of PAHs including five- and seven-ring compounds in liquid medium. The next step in the development of a bioremediation process is to determine the performance of the microorganisms in a soil environment. Characterising the performance of PAH-degraders at least in ideal soil matrices prior to field trials may help reduce the incidence of poor *in situ* bioremediation. Community five was selected instead of one of the *St. maltophilia* strains because microbial communities are often more versatile in their metabolic capabilities, offer greater genetic stability and are less fastidious than pure cultures. Community five was inoculated into uncontaminated soil spiked with PAHs. The uncontaminated soil for bioaugmentation studies was obtained from St Albans, Melbourne. The soils were sieved (2 mm mesh) and the pH was adjusted from 4.7 to

Table 5.1. Physical and chemical characteristics of soils used in the PAH-spiking experiments.

Parameter	Value
Physical Characteristics:	
Texture	Fine Sandy Loam
% Sand	93
% Clay	11.6
% Silt	<0.5
Chemical Characteristics:	
pH	6.7
% Carbon	7.4
% Nitrogen	<0.1
Calcuim, ppm	23,000
Magnesium, ppm	2,800
Phosphorus, ppm	340
Potassium, ppm	780
Sodium, ppm	590
Manganese, ppm	150
Iron, ppm	2,000
Aluminium, ppm	7,500
Cadmium, ppm	<1
Copper, ppm	11
Lead, ppm	8
Nickle, ppm	19
Zinc, ppm	32
Cation exchange capacity	29

6.7 by the addition of garden lime. The physical and chemical characteristics of the soil were analysed by the National Analytical Laboratories Pty, Ltd, Melbourne (Table 5.1). The soil was a sandy loam containing a small percentage of clay (11.6%). Low concentrations of heavy metals and nitrogen were present in the soil, however, no extractable PAHs were detected. PAHs were added to the soils by dissolving the compounds in dichloromethane, stirring the soil/solvent slurry to effect even distribution of the PAHs and letting the solvent evaporate before inoculation with pyrene-grown community five.

5.2.1 Individual PAHs

Initial experiments to test the degradation of PAHs by community five in uncontaminated soils were performed using sterile or unsterile soils spiked with phenanthrene or pyrene (100 mg/kg soil). Community five was grown in BSM containing pyrene (250 mg/l). After the complete degradation of pyrene (7-10 days), cells were harvested by centrifugation, washed and resuspended in double-strength BSM to achieve a 10-fold concentration in biomass. Aliquots of the cell suspension were added to the PAH-spiked soils. The controls consisted of spiked sterile and unsterile soils without inoculation with community five and inoculated soil without the addition of PAHs.

Greater than 85% of the added phenanthrene and pyrene was recovered from the control soil cultures after 42 days incubation (Figure 5.1 and 5.2) Presumably, the decrease in extractable PAHs during the incubation time arose due to abiotic losses caused by adsorption of phenanthrene or pyrene to soil particles and photolytic decomposition. In contrast, in soils inoculated with community five, phenanthrene (Figure 5.1) and pyrene (Figure 5.2) were degraded to undetectable levels after 21 days; similar degradation rates were observed for each PAH added to sterile and unsterile soil.

The degradation of these compounds by community five corresponded with an increase in microbial numbers: after 21 days, microbial numbers had increased from the initial 9.5×10^5 cells/g soil to 4.5×10^7 cells/g soil in the presence of phenanthrene or pyrene (Figure 5.1 and 5.2). Microbial numbers then remained constant until the end of the incubation period (42 days). When community five was inoculated into soils lacking PAH supplementation, there was a slight increase in microbial numbers (Figure 5.1), presumably due to inocula growth on the organic material in the soil. The indigenous soil microbial population was low (5×10^3 cells/g soil) (Figure 5.1), which was probably due to soil pretreatment (drying), PAH addition to the unconditioned

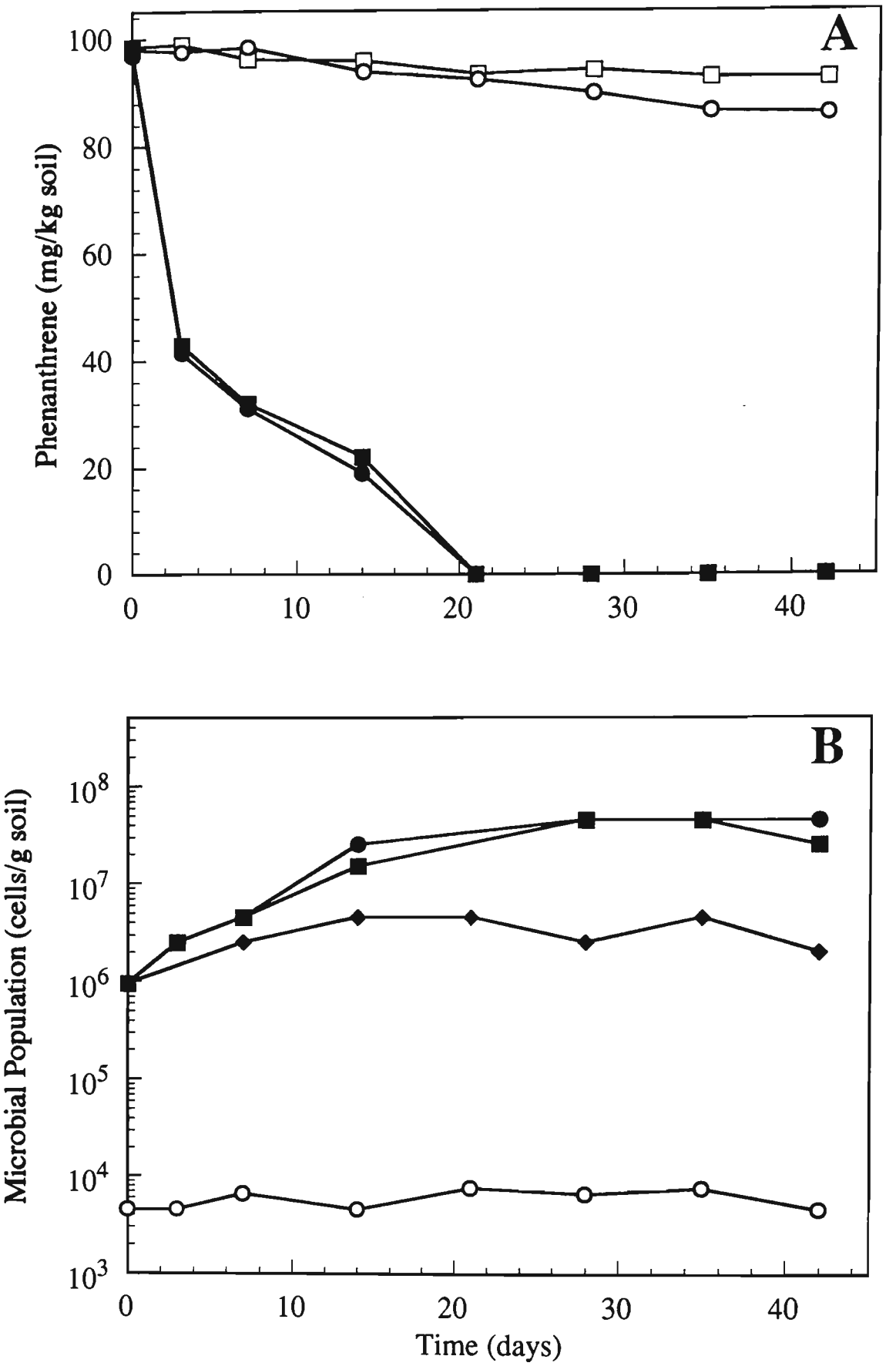


Figure 5.1. Time course for phenanthrene concentration (A) and microbial population (B) in soil spiked with phenanthrene. Pyrene-grown community five was inoculated into sterile (■) and unsterile soil (●). Uninoculated controls are shown for sterile (□) and unsterile (○) soil. Inoculated sterile soil without added phenanthrene (◆) is also shown.

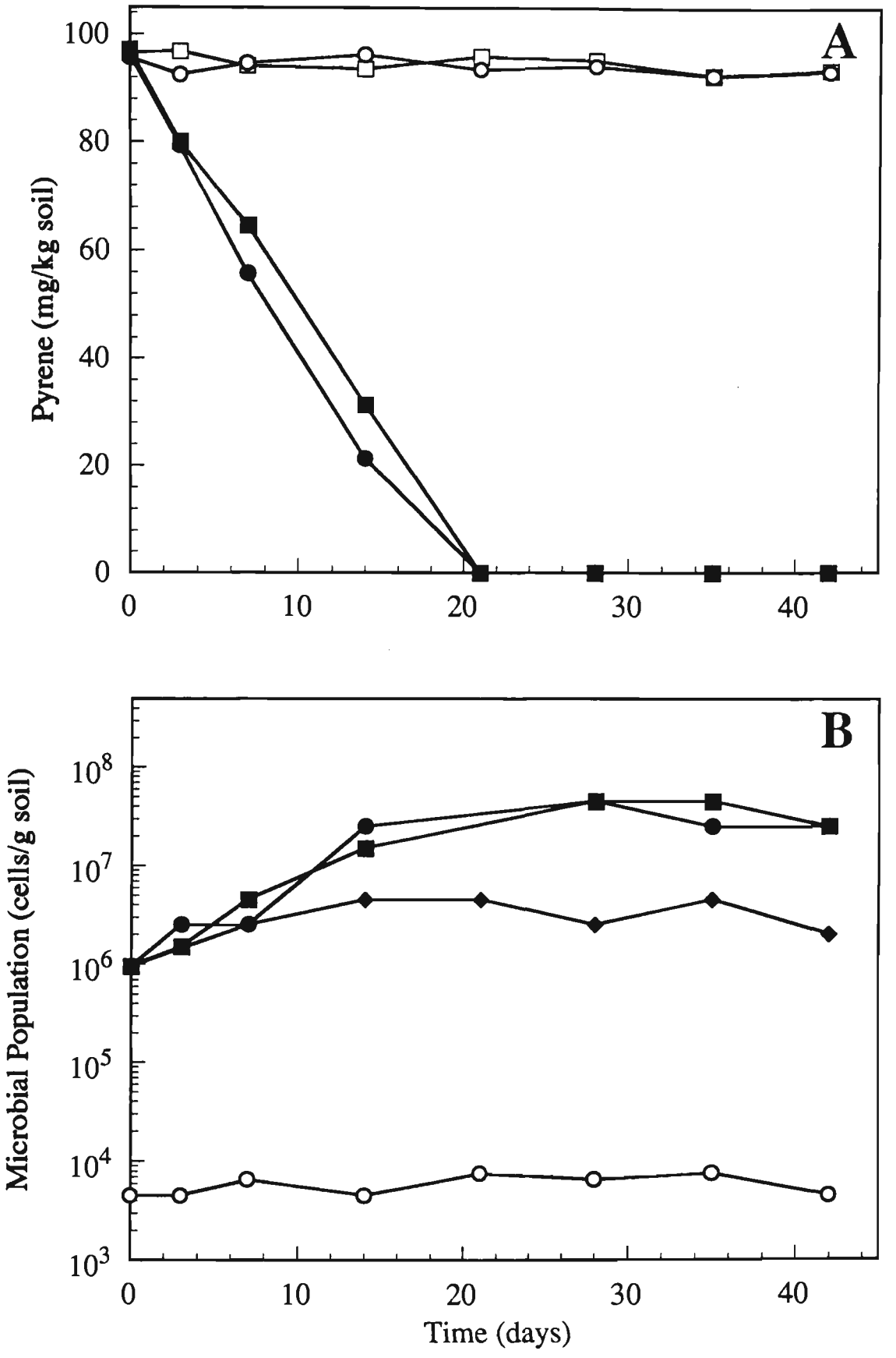


Figure 5.2. Time course for pyrene concentration (A) and microbial population (B) in soil spiked with pyrene. Pyrene-grown Community five was inoculated into sterile (■) and unsterile soil (●). Uninoculated controls are shown for sterile (□) and unsterile (○) soil. Inoculated sterile soil without added pyrene (◆) is also shown.

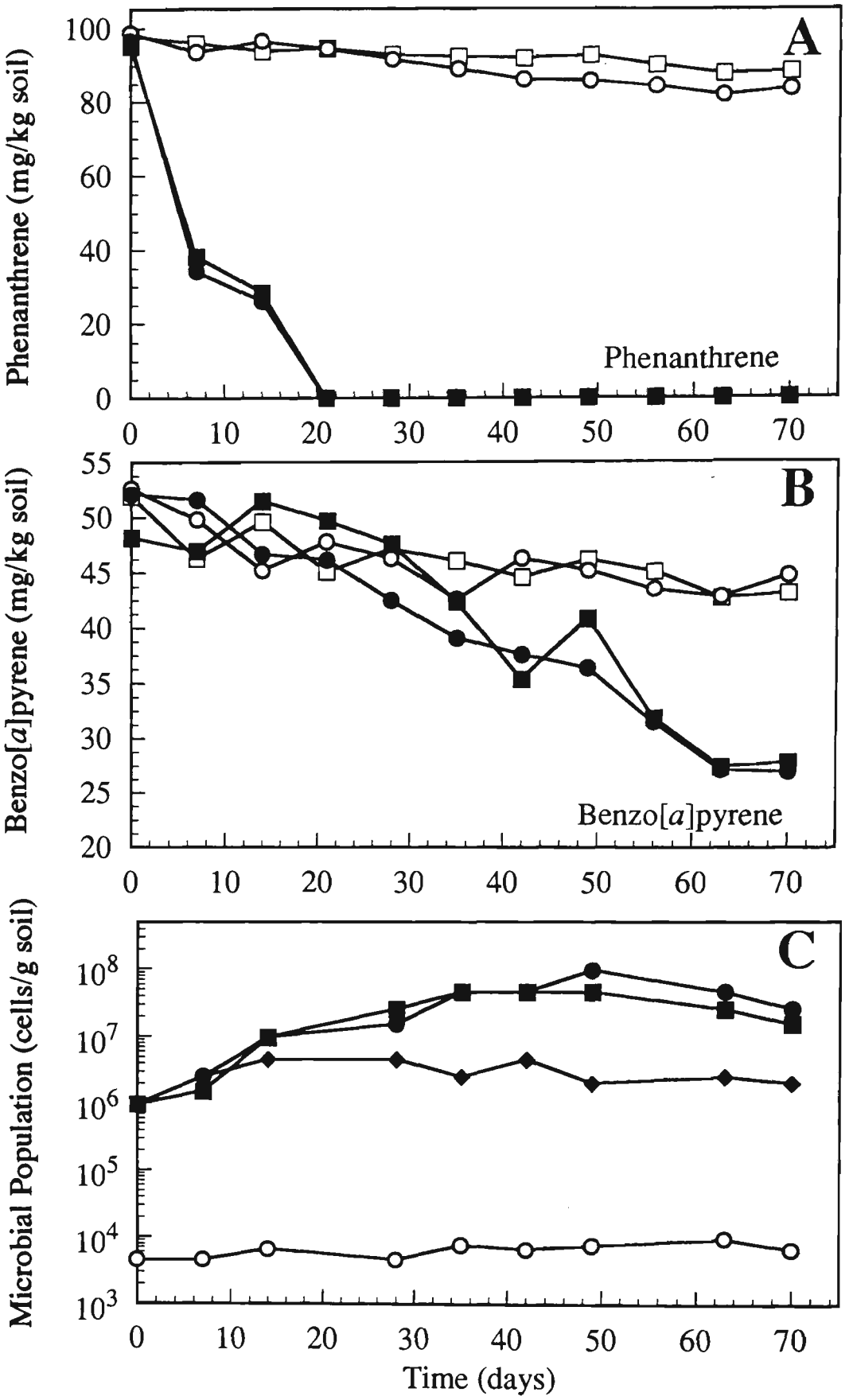


Figure 5.3. PAH concentration (A and B) and microbial numbers (C) in soil spiked with phenanthrene (A) and benzo[*a*]pyrene (B) inoculated with pyrene-grown community five. The panels represent the concentration profile of each PAH in soil cultures containing added phenanthrene and benzo[*a*]pyrene. Community five was inoculated into sterile (■) and unsterile (●) soil. Uninoculated controls are also shown for sterile (□) and unsterile (○) soil as well as inoculated sterile soil without PAH addition (◆).

population and the addition of small quantities of dichloromethane for the distribution of PAHs.

5.2.2 PAHs in Paired Combinations

It was shown in liquid cultures that the degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by low cell populations of community five was restricted by the inability of the isolates to grow on these compounds (Section 4.2.1). One method used to overcome this limitation was to supplement cultures containing a single five-ring compound with either phenanthrene or pyrene; the lower molecular weight PAH served the purpose of supporting microbial growth (Section 4.2.3.2). In the work reported in this section, phenanthrene or pyrene were supplemented into soil containing benzo[*a*]pyrene or dibenz[*a,h*]anthracene to evaluate whether degradation of the latter compounds in a soil environment was stimulated.

The rate of phenanthrene degradation in the presence of benzo[*a*]pyrene or dibenz[*a,h*]anthracene was similar to experiments reported in the previous section where phenanthrene was supplied as the sole PAH (Figure 5.1, 5.3 and 5.4). However, pyrene degradation in the presence of the five-ring compounds was slightly slower compared to when pyrene was supplied as the sole PAH (Figure 5.2, 5.5 and 5.6) taking 28 days to be degraded to undetectable levels compared to 21 days when present as a single PAH. Microbial numbers of community five in soils containing phenanthrene and benzo[*a*]pyrene or dibenz[*a,h*]anthracene increased until day 35, reaching a maximum of 4.5×10^7 cells/g soil (two order of magnitude increase) (Figure 5.3 and 5.4). Microbial counts decreased slightly over the remaining incubation period, resulting in 2.5×10^7 cells/g soil for benzo[*a*]pyrene and dibenz[*a,h*]anthracene soil after 70 days. Similar growth curves were observed when pyrene was used as the growth substrate (Figure 5.5 and 5.6). Growth of community five occurred during the first 42 days, with microbial numbers reaching a maximum of 4.5×10^7 cells/g soil in benzo[*a*]pyrene soil and dibenz[*a,h*]anthracene soil. Over the remaining incubation period, microbial numbers decreased (2.5×10^7 cells/g soil).

A lag period of approximately 21-28 days occurred before the onset of benzo[*a*]pyrene (Figure 5.3) and dibenz[*a,h*]anthracene (Figure 5.4) degradation in the presence of phenanthrene. Longer lag periods were observed when the five-ring compounds were in the presence of pyrene; a lag period of approximately 42 days occurred before the onset of benzo[*a*]pyrene degradation (Figure 5.5), where as dibenz[*a,h*]anthracene degradation (Figure 5.6) was proceeded by a lag period of 28-35 days. Degradation of

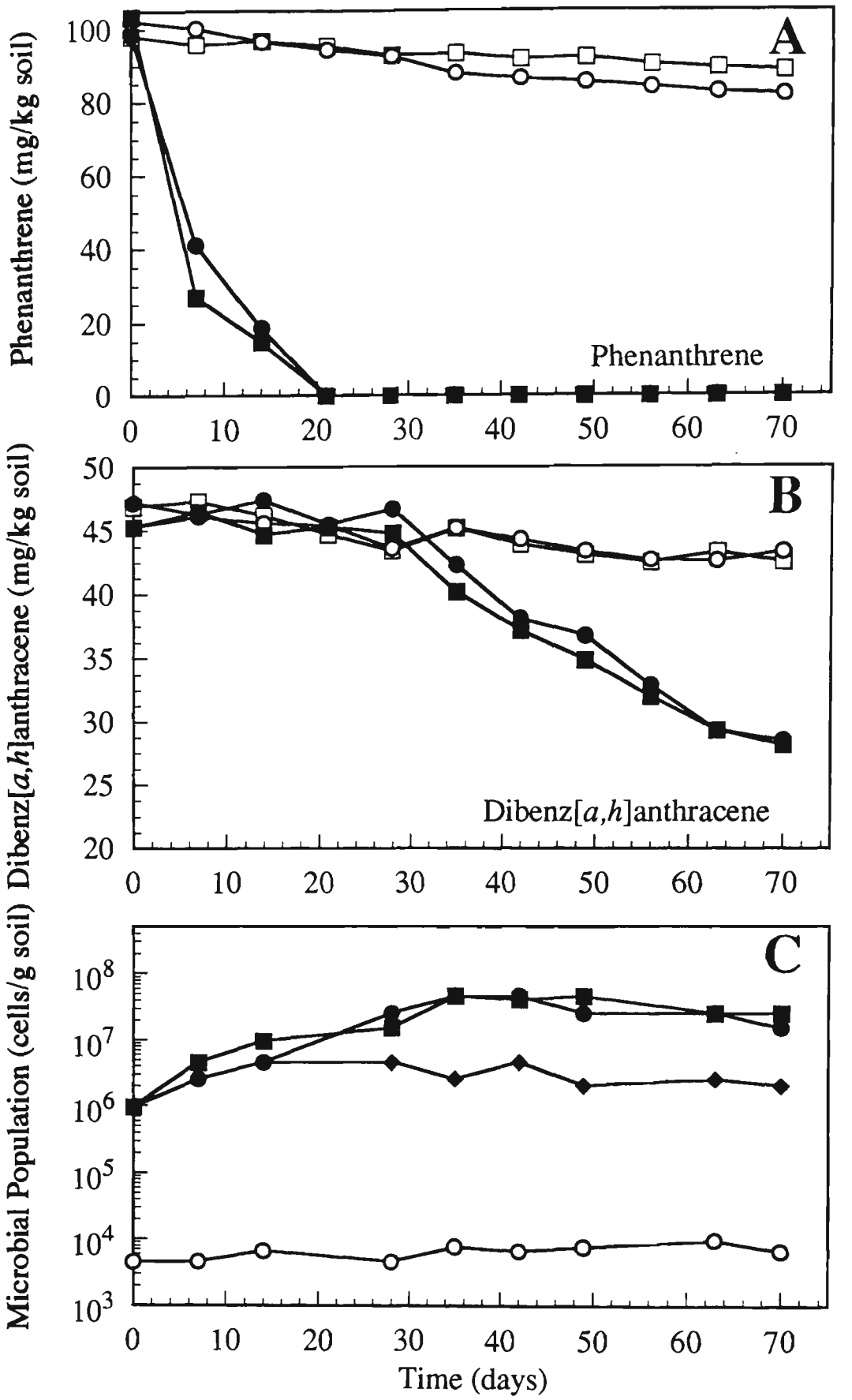


Figure 5.4. PAH concentration (A and B) and microbial numbers (C) in soil spiked with phenanthrene (A) and dibenz[*a*,*h*]anthracene (B) inoculated with pyrene-grown community five. The panels represent the concentration profile of each PAH in soil cultures containing phenanthrene and dibenz[*a*,*h*]anthracene. Community five was inoculated into sterile (■) and unsterile (●) soil. Uninoculated controls are also shown for sterile (□) and unsterile (○) soil as well as uninoculated soil without PAH addition (◆).

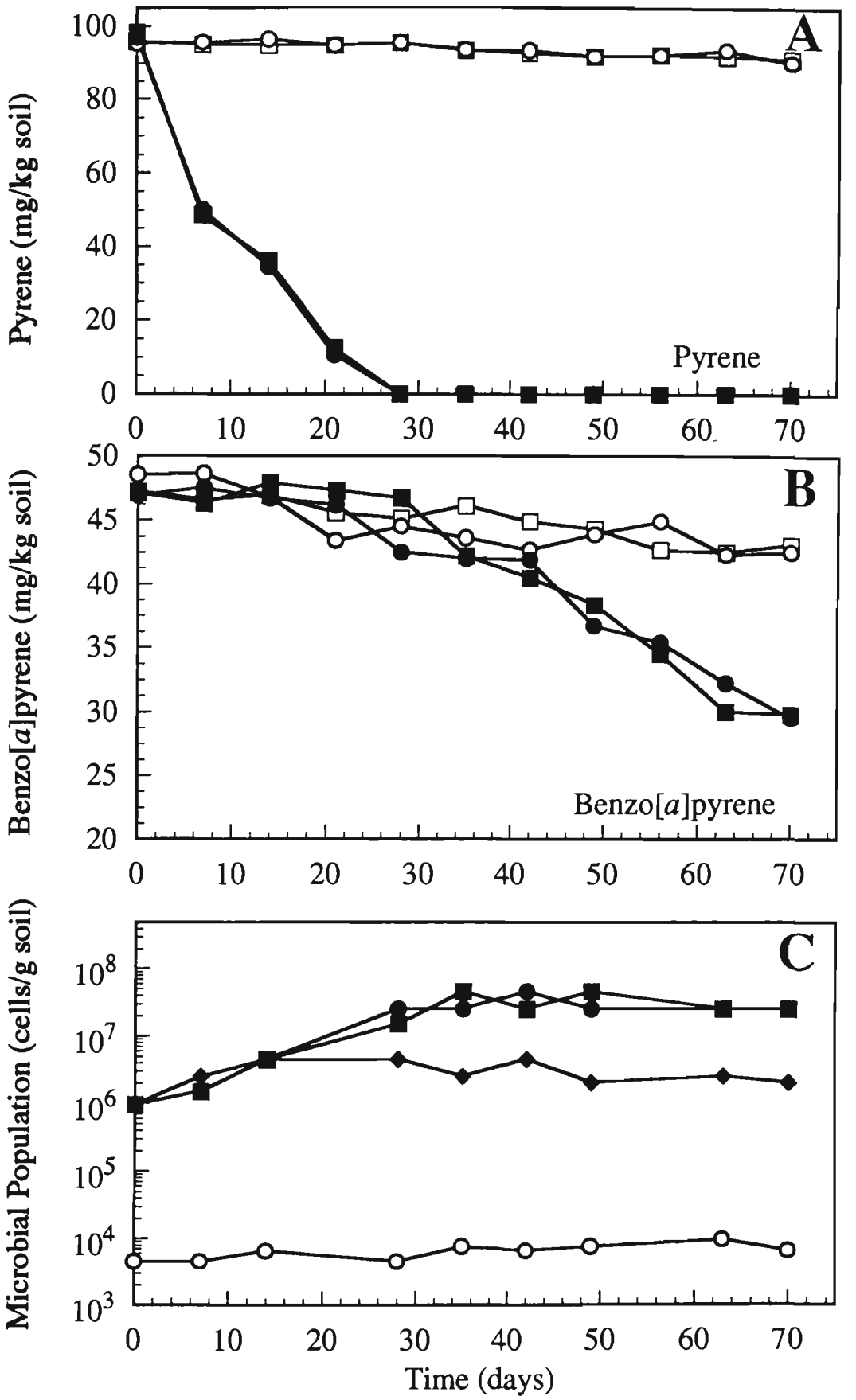


Figure 5.5. PAH concentration (A and B) and microbial numbers (C) in soil spiked with pyrene and benzo[*a*]pyrene inoculated with pyrene-grown community five. The panels represent the concentration profile of each PAH in soil cultures containing pyrene and benzo[*a*]pyrene. Community five was inoculated into sterile (■) and unsterile (●) soil. Uninoculated controls are also shown for sterile (□) and unsterile (○) soil as well as uninoculated soil without PAH addition (◆).

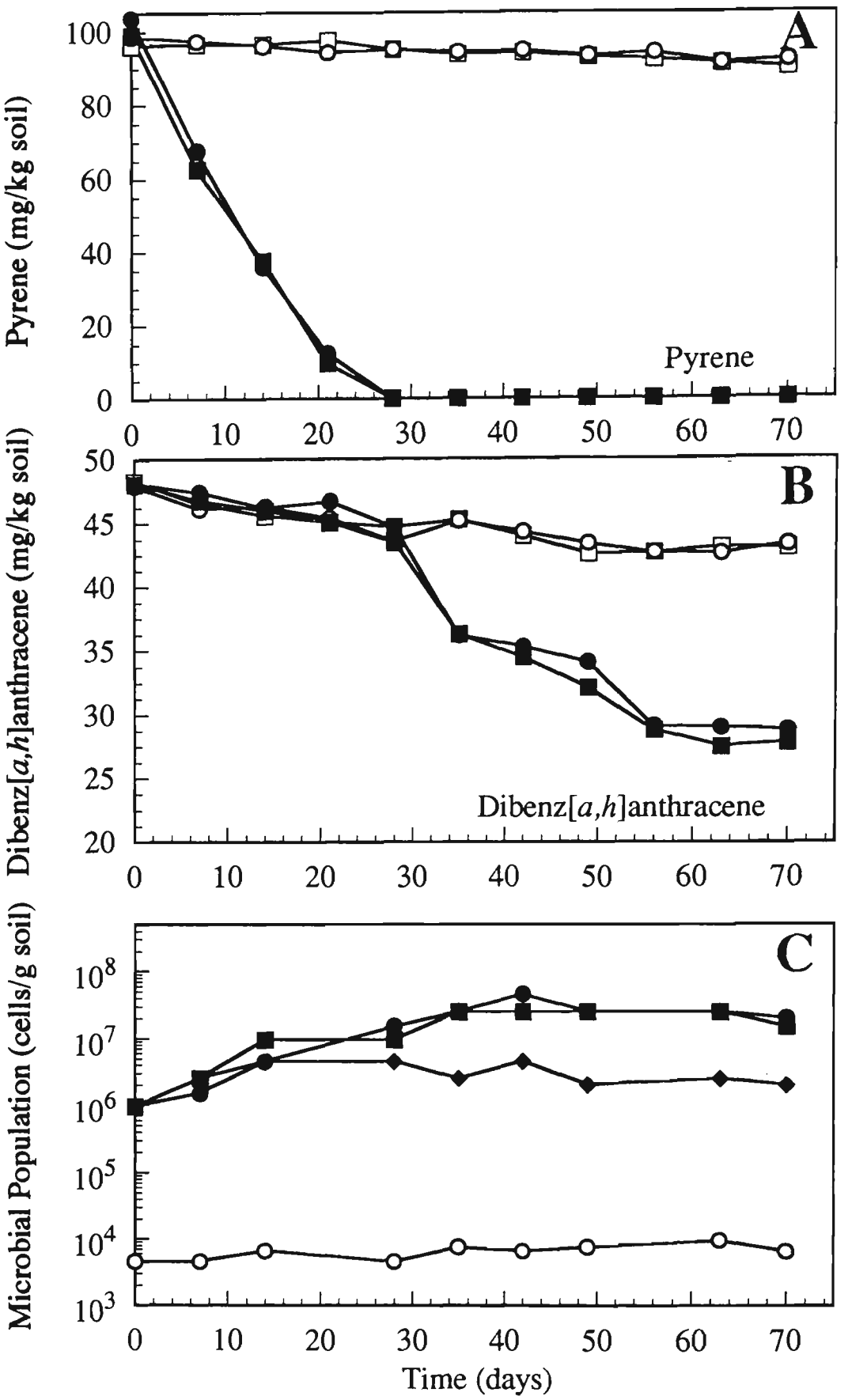


Figure 5.6. PAH concentration (A and B) and microbial numbers (C) in soil containing pyrene (A) and dibenz[*a*,*h*]anthracene (B) inoculated with pyrene-grown community five. The panels represent the concentration profile of each PAH in soil cultures containing pyrene and dibenz[*a*,*h*]anthacene. Community five was inoculated into sterile (■) and unsterile (●) soil. Uninoculated controls are also shown for sterile (□) and unsterile (○) soil as well as uninoculated sterile soil without PAH addition (◆).

the five-ring compounds was slow, however, significant decreases (33-36%) in their respective concentrations were observed in both phenanthrene- and pyrene-supplemented cultures after 63 days; degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene ceased after this time period.

5.2.3 PAH Mixture

Community five was also evaluated for its ability to degrade a synthetic PAH mixture in soil. The PAH cocktail was formulated to simulate the range and concentrations of PAHs found at contaminated sites. The cocktail contained 50 mg/kg soil of fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene and 20 mg/kg soil of coronene. Abiotic loss of PAHs was distinguished by using controls consisting of uninoculated sterilised or unsterilised soil containing the synthetic mixture (see Figure 5.7).

The extraction of soils containing the PAH mixture was able to recover approximately 70% of the added fluorene, which was probably due to volatilisation of this compound during incubation. Coronene could not be recovered from the soils after the first sample, presumably due to adsorption of this hydrophobic compound onto soil components. Community five was able to degrade all PAHs significantly over the 70 day incubation period (Figure 5.7). Fluorene, phenanthrene and pyrene were degraded to undetectable levels after 35 days. Fluoranthene degradation commenced after a lag period of 14 days and continued until day 70, resulting in a 70% decrease in fluoranthene concentration. A lag period of 35 days occurred before the onset of benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. Significant decreases in the concentration of these compounds (40, 28 and 33% respectively) were observed up to 70 days (Figure 5.7).

Microbial numbers in the inoculated cultures containing the PAH mixture increased rapidly during the initial stages of incubation, reaching a maximum population size of 9.5×10^7 cells/g soil after 28 days (Figure 5.8). The microbial population decreased slightly over the remaining incubation period, resulting in 4.5×10^7 cells/g soil after 70 days.

5.3 DEGRADATION OF PAHs IN PAH-CONTAMINATED SOIL

The degradation results from the PAH-spiked soil experiments (section 5.2) demonstrated that community five was capable of degrading PAH compounds in a soil

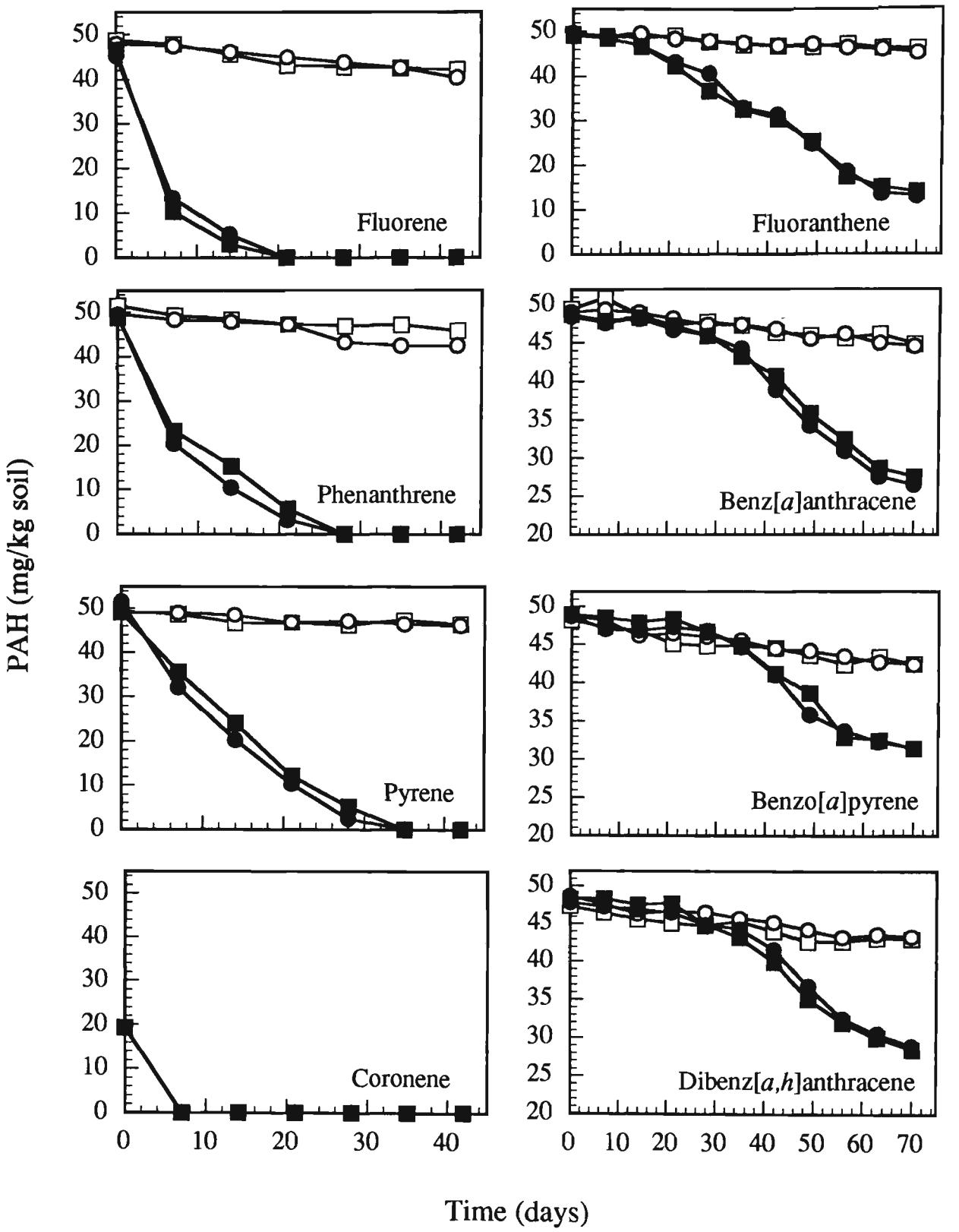


Figure 5.7. PAH concentration in soil spiked with a PAH mixture inoculated with pyrene-grown community five. The panels represent the concentration profile of each PAH in soil cultures containing all of the above PAHs. Community five was inoculated into sterile (■) and unsterile (●) soil. Uninoculated controls are also shown for sterile (□) and unsterile (○) soil.

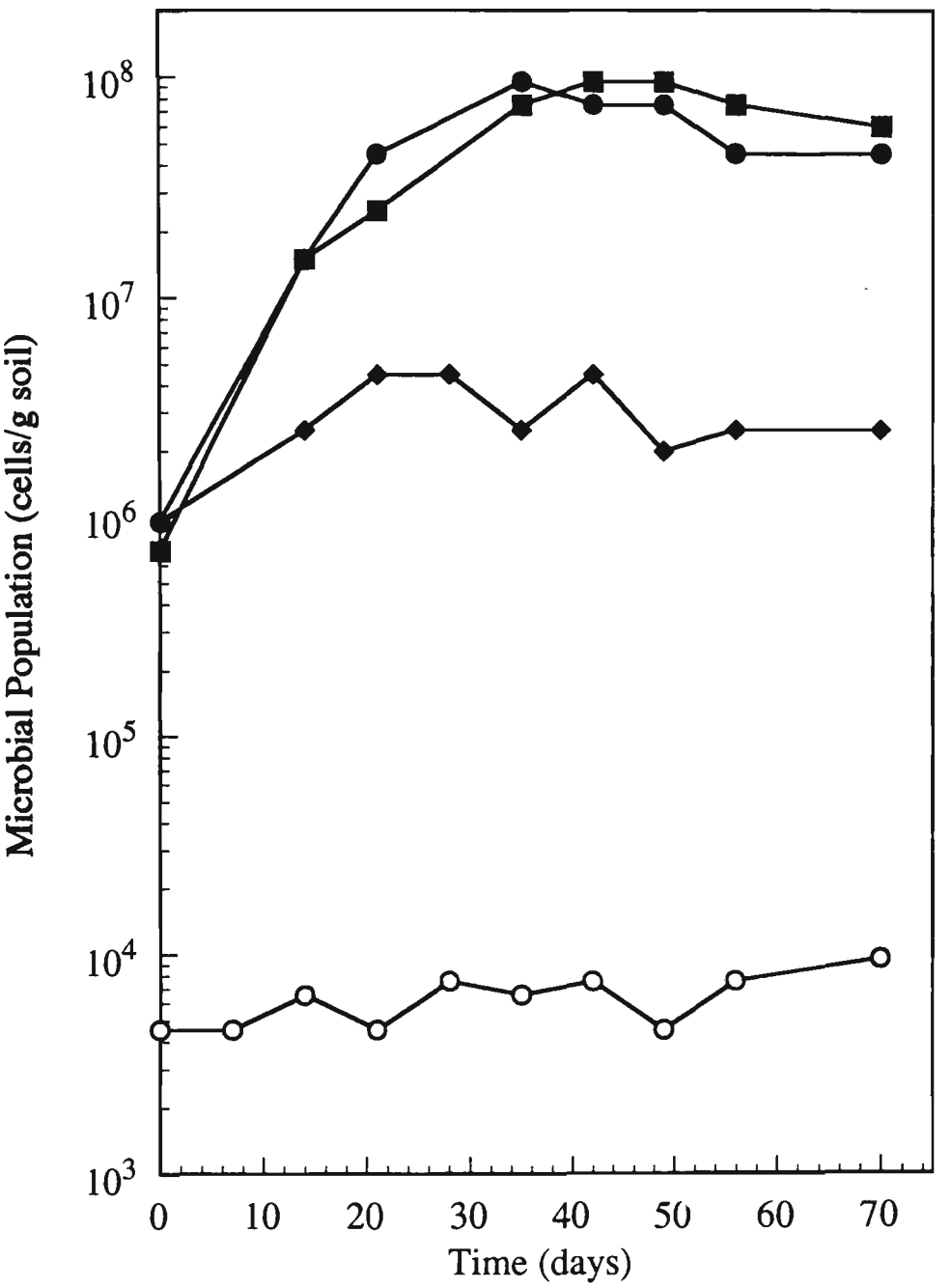


Figure 5.8. Microbial population in soils spiked with a PAH mixture (see Figure 5.7). Community five was inoculated into sterile (■) and unsterile (●) soil. Controls are shown for uninoculated unsterile (O) soil and inoculated sterile soil without added PAHs (◆).

Table 5.2. Chemical characteristics of PAH-contaminated soil.

Parameter	Value
Chemical Characteristic:	
pH	5.3
% Carbon	26
% Nitrogen	<0.1
Calcium, ppm	170
Magnesium, ppm	110
Phosphorus, ppm	750
Potassium, ppm	110
Sodium, ppm	50
Manganese, ppm	110
Iron, ppm	3,100
Aluminium, ppm	1,000
Cadmium, ppm	<1.0
Copper, ppm	190
Lead, ppm	570
Nickel, ppm	80
Zinc, ppm	260
Cation Exchange Capacity	350
Total Petroleum Hydrocarbons (ppm):	
C6-C9	66
C10-C14	350
C15-C28	6,700
C29-C36	1,300
BTEX (ppm):	
Benzene	0.07
Toluene	0.12
Ethylbenzene	0.03
Xylenes	0.28
Total Polychlorinated Biphenyls, ppm	<0.1
Chlorinated Hydrocarbons (ppm):	
Trichloromethane	0.007
1,2-Dichloroethylene	0.006
Polycyclic Aromatic Hydrocarbons (ppm):	
Naphthalene	186.2
Acenaphthene	42.7
Fluorene	86.9
Phenanthrene	156.0
Anthracene	53.3
Fluoranthene	136.6
Pyrene	98.6
Benz[*a*]anthracene	32.7
Benzo[*a*]pyrene	15.1
Dibenz[*a,h*]anthracene	12.4

matrix. However, PAH degradation was under ideal conditions: the soil was spiked with PAHs thus providing potentially more bioavailable compounds; it did not contain heavy metals or other contaminants that could possibly inhibit the inoculated microorganisms and the inoculum did not have to compete with the indigenous microbial population due to their low numbers. To assess the ability of community five to degrade PAHs in a soil matrix, further degradation experiments were performed with community five in PAH-contaminated soil.

The PAH-contaminated soil was obtained from Dr Brent Davey, Australian Defence Industry, Environmental Services, Melbourne. The soil was sieved (2 mm mesh) and the pH was adjusted from 5.3 to 7.1 by the addition of garden lime. The physical and chemical characteristics of the soil were analysed by the National Analytical Laboratories Pty, Ltd, Melbourne (Table 5.2). The soil had a high carbon loading (26%) and was low in nitrogen (<0.1%). Some heavy metals were present, namely lead, at a concentration of 570 mg/kg. Total petroleum hydrocarbons were in the order of 8,400 mg/kg and there were trace amounts of benzene, toluene, ethylbenzene, xylene and chlorinated hydrocarbons. PAH concentration was approximately 820 mg/kg with the major components being naphthalene, phenanthrene, fluoranthene and pyrene (Table 5.2).

Inocula for the soil studies were prepared by growing community five in the CYEM medium formulated in Section 4.4. After three days growth, the cells were collected by centrifugation, washed and resuspended in double strength BSM to achieve a 10-fold concentration in biomass. Aliquots of the cell suspension were added to PAH-contaminated soil. CYEM-grown community five was chosen as the inoculum for these experiments in preference to pyrene-grown community five as the growth medium was shown to be suitable for mixed PAH degradation plus it was an inexpensive medium for future bioremediation use. PAH degradation was also determined after adding the inoculum as well as yeast extract to the soil (1 g/kg). Yeast extract was added to the soil as it was seen to enhance the degradation of creosote components by CYEM-grown community five in liquid medium (see Section 4.4.5.2). The degradation of PAHs by the indigenous microflora was also determine with and without yeast extract supplementation. The controls consisted of soils with or without inoculation killed with mercuric chloride. The inoculated killed control served to determine the effect of adsorption of PAHs to the inoculated biomass. The percentage decrease in the concentration of the PAHs over the incubation period was calculated by using the PAH concentrations from the killed uninoculated control compared to the respective cultures. In addition, Ames and Microtox™ tests were performed to

Table 5.3. Recovery of PAHs from mercuric chloride killed PAH-contaminated soil inoculated with CYEM-grown community five.

PAH	PAH Concentration (mg/kg)[a]							
	Initial Conc.	7 Days	28 Days	48 Days	63 Days	91 Days	$HgCl_2$ Control (91 Days)[b]	% PAH Decrease[c]
NAPH	186.2±11.4	181.5±10.5	180.3±9.9	174.6±14.2	166.9±7.9	163.8±8.9	161.3±10.2	-
ACE	42.7±5.2	40.6±3.5	38.9±5.5	41.4±4.1	40.2±3.7	38.6±2.1	36.4±4.3	-
FLU	86.9±7.1	85.6±8.9	84.3±10.2	81.3±6.7	78.9±7.3	75.1±3.8	76.9±4.4	2.3
PHEN	156.0±12.3	152.5±9.2	144.9±6.8	145.9±12.1	143.9±7.8	139.9±10.2	141.6±8.9	1.2
ANTH	53.3±4.1	52.5±5.3	55.7±4.7	54.2±3.1	52.3±3.9	52.1±5.5	51.6±2.7	-
FA	136.6±11.2	139.5±10.2	141.5±7.8	134.6±8.4	130.3±9.9	131.3±12.1	129.5±10.2	-
PYR	98.6±5.3	94.3±9.3	96.7±7.8	93.2±5.3	94.5±10.2	92.9±6.6	94.2±8.0	1.9
BA	32.7±4.5	34.2±1.2	31.4±3.5	33.7±2.8	32.5±4.3	31.9±1.9	33.7±2.9	5.3
B[a]P	15.1±1.5	14.3±3.2	14.7±2.3	15.3±1.2	15.1±0.9	15.4±1.3	16.7±1.5	-
DBA	12.4±0.5	10.8±1.5	11.9±0.5	12.3±0.6	11.8±1.3	12.1±1.4	11.9±0.9	-
Sum PAH	820.5	805.8	800.3	786.5	766.4	753.1	753.8	-

[a]Data reported are the average of triplicate samples.

[b]PAH concentration in mercuric chloride killed soil cultures after 91 days.

[c]The percentage PAH decrease over the incubation period was calculated with reference to the mercuric chloride killed control.

determine whether bioaugmentation of PAH-contaminated soil results in a reduction in the toxicity or mutagenicity of aqueous and organic soil extracts.

5.3.1 PAH Concentration in Soil

Abiotic degradation of PAHs in mercuric chloride killed soils was restricted to the lower molecular weight compounds; a 9.2-14.8% decrease in the concentration of naphthalene, acenaphthene, fluorene and phenanthrene was observed after 91 days (Table 5.3). A change in PAH concentration of less than 5% was observed for the remaining compounds.

Degradation of PAHs by the indigenous microflora was restricted to the lower molecular weight compounds (Table 5.4). Naphthalene (23.9%) and fluorene (19.2%) were degraded to the greatest extent, while a 14-15% decrease in the concentration of acenaphthene and phenanthrene was observed after 91 days. No significant decrease in the concentration of anthracene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene was observed. The indigenous microbial population increased in size over the incubation period from an initial level of 3.5×10^5 cells/g soil (Figure 5.9). Microbial numbers rose to 2.5×10^6 cells/g soil after 28 days and then remained constant until the end of the incubation period. The increase in microbial number of the indigenous microflora was probably due to the addition of inorganic nutrients and an increased incubation temperature.

The addition of yeast extract to the soil culture resulted in the stimulation of low molecular weight PAH degradation by the indigenous microorganisms (Table 5.5). A 36.6% decrease in the concentration of naphthalene and decreases of 26.9-32.0% for acenaphthene, fluorene and phenanthrene were observed after 91 days. A slight reduction in the concentration of anthracene (5.2%) was observed, however, no significant decrease in the concentration of the high molecular weight PAHs (four- and five-ring compounds) occurred. Yeast extract addition also stimulated the growth of the indigenous microflora. After 28 days, microbial numbers increased from 3.5×10^5 cells/g soil to 2.5×10^7 cells/g soil (Figure 5.9). Microbial numbers decreased over the remaining incubation period resulting in a final population size of 9.5×10^6 cells/g soil.

Inoculation of CYEM-grown community five into PAH-contaminated soil resulted in significant decreases in the concentration of all PAHs over the incubation period (Table 5.6). Greater than 86% of naphthalene, acenaphthene, fluorene and phenanthrene were degraded after 91 days, while anthracene, fluoranthene and pyrene were degraded to

Table 5.4. Degradation of PAHs from PAH-contaminated soil by the indigenous microflora.

PAH	PAH Concentration (mg/kg)[a]							
	Initial Conc.	7 Days	28 Days	48 Days	63 Days	91 Days	$HgCl_2$ Control (91 Days)[b]	% PAH Decrease[c]
NAPH	186.2±11.4	183.4±9.6	170.4±12.6	155.2±11.6	131.6±9.9	122.8±10.2	161.3±10.2	23.9
ACE	42.7±5.2	41.9±4.5	38.6±3.2	35.4±3.8	33.7±3.6	31.1±2.9	36.4±4.3	14.6
FLU	86.9±7.1	86.7±10.2	84.7±7.8	77.4±9.4	67.2±5.6	62.1±8.5	76.9±4.4	19.2
PHEN	156.0±12.3	160.3±15.3	155.3±12.4	143.5±11.7	136.7±9.9	121.5±13.2	141.6±8.9	14.2
ANTH	53.3±4.1	52.5±4.3	53.2±3.4	51.4±2.9	52.3±3.5	49.9±5.7	51.6±2.7	3.3
FA	136.6±11.2	134.8±13.2	137.4±11.7	140.3±10.4	133.5±5.9	130.7±10.0	129.5±10.2	-
PYR	98.6±5.3	99.4±10.3	97.8±8.9	95.3±9.3	94.3±7.9	91.0±10.3	94.2±8.0	3.4
BA	32.7±4.5	32.8±4.3	35.3±1.4	32.7±2.7	33.7±4.1	32.6±2.2	33.7±2.9	3.3
B[a]P	15.1±1.5	15.3±1.3	15.4±0.9	14.7±0.6	15.1±1.1	15.0±1.2	16.7±1.5	0.7[d]
DBA	12.4±0.5	11.9±2.1	12.4±1.2	12.7±1.4	11.4±0.9	12.3±1.1	11.9±0.9	-
Sum PAH	820.5	819.0	800.5	758.6	709.5	669.0	753.8	11.3

[a]Data reported are the average of triplicate samples.

[b]PAH concentration in mercuric chloride killed soil cultures after 91 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.

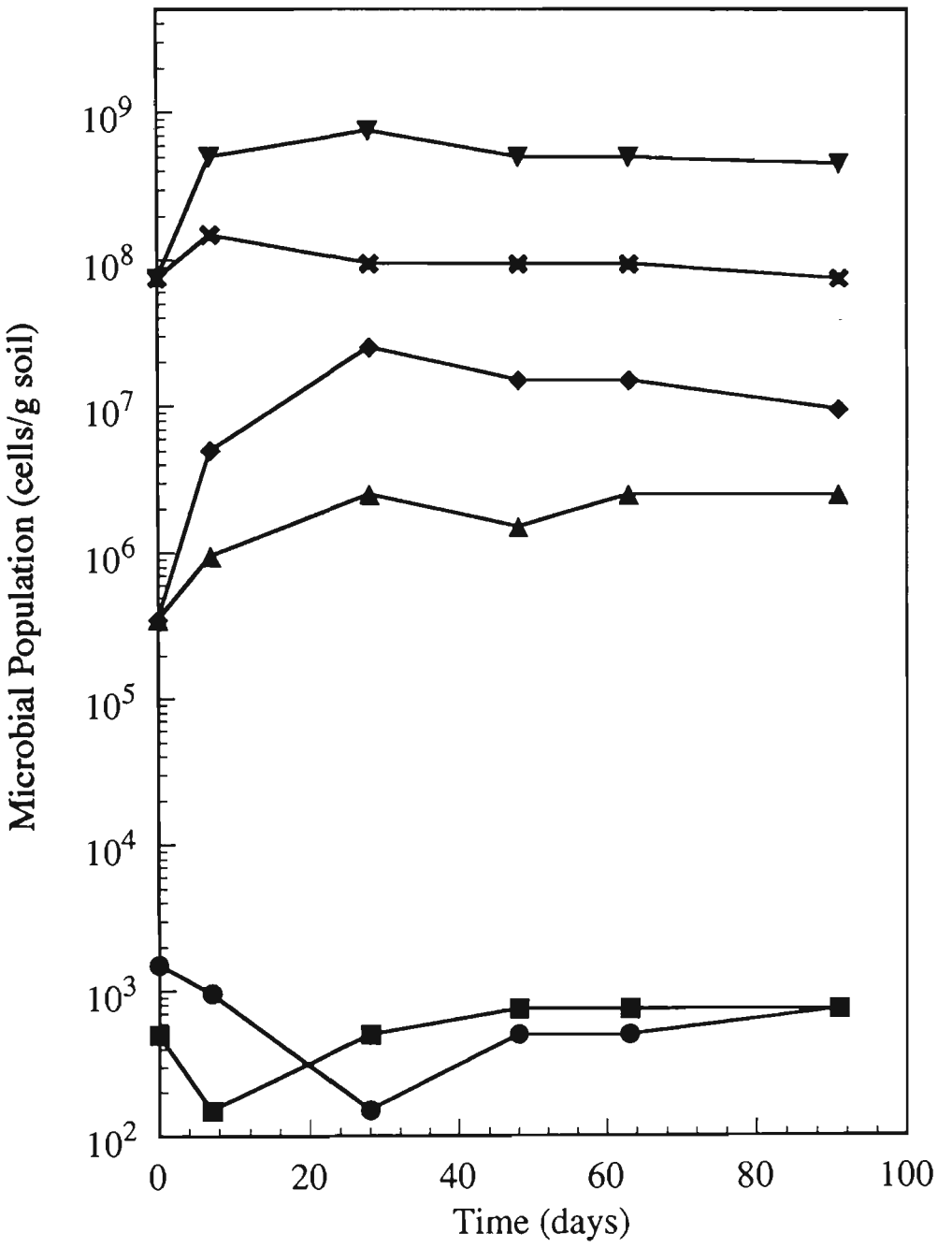


Figure 5.9. Microbial populations in PAH-contaminated soil. CYEM-grown community five was inoculated into soil with (▼) and without (✖) yeast extract (1 g/kg) supplementation. The growth of the indigenous microbial population was also assessed with (◆) and without (▲) yeast extract. Mercuric chloride killed controls are shown for uninoculated (■) and inoculated (●) soils.

Table 5.5. Degradation of PAHs from PAH-contaminated soil by the indigenous microflora supplemented with yeast extract (1 g/kg).

PAH	PAH Concentration (mg/kg)[a]							
	Initial Conc.	7 Days	28 Days	48 Days	63 Days	91 Days	$HgCl_2$ Control (91 Days)[b]	% PAH Decrease[c]
NAPH	186.2±11.4	170.5±14.5	159.4±9.9	123.2±12.2	112.6±11.6	102.3±7.8	161.3±10.2	36.6
ACE	42.7±5.2	40.4±3.4	35.4±4.2	30.5±2.8	27.1±4.8	26.4±3.1	36.4±4.3	27.5
FLU	86.9±7.1	83.4±8.8	75.1±6.9	64.8±8.2	58.5±9.0	52.3±6.7	76.9±4.4	32.0
PHEN	156.0±12.3	153.7±12.3	149.9±10.5	145.6±12.3	122.3±9.7	103.5±11.3	141.6±8.9	26.9
ANTH	53.3±4.1	53.5±3.4	42.9±4.1	52.8±4.0	51.5±3.9	48.9±4.2	51.6±2.7	5.2
FA	136.6±11.2	136.2±11.3	133.7±9.8	134.5±9.4	129.9±10.3	126.7±11.2	129.5±10.2	2.2
PYR	98.6±5.3	100.3±8.9	95.6±12.1	93.7±10.5	94.0±11.2	92.4±7.9	94.2±8.0	1.9
BA	32.7±4.5	33.7±3.1	32.6±3.6	33.1±2.6	32.7±2.9	32.8±3.2	33.7±2.9	2.7
B[a]P	15.1±1.5	16.2±1.7	16.1±1.4	15.8±2.0	14.9±1.5	15.4±0.9	16.7±1.5	-[d]
DBA	12.4±0.5	11.8±1.6	11.9±1.3	12.1±2.1	13.2±1.9	12.0±1.0	11.9±0.9	-
Sum PAH	820.5	799.7	752.6	706.1	656.7	612.7	753.8	18.7

[a]Data reported are the average of triplicate samples.

[b]PAH concentration in mercuric chloride killed soil cultures after 91 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 5.6. Degradation of PAHs from PAH-contaminated soil inoculated with CYEM-grown community five.

PAH	PAH Concentration (mg/kg)[a]							
	Initial Conc.	7 Days	28 Days	48 Days	63 Days	91 Days	$HgCl_2$ Control (91 Days)[b]	% PAH Decrease[c]
NAPH	186.2±11.4	145.6±12.1	89.4±6.3	23.3±4.1	12.1±1.8	7.8±0.4	161.3±10.2	95.2
ACE	42.7±5.2	38.2±2.9	22.1±3.2	10.3±1.5	5.1±0.3	4.2±0.4	36.4±4.3	88.5
FLU	86.9±7.1	75.3±6.3	58.9±4.5	21.3±1.9	12.4±0.9	4.7±0.2	76.9±4.4	93.9
PHEN	156.0±12.3	141.2±10.9	99.5±11.3	54.3±4.3	33.9±2.5	19.5±0.8	141.6±8.9	86.2
ANTH	53.3±4.1	50.3±5.7	45.7±4.3	32.5±3.9	24.8±3.4	22.5±2.1	51.6±2.7	56.4
FA	136.6±11.2	129.0±10.0	120.9±9.4	111.2±7.8	79.6±8.4	62.6±5.9	129.5±10.2	51.7
PYR	98.6±5.3	92.1±5.7	84.3±7.2	66.3±6.4	46.8±3.6	39.9±2.9	94.2±8.0	57.6
BA	32.7±4.5	33.1±2.6	32.4±3.1	30.1±1.9	28.8±2.0	22.7±1.3	33.7±2.9	32.6
B[a]P	15.1±1.5	15.3±1.5	15.3±1.2	14.7±0.8	14.1±1.2	11.6±1.1	16.7±1.5	25.2[d]
DBA	12.4±0.5	12.2±1.1	12.1±0.4	12.4±1.0	11.9±0.9	9.7±0.7	11.9±0.9	18.5
Sum PAH	820.5	732.3	580.9	376.4	269.5	205.2	753.8	72.8

[a]Data reported are the average of triplicate samples.

[b]PAH concentration in mercuric chloride killed soil cultures after 91 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 5.7. Degradation of PAHs from PAH-contaminated soil inoculated with CYEM-grown community five supplemented with yeast extract (1 g/l).

PAH	PAH Concentration (mg/kg)[a]							
	Initial Conc.	7 Days	28 Days	48 Days	63 Days	91 Days	$HgCl_2$ Control (91 Days)[b]	% PAH Decrease[c]
NAPH	186.2±11.4	122.3±14.2	33.5±4.6	12.1±2.1	8.9±0.4	4.5±0.3	161.3±10.2	97.2
ACE	42.7±5.2	30.2±2.3	14.7±1.2	6.7±0.3	4.2±0.2	2.4±0.2	36.4±4.3	93.4
FLU	86.9±7.1	67.3±5.6	43.2±2.1	14.2±1.4	5.2±0.2	3.9±0.1	76.9±4.4	94.9
PHEN	156.0±12.3	135.2±14.2	86.4±11.9	44.5±3.5	23.2±1.3	14.5±0.9	141.6±8.9	89.8
ANTH	53.3±4.1	49.9±3.4	42.1±3.1	29.5±2.9	22.4±1.4	20.1±1.1	51.6±2.7	61.0
FA	136.6±11.2	125.9±11.2	103.5±9.7	89.4±5.6	66.8±5.2	52.6±3.9	129.5±10.2	59.4
PYR	98.6±5.3	85.2±8.4	69.4±5.3	54.2±4.6	40.2±3.2	30.3±3.1	94.2±8.0	67.8
BA	32.7±4.5	32.4±1.9	31.3±1.8	31.6±2.1	25.8±2.3	21.6±1.7	33.7±2.9	35.9
B[a]P	15.1±1.5	15.6±1.2	15.3±1.4	14.2±0.8	12.2±0.6	10.9±0.7	16.7±1.5	27.8[d]
DBA	12.4±0.5	12.5±1.4	12.5±1.1	12.1±0.9	10.9±0.8	9.4±0.6	11.9±0.9	21.0
Sum PAH	820.5	676.5	451.9	308.5	219.8	170.2	753.8	76.4

[a]Data reported are the average of triplicate samples.

[b]PAH concentration in mercuric chloride killed soil cultures after 91 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.

similar extents (51.7-57.6%). A lag period of 48-63 days was observed before the onset of benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. However, significant decreases in the concentration of these compounds (32.6, 25.2 and 18.5% respectively) were observed after 91 days. Inoculation of community five into the PAH-contaminated soil resulted in an initial population size of 7.5 x 10^7 cells/g soil (Figure 5.9). Microbial numbers rose during the first seven days of incubation (1.5 x 10^8 cells/g soil) but then decreased over the remaining incubation period resulting in a final population size of 7.5 x 10^7 cells/g soil.

The addition of yeast extract to inoculated soil resulted in small increases (2-10%) in the amount of individual PAHs degraded compared to augmented soils lacking supplementation after 91 days (Table 5.7). However, nutrient supplementation increased the rate of degradation of naphthalene, acenaphthene, fluorene and phenanthrene during the first 28 days of incubation. The amount of PAHs degraded by community five in the presence of yeast extract was 58% greater for naphthalene, 36% greater for acenaphthene, 56% greater for fluorene and 23% greater for phenanthrene after 28 days. The rate of fluoranthene, pyrene and benzo[*a*]pyrene degradation was also enhanced by the addition of yeast extract, however, no significant increase in the rate of dibenz[*a,h*]anthracene degradation was observed (Table 5.6 and 5.7). The addition of yeast extract to inoculated soil resulted in an increase in microbial numbers from 7.5 x 10^7 cells/g soil to 7.5 x 10^8 cells/g soil after 28 days (Figure 5.9). Microbial numbers declined slightly over the remaining incubation period resulting in a final population size of 5.0 x 10^8 cells/g soil.

5.3.2 Mutagenicity of PAH-Contaminated Soil

The mutagenic potential of PAH-contaminated soil was determined over the incubation period using the Ames Test. A reduction in contaminant concentration may not guarantee a reduction in the mutagenic potential of the soil. The microbial degradation of soil contaminants may result in the production of intermediate products that exhibit stronger mutagenic properties than the parent compound.

5.3.2.1 *Dose-Response Curve*

The mutagenic activity of extracts of organics from PAH-contaminated soil was tested at a number of dose levels to determine whether *S. typhimurium* strains TA98 and TA100 exhibited a dose-related response to soil extracts. Soils (1.0 g) were extracted with dichloromethane (50 ml) (see Section 2.7.3.5) and aliquots of the extracts (0.1,

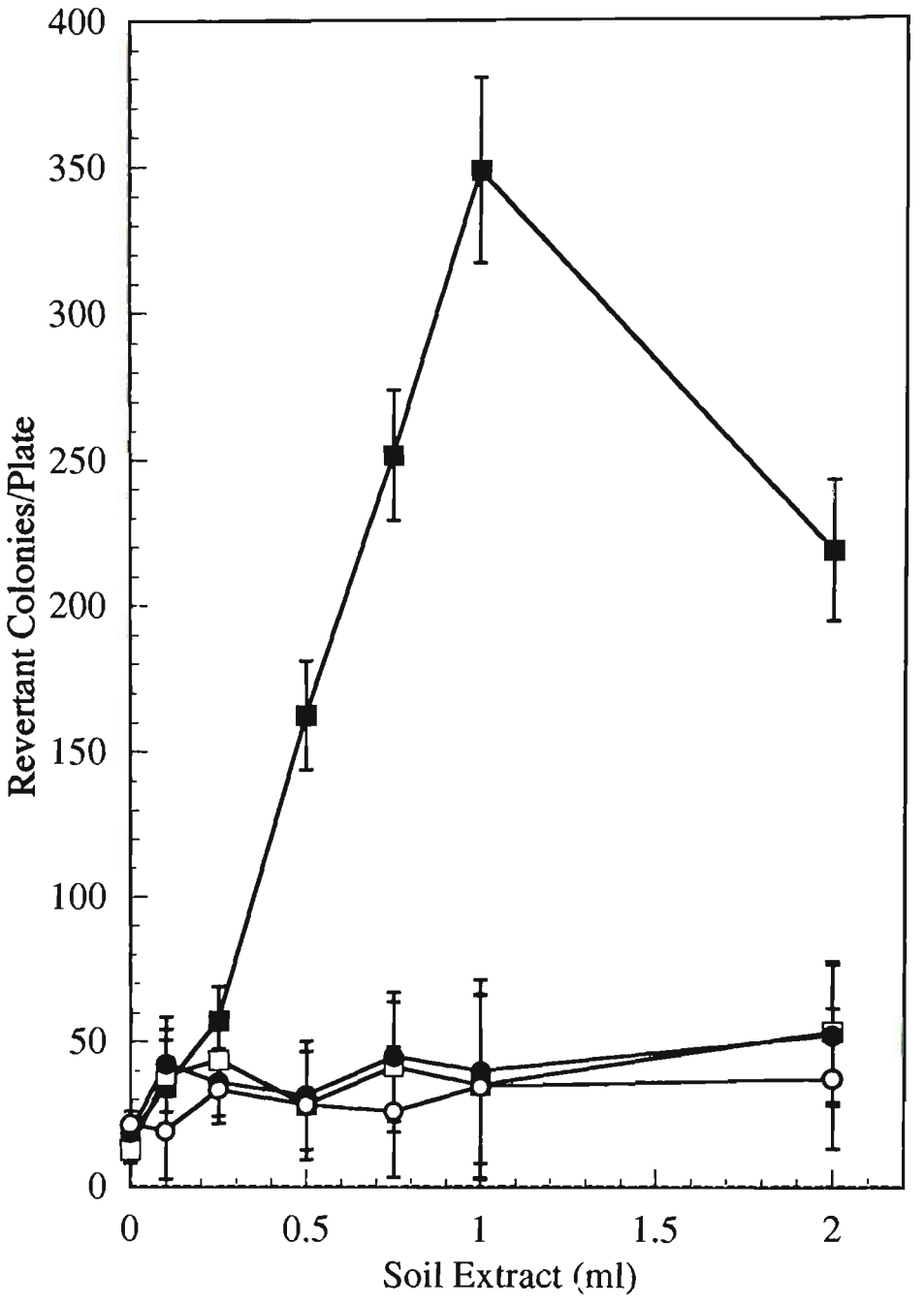


Figure 5.10. Dose related mutagenic response of *S. typhimurium* strains TA98 and TA100 to various amounts of PAH-contaminated soil extracts. Mutagenicity assays were performed in the presence (●, ■) and absence (○, □) of the S9 fraction for TA 98 and TA100 respectively. Soil (1 g) was extracted with 50 ml DCM. Aliquots of the soil extacts (0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 ml) were solvent exchanged with DMF and mutagenicity assays performed.

0.25, 0.5, 0.75, 1.0 and 2.0 ml) were solvent exchanged with dimethylformamide (0.1 ml). *S. typhimurium* strains TA98 and TA100 were exposed to various concentrations of the soil extracts, with and without the addition of the mammalian microsomal preparation (S9 fraction) using DMF as the control.

S. typhimurium strain TA98 did not show a dose-related response to the soil extracts at the concentrations tested in the presence or absence of the S9 fraction (Figure 5.10). However, the dose-response curve demonstrated the mutagenic potential of the soil extracts toward strain TA100 in the presence of the S9 fraction. The soil extracts displayed a dose-related mutagenic response (Figure 5.10), however, toxic effects, exhibited by decreasing revertant colony numbers and a sparser background lawn of autotrophic bacteria were noticed when testing at the highest concentration (2.0 ml soil extract). No dose-related response was observed for strain TA100 in the absence of the S9 fraction.

5.3.2.2 *Mutagenic Activity of PAH-Contaminated Soil Extracts*

The *Salmonella* reversion assay was used to determine the mutagenic potential of organic PAH-contaminated soil extracts after 3, 48 and 91 days from soils incubated with the indigenous microflora, indigenous microflora with yeast extract, community five and community five with yeast extract. Aliquots of the soil extracts (1.0 ml) were solvent exchanged into dimethylformamide (0.1 ml) and the mutagenic response was tested with strain TA100 with and without the addition of the S9 fraction. Mutagenicity tests were performed without the addition of the S9 fraction (even though there was no dose-related mutagenic response observed without S9) to determine if degradation of the soil components resulted in the formation of direct acting mutagenic compounds.

In the absence of the S9 fraction, there was no significant difference in the number of revertant colonies of TA100 exposed to soil extracts taken over the time course (Figure 5.11). Incubation of soils with the indigenous microflora or community five in the presence or absence of yeast extract did not result in the formation of direct acting mutagenic compounds. However, in the presence of the S9 fraction, the number of revertant colonies varied depending on the decrease in the concentration of the PAHs over the incubation period. Small decreases in the concentration of PAHs were observed in soil containing the indigenous microflora, however, no significant decrease in the mutagenic potential of soil extracts occurred (Figure 5.12). After 91 days, soil extracts exerted a mutagenic response corresponding to 315.6±42.6 revertant colonies per plate compared to 341.6±30.2 revertant colonies per plate at the start of the

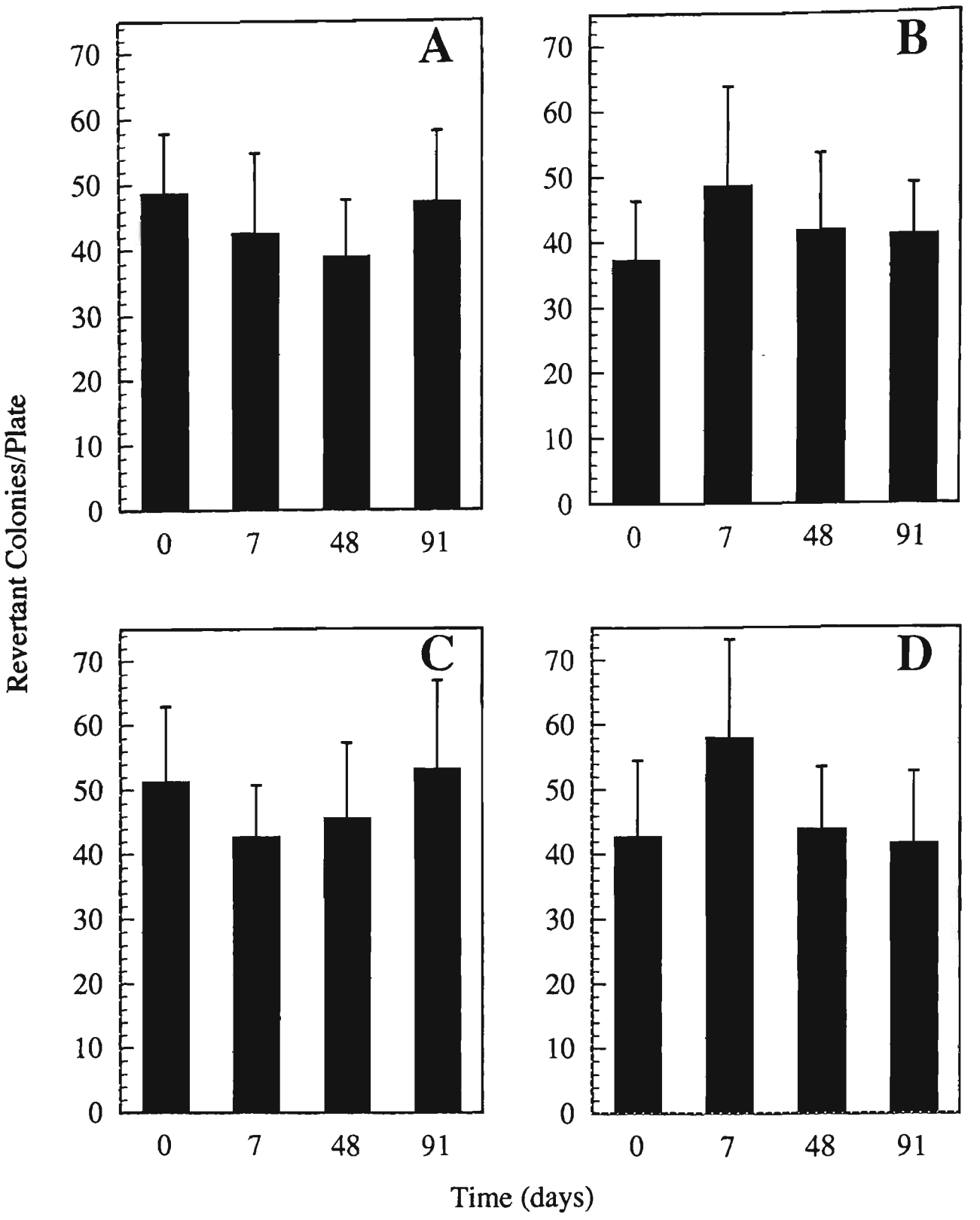


Figure 5.11. Mutagenic potential of PAH-contaminated soil extracts towards *S. typhimurium* strain TA100 in the absence of the S9 fraction. The graphs illustrate the mutagenicity of soils incubated with the indigenous microbial population (A), the indigenous population and yeast extract (B), inoculated CYEM-grown community five (C) and inoculated CYEM-grown community five and yeast extract (D).

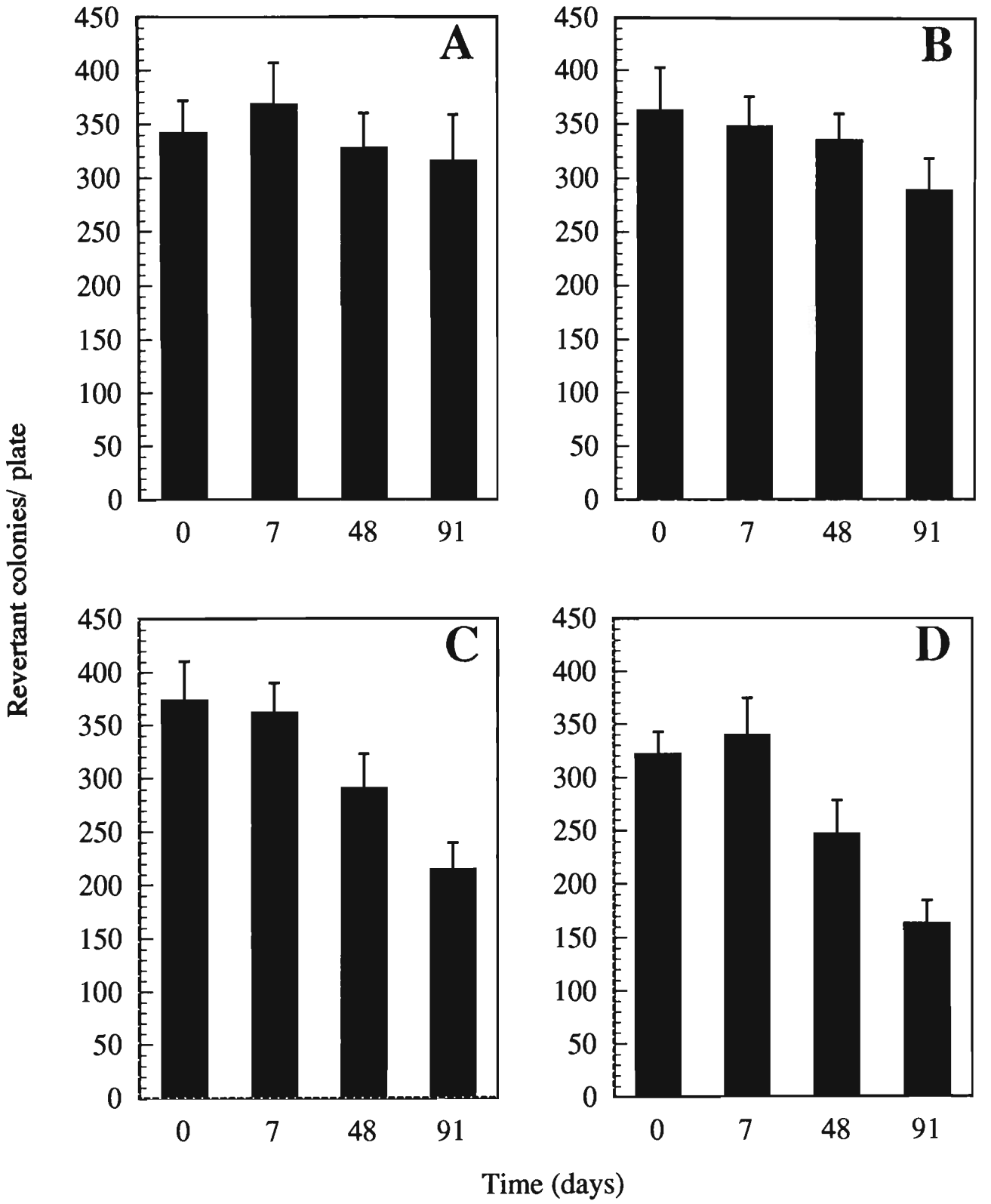


Figure 5.12. Mutagenic potential of PAH-contaminated soil extracts towards *S. typhimurium* strain TA100 in the presence of the S9 fraction. The graphs illustrate the mutagenic potential of soils incubated with the indigenous microbial population (A), the indigenous population and yeast extract (B), inoculated CYEM-grown community five (C) and inoculated CYEM-grown community five and yeast extract (D).

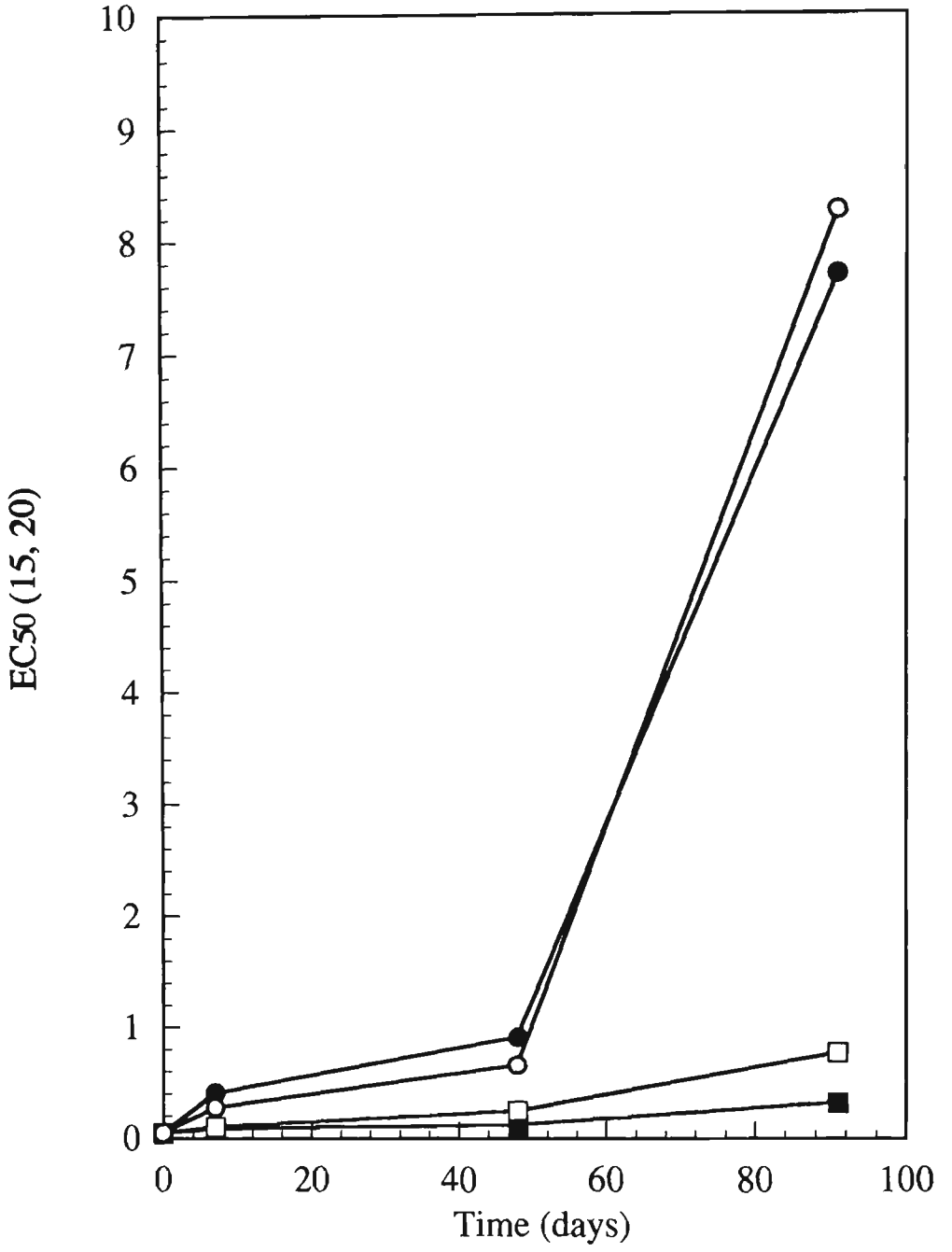


Figure 5.13. Microtox™ toxicity of the water soluble fraction of PAH-contaminated soil incubated with the indigenous microflora (■), indigenous microflora and yeast extract (1 g/kg) (□), CYEM-grown community five (●) and CYEM-grown community five and yeast extract (1 g/kg) (○). EC50 (15, 20) denotes the effective concentration (v/ml) of water soluble soil extract that reduces *P. phosphoreum* light emission by 50% 15 minutes after exposure to the extract at 20°C.

incubation period. In yeast extract supplemented PAH-contaminated soils, a 20% reduction in the mutagenic potential of soil extracts was observed (Figure 5.12). After 91 days, the number of revertant colonies had decreased from 362.4±40.1 at the start of the incubation period to 289.6±29.6.

Bioaugmentation of PAH-contaminated soil with CYEM-grown community five resulted in significant decreases in the concentration of all PAH compounds present in the soil. The reduction in the concentration of the PAHs corresponded to a reduction in the mutagenic potential of soil extracts. Inoculation and incubation of soil with community five resulted in a 43% decrease in the mutagenic potential of soil extracts after 91 days (Figure 5.12); the number of revertant colonies decreased from 372.9±37.2 to 213.7±25.3. When yeast extract was supplemented, the mutagenic potential of soil extracts decreased by 49% after 91 days (321.6±20.9 to 162.9±21.3) (Figure 5.12).

5.3.2.3 *Toxicity of PAH-Contaminated Soil Extracts*

The acute toxicity of aqueous soil extracts from the above PAH-contaminated soil incubations (excluding the mercuric chloride killed controls) was assessed using the modified Microtox™ assay described in section 2.4.7. Aqueous soil extracts of samples taken over the time course were obtained by shaking soil (5 g) with distilled water (20 ml) for two hours at 22°C. The extracted fluids were centrifuged (3,000 rpm for 15 minutes) and the supernatants assessed for toxicity towards *P. phosphoreum*. The EC_{50} (effective concentration at which a 50% decrease in light output is observed) for each sample was determined using a number of soil extract dilutions. EC_{50}s were determined using the formula outlined in Appendix 3.

Microtox™ results from the PAH-contaminated soil studies indicated a detoxification of the water soluble fraction of the soils with increased incubation time. At the start of the incubation period, the EC_{50} (15 minutes, 20°C) was 0.045 ml, *i.e.* a 50% reduction in *P. phosphoreum* light output was observed when the organism was exposed to 0.045 ml aqueous soil extract diluted in 1 ml 3% NaCl for 15 minutes at 20°C (Figure 5.13). Incubation of the soil with the indigenous microflora, with or without yeast extract supplementation, resulted in a decrease in the toxicity of the water soluble fraction. After 91 days, the EC_{50} for soils incubated with the indigenous microflora increased seven-fold (0.315 ml) and when incubated in the presence of yeast extract resulted in a 17-fold increase (0.764 ml) (Figure 5.13).

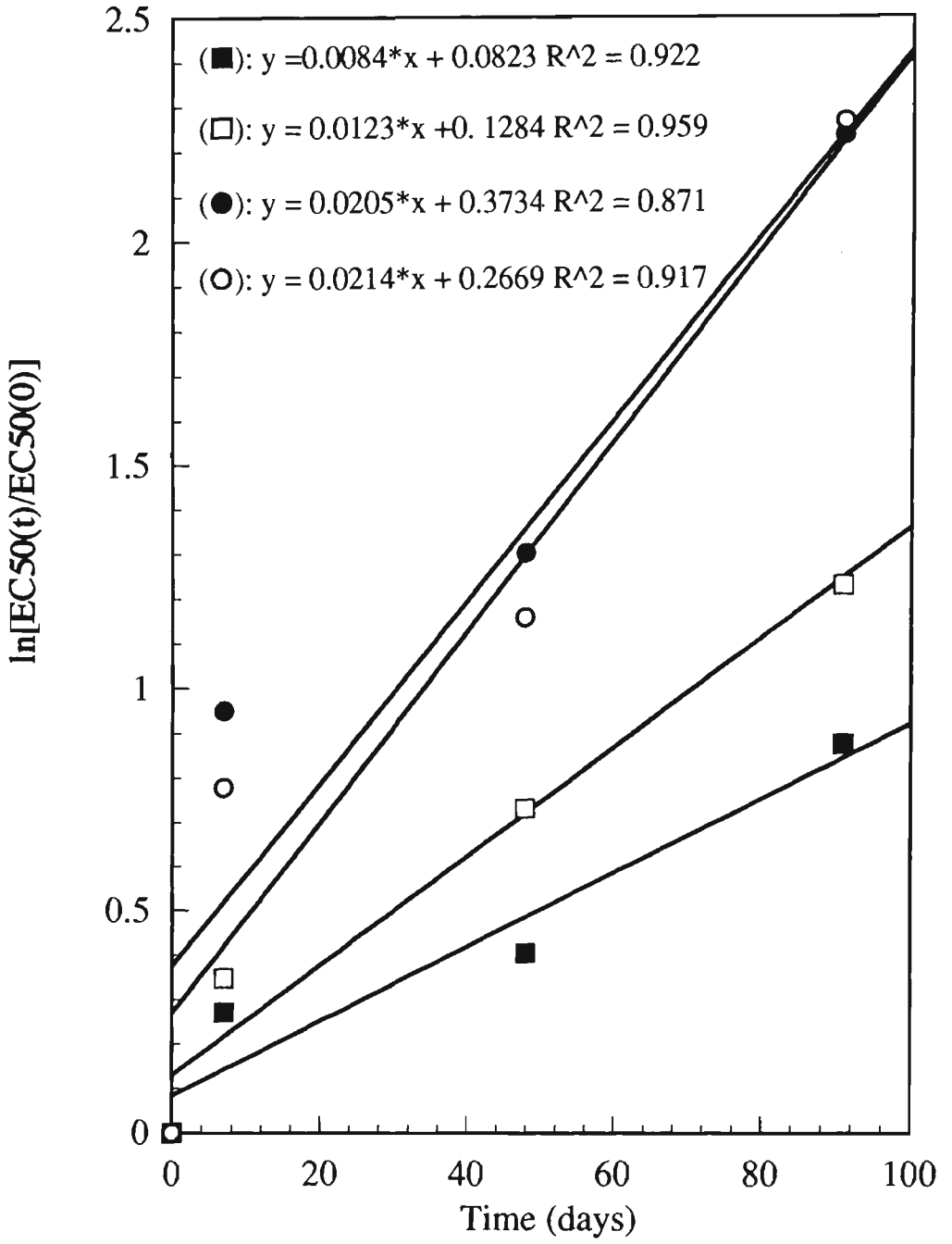


Figure 5.14. Rate of detoxification of PAH-contaminated soil by indigenous microflora (■), indigenous microflora and yeast extract (1 g/kg) (□), inoculated CYEM-grown community five (●) and inoculated CYEM-grown community five and yeast extract (1 g/kg) (○).

Inoculation of PAH-contaminated soil with CYEM-grown community five resulted in enhanced reductions in the toxicity of soil extracts compared to the indigenous microflora. After 91 days, the EC_{50} of community five inoculated soils was 170-fold greater (7.691 ml) than the initial EC_{50} value. Supplementation of yeast extract to inoculated soils resulted in an EC_{50} value 184-fold greater (8.260 ml) than the time zero value (Figure 5.13).

Figure 5.14 shows the rate of detoxification of the water soluble fraction of PAH-contaminated soil by the indigenous microflora and inoculated community five. Detoxification rates were significantly increase when the PAH-contaminated soil was inoculated with CYEM-grown community five. The rate of PAH-contaminated soil detoxification by the indigenous microflora was increased when yeast extract was added to the soil, where as supplementation of yeast extract to community five inoculated soil did not significantly increase the rate of detoxification.

5.4 LIQUID AND SOIL MATRICES: A COMPARISON OF PAH DEGRADATION PERFORMANCES BY COMMUNITY FIVE

Many evaluation studies on PAH degradation by microbial isolates are conducted in liquid media due to the ease of handling, the ability to accurately define the microbial environment and greater control over various environmental parameters. However, when inoculated into PAH-polluted soils, the performance of these PAH-degraders will be influenced by, among other environmental factors, the change in the nature of the biological matrix. It is generally accepted that microbial performance in soil will be restricted by factors such as the availability of nutrients, the presence of other organics, oxygen diffusion rates, moisture content, the extent of mixing and sorption of the pollutant to soil particles rendering them less biologically available, however, the extent to which this change from liquid to soil matrix affects PAH degradation has not been evaluated.

The ability of community five to degrade a spectrum of PAHs in both liquid and soil media provides an opportunity to evaluate the change in magnitude of their PAH-degrading performances when transferred between the two matrices. This section attempts to measure this change in PAH-degrading performance on a cellular basis by comparing the specific degradation rate, defined as mg PAH degraded/[10^{10} cells]/day, of community five in liquid and soil matrices. The specific degradation rate of phenanthrene, pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene was evaluated when supplied as a PAH mixture in liquid and soil cultures.

Table 5.8. Degradation of a PAH mixture (phenanthrene, pyrene, dibenz[*a*,*h*]anthracene and benzo[*a*]pyrene) by pyrene-grown community five in liquid and soil medium.

Medium	Incubation Time (days)	PAH Concentration	PAH	% Decrease[a]
Liquid	42	50 mg/l	PHEN	100[b]
			PYR	100[b]
			B[*a*]P	16.6
			DBA	38.9
Sterile Soil	70	50 mg/kg	PHEN	100[b]
			PYR	100[b]
			B[*a*]P	30.6
			DBA	35.6

[a]These values represent the mean percentage of PAHs degraded in the experimental cultures from three separate incubations.

[b]The compound was not detected at day 28.

In PAH-spiked soil, phenanthrene and pyrene were degraded to undetected levels after 28 days (Table 5.8). A lag period of 35 days was observed before the onset of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation, however, their degradation continued until day 70. Significant decreases in benzo[*a*]pyrene (22.4-25.6%) and dibenz[*a,h*]anthracene (30.0-30.8%) concentrations by community five were observed. Growth of the community reached a maximum cell population after 35 days (7.5×10^7 cells/g soil) then numbers decreased slightly (to 2.5×10^7 cells/g soil) over the remaining incubation period (Table 5.8).

The degradation of the above PAH mixture was also performed in liquid BSM (Table 5.8). Phenanthrene and pyrene were degraded to undetectable levels after 28 days. A lag period of 28 days occurred before the onset of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation. Degradation of the five-ring compounds resulted in concentration decreases of 18.7% and 42.8% for benzo[a]pyrene and dibenz[*a,h*]anthracene respectively. Microbial numbers were low in cultures containing the PAH mixture; community five reached a maximum population size of 4.4×10^6 cell/ml after 28 days, which remained constant over the remaining incubation period (Table 5.8).

The specific rates of benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation were calculated on a cellular basis and are expressed as mg PAH degraded/(10^{10} cells)/day (Table 5.9). Calculations were performed for incubations in sterile soil so that there would be no contribution to the degradation process by the indigenous microflora. The specific degradation rates were determined from the data collected during the period when the microbial populations in both liquid and soil media were relatively constant. The specific degradation rates of both benzo[*a*]pyrene and dibenz[*a,h*]anthracene were substantially greater in liquid media compared to a soil matrix. The rate of PAH degradation was 10-fold greater for benzo[*a*]pyrene and 17-fold greater for dibenz[*a,h*]anthracene in liquid media.

5.5 DISCUSSION

5.5.1 Degradation of PAHs in Soil

The potential use of microorganisms to clean up contaminated soil, sediment and water has been recognised for some time. Bioremediation has gained acceptance as a viable

Table 5.9. Specific degradation rates of benzo[*a*]pyrene and dibenz[*a,h*]anthracene in a PAH mixture (PHEN, PYR, B[*a*]P and DBA) by community five in liquid and soil culture.

Medium	Incubation Time (days)	Lag Phase (days)	Population Size	Amount of PAH degraded[a]	Specific Degradation Rate (mg PAH degraded/[10^{10} cells]/day)[b]
Liquid	42	28	4.4 x 10^6 cells/ml	0.63 mg B[*a*]P/l/ day	1.43
	42	28	4.4 x 10^6 cells/ml	1.39 mg DBA/l/day	3.16
Sterile Soil	70	35	2.5 x 10^7 cells/g soil	0.35 mg B[*a*]P/kg/soil/day	0.14
	70	35	2.5 x 10^7 cells/g soil	0.44 mg DBA/kg/soil/day	0.17

[a]These values represent the mean of the amount of PAH degraded in the experimental cultures from three separate incubations. The standard deviation for all recovered PAHs was less than five percent.

[b]The degradation rates were calculated for the amounts of B[*a*]P and DBA degraded in the total amount of culture medium after the initial degradation lag phase for each cmpound. Rates have been normalised to account for abiotic losses in the control cultures.

technology and if used prudently, can provide efficient, inexpensive and environmentally safe clean up of waste material. However, one of the major limitations in developing bioremediation is the transition from the degradative performance in liquid media to a soil matrix. Any bioremediation effort must provide a scientifically valid demonstration of the processes effectiveness (Pritchard *et al.*, 1992). Essentially, the process must demonstrate that the removal of the contaminant is primarily attributable to bioremediation and not other processes and that the degradation rates are sufficiently faster than the natural rates (Pritchard *et al.*, 1992). In addition, by-products from the degradation of the contaminants must be less toxic than the parent compound and augmented microorganisms must be able to compete with the indigenous microorganisms and maintain their degradative capabilities.

Bioaugmentation, the addition of microorganisms with known metabolic capabilities to contaminated soil, has been used as a remediation technology to enhance the degradation rates in recently contaminated soils, where the indigenous microbial population is low or where the indigenous microbial population is lacking the metabolic capabilities to degrade the contaminants. Bioaugmentation has been shown to be effective in the clean up of petroleum wastes (Forsyth *et al.*, 1995), PAHs (Kastner and Mahro, 1996; Thibault *et al.*, 1996, Banerjee *et al.*, 1995; Trzesickia-Mlynarz and Ward, 1996) and chlorinated compounds (Baud-Grasset and Vogel, 1995; Edgehill 1995).

Previous studies on the degradation of PAHs in contaminated soil by indigenous microorganisms both with and without biostimulation (Erickson *et al.*, 1993; Grosser *et al.* 1991; Kastner *et al.*,1995; Park *et al.*, 1990) have demonstrated the microbial degradation of low molecular weight PAH compounds, but the concentration of high molecular weight PAH compounds has been largely unaffected. This had led to the postulation that a more effective and complete soil remediation may be achieved by inoculating the soil with microorganisms capable of degrading high molecular weight PAHs (Wilson and Jones, 1993). A major limitation in the bioaugmentation of PAH-contaminated sites is the paucity of microbial isolates described in the literature which are capable of degrading high molecular weight PAHs, especially five-ring compounds. The microorganisms isolated from community five displayed some of the characteristics necessary for its use as an inoculum for the remediation of a PAH-contaminated sites. The previous chapter established the potential of community five as a microbial inoculum for PAH degradation by demonstrating its ability in liquid media to degrade four-, five- and seven-ring PAHs either as individual substrates or as part of a PAH mixture. Furthermore, the degradation rate of benzo[*a*]pyrene by

community five was substantially increased in the presence of low molecular weight PAH compounds. The next step in assessing whether these microorganisms are suitable for the bioaugmentation of PAH-polluted sites is to characterise their performance in a soil matrix. Community five was chosen for such soil studies and the ability of the indigenous soil populations to degrade PAHs was also assessed to determine whether stimulation of these organisms could result in a decrease in PAH concentration.

5.5.1.1 *PAH Degradation by the Indigenous Microflora*

The indigenous soil microorganisms in the PAH-spiked soil study were unable to degrade PAHs added to the soil either as individual compounds or as part of a mixture. It should be noted that the indigenous microbial population numbers were relatively low, which may reflect the prior treatment of the test soil used and a decrease in indigenous numbers due to the addition of PAHs and DCM. Although higher numbers of indigenous microorganisms were observed in the control PAH-contaminated soil (3.5×10^5 cells/g soil), only a small reduction in the concentration of naphthalene, acenaphthene, fluorene and phenanthrene (14-24%) was observed after 91 days. The addition of yeast extract resulted in an increase in microbial numbers and an increase in the amount of two- and three-ring PAHs degraded, however, it had no effect on the stimulation of four- and five-ring PAH degradation. This illustrates that a low microbial population was not responsible for the lack of high molecular weight PAH degradation by the indigenous soil microorganisms. These microorganisms probably lack the metabolic capabilities to degraded the high molecular weight PAHs or components in the soil inhibit the expression of these capabilities.

Previous soil studies by Mueller *et al.* (1991a) and Park *et al.* (1990) have observed the degradation of the lower molecular weight PAHs by the indigenous soil microflora. Although the indigenous microorganisms were capable of degrading the two- and three-ring PAHs, the high molecular weight compounds (four-, five- and six-ring PAHs) were resistant to microbial attack.

5.5.1.2 *Pyrene Degradation by Community Five*

The inoculation of pyrene-spiked soil with community five resulted in the degradation of all the added pyrene (100 mg/kg soil) after 21 days. In addition, when community five was inoculated into soil spiked with a PAH mixture or PAH contaminated soil, the microbial community was capable of degrading pyrene when present as part of a

complex PAH mixture in soil. Although pyrene is a major component of most PAH-contaminated sites, few other studies have investigated the degradation of this compound in bioaugmented soil. Grosser *et al.* (1991) observed a 55% increase in the degradation rate of pyrene in soil from a coal gasification plant when this was inoculated with pyrene-degrading bacteria, compared to uninoculated soil. Pyrene degradation was based on $^{14}CO_2$ production from a small quantity of added radiolabelled pyrene. However, the total amount of pyrene and other PAHs in the soil was not determined nor was microbial growth and survival measured. Also, the effect of the microbial inoculum on the degradation of the other PAH compounds in the soil was not investigated.

Thibault *et al.* (1996) also observed an increase in the degradation of pyrene in soil when a microbial inoculum was added. PAH-contaminated soil was supplemented with ^{14}C-pyrene and the mineralisation of the compound by the indigenous microorganisms and an inoculum was monitored. Mineralisation of pyrene by the indigenous microflora was minimal; approximately 5% of the ^{14}C was detected as $^{14}CO_2$ after 42 days. However, inoculation of the soil with a pyrene-degrading microorganism resulted in 48% of the ^{14}C being detected as $^{14}CO_2$. Although the effectiveness of the microbial inoculum to degrade the radiolabelled pyrene in soil was demonstrated, Thibault *et al.* (1996) study had similar limitations as that of Grosser *et al.* (1991). The results from the PAH-spiking experiments and PAH-contaminated soil studies demonstrated that bioaugmentation of community five into these soils results in significant decreases in the concentration of pyrene over time. In addition, the microbial community was capable of degrading pyrene when present in a complex mixture and was capable of maintaining high microbial numbers in these soils.

The degradation rate of pyrene in spiked soils was substantially lower when in the presence of other PAH compounds (1.43 mg/kg soil/day) compared to its use as a single substrate (5 mg/kg soil/day) *i.e.* a 71% decrease (see Figure 5.2 and 5.7). This effect on the pyrene degradation rate by community five was also observed in liquid media. Presumably, this is due to the concurrent use of other PAH substrates, such as fluorene and phenanthrene, since microbial growth was similar in both the pyrene and mixed PAH cultures, or inhibition of pyrene-degrading bacteria in the consortium by the higher molecular weight PAHs. This effect should be accounted for in studies which examine the degradation rates of single PAHs to predict the performance of microbial isolates or consortia to be used in the field.

5.5.1.3 *Degradation of PAH Mixtures by Community Five*

For bioaugmentation to be effective, the inoculated PAH-degraders must have the ability to degrade the full spectrum of PAH compounds in a soil matrix for the inoculum to be used in remediation of PAH-contaminated sites. Although the effectiveness of bioaugmentation has been demonstrated for soil slurries (Mueller *et al.*, 1991b), aqueous sediments (Shiaris, 1989b) and soils contaminated with low molecular weight PAHs (Trzesicka-Mlynarz and Ward, 1996; Banerjee *et al.*, 1994; Kastner and Mahro, 1996), there are relatively few studies on the bioaugmentation of soil for the removal of high molecular weight PAHs. A number of soil studies that have investigated the effect of bioaugmentation on PAH degradation in a PAH mixture have concluded that both the indigenous microflora and the inoculum were effective in removing the lower molecular weight PAHs, however, the four- and five-ring compounds were recalcitrant to microbial attack (Mueller *et al.*, 1991a; Park *et al.*, 1990). In one study, however, Erickson *et al.* (1993) observed that the microbial inoculum was ineffective in removing PAHs in soil from a manufactured gas plant site. The failure of the introduced microorganisms to enhance PAH degradation in the work of Erickson *et al.* (1993) may have been due to the use of a poorly adapted inoculum.

In contrast, this work showed that the introduced PAH-degrading microorganisms removed both low and high molecular weight PAH compounds from PAH-spiked soil and PAH-contaminated soil. In addition, significant decreases in the concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene were observed in both soil matrices. Previous experiments with community five adapted on pyrene have demonstrated the ability of this consortium to degrade, but not grow on, five-ring PAH compounds in liquid culture (see Chapter 4). However, the inability of community five to grow on these PAHs as sole carbon and energy sources does not preclude its use for the remediation of PAH-polluted sites, since these sites, as typified by the PAH-contaminated soil, usually contain low molecular weight PAH compounds which can be used as growth substrates by the microbial consortium.

The ability of community five to degrade both benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the presence of phenanthrene or pyrene (spiked soils) or as part of a PAH mixture (PAH-contaminated soil) demonstrated the ability of the microbial community to degrade the five-ring PAHs in the presence of growth-supporting PAHs. Benzo[*a*]pyrene has been shown to be degraded by indigenous microorganisms and by inoculated microorganisms in sediment (Shiaris, 1989b; Heitkamp and Cerniglia, 1989), however, few studies have demonstrated the

degradation of benzo[*a*]pyrene in soil. Aprill *et al.* (1990) evaluated the bioremediation of two types of wood preserving and petroleum refining wastes in soil. Although there was a 97% removal of the two- and three-ring PAH compounds, no detectable degradation of the five-ring PAHs occurred after 354 days. The degradation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by indigenous soil microorganisms was observed by Weissenfels *et al.* (1990b). Contaminated soil from a wood impregnation plant was prepared in a trickling column, circulating a mineral salts medium for eight weeks. A 29% decrease in benzo[*a*]pyrene concentration (from 94 mg/kg soil) and a 17% decrease in dibenz[*a,h*]anthracene concentration (from 10 mg/kg soil) was observed over the incubation period. However, in soils obtained from a coking plant which contained a higher proportion of high molecular weight PAHs, no significant decrease in the concentration of any of the PAHs was observed over the eight week period.

In another study, Pott and Henrysson (1995) compared the degradation rates of two-, three-, four- and five-ring PAHs in soil by indigenous microorganisms. Naphthalene was degraded to undetectable levels after 50 days (from an initial concentration of 12 mg/kg soil), anthracene was degraded at a rate of 0.36-0.57 mg/kg soil/day and benz[*a*]anthracene was degraded at a rate of 0.22-0.55 mg/kg soil/day. However, no significant difference in the rate of removal of the five-ring PAH, dibenz[*a,h*]anthracene was observed in the treated soil compared to the controls. An increase in the water content of the soil or the addition of nutrients did not stimulate the degradation of dibenz[*a,h*]anthracene. Pott and Henrysson (1995) concluded that the rate of PAH removal was highly dependent on the number of fused benzene rings of the PAH compound.

The rate of PAH removal by community five in the PAH-contaminated soil was also dependent on the number of fused benzene rings of the PAH compound. The rate of PAH removal over 91 days was 1.96 mg/kg soil/day for naphthalene, 1.50 mg/kg soil/day for phenanthrene, 0.65 mg/kg soil/days for pyrene and 0.04 mg/kg soil/day for benzo[*a*]pyrene. This demonstrates that the rate of PAH degradation by community five also is highly dependent on the size of the PAH compound and the importance of optimising bioremediation methods based on the degradation rates of high molecular weight PAHs instead of the total PAH concentration.

The addition of yeast extract to PAH-contaminated soil resulted in a small increase in the amount of PAHs degraded by both the indigenous microbial population and inoculated community five. Although only a small increase in PAH degradation was

observed in the inoculated soils, the degradation rate of the low molecular weight PAHs was increased in the presence of yeast extract. After 28 days, the rate of PAH removal was 57% greater for naphthalene, 35% for acenaphthene, 56% for fluorene, 23% for phenanthrene, 48% for anthracene, 111% for fluoranthene and 104% for pyrene in the presence of yeast extract. The increased degradation rate was probably due to the increased population size due to the growth of the microorganisms on the yeast extract.

5.5.1.4 *Survival of Community Five in Soil*

A key factor in the rapid removal of PAHs from contaminated soil is a high population of active PAH-degrading microorganisms. In the spiked soil experiments, such populations occurred 20-35 days after inoculation when the test soil contained single or mixed PAHs; this high microbial population was sustained over the incubation period trialed. In the PAH-contaminated soil, high microbial numbers were maintained throughout the incubation period after inoculation with community five. Microbial numbers increased an order of magnitude in inoculated soils when yeast extract was added to the soil. High growth and survival rates are desirable characteristics in an introduced microbial population but are not always guaranteed with PAH degraders. Trzesicka-Mlynarz and Ward (1996) demonstrated the enhanced degradation of fluoranthene in inoculated soil, however, the introduced bacteria declined in numbers from the first day of the incubation period. In sterile inoculated soil, fluoranthene concentration decreased by 46% (7.05 mg/kg soil to 3.8 mg/kg soil) after 9 weeks, however, microbial numbers decreased from 2.1×10^7 cfu/g soil to 7.3×10^4 cfu/g soil. Trzesicka-Mlynarz and Ward (1996) accounted for the decrease in microbial numbers by the limitation of an essential nutrient which stopped synthesis of cell molecules, limiting endogenous metabolism which led to the loss of cell viability. This suggests that a high inoculum size, or reseeding the soil after a period of time, would be required to ensure adequate fluoranthene removal rates at a PAH-polluted site. In the spiked soil, community five grew effectively on the PAHs when they were supplied as individual compounds, as pairs or as part of a mixture. In addition, some growth was observed when community five was inoculated in soil without PAH supplementation, indicating that other organic components within the soil can support and maintain a microbial population. In the PAH-contaminated soil, microbial numbers remained constant after inoculation, indicating that community five could survive in the soil matrix and compete for nutrients with the indigenous microbial population.

Although the indigenous microbial population in the PAH-contaminated soil was relatively large, [2.5×10^6 cells/g soil (after 28 days) and 2.5×10^7 cells/g soil in the

presence of yeast extract (after 28 days)], substantial decreases in PAH concentration were not observed. The indigenous microbial population may in fact utilise other components in the soil as growth substrates before the PAHs or the microorganisms may have limited PAH-degrading abilities. As such, the high indigenous microbial populations did not result in high removal rates of PAHs.

5.5.2 Detoxification of PAH-Contaminated Soil

A majority of research conducted into the microbial detoxification of PAHs has involved the use of complex waste mixtures (*e.g.* creosote waste, separator sludge solids) which have been combined with uncontaminated soil (Symons and Sims, 1988; Aprill *et al.*, 1990; Donnelly *et al.*, 1987). The disappearance of PAHs, mutagenic potential of soil extracts and toxicity of the water soluble fraction of the soil were determined over the incubation period. Although a number of these studies demonstrated a reduction in the mutagenicity or toxicity of soil extracts and soil leachates, an understanding of how these reductions occurred is still unclear. Control soil reactors or columns consisted of experimental soil without the application of the waste material and the extent of chemical loss was determined by comparing the amount initially added to soils and the amount that was recovered at various time periods. The decrease in the concentration of the PAHs may have occurred due to a number of mechanisms including microbial, chemical or photodegradation, hydrolysis, volatilisation or a combination of the above. However, these mechanisms were not identified. In addition, the corresponding reduction in mutagenicity or toxicity may have also occurred due to any one of these processes. No attempt was made to isolate or identify indigenous soil microorganisms that may have contributed to the degradation of the PAHs or to differentiate between biological and chemical or physical transformations. The dearth of research into microbial detoxification of PAHs provides a poor framework for understanding the mechanisms involved in such processes, which may be crucial in developing successful strategies for bioremediation.

5.5.2.1 *Mutagenicity*

In this study, incubation of PAH-contaminated soil with the indigenous microflora resulted in a small decrease in the concentration of PAHs over the incubation period. Although a decrease in the concentration of naphthalene, acenaphthene, fluorene and phenanthrene occurred, no significant decrease in the mutagenic potential of soil extracts was observed. Lower molecular weight PAHs such as the aforementioned are non-carcinogenic (Aprill *et al.*, 1990) and do not exhibit a dose-related mutagenic

response in the *Salmonella* mutagenicity assay. As such, small reductions in the concentration of these compounds do not influence the overall mutagenic potential of soil extracts. However, when yeast extract was supplemented to PAH-contaminated soil, a reduction in the mutagenic potential of soil extracts (20%) was observed. The microbial degradation of the lower molecular weight PAHs was increased, however, the addition of yeast extract did not stimulate the degradation of the four- and five-ring PAHs. Presumably, the reduction in the mutagenic potential of soil extracts was due to the degradation of other mutagenic components in the soil by the indigenous microflora when stimulated with yeast extract. Yeast extract may act as a substrate for the cometabolism of these soil contaminants.

In contrast to the indigenous microflora soil cultures, significant decreases in the concentration of all PAHs were observed when PAH-contaminated soil was inoculated with CYEM-grown community five with or without yeast extract supplementation. The decrease in the concentration of PAHs corresponded to a decrease in the mutagenic potential of soil extracts. After 91 days, the mutagenic potential of soil extracts was reduced by 43% in soils inoculated with community five and by 49% in soils inoculated with community five supplemented with yeast extract. Similar results were observed by Aprill *et al.* (1990) when wood preserving or petroleum refining wastes were applied to soil; the decrease in PAH concentration corresponded to a reduction in the mutagenic potential of soil extracts. Aprill *et al.* (1990) accounted for the decrease in mutagenic potential of soils by the apparent decrease in the concentration of the carcinogenic and co-carcinogenic PAHs. Microbial transformations may reduce the toxicity of a compound by breaking an aromatic ring, removing substitute groups or by substitutions that produce a less reactive product. The degradation of PAHs by community five must result in either the mineralisation of the compounds or transformation of the PAHs to by-products that exhibit decreased mutagenic properties compared to the parent compounds.

5.5.2.2 *Toxicity*

Although no significant decrease in the mutagenic potential of soil extracts was observed when PAH-contaminated soil was incubated with the indigenous microflora, a seven-fold reduction in the toxicity of the water soluble fraction of the soil occurred after 91 days. The reduction in toxicity was enhanced when the soil was supplemented with yeast extract (17-fold reduction). The main focus of this study was to determine the PAH-degradative capabilities of the indigenous and augmented microorganisms in contaminated soil and how this affects the mutagenic potential and toxicity of soil

extracts. However, the soil used in this study contained a variety of other contaminants. These compounds also exert toxic and mutagenic responses. Although the indigenous microflora were limited in their ability to degrade the PAH component of the soil, the microorganisms reduced the toxicity of the water soluble fraction. This may be due to the preferential degradation of some of the more water soluble contaminants of the soil.

Inoculation of community five into PAH-contaminated soil significantly enhanced the detoxification of the water soluble fraction of the soil. EC_{50}s increased 170-fold and 184-fold after 91 days for soils inoculated with community five and community five supplemented with yeast extract respectively. The increase in the rate of detoxification by community five compared to the indigenous microflora is most likely due to the broad substrate range of community five (see Chapter 3) and its ability to degrade the more water soluble contaminants of the soil as well as PAHs. The degradation of hazardous wastes may not always result in the detoxification of the compounds. Aprill *et al.* (1990) observed an increase in toxicity of the water soluble fraction of soil amended with petroleum separator sludge waste. Although a 29% decrease in the concentration of the separator sludge was observed after 340 days, the toxicity increased 4.5-fold. Aprill *et al.* (1990) suggested that the accumulation of polar metabolites of the petroleum separator sludge waste contributed to the increase in toxicity.

The results from the PAH degradation studies in soil as well as the liquid culture results (see Chapter 4) demonstrate that community five possesses a number of characteristics that are essential for successful bioaugmentation of PAH-contaminated sites. These characteristics include:

(i) a broad substrate range;
(ii) the ability to degrade high and low molecular weight PAHs as individual compounds or as part of a PAH mixture;
(iii) the production of low concentrations of water soluble by-products from the degradation of PAHs;
(iv) the ability to reduce the mutagenic potential of PAH-contaminated soil;
(v) the ability to reduce the toxicity of the water soluble fraction of PAH-contaminated soil; and
(vi) the ability to maintain a high microbial population when inoculated into soil.

5.5.3 Comparison of PAH Degradation Rates in Liquid and Soil Cultures

Although soil is generally regarded as a poor matrix for PAH degradation, no data exists which gives a proper comparison of PAH degradation rates between liquid and soil media. This type of information is important given that most published work on PAH degradation is performed in liquid culture with the potential application being for soil remediation. In this work the specific PAH degradation rate [mg PAH degraded/(10^{10} cells)/day] in the two media types was determined. It was found that microbial activity toward benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation was 10-17 times greater in liquid medium compared to the soil matrix. Presumably the principal reasons for this difference are poor oxygen diffusion rates, the presence of other organic material in the soil, sorption of the PAHs to soil particles and nutrient availability.

PAH degradation rates are highly influenced by oxygen availability. Hambrick *et al.* (1980) observed that hydrocarbon degradation rates decreased with decreasing redox potential. After 35 days incubation at a redox potential of -220 mV, only 0.4% of added ^{14}C-naphthalene was mineralised. However, when oxygen was introduced to the incubation vessel and the redox potential was increased to +130 mV, 22.6% of the naphthalene was mineralised after 35 days. Milhelcic and Luthy (1988) also observed that the degradation of naphthalene and acenaphthene by indigenous soil microorganisms increased when the cultures were incubated under aerobic conditions compared to anaerobic conditions. Soil texture, or porosity, can significantly influence the oxygen availability and oxygen diffusion rates. In soils containing clay aggregates, micro-environments may form providing a localised area for microbial activity. Soils containing high clay contents may also be subject to water logging which limits oxygen diffusion rates. In contrast, in laboratory studies using liquid media, aeration is often supplied by incubating the cultures on a shaking incubator, which adequately supplied oxygen to the entire culture.

Organic material in the soil may inhibit PAH degradation due to the microorganisms utilising these compounds as a growth source or by limiting the bioavailability of the PAHs. PAHs are hydrophobic compounds and tend to sorb onto soil organic material. This may limit the availability of the compounds to microbial attack and ultimately reduces the degradation rate (Rasaih *et al.*, 1992).

Nutrient availability, in particular nitrogen and phosphorus, can inhibit the rate and extent of PAH degradation. Contaminated soils and water are usually nutrient limited

and therefore fertiliser applications are necessary for the stimulation of PAH degradation. One of the most publicised examples of the enhancement of bioremediation by nutrient application was the clean up of crude oil contaminated coastline as a consequence of the Exxon Valdez oil spill in Prince William Sound, Alaska (Pritchard *et al.*, 1992).

Some of these limitations with soil can be addressed by surfactant or compost addition which is an economical means of improving PAH degradation rates (Aronstein *et al.*, 1991; Thibault *et al.*, 1996; Kastner and Mahro, 1996). The addition of surfactants can increase the bioavailability of PAHs by increasing the solubility of the PAHs in the aqueous phase. Surfactants have been shown to enhance desorption and solubilisation of PAHs with appreciable desorption in excess of the critical micelle concentration (Wilson and Jones, 1993). Thibault *et al.* (1996) demonstrated that Witconol SN70, a non ionic surfactant, was effective in partitioning pyrene into the hydrophobic core of the micelle. The enhanced solubilisation of pyrene resulted in increased pyrene degradation rates by the inoculated pyrene-degrading microorganisms. Phenanthrene degradation rates in mineral soils by indigenous microorganisms were also enhanced in the presence of the non-ionic alcohol ethoxylate surfactant Alfonic 810-60 (Aronstein *et al.*, 1991). Nearly 50% of the added phenanthrene was mineralised after 495 hours in the presence of the surfactant compared to only 4.8% in the absence of the surfactant.

Kastner and Mahro (1996) described the enhanced degradation of PAHs by the addition of a organic matrix of compost. The compost used by Kastner and Mahro (1996) was thought to improve PAH degradation (phenanthrene, anthracene, fluoranthene and pyrene) by cometabolic or unspecified oxidative metabolism since the compost microflora was lacking in PAH degraders. However, stimulation of the PAH degradation rate by the compost would only occur for the PAH compounds that can be degraded by the indigenous microflora. The degradation of high molecular weight PAHs such as benzo[*a*]pyrene and dibenz[*a,h*]anthracene would not be expected to be increased.

These results suggest that effective remediation of soil contaminated with PAHs may be achieved by a combination of composting, surfactant addition and augmentation with high molecular weight PAH-degraders. The present study demonstrates the efficacy of inoculating PAH-contaminated soil with high molecular weight PAH-degrading bacteria for the degradation of all PAH compounds. However, it is recognised that the degradation performance of any inoculum will depend on soil types and other environmental conditions which may not be easily controlled in the field.

5.5.4 Conclusion

Research reported in this chapter demonstrated the degradation of high molecular weight PAHs in a soil matrix by community five. The decrease in the concentration of PAHs in the PAH-contaminated soil resulted in a reduction in mutagenic potential of organic soil extracts and a reduction in toxicity of aqueous soil extracts. Furthermore, it was demonstrated that community five could maintain high microbial numbers when inoculated into PAH-contaminated soil. The following chapter reports on the isolation and identification of metabolites from pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation by *St. maltophilia* strains.

CHAPTER 6

KINETICS OF PAH DEGRADATION AND METABOLITE FORMATION

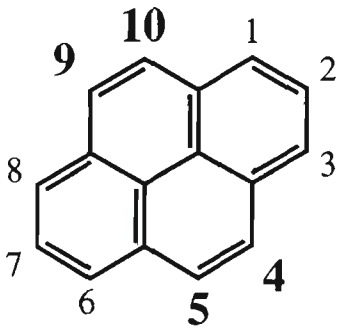
CHAPTER 6

KINETICS OF PAH DEGRADATION AND METABOLITE FORMATION

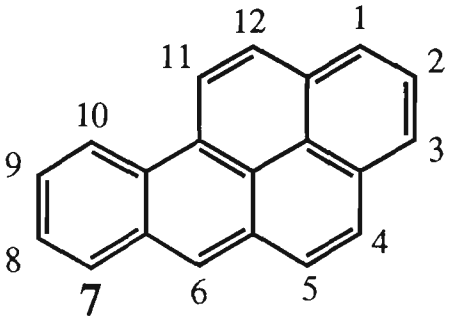
6.1 INTRODUCTION

Although the physiology of PAH biodegradation has been studied extensively, the biochemical principals underlying microbial PAH catabolism has been studied to a lesser extent. The ultimate aim of bioremediation is to reduce the potential toxicity of environmental contaminants by degrading them to harmless constituents such as carbon dioxide and water (Wilson and Jones, 1993). However, the biological degradation of PAH compounds often results in the partial degradation of the compounds, hence the formation and accumulation of intermediate products. Metabolites arising from PAH catabolism including dihydrodiols, phenols and arene oxides have been identified as having carcinogenic and mutagenic properties (Datta and Samanta, 1988). As such, these metabolites pose an even greater risk to the environment than the parent compounds due to their increased polarity, water solubility and mobility. The determination of PAH degradation pathways will lead to improved predictions of the detoxification of PAH compounds and the fate of metabolites in the environment. Identifying and understanding the biotransformation and biodegradation of these compounds will contribute to improved control, assessment and bioremediation performance, which will result in efficient and effective biological processes for the decontamination of PAH-polluted sites.

The microbial degradation of low molecular weight PAHs such as naphthalene (Kiyohara and Nagao, 1978), fluorene (Monna *et al.*, 1993; Trenz *et al.*, 1994; Grifoll *et al.*, 1992, 1994, 1995; Casellas *et al.*, 1997) and phenanthrene (Kiyohara and Nagao, 1978; Guerin and Jones, 1988b) have been well documented and the biochemical pathways have been elucidated. However, less is known about the metabolism of higher molecular weight PAHs such as pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene due to the scarcity of microorganisms capable of degrading these compounds. The few studies that have been concerned with determining the degradative pathway of pyrene have involved the catabolic capabilities of Gram positive microorganisms, namely *Mycobacterium* species (Heitkamp *et al.*, 1988b), *Mycobacterium* strain RJGII-135 (Schneider *et al.*, 1996) and *Rhodococcus* strain UW1 (Walter *et al.*, 1991). The initial oxidation products of benzo[*a*]pyrene have been



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



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

Figure 6.1. The position and number of labelled ^{14}C on pyrene and benzo[*a*]pyrene used in radiolabelled degradation experiments. The bold numerals indicate the position of the labelled carbons.

determined (Schneider *et al.*, 1996; Gibson *et al.*, 1975), however, metabolic intermediates from the degradation of dibenz[*a,h*]anthracene have not been identified.

The results from Chapter 4 illustrated that the isolated *St. maltophilia* strains VUN 10,001, VUN 10,002 and VUN 10,003 were capable of utilising pyrene as a growth substrate; the degradation of pyrene resulted in a concomitant increase in cellular protein and the accumulation of low concentrations of RE intermediate compounds. Although the *St. maltophilia* strains were not capable of growing on the five-ring compounds as sole carbon and energy sources, pure cultures were shown to degrade benzo[*a*]pyrene and dibenz[*a,h*]anthracene when inocula contained high cell numbers. The ability of strains VUN 10,001, VUN 10,002 and VUN 10,003 to degrade the high molecular weight PAHs provided an opportunity to explore and understand the biological mechanisms involved in the degradation of these compounds. The research described in this chapter was undertaken to take some preliminary steps towards defining the biochemical pathways that are involved in catabolism of high molecular weight PAHs by *St. maltophilia.*

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

1. determine the extent to which the strains described in Chapter 3 degraded pyrene and benzo[*a*]pyrene by using radiolabelled PAH compounds; and

2. define some catabolic steps in the metabolic pathways involved in the degradation of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene by strains VUN 10,001, VUN 10,002 and VUN 10,003.

6.2 EXPERIMENTAL APPROACH TO DETERMINING DEGRADATION OF RADIOLABELLED PAHs

The fate of biodegraded PAHs can be investigated by performing degradation experiments using PAHs containing ^{14}C-labelled carbon. This method provides a means for determining the distribution and quantity of ^{14}C in culture fractions (*e.g.* gaseous, aqueous or organic phases and cells) so that the proportion of PAH mineralised (converted to CO_2), converted to polar metabolites and incorporated into cellular material can be established. Figure 6.1 illustrates the positions of the ^{14}C labels of pyrene and benzo[a]pyrene used in the work described in this chapter.

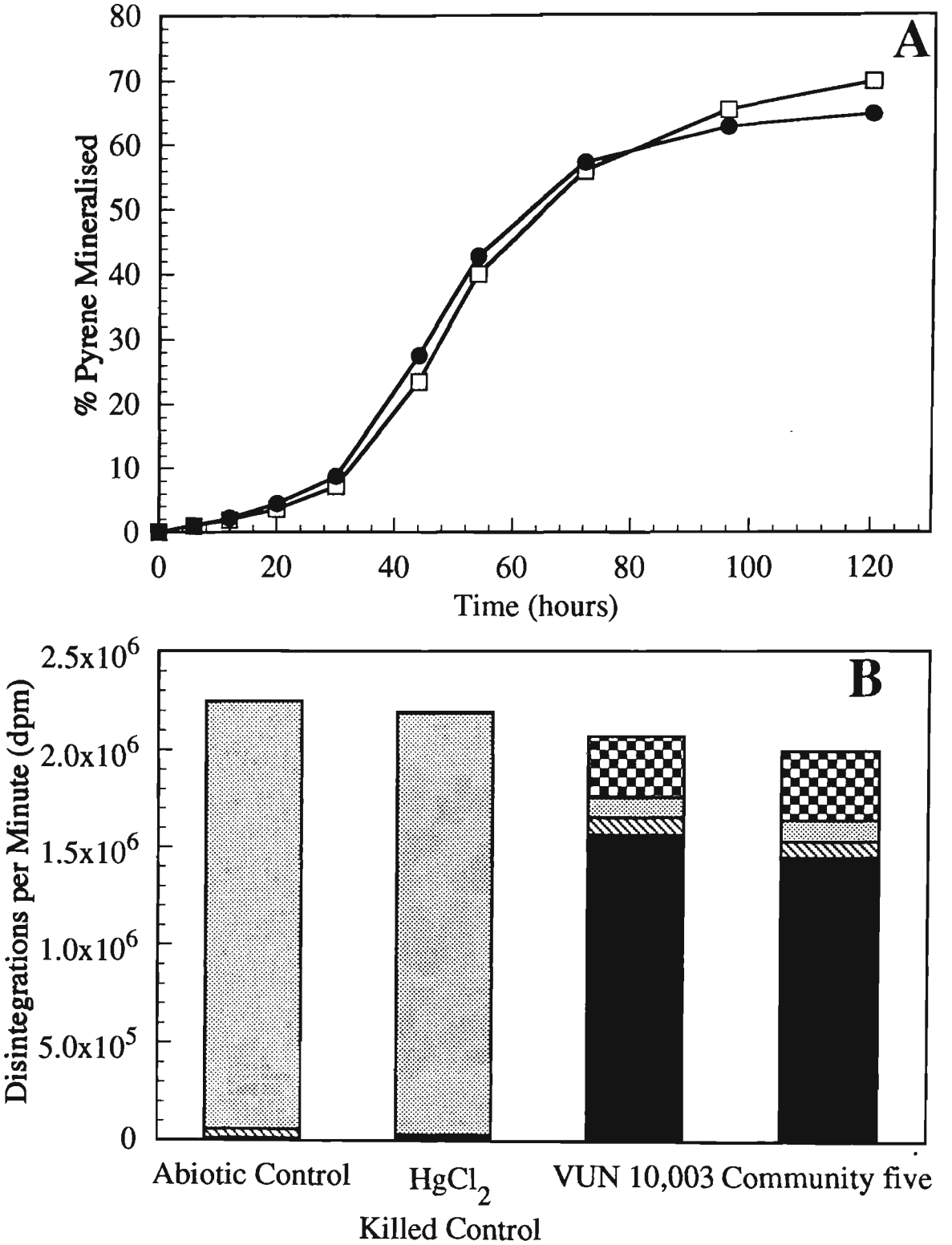


Figure 6.2. Fate of ^{14}C-pyrene (A) following incubation of community five (●) and strain VUN 10,003 (□) in BSM containing 250 mg/l of unlabelled pyrene and 1.0 μCi of [4,5,9,10-^{14}C]-pyrene. Community five and strain VUN 10,003 were inoculated into the medium after growth on pyrene to achieve a final population size of 9.5 x 10^7 cells/ml. The distribution of the recovered labelled carbon (B) in the organic phase (▩), aqueous phase (▨), gaseous phase (■) and cell debris (◪) was determined at the end of the incubation period (120 hours).

Degradation experiments were prepared in biometer flasks, containing a carbon dioxide trap (NaOH) in a side arm tube. The flasks enable carbon dioxide production to be monitored at frequent intervals for prolonged periods. Inocula containing high cell numbers of either community five or strain VUN 10,003 suspended in BSM were added to flasks containing 250 mg/l of unlabelled pyrene or 50 mg/l of unlabelled benzo[*a*]pyrene. The respective cultures were supplemented with 1.0 μCi of [4,5,9,10-^{14}C]-pyrene (58.7 mCi/mmol) or 7-^{14}C-benzo[a]pyrene (26.6 mCi/mmol). The production of $^{14}CO_2$ over the incubation period was monitored by assaying the NaOH ($^{14}CO_2$ trap) for radioactivity. Fresh NaOH was added to the side arm tube after each sample. At the end of the incubation period, the distribution of ^{14}C in the aqueous phase, organic phase and cells was also determined (see Section 2.7.8). The distribution of ^{14}C-residues was calculated with reference to the total radioactivity recovered from the respective control samples.

Pyrene degradation experiments were also prepared in contaminated soil to assess the degradation potential of the indigenous microflora and inoculated microorganisms. Soils (20 g) were added to biometer flasks and inoculated with community five (2 ml). The cultures were supplemented with 1.0 μCi of [4,5,9,10-^{14}C]-pyrene (58.7 mCi/mmol) and the production of $^{14}CO_2$ was monitored over the incubation period (48 days). After incubation, the soils were extracted with dichloromethane to determine the residual ^{14}C.

6.2.1 ^{14}C-Pyrene Degradation

Pyrene was rapidly mineralised to $^{14}CO_2$ by community five and strain VUN 10,003 in liquid culture after an initial lag period of 10 hours. The pyrene-grown cultures of community five and strain VUN 10,003 mineralised 64.5% and 70.5% of the supplied pyrene respectively after 120 hours (Figure 6.2). The distribution of the remaining labelled carbon into the organic phase, aqueous phase and cell debris was similar for community five and strain VUN 10,003: 5.3-5.5% of the ^{14}C was recovered in the organic phase, 4.1% in the aqueous phase and 17.4-17.8% of the ^{14}C was recovered from the cell debris (Figure 6.2). These results are indicative of the low amount of polar and non-polar metabolites produced by these cultures. The higher amount of labelled carbon detected in the cell debris is an indication of the proportion of pyrene carbon that was incorporated into the cellular material. Abiotic pyrene degradation was found to be minimal: labelled carbon from the uninoculated cultures or those containing mercuric chloride killed inocula was detected in small amounts in the aqueous phase

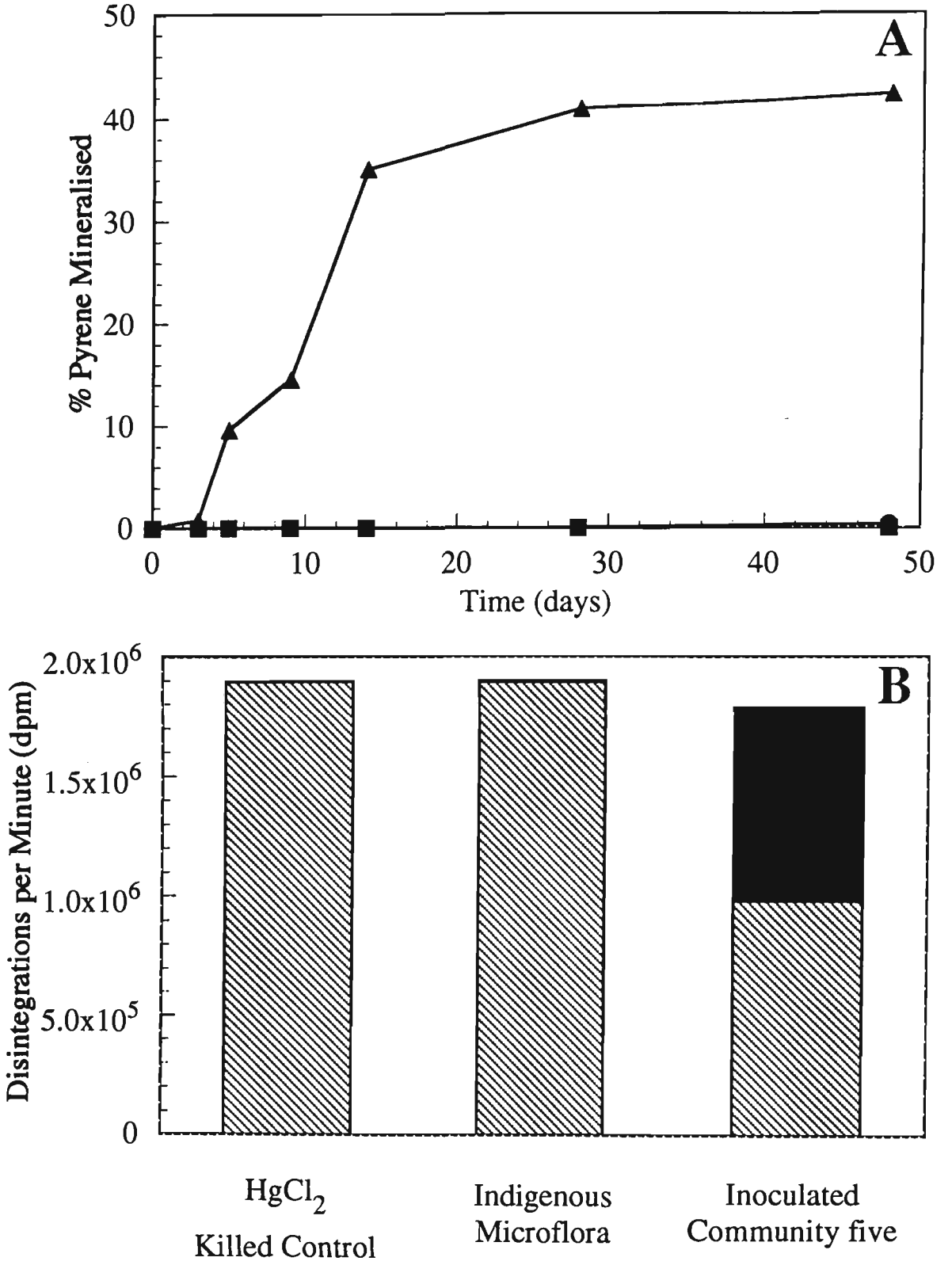


Figure 6.3. Fate of ^{14}C-pyrene (A) in PAH-contaminated soil after incubation with the indigenous soil microflora (●) and inoculated community five (▲). Community five was inoculated into PAH-contaminated soil spiked with 1.0 µCi of [4,5,9,10-^{14}C]-pyrene. CYEM-grown community five was added to achieve a final population size of 7.5 x 10^7 cell/g soil. The evolution of $^{14}CO_2$ in mercuric chloride killed control cultures, containing community five (■), is also shown. The distribution of the recovered labelled carbon in the organic phase (▨) and the gaseous phase (■) after 48 days is shown (B).

(0.9-2.1%) and as $^{14}CO_2$ (0.5-0.6%); 88.8 to 96.4% of the radioactivity was recovered from the inoculated flasks with reference to the uninoculated cultures.

The degradation of pyrene by indigenous soil microorganisms and inoculated pyrene-degrading microorganisms was assessed in PAH-contaminated soil (Figure 6.3). Control soils consisted of inoculated soils killed with mercuric chloride. Abiotic degradation of pyrene in control soils was negligible; 0.06% of the label was detected as $^{14}CO_2$. Degradation of pyrene by the indigenous microorganisms was also minimal: approximately 0.5% of the label was detected as $^{14}CO_2$. Pyrene was mineralised by community five after an initial lag period of three days. The CYEM-grown community five mineralised 40% of the pyrene to $^{14}CO_2$ after 28 days (Figure 6.3). After 48 days, pyrene mineralisation increased marginally to 42%. The remaining label was recovered from the soil matrix; 94% of the radioactivity was recovered from the inoculated flasks with reference to the mercuric chloride killed cultures.

6.2.2 ^{14}C-Benzo[*a*]pyrene Degradation

Degradation experiments using benzo[*a*]pyrene, inoculated with community five and strain VUN 10,003 demonstrated that less than 0.25% of the total radioactivity was recovered as $^{14}CO_2$ after 70 days (Figure 6.4). The majority of the ^{14}C (81.7-83.1%) was recovered from the organic phase as undegraded substrate and/or non-polar extractable metabolites. The distribution of the remaining labelled carbon into the aqueous phase and cell debris was similar for community five and strain VUN 10,003: 4.8-6.5% of the ^{14}C was recovered in the aqueous phase and 11.6-12.0% of the ^{14}C was recovered from the cell debris (Figure 6.4). Abiotic benzo[*a*]pyrene degradation was found to be minimal. Labelled carbon from the uninoculated cultures or those containing mercuric chloride killed inocula, was detected in small amounts in the aqueous phase (0.5-0.6%), cell debris (0.5%) and as $^{14}CO_2$ (0.1%); 90.7% of the radioactivity was recovered from the inoculated flasks with reference to the uninoculated cultures.

6.3 ISOLATION OF PAH METABOLITES BY THIN LAYER CHROMATOGRAPHY (TLC)

The small quantities of metabolites produced from the degradation of pyrene and benzo[*a*]pyrene from the cultures in section 6.2.1 and 6.2.2 were not sufficient for the isolation and identification of these compounds. For the isolation of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites, large scale cultures (4 litres) of

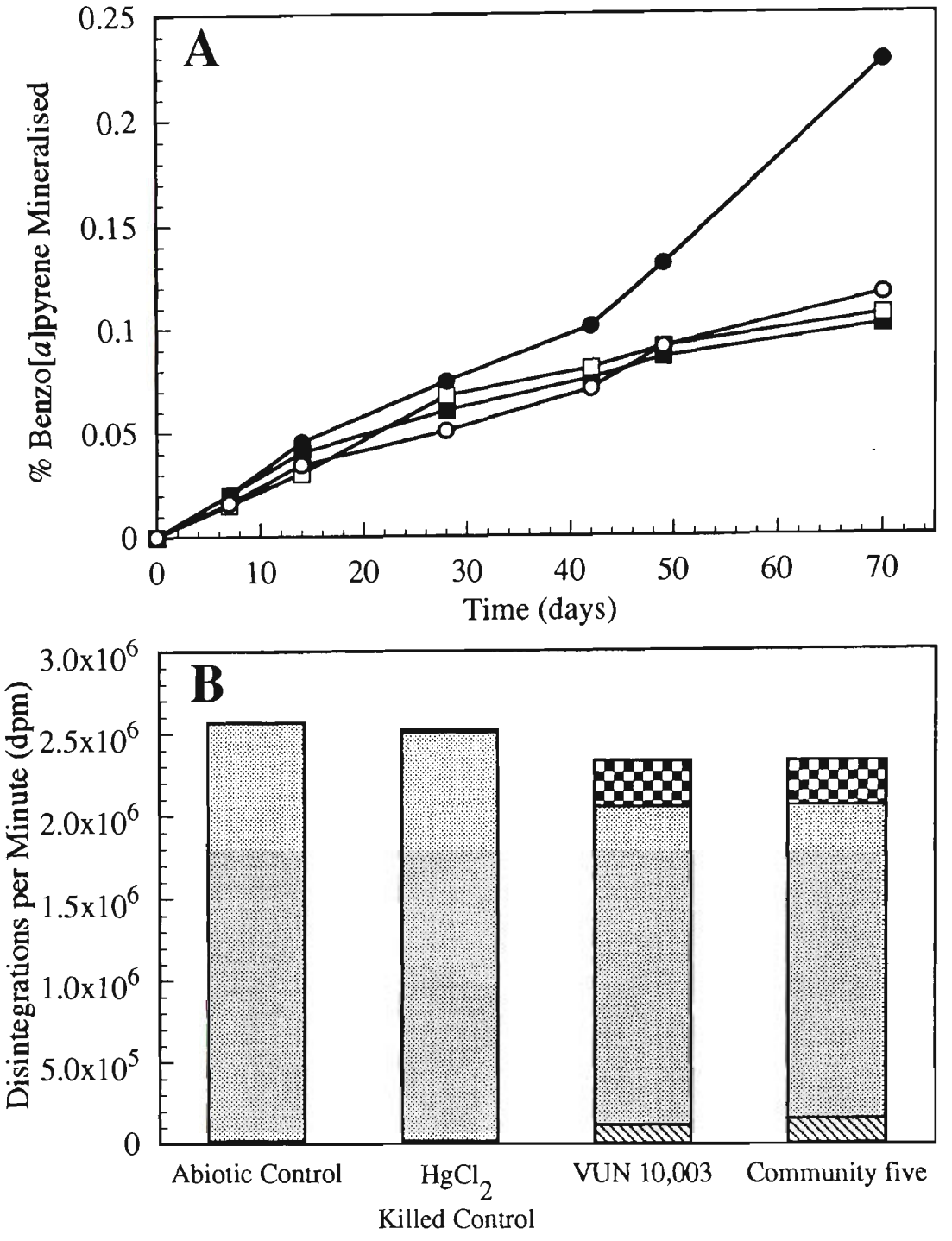


Figure 6.4. Fate of ^{14}C-benzo[*a*]pyrene (A) following incubation of community five (●) and strain VUN 10,003 (O) in BSM containing 50 mg/l of unlabelled benzo[*a*]pyrene and 1.0 µCi of 7-^{14}C-benzo[*a*]pyrene. Community five and strain VUN 10,003 were inoculated into the medium after growth on pyrene to achieve a final population size of 9.5 x 10^7 cells/ml. The evolution of $^{14}CO_2$ by abiotic (■) and mercuric chloride killed cell controls (□) is also shown. The distribution of the recovered labelled carbon in the organic phase (▨), aqueous phase (▧), gaseous phase (■) and cell debris (▪) after 70 days is shown (B).

strains VUN 10,001, VUN 10,002 and VUN 10,003 were prepared with the respective unlabelled PAH. Culture broths were collected and extracted with dichloromethane to recover water soluble metabolites resulting from the degradation of the parent compound. Samples were taken at various stages during the incubations to differentiate between transitory metabolites and those that accumulated over the entire incubation period. Supernatants were extracted at neutral pH as well as at pH 2, in order to recover acidic metabolites. Previous work has demonstrated that the acidification of culture supernatants facilitates greater extraction efficiency of acid metabolites such as phthalic acid, succinic acid and salicylic acid (Heitkamp *et al.*, 1988b; Guerin and Jones, 1988b).

The crude extracts were pooled, vacuum concentrated and developed on silica gel 60 TLC plates in order to separate, visualise and purify PAH metabolites. Metabolite separation was achieved using a three-phase solvent system. Undegraded PAH was separated from non-polar and more polar metabilotes using a benzene:hexane mixture (1:1, v:v). Ring oxidation products were eluted using hexane:acetone (8:2, v:v), while benzene:acetone:acetic acid (85:15:5, v:v:v) was used to separate the highly polar metabolites (Guerin and Jones, 1988b).

6.3.1 Metabolites of Pyrene Degradation

Over 20 different metabolite bands were resolved by TLC from the crude pyrene supernatant extracts of strains VUN 10,001, VUN 10,002 and VUN 10,003. The chromatographic mobility (R*f*) of individual metabolites varied from 0.22, for the most polar compound to 0.96 for ring oxidation products or non-polar metabolites. "Major" metabolites were identified by a high band intensity and large band size on the TLC plates. Although there was a slight difference in the number and intensity of metabolite bands seen for each strain, all three accumulated similar major metabolites from the degradation of pyrene. Metabolite banding profiles changed over the incubation period with the appearance and disappearance of various compounds (Figure 6.5). Seven major metabolites were isolated, excised from the TLC plates and purified by preparative TLC (Figure 6.6). All seven metabolites were still detected at the end of the incubation period. Table 6.1 shows the R*f* values and colour of purified pyrene metabolites.

Table 6.1. *Rf* values and colour of metabolites isolated from the degradation of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 by preparative TLC.

	Metabolites purified from pyrene degradation:	
Metabolite Number	*Rf* Value	Colour[a]
M5	0.22	yellow
M6	0.28	white
M9	0.53	purple
M12	0.71	yellow
M14	0.81	blue
M15	0.91	blue
M16	0.96	pale yellow

[a] The colour of metabolites was observed under UV light (302 nm).

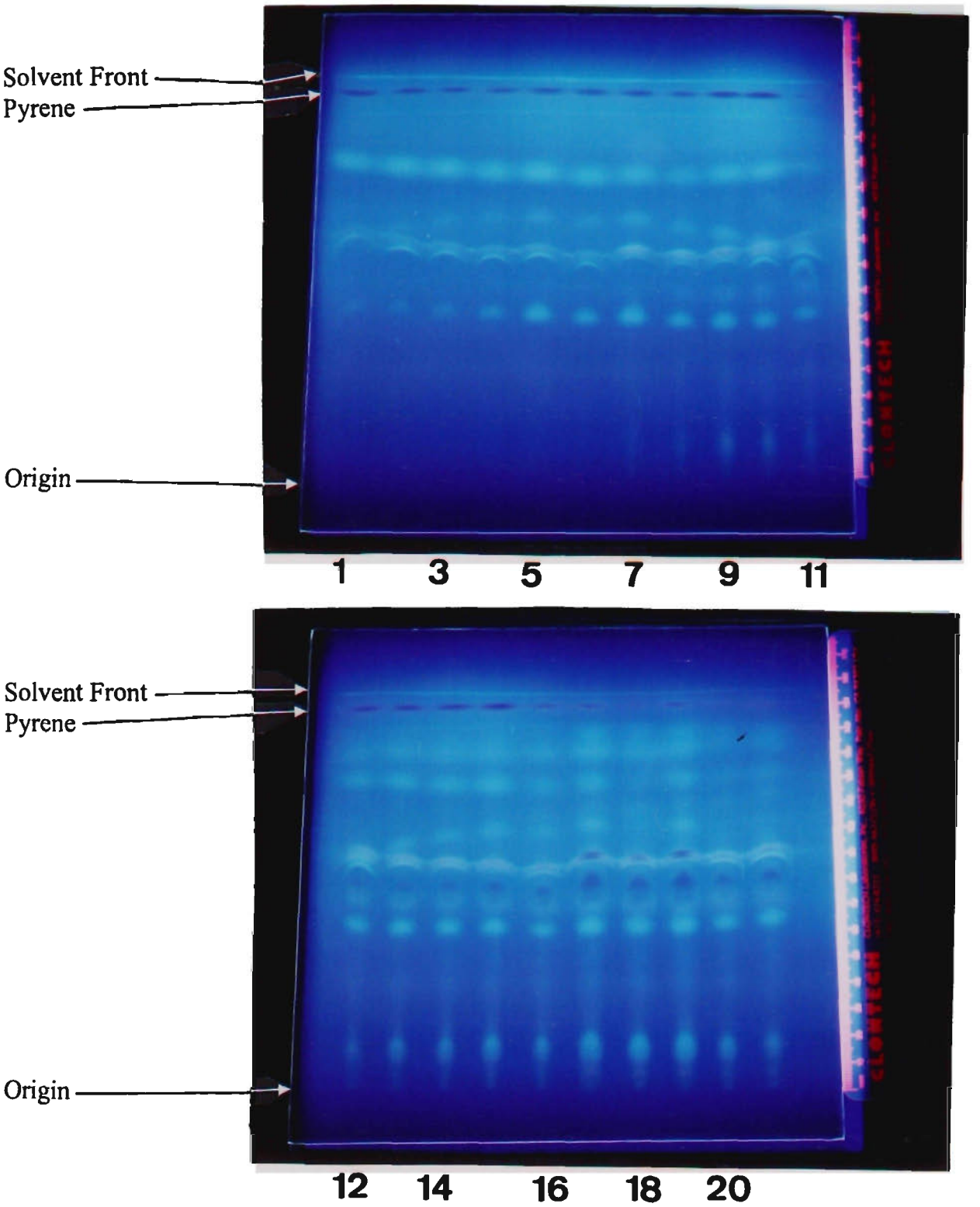


Figure 6.5. TLC separation and visualisation (302 nm) of pyrene metabolites extracted from pyrene medium inoculated with strain VUN 10,003. Culture fluid was removed and extracted at regular intervals over a 120 hour incubation period. Extracts (5 µl) were applied at the origin and metabolites were separated using a three phase solvent system; benzene:hexane (1:1 v/v), hexane:acetone (8:2 v/v) and benzene:acetone:acetic acid (85:15:5 v/v/v).

Lane 1: 4 h	Lane 5: 20 h	Lane 9: 36 h	Lane 13: 52 h	Lane 16: 64 h	Lane 19: 96 h
Lane 2: 8 h	Lane 6: 24 h	Lane 10: 40 h	Lane 14: 56 h	Lane 17: 68 h	Lane 20: 120 h
Lane 3: 12 h	Lane 7: 28 h	Lane 11: 44 h	Lane 15: 60 h	Lane 18: 72 h	Lane 21: 120 h
Lane 4: 16 h	Lane 8: 32 h	Lane 12: 48 h			

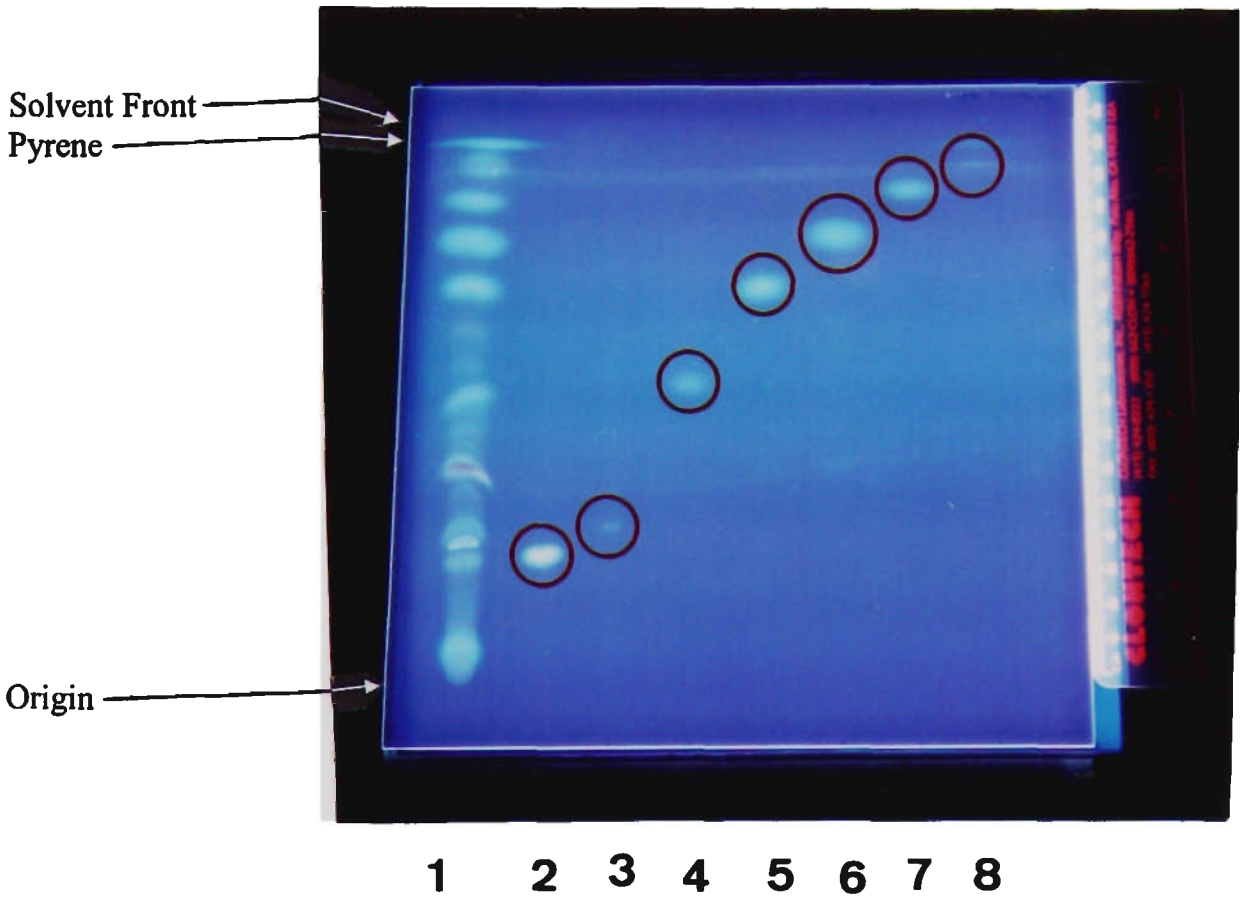


Figure 6.6. TLC of strain VUN 10,003 pyrene crude extract (120 hours) and purified pyrene metabolites. The crude extract and pyrene metabolites (5 µl) were applied at the origin and separated using the three phase solvent system described in Figure 6.5. Metabolites were observed under UV light (302 nm).

Lane 1: crude extract (120 hours)	Lane 5: M12
Lane 2: M5	Lane 6: M14
Lane 3: M6	Lane 7: M15
Lane 4: M9	Lane 8: M16

6.3.2 Metabolites of Benzo[*a*]pyrene Degradation

The degradation of benzo[*a*]pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 produced approximately 15 distinct metabolite bands on the TLC plates when viewed inder UV light (Figure 6.7). Nine metabolites (Figure 6.7), with chromatographic mobilities (R*f*) ranging from 0.19 to 0.97 were purified from the benzo[*a*]pyrene crude extracts. No benzo[*a*]pyrene metabolites were observed from the samples taken after two weeks incubation, however, subsequent samples (taken after four, six, eight, 10 and 12 weeks) showed intermediate products. The number of benzo[*a*]pyrene intermediate products observed did not change over the remaining incubation period, but their intensity did increase. The R*f* values and colour of purified benzo[*a*]pyrene metabolites are shown in Table 6.2.

6.3.2 Metabolites of Dibenz[*a,h*]anthracene Degradation

The degradation of dibenz[*a,h*]anthracene by strains VUN 10,001, VUN 10,002 and VUN 10,003 produced approximately 15 distinct metabolite bands on the TLC plates (Figure 6.8). Eight metabolites (Figure 6.8), with chromatographic mobilities (R*f*) ranging from 0.19 to 0.99 were purified from the dibenz[*a,h*]anthracene crude extracts. Similarly to benzo[a]pyrene cultures, no dibenz[*a,h*]anthracene metabolites were observed from the two week sample, however, subsequent samples (taken after four, six, eight, 10 and 12 weeks) showed intermediate products. The number of dibenz[*a,h*]anthracene intermediate products observed did not change over the remaining incubation period, but their intensity did increase. The R*f* values and colour of purified dibenz[*a,h*]anthracene metabolites are outlined in Table 6.2.

6.4 IDENTIFICATION OF PAH METABOLITES

6.4.1 HPLC Analysis of PAH Metabolites

The purified PAH metabolites described in Section 6.3 were analysed by reverse phase high performance liquid chromatography (HPLC). The aims of the HPLC analysis were three-fold. Firstly, to check the purity of the isolated compounds as HPLC provides better resolution and separation than TLC. Secondly, by matching the retention times of the isolated PAH metabolites to commercially available suspected PAH intermediate compounds, an insight into the compounds identity would be assertained. Finally, by determining the absorption spectra of the PAH intermediate compounds, specific absorption peaks can be related to known structures.

Table 6.2. R*f* values and colour of metabolites isolated from the degradation of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene by strains VUN 10,001, VUN 10,002 and VUN 10,003 by preparative TLC.

	Metabolites purified from:			
	Benzo[*a*]pyrene		Dibenz[*a*,*h*]anthracene	
Metabolite Number	Rf Value	Colour[a]	Rf Value	Colour[a]
1	0.19	yellow	0.19	yellow
2	0.49	pale orange	0.55	pale lime
3	0.66	orange/red	0.63	orange
4	0.70	pale orange	0.83	blue
5	0.74	yellow	0.89	orange
6	0.76	blue	0.91	orange/red
7	0.89	pale lime	0.975	blue
8	0.96	blue	0.99	yellow/green
9	0.97	orange	-	-

[a] The colour of metabolites was observed under UV light (302 nm).

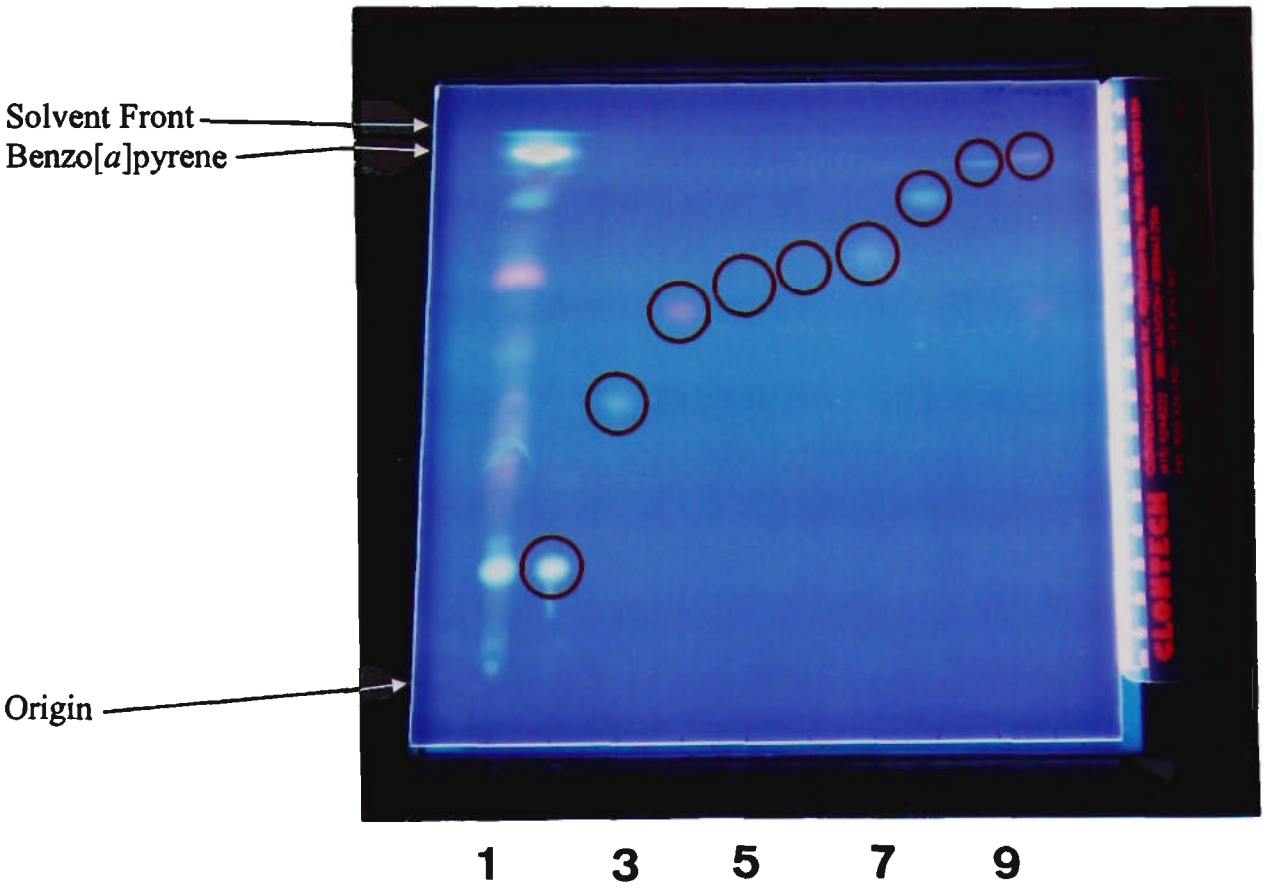


Figure 6.7. TLC of strain VUN 10,003 benzo[*a*]pyrene crude extract (12 weeks) and purified benzo[*a*]pyrene metabolites. The crude extract and benzo[*a*]pyrene metabolites (5 µl) were applied at the origin and separated using the three phase solvent system described in Figure 6.5. Metabolites were observed under UV light (302 nm).

Lane 1: crude extract (12 weeks)
Lane 2: B[a]P1
Lane 3: B[a]P2
Lane 4: B[a]P3
Lane 5: B[a]P4
Lane 6: B[a]P5
Lane 7: B[a]P6
Lane 9: B[a]P7
Lane 9: B[a]P8
Lane 10: B[a]P 9

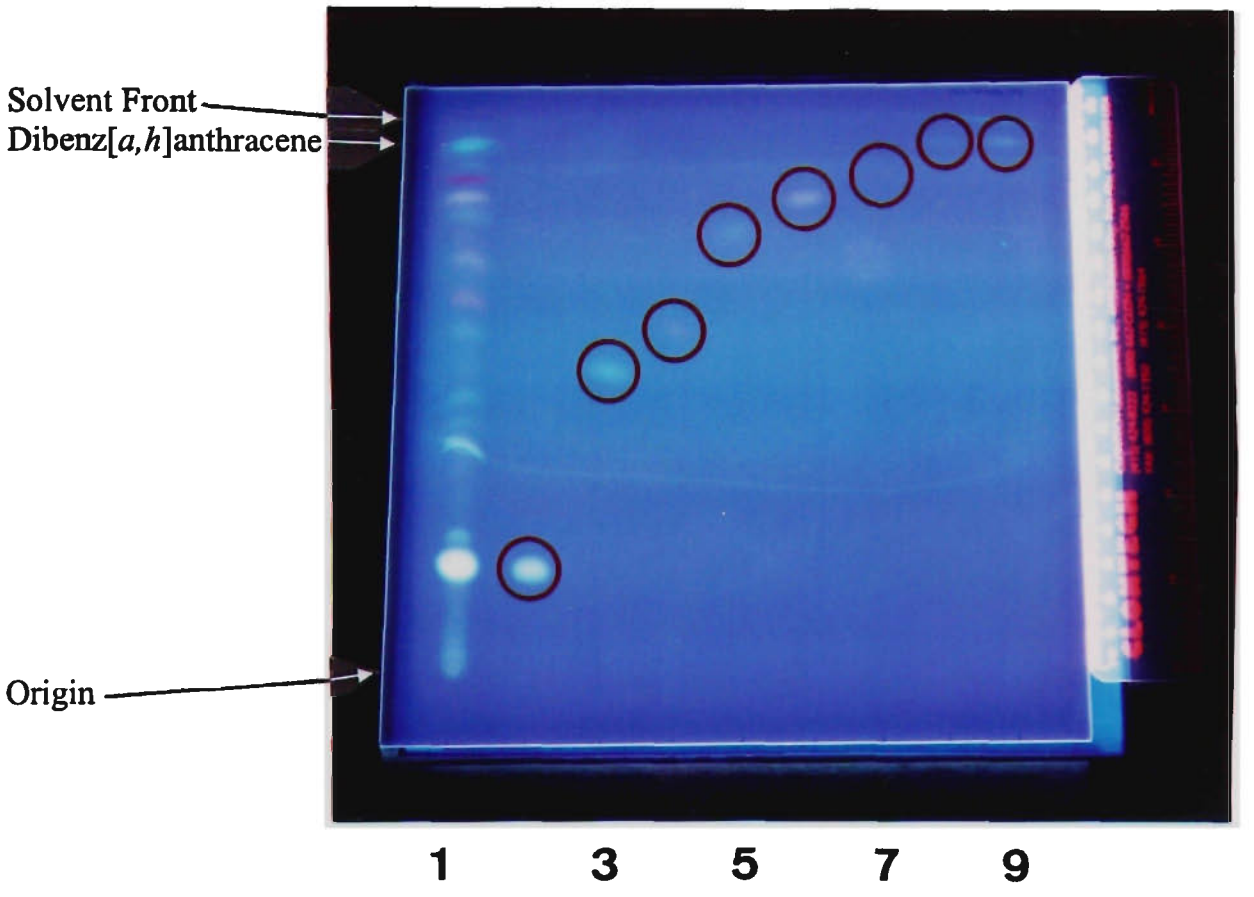


Figure 6.8. TLC of strain VUN 10,003 dibenz[*a*,*h*]anthracene crude extract (12 weeks) and purified dibenz[*a*,*h*]anthracene metabolites. The crude extract and dibenz[*a*,*h*]anthracene metabolites (5 µl) were applied at the origin and separated using the three phase solvent system described in Figure 6.5. Metabolites were observed under UV light (302 nm).

Lane 1: crude extract (12 weeks)
Lane 2: DBA1
Lane 3: DBA2
Lane 4: DBA3
Lane 5: DBA4
Lane 6: DBA5
Lane 7: DBA6
Lane 9: DBA7
Lane 9: DBA8

Separation of standards and unknown compounds was achieved using a Spherex 5 µm C18 column. PAH metabolites and standard compounds (cinnamic acid, gentisic acid, phthalic acid, salicylic acid, 1,2-dihydroxynaphthalene, 2-carboxybenzaldehyde, catechol, *p*-hydroxybenzoic acid, 1-hydroxypyrene, protocatechuic acid) were resolved using a methanol-water gradient system as the mobile phase (Heitkamp *et al.*, 1988b; Guerin and Jones, 1988b). Compounds were scanned at a wavelength between 190 and 367 nm using a diode array detector to determine the UV absorption maxima.

6.4.1.1 *HPLC Analysis of Pyrene Metabolites*

HPLC analysis of the isolated pyrene metabolites confirmed that metabolites M5, M6, M9, M12, M14 and M15 were pure; chromatograms of each of the metabolites contained only single peaks. The HPLC elution profile of these metabolites is shown in Figure 6.9. The chromatogram of metabolite M16 contained two peaks, with HPLC retention times of 29.4 and 31.2 minutes (Figure 6.10). The two compounds were assigned the reference names M16a and M16b respectively. The preliminary identification of the pyrene metabolites was not possible, as none of the metabolites had similar retention times to those of the standards (Table 6.3). No other commercially available compounds of potential pyrene metabolites were available. The absorption maxima of pyrene metabolites were determined and are shown in Table 6.4. An absorption maxima of 256 nm, as observed from metabolites M5 and M9, is indicative of aromatic chromophores. A conjugated alkene chromophore (217 nm) was observed in metabolite M12, as well as a carbonyl group (absorption maxima at 280 nm). The absorption maxima of the other pyrene metabolites were not similar to other common organic chromophores. HPLC analysis of uninoculated pyrene medium did not show any peaks with retention times matching those of the isolated pyrene metabolites (Figure 6.9).

6.4.1.2 *HPLC Analysis of Benzo[a]pyrene Metabolites*

Due to the low yield of benzo[*a*]pyrene metabolites following incubation of strains VUN 10,001, VUN 10,002 and VUN 10,003 with benzo[*a*]pyrene, HPLC analysis of the crude extracts failed to detect intermediate compounds. However, metabolites isolated by preparative TLC then concentrated resulted in low concentrations of individual benzo[*a*]pyrene metabolites which were then analysed individually by HPLC. HPLC analysis of the isolated compounds confirmed the purity of metabolites B[a]P1, B[a]P2, B[a]P3, B[a]P4, B[a]P5 and B[a]P6, however, metabolites B[a]P7,

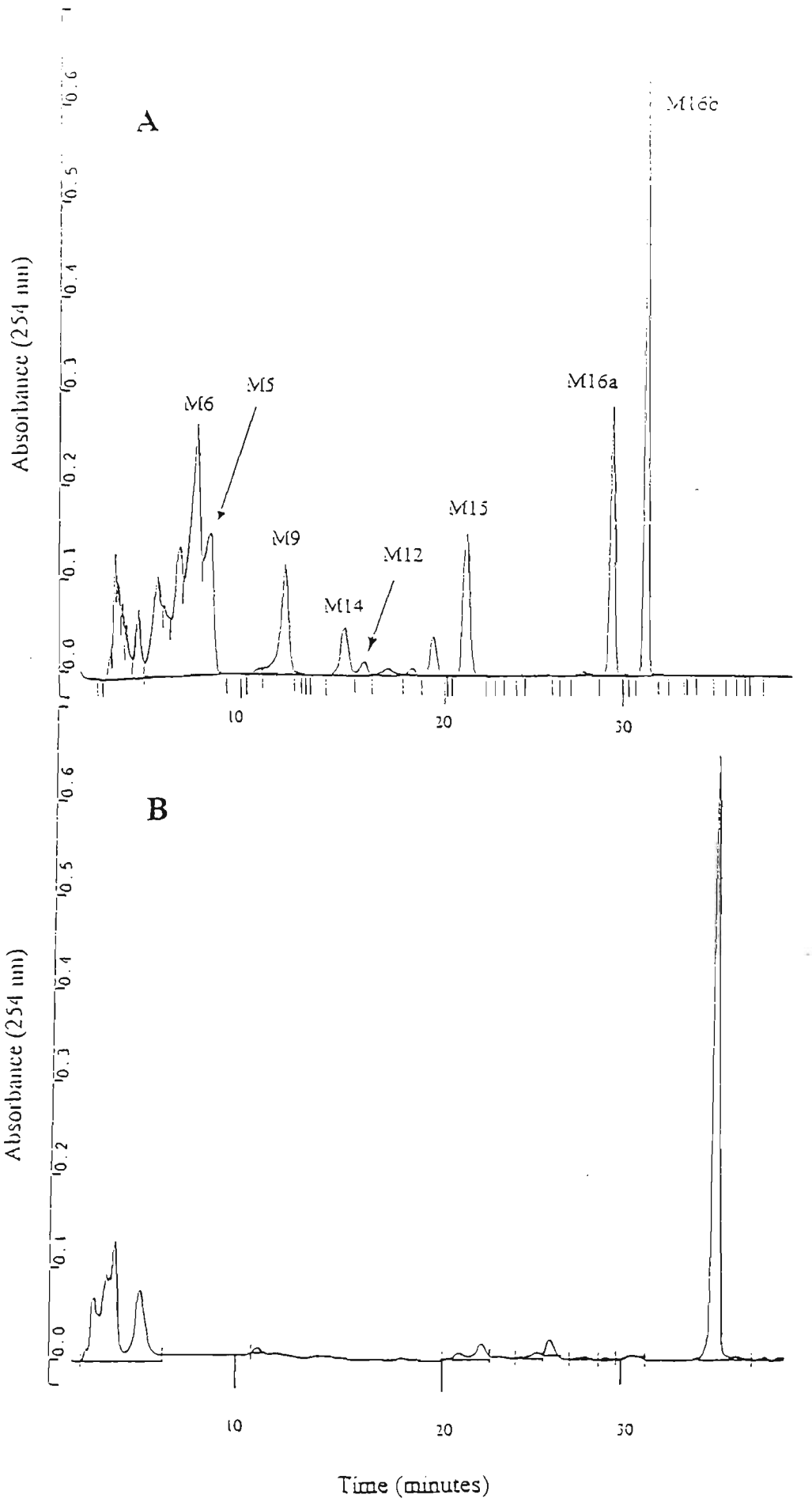


Figure 6.9. HPLC elution profile of pyrene and pyrene metabolites extracted from medium inoculated with strain VUN 10,003 (A) and uninoculated pyrene medium. Cultures were extracted after 72 hours incubation. Pyrene and the metabolites were separated on a Spherex 5 μm C18 column using a linear gradient of methanol-water (50% to 100% v/v, 30 minutes). Compounds were detected at a wavelength of 254 nm.

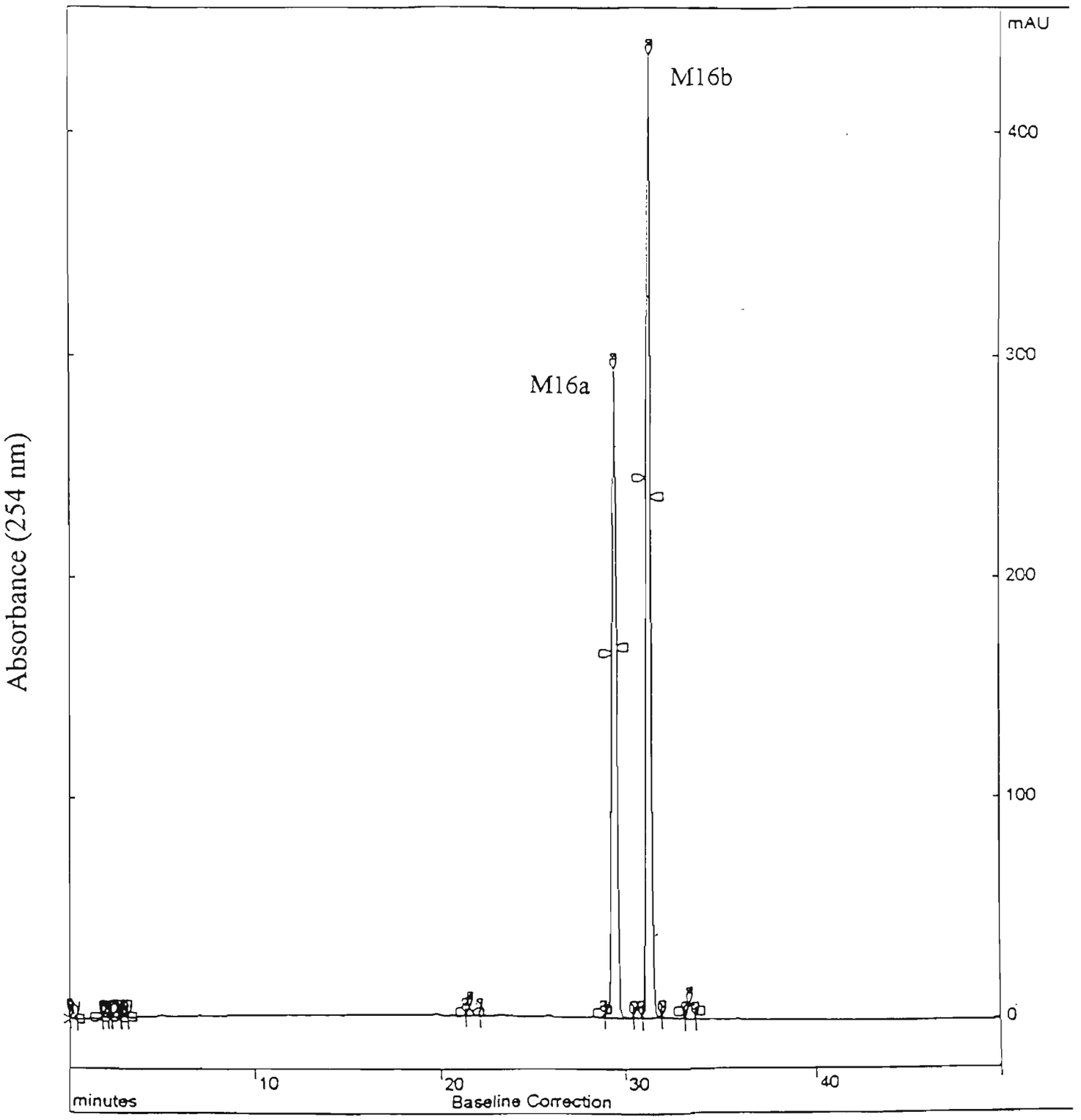


Figure 6.10. HPLC elution profile of pyrene metabolite M16 isolated from pyrene crude extracts of strain VUN 10,003. Two distinct peaks were observed after HPLC analysis of metabolite M16. They were given the reference names M16a and M16b.

Table 6.3. HPLC retention times of standards which could be produced from PAH degradation. Compounds were separated on a Spherex 5 μm C18 column using a linear gradient of methanol-water (50% to 100% v/v, 30 minutes). Compounds were detected at a wavelength of 254 nm.

Compound	Retention Time (minutes)
Catechol	10.34
2-Carboxybenzaldehyde	5.21
Cinnamic acid	15.94
1,2-Dihydroxynaphthalene	24.45
Gentisic acid	4.30
p-Hydroxybenzoic acid	5.66
1-Hydroxypyrene	32.23
Phthalic acid	4.85
Protocatechuic acid	4.97
Pyrene	35.85
Salicylic acid	8.13

Table 6.4. UV absorption maxima and retention times of purified pyrene metabolites. Metabolites were separated on a Spherex 5 μm C18 column using a linear gradient of methanol-water (50% to 100% v/v, 30 minutes). Compounds were scanned at a wavelenght between 190 and 367 nm using a diode array detector to determine the UV absorption maxima.

Metabolite	Absorption Maxima	Retention Time
M5	256.79, 348.97	7.55
M6	218.31, 270.90, 323.37	6.02
M9	256.1	11.67
M12	217.41, 253.44, 280.36	15.77
M14	264.48, 272.08, 306.78, 319.22, 356.58	14.83
M15	235.87, 248.14, 272.15, 280.16, 304.58	21.50
M16a	236.67, 259.52, 268.30, 316.93, 332.55	29.40
M16b	259.15, 282.87, 301.81, 313.90, 338.72	31.28

Table 6.5. UV absorption maxima and retention times of purified benzo[*a*]pyrene metabolites. Metabolites were separated on a Spherex 5 μm C18 column using a linear gradient of methanol-water (50% to 100% v/v, 30 minutes). Compounds were scanned at a wavelenght between 190 and 367 nm using a diode array detector to determine the UV absorption maxima.

Metabolite	Absorption Maxima	Retention Time
B[a]P 1	256.83, 331.80, 347.40, 356.49	8.92
B[a]P 2	271.74, 334.42	24.62
B[a]P 3	324.25, 343.29, 361.76	22.20
B[a]P 4	313.73, 352.13	27.21
B[a]P 5	285.09, 328.78, 339.80, 349.55, 360.75	30.28
B[a]P 6	259.08, 282.07, 302.24, 310.92	32.24
B[a]P 7	Not detected	-
B[a]P 8	Not detected	-
B[a]P 9	Not detected	-

B[a]P8 and B[a]P9 could not be detected, presumably due to their low concentrations. The retention times of the benzo[*a*]pyrene metabolites did not match any of the standard compounds which were potential metabolites of benzo[*a*]pyrene. Table 6.5 shows the absorption maxima and retention times of purified benzo[*a*]pyrene metabolites. Metabolite B[a]P1 had an absorption maxima (256 nm) similar to an aromatic chromophore. The absorption maxima of the remaining benzo[*a*]pyrene metabolites were not similar to other common organic chromophores. HPLC analysis of extracts obtained from uninoculated benzo[*a*]pyrene medium revealed only one peak which corresponded to benzo[*a*]pyrene.

6.4.1.3 *HPLC Analysis of Dibenz[a,h]anthracene Metabolites*

Due to the low yield of dibenz[*a,h*]anthracene metabolites following incubation of strains VUN 10,001, VUN 10,002 and VUN 10,003 with dibenz[*a,h*]anthracene, HPLC analysis of the crude extracts failed to detect intermediate compounds. However, metabolites isolated by preparative TLC then concentrated resulted in low concentrations of individual dibenz[*a,h*]anthracene metabolites which were then analysed individually by HPLC. HPLC analysis of the isolated compounds confirmed the purity of metabolites DBA1 and DBA5, however, metabolites DBA2, DBA3, DBA4, DBA6, DBA7 and DBA8 could not be detected, presumably due to their low concentrations. The retention times of the dibenz[*a,h*]anthracene metabolites did not match any of the standard compounds which were potential metabolites of dibenz[*a,h*]anthracene. Table 6.6 shows the absorption maxima and retention times of purified dibenz[*a,h*]anthracene metabolites. Metabolite DBA1 had an absorption maxima at 214 nm and 256 nm which are similar to an aromatic and an amido chromophore. The absorption maxima of metabolite DBA5 were not similar to other common organic chromophores. HPLC analysis of extracts obtained from uninoculated dibenz[*a,h*]anthracene medium revealed only one peak which corresponded to dibenz[*a,h*]anthracene.

6.4.2 GC-MS Analysis of PAH Metabolites

The purified PAH metabolites described in Section 6.3 were analysed by gas chromatography-mass spectrometry (GC-MS). The aim of the GC-MS analysis was to elucidate the molecular structure of the PAH intermediate compounds. Knowledge of the mass of the molecular ion and its major fragment ions is frequently sufficient to enable the structure of the parent compound to be deduced. The majority of ions produced during the initial ionisation procedure have a single positive charge, *i.e.* one

Table 6.6. UV absorption maxima and retention times of purified dibenz[*a*,*h*]anthracene metabolites. Metabolites were separated on a Spherex 5 μm C18 column using a linear gradient of methanol-water (50% to 100% v/v, 30 minutes). Compounds were scanned at a wavelenght between 190 and 367 nm using a diode array detector to determine the UV absorption maxima.

Metabolite	Absorption Maxima	Retention Time
DBA 1	214.24, 256.75, 347.67	8.98
DBA 2	Not detected	-
DBA 3	Not detected	-
DBA 4	Not detected	-
DBA 5	197.94, 259.28, 283.06, 301.85, 313.66	32.08
DBA 6	Not detected	-
DBA 7	Not detected	-
DBA 8	Not detected	-

electron is removed from the molecule or fragment so that the mass to charge ratio is numerically equal to the mass.

The separation of individual PAH metabolites was achieved using a BPX-5 capillary column, using a temperature from 100°C to 300°C at 10°C/minute. The MS was operated in electron impact mode with an electron energy of 70 eV over a scan range of 45-400 Da. Compounds that could not be ionised were derivatised by BSTFA plus 1% TMCS (silylating reagent) or by methylation.

6.4.2.1 *GC-MS Analysis of Pyrene Metabolites*

6.4.2.1.1 *Metabolite M14*

Metabolite M14 had a GC retention time of 18.4 minutes (Figure 6.11). GC-MS analysis of M14 showed that the compound had a molecular ion (M^+) at m/z 196 and fragment ions at m/z 168 (M^+ -28) and m/z 139 (M^+ -57) (Figure 6.12). The fragment ions indicated losses of a -CO group (M^+ -28) as well as a -CO group plus a -COH group (M^+ -57). The mass spectral analysis is consistent with a molecular formula of $C_{13}H_8O_2$ and an aromatic hydrocarbon containing single keto and hydroxyl moieties (Heitkamp *et al.*, 1988b). The chromatographic characteristics, molecular weight and mass spectral fragmentation pattern of metabolite M14 were similar to those reported by Heitkamp *et al.* (1988b) (Figure 6.12). Metabolite M14, formed from the degradation of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 was identified as 4-hydroxyperinaphthenone.

6.4.2.1.2 *Metabolite M15*

Metabolite M15 had a GC retention time of 19.6 minutes (Figure 6.11). M15 had a molecular ion (M^+) at m/z 222 and fragment ions at m/z 205 and m/z 177. The major ion fragments represented probable losses of an -OH group (M^+ -17) and a -COOH group (M^+ -45). Minor fragment ions were also observed at m/z 194 (M^+ -28), m/z 165 (M^+ -57) and m/z 151 (M^+ -71), representing probable losses of a $-CH_2{=}CH_2$ from an aromatic ring (M^+ -28), the loss of a -C from m/z 177 and the loss of -CHCCOOH plus a -H (M^+ -71) from an accompanying hydrogen shift respectively (Heitkamp *et al.*, 1988b).

The derivatised metabolite (methylated) had a molecular ion (M^+) at m/z 236, representing a mass increase of 14 mass units over the underivatised compound.

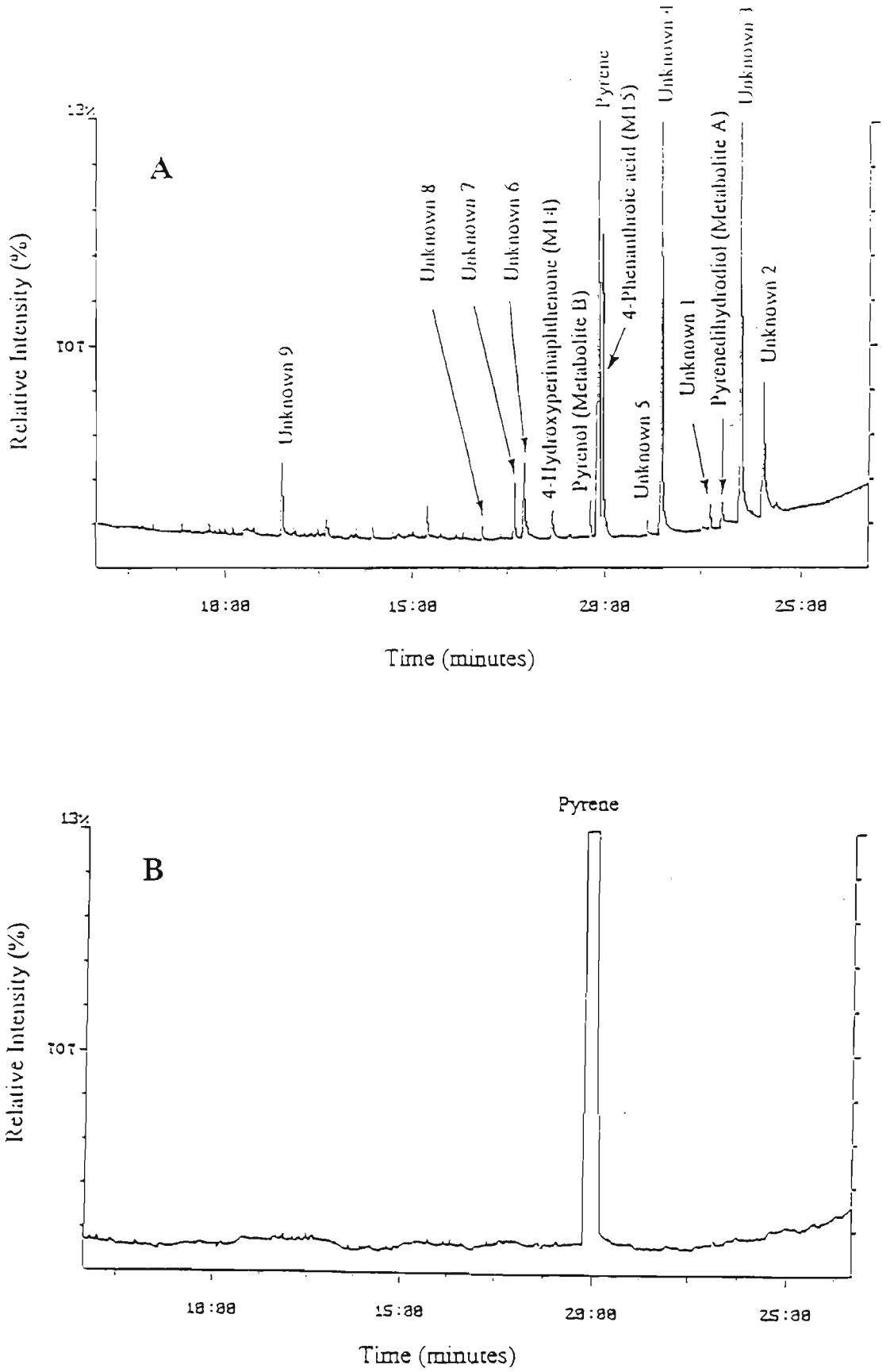


Figure 6.11. GC-MS profile of pyrene and pyrene metabolites extracted from medium inoculated with strain VUN 10,003 (A) and uninoculated pyrene medium (B). Samples were extracted after 72 hours incubation. Pyrene and the unknown compounds were separated on a BPX-5 capillary column using a temperature from 100°C to 300°C at 10°C/minute. The MS was operated over a scan range of 45-400 Da.

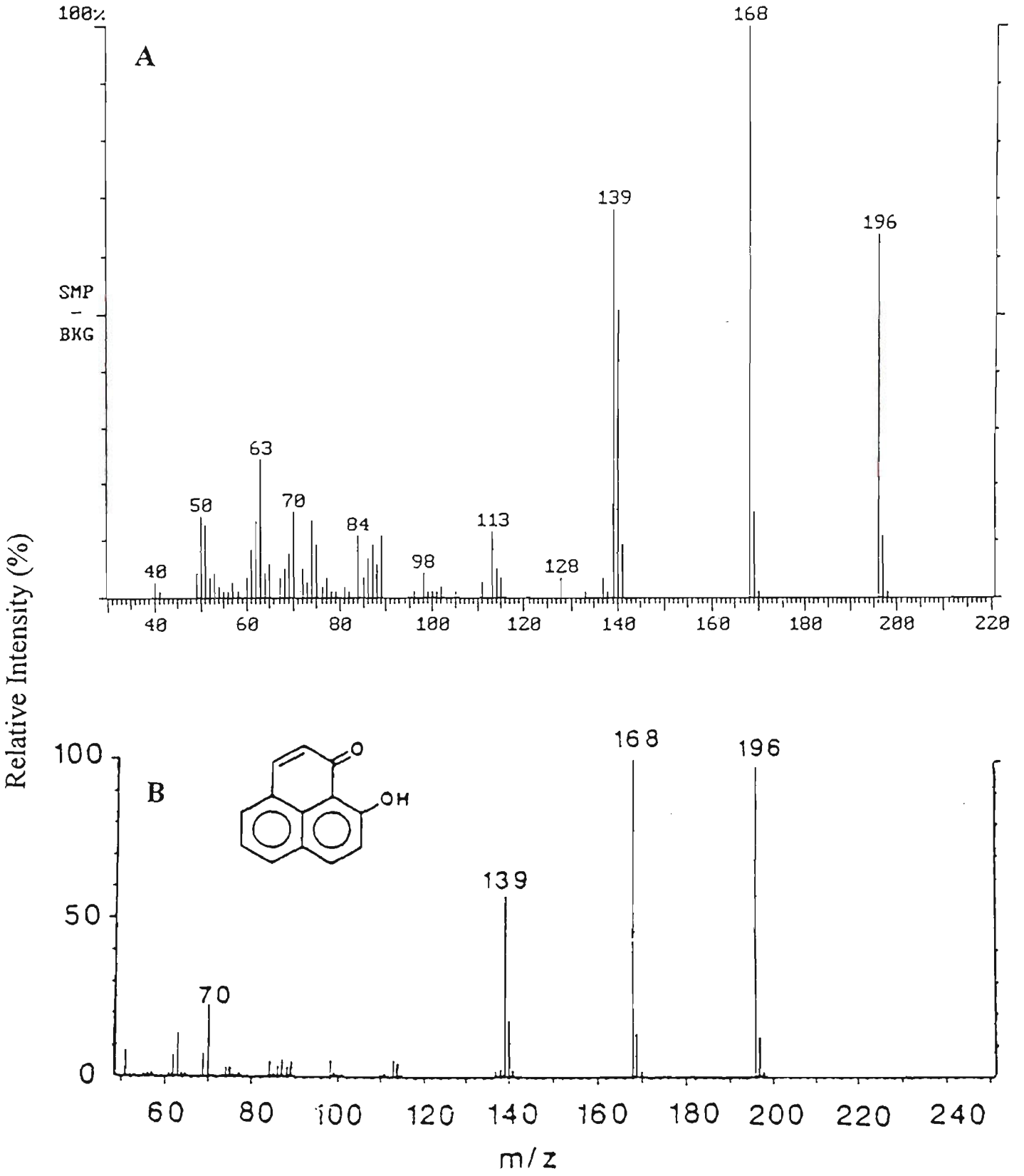


Figure 6.12. Mass spectra of 4-hydroxyperinaphthenone (Metabolite M14) formed from the degradation of pyrene by strain VUN 10,003 (A) compared to a 4-hydroxyperinaphthenone formed by *Mycobacterium* species as reported by Heitkamp *et al.* (1988b).

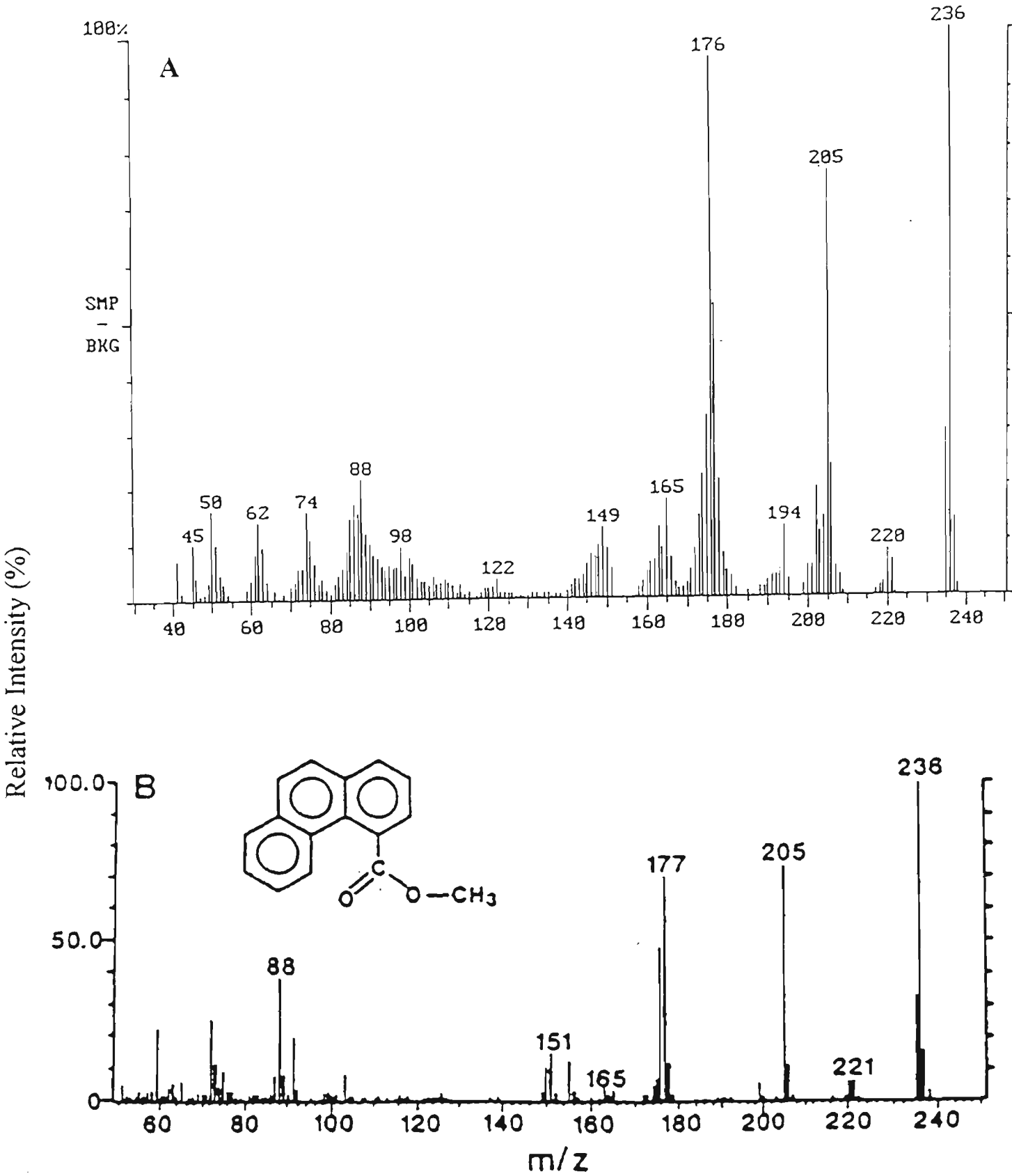


Figure 6.13. Mass spectra of methylated 4-phenanthroic acid (Metabolite M15) formed from the degradation of pyrene by strain VUN 10,003 (A) compared to methylated 4-phenanthroic acid formed by *Mycobacterium* species as reported by Heitkamp *et al.* (1988b).

Fragment ions were observed at m/z 221 (M+ -15), m/z 205 (M+ -31) and m/z 177 (M+ -59) (Figure 6.13). The fragment ions represented the loss of a $-CH_3$ group (M+ -15), the loss of a $-OCH_3$ unit (M+ -31) and the loss of a $-COOCH_3$ unit (M+ -59). Metabolite M15 was given the molecular formula of $C_{15}H_{10}O_2$. The chromatographic characteristics, molecular weight and mass spectral fragmentation pattern of metabolite M15 were similar to those reported by Heitkamp *et al.* (1988b) (Figure 6.13). Metabolite M15, formed from the degradation of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 was identified as 4-phenanthroic acid.

Metabolites M5, M6, M9, M12, M16a and M16b were unable to be resolved by GC-MS, presumably due to the inability of the compounds to volatilise or ionise. Derivatisation of the metabolites with BSTFA + 1% TMCS (silylating reagent) was performed to prepare volatile and thermally stable compounds for GC-MS. The silylation reaction results in the replacement of a labile hydrogen with a $Si(CH_3)_3$ group. A number of derivatisation reaction times and reaction temperatures were tried with metabolites M5, M6, M9, M12, M16a and M16b, however, none of the combinations resulted in derivatised compounds that could be detected by GC-MS.

6.4.2.2 *GC-MS Analysis of Pyrene Crude Extracts*

The crude pyrene extracts, from which metabolites M5, M6, M9, M12, M14, M15, M16a and M16b were purified, were also analysed by GC-MS. TLC may have failed to separate or resolve some pyrene metabolites which may otherwise be separated by capillary column GC and detected by MS. In addition, analysis of crude extracts from the time course incubations may have resolved metabolites that were transiently accumulated or accumulated at low concentrations.

6.4.2.2.1 *Metabolite A*

Metabolite A could not be purified from the pyrene crude extracts of strains VUN 10,001, VUN 10,002 and VUN 10,003 by TLC, however, it was detected at low concentrations by GC-MS (Figure 6.11). The compound had a molecular ion (M^+) at m/z 236 and a base peak at m/z 218. This represented a loss of an H_2O unit (M^+ -18). Fragmentation ions were detected at m/z 189 (M^+ -47), m/z 176 (M^+ -60) and m/z 94 (M^+ -142) (Figure 6.14). The mass spectral fragmentation pattern was similar to authentic pyrene *cis*-4,5-dihydrodiol and a *Mycobacterium* species pyrene metabolite observed by Heitkamp *et al.* (1988b) (Figure 6.14). Metabolite A was identified as a

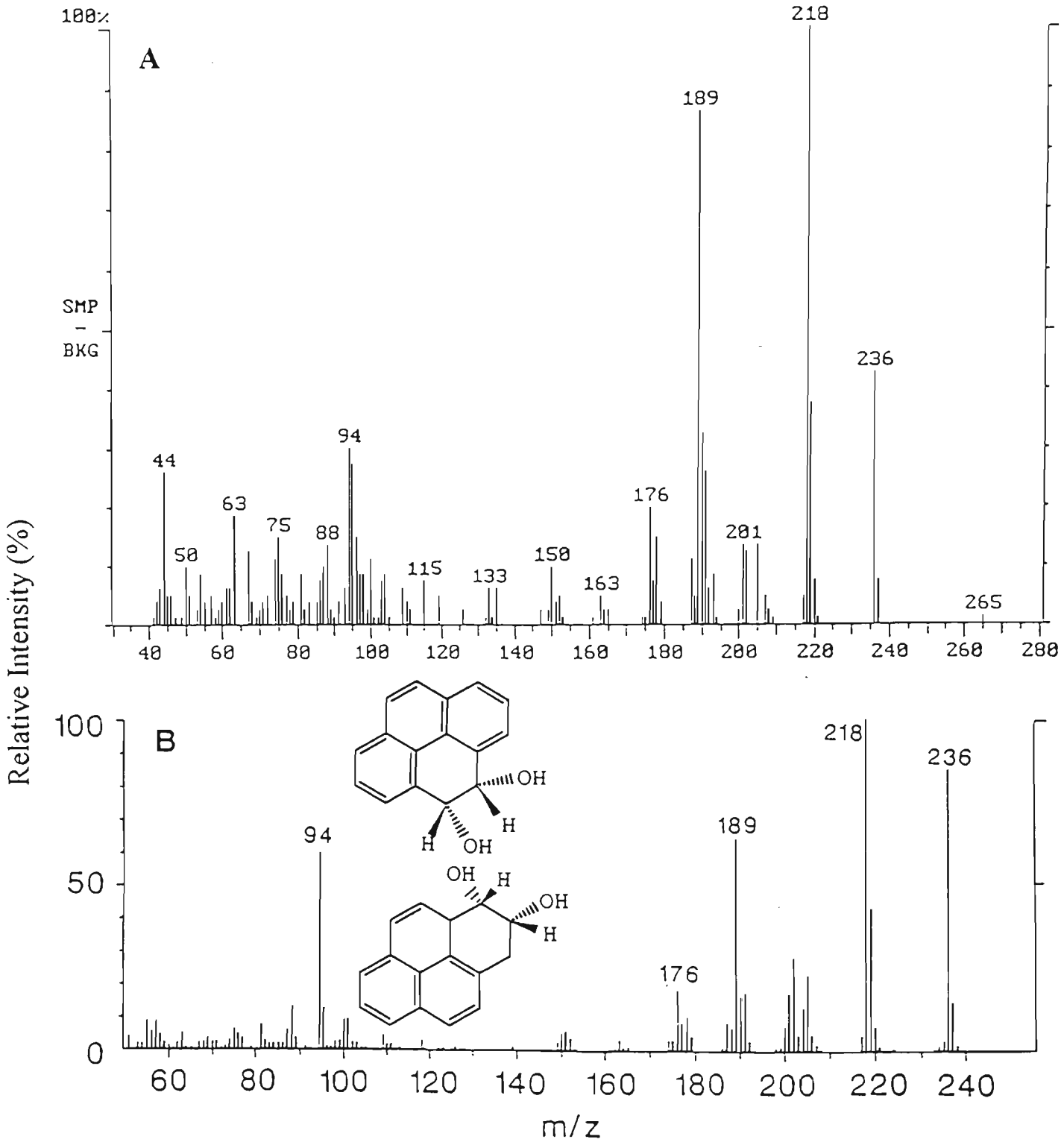


Figure 6.14. Mass spectra of pyrenedihydrodiol (Metabolite A) formed from the degradation of pyrene by strain VUN 10,003 (A) compared to a pyrenedihydrodiol formed by *Mycobacterium* species as reported by Heitkamp *et al.* (1988b).

pyrenedihydrodiol, however, GC-MS analysis did not allow for the determination of the absolute stereochemistry of the dihydrodiol.

6.4.2.2.2 *Metabolite B*

Metabolite B could not be purified from strains VUN 10,001, VUN 10,002 and VUN 10,003 pyrene crude extracts by TLC, however, it was detected at low concentrations by GC-MS (Figure 6.11). The compound had a molecular ion (M^+) at m/z 218 and a fragment ion at m/z 189 (M^+ -29), representing the loss of a -CHO group (Figure 6.15). The GC-MS retention time and the mass spectral fragmentation pattern were identical to those of authentic 1-hydroxypyrene (Figure 6.15). Metabolite B was identified as a pyrenol, however, GC-MS analysis did not allow for the determination of the absolute stereochemistry of the compound.

In addition to metabolites A and B, analysis of strains VUN 10,001, VUN 10,002 and VUN 10,003 crude pyrene extracts resulted in the detection of nine unknown compounds. The observed masses of the unknown compounds ranged from 149 to 262 mass units. The observed masses and major ion fragments of the unknown pyrene metabolites (Figure 6.11) are shown in Table 6.7. GC-MS analysis of uninoculated pyrene medium did not show any peaks with retention times matching those of the unknown pyrene metabolites (Figure 6.11).

6.4.2.3 *GC-MS Analysis of Benzo[a]pyrene Metabolites*

The benzo[*a*]pyrene metabolites, B[a]P1, B[a]P2, B[a]P3, B[a]P4, B[a]P5, B[a]P6, B[a]P7, B[a]P8 and B[a]P9 isolated by TLC, could not be resolved by GC-MS, presumably due to the inability of the compounds to volatilise or ionise. To overcome this problem, derivatisation of the metabolites with BSTFA plus 1% TMCS (silylating reagent) was performed to prepare volatile and thermally stable compounds for GC-MS. A number of derivatisation reaction times and reaction temperatures were tried with the benzo[*a*]pyrene metabolites, however, none of the combinations resulted in derivatised compounds that could be detected by GC-MS.

6.4.2.4 *GC-MS Analysis of Benzo[a]pyrene Crude Extracts*

Because the isolated benzo[*a*]pyrene metabolites could not be analysed by GC-MS, analysis of benzo[*a*]pyrene crude extracts was performed in an attempt to identify metabolites produced by strains VUN 10,001, VUN 10,002 and VUN 10,003. Two

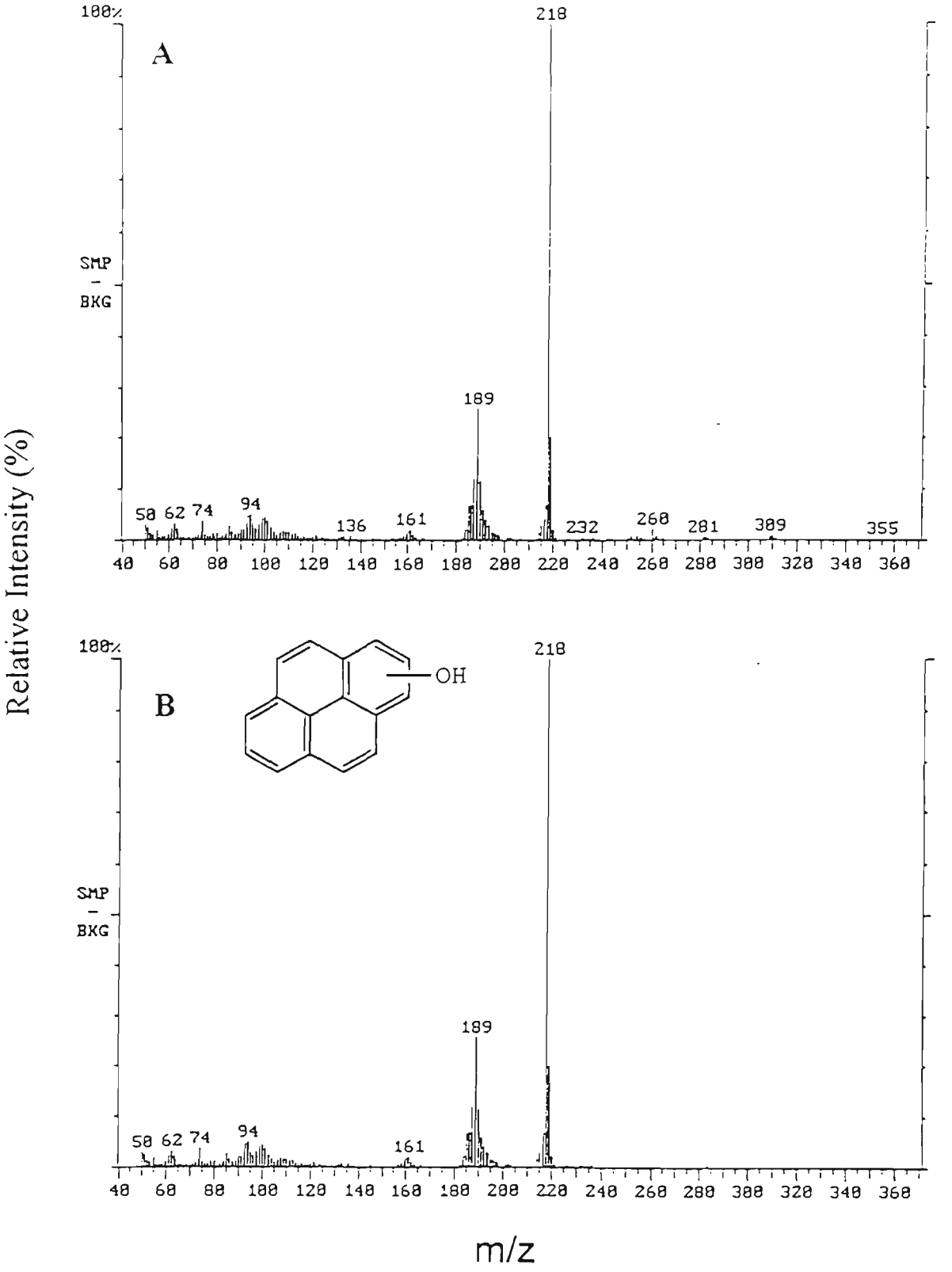


Figure 6.15. Mass spectra of pyrenol (Metabolite B) formed from the degradation of pyrene by strain VUN 10,003 (A) compared to authentic 1-hydroxypyrene (B) obtained from Sigma Chemical Company.

Table 6.7. Mass spectral characteristics of unknown pyrene metabolites shown in Figure 6.11.

Compound	Retention Time	Molecular Ion Observed Mass	Major Fragments
Pyrene	19.51	202	202
Unknown 1	22.42	262	262, 247 (-CH_3 loss), 219 (further -CO loss), 201, 191, 176 (further -CH_3 loss)
Unknown 2	24.02	254	254, 210, 182 (-CO loss), 137 (further -COOH loss)
Unknown 3	23.28	248	248, 204, 176 (-CO loss)
Unknown 4	21.30	220	220, 192 (-CO loss), 161 (further -OCH_3 loss)
Unknown 5	21.08	226	226, 198 (-CO loss), 170 (further -CO loss), 126
Unknown 6	17.57	198	198, 170 (-CO loss), 156, 141 (further -CH_3 loss), 128
Unknown 7	17.41	149	149
Unknown 8	16.52	182	182, 153, 126
Unknown 9	11.31	152	152, 134 (-CO loss)

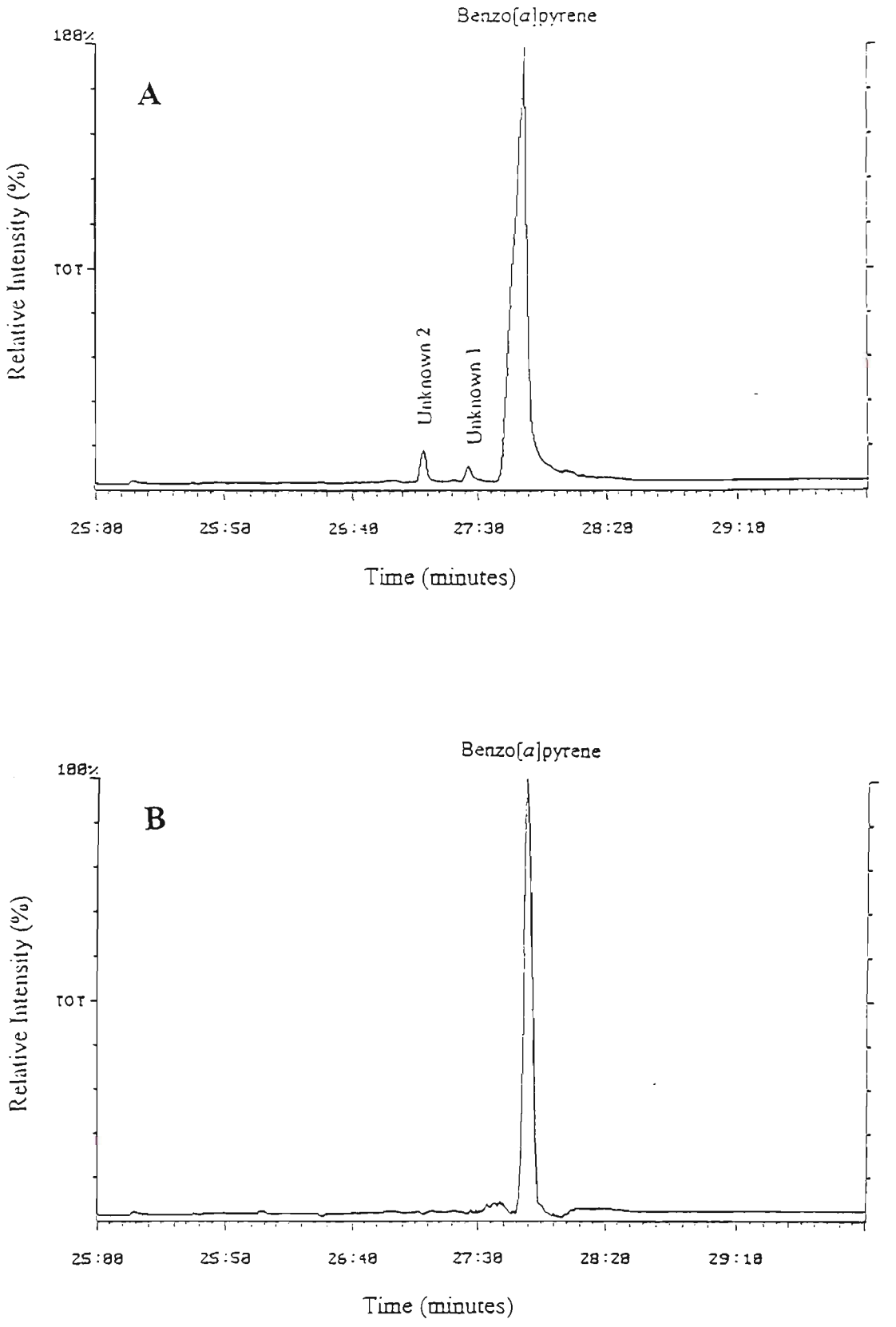


Figure 6.16. GC-MS profile of benzo[*a*]pyrene and benzo[*a*]pyrene metabolites extracted from medium inoculated with strain VUN 10,003 (A) and uninoculated benzo[*a*]pyrene medium (B). Samples were extracted after 12 weeks incubation. Benzo[*a*]pyrene and the unknown compounds were separated on a BPX-5 capillary column using a temperature from 100°C to 300°C at 10°C/minute. The MS was operated over a scan range of 45-400 Da.

unknown compounds were detected in benzo[*a*]pyrene crude extracts from cultures incubated with each of the three strains. The unknown compounds eluted from the column before benzo[*a*]pyrene, however, their retention times were within 0.4 of a minutes of benzo[*a*]pyrene (Figure 6.16). In addition, the observed masses of the unknown compounds were 7 mass units (unknown 1) and 5 mass units (unknown 2) greater than benzo[*a*]pyrene (252 mass units). The observed masses and major ion fragments of the unknown benzo[*a*]pyrene metabolites are shown in Table 6.8. GC-MS analysis of uninoculated benzo[*a*]pyrene medium did not show any peaks with retention times matching those of the unknown benzo[*a*]pyrene metabolites (Figure 6.16).

6.4.2.5 *GC-MS Analysis of Dibenz[a,h]anthracene Metabolites*

The results obtained for the GC-MS analysis of TLC-isolated dibenz[*a,h*]anthracene metabolites were similar to those obtained for the benzo[*a*]pyrene metabolites; the metabolites could not be resolved by GC-MS. Similarly, derivatisation of dibenz[*a,h*]anthracene metabolites by silyation did not result in compounds that could be detected by GC-MS.

6.4.2.6 *GC-MS Analysis of Dibenz[a,h]anthracene Crude Extracts*

Due to the inability of the isolated dibenz[*a,h*]anthracene metabolites to be detected by GC-MS, analysis of dibenz[*a,h*]anthracene crude extracts was performed in an attempt to identify metabolites produced by strains VUN 10,001, VUN 10,002 and VUN 10,003. Five unknown compounds were detected in dibenz[*a,h*]anthracene crude extracts from cultures incubated with each of the three strains. The observed masses of the unknown compounds ranged from 170 to 308 mass units and the mass of the unknown compounds increased with increasing GC retention times. The observed masses and major ion fragments of the unknown dibenz[*a,h*]anthracene metabolites (Figure 6.17) are shown in Table 6.9. GC-MS analysis of uninoculated dibenz[*a,h*]anthracene medium did not show any peaks with retention times matching those of the unknown dibenz[*a,h*]anthracene metabolites (Figure 6.17).

6.4.3 Proton NMR Analysis of PAH Metabolites

Proton NMR of the isolated PAH metabolites was conducted in an attempt to identify the intermediate compounds that could not be detected by GC-MS, to confirm the identity of the pyrene metabolites identified by GC-MS and to determine the absolute stereochemistry of the identified compounds. Metabolites were solvent exchanged

Table 6.8. Mass spectral characteristics of unknown benzo[*a*]pyrene metabolites shown in Figure 6.16.

Compound	Retention Time	Molecular Ion Observed Mass	Major Fragments
Benzo[*a*]pyrene	27.47	252	252
Unknown 1	27.26	259	259, 213, 128
Unknown 2	27.08	257	257, 239 (loss of -CO), 228

from methanol to DCM-d_2. Methanol was removed by evaporation under a gentle stream of nitrogen and compounds were dissolved in DCM-d_2. NMR spectra were recorded on a Bruker DPX 300 spectrometer recording ^{1}H NMR at 300 MHz.

Proton NMR of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites was not successful as the yield of metabolites obtained from degradation experiments was not sufficient for NMR analysis.

6.5 DISCUSSION

6.5.1 Pyrene Degradation

6.5.1.1 *Mineralisation of Pyrene*

Pyrene was rapidly mineralised by community five and a pure culture of strain VUN 10,003 when the compound was supplied as the sole carbon and energy source. Only a small fraction of the label (4.1%) was detected in the aqueous phase, representing water soluble metabolites, where as 64.5-70.5% of ^{14}C was recovered in the gaseous phase ($^{14}CO_2$). Pyrene has previously been shown to be mineralised by *Mycobacterium* and *Rhodococcus* species (Heitkamp *et al.*, 1988a; Walter *et al.*, 1991; Schneider *et al.*, 1996). Walter *et al.* (1991) demonstrated the ability of *Rhodococcus* strain UW1 to mineralise ^{14}C-pyrene. Strain UW1 mineralised 72% of the pyrene after 14 days. The remaining label was detected in the aqueous phase (25%), representing water soluble metabolites and cell associated residues while 3% of the ^{14}C residue was detected as undegraded pyrene. A *Mycobacterium* species mineralised 52.4% of ^{14}C-pyrene after 96 hours incubation (Heitkamp *et al.*, 1988a). The degradation of pyrene resulted in the formation of a number of water soluble metabolites. After 96 hours, 2.4% of the label was detected as undegraded pyrene, while the remaining label (45.2%) was distributed between six metabolites. Similar results were obtained by Schneider *et al.* (1996) when pyrene was incubated with *Mycobacterium* strain RJGII 135, isolated from an abondoned coal gasification site. Approximately 45% of the pyrene was mineralised after 4 hours incubation; 9.5% of the pyrene was undegraded while the remaining label (49.1%) was distributed between four metabolites.

In contrast to the *Mycobacterium* strains, community five and strain VUN 10,003 accumulated only a small portion of the ^{14}C label as water soluble compounds (4% compared to 45-49%). The water soluble fraction of a degradation process is of great

Table 6.9. Mass spectral characteristics of unknown dibenz[*a,h*]anthracene metabolites shown in Figure 6.17.

Compound	Retention Time	Molecular Ion Observed Mass	Major Fragments
DBA	31.51	278	278
Unknown 1	33.10	308	308, 281, 253 (loss of -CO)
Unknown 2	28.34	281	281, 266 (loss of CH_3), 208
Unknown 3	27.46	253	253, 225 (loss of -CO),126
Unknown 4	23.21	250	250, 232, 218, 189
Unknown 5	17.16	170	170, 141, 77

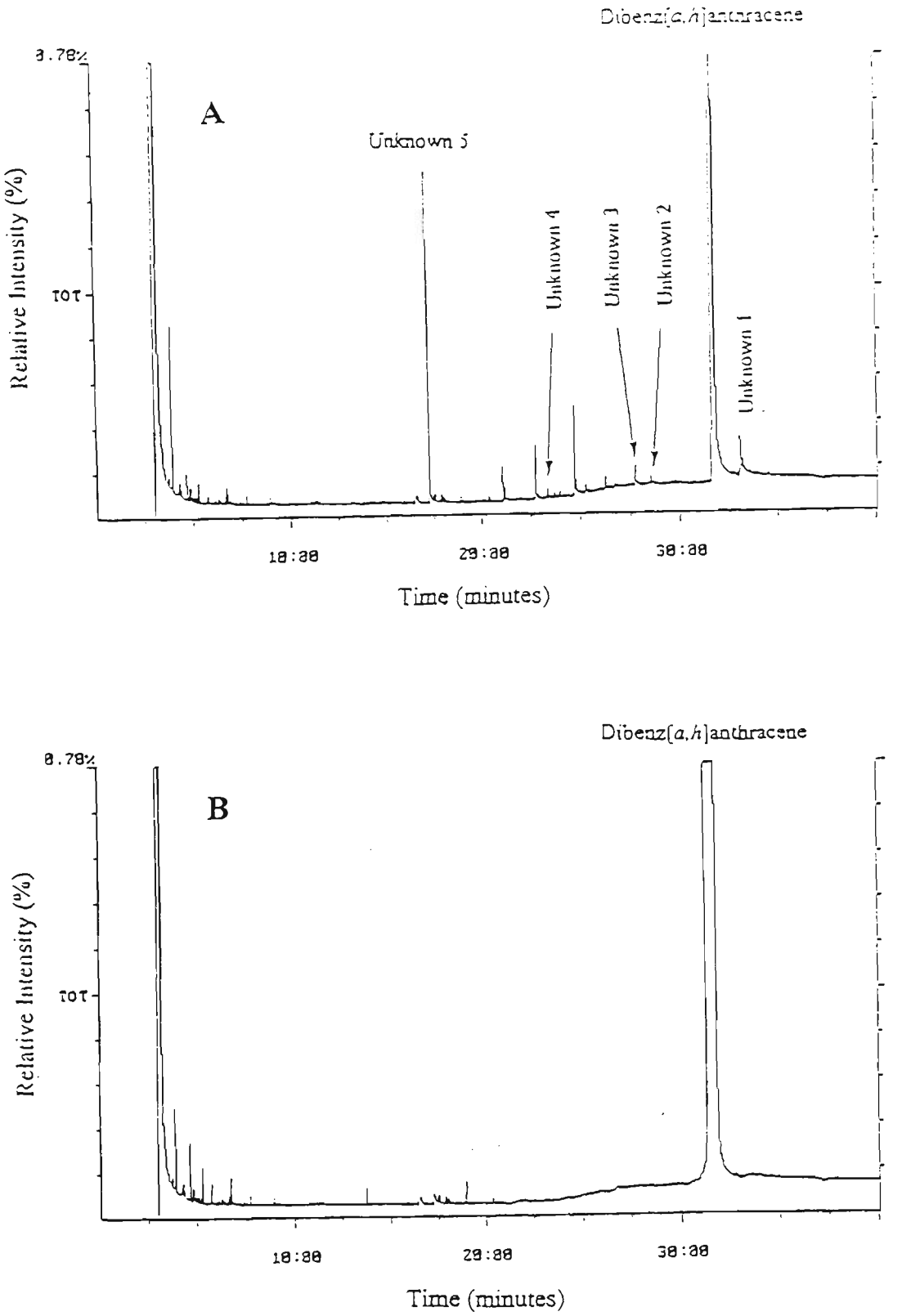


Figure 6.17. GC-MS profile of dibenz[*a,h*]anthracene and dibenz[*a,h*]anthracene metabolites extracted from medium inoculated with strain VUN 10,003 (A) and uninoculated dibenz[*a,h*]anthracene medium (B). Samples were extracted after 12 weeks incubation. Dibenz[*a,h*]anthracene and the unknown compounds were separated on a BPX-5 capillary column using a temperature from 100°C to 300°C at 10°C/minute. The MS was operated over a scan range of 45-400 Da.

interest as the incomplete degradation of a compound may generate by-products that are more toxic than the parent compound (Dasappa and Loehr, 1991). These by-products may pose a greater threat to the environment due to their increased solubility and mobility. The extensive utilisation of pyrene (*i.e.* conversion to CO_2, H_2O and biomass) by community five and strain VUN 10,003 is advantageous as it limits the potential adverse environmental effects of metabolite accumulation and interactions with other organisms.

The extent of pyrene degradation was also assessed in a soil matrix. Radiolabelled pyrene was spiked into PAH-contaminated soil to assess the pyrene degrading potential of the indigenous microflora as well as an inoculated pyrene-degrading microbial community. The indigenous microbial population was unable to mineralise the added ^{14}C-pyrene resulting in 99.5% of the ^{14}C being recovered from the soil matrix. In a previous study, Grosser *et al.* (1991) reported the microbial degradation of pyrene by the indigenous microflora using soils collected from three abondoned coal gasification plants, where the chemical and microbiological charactersitics varied between site. After 60 days, the levels of indigenous microbial pyrene mineralisation in the soil ranged from 10% to 48% depending on the soil type. Pyrene mineralisation was the greatest in the soils containing the lowest total organic carbon content. Failure of the indigenous microbial population in this study to mineralise pyrene may have been a result of:

(i) lack of a subpopulation with the necessary genes/biochemistry to effect degradation;
(ii) other compounds present in the soil being used as a carbon source in preference to pyrene (necessary biochemisty present, however, catabolic repression of genes);
(iii) inhibitory or toxic compounds contained in the soil;
(iv) lack of essential nutrients; or
(v) partial degradation of ^{14}C-pyrene leading to the formation of intermediates which accumulate in the soil rather than mineralisation occurring.

The introduction of a pyrene-degrading microbial community to the ^{14}C-pyrene-spiked PAH-contaminated soil resulted in the mineralisation of the compound. After 48 days, 42% of the recovered ^{14}C was detected as $^{14}CO_2$. Similar results were observed by Grosser *et al.* (1991). The reintroduction of a pyrene-degrading microorganism isolated from the soil resulted in the enhanced mineralisation of pyrene; 55% of the ^{14}C was

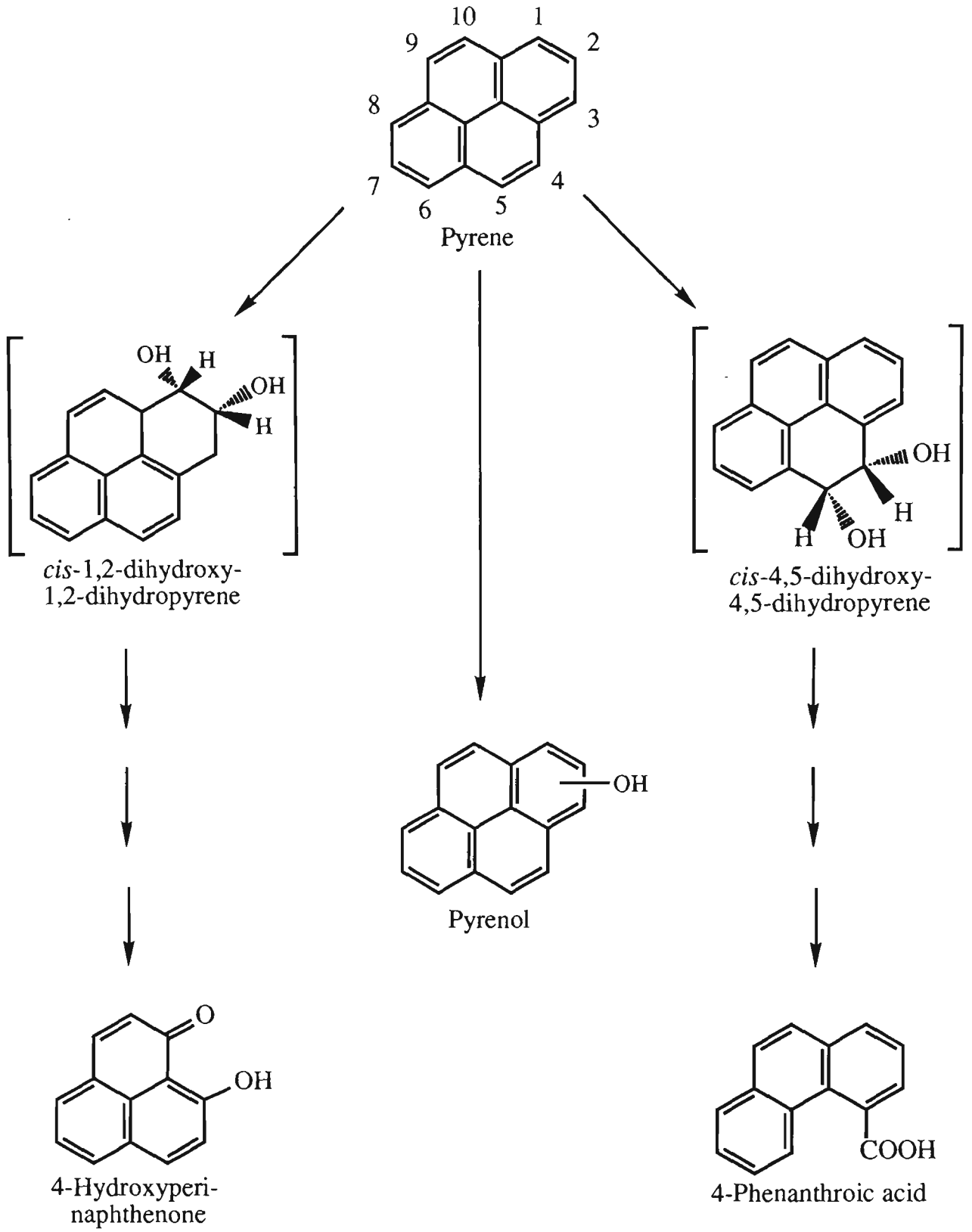


Figure 6.18. Proposed pathway for the metabolism of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003. The structures in brackets represent the most likely structure of the dihydrodiols, however, the absolute stereochemistry could not be determined. Multiple arrows between metabolites indicate multiple steps, not single reactions.

recovered as $^{14}CO_2$ within 2 days compared to a level of 1% for the indigenous population.

The rate of pyrene mineralisation by community five was substantially slower in the soil matrix compared to the liquid medium. The decreased degradation rate and the extent of mineralisation of pyrene in the soil matrix may be due to the decreased bioavailability of the compound. The total organic carbon present in the soil may have played a role in the availability of the compound to the microorganisms. The amount of total organic carbon is known to affect the adsorption and desorption of many hydrophobic compounds (Grosser *et al.*, 1991). Other factors that may have reduced the PAH-degradation rate have previously been discussed in Chapter 5 (see Section 5.5.3).

Although the results obtained from experiments conducted with radiolabelled compounds spiked into contaminated soil provide an insight into the degradative potential of the indigenous or inoculated microorganisms, these results are hard to interpret in terms of predicting mineralisation *in situ*. Freshly added ^{14}C-substrate may be more available for degradation than the resident material due to the effects of binding and adsorption to organic material over time. Consequently, degradation rates of ^{14}C-substrates may give an inflated estimate of the biodegradability of soil contaminants.

6.5.1.2 *Pyrene Metabolites*

Although pyrene is not considered genotoxic (Pothuluri and Cerniglia, 1994), it has a chemical structure which is found in several carcinogenic PAHs (1-nitropyrene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene) and as such is a good model compound for PAH metabolism studies.

Analysis of samples taken from pyrene cultures inoculated with the three *St. maltophilia* strains provided sufficient information to construct a possible pyrene catabolic pathway. Figure 6.18 outlines a proposed pathway for the metabolism of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003. The pathway was constructed with reference to the pyrene catabolic pathways proposed by Heitkamp *et al.* (1988b) and Schneider *et al.* (1996). Although preparative TLC could not isolate and purify a ring oxidation product of pyrene, direct analysis of crude pyrene supernatant extracts of strains VUN 10,001, VUN 10,002 and VUN 10,003 resulted in the identification of a pyrene dihydrodiol (Metabolite A). Isolation of the dihydrodiol

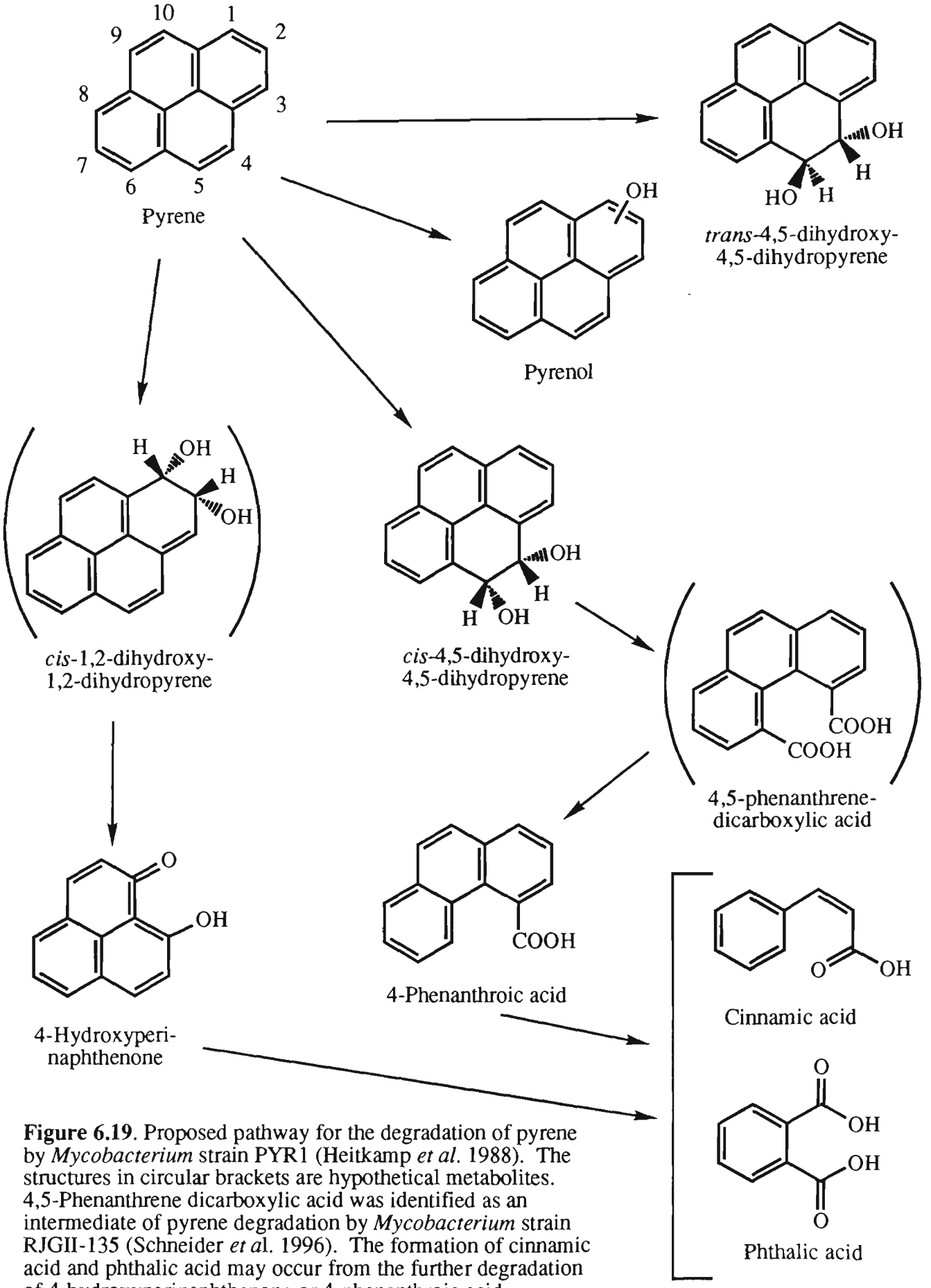


Figure 6.19. Proposed pathway for the degradation of pyrene by *Mycobacterium* strain PYR1 (Heitkamp *et al.* 1988). The structures in circular brackets are hypothetical metabolites. 4,5-Phenanthrene dicarboxylic acid was identified as an intermediate of pyrene degradation by *Mycobacterium* strain RJGII-135 (Schneider *et al.* 1996). The formation of cinnamic acid and phthalic acid may occur from the further degradation of 4-hydroxyperinaphthenone or 4-phenanthroic acid.

may be possible by TLC, however, due to the compound being a minor component of the metabolite fraction it was not excised from the TLC plates. Pyrene dihydrodiol may not accumulate to high concentrations in the culture medium due to the rapid ring fission of the compound. The mass spectral fragmentation pattern of the dihydrodiols was similar to that previously reported by Heitkamp *et al.* (1988b), however, the absolute stereochemistry of the dihydrodiol could not be determined due to the yield of the metabolite even from large scale cultures was not sufficient for NMR analysis. Consequently, it is not clear whether the ring cleavage occurred at the 1,2- or the 4,5-position.

Heitkamp *et al.* (1988b) proposed that the initial oxidation of pyrene by a *Mycobacterium* species occurred at the 4,5- position, resulting in the formation of 4,5-dihydroxy-4,5-dihydropyrene. Although pyrene-1,2-dihydrodiol was not detected as a ring oxidation metabolite, the formation of 4-hydroxyperinaphthenone, an isolated ring fission metabolite, probably resulted from the ring oxidation and cleavage of 1,2-dihydroxy-1,2-dihydropyrene (Figure 6.19). Two analogous pathways were proposed for the initial oxidation and ring fission of pyrene by *Rhodococcus* strain UW1 (Walter *et al.*, 1991). Walter *et al.* (1991) failed to determine the configuration of metabolite I ($C_{16}H_{10}O_4$), however, they proposed that the initial oxidation of pyrene occurred at either the 1,2- or the 4,5- position (Figure 6.20). Oxidation of pyrene at the 1,2-position seems likely since PAHs with similar structural configurations are attacked at this position. A number of studies have indicated that bacteria initially oxidise naphthalene and phenanthrene by incorporating molecular oxygen into the aromatic molecule to form 1,2- dihydrodiols (Kelly *et al.*, 1990; Cerniglia and Heitkamp, 1989; Pothuluri and Cerniglia, 1994; Cerniglia, 1984a; Cox and Williams, 1980).

The formation of dihydrodiols is indicative of dioxygenase enzyme systems (Cerniglia, 1992). Procaryotes are known to utilise dioxygenase enzymes to incorporate two atoms of oxygen into aromatic hydrocarbons, which results in the production of dihydrodiols with a *cis* formation. Both *cis* and *trans* pyrene dihydrodiols were detected from the degradation of pyrene by a *Mycobacterium* species (Heitkamp *et al.*, 1988b), suggesting that the organism was capable of multiple pathways for the initial oxidative attack on the compound. Heitkamp *et al.* (1988b) proposed that in addition to the dioxygenase enzyme system, a monooxygenase catalysed reaction was responsible for the formation of *trans* pyrene dihydrodiol.

Pyrenol (Metabolite B) was also identified as an initial ring oxidation product of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003. The compound could not be

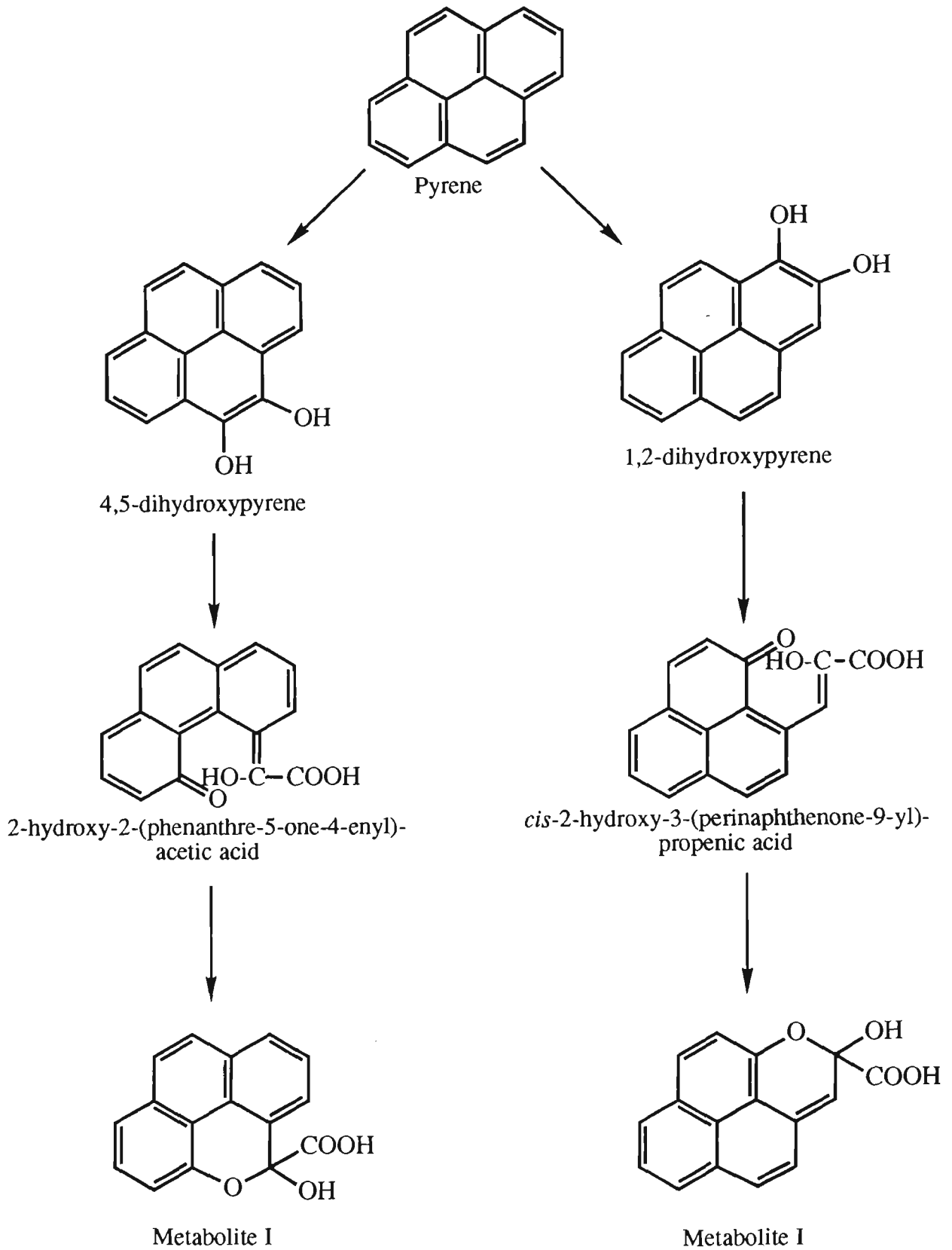


Figure 6.20. Proposed pathway for the initial oxidation of pyrene by *Rhodococcus* strain UW1 (Walter *et al.* 1991). The absolute stereochemistry of metabolite I could not be determined so two possible structures were proposed.

isolated from TLC plates, however, it was detected in crude pyrene extracts. Pyrenol may not accumulate to significant concentrations in the culture medium due to the formation of pyrenol being a minor degradative pathway of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 or the compound may be rapidly degraded to other products. Pyrenol was detected as a minor metabolite of pyrene degradation by a *Mycobacterium* species (Heitkamp *et al.*, 1988b). However, it was unclear whether the formation of pyrenol resulted from the oxidative metabolism of pyrene by the *Mycobacterium* species or from the non-enzymatic dehydration of pyrene dihydrodiols. The position of the hydroxyl moiety was unable to be determined due to the inability to isolate and purify the compound for NMR analysis. 1-Hydroxypyrene has been reported as a fungal metabolite of pyrene (Cerniglia *et al.*, 1986) and a mammalian metabolite of pyrene (Keimig *et al.*, 1983).

In contrast to bacteria, mammals and fungi oxidise PAHs via cytochrome P450 monooxygenase and epoxide hydrolase catalysed reactions to *trans* dihydrodiols (Cerniglia and Heitkamp, 1989; Cerniglia and Gibson, 1980; Cerniglia *et al.*, 1980; Cooper *et al.*, 1983). Arene oxides can also undergo isomerisation to form phenols which can be conjugated with sulphur, glucuronic acid, glucose and glutathione. Mammalian and fungal metabolism of pyrene results in the formation of 1-pyrenol (Jacob *et al.*, 1982; Keimig *et al.*, 1983), 1,6- and 1,8-pyrenediols and 1,6- and 1,8-pyrenequinones (Wunder *et al.*, 1994; Launen *et al.*, 1995; Okamoto and Yoshida, 1981), which may undergo secondary metabolism to form 1-pyrenyl sulphate and 1-hydroxy-8-pyrenyl sulphate (Figure 6.21). Fungi hydroxylate PAHs as an initial action for the detoxification of the compound, where as bacteria oxidise PAHs to dihydrodiols as a prelude to ring fission and assimilation (Cerniglia, 1992).

cis-Dihydrodiols are further metabolised by bacteria after rearomatisation through *cis* dihydrodiol dehydrogenase (Cerniglia, 1984b). This yields dihydroxylated derivatives which are further metabolised by the enzymatic cleavage of the aromatic ring. Two ring fission products (M14 and M15) were isolated from the culture supernatants of strains VUN 10,001, VUN 10,002 and VUN 10,003 and identified as 4-hydroxyperinaphthenone and 4-phenanthroic acid. It is likely that the formation of these metabolites resulted from the ring fission of two separate ring oxidation products. 4-Hydroxyperinaphthenone probably resulted from the ring oxidation and cleavage of the alpha ring of pyrene (Heitkamp *et al.*, 1988b). 1,2-Dihydroxy-1,2-dihydropyrene was not detected in crude extracts which may be due to rapid ring fission of the compound preventing the accumulation of pyrene 1,2-dihydrodiol in the culture medium. The ring fission of 4,5-dihydroxy-4,5-dihydropyrene probably resulted in the

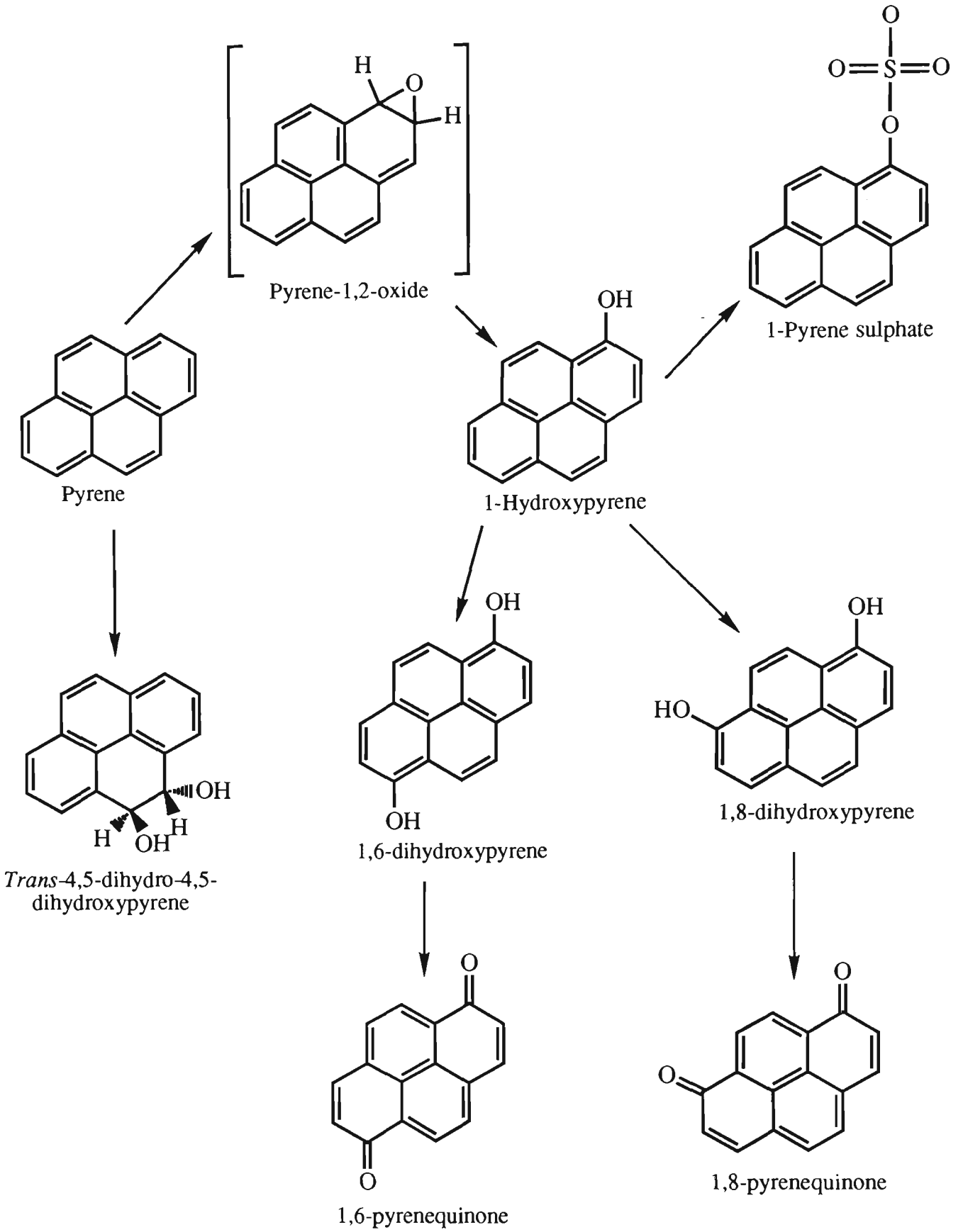


Figure 6.21. Proposed pathway for the degradation of pyrene by *Crinipellis stipitaria* (adapted from Lange *et al.* 1994).

formation of 4-phenanthroic acid. Both Heitkamp *et al.* (1988b) and Schneider *et al.* (1996) isolated 4-phenanthroic acid from the degradation of pyrene by *Mycobacterium* species. It was proposed that the formation of 4-phenanthroic acid resulted from a 1-carbon excision from the K-region of pyrene, however, the mechanism of this reaction is not known. A similar mechanism was observed during the degradation of fluoranthene by *A. denitrificans* strain WW1 (Weissenfels *et al.*, 1991a). After the initial hydroxylation of fluoranthene at the 9,10- position, 7-hydroxy-8-acenaphthylenealdehyde was expected as the result of an aldolase reaction on the ring fission product. Instead, 7-hydroxyacenaphthylene occurred by a 1-carbon excision from the aromatic aldehyde. Weissenfels *et al.* (1989) also observed this reaction during fluorene degradation by *P. paucimobilis*.

Other reported ring fission products of pyrene, such as phthalic and cinnamic acids (Heitkamp *et al.*, 1988b), were not detected in pyrene supernatant extracts of strains VUN 10,001, VUN 10,002 and VUN 10,003. These compounds may be produced from the degradation of pyrene, however, they may not accumulate in the culture medium due to their rapid utilisation by the organisms. Substrate utilisation tests described in Chapter 3 illustrated that the three strains were capable of utilising cinnamic and phthalic acids as growth sources.

The degradation of ^{14}C-pyrene by *St. maltophilia* strain VUN 10,003 demonstrated that the majority of the substrate added was converted to $^{14}CO_2$. This finding may have implications on deciding what is the key metabolic pathway of pyrene by these strains. As a sole carbon and energy source, utilisation of pyrene may result in cell growth, CO_2 or the accumulation of metabolic by-products. As the majority of the ^{14}C was detected as $^{14}CO_2$, it seems appropriate that the major catabolic pathway of pyrene by the *St. malotphilia* strains results in the mineralisation of the compound while the accumulation of by-products may only be via a minor pathway. Multiple pathways for the degradation of pyrene have been observed for a *Mycobacterium* species (Heitkamp *et al.*, 1988b). Heitkamp *et al.* (1988b) observed that pyrene was degraded to *trans*- and *cis*-dihydrodiols by a *Mycobacterium* species. The organism possessed both mono- and dioxygenase enzyme systems that catalysed the formation of the pyrenedihydrodiols. The *cis*-pyrenedihydrodiol was further metabolised, however, the concentration of the *trans*-pyrenedihydrodiol peaked after 48 hours and persisted throughout the experiment. The persistence of the pyrene by-products isolated by preparative TLC from *St. maltophilia* cultures may be indicative of a minor pyrene metabolic pathway. The accumulation of low concentrations of these by-products (including 4-phenanthroic acid and 4-hydroxyperinaphthenone) may occur due to the

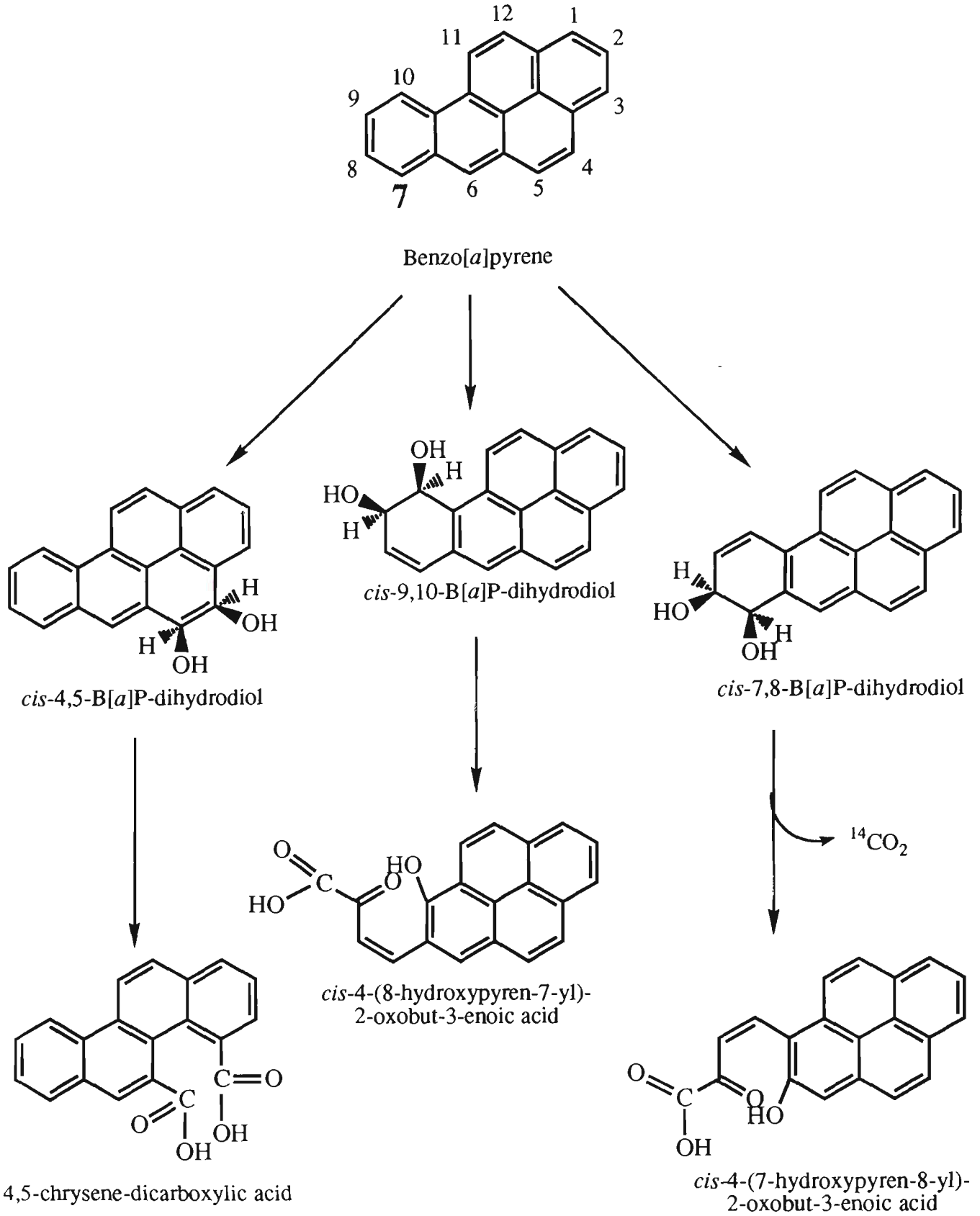


Figure 6.22. The proposed fate of ^{14}C from the degradation of 7-^{14}C-benzo[*a*]pyrene by strain VUN 10,003. The labelled carbon would be detected as $^{14}CO_2$ if benzo[*a*]pyrene is oxidised at the 7,8- position or if the ring oxidation products (4,5- and 9,10-dihydrodiols) are extensively degraded.

inability of the organisms to synthesis to appropriate enzymes for their further degradation. In addition, 4-phenanthroic acid may not be degraded because of molecular overcrowding due to bay region substitution (Rutherford and Newman, 1957). Future research on pyrene degradation by *St. maltophilia* strains should investigate the catabolic pathway leading to CO_2 production. This would involve the isolation of metabolites proceeding the initial degradation lag period (10 hours) as well as other strategies outlined in Section 6.5.4.

6.5.2 Benzo[*a*]pyrene Degradation

6.5.2.1 *Benzo[a]pyrene Mineralisation*

Degradation experiments using [^{14}C]-benzo[*a*]pyrene demonstrated that community five and strain VUN 10,003 failed to mineralise the compound as $^{14}CO_2$ was not detected, however, benzo[*a*]pyrene was degraded to polar metabolites (4.8-6.5%) and the label was detected in cellular material (11.6-12.0%). Given that [^{14}C]-benzo[*a*]pyrene was only labelled at the C7 position, it seems likely that ring cleavage may have occurred at the 9,10- position since the label was detected in the aqueous phase and cellular material, but not in the gaseous phase. This statement is based on the premise that ring cleavage at the 7,8- position would result in the evolution of $^{14}CO_2$ whereas ring cleavage at the 4,5- position would not result in the label being detected as $^{14}CO_2$ or polar metabolites unless benzo[*a*]pyrene was degraded extensively (Figure 6.22). Gibson *et al.* (1975) reported the oxidation of benzo[*a*]pyrene at the 9,10-position by *Beijernickia* strain B836. Benzo[*a*]pyrene failed to induce significant oxgenase activity in cells of *Beijernickia* strain B836. However, after growth on succinate and biphenyl, strain B836 oxidised benzo[*a*]pyrene to a polar product. Oxidation at the 9,10- position resulted in the formation of *cis*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene. In addition, Schneider *et al.* (1996) proposed that *Mycobacterium* strain RJGII-135 oxidised benzo[*a*]pyrene at the 4,5-, 7,8- and 9,10-positions. Degradation experiments with [7,10-]^{14}C-benzo[*a*]pyrene demonstrated that the majority of the label was recovered as undegraded benzo[*a*]pyrene (61%), however, *Mycobacterium* strain RJGII 135 producted six metabolites after 32 days incubation (28% of the total ^{14}C). The 7,8-benzo[*a*]pyrene dihydrodiol was isolated by HPLC fraction collection, however, the 4,5- and the 9,10- benzo[*a*]pyrene dihydrodiols were proposed ring oxidation products on the basis of the isolation of 4,5-chrysene-dicarboxylic acid and 7,8-dihydro-pyrene-7-carboxylic acid. Although strains B836 and RGJII 135 were able to degrade benzo[*a*]pyrene, mineralisation of the compound did not occur. The formation of 7,8-dihydro-pyrene-7-carboxylic acid and 7,8-dihydro-

pyrene-8-carboxylic acid by strain RGJII 135 would result in a small proportion of ^{14}C label occurring as $^{14}CO_2$, however, the metabolite persisted in the medium.

6.5.2.2 *Benzo[a]pyrene Metabolites*

The degradation of benzo[*a*]pyrene has been reported for bacteria (Gibson *et al.*, 1975; Schneider *et al.*, 1996; Juhasz *et al.*, 1996), fungi (Collins *et al.*, 1996; Sanglard *et al.*, 1986; Cerniglia and Gibson, 1979; Haemmerli *et al.*, 1986; Ghosh *et al.*, 1983), alga (Cody *et al.*, 1984; Warshawshy *et al.*, 1988, 1990; Lindquist and Warshawshy, 1985) and mammalian systems (Cooper *et al.*, 1983). The characterisation of benzo[*a*]pyrene metabolites is important since the carcinogenic properties of benzo[*a*]pyrene are only expressed after the compound has been partially degraded (Cerniglia and Gibson, 1980). Degradation of benzo[*a*]pyrene by microsomal enzymes results in the formation of (+)-7ß, 8∂-dihydroxy-9∂, 10∂-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, which has been implicated as the ultimate carcinogenic form of benzo[*a*]pyrene (Cerniglia and Gibson, 1980). A prerequisite for understanding the mechanism of PAH carcinogensis is a knowledge of the profile of metabolites formed and factors regulating their formation (Datta and Samanta, 1988).

Nine metabolites were isolated by preparative TLC from degradation experiments with benzo[*a*]pyrene and strains VUN 10,001, VUN 10,002 and VUN 10,003. Analysis and identification of intermediate compounds by GC-MS and NMR proved to be difficult due to problems associated with volatilising, ionising and derivatising of the compounds. Consequently, the identity of the isolated compounds could not be determined. The major limitation in identifying benzo[*a*]pyrene metabolites in this study was the low yield of potential benzo[*a*]pyrene breakdown products. A larger yield of metabolites was required in order to test different derivatising and analytical protocols. Future research on benzo[*a*]pyrene degradation by *St. maltophilia* strains should address the issues of metabolite yield, derivatising protocols as well as other strategies outlined in Section 6.5.4.

The processes involved in the degradation of benzo[*a*]pyrene are not well understood. To date, only two studies have reported structures of metabolites produced from the microbial transformation of the compound. Gibson *et al.* (1975) and Schneider *et al.* (1996) identified benzo[*a*]pyrene dihydrodiols from the degradation of benzo[*a*]pyrene by *Beijernickia* strain B836 and *Mycobacterium* strain RJGII 135. It has been proposed that benzo[*a*]pyrene can be oxidised at the 4,5-, 7,8- and 9,10- positions (Gibson *et al.*, 1975; Schneider *et al.*, 1996) resulting in *cis*-benzo[*a*]pyrene dihydrodiols. *Ortho*

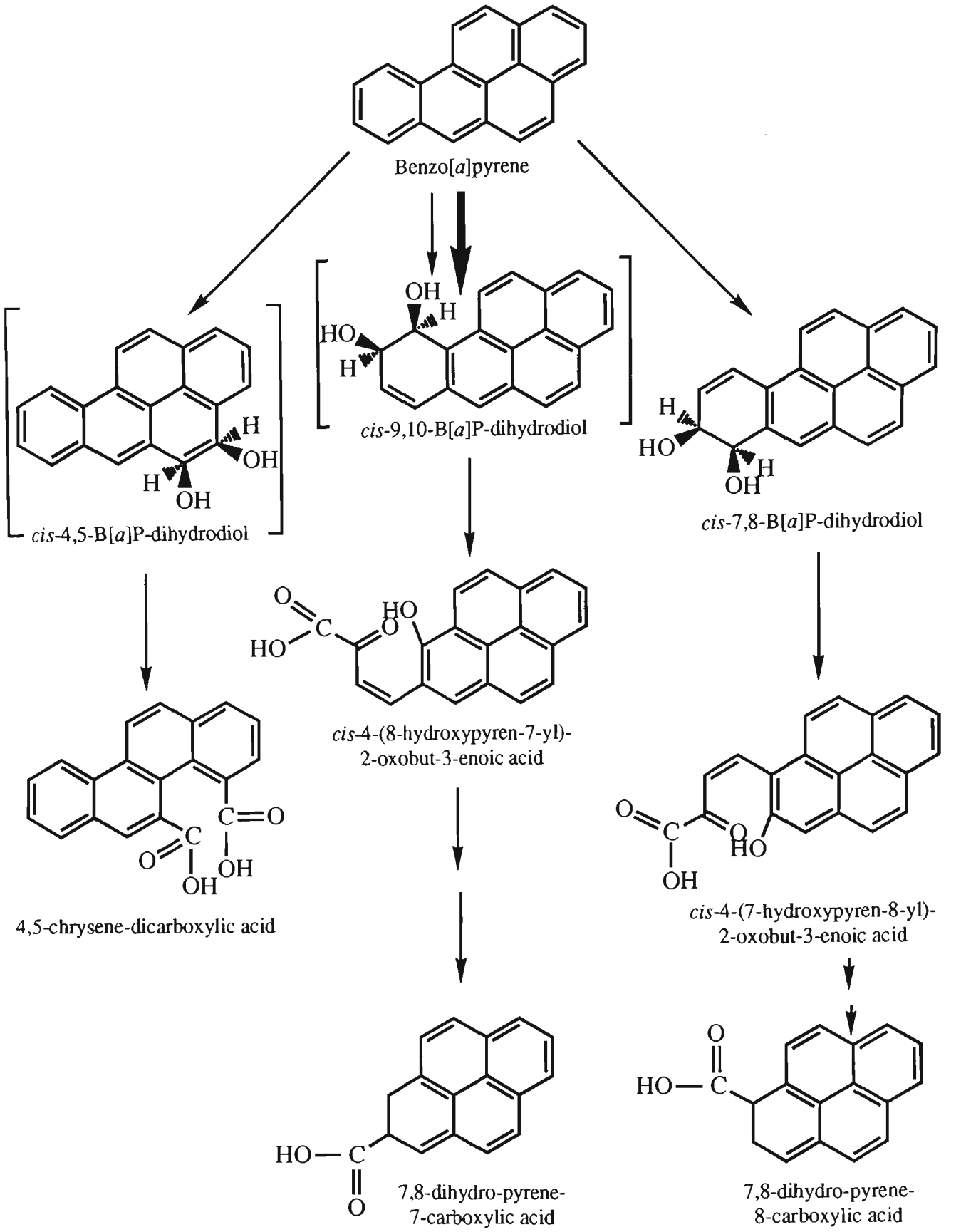


Figure 6.23. Proposed pathway for the degradation of benzo[*a*]pyrene by *Mycobacterium* strain RJGII-135 (Schneider *et al.* 1996) and *Beijernickia* strain B836 (Gibson *et al.* 1975). The compounds in brackets are hypothetical intermediates proposed by Schneider *et al.* (1996). Arrows between metabolites indicate multiple steps, not single reactions. The pathway for the degradation of benzo[*a*]pyrene by *Beijernickia* strain B836 is indicated by the bold arrow.

fission of the 4,5-benzo[*a*]pyrene dihydrodiol leads to the formation of 4,5-chrysene-dicarboxylic acid, while *meta* fission of the 7,8- and 9,10-benzo[*a*]pyrene dihydrodiols results in the formation of *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid and *cis*-4-(8-hydroxypyren-7-yl)-2-oxobut-3-enoic acid respectively. Further metabolism of the meta fission products would produce 7,8-dihydro-pyrene-8- and 7,8-dihydro-pyrene-7- carboxylic acids, however, this would involve several steps (Schneider *et al.*, 1996) (Figure 6.23).

Selanastrum capricornutum, a green alga, has also been shown to produce *cis*-benzo[*a*]pyrene dihydrodiols (4,5-, 7,8- and 11,12-benzo[*a*]pyrene dihydrodiols) (Lindquist and Warshawsky, 1985; Warshawsky *et al.*, 1988), however, the mammalian and fungal metabolism results in *trans* dihydrodiols (4,5-, 7,8- and 9,10-), benzo[*a*]pyrene quinones (1,6- 3,6- and 6,12-), benzo[*a*]pyrene phenols (3- and 9-) and sulphur and glucuronide conjugated derivatives (Ghosh *et al.*, 1983; Lindquist and Warshawsky, 1985; Warshawsky *et al.*, 1988; Datta and Samanta, 1988; Cerniglia and Gibson, 1979, 1980; Haemmerli *et al.*, 1986; Sanglard *et al.*, 1986).

6.5.3 Dibenz[*a,h*]anthracene Degradation

The microbial degradation of dibenz[*a,h*]anthracene has received little review because of its recalcitrance to microbial attack. Dibenz[*a,h*]anthracene has been reported to be degraded (Ye *et al.*, 1996; Juhasz *et al.*, 1996, 1997), however, the mechanisms for its degradation have not been elucidated. High initial cell density experiments with *St. maltophilia* strains (see Section 4.2.3.1) demonstrated the ability of the soil isolates to degrade dibenz[*a,h*]anthracene as a sole carbon source. Over a 63 day incubation period, 10-14 mg/l (20-30%) of dibenz[*a,h*]anthracene was degraded after a degradation lag period of 21 days. Although the resting cell experiments by Ye *et al.* (1996) demonstrated the microbial degradation of dibenz[*a,h*]anthracene, metabolites produced from the degradation of the compound were not identified and the extent of dibenz[*a,h*]anthracene degradation was not determined. In order to gain an insight into the mechanisms of dibenz[*a,h*]anthracene degradation, experiments were prepared with strains VUN 10,001, VUN 10,002 and VUN 10,003.

Preparative TLC of supernatants from dibenz[*a,h*]anthracene cultures resulted in the separation, visualisation and isolation of eight UV fluorescent metabolite bands. As seen for the benzo[*a*]pyrene metabolites, analysis of the isolated dibenz[*a,h*]anthracene compounds by GC-MS and proton NMR proved unsuccessful due to the problems associated with volatilising, ionising and derivatising of the compounds. The low yield

of dibenz[*a,h*]anthracene metabolites was one of the major limitations in identifying the compounds isolated. A larger yield of metabolites was required in order to test different derivatising and analytical protocols. Furture research on dibenz[*a,h*]anthracene degradation by *St. maltophilia* strains should address the issues of metabolite yield, derivatising protocols as well as other strategies outlined in Section 6.5.4.

The initial oxidative attack on dibenz[*a,h*]anthracene may occur at the 1,2- or the 8,9-position. Structurally-related compounds, such as benz[*a*]anthracene and phenanthrene, have been shown to produce ring oxidation products at these positions (Mahaffey *et al.*, 1988). In particular, *Beijernickia* strain B1 was shown to oxidise benz[*a*]anthracene after induction with phenyl, m-xylene and salicylate. Oxidation of benz[*a*]anthracene occurred at the 1,2-, 8,9- and 10,11- positions, however, the 1,2-benz[*a*]anthracene dihydrodiol was the predominant isomer. *Cis*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene was metabolised through a number of steps to form the ring oxidation product 1-hydroxy-2-anthroic acid. Presumably, ring cleavage of *cis*-1,2-dihydroxy-1,2-dihydrodibenz[*a,h*]anthracene would result in the formation of 1-hydroxy-2-benz[*h*]anthroic acid where as ring cleavage of *cis*-8,9-dihydroxy-8,9-dihydrodibenz[*a,h*]anthracene would produce of 8-hydroxy-9-benz[*a*]anthroic acid. These metabolites may occur if the microorganism is able to synthesise enzymes responsible for ring oxidation and cleavage.

The mammalian metabolism of dibenz[*a,h*]anthracene has been studied in rat liver preparations (Sims, 1970; Selkirk *et al.*, 1971; Boyland and Sims, 1965), mice (Heidelberger and Weist, 1951), rabbits (Boyland *et al.*, 1941) and in cell cultures (Huberman *et al.*, 1971). These metabolism studies have isolated dibenz[*a,h*]anthracene dihydrodiols, dibenz[*a,h*]anthracene phenols, dibenz[*a,h*]anthracene epoxides and aromatic acids as mammalian metabolites of dibenz[*a,h*]anthracene. The initial hydroxylation of dibenz[*a,h*]anthracene was found to occur at the 1,2-, 3,4- and 5,6- positions resulting in dihydrodiols with the respective stereochemistries. 3- and 4-hydroxydibenz[*a,h*]anthracene have also been isolated from rat liver preparations and cell cultures incubated with dibenz[*a,h*]anthracene. Heidelberger and Weist (1951) demonstrated the formation of 5-hydroxy-1,2-naphthalic acid from the metabolism of dibenz[*a,h*]anthracene by female albino mice. The acid was found in the faeces and liver of mice injected intravenously with the PAH. In addition, the metabolic product was also found found in the epidermis of mice to which dibenz[*a,h*]anthracene had been treated to the shin. The authors proposed that in order for 5-hydroxy-1,2-naphthalic acid to be produced, the central ring of

dibenz[*a,h*]anthracene must be metabolically cleaved and this could only occur if hydroxyl groups were present on the terminal rings.

Although strains VUN 10,001, VUN 10,002 and VUN 10,003 were capable of degrading benzo[*a*]pyrene and dibenz[*a,h*]anthracene when inocula contained high cell numbers, degradation of the five-ring compounds did not result in the production of biomass (see Section 4.2.3.1). Degradation experiments with [^{14}C]-benzo[*a*]pyrene illustrated that community five and strain VUN 10,003 were not capable of mineralising the compound. It is apparent from these experiments that the *St. maltophilia* strains cannot degrade benzo[*a*]pyrene to organic acids and aldehydes (succinic, fumaric, pyruvic, acetic) which may then be utilised for cellular growth. The inability of the three strains to extensively degrade benzo[*a*]pyrene may be due to a number of factors. Although benzo[*a*]pyrene and dibenz[*a,h*]anthracene ring oxidation may occur due to the non-specific dioxygenases produced by strains VUN 10,001, VUN 10,002 and VUN 10,003, further degradation of the ring oxidation products may not occur because the enzymes involved in the catabolic pathway are not being induced. In addition, the production and accumulation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites may in fact repress the induction of enzymes which are necessary for the further catabolism of the five-ring compounds.

6.5.4 Strategies for Pyrene, Benzo[*a*]pyrene and Dibenz[*a,h*]anthracene Metabolite Identification

Future work is required for the determination of the major catabolic pathway of pyrene by strains VUN 10,001, VUN 10,002 and VUN 10,003 as well as the isolation and identification of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites. One major limitation involved in this work is the commercial availability of radiolabelled compounds (limited in the number and positioning of the ^{14}C label) and the cost involved in producing custom-labelled compounds. However, less expensive strategies can be adopted for metabolite isolation and identification.

(i) Degradation experiments using differentially-labelled PAHs could be used for determining the position of initial PAH hydroxylation. One major limitation with the benzo[a]pyrene study was that the only commercially available ^{14}C-labelled compound was 7-^{14}C-benzo[*a*]pyrene. The formation of $^{14}CO_2$ would indicate hydroxylation at the 7,8- position, however, it may not indicate mineralisation of the compound. Hydroxylation may occur and the resultant compound may not be further

degraded. Degradation experiments using uniformly labelled PAHs would give a definitive result of the proportion of the carbon converted to CO_2, biomass or by-products. Radiolabelled experiments with ^{14}C-dibenz[*a,h*]anthracene were unable to be performed due to the ^{14}C-compound not being commercially available.

(ii) The use of uniformly labelled PAH compounds would also assist in quantifying the rise and fall in metabolite concentrations. Metabolites could be separated from culture extracts by TLC and the distribution of the ^{14}C label could be monitored.

(iii) Large scale degradation experiments are required for the isolation and purification of adequate quantities of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites. In this study, 500 ml samples were taken at various time periods, extracted and analysed by TLC. Metabolites were isolated, however, adequate quantities were not obtained for further analysis. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene experiments need to be scaled up in order to purify sufficient amounts of the respective metabolites.

(iv) Preparative TLC of supernatant crude extracts from *St. maltophilia* strains incubated in BSM containing pyrene resulted in the isolation of seven pyrene by-products. These compounds persisted throughout the incubation period. The degradation of ^{14}C-pyrene by the pyrene-enriched microorganisms proceeded after an initial lag period of 10 hours, however, approximately 70% of the ^{14}C was detected as $^{14}CO_2$. Presumably, the compounds isolated by preparative TLC are those that comprise a minor metabolic pathway of pyrene. Analysis of crude extracts at an early stage of degradation (after 10 hours) may isolate intermediate compounds that comprise the major degradative pathway of pyrene (*i.e.* compounds further degraded to CO_2) if sufficient volumes of culture fluid are used.

(v) The ability of strains VUN 10,001, VUN 10,002 and VUN 10,003 to utilise potential pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene intermediate products could be demonstrated by supplying these compounds as sole carbon and energy sources. The origin of polar single ringed metabolites (phthalic and cinnamic acids) may be resolved in studies in which large quantities of ring fission products are synthesised and utilised as substrates

for microbial metabolism. Unfortunately, a number of these compounds are not commercially available and would have to be synthesised.

(vi) The derivatisation of benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites by BSTFA plus 1% TMCS was unsuccessful; silylation failed to produce compounds that could be detected by GC-MS. Alternative derivatising methods, such as acetylation, may be required to produce volatile benzo[*a*]pyrene and dibenz[*a,h*]anthracene for GC-MS analysis.

6.5.5 Conclusion

Research reported in this chapter demonstrated the rapid degradation of pyrene to CO_2 by community five and *St. maltophilia* strain VUN 10,003. Only a small proportion of the label (4%) was detected in the aqueous phase indicating the low amount of polar metabolites produced by these cultures. Benzo[*a*]pyrene was not mineralised, however, 4.8-6.5% of the ^{14}C was detected in the aqueous phase. GC-MS analysis of TLC-isolated pyrene metabolites identified a pyrenedihydrodiol and pyrenol as ring oxidation products of pyrene and 4-hydroxyperinaphthenone and 4-phenanthroic acid as ring fission products. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites could be isolated by preparative TLC, however, their structure could not be determined due to the low yields of the compounds. The final chapter of this thesis reports the major findings of the research as well as recommedations for future work.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

7.2 RECOMMENDATIONS FOR FUTURE WORK

7.2.1 Molecular Characterisation of *St. maltophilia* Strains

7.2.2 Development of DNA Probes for the Detection of High Molecular Weight PAH-Degrading Genotypes

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

The research described in this thesis investigated the microbial degradation of high molecular weight PAHs. To date, few reports in the literature have described the microbial degradation of five-ring compounds. This work concentrated on three strains of *St. maltophilia* (strains VUN 10,001, VUN 10,002 and VUN 10,003) and a microbial community (community five) isolated on pyrene from soil collected from a former manufacturing gas plant.

The enrichment of bacteria from PAH-contaminated soil resulted in five microbial communities capable of growing on phenanthrene and pyrene as sole carbon and energy sources. The growth of microbial communities on pyrene is quite noteworthy as until recently four-ring compounds have been considered as rather recalcitrant against microbial degradation. Three pure cultures were isolated from community five with the ability to grow on pyrene as a sole source of carbon and energy. The organisms were identified as strains of *St. maltophilia* based on 16SrRNA gene sequence analysis. This is the first report of a Gram negative organisms capable of utilising pyrene as a growth source. In addition, there are few reports of the degradation of aromatic compounds by *St. maltophilia* strains. The organism has previously been shown to degrade toluene, xylene and 1-naphthoic acid. Although the *St. maltophilia* strains were almost identical in their PAH degradative profiles, biochemical characteristics and 16SRNA gene sequences, the strains could be differentiated by ribotyping and pulse field gel electrophoresis. All three strains showed different ribotyping patterns, indicating different copies of the 16SrRNA gene. The aquisition of extra gene copies may have occurred through lateral gene transfer between the strains.

Community five and the *St. maltophilia* strains could grow on and degrade fluorene, phenanthrene and pyrene when supplied as sole carbon and energy sources. Small decreases in the concentration of fluoranthene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were observed, however, little growth occurred. It appeared that the strains could not utilise these compounds as growth substrates and that the amount degraded was limited by the size of the microbial population. Degradation of the high molecular weight PAHs was demonstrated when cultures were inoculated with high

initial cell numbers of community five or the *St. maltophilia* strains. Furthermore, it was demonstrated that the pyrene-enriched organisms could degrade coronene, a seven-ring PAH. There have been no previous reports of the bacterial degradation of this compound. When high cell numbers of community five and the *St. maltophilia* isolates were inoculated into media containing both low and high molecular weight PAHs (three- to seven-rings), the organisms were able to simultaneously degrade all the PAHs present. Furthermore, improved degradation of the five- and seven-ring compounds was observed presumably due to increased metabolic activity due to the presence of the more easily degradable low molecular weight compounds. Although many authors have observed the sequential degradation of PAHs in a PAH mixture, the concurrent degradation of all PAHs by community five and the *St. maltophilia* isolates indicated that the catabolic pathways for these compounds are not repressed by the presence of more easily metabolised PAH compounds. Induction of PAH degrading ability appears to be involved with these organisms as growth of community five and the *St. maltophilia* strains on PYEG did not allow for the consequent degradation of pyrene, benzo[*a*]pyrene or dibenz[*a,h*]anthracene.

Although the *St. maltophilia* strains could degrade benzo[*a*]pyrene and dibenz[*a,h*]anthracene, degradation was limited to 10-15 mg/l. Metabolite or by-product repression was found to be responsible for the inhibition of five-ring PAH degradation. Cells that were exposed to the five-ring metabolites were still capable of degrading benzo[*a*]pyrene and dibenz[*a,h*]anthracene in the presence of pyrene when transferred to fresh media. This indicates that the cells are still metabolically active and the loss of plasmid/genetic material did not occur.

A medium containing creosote and yeast extract (CYEM) was developed for preparing community five inocula for PAH degradation. CYEM provided a medium which supported and maintained the pyrene-degrading capacity of the community. In addition, biomass yields were attained in half the time compared to pyrene-grown cells and the degradative performance was comparable to pyrene-grown cells. The medium was prepared using inexpensive sources of PAHs (creosote) and fermentation wastes (yeast extract) which is economical when large amounts of biomass are required for bioremediation.

Inoculation of community five into PAH-contaminated soil resulted in significant decreases in the concentration of both low and high molecular weight PAH compounds. Previous researchers have reported the bioremediation of two- to four-ring PAHs in a soil matrix, however, few have demonstrated a reduction in the concentration of the

higher molecular weight compounds. Community five degraded up to 73% of the total PAHs in the soil, while an 18-25% decrease in the concentration of benzo[*a*]pyrene and dibenz[*a,h*]anthracene were observed after 91 days. In addition, the decrease in the concentration of PAHs corresponded to a reduction in the mutagenic potential of organic extracts of soil and a decrease in the toxicity of aqueous extracts of soil. These results demonstrate that community five possesses a number of characteristics that are essential for successful bioremediation of PAH-contaminated sites.

Community five and *St. maltophilia* strain VUN 10,003 were capable of rapidly mineralising pyrene, however, benzo[*a*]pyrene mineralisation was minimal. Athough the organisms were unable to mineralise benzo[a]pyrene, ^{14}C was detected in the aqueous phase and cellular material indicating the production of water soluble metabolites and incorporation of carbon into the cell wall.

Pyrenedihydrodiol, pyrenol, 4-hydroxyperinaphthenone and 4-phenanthroic acid were identified as pyrene metabolites of *St. maltophilia* strains. The persistence of 4-hydroxynaphthenone and 4-phenanthroic acid in the culture medium over the time course period suggests that these compounds are by-products of a minor pyrene catabolic pathway as the majority of ^{14}C-pyrene was converted to $^{14}CO_2$. Benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites could be isolated by preparative TLC, however, their structures could not be determined.

The research reported in this thesis demonstrated the high molecular weight PAH degrading ability of *St. maltophilia* strains in liquid and soil matrices. It demonstrated the detoxification of PAH compounds, provided an insight into the regulation of PAH metabolism, *i.e.* induction of PAH-degrading ability, concurrent degradation of PAHs, cometabolism of high molecular compounds, and showed the inhibition of high molecular weight PAH degradation as a result of by-product accumulation. Preliminary steps were also taken into identifying catabolic by-products of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene.

7.2 RECOMMENDATIONS FOR FUTURE WORK

7.2.1 Molecular Characterisation of *St. maltophilia* Strains

Further work needs to be performed on the molecular characterisation of the *St. maltophilia* strains. This would include the identification of genes encoding high

molecular weight PAH degradation. Most often degradative pathways are specified by genes located on large plasmids (Kurkela *et al.*, 1988). Several such plasmids have shown to confer the ability to utilise naphthalene as a sole carbon and energy source (Dunn and Gunsalus, 1973; Rossello-Mora *et al.*, 1994; Zuniga *et al.*, 1981). As such, catabolic plasmids need to be demonstrated and mapped. In addition, the organisation and regulation of these genes, including the structure and promotor regions of the genes need to be identified. The isolation of high molecular weight PAH-degrading genes of the *St. maltophilia* strains is an important step towards understanding the ability of the microorganisms to degrade four-, five-, and seven-ring PAHs and in developing strains with even greater degradative potentials for use in bioremediation.

7.2.2 Development of DNA Probes for the Detection of High Molecular Weight PAH-Degrading Genotypes

The development of specific DNA probes for the detection of high molecular weight PAH-degrading microorganisms in the environment has several advantages over conventional enrichment and isolation techniques. These advantages include the potential for detecting very small numbers of target organisms in the environment, a more rapid method for the quantification of PAH degraders in a given sample and elimination of culturing cells, making possible the detection of non-culturable but potentially viable organisms. In addition, specific microbial genotypes could be tracked within a microbial community, which would provide an insight into the population dynamics of specific populations within complex communities. DNA probes have been developed for the detection of naphthalene degraders in the environment and for specific microbial genotypes (Pickup, 1991; Diels and Mergeay, 1990; Fleming *et al.*, 1993). The use of probes for the detection of high molecular weight PAH-degraders in a given sample would provide a rapid method for assessing the potential of bioremediation to remediate soils contaminated with these compounds. DNA probes could also be used applied for monitoring the efficacy of bioremediation.

7.2.3 Enhancing the PAH-Degrading Ability of *St. maltophilia* strains

Although *St. maltophilia* strain VUN 10,003 had the ability to degrade benzo[*a*]pyrene and dibenz[*a,h*]anthracene, degradation was limited to 10-15 mg/l: by-products of benzo[*a*]pyrene and dibenz[*a,h*]anthracene catabolism inhibited further degradation of the five-ring compounds. Increasing the substrate range of the microorganism may result in the metabolism of the inhibitory by-products and consequently further degradation of the five-ring compounds. Cloning and expression of genes encoding

other dioxygenases (1,2-, 4,5-, 9,10-dioxygenases) in *St. maltophilia* strains may enhance the substrate range of the strains and increase five-ring degradation performance. In addition, cloning and expression of other traits into *St. maltophilia* strains may enhance the PAH-degrading ability of the strains in soil. Many PAH-contaminated soils contain high concentrations of heavy metals such as cadmium, cobalt, copper, lead, mercury and zinc. Cloning and expression of genes that encode microbial responses to pollutants, *e.g. mer* genes and Hg^{2+} resistance (Barkay *et al.*, 1989) or *czc* genes and Cd^{2+}, Co^{2+} and Zn^{2+} resistance (Diels and Mergeay, 1990), may result in strains that can degrade PAHs in the presence of high concentrations of heavy metals.

7.2.4 Degradation of Dibenz[*a,h*]anthracene and Coronene Using Radiolabelled Compounds

Degradation studies using radiolabelled PAHs demonstrated that VUN 10,003 and community five were capable of mineralising pyrene, however, they were unable to mineralise benzo[*a*]pyrene. The degradation of benzo[*a*]pyrene resulted in a small proportion of the label being detected in the aqueous phase and in cellular material. To gain a better understanding of the degradative fate of other high molecular weight PAHs, such as dibenz[*a,h*]anthracene and coronene, degradation studies should be performed with the *St. maltophilia* strains and radiolabelled PAHs. The major limitation in performing such experiments is the cost of the radiolabelled PAHs. ^{14}C-labelled dibenz[*a,h*]anthracene and coronene are not "off the shelf" chemicals and are consequently prohibitively expensive. However, the use of uniformly labelled PAH compounds would also assist in isolating metabolites or by-products of PAH degradation and in quantifying the concentration of metabolites.

7.2.5 Identification of High Molecular Weigh PAH Metabolites

Although the work in Chapter 6 identified some metabolites produced during the initial ring oxidation and cleavage of pyrene, a more detailed study is required for the identification of other metabolites so that the complete pyrene degradative pathway can be revealed. Studies on the metabolism of benzo[*a*]pyrene and dibenz[*a,h*]anthracene by *St. maltophilia* strains demonstrated that metabolites produced from these compounds can be isolated, however, due to the small quantities recovered, the identity of the metabolites could not be assertained. A number of strategies for the identification of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene metabolites were outlined in Section 6.5.4. These strategies include differentially and uniformally ^{14}C-

labelled PAH degradation studies, large scale benzo[*a*]pyrene and dibenz[*a,h*]anthracene degradation studies, studying the degradation of potential high molecular weight PAH by-products and different derivatisation methods for the production of volatile compounds for GC-MS analysis. In addition, the degradative pathway for coronene by the *St. maltophilia* strains has not been studied.

7.2.6 Scale-up of Soil Bioremediation

Inoculation of CYEM-grown community five into PAH-contaminated soil resulted in significant decreases in the concentration of all PAHs (high and low molecular weight compounds) over a 91 day period. Microbial numbers remained relatively steady over the incubation period. In addition, inoculation of PAH-contaminated soil with CYEM-grown community five resulted in a reduction in the mutagenic potential of soil organic extracts and detoxification of the water soluble fraction of the soil. The degradative performance of community five in PAH-contaminated soil warrants investigation of the efficiency of this on a larger scale. Prepared bed (on-site) or bioreactor treatments seem more appropriate than *in situ* treatment as factors affecting degradation can be controlled more effectively. Prepared beds can be managed to provide nutrient and oxygen addition and the control of pH and moisture. Bioreactors offer the advantage of running the process under optimal conditions, however, running costs are greater than on-site treatments. Treatability and pilot-scale studies are important to minimise problems associated with application in the field and such studies would be an obvious extension of this thesis.

APPENDICES

Appendix 1 Biochemical Tests

Appendix 2 Degradation of PAHs in liquid medium by community five *and St. maltophilia* isolates.

Appendix 3 Calculation of Gamma and EC_{50} for Microtox™ assays.

APPENDIX 1

BIOCHEMICAL TESTS (MacFadden, 1980; Palleroni, 1984)

Citrate Test

Medium: Simmons Citrate Medium (pH 6.9)

$MgSO_4$	0.2 g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Sodium Citrate	2.0 g
NaCl	5.0 g
Agar	15.0 g
Bromothymol Blue	0.08 g
Distilled Water	1000 ml

pH Indicator- Bromothymol Blue
(a) Acid: yellow colour pH 6.0
(b) Alkaline: deep blue pH 7.6
(c) Uninoculated medium: pH 6.9 green colour

Inoculate slant by "fishtail" streak and incubate at 35°C for 24 to 48 hours. A positive test results with growth and an intense blue colour on the slant.

Decarboxylase Test

Medium: Falkow decarboxylase broth (pH 6.8)

Peptone	5.0 g
Yeast Extract	3.0 g
Glucose	1.0 g
L-Lysine or L-Arginine	5.0 g
Bromocresol Purple	0.02 g
Distilled Water	1000 mL

pH Indicator- Bromocresol Purple
(a) Acid: yellow colour pH 5.2
(b) Alkaline: deep blue pH 6.8
(c) Uninoculated medium: pH 6.8 deep brilliant purple colour

Inoculate broth with a light inoculum and incubate at 35°C for 24 to 48 hours. A positive result is indicated by a turbid purple to a faded yellow purple colour. A negative result is indicated by a bright clear yellow colour (only glucose fermented).

Gelatin Hydrolysis

Medium: Nutrient Broth containing Gelatin

Sterile Nutrient Broth	1000 ml
Davis Gelatin	120 g

Autoclave and dispense (15 ml) into sterile MacCartney bottles. Media is stab inoculated and incubated at 37°C for 24-48 hours (up to 30 days). Cultures are transferred to 4°C for 4 hours before reading the results. A positive reaction is indicated by liquefaction of the gelatin.

Indole Test
Medium: Peptone Water

Peptone	10.0 g
NaCl	5.0 g
Distilled Water	1000 ml

Kovac's Reagent

Amyl alcohol	75 ml
p-dimethylaminobenzaldehyde	5.0 g
Concentrated HCl	25 ml

Cultures are inoculated into Peptone water and incubated at 37°C for 18 hours. Kovac's reagent (5 ml) is added to the cultures and shaken gently. A positive result is indicated by a pink to red colour. No colour change indicates a negative result.

Methyl Red Test
Medium: Glucose Phosphate Peptone Water (pH 7.6)

Peptone	5.0 g
K_2HPO_4	5.0 g
Glucose	5.0 g
Distilled Water	1000 ml

The inoculated medium is incubated for 2-4 days at 37°C. After incubation, methyl red is added to the culture. A positive result is indicated by a red colour. A yellow colour indicates a negative result.

Urease Production
Medium: Urease Medium (pH 6.8)

Peptone	1.0 g
KH_2PO_4	2.0 g
NaCl	5.0 g
0.5% Phenol Red	2.4 ml
Agar	12 g
Distilled Water	1000 ml

Autoclave at 121°C for 20 minutes, cool to 50°C then add 2.5 ml sterile 20% glucose and 25 ml filter sterilised 40% urea.

Inoculate the media with a heavy inoculum. Incubate at 37°C overnight (up to 7 days). Hydrolysis is indicated by a pink colour.

Voges-Proskauer Test
Medium: Glucose Phosphate Peptone Water (pH 7.6)

Peptone	5.0 g
K_2HPO_4	5.0 g
Glucose	5.0 g
Distilled Water	1000 ml

Inoculate the media and incubate for 2-4 days at 37°C. After incubation add 1.0 ml of the culture to 0.4 ml 40% KOH and a knife point of creatin (guanidine) and mix. A positive result is indicated by a pale pink colour. No colour change indicates a negative result.

Starch Hydrolysis
Medium: Starch Agar (pH 7.2)

Nutrient Broth N° 2	25 g
Yeast extract	3.0 g
Agar	12 g
Distilled Water	1000 ml

Starch Solution
Add potato starch (5% w/v) to distilled water. Dissolve the starch slurry in a boiling waterbath then autoclave (121°C for 15 minutes).

Iodine solution
1% (w/v) iodine and 20% (w/v) KI.

Sterilise the medium by autoclaving (121°C for 15 minutes), then add 0.65% (w/v) starch solution and pour plates.

Test organisms are spot inoculated onto the surface of dried plates and incubated for an appropriate time to give good growth (37°C). Starch hydrolysing organisms produce cloudy zones around the colonies. When the plates are flooded with iodine solution the zones become clear.

Reactions in Litmus Milk
Medium: Litmus Milk (pH 6.8)

Skin Milk	100 g
Litmus powder	0.75 g
Distilled Water	1000 ml

Autoclave at the medium at 121°C for 20 minutes. After inoculation, incubate the cultures at 37°C for 18-24 hours (longer periods of up to 14 days may be necessary).

Results

(1) Acid formation: litmus milk turns a pink colour due to the production of acid from lactose.
(2) Acid and clot: the litmus turns a pink colour and enough acid is produced to clot the casein.
(3) Reduction: the litmus milk becomes colourless.
(4) Curdling of the casein: Clotting of the casein may occur as a result of rennet-like enzymes. Little or no acid is produced.
(5) Casein decomposition: The medium may clear due to the activity of proteolytic enzymes. This frequently occurs at the top of the meduim.
(6) Alkaline reaction: Bluish purple colour.

Hydrogen sulphide production
Medium: Triple Sugar Iron Agar

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20 g
Lactose	10 g
Sucrose	10 g
Glucose	1.0 g
$FeSO_4$	0.2 g
$Na_2S_2O_3$	0.3 g
NaCl	5.0 g
Agar	12 g
Phenol Red	0.024 g
Distilled Water	1000 ml

Dissolve ingredients in distilled water by gently heating. Dispense into MacCartney bottles and autoclave at 121° for 20 minutes. Cool media in a slanted position with deep butts. Inoculate medium with an inoculating needle by (1) fishtail slant and (2) stab butt. The production of hydrogen sulphite is indicated by blackening of the medium.

ONPG Test
Medium: ONPG Medium

Sterile 1% peptone water	75 ml
Sterile ONPG solution	25 ml

ONPG Solution
o-nitrophenyl-B-D-galacto-pyranoside 0.6 g
0.01 M Na_2HPO_4 Buffer 100 ml
Dissolve at room temperature and sterilise by filtration.

Inoculate medium and incubate at 37°C for 24 hours. A positive result is indicated by a bright yellow colour.

Catalase test

After growth on NA (2 days) organisms are transfered onto a glass microscope slide covered with 3% hydrogen peroxide. A positive result is indicated by the evolution of gas bubbles within two minutes.

Oxidase test

After growth on NA (2 days) organisms are smeared onto filter paper moistened with 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride (aqueous solution). A positive reaction is indicated by the development of a purple colour within 10 seconds.

Growth with 6.5% NaCl
Medium: Peptone Water containing NaCl

Peptone	10.0 g
NaCl	65 g
Distilled Water	1000 ml

Inoculated cultures are incubated at 30°C for up to 7 days. Growth is determined by visual observations of turbidity.

Growth at 42°C

Microorganisms are inoculated into Peptone water and incubated at 42°C for up to 7 days. Growth is determined by visual observations of turbidity relative to uninoculated controls.

O-F Glucose
Medium: Hugh and Leifson Medium

Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Agar	3.0 g
1% aqueous Bromothymol blue	3.0 ml
Distilled Water	1000 ml

Sterilise by autoclaving then add sterile glucose to the cooled base medium at a concentration of 1%.

Stab inoculate the medium with the test organism and overlay one tube with approximately 2 ml of sterile paraffin oil for anaerobic conditions. An acidic (yellow) change at or near the surface indicates that the substrate is being oxidised by aerobic bacteria. Uniform acidity indicates that facultative anaerobic organisms are both oxidising and fermenting the substrate. Acidity in the tube sealed with paraffin indicates fermentative activity.

Nitrate Reduction
Medium: Nitrate Peptone Water

Nutrient Broth N° 2	25 g
KNO_3	1.0 g
Distilled Water	1000 ml

Reagents
Solution A:

Sulphanilic acid	0.8 g
5 N acetic acid	100 ml

Solution B:

a-naphthylamine	0.5 g
5 N acetic acid	100 ml

Dispense the Nitrate peptone water into Bijou bottles containing Durham tubes and autoclave. Inoculate and incubate for 24-48 hours.

Spot test: To one drop of solution A and solution B add a large drop of the culture. A red colour indicates that nitrite is present. No colour change indicates a negative result. A negative result is confirmed by the chemical reduction of nitrate test. A small amount of zinc dust is added to the test that gave the negative result for nitrite. An immediate red colour indicates the presence of previously unreduced nitrate.

Tween 80 Hydrolysis

Nutrient agar is supplemented with 0.01% $CaCl_2$ and 1% Tween 80. Plates are inoculated with the test organisms and incubated at 30°C for up to 7 days. Opaque zones surrounding colonies indicate the hydrolysis of the Tween.

Growth on MacConkey agar

MacConkey agar plates (Oxoid) were inoculated and incubated for 2-4 days at 30°C.

Fluorescent Pigments
Medium: King's A (pH 7.2)

Bacto Peptone	20.0 g
Glycerol	15 ml
K_2SO_4	10.0 g
$MgCl_2.6H_20$	1.4 g
Distilled Water	985 ml

Dissolve the ingredients by boiling. Dispense as 10 ml volumes in McCartney bottles and autoclave at 121°C for 15 minutes. Inoculate the medium and incubate at 30°C for 24-28 hours. Observed the inoculated medium for fluorescent pigments.

Motility

Using cavity slides, nutrient broth cultures in the early logarithmic phase were examined for motility by the hanging drop method (Sherman, 1967).

Phenylalanine Deaminase
Medium: Phenylalanine Agar (pH 7.4)

Yeast Extract	3.0 g
DL-phenylalanine	2.0 g
Na_2HPO_4	1.0 g
NaCl	5.0 g
Agar	12.0g

Dispense the medium into McCartney bottles and autoclave at 121°C for 10 minutes. Solidify the medium as slants. Inoculate the medium with a heavy inoculum and incubate at 30°C for 24 hours. Following incubation add 4 to 5 drops of 10% ferric chloride solution to the agar slant. A positive test results in the development of a green colour in the syneresis fluid and the slant.

Test for Depolymerase (DNase)
Medium: DNase agar (Oxoid)

Spot inoculate the organism on the surface of the agar and incubate at 30°C for 24 hours. Following incubation, flood the plates with HCl. DNase positive colonies are surrounded by clear zones.

Aromatic Ring Cleavage

Cultures are grown in BSM agar containing 0.1% sodium *p*-hydroxybenzoate as the carbon source. Colonies are removed from the agar and resuspended in 2 ml of 0.02M Tris buffer (2-amino-2-hydroxymethyl-1,3-propanediol), pH 8. Toluene (0.5 ml) and sodium protocatechuate (3.5 mg) are added to the cell suspension and agitated for 30 seconds. A yellow colour within a few minutes indicates *meta* cleavage. If no colour appears, shake the tubes for 1 hour at 30°C. Add 1.0 g of $(NH4)2SO4$, 1 drop of 1.0% sodium nitroprusside (nitroferricycanide), and 0.5 ml of ammonia solution (specific gravity, 0.880, or 28 to 30%). A purple colour indicates *ortho* cleavage.

Accumulation of poly-ß-hydroxybutyrate
Medium: Poly-ß-hydroxybutyrate Medium (PHB medium)

Part A	900 ml
Part B	100 ml

Part A (pH 7.2)

$K_2HPO_4.3H_2O$	0.6 g
KH_2PO_4	0.2 g
$MgSO_4.7H_2O$	0.2 g
$(NH_4)_2SO_4$	0.2 g

Agar	15 g
Distilled Water	900 ml

Part B

Glucose	10.0 g
Distilled Water	100 ml

Autoclave Parts A and B at 121°C for 15 minutes. Once the components have cooled to 50°C, aseptically combine Parts A and B, mix and pour plates. Inoculate plates and incubate at 30°C for 24 hours. The production of poly-ß-hydroxybutyrate is determined by staining cells with sudan black; cells that accumulate poly-ß-hydroxybutyrate with contain stained sections.

APPENDIX 2

Degradation of PAHs in Liquid Culture by Community Five and *St. maltophilia* Isolates

Table 1. Degradation of individual PAHs by community five, VUN 10,002 and VUN 10,003 in liquid medium. High initial cell density inocula were added to BSM containing individual PAHs as sole carbon and energy sources.

		Community five			VUN 10,002			VUN 10,003		
PAH	Initial Conc. (mg/l)	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b]	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b]	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b]
PYR	250	4.0	3	1093.3[d]	2.4	5	1100.4[d]	7.3	4	809.2[d]
FA	100	46.9	14	75.9[e]	55.3	14	106.4[f]	52.9	14	67.3[e]
BA	100	69.3	14	43.9[e]	72.5	14	65.5[f]	73.8	14	37.4[e]
B[a]P	50	40.6	42 (21)[c]	9.0[e]	40.4	42 (21)	9.2[e]	39.3	42 (21)	17.0[f]
DBA	50	39.2	42 (21)	10.3[e]	42.9	42 (21)	6.8[e]	39.7	42 (21)	16.4[f]
COR	20	9.9	42 (14)	7.2[e]	9.9	42 (14)	7.2[e]	9.1	42 (14)	13.0[f]

[a]These values represent the mean of the final PAH concentration in the experimental cultures for three separate incubations. The standard deviation for all recovered PAHs was less than five percent.

[b]The degradation rates were calculated after the initial degradation lag phase for each compound. Degradation rates have been normalised to account for abiotic losses in the control cultures.

[c]The value in brackets represents the degradation lag period in days.

[d]The cell population was constant at 7.5×10^7 cells/ml.

[e]The cell population decreased linearly from 7.5×10^7 to 2.5×10^7 cells/ml. The degradation rate was calculated using an average figure of 5.0×10^7 cells/ml.

[f]The cell population decreased linearly from 4.5×10^7 to 1.5×10^7 cells/ml. The degradation rate was calculated using an average figure of 3.0×10^7 cells/ml.

Table 2. Degradation rates of phenanthrene, pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene in a PAH mixture (PPDB) by community five , VUN 10,002 and VUN 10,003 in liquid media. High initial cell density inocula were added to BSM containing the PAH mixture as the sole carbon and energy source.

		Final Conc. (mg/l)[a]				Degradation Rate (mg/l/[10^9 cells]/day)[c]		
PAH	Initial Conc. (mg/l)	Community five	VUN 10,002	VUN 10,003	Lag Phase (days)	Community five[d]	VUN 10,002[d]	VUN 10,003[e]
PHEN	50	1.0 (14)[b]	2.4 (14)	1.4 (14)	0	70.0	68.0	99.2
PYR	50	6.8 (14)	1.0 (21)	4.0 (14)	0	61.7	46.7	93.9
B[a]P	50	32.7 (42)	35.6 (42)	33.8 (42)	14	12.4	10.3	16.5
DBA	50	31.0 (42)	33.9 (42)	32.1 (42)	14	13.6	11.5	18.3

[a]These values represent the mean of the final PAH concentration in the experimental cultures for three separate incubations. The standard deviation for all recovered PAHs was less than five percent.

[b]The values in the brackets represents the time period used to calculate degradation rates.

[c]The degradation rates were calculated after the initial degradation lag phase for each compound. Degradation rates have been normalised to account for abiotic losses in the control cultures.

[d]The cell population decreased linearly from 7.5 x 10^7 to 2.5 x 10^7 cells/ml. The degradation rate was calculated using an average figure of 5.0 x 10^7 cells/ml.

[e]The cell population decreased linearly from 4.5 x 10^7 to 2.5 x 10^7 cells/ml. The degradation rate was calculated using an average figure of 3.5 x 10^7 cells/ml.

Table 3. Degradation of a PAH mixture (FC) by community five, VUN 10,002 and VUN 10,003 in liquid medium. High initial cell density inocula were added to BSM containing the PAH mixture as the sole carbon and energy source.

		Community five			VUN 10,002			VUN 10,003		
PAH	Initial Conc. (mg/l)	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b,d]	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b,d]	Final Conc. (mg/l)[a]	Time (days)	Deg. Rate (mg/l/[10^9 cells] /day)[b,d]
FLU	50	2.5	42	19.5	3.5	42	22.1	5.0	42	21.4
PHEN	50	14.8	42	15.3	9.0	42	19.5	4.5	42	21.7
PYR	50	14.3	42 (7)[c]	20.0	19.0	42 (7)	17.7	13.0	42 (7)	40.0
FA	50	19.5	42	14.0	18.0	42 (7)	18.3	14.0	42	17.1
BA	50	33.7	42 (7)	9.3	34.5	42 (7)	11.1	31.5	42 (7)	10.6
B[a]P	50	32.1	42 (14)	12.8	38.5	42 (14)	8.2	33.0	42 (14)	21.1
DBA	50	34.9	42 (14)	10.8	36.6	42 (14)	9.6	31.8	42 (14)	13.0
COR	20	12.7	42 (14)	5.2	14.6	42 (14)	3.9	13.2	42 (14)	4.9

[a]These values represent the mean of the final PAH concentration in the experimental cultures for three separate incubations. The standard deviation for all recovered PAHs was less than five percent.

[b]The degradation rates were calculated after the initial degradation lag phase for each compound. Degradation rates have been normalised to account for abiotic losses in the control cultures.

[c]The value in brackets represents the degradation lag period in days.

[d]The cell population decreased linearly from 7.5×10^7 to 2.5×10^7 cells/ml. The degradation rate was calculated using an average figure of 5.0×10^7 cells/ml.

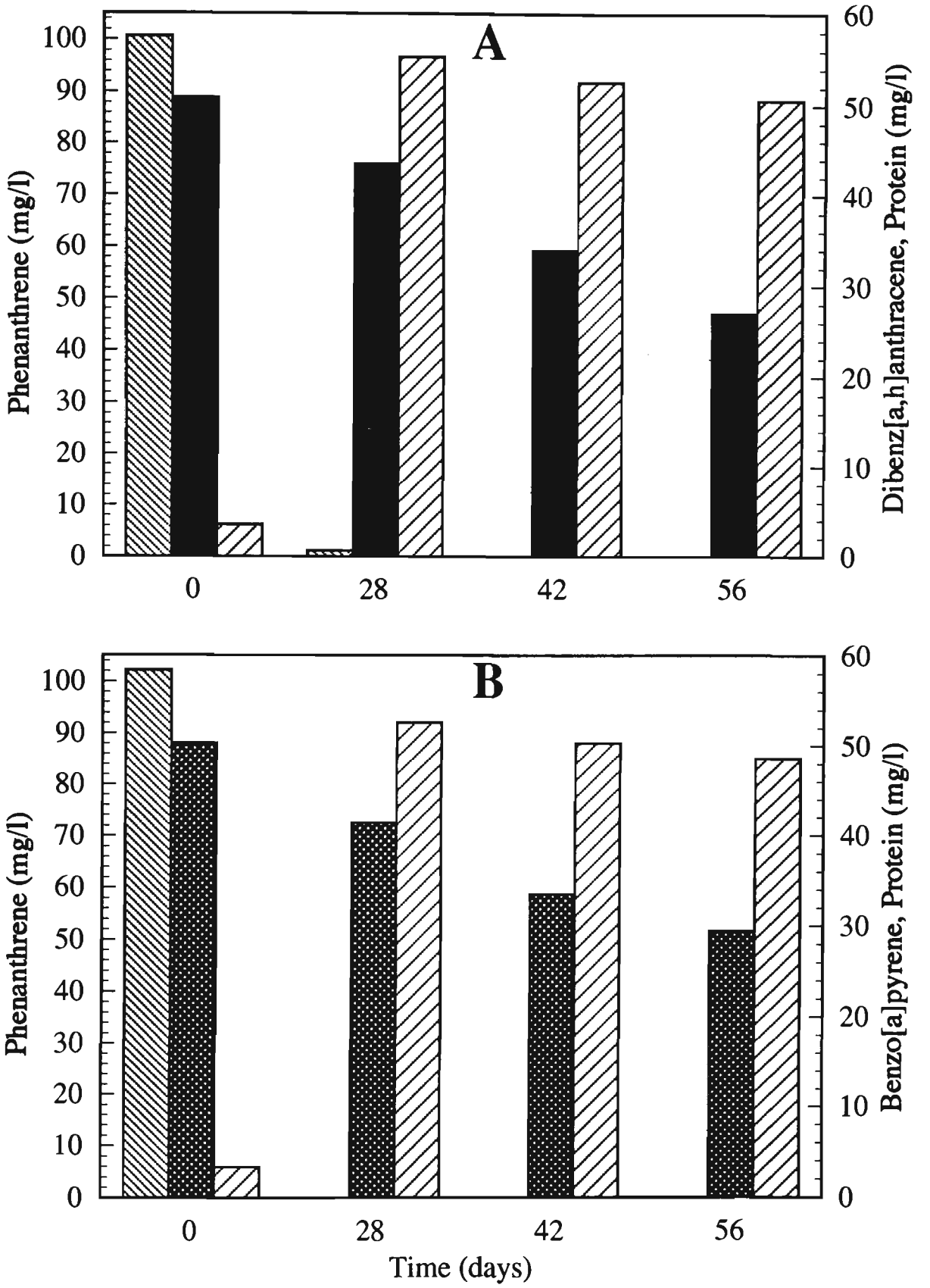

Figure 1. Effect of phenanthrene (▨) on the degradation of dibenz[*a*,*h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by community five. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of community five. Protein concentrations (▨) were determined as described in the material and methods.

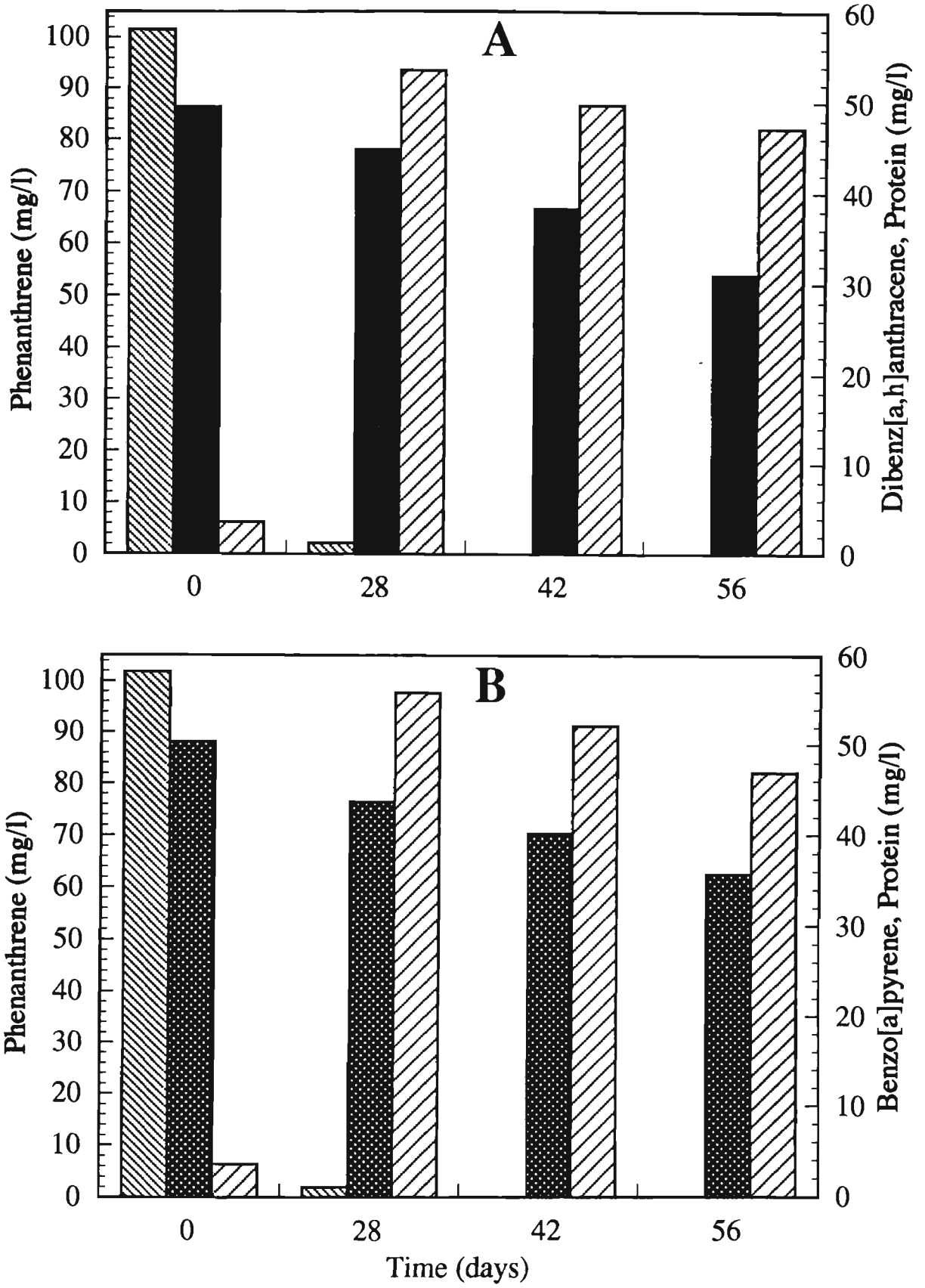

Figure 2. Effect of phenanthrene (▨) on the degradation of dibenz[*a*,*h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,002. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,002. Protein concentrations (▨) were determined as described in the material and methods.

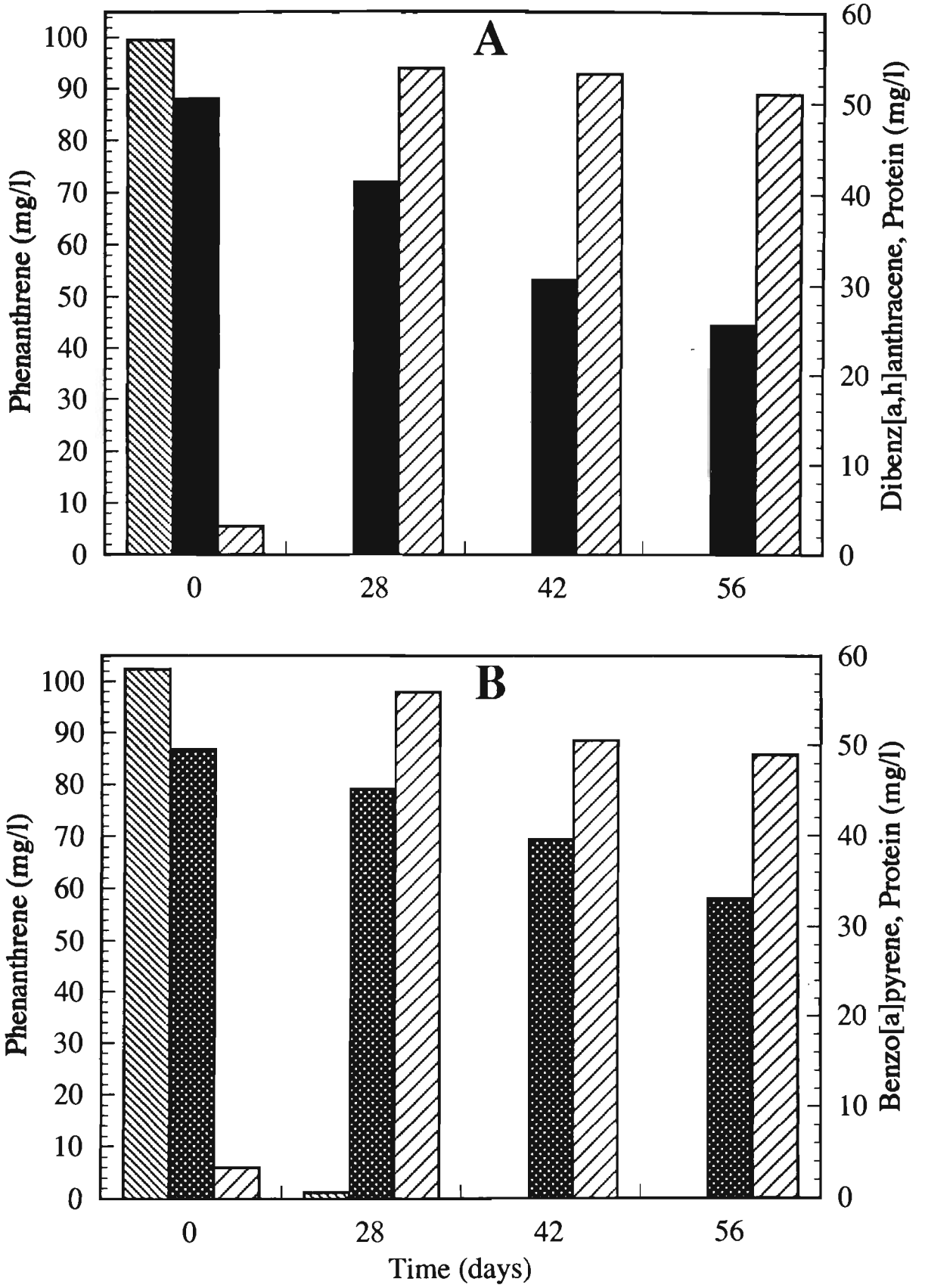

Figure 3. Effect of phenanthrene (▨) on the degradation of dibenz[*a,h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,003. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,003. Protein concentrations (▨) were determined as described in the material and methods.

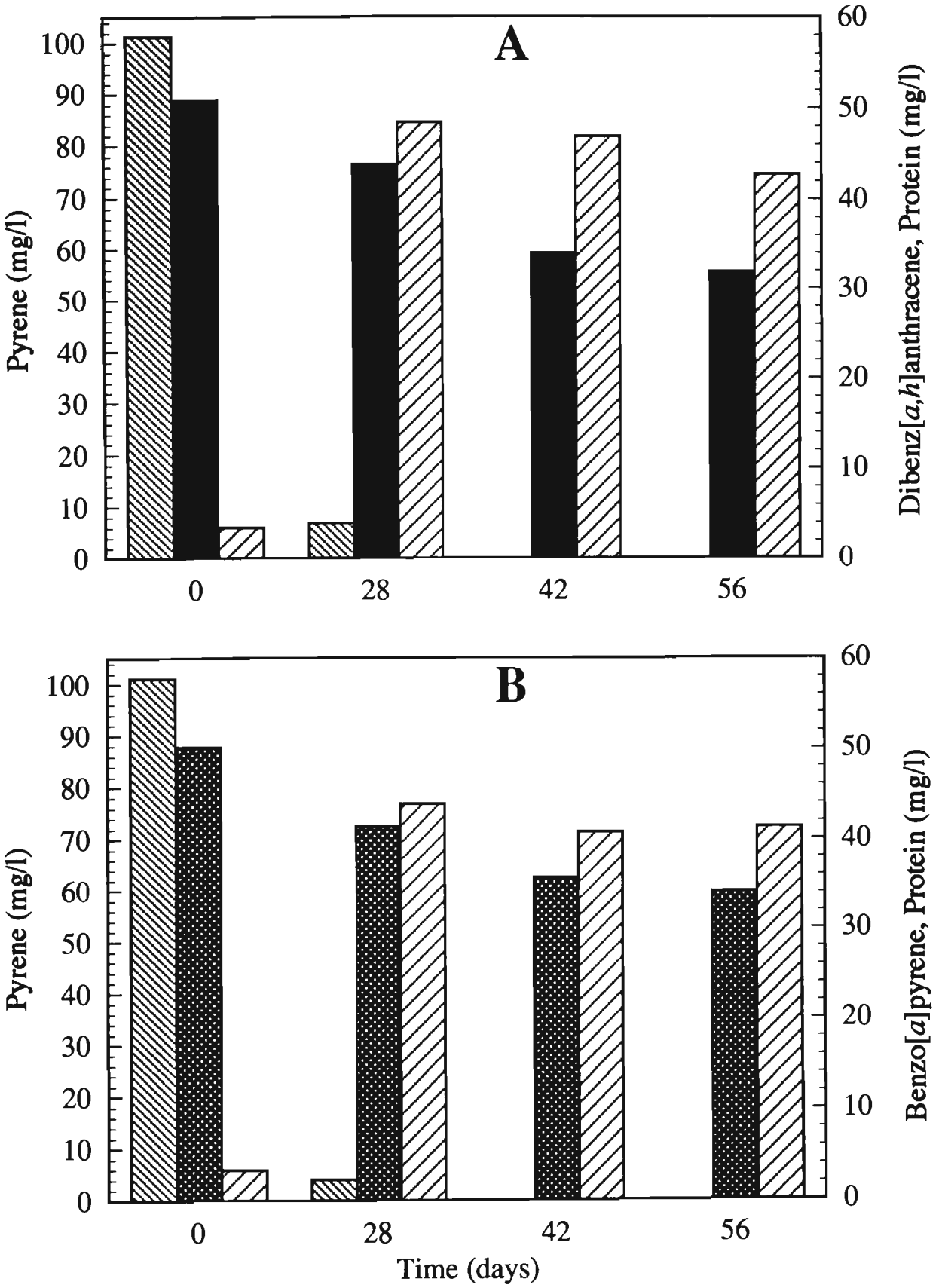

Figure 4. Effect of pyrene (▨) on the degradation of dibenz[*a*,*h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,001. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,001. Protein concentrations (▨) were determined as described in the material and methods.

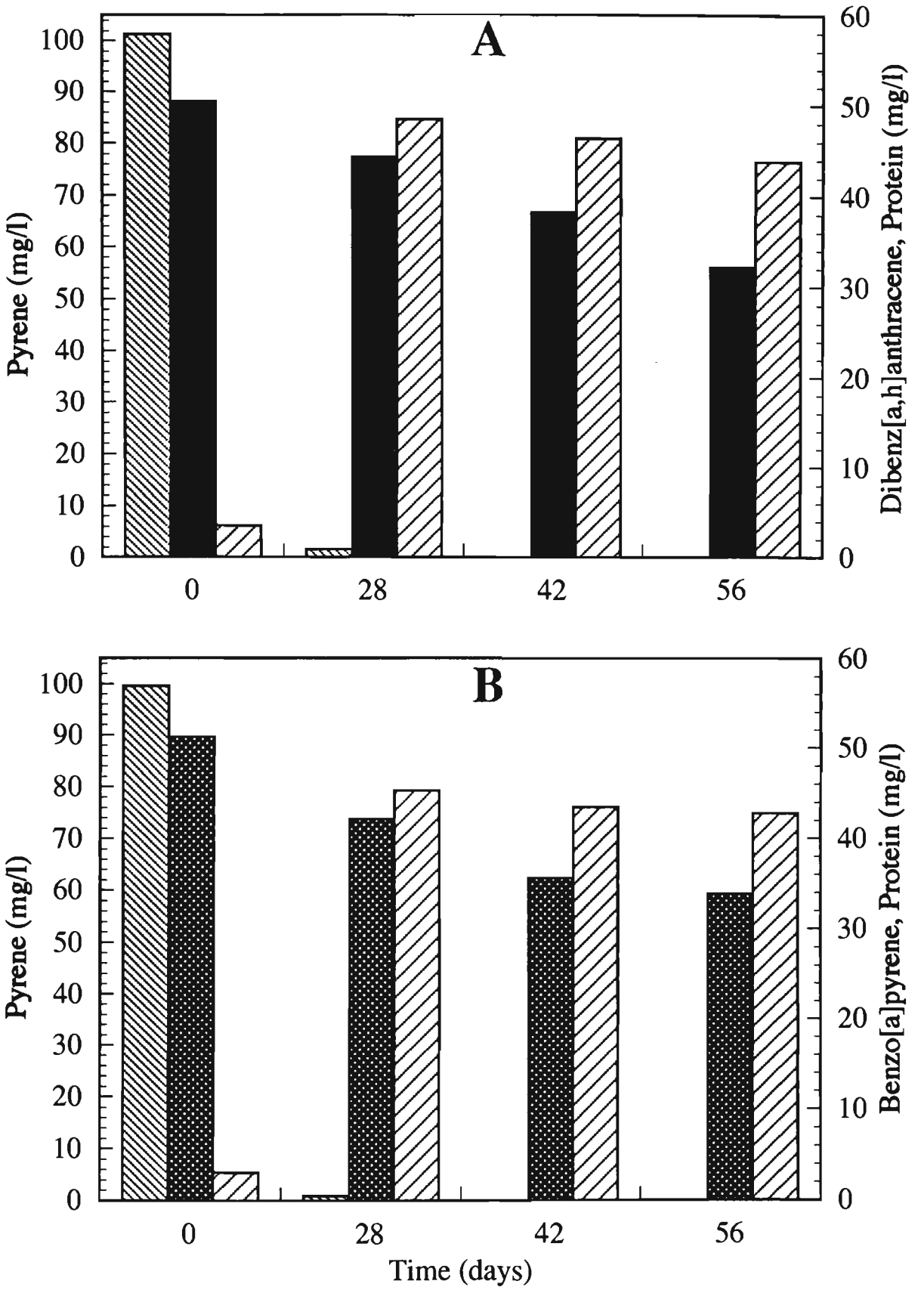

Figure 5. Effect of pyrene (▨) on the degradation of dibenz[*a*,*h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,002. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,002. Protein concentrations (▨) were determined as described in the material and methods.

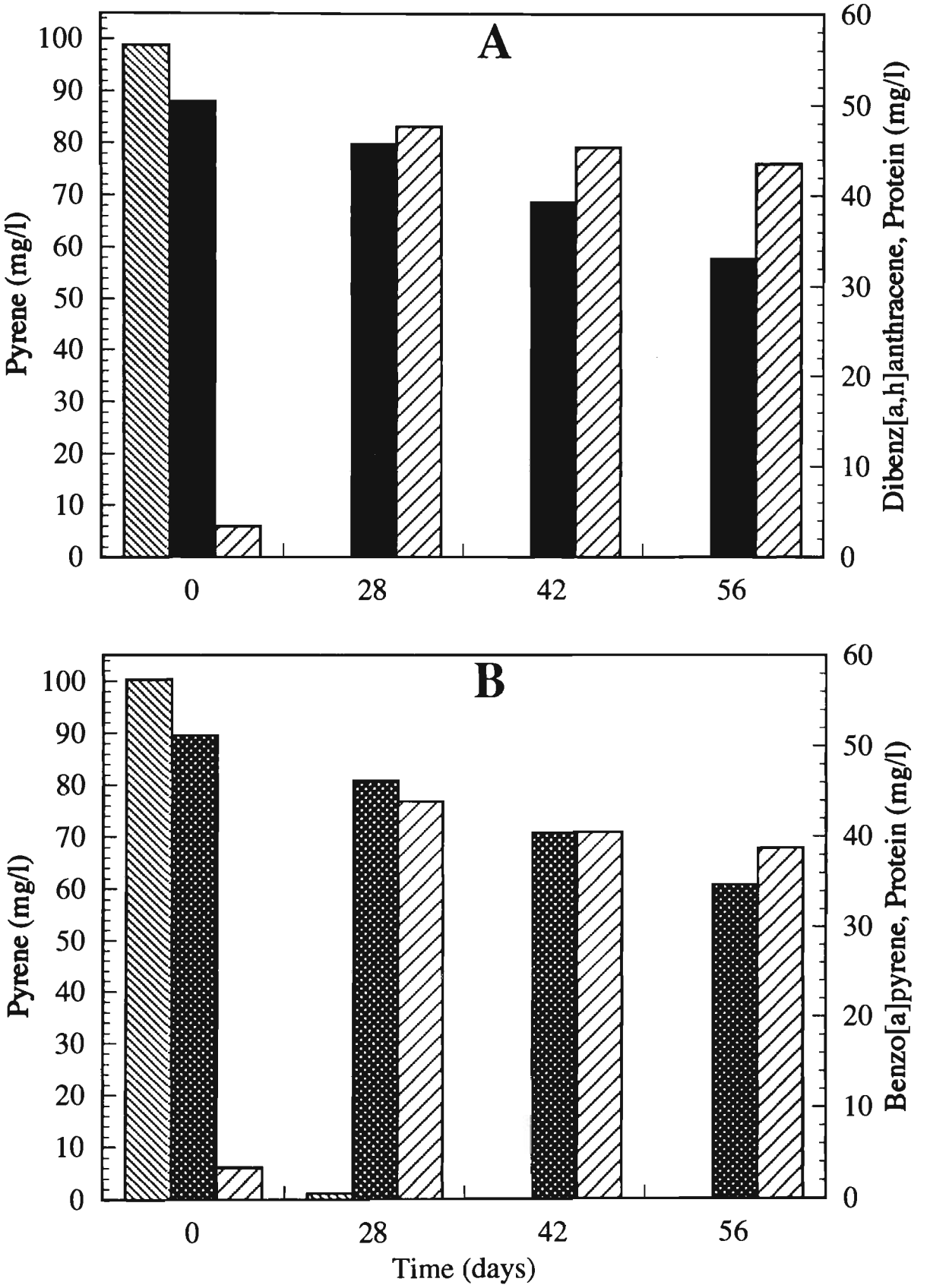

Figure 6. Effect of pyrene (▨) on the degradation of dibenz[*a,h*]anthracene (■) (A) or benzo[*a*]pyrene (▩) (B) by VUN 10,003. PAH containing media was inoculated with a 1% unwashed pyrene-grown inoculum of VUN 10,003. Protein concentrations (▨) were determined as described in the material and methods.

APPENDIX 3

Calculation of Gamma and EC_{50} for Microtox™ Assays (Ribo and Kaiser, 1987)

The result of Microtox™ assays are usually expressed as the effective concentation of a toxicant at which there is a 50% decrease in the bioluminescence of *P. phosphoreum* (EC_{50}). As the total light output of any bacterial suspensions decreases with time (Ribo and Kaiser, 1987), there is a need to correct for the natural drift in light emission. This is done using the "Blank Ratio" which is the ratio of the actual readings for the control solutions without toxicant at the start of the assay and at each time a recording is made.

$$BR_t = \frac{I[0]_t}{I[0]_o}$$

where:

BR: Blank ratio (one value for each exposure time).
$I[0]_o$, $I[0]_t$: Blank reading at time *0* and *t*.

It has been widely accepted that the GAMMA function is used as the bioassay response parameter (Ribo and Kaiser, 1987). The GAMMA function is defined as the ratio of light lost to the light remaining. GAMMA is calculated using:

$$\text{GAMMA} = \frac{BR_t \cdot I[c]_o - I[c]_t}{I[c]_t}$$

where:

$I[0]_o$, $I[0]_t$: Light intensity readings for a "toxic" solution at concentration [c] at times *0* and *t*.

When plotting log GAMMA values against the logarithm of the corresponding concentrations, the EC_{50} of a toxicant can be determined at a given time point. The EC_{50} is the concentration at which GAMMA = 1.0.

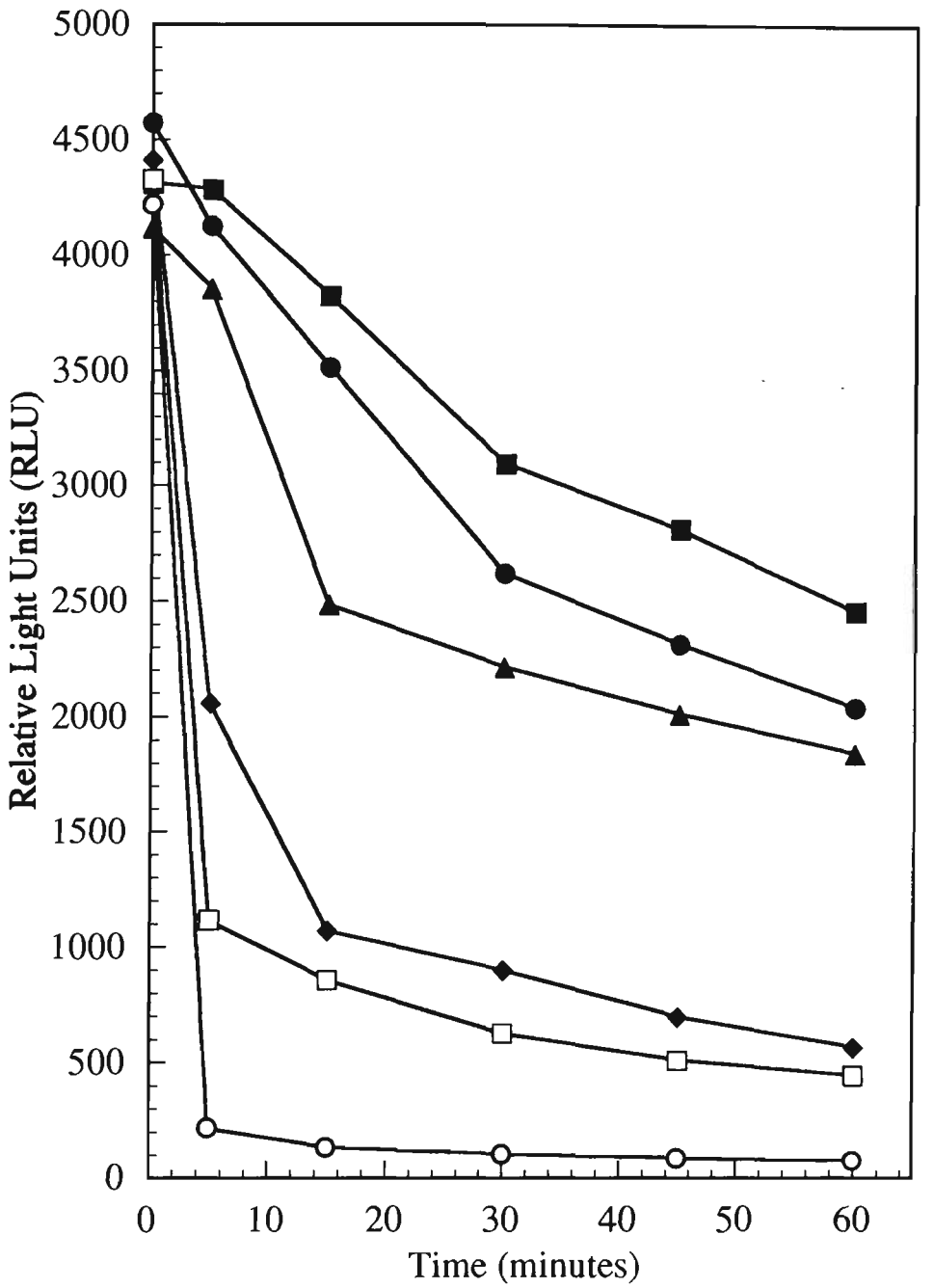

Figure 1. Toxic response of aqueous soil extracts from PAH-contaminated soil towards *P. phosphoreum*. The light output of *P. phosphoreum* was determined after 0, 5, 15, 30, 45 and 60 minutes exposure to undiluted (O), 1 in 2 (□), 1 in 10 (◆), 1 in 50 (▲) and 1 in 100 (●) diluted aqueous soil extracts. The light output of *P. phosphoreum* in 3% NaCl without additives (■) is also shown.

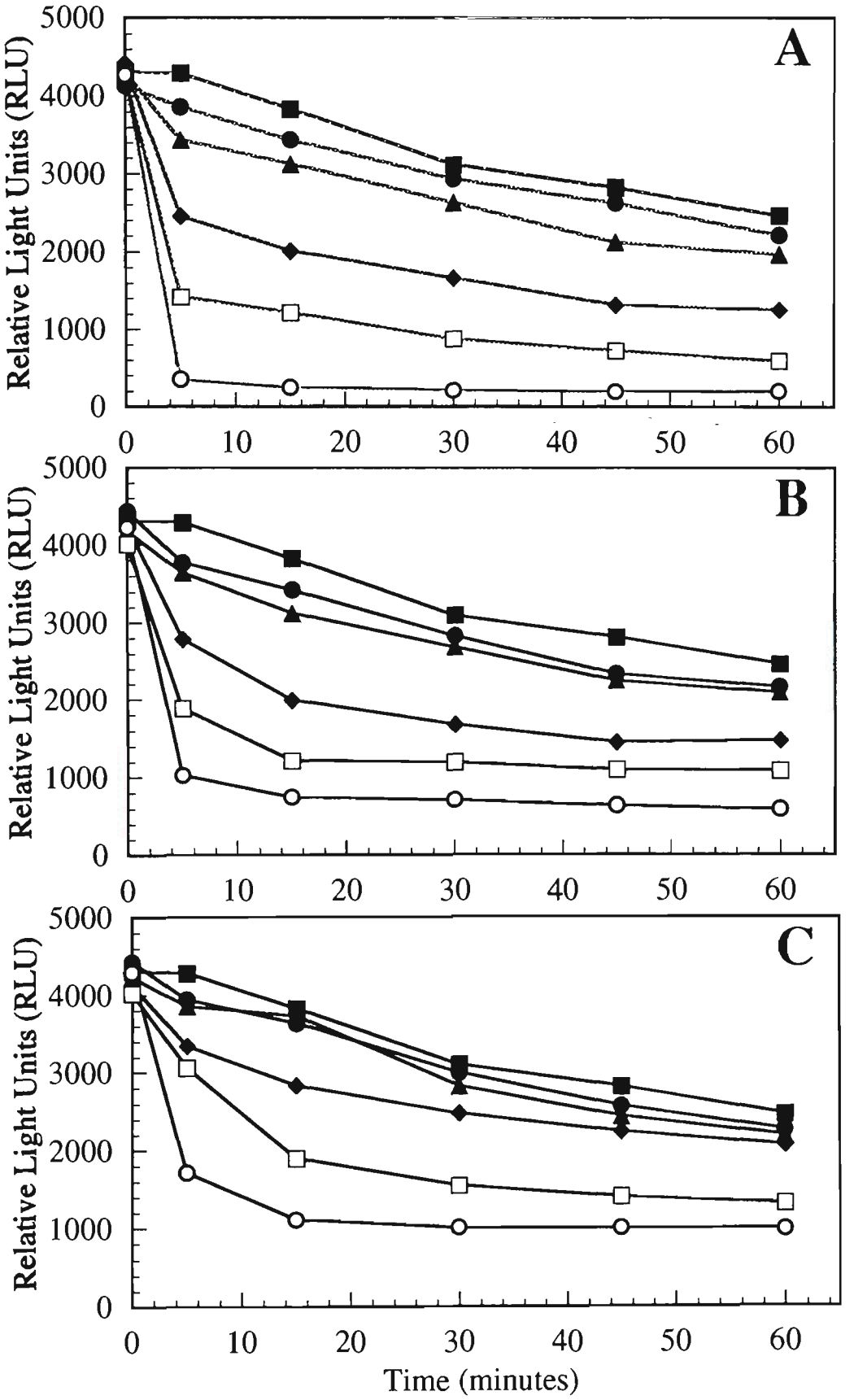

Figure 2. Toxic response of *P. phosphoreum* towards aqueous soil extracts of PAH-contaminated soil incubated with the indigenous soil microflora after 7 (A), 48 (B) and 91 days (C). The light output of *P. phosphoreum* was determined after 0, 5, 15, 30, 45 and 60 minutes exposure to undiluted (O), 1 in 2 (□), 1 in 10 (◆), 1 in 50 (▲) and 1 in 100 (●) 3% NaCl diluted aqueous soil extracts. The light output of *P. phosphoreum* in 3% NaCl without additives (■) is also shown.

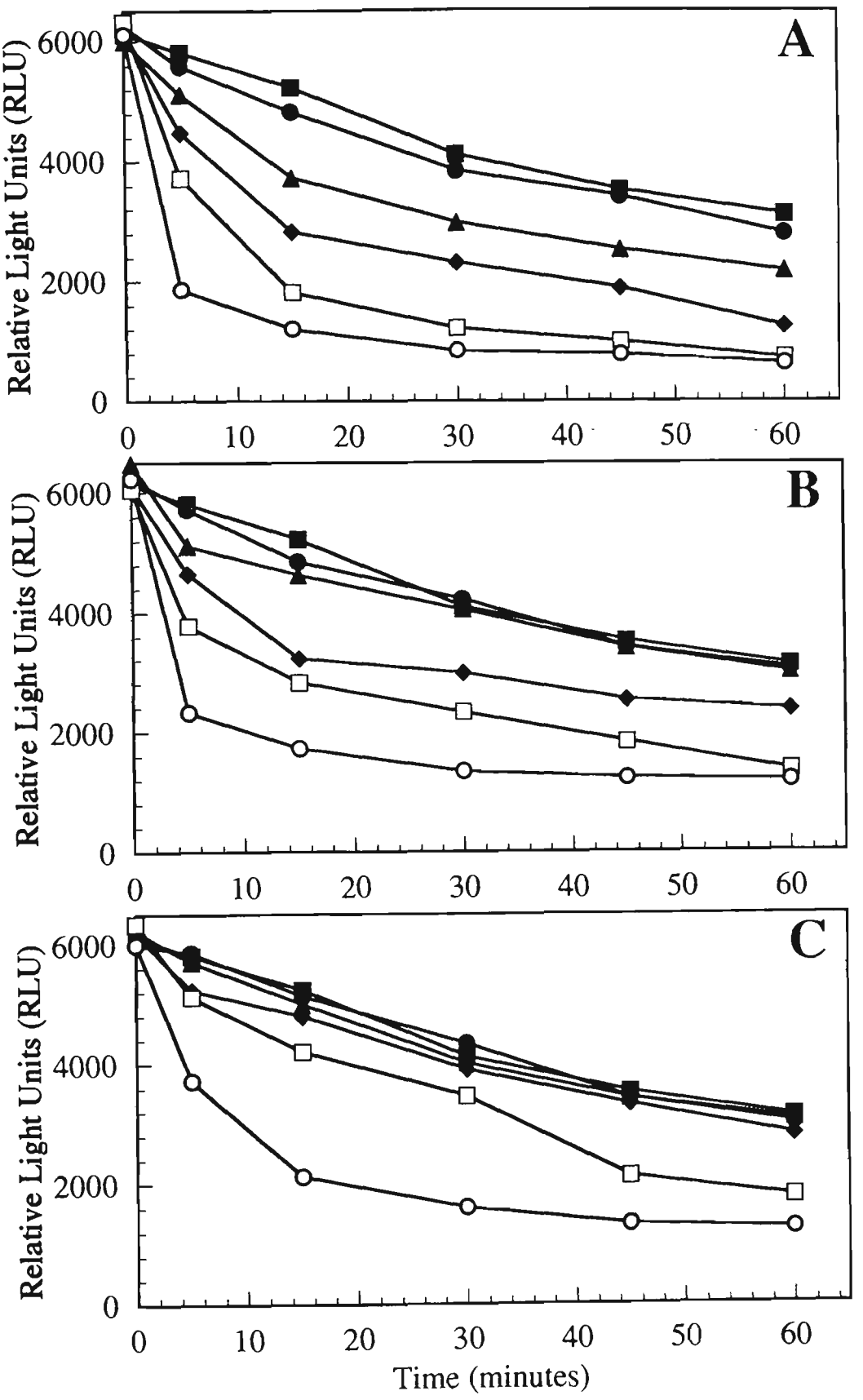

Figure 3. Toxic response of *P. phosphoreum* towards aqueous soil extracts of PAH-contaminated soil incubated with the indigenous soil microflora and yeast extract (1 g/kg) after 7 (A), 48 (B) and 91 days (C). *The light output of P. phosphoreum* was determined after 0, 5, 15, 30, 45 and 60 minutes exposure to undiluted (○), 1 in 2 (□), 1 in 10 (◆), 1 in 50 (▲) and 1 in 100 (●) 3% NaCl diluted aqueous soil extracts. The light output of *P. phosphoreum* in 3% NaCl without additives (■) is also shown.

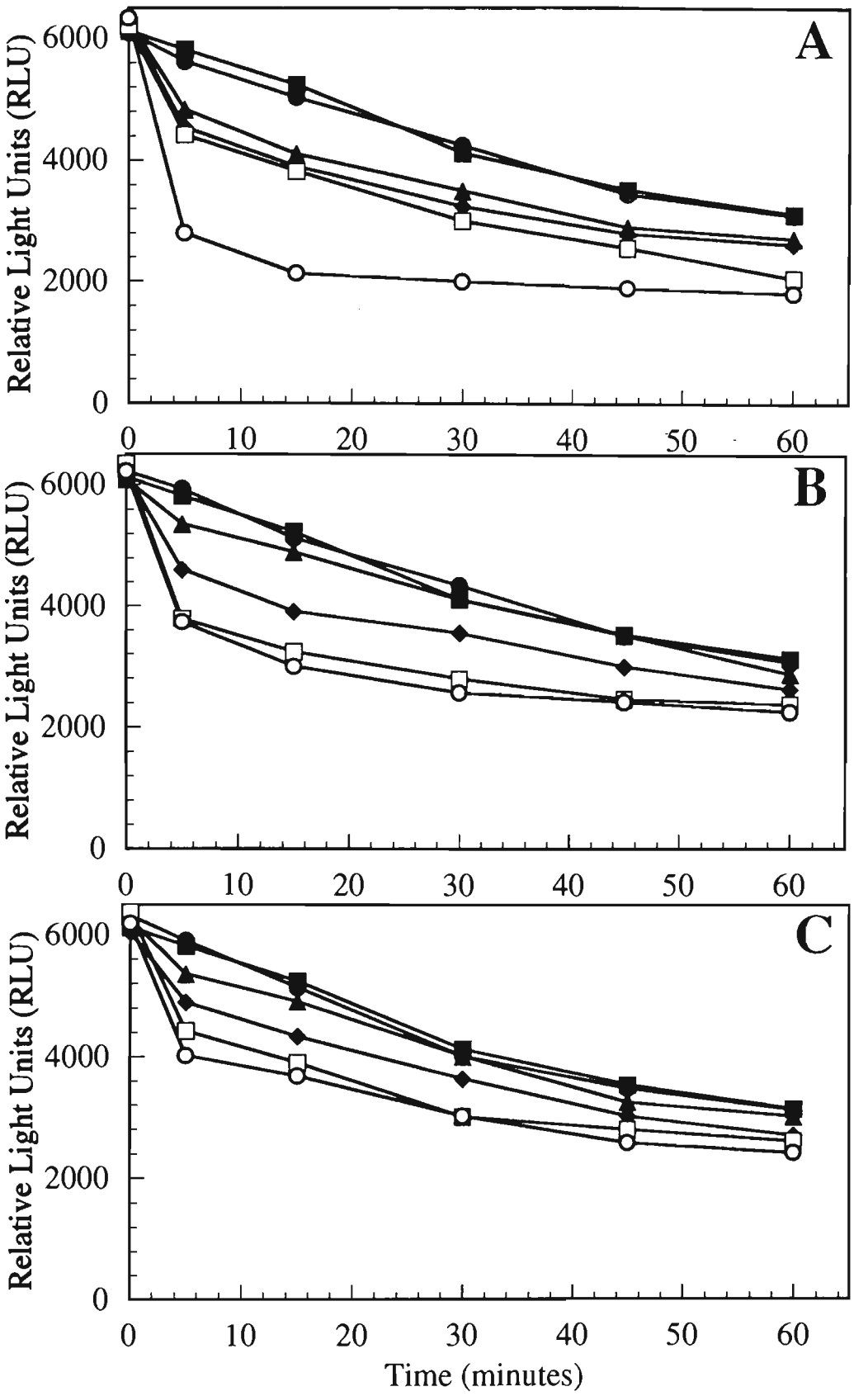

Figure 4. Toxic response of *P. phosphoreum* towards aqueous soil extracts of PAH-contaminated soil inoculated with CYEM-grown community five after 7 (A), 48 (B) and 91 days (C). The light output of *P. phosphoreum* was determined after 0, 5, 15, 30, 45 and 60 minutes exposure to undiluted (O), 1 in 2 (□), 1 in 10 (◆), 1 in 50 (▲) and 1 in 100 (●) 3% NaCl diluted aqueous soil extracts. The light output of *P. phosphoreum* in 3% NaCl without additives (■) is also shown.

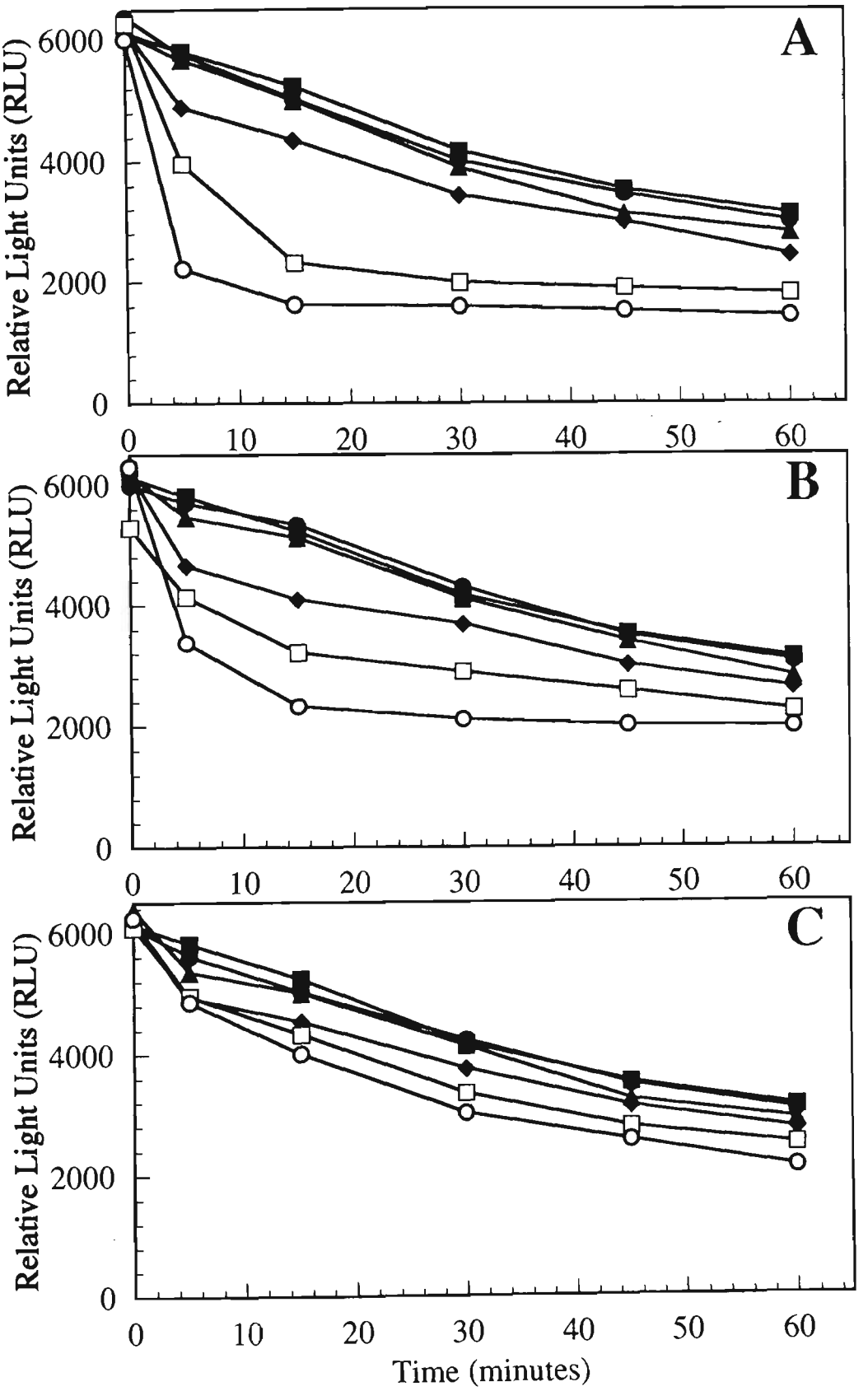

Figure 5. Toxic response of *P. phosphoreum* towards aqueous soil extracts of PAH-contaminated soil inoculated with CYEM-grown community five and yeast extract (1 g/kg) after 7 (A), 48 (B) and 91 days (C). *The light output of P. phosphoreum* was determined after 0, 5, 15, 30, 45 and 60 minutes exposure to undiluted (○), 1 in 2 (□), 1 in 10 (◆), 1 in 50 (▲) and 1 in 100 (●) 3% NaCl diluted aqueous soil extracts. The light output of *P. phosphoreum* in 3% NaCl without additives (■) is also shown.

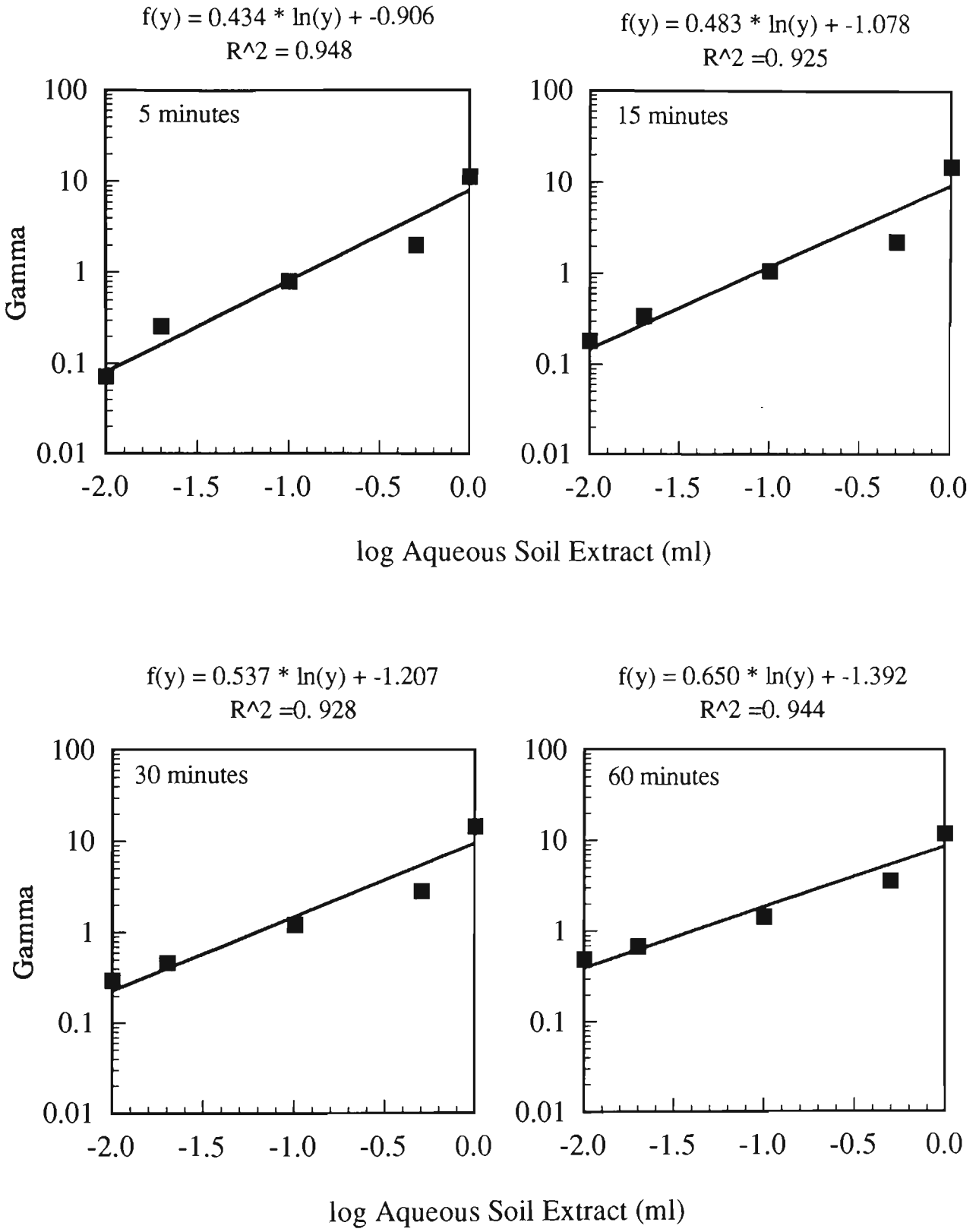

Figure 6. Calculation of the Microtox™ EC50 value of PAH-contaminated soil incubated with the indigenous microflora for 7 days. *P. phosphoreum* was exposed to various dilutions of the soil extract for 5, 15, 30, 45 and 60 minutes. Gamma was calculated according to the formula described in Appendix 3. The EC50 is the value at which Gamma = 1.0.

BIBLIOGRAPHY

Aamand, J., G. Bruntse, M. Jepsen, C. Jorgensen and B. K. Jensen (1995). Degradation of PAHs in Soil by Indigenous and Inoculated Bacteria. *In*: R. E. Hinchee, J. Fredrickson and B. C. Alleman (eds), *Bioaugmentation for Site Remediation*. Battelle Press, Ohio, pp 121-127.

Akhtar, M. N., D. R. Boyd, N. J. Thomas, M. Koreeda, D. T. Gibson, V. Mahadevan and D. M. Jerina (1975). Absolute Stereochemistry of the Dihydroanthracene *Cis*- and *Trans*-1,2-Diols Produced from Anthracene by Mammals and Bacteria. *Journal of the Chemists Society, Perkin Transcripts* **1**: 2506-2511.

Albaiges, J., R. W. Frei and E. Merian (1983). *Chemistry and Analysis of Hydrocarbons in the Environment*. Gordon and Breach Science Publishers, New York, pp 177-190.

Allen, C. C. R., D. R. Boyd, M. J. Larkin, K. A. Ried, N. D. Sharma and K. Wilson (1997). Metabolism of Naphthalene, 1-Naphthol, Indene and Indole by *Rhodococcus* sp. Strain NCIMB 12038. *Applied and Environmental Microbiology* **63**: 151-155.

Allport, D. C. and J. D. Bu'Lock (1960). Biosynthetic Pathways in *Daldinia concentrica*. *Journal of Chemist Society london*. **1960**:654-662.

Amacher, D. E., S. C. Paillet, G. N. Turner, V. A. Ray and D. S. Salsburg (1980). Point Mutations at the Thymidine Kinase Locus in L5178Y Mouse Lymphoma Cells. II. Test Validation and Interpretation. *Mutation Research* **72**:447-474.

Amacher, D. E. and G. N. Turner (1980). Promutagen Activation by Rodent-Liver Postmitochondrial Fractions in the L5178Y/TK Cell Mutation Assay. *Mutation Research* **74**: 485-501.

Ames, B. N. (1972). A Bacterial System for Detecting Mutagens and Carcinogens. *In* H. E. Sutton and M. I. Harris (eds), *Mutagenic Effects of Environmental Contaminants*. Academic Press, New York, pp 57-66.

Anderson, W. C., T. H. Belt and P. W. Tunnicliffe (1993). Wastech spotlights remediation. *Water Environment and Technology* **October 1993**: 53-55.

Andrews, A. W., L. H. Thibault and W. Lijinsky (1978). The Relationship Between Carcinogenicity and Mutagenicity of Some Polynuclear Hydrocarbons. *Mutation Research* **51**: 311-318.

Aprill, W., R. C. Sims, J. L. Sims and J. E. Matthews (1990). Assessing Detoxification and Degradation of Wood Preserving and Petroleum Wastes in Contaminated Soil. *Waste Management and Research* **8**: 45-65.

Aranha, H. G. and L. R. Brown (1981). Effect of Nitrogen Source on End Products of Naphthalene Degradation. *Applied and Environmental Microbiology* **42**(1): 74-78.

Aronstein, B. N., Y. M. Calvillo and M. Alexander (1991). Effects of Surfactants at Low Concentrations on the Desorption and Biodegradation of Sorbed Aromatic Compounds in Soil. *Environmental Science and Technology* **25**: 1728-1731.

Aronson, B. D., M. Levinthal and R. L. Somerville (1989). Activation of a Cryptic Pathway for Threonine Metabolism Via Specific IS3-Mediated Alteration of Promoter Sturcture in *Escherichia coli. Journal of Bacteriology* **171**: 5503-5511.

Ashby, J. and B. Kilby (1981). Summary Report on the Performance of Bacterial Repair, Phage Induction, Degranulation and Nuclear Enlargement Assays. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program*. Progress in Mutation Research, Volume 1, New York, Elsevier/North Holland, pp. 33-48.

Atlas, R. M. (1981). Microbial Degradation of Petroleum Hydrocarbons; an Environmental Perspective. *Microbiological Reviews* **45**(3):180-209.

Atlas, R. M. and R. Bartha (1972). Biodegradation of Petroleum in Seawater at Low Temperatures. *Canadian Journal of Microbiology* **18**:1851-1855.

Atlas, R. M. and R. Bartha (1973). Simulated Biodegradation of Oil Slicks Using Oleophilic Fertilisers. *Environmental Science and Technology* **7**: 538-541.

Atlas, R. M. and R. Bartha (1981). *Microbial Ecology; Fundamentals and Applications*. Addison-Wesley Publishing Company, Massechusetts, pp 423-427.

Baker, R. S. U., A. M. Bonin, I. Stupans and G. M. Holder (1980). Comparison of Rat and Geinea Pig as Sources of the S9 Fraction in the Salmonella/Mammalian Microsome Mutagenicity Test. *Mutation Research* **71**: 43-52.

Balani, S. K., P. J. Van Blanderen, N. Shirai and D. M. Jerina (1986). Resolution and Absolute Configuration of *K*-region *Trans*-dihydrodiols from Polycyclic Aromatic Hydrocarbons. *Jourrnal of Organic Chemistry* **51**: 1773-1778.

Banerjee, D.K., P. M. Fedorak, A. Hashimoto, J. H. Masliyah, M. A. Pickard and M. R. Gray (1995) Monitoring the Biological Treatment of Anthracene-Contaminated Soil in a Rotating-Drum Bioreactor. *Applied Microbiology and Biotechnology* **43**: 521-528.

Barbosa, A. M., R. F. H. Dekker and G. E. St Hardy (1996). Veratryl Alcohol as an Inducer of Laccase by an Ascomycete, *Botryosphaeria* sp., When Screened on the Polymeric Dye Poly R-478. *Letters in Applied Microbiology* **23**: 93-96.

Barkay, T., C. Liebert and M. Gillman (1989). Hybridisation of DNA Probes with Whole-Community Genome for Detection of Genes that Encode Microbial Responses to Pollutants: *mer* Genes and Hg^{2+} Resistance. *Applied and Environmental Microbiology* **55**: 1574-1577.

Barnsley, E. A. (1975a). The Induction of the Enzymes of Naphthalene Metabolism in Pseudomonads by Salicylate and 2-Aminobenzoate. *Journal of General Microbiology* **88**: 193-196.

Barnsley, E. A. (1975b). The Bacterial Degradation of Fluoranthene and Benzo[*a*]pyrene. *Canadian Journal of Microbiology* **21**: 1004-1008.

Barnsley, E. A. (1983a). Bacterial Oxidation of Naphthalene and Phenanthrene. *Journal of Bacteriology* **153**(2):1069-1071.

Barnsley, E. A. (1983b). Phthalate Pathway of Phenanthrene Metabolism: Formation of 2' Carboxybenzalpyruvate. *Journal of Bacteriology* **154**:113-117.

Barratt, P. and P. Harold (1991) *In Situ* Biological Treatment of Contaminated Land-Feasibility Studies and Treatment of a Creosote Contaminated Site. *In*: M. C. R. Davies (ed.), *Land Reclamation, An End to Dereliction?* Elsevier Applied Science, New York, pp. 336-346.

Bartsch, H., C. Malaveille, A. M. Camus, G. Martel-Planche, G. Brun, A. Hautefeuille, N. Sabadie, A. Barbin, T. Kuroki, C. Drevon, C. Piccoli and R. Montesano (1980). Validation and Comparative Studies on 180 Chemicals with *S. typhimurium* Strains and V79 Chinese Hamster Cells in the Presence of Various Metabolising Systems. *Mutation Research* **76**: 1-50.

Baud-Grasset, S., F. Baud-Grasset, J. M. Bifulco, J. R. Meier and T-H. Ma (1993). Reduction of Genotoxicity of a Creosote Contaminated Soil After Fungal Treatment Determined by the Tradescantia-Micronucleus Test. *Mutation Research* **303**: 77-82.

Baud-Grasset, F. and T. M. Vogel (1995). Bioaugmentation: Biotreatment of Contaminated Soil by Adding Adapted Bacteria. *In*: R. E. Hinchee, J. Fredrickson and B. C. Alleman (eds), *Bioaugmentation for Site Remediation*. Battelle Press, Ohio, pp 39-48.

Bauer, J. E. and D. G. Capone (1985). Degradation and Mineralisation of the Polycyclic Aromatic Hydrocarbons Anthracene and Naphthalene in Intertidal Marine Sediment. *Applied and Environmental Microbiology* **50**(1):81-90.

Bauer, J. E. and D. G. Capone (1988). Effects of Co-Occurring Aromatic Hydrocarbons on Degradation of Individual PAHs in Marine Sediment Slurries. *Applied and Environmental Microbiology* **54**(7):1649-1655.

Beacham, I. R. (1987). Silent Genes in Procaryotes. *FEMS Microbiology Reviews* **46**: 409-417.

Beam, H. W. and J. J. Perry (1973). Cometabolism as a Factor in Microbial Degradation of Cycloparaffinic Hydrocarbons. *Archives of Microbiology* **91**:87-90.

Bewley, R., B. Ellis, P. Theile, I. Viney and J. Rees (1989). Microbial Clean up of Contaminated Soil. *Chemistry and Industry* **23**: 778-783.

Bezalel, L., Y. Hadar and C. E. Cerniglia (1996). Mineralisation of Polycyclic Aromatic Hydrocarbons by the White Rot Fungus *Pleutotus ostreatus*. *Applied and Environmental Microbiology* **62**: 292-295.

Bingham, E. and H. L. Falk (1969). Environmental Carcinogens. The Modifying Effect of Cocarcinogens on the Threshold Response. *Archives of Environmental Health* **19**: 779-783.

Bjorseth, A., G. Lunde and A. Lindskog (1979). Long-Range Transport of Polycyclic Aromatic Hydrocarbons. *Atmospheric Environment* **13**: 45-53.

Blackburn, J. W. and W. R. Hafker (1993). The Impact of Biochemistry, Bioavailability and Bioactivity on the Selection of Bioremediation Techniques. *Trends in Biotechnology* **11**: 328-333.

Blom, A., W. Harder and A. Martin (1992). Unique and Overlapping Pollutant Stress Proteins of *Escherichia coli*. *Applied and Environmental Microbiology* **58**: 331-334.

Blumer, M. (1976). Polycyclic Aromatic Compounds in Nature. *Scientific America* **March 1976**: 35-45.

Boethling, R. S. and M. Alexander (1979a). Effect of Concentration of Organic Chemicals on their Biodegradation by Natural Microbial Communities. *Applied and Environmental Microbiology* **37**(6):1211-1216.

Boethling, R. S. and M. Alexander (1979b). Microbial Degradation of Organic Compounds at Trace Levels. *Environmental Science and Technology* **13**(2):989-991.

Bogardt, A. H. and B. B. Hemmingsen (1992). Enumeration of Phenanthrene Degrading Bacteria by an Overlay Technique and Its Use in Evaluation of Petroleum-Contaminated Sites. *Applied and Environmental Microbiology* **58**:2579-2582.

Bos, R. P., W. J. C. Prinsen, J. G. M. van Rooy, F. J. Jongeneelen, J. L. G. Theuws and P. T. Henderson (1987). Fluoranthene, a Volitile Mutagenic Compound, Present in Creosote and Coal Tar. *Mutation Research* **187**: 119-125.

Bos, R. P., J. L. G. Theuws C. M. Leijdekkers and P. T. Henderson (1984). The Presence of Mutagenic Polycyclic Aromatic Hydrocarbons Benzo[*a*]pyrene and Benz[*a*]anthracene in Creosote P1. *Mutation Research* **130**: 153-158.

Bouchez, M., D. Blanchet and J. P. Vandecasteele (1995). Degradation of Polycyclic Aromatic Hydrocarbons by Pure Strains and by Defined Strain Associations: Inhibition Phenomena and Cometabolism. *Applied Microbiology and Biotechnology* **43**: 156-164.

Bouwer, E., Durant, N., Wilson, L., Zhang, W. and Cunningham, A. (1994) Degradation of Xenobiotic Compounds *In Situ*: Capabilities and Limits. *FEMS Microbiology Reviews* **15**: 307-317.

Bowen, H. G. M (1996). Trace elements in Biochemistry. New York, U.S.A, Academic Press

Box, J. D. (1983). Investigation of the Folin-Ciocalteau Phenol Reagent for the Determination of Polyphenolic Substances in Natural Waters. *Water Research* **17**: 511-525.

Boyland, E. and H. Burrows (1935). The Experimental Production of Sarconoma in Rats and Mice by a Colloidal Aqueous Solution of 1:2 5:6-Dibenzanthracene. *Journal of Pathology and Bacteriology* **41**: 231-238

Boyland, E., A. A. Levi, E. H. Mawson and E. Roe (1941). Metabolism of Polycyclic Compounds: Production of a Dihydroxy-1:2:5:6-Dibenzanthracene from 1:2:5:6-Dibenzanthracene. *Biochemical Journal* **35**: 184-191.

Boyland E. and P. Sims (1965). The Metabolism of Benz[*a*]anthracene and Dibenz[*a,h*]anthracene and their 5,6-Epoxy-5,6-Dihydro Derivatives by Rat-Liver Homogenates. *Biochemical Journal* **97**: 7-16.

Boyland, E. and P. Sims (1967). The Carcinogenic Activities in Mice of Compounds Related to Benzo[*a*]anthracene. *International Journal of Cancer* **2**: 500-504.

Boyland, E. and G. Wolf (1950). Metabolism of Polycyclic Compounds. 6. Conversion of Phenanthrene into Dihydroxydihydrophenanthrenes. *Biochemistry Journal* **47**: 64-69.

Brodkorb, T. S. and R. L. Legge (1992). Enhanced Biodegradation of Phenanthrene in Oil Tar-Contaminated Soils Supplemented with *Phanerochaete chysosporium*. *Applied and Environmental Microbiology* **58**:3117-3121.

Bruice, P. Y., T. C. Bruice, P. M. Dansette, H. G. Selander, H. Yagi and D. M. Jerina (1976). Comparison of the Mechanism of Solvolysis and Rearrangement of *K*-Region vs. Non-*K*-Region Arene Oxides of Phenanthrene. Comparative Solvolytic Rate Constants of *K*-Region and Non-*K*-Region Arene Oxides. *Journal of American Chemist Society* **98**: 2965-2973.

Buening, M. K., W. Levin, J. M. Karle, H. Yagi, D. M. Jerina and A. H. Conney (1979). Tumourigenicity of Bay Region Epoxides and Other Derivatives of Chrysene and Phenanthrene in Newborn Mice. *Cancer Research* **39**: 5063-5068.

Bumpus, J. A. (1989). Biodegradation of Polycyclic Aromatic Hydrocarbons by *Phanerochaeta chrysosporium*. *Applied and Environmental Microbiology* **55**:154-158.

Bumpus, J. A., M. Tien, D. Wright and S. D. Aust (1985). Oxidation of Persistent Environmental Pollutants by a White Rot Fungus. *Science* **228**: 1434-1436.

Burd, G. and O. P. Ward (1996). Involvement of a Surface-Active High Molecular Weight Factor in Degradation of Polycyclic Aromatic Hydrocarbons by Pseudomonas marginalis. *Canadian Journal of Microbiology* **42**: 791-797.

Cameron, D. W., R. I. T. Cromartie and A. R. Todd (1964). Colouring Matters of the Aphididae. Part XVI. Reconsideration of the Structure of the Erythoaphins. *Journal of the Chemist Society, London*. **1964**: 48-50.

Cantor, C. R., A. Gaal and C. L. Smith (1988). High-Resolution Separation and Accurate Size Determination in Pulsed Field Gel Electrophoresis of DNA. 3. Effect of Electrical Field Shape. *Biochemistry* **27**: 9216-9221.

Casellas, M., M. Grifoll, J. M. Bayona and A. M. Solanas (1997). New Metabolites in the Degradation of Fluorene by *Arthrobacter* sp. Strain F101. *Applied and Environmental Microbiology* **63**: 819-826.

Casto, B. C., N. Janosko and J. A. DiPaolo (1977). Development of a Focus Assay Model for Transformation of Hamster Cells *In-Vitro* by Chemical Carcinogens. *Cancer Research* **37**: 3508-3515.

Cerniglia, C. E. (1982). Initial Reactions in the Oxidation of Anthracene by *Cunninghamella elegans*. *Journal of General Microbiology* **128**: 2055-2061.

Cerniglia, C. E. (1984a). Microbial Metabolism of Polycyclic Aromatic Hydrocarbons. *Advances in Applied Microbiology* **30**:31-71.

Cerniglia, C. E. (1984b). Microbial Transformations of Aromatic Hydrocarbons. *In*: R.M. Atlas (ed.), *Petroleum Microbiology*. Macmillan Publishing Co., New York, pp 99-128.

Cerniglia, C. E. (1992). Biodegradation of Polycyclic Aromatic Hydrocarbons. *Biodegradation* **3**: 351-368.

Cerniglia, C. E. and S. A Crow (1981). Metabolism of Aromatic Hydrocarbons by Yeasts. *Archives of Microbiology* **129**: 9-13.

Cerniglia, C. E. and D. T. Gibson (1977). Metabolism of Naphthalene by *Cunninghamella elegans*. *Applied and Environmental Microbiology* **34**:366-370.

Cerniglia, C. E. and D. T. Gibson (1979). Oxidation of Benzo[*a*]pyrene by the Filamentous Fungus *Cunninghamella elegans*. *Journal of Biological Chemistry* **254**: 12174-12180.

Cerniglia, C. E. and D. T. Gibson (1980). Fungal Oxidation of Benzo[*a*]pyrene and (±)-Trans-7,8-Dihydroxy-7,8-Dihydrobenzo[*a*]pyrene. *Journal of Biological Chemistry* **255**: 5159-5163.

Cerniglia, C. E. and M. A. Heitkamp (1989). Microbial Degradation of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. *In* U Varanasi (ed) Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment, CRC Press, Inc., pp 42-64.

Cerniglia, C. E. and S. K. Yang (1984). Stereoselective Metabolism of Anthracene and Phenanthrene by the Fungus *Cunninghamella elegans*. *Applied and Environmental Microbiology* **47**: 119-124.

Cerniglia, C. E., W. L. Campbell, J. P. Freeman and F. E. Evans (1989). Identification of a Novel Metabolite in Phenanthrene Metabolism by the Fungus *Cunninghamella elegans*. *Applied and Environmental Microbiology* **55**:2275-2279.

Cerniglia, C. E., R. H. Dodge and D. T. Gibson (1980d). Studies on the Fungal Oxidation of Polycyclic Aromatic Hydrocarbons. Bot. Mar. **23**: 121-124.

Cerniglia, C. E., J. P. Freeman and R. K. Mitchum (1982b). Glucuronide and Sulphate Conjugation in the Fungal Metabolism of Aromatic Hydrocarbons. *Applied and Environmental Microbiology* **43**:1070-1075.

Cerniglia, C. E., P. P. Fu and S. K. Yang (1982c). Metabolism of 7-Methylbenz[*a*]anthracene and 7-Hydroxymethylbenz[*a*]anthracene by *Cumminghamella elegans*. *Applied and Environmental Microbiology* **44**:682-689.

Cerniglia, C. E., D. T. Gibson and C. Van Baalen (1979). Algal Oxidation of Aromatic Hydrocarbons: Formation of 1-Naphthol from Naphthalene by *Agmenellum quadruplicatum* strain PR-6. *Biochemical and Biophysical Research Communications* **88**: 50-58.

Cerniglia, C. E., D. T. Gibson and C. Van Baalen (1980c). Algal Oxidation of Naphthalene. *Journal of General Microbiology* **116**: 495-500.

Cerniglia, C. E., D. T. Gibson and C. Van Baalen (1982a). Aromatic Hydrocarbon Oxidation by Diatoms Isolated from the Kachemak Bay Region of Alaska. *Journal of General Microbiology* **128**: 987-990.

Cerniglia, C. E., R. L. Herbert, R. H. Dodge, P. J. Szaniszlo and D. T. Gibson (1978). Fungal Transformation of Naphthalene. *Archives of Microbiology* **117**: 135-143.

Cerniglia, C. E., D. W. Kelly, J. P. Freeman and D. W. Miller (1986). Microbial Metabolism of Pyrene. *Chemical-Biological Interactions* **57**: 203-216.

Cerniglia, C. E., W. Mahaffey and D. T. Gibson (1980a). Fungal Oxidation of Benzo[*a*]pyrene: Formation of (-)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by *Cunninghamella elegans*. *Biochemical and Biophysical Research Communications* **94**: 226-232.

Cerniglia, C. E., C. Van Baalen and D. T. Gibson (1980b). Metabolism of Naphthalene by the Blue-Green Alga, *Oscillatoria* sp., Strain JCM. *Journal of General Microbiology* **116**: 485-495.

Chapman, P. J. (1979). Degradation Mechanisms. *In:* A. W. Bourquin and P. H. Pritchard (eds) *Proceedings of the Workshop: Microbial Degradation of pollutants in Marine Environments.* U.S. Environmental Protection Agency, Florida, pp 28-66.

Chatfield, L. K. and P. A. Williams (1986). Naturally Occuring TOL Plasmids in Pseudomonas Strains Carrying Either Two Homologous or Two Nonhomologous Catechol 2,3-Oxygenase Genes. *Journal of Bacteriology* **168**: 878-885.

Chaturapit, S. and G. M. Holder (1978). Studies on the Hepatic Microsomal Metabolism of [^{14}C] Phenanthrene. *Biochemical Pharacology* **27**: 1865-1871.

Clarkson, W. W., C. P. Yang and A. R. Harker (1993). 2,4-D Degradation in Monoculture Biofilm Reactors. *Water Research* **27**: 1275-1284.

Cody, T. E., M. J. Radike and D. Warshawsky (1984). The Phototoxicity of Benzo[*a*]pyrene in the Green Alga *Selenastrum capricornutum. Environmental Research* **35**: 122-132.

Colby, J., D. I. Stirling and H. Dalton (1977). The Soluble Methane Mono-oxygenase of *Methylococcus capsulatus* (Bath). Its Ability to Oxygenate *n*-Alkanes, *n*-Alkenes, Ethers and Alicyclic Aromatic and Heterocyclic Compounds. *Biochemical Journal* **165**:395-402.

Colla, A., A. Fiecchi and V. Treccani (1959). Ricerche sul Metabolismo Ossidativo Microbico dell'Antracene e del Fenantrene. *Annals of Microbiology* **9**: 87-91.

Collins, P. J., M. J. J. Kotterman, J. A. Field and A. D. W. Dobson (1996). Oxidation of Anthracene and Benzo[*a*]pyrene by Laccases from *Trametes versicolor. Applied and Environmental Microbiology* **62**: 4563-4567.

Coombs, M. M., C. Dixon and A. M. Kissonerghis (1976). Evaluation of the Mutagenicity of Compounds of Known Carcinogenicity, Belonging to the Benz[*a*]anthracene, Chrysene and Cyclopenta[*a*]phenanthrene Series, Using Ame's Test. *Cancer Research* **36**: 4525-4529.

Cooper, C. S., P. L. Grover and P. Sims (1983). The Metabolism and Activation of Benzo[*a*]pyrene. *Progress in Drug Metabolism* **7**: 295-396.

Cox, D. P. and Williams, L. (1980) Biological Process for Converting Naphthalene to *cis*-1,2-dihydroxy-1,2,dihydronaphthalene. *Applied and Environmental Microbiology* **39**, 320-326.

Dasappa, S. M. and R. C. Loehr (1991). Toxicity Reduction in Contaminated Soil Bioremediation Processes. *Water Research* **9**: 1121-1130.

Datta, D. and T. B. Samanta (1988). Effects of Inducers of Metabolism of Benzo[*a*]pyrene *In Vivo* and *In Vitro*: Analysis by High Pressure Liquid Chromatography. *Biochemical and Biophysical Research Communications* **155**: 493-502.

Davies, J. I. and W. C. Evans (1964). Oxidative Metabolism of Naphthalene by Soil Pseudomonads. *Biochemical Journal* **91**: 251-261.

de Serres, F. J. and J. Ashby (1981). Evaluation of short Term Tests for Carcinogens. Report of the International Collaborative Program. Progress in Mutation Resarch, Volume 1, Amsterdam, Elsevier/North-Holland.

de Serres, F. J. and G. R. Hoffmann (1981). Summary Report on the Performance of Yeast Assays. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program.* Progress in Mutation Research, Volume 1, New York, Elsevier/North Holland, pp. 68-76.

Delaune, R. D., W. H. Patrick and M. E. Casselman (1981). Effects of Sediment pH and Redox Conditions on Degradation of Benzo[a]pyrene. *Marine Pollution Bulletin* **12**:251-253.

Devoret, R. (1979). Bacterial Test for Potential Carcinogens. *Scientific American* **241**:28-37.

Dibble, J. T. and R. Bartha (1979). Effect of Environmental Parameters on the Biodegradation of Oil Sludge. *Applied and Environmental Microbilogy* **37**:729-739.

Dice, L. R. (1945). Measurements of the Amounts of Ecological Association Between Species. *Ecology* 26: 297-302.

Diels, L. and M. Mergeay (1990). DNA Probe-Mediated Detection of Resistant Bacteria from Soils Highly Polluted by Heavy Metals. *Applied and Environmental Microbiology* **56**: 1485-1491.

DiGrazia, P. M., J. M. H. King, J. W. Blackburn, B. A. Applegate, P. R. Bienkowski, B. L. Hilton and G. S. Sayler (1991). Dynamic Response of Naphthalene Biodegradation in a Continuous Flow Soil Slurry Reactor. *Biodegradation* **2**: 81-91.

Dipple, A. (1976). Polynuclear Aromatic Carcinogens. Chemical Carcinogens. *In*: C. E. Searle (ed.), *ASC Monograph 172*, American Chemical Society, Washington, DC.

Dipple, A. and C. A. H. Bigger (1991). Mechanism of Action of Food-Associated Polycyclic Aromatic Hydrocarbon Carcinogens. *Mutation Research* **259**: 263-276.

Dipple, A., R. C. Moschel and C. A. Bigger (1984). Polynuclear Aromatic Carcinogens. *In*: C. E. Searle (ed.), *Chemical Carcinogens*, Vol 1, 2nd Edition. American Chemist society, Washington, DC, pp41-163.

Donnelly, K. C., P. Davol, K. W. Brown, M. Estiri and J. C. Thomas (1987). Mutagenic Activity of Two Soils Amended with a Wood-Preserving Waste. *Environmental Science and Technology* **21**: 57-64.

Dua, R. D and S. Meera (1981). Purification and Characterisation of Naphthalene Oxygenase from *Corynebacterium renale. European Journal of Biochemistry* **120**: 461-465.

Dunn, N. W. and I. C. Gunsalus (1973). Transmissible Plasmid Coding Early Enzymes of Naphthalene Oxidation in *Pseudomonas putida. Journal of Bacteriology* **114**: 974-979.

Dzombak, D. A and R. G. Luthy (1984). Estimating Adsorption of Polycyclic Aromatic Hydrocarbons on Soil. *Soil Science* **137**: 292-298.

Edgehill, R. U. (1995). Removal of Pentachlorophenol from Soil by *Arthrobacter* Strain ATCC 33790. *In:* R. E. Hinchee, J. Fredrickson and B. C. Alleman (eds), *Bioaugmentation for Site Remediation*. Battelle Press, Ohio, pp 85-90.

Ellis, B., P. Harold and H. Kronberg (1991). Bioremediation of a Creosote Contaminated Site. *Environmental Technology* **12**: 447-459.

Elnabarawy, M. T., R. R. Robideau and S. A. Beach (1988). Comparison of Three Toxicity Test Procedures: Microtox, Polytox and Activated Sludge Respiration Inhibition. *Toxicity Assessment: An International Quarterly* **3**: 361-370.

Ensley, B. D. and B. E. Haigler (1990). Naphthalene Dioxygenase from *Pseudomonas* NCIB 9816. *In:* M. E. Lidstrom (ed.), Methods in Enzymology Volume 188, Hydrocarbons and Methylotrophy, Academic Press Inc., pp 46-95.

EPA (1992) Ultrasonic extraction of nonvolatile and semivolatile organics from solids. In: EPA Methods (pp 3550A/1-13).

Erickson, D. C., Loehr, R. C.and Neuhauser, E. F. (1993) PAH Loss During Bioremediation of Manufactured Gas Plant Site Soil. *Water Research* **27**, 911-919.

Escuadra, M. D. (1992). Genetic and Physical Analysis of *Pseudomonas solanacearum* Strains PS01and PS1000. Ph D Thesis. Dept. of Genetics and Developmental Biology, Monash University, Australia.

Evans, W. C., H. N. Fernley and E. Griffiths (1965). Oxidation Metabolism of Phenanthrene and Anthracene by Soil Pseudomonads. *Biochemical Journal* **95:**819-831.

Evans, P. J., D. T. Mang and L. Y. Young (1991). Degradation of Toluene and *m*-Xylene and Transformation of *o*-Xylene by Denitrifying Enrichment Cultures. *Applied and Environmental Microbiology* **57**(2):450-454.

Fahmy, O. G. and M. J. Fahmy (1973). Oxidative Activation of Benz[*a*]anthracene and Methylated Derivatives in Mutagenesis and Carcinogenesis. *Cancer Research* **33**: 2354-2361.

Fedorak, P. M., J. M. Foght and D. W. S. Westlake (1982). A Method for Monitoring Mineralisation of ^{14}C-Labelled Compounds in Aqueous Samples. *Water Research* **16**: 1285-1290.

Ferris, J. P., Fasco, M. J., F. L. Stylianopoulou, D. M. Jerina, J. W. Daly and A. M. Jeffrey (1973). Mono-Oxygenase Activity in *Cunninghamella bainieri*: Evidence for a Fungal System Similar to Liver Microomes. *Archives of Biochemistry and Biophysiology* **156**: 97-103.

Fiechter, A. (1993). Function and Synthesis of Enzymes Involved in Lignin Degradation. *Journal of Biotechnology* **30**: 49-55.

Field J. A., E. de Jong, G. F. Costa and J. A. M. de Bont (1992). Biodegradation of Polycyclic Aromatic Hydrocarbons by New Isolates of White Rot Fungi. *Applied and Environmental Microbiology* **58**:2219-2226.

Fleming, J. T., J. Sanseverine and G. S. Sayler (1993). Quantitative Relationship Between Naphthalene Catabolic Gene Frequency and Expression in Predicting PAH Degradation in Soils at Town Gas Manufacturing Sites. *Environmental Science and Technology* **27**: 1068-1074.

Foght, J. M., P. M. Fedorak and D. W. S Westlake (1990). Mineralisation of [^{14}C] Hexadecane and [^{14}C] Phenanthrene in Crude Oil; Specificity Among Bacterial Isolates. *Canadian Journal of Microbiology* **36**:169-175.

Foght, J. M. and D. W. S. Westlake (1988). Degradation of Polycyclic Aromatic Hydrocarbons and Aromatic Heterocycles by a Pseudomonas species. *Canadian Journal of Microbiology* **34**:1135-1141.

Forsyth, J. V., Y. M. Tsao and R. D. Bleam (1995). Bioremediation: When is Augmentation Needed? *In*: R. E. Hinchee, J. Fredrickson and B. C. Alleman (eds), *Bioaugmentation for Site Remediation*. Battelle Press, Ohio, pp 1-14.

Foster, J. W. (1962). Hydrocarbons as Substrates for Microorganisms. *Antonie van Leeuwenhoek Journal of Microbial Serology* **28**: 241-274.

Fox, G. E., J. D. Wisotzky and P. Jurshuk Jr. (1992). How Close is Close: 16SrRNA Sequence Identity May Not Be Sufficient to Guarantee Species Identity. *International Journal of Systematic Bacteriology* **42**: 66-170.

Freeman, D. J. and F. C. R. Cattell (1990). Woodburning as a Source of Atmospheric Polycyclic Aromatic Hydrocarbons. *Environmental Science and Technology* **24**: 1581-1585.

Fujikawa, K., F. L. Fort, K. Samejima and Y. Sakamoto (1993). Genotoxic Potency in *Drosophila melanogaster* of Selected Aromatic Amines and polycyclic Aromatic

Hydrocarbons as Assayed in the DNA Repair Test. *Mutation Research* **290**: 175-182.

Gadd, G. M. (1991). Microbial Biosorption of Heavy Metals and Radionuclides for Environmental Protection. *In*: Heavy Metals in the Environment, J. G. Farmer (ed.), CEP Consultants, Edinburgh, U.K, pp 403-412.

Gamlin, L. and B. Price (1988). Bonfires and Brimstone. *New Scientist* Nov 1988 **1637**: 48-51.

Garcia-Valdes, E., E. Cozar, R. Rotger, J. Lalucat and J. Ursing (1988). New Naphthalene-Degrading Marine *Pseudomonas* Strains. *Applied and Environmental Microbiology* **54**: 2478-2485.

Ghosh, D. K., D. Datta, T. B. Samanta and A. K. Mishra (1983). Microsomal Benzo[*a*]pyrene Hydroxylase in *Aspergillus ochraceus* TS: Assay and Characterisation of the Enzyme System. *Biochemical and Biophysical Research Communications* **113**: 497-505.

Ghosh, D. K. and A. K. Mishra (1983). Oxidation of Phenanthrene by a Strain of Micrococcus: Evidence of a Protocatechuate Pathway. *Current Microbiology* **9**:219-224.

Gibson, D. T., V. Mahadevan, R. M. Jerina, H. Yagi and H. J. C. Yeh (1975). Oxidation of the Carcinogens benzo[*a*]pyrene and Benzo[*a*]anthracene to Dihydrodiols by a Bacterium. *Science* **189**: 295-297.

Gibson, D. T. and V. Subramanian (1984). Microbial Degradation of Aromatic Hydrocarbons. *In*: Gibson D.T. (ed.), *Microbial Degradation of Organic Compounds* , Marcel Dekker, New York, pp.181-252.

Ginn, J. S., R. C. Sims and I. P. Murarka (1995). In Situ Bioremediation (Natural Attenuation) at a Gas Plant Waste Site. *In*: R. E. Hinchee, J. T. Wilson and D. C. Downey (eds), *Intrinsic Bioremediation*. Battelle Press, Ohio, pp 153-162.

Goldschmidt, B. M., C. Katz and B. L. Van Duuren (1973). The Cocarcinogenic Activity of Non-Carcinogenic Aromatic Hydrocarbons. *Proceedings of the American Association of Cancer Research* **17**: 84.

Gordillo, M. E., K. V. Singh, C. J. Baker and B. E. Murray (1993). Typing of Group B Streptococci: Comparison of Pulse Field gel Electrophoresis and Conventional Electrophoresis. *Journal of Clinical Microbiology* **31**: 1430-1434.

Gottschalk, G. (1986). *Bacterial Metabolism*. Springer-Verlag Inc., New York, pp 154-159.

Greenberg, A., F. Darack, R. Harkov, P. Lioy and J. Daisey (1985). Polycyclic Aromatic Hydrocarbons in New Jersey: A Comparison of Winter and Summer Concentrations Over a Two Year Period. *Atmospheric Environment* **19**: 1325-1339.

Grifoll, M., M. Casellas, J. M. Bayona and A. M. Solanas (1992). Isolation and Characterisation of a Fluorene-Degrading Bacterium: Identification of Ring Oxidation and Ring Fission Products. *Applied and Environmental Microbiology* **58**:2910-2917.

Grifoll, M., S. A. Selifonov and P. J. Chapman (1994). Evidence for a Novel Pathway in the Degradation of Fluorene by *Pseudomonas* sp. Strain F274. *Applied and Environmental Microbiology* **60**: 2438-2449.

Grifoll, M., Selifonov, S. A., Gatlin, C. V. and Chapman, P. J. (1995) Actions of a Versatile Fluorene-Degrading Bacterial Isolate on Polycyclic Aromatic Compounds. *Applied and Environmental Microbiology* **61**, 3711-3723.

Grosser, R. J., D. Warshawsky and J. R. Vestal (1991). Indigenous and Enhanced Mineralisation of Pyrene, Benzo[*a*]pyrene and Carbazole in Soil. *Applied and Environmental Microbiology* **57**:3462-3469.

Grund, E., B. Denecke and R. Eichenlaub (1992). Naphthalene Degradation Via Salicylate and Gentisate by *Rhodococcus* sp. Strain B4. *Applied and Environmental Microbiology* **58**: 1874-1877.

Guerin, W. F. and S. A. Boyd (1992) Differential Bioavailability of Soil-Sorbed Naphthalene to Two Bacterial Species. *Applied and Environmental Microbiology* **58**, 1142-1152.

Guerin, W. F and G. E. Jones (1988a). Two Stage Mineralisation of Phenanthrene by Estuarine Enrichment Cultures. *Applied and Environmental Microbiology* **54**:929-936.

Guerin, W. F and G. E. Jones (1988b). Mineralisation of Phenanthrene by a *Mycobacterium* sp. *Applied and Environmental Microbiology* **54**(4):937-944.

Guerin, W. F. and G. E. Jones (1989). Estuarine Ecology of Phenanthrene-Degrading Bacteria. *Estuarine Coastal Shelf Science* **29**: 115-130.

Hadler, H. I., V. Darchun and K. Lee (1959). Initiation and Promotion Activity of Certain Polynuclear Hydrocarbons. *Journal of the National Cancer Institute* **23**: 1383

Haemmerli, S. D, M. S. A. Leisola, D. Sanglard and A. Fiechter (1986). Oxidation of Benzo[*a*]pyrene by Extracellular Liginase of *Phanerochaete chrysosporium*. *The Journal of Biological Chemistry* **261**: 6900-6903.

Hambrick, G. A., R. D. DeLaune and W. H. Patrick (1980). Effects of Estuarine Sediment pH and Oxidation-Reduction Potential on Microbial Hydrocarbon Degradation. *Applied and Environmental Microbiology* **40**:365-369.

Hammel, K. E., Z. G. Gai, B. Green and M. A. Moen (1992). Oxidative Degradation of Phenanthrene by the Ligninolytic Fungus *Phanerochaete chrysosporium. Applied and Environmental Microbiology* **58**: 1831-1838.

Hammel, K. E., B. Green and W. Z. Gai (1991). Ring Fission of Anthracene by a Eukaryote. *Proceeding of the National Academy of Science* **88**: 10605-10608.

Hammel, K. E., B. Kalyanaraman and T. K. Kirk (1986). Oxidation of Polycyclic Aromatic Hydrocarbons and Dibenzo[*p*]dioxins by *Phanerochaete chrysosporium* Liginase. *Journal of Biological Chemistry* **261**: 16948-16952.

Hamzah, R. Y. and B. S. Al-Baharna (1994) Catechol ring cleavage in *Pseudomonas cepacia*: The simultaneous induction of *ortho* and *meta* pathways. *Applied Microbiology and Biotechnology* **41**, 250-256.

Harayama, S., P. R. Lehrbach and K. N. Timmis (1984). Transposon Mutagenesis Analysis of Meta-cleavage Pathway Operon Genes of the TOL Plasmid of *Pseudomonas putida* mt-2. *Journal of Bacteriology* **160**: 251-255.

Harayama, S., M. Rekik, A. Wasserfallen and A. Bairoch (1987). Evolutionary Relationships Between Catabolic Pathways for Aromatics: Conservation of Gene Order and Nucleotide Sequences of Catechol Oxidation Genes of pWW0 and NAH7 Plasmids. *Molecular General Genetics* **210**: 241-247.

Harkov, R., A. Greenberg, F. Darack, J. M. Dalsey and P. J. Lioy (1984). Summer Time Variations in Polyaromatic Hydrocarbons at Four Sites in New Jersey. *Environmental Science and Technology* **18**: 287-291.

Haugen, D. A. and M. J. Peak (1983). Mixtures of Polycyclic Aromatic Compounds Inhibit Mutagenesis in the Salmonella/Microsome Assay by Inhibition of Metabolic Activation. *Mutation Research* **116**: 257-269.

Heidelberger, C. and W. G. Wiest (1951). The Metabolic Degradation in the Mouse of 1,2,5,6-Dibenzanthracene-9,10-^{14}C. II. 5-Hydroxy-1,2-Naphthalic Acid, a New Metabolite. *Cancer Research* **11**: 511-518.

Heitkamp, M. A. and C. E. Cerniglia (1987). Effects of Chemical Structure and Exposure on the Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Freshwater and Estuarine Ecosystems. *Environmental Toxicology and Chemistry* **6**: 35-46.

Heitkamp, M. A. and C. E. Cerniglia (1988). Mineralisation of Polycyclic Aromatic Hydrocarbons by a Bacterium Isolated from Sediment Below and Oil Field. *Applied and Environmental Microbiology* **54**(6):1612-1614.

Heitkamp, M. A. and C. E. Cerniglia (1989). Polycyclic Aromatic Hydrocarbon Degradation by a *Mycobacterium* sp. in Microcosms Containing Sediment and Water From a Pristine Ecosystem. *Applied and Environmental Microbiology* **55**(8):1969-1973.

Heitkamp, M. A., W. Franklin and C. E. Cerniglia (1988a) Microbial Metabolism of Polycyclic Aromatic Hydrocarbons: Isolation and Characterisation of a Pyrene-Degrading Bacterium. *Applied and Environmental Microbiology* **54**:2549-2555.

Heitkamp, M. A., J. P. Freeman, and C. E. Cerniglia (1987). Naphthalene Biodegradation in Environmental Microcosms: Estimates of Degradation Rates and Characterisation of Metabolites. *Applied and Environmental Microbiology* **53**:129-136.

Heitkamp, M. A., J. P. Freeman, D. W. Miller and C. E. Cerniglia (1988b). Pyrene Degradation by a *Mycobacterium* sp.: Identification of Oxidation and Ring Fission Products. *Applied and Environmental Microbiology* **54**:2256-2265.

Herbes, S. E. and L. R. Schwall (1978). Microbial Transformation of PAHs in Pristine and Petroleum Contaminated Sediments. *Applied and Environmental Microbiology* **35**(2):306-316.

Hermann, M. (1981). Synergistic Effects of Individual Polycyclic Aromatic Hydrocarbons on the Mutagenicity of their Mixtures. *Mutation Research* **399**-409.

Hermann, M., O. Chaude, N. Weill, H. Bedouelle and M. Hofnung (1980). Adaptation of the Salmonella/Mammalian Microsome Test to the Determination of the Mutagenic Properties of Mineral Oils. *Mutation Research* **77**: 327-339.

Holloway, B.W., M. D. Escuadra, A. F. Morgan, R. Saffrey and V. Krishnapillai (1992). The New Approaches to Whole Genome Analysis of Bacteria. *FEMS Microbiology Letters* **100**:101-106.

Hollstein, M., J. McCann, F. A. Angelosanto and W. W. Nichols (1979). Short-Term Tests for Carcinogens and Mutagens. *Mutation Research* **65**: 133-226.

Horton, A. W. and G. M. Christian (1974). Cocarcinogenesis Verses Incomplete Carcinogenic Activity Among Aromatic Hydrocarbons: Contrast Between Chrysene and Benzo[*b*]triphenylene. *Journal of the National Cancer Institute* **53**: 1017-1020.

Horvath, R. S. (1972). Microbial Cometabolism and the Degradation of Organic Compounds in Nature. *Bacteriological Reviews* **36**: 146-155.

Huber, I. and S. Selenska-Pobell (1994). Pulse Field Gel Electrophoresis Fingerprinting, Genome Size Estimation and rrn Loci Number in *Rhizobium galegae*. *Journal of Applied Bacteriology* **77**: 528-533.

Huberman, E., J. K. Selkirk and C. Heidelberger (1971). Metabolism of Polycyclic Aromatic Hydrocarbons in Cell Cultures. *Cancer Research* **31**: 2161-2167.

Huggins, C. and N. C. Yang (1962). Induction and Extinction of Mammary Cancer. *Science* **137**: 257-262.

Hugh, R. and E. Ryschenkow (1961). *Pseudomonas maltophilia*, an *Alcaligenes*-like species. *Journal of General Microbiology* **26**: 123-132.

Huis in't Veld, M. G. A., J. Werners, J. J. van Veen and H. J. Doddema (1995). Biological PAH Degradation in Dreged Sludges. *In:* R. E. Hinchee, R. E. Hoeppel and D. B. Anderson (eds), *Bioremediation of Recalcitrant Organics*. Battelle Press, Columbus, pp 17-22.

Huisman, O. and R. d'Ari (1981). An Inducible DNA Replication-Cell Division Coupling Mechanism in *E. coli*. *Nature* **290**: 797-799.

Hulbert, M. H. and S. Krawiec (1977). Co-metabolism: A Critique. *Journal of Theroretical Biology* **69**:187-291.

Huling, S. G., D. F. Pope, J. E. Matthews, J. L. Sims, R. C. Sims and D. L. Sorenson (1995). Wood Preserving Waste-Contaminated Soil: Treatment and Toxicity Response. *In:* R. E. Hinchee, R. E. Hoeppel and D. B. Anderson (eds), *Bioremediation of Recalcitrant Organics*. Battelle Press, Columbus, pp 101-109.

IARC (1983). Monographs on the Evaluation of Carcinogenic Risk to Humans. *Volume 32, Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data*. International Agency for Research on Cancer, IARC Press, France.

Jacob, J., G. Grimmer, G. Raab and A. Schmoldt (1982). The Metabolism of Pyrene by Rat Liver Microomes and the Influence of Various Mono-Oxygenase Inducers. *Xenobiotica* **12**: 45-53.

Jeenes, D. J., W. Reineke, H. J. Knackmuss and P. A. Williams (1982). TOL Plasmid pWW0 in Constructed Halobenzoate-Degrading *Pseudomonas* strains: Enzyme Regulation and DNA Structure. *Journal of Bacteriology* **150**: 180-187.

Jeffrey, A. M., H. J. C. Yeh, D. M. Jerina, R. T. Patel, J. F. Davey and D. T. Gibson (1975). Initial Reaction in the Oxidation of Naphthalene by *Pseudomonas putida*. *Biochemistry* **14**: 575-584.

Jerina, D. M., H. Selander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan and D. T. Gibson (1976). Dihydrodiols from Anthracene and Phenanthrene. *Journal of the American Chemists Society* **98**: 5988-5996.

Jobson, A., M. McLaughlin, F. D. Cook and D. W. S. Westlake (1974). Effect of Amendments on the Microbial Utilisation of Oil Applied to Soil. *Applied Microbiology* **27**: 166-171.

Johnson, A. C., F. P. Larson, D. F. Gadbois and A. W. Humason (1985). The Distribution of Polycyclic Aromatic Hydrocarbons in the Surficial Sediments of Penobscot Bay (Maine, USA) in Relation to Possible Sources and to Other Sites Worldwide. *Marine Environmental Research* **15**: 1-16.

Jones, K. C., J. A. Stratford, P. Tidridge, K. S. Waterhouse and A. E. Johnston (1989a). Polynuclear Aromatic Hydrocarbons in an Agricultural Soil: Long-Term Changes in Profile Distribution. *Environmental Pollution* **56**: 337-351.

Jones, K. C., J. A. Stratford, K. S. Waterhouse, E. T. Furlong, W. Giger, R. A. Hites, C. Schaffner and A. E. Johbston (1989b). Increases in the Polynuclear Aromatic hydrocarbon Content of an Agricultural Soil Over the Last Century. *Environmental Science and Technology* **23**: 95-101.

Jones, K. C., J. A. Stratford, K. S. Waterhouse and N. B. Vogt (1989c). Organic Contaminants in Welsh Soil: Polynuclear Aromatic Hydrocarbons. *Environmental Science and Technology* **23**: 540-550.

Jotz, M. M. and A. D. Mitchell (1981). Effects of 20 Coded Chemicals in the Forward Mutation Frequency at the Thymidine Kinase Locus in L5178Y Mouse Lymphoma Cell. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program.* Progress in Mutation Research, Volume 1, New York, Elsevier/North Holland, pp. 580-593.

Juhasz, A. L., M. L. Britz and G. A. Stanley (1996) Degradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons by *Pseudomonas cepacia. Biotechnology Letters* **18**: 577-582.

Juhasz, A.L., M. L. Britz and G. A. Stanley (1997) Degradation of Fluoranthene, Pyrene, Benz[*a*]anthracene and Dibenz[*a,h*]anthracene by *Burkholderia cepacia. Journal of Applied Microbiology* **83**: 189-198.

Kaden, D. A., R. A. Hites and W. G. Thilly (1979). Mutagenicity of Soot and Associated Polycyclic Aromatic Hydrocarbons to *Salmonella typhimurium. Cancer Research* **39**: 4152-4159.

Kastner, M., M. Breuer-Jammali and B. Mahro (1994). Enumeration and Characterisation of the Soil Microflora from Hydrocarbon-Contaminated Soil Sites able to Mineralise Polycyclic Aromatic Hydrocarbons (PAH). *Applied Microbiology and Biotechnology* **41**: 267-273.

Kastner, M., S. Lotter, J. Heerenklage, M. Breuer-Jammali, R. Stegmann and B. Mahro (1995) Fate of ^{14}C-Labelled Anthracene and Hexadecane in Compost-Manured Soil. *Applied Microbiology and Biotechnology* **43**: 1128-1135.

Kastner, M. and B. Mahro (1996) Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Soils Affected by the Organic Matrix of Compost. *Applied Microbiology and Biotechnology* **44**: 668-675.

Keimig, S. D., K. W. Kirby, D. P. Morgan, J. E. Keiser and T. D. Hubert (1983). Identification of 1-Hydroxypyrene as a Major Metabolite of Pyrene in Pig Urine. *Xenobiotica* **13**: 415-420.

Kelly, I. and C. E. Cerniglia (1991). The Metabolism of Phenanthrene by a Species of *Mycobacterium*. *Journal of Industrial Microbiology* **7**: 19-26.

Kelly I., J. P. Freeman and C. E. Cerniglia (1990). Identification of Metabolites from Degradation of Naphthalene by a *Mycobacterium* sp. *Biodegradation* **1**: 283-290.

Kelly I., J. P. Freeman, F. E. Evans and C. E. Cerniglia (1991). Identification of a Carboxylic Acid Metabolite from the Catabloism of Fluoranthene by *Mycobacterium* sp. *Applied and Environmental Microbiology* **57**(3):636-641.

Kelly I., J. P. Freeman, F. E. Evans and C. E. Cerniglia (1993). Identification of Metabolites from the Degradation of Fluoranthene by *Mycobacterium* sp. Strain PYR-1. *Applied and Environmental Microbiology* **59**(3):800-806.

Kennaway, E. L. and I. Hieger (1930). Carcinogenic Substances and their Fluorescent Spectra. *Journal of British Medicine* **1**: 1044-1046.

Kenyon, C. J. and G. C. Walker (1980). DNA-Damaging Agents Stimulate Gene Expression at Specific Loci in *Escherichia coli*. *Proceedings of the National Academy of Science, USA* **77**: 2819-2823.

Keuth, S. and H. J. Rehm (1991). Biodegradation of Phenanthrene by *Arthrobacter polychromogenes* Isolated From a Contaminated Soil. *Applied Microbiology and Biotechnology* **34**: 804-808.

King, J. M. H., P. M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer and G. S. Sayler (1990). Rapid, Sensitive Bioluminescent Reporter Technology for Naphthalene Exposure and Biodegradation. *Nature* **249**: 778-781.

Kiyohara, H. and K. Nagao (1978). The Catabolism of Phenanthrene and Naphthalene by Bacteria. *Journal of General Microbiology* **105**:69-75.

Kiyohara, H., K. Nagao, K. Kouno and K. Yano (1982b). Phenanthrene-Degrading Phenotype of *Alcaligenes faecalis* AFK2. *Applied and Environmental Microbiology* **43**:458-461.

Kiyohara, H., K. Nagao and R. Nomi (1976). Degradation of Phenanthrene Through *o*-Phthalate by an *Aeromonas sp*. *Agricultural Biology and Chemistry* **40**: 1075-1082.

Kiyohara, H., K. Nagao and K. Yana (1982a). Rapid Screen for Bacteria Degrading Water Insoluble, Solid Hydrocarbons on Agar Plates. *Applied and Environmental Microbiology* **43**:454-457.

Kiyohara, H., N. Takizawa, H. Date, S. Torigoe and K. Yano (1990). Characterisation of a Phenanthrene Degradation Plasmid from *Alcaligenes faecalis* AFK2. *Journal of Fermentation Bioengineering* **69**: 54-56.

Klein, M. (1960). A Comparison of the Initiating and Promoting Actions of 9,10-Dimethyl-1,2-Benzanthracene and 1,2,5,6-Dibenzanthracene in Skin Tumourigenesis. *Cancer Research* **20**: 1179

Klein, M. (1963). Susceptibility of Strain B6AF$_1$/J Hybrid Infant Mice to Tumourigenesis with 1,2-Benzanthracene, Deoxycholic Acid and 3-methylcholanthrene. *Cancer Research* **23**: 1701

Kohler, A., M. Schuttoff, D. Bryniok and H-J. Knackmuß (1994). Enhanced Biodegradation of Phenanthrene in a Biphasic Culture System. *Biodegradation* **5**: 93-103.

Koreeda, M., M. N. Akhtar, D. R. Boyd, J. D. Neill, D. T. Gibson and D. M. Jerina (1978). Absolute Stereochemisrty of *Cis*-1,2-, *Trans*-1,2 and *Cis*-3,4-Dihydrodiol Metabolites of Phenanthrene. *Journal of Organic Chemistry* **43**: 1023-1029.

Kuhm, A. E., A. Stolz and H. J. Knackmuss (1991). Metabolisn of Naphthalene by the Biphenyl-Degrading Bacterium *Pseudomonas paucimobilis* Q1. *Biodegradation* **2**: 115-120.

Kurkela, S., H. Lehvaslaiho, E. T. Palva and T. H. Teeri (1988). Cloning, Nucleotide Sequence and Characterisation of Genes Encoding Naphthalene Dioxygenase of *Pseudomonas putida* Strain NCIB 9816. *Gene* **73**: 355-362.

Laflamme, R. E. and R. A. Hites (1978). The Global Distribution of Polycyclic Aromatic Hydrocarbons in Recent Sediments. *Geochimica et Cosmochimica Acta* **42**: 289-303.

Laflamme, R. E. and R. A. Hites (1979). Tetra- and Pentacyclic, Naturally Occurring, Arpmatic Hydrocarbons in Recent Sediments. *Geochimica et Cosmochimica Acta* **43**: 1687-1691.

Lake, R. S., M. L. Kropko, M. R. Pezzutti, R. H. Shoemaker and H. J. Igel (1978). Chemical Induction of Unscheduled DNA Synthesis in Human Skin Epithelial Cell Cultures. *Cancer Research* **38**: 2091-2098.

Lake, J. L., C. Norwood, C. Dimock and R. Bowen (1979). Origins of Polycyclic Aromatic Hydrocarbons in Estuarine Sediments. *Geochimica et Cosmochimica Acta* **43**: 1847-1854.

Lange, B., S. Kremer, O. Sterner and H. Anke (1994). Pyrene Metabolism in *Crinipellis stipitaria*: Identification of *Trans*-4,5-Dihydro-4,5-Dihydroxypyrene and 1-Pyrenylsulphate in Strain JK364. *Applied and Environmental Microbiology* **60**: 3602-3607.

Larson, R. J. (1979). Estimation of Biodegradation Potential of Xenobiotic Organic Chemicals. *Applied and Environmental Microbiology* **38**: 1153-1161.

Launen, L., L. Pinto, C. Wiebe, E. Kiehlmann and M. Moore (1995). The Oxidation of Pyrene and Benzo[*a*]pyrene by Nonbasidiomycete Soil Fungi. *Canadian Journal of Microbiology* **41**: 477-488.

LaVoie, E. J., L. Tulley-Freiler, V. Bedenko and D. Hoffmann (1981). Mutagenicity, Tumour-Initiating Activity and Metabolism of Methylphenanthrenes. *Cancer Research* **41**: 3441-3447.

Leadbetter, E. R. and J. W. Foster (1959). Oxidation Products formed from Gaseous Alkanes by the Bacterium *Pseudomonas methanica*. *Archives of Biochemistry and Physiology* **82**: 491-492.

Leblond, P., F. X. Francou, J-M. Simonet and B. Decaris (1990). Pulse Field Gel Electrophoresis Analysis of the Genome of *Streptomyces ambofaciens* Strains. *FEMS Microbiology Letters* **72**: 79-88.

Lessie, T. G., M. S. Wood, A. Byrne and A. Ferrante (1990). Transposable Gene-Ativating Elements in *Pseudomonas cepacia*. *In*: S. Silver, A. M. Chakrabarty, B. Iglewski and S. Kaplan (ed.), *Pseudomonas*: *Biotransformations, Pathogenesis and Evolving Biotechnology*. American Society for Microbiology, Washington, D.C, pp 279-292.

Lewis, D. L., H. P. Kollig and R. E. Hodson (1986). Nutrient Limitation and Adaption of Microbial Populations to Chemical Transformations. *Applied and Environmental Microbiology* **51**(3):598-603.

Lijinsky, W. (1991). The Formation and Occurence of Polynuclear Aromatic Hydrocarbons Associated With Food. *Mutation Research* **259**: 251-262.

Lin, W. S. and M. Kapoor (1979). Induction of Aryl Hydrocarbon Hydroxylase in *Neurospora crassa* by Benzo[*a*]pyrene. *Current Microbiology* **3**: 177-181.

Lindquist, B. and D. Warshawsky (1985). Identification of the 11,12-Dihydro-11,12-dihydroxybenzo[*a*]pyrene as a Major Metabolite by the Green Alga *Selenastrum capricornutum* **130**: 71-75.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry* **193**: 267-273.

Ma, T. H. (1982a). Vicia Cytogenetic Tests for Environmental Mutagens. A Report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research* **99**: 257-271.

Ma, T. H. (1982b). Tradescantia Cytogenic Tests (Root-Tip Mitosis, Pollen Mitosis, Pollen Mother-Cell Meiosis). A Report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research* **99**: 293-302.

Ma, T. H., M. M. Harris, V. A. Anderson, I. Ahmed, K. Mohammad, J. L. Bare and G. Lin (1984). Tradescantia-Micronucleus (Trad-MCN) Test on 140 Health-Related Agents. *Mutation Research* **138**: 157-167.

Ma, T. H., A. H. Sparrow, L. A. Schairer and A. F. Nauman (1978). Effects of 1,2-Dibromoethane (DBE) on Meiotic Chromosomes of Tradescantia. *Mutation Research* **58**: 251-258.

MacDonald, R. and J. Kalmakoff (1995). Comparison of Fulsed Field Gel Electrophoresis DNA Fingerprints of Field Isolates of the Entomopathogen *Bacillus popilliae*. *Applied and Environmental Microbiology* **61**: 2446-2449.

MacFadden, J. F. (1980). *Biochemical Tests for the Identification of Medical Bacteria.* Williams and Wilkins, London.

Mahaffey, W. R., D. T. Gibson and C. E. Cerniglia (1988). Bacterial Oxidation of Chemical Carcinogens: Formation of Polycyclic Aromatic Acids from Benz[*a*]anthracene. *Applied and Environmental Microbiology* **54**(10):2415-2423.

Mallakin, A. and O. P. Ward (1996). Degradation of BTEX Compounds in Liquid Media and in Peat Biofilters. *Journal of Industrial Microbiology* **16**: 309-318.

Manilal, B. and M. Alexander (1991). Factors affeting the Microbial Degradation of Phenanthrene in Soil. *Applied Microbiology and Biotechnology* **35**: 401-405.

Marinez-Murcia, A.J., S. Benloch and M. D. Collins (1992). Phylogenetic Interrelationships of Members of the Genera *Aeromonas* and *Plesiomonas* as Determined by 16S ribosomal DNA Sequencing: Lack of Congruence With Results of DNA-DNA Hybridisation. *International Journal of Systematic Bacteriology* **42**: 412-421.

Maron, D. M. and B. N. Ames (1983). Revised Methods for the Salmonella Mutagenicity Test. *Mutation Research* **113**: 173-215.

Martin, C. N., A. C. McDermid and R. C. Garner (1978). Testing of Known Carcinogens and Noncarcinogens for their Ability to Indice Unscheduled DNA Synthesis in HeLa Cells. *Cancer Research* **38**: 2621-2627.

Maslow, J. N., M. E. Mulligan and R. D. Arbeit (1993). Molecular Epidemiology: Application and Contemporary Techniques to Typing Microorganisms. *Clinical Infectious Diseases* **17**: 153-164.

McCarthy, J. F. and B. D. Jimenez (1985). Interaction Between Polycyclic Aromatic Hydrocarbons and Dissolved Humic Material: Binding and Dissolution. *Environmental Science and Technology* **19**: 1072-1076.

McClelland, M., R. Jones, Y. Patel and M. Nelson (1987). Restriction Endonucleases for Pulsed Field Mapping of Bacterial Genomes. *Nucleic Acid Research* **15**: 5985-6005.

McGhee, I. and R. G. Burns (1995). Biodegradation of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and 2-Methyl-4-chlorophenoxyacetic Acid (MCPA) in Contaminated Soil. *Applied Soil Ecology* **2**: 143-154.

Means, J. C., S. G. Wood, J. J. Hassett and W. L. Banwart (1980). Sorption of Polynuclear Aromatic Hydrocarbons by Sediments and Soils. *Environmental Science and Technology* **14**: 1524-1528.

Menn, F. M., B. M. Applegate and G. S. Sayler (1993). NAH Plasmid-Mediated Catabolism of Anthracene and Phenanthrene to Naphthoic Acids. *Applied and Environmental Microbiology* **59**(6): 1938-1942.

Menzies, C. A., B. B. Potocki and J. Santodonato (1992). Exposure to Carcinogenic PAHs in the Environment. *Environmental Science and Technology* **26**: 1278-1284.

Mersch-Sundermann, V., S. Mochayedi and S. Kevekordes (1992). Genotoxicity of polycyclic Aromatic Hydrocarbons in *Escherichia coli* PQ37. *Mutation Research* **278**: 1-9.

Mihelcic, J. R. and R. G. Luthy (1988). Degradation of Polycyclic Aromatic hydrocarbon Compounds Under Various Redox Conditions in Soil-Water Systems. *Applied and Environmental Microbiology* **54**(5):1182-1187.

Monna, L., T. Omori and T. Kodama (1993). Microbial Degradation of Dibenzofuran, Fluorene and Dibenzo-*p*-Dioxin by *Staphlococcus auriculans* DBF63. *Applied and Environmental Microbiology* **59**(1):285-289.

Moore, E. R. B., A. S. Kruger, L. Hauben, S. E. Seal, R. de Baere, R. de Wachter, K. N. Timmis and J. Swings (1997). 16SrRNA Gene Sequence Analyses and Inter- and Intrageneric Relationships of *Xanthomonas* Species and *Stenotrophomonas maltophilia*. *FEMS Microbiology Letters* **151**: 145-153.

Morgan, P., S. T. Lewis and R. J. Watkinson (1991). Comparison of Abilities of White-Rot Fungus to Mineralise Selective Xenobiotic Compounds. *Applied Microbiology and Biotechnology* **34**: 693-696.

Mott, S. C., P. H. Groenevelt and R. P Voroney (1990). Biodegradation of a Gas Oil Applied to Aggregates of Different Sizes. *Journal of Environmental Quality* **19**: 257-260.

Mueller, J. G., Chapman, P. J. and Pritchard, P. H. (1989a) Creosote-Contaminated Sites. *Environmental Science and Technology* **23**, 1197-1201.

Mueller, J. G., P. J. Chapman and P. H. Pritchard (1989b) Action of a Fluoranthene-Utilising Bacterial Community on Polycyclic Aromatic Hydrocarbon Components of Creosote. *Applied and Environmental Microbiology* **55**, 3085-3090.

Mueller, J. G., P. J. Chapman, and P. H. Pritchard (1990a). Action of a Fluoranthene-Utilising Bacterial Community on Polycyclic Aromatic Hydrocarbon Components of Creosote. *Applied and Environmental Microbiology* **55**(12):3085-3090.

Mueller, J. G., P. J. Chapman, B. O. Blattmann and P. H. Pritchard (1990b). Isolation and Characterisation of a Fluroanthene Utilising Strain of *Pseudomonas paucimobilis Applied and Environmental Microbiology* **56**(4):1079-1086.

Mueller, J. G., S. E. Lantz, B. O. Blattmann and P. J Chapman (1991a). Bench-Scale Evaluation of Alternative Biological Treatment Process for the Remediation of Pentachlorophenol and Creosote Contaminated Materials: Solid Phase Bioremediation. *Environmental Science and Technology* **25**: 1045-1055.

Mueller, J. G., S. E. Lantz, B. O. Blattmann and P. J Chapman (1991b). Bench-Scale Evaluation of Alternative Biological Treatment Process for the Remediation of Pentachlorophenol and Creosote Contaminated Materials: Slurry Phase Bioremediation. *Environmental Science and Technology* **25**: 1055-1061.

Mueller, J. G., S. E. Lantz, D. Ross, R. J. Colvin, D. P. Middaugh and P. H. Pritchard (1993). Strategy Using Bioreactors and Specially Selected Microorganisms for Bioremediation of Groundwater Contaminated with Creosote and Pentachlorophenol. *Environmental Science andTechnology* **27**: 691-698.

Mueller, J. G., D. P. Middaugh, S. E. Lantz and P. J. Chapman (1991c). Biodegradation of Creosote and Pentachlorophenol in Contaminated Groundwater: Chemical and Biological Assessment. *Applied and Environmental Microbiology* **57**(5):1277-1285.

Narro, M. L., C. E. Cerniglia, C. Van Baalen and D. T. Gibson (1992a). Metabolism of Phenanthrene by the Cyanobacterium *Agmenellum quadruplicatum* PR-6. *Applied and Environmental Microbiology* **58**(4):1352-1359.

Narro, M. L., C. E. Cerniglia, C. Van Baalen and D. T. Gibson (1992b). Evidence of NIH Shift in Naphthalene Oxidation by the Marine Cyanobacterium, *Oscillatoria* Species Strain JCM. *Applied and Environmental Microbiology* **58**: 1360-1363.

Nishioka, M., H. C. Chang and M. L. Lee (1986). Structural Charactristics of Polycyclic Aromatic Hydrocarbon Isomers in Coal Tars and Combustion Products. *Environmental Science and Technology* **20**: 1023-1027.

Nylund, L., P. Heikkila, M. Hameila, L. Pyy, K. Linnainmaa and M. Sorsa (1992). Genotoxic Effects and Chemical Composition of Four Creosotes. *Mutation Research* **265**: 223-236.

Odu, C. T. I. (1978). The Effect of Nutrient Application and Aeration on Oil Degradation in Soil. *Environmental Pollution* **15**: 235-240.

Okamoto, H. and D. Yoshida (1981). Metabolic Formation of Pyrenequinones as Enhancing Agents of Mutagenicity in *Salmonella*. *Cancer Letter* **11**: 215-220.

Osborne, D. J., R. W. Pickup and P. A. Williams (1988). The Presence of Two Complete Homologous *Meta* Pathway Operons on TOL Plasmid pWW53. *Journal of General Microbiology* **134**: 2965-2975.

Paika, I. J., M. T. Beauchesne, M. Randall, R. Schreck and S. A. Latt (1981). *In Vivo* SCE Analysis of 20 Coded Compounds. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program. Progress in Mutation Research*, Volume 1, New York, Elsevier/North Holland, pp. 672-681.

Pal, K. (1981). The Induction of Sister-Chromatid Exchanges in Chinese Hamster Ovary Cells by *K*-Region Epoxides and Some Dihydrodiols Derived from Benz[*a*]anthracene, Dibenz[*a,c*]anthracene and Dibenz[*a,h*]anthracene. *Mutation Research* **84**: 389-398.

Palleroni, N. J. (1984). Section 4, Gram-Negative Aerobic Rods and Cocci, Family Pseudomonadaceae. In: (N. R. Krieg and J. G. Holt, *eds*) *Bergey's Manual of Systematic Bacteriology* Volume 1, pp 141-199.

Palleroni, N.J. and J. F. Bradbury (1993). *Stenotrophomonas*, a New Bacterial Genus for *Xanthomonas maltophilia*. *International Journal of Systematic Bacteriology* **43**: 606-609.

Park, K. S., R. C. Sims and R. Dupont. (1990) Transformations of PAHs in soil systems. *Journal of Environmental Engineering* (ASCE) **116**:632-640.

Parker, L. L., P. W. Betts and B. G. Hall (1988). Activation of a Crytic Gene by Excision of a DNA Fragment. *Journal of Bacteriology* **170**: 218-222.

Pavon, V. and N. Gaju (1997). Genomic Relationship of Five Species of the Genus Chromatium by Analysis of Large Restriction Fragments (Macrorestriction Analysis) Using Pulsed Field Gel Electroporesis. International J*ournal of Systematic Bacteiology* **47**: 586-589.

Pelkonen, O. and D. W. Nebert (1982). Metabolism of Polycyclic Hydrocarbons: Etiologic Role in Carcinogenesis. *Pharmacology Review* **34**: 189-222.

Perkin Elmer (1992). *AmpliTaq® DNA Polymerase*. Technical Bulletin. Roche Molecular Systems Inc. pp 1-4.

Perry, J. J. (1979). Microbial Oxidations Involving Hydrocarbons. *Microbiological Reviews* **43**:59-72.

Petrilli, F. L., G. P. De Renzi and S. De Flora (1980). Interaction Between Polycyclic Aromatic Hydrocarbons, Crude Oil and Oil Dispersants in the Salmonella Mutagenesis Assay. *Carcinogenesis* **1**: 51-56.

Phale, P. S., M. C. Mahajan and C. S. Vaidyanathan (1995). A Pathway for Biodegradation of 1-Naphthoic Acid by *Pseudomonas maltophilia* CSV89. *Archives of Microbiology* **163**: 42-47.

Phillips, D. H. (1983). Fifty Years of Benzo[*a*]pyrene. *Nature* **303**: 486-472.

Pickup, R. W. (1991). Development of Molecular Methods for the Dectection of Specific Bacteria in the Environment. *Journal of General Microbiology* **137**: 1009-1019.

Pienta, R. J., J. A. Poiley, and W. B. Lebherz (1977). Morphological Transformations of Early Passage Golden Syrian Hanster Embryo Cells Derived from Cryopreserved Primary Cultures in a Reliable *In-Vitro* Bioassay for Identifying Diverse Carcinogens. *International Journal of Cancer* **19**: 642-655.

Pierce (1995). N, O-bis(Trimethylsilyl)trifluoroacetamide with Trimethylchlorosilane. Pierce Chemical Company, pp 1-7.

Pollard, S. J. T., S. E. Hurdey and P. M. Fedorak (1994). Bioremediation of Petroleum- and Creosote-Contaminated Soils: A Review of Constraints. *Waste Management and Research* **12**: 173-194.

Pollia, J. A. (1941). Investigations on the Possible Carcinogenic Effects of Anthracene and Chrysene and Some of their Compounds. II. The Effect of Subcutaneous Injection in Rats. *Journal of Industrial Hygene and Toxicology* **23**: 449-451.

Pothuluri, J. V. and C. E. Cerniglia (1994). Microbial Metabolism of Polycyclic Aromatic Hydrocarbons. *In:* G. R. Chaudhry (ed.), *Biological degradation and bioremediation of toxic chemicals*. pp92-124.

Pothului, J. V., J. P. Freeman, F. E. Evans and C. E. Cerniglia (1990). Fungal Transformation of Fluoranthene. *Applied and Environmental Microbiology* **56**: 2974-2983.

Pothului, J. V., J. P. Freeman, F. E. Evans and C. E. Cerniglia (1992b). Fungal Metabolism of Acenaphthene by *Cunninghamella elegans*. *Applied and Environmental Microbiology* **58**: 3654-3659.

Pothuluri, J. V., R. H. Heflich, P. P. Fu and C. E. Cerniglia (1992a). Fungal Metabolism and Detoxification of Fluoranthene. *Applied and Environmental Microbiology* **58**(3):937-941.

Pott, B. M. and T. Henrysson (1995). *Ex Situ* Bioremediation of Polycyclic Aromatic Hydrocarbons in Laboratory Systems. *In*: R. E. Hinchee, R. E. Hoeppel and D. B. Anderson (eds), *Bioremediation of Recalcitrant Organics*. Battelle Press, Ohio, pp 39-44.

Priest, F. and B. Aust (1993). *Modern Bacterial Taxonomy*. Chapman and Hall, Ltd, London.

Pritchard, P. H., J. G. Mueller, J. C. Rogers, F. V. Kremer and J. A. Glaser (1992). Oil Spill Bioremediation: Experiences, Lessons and Results from the Exxon Valdez Oil Spill in Alaska. *Biodegradation* **3**: 315-335.

Promega (1993). *Wizard PCR Preps DNA Purification System for Rapid Purification of DNA Fragments*. Technical Bulletin, pp 1-4.

Quillardet, P. and M. Hofnung (1985). The SOS Chromotest: A Colourimetric Bacterial Assay for Genotoxins- Procedures. *Mutation Research* **147**: 65-78.

Quillardet, P., O. Huisman, R. d'Ari and M. Hofnung (1982). SOS Chromotest: A Direct Assay of Induction of an SOS Function in *Escherichia coli* K-12 to Measure Genotoxicity. *Proceedings of the National Academy of Science, USA* **79**: 5971-5975.

Ramdahl, T., I. Alfheim, S. Rustad and T. Olsen (1982). Chemical and Biological Characterisation of Emissions from Small Residential Stoves Burning Wood and Charcoal. *Chemosphere* **11**: 601-611.

Ramos, J. L. and K. N. Timmis (1987). Experimental Evolution of Catabolic Pathways of Bacteria. *Micobiological Science* **4**: 228-237.

Ramos, J. L., A. Stolz, W. Reineke and K. N. Timmis (1986). Altered Effector Specificities in Regulators of Gene Expression: TOL Plasmid xylS Mutants and their use to Engineer Expansion of the Range of Aromatics Degraded by Bacteria. *Proceedings of the National Academy of Science USA* **83**: 8467-8471.

Ramos, J. L., A. Wasserfallen, K. Rose and K. N. Timmis (1987). Redesigning Metabolic Routes: Manipulation of TOL Plasmid Pathway for Catabolism of Alkylbenzoates. *Science* **235**: 593-596.

Rasaih, V., R. P. Voroney and R. G. Kachanoski (1992). Bioavailability of Stabilised Oily Waste Organics in Ultrasonified Soil Aggregates. *Water, Air and Soil Pollution* **63**: 179-186.

Ribo, J. M. and K. L. E. Kaiser (1987). *Photobacterium phosphoreum* Toxicity Bioassay. I. Test Procedures and Applications. *Toxicity Assessment: An International Quarterly* **2**: 305-323.

Robinson, D. E. and A. D. Mitchell (1981). Unscheduled DNA Synthesis Response of Human Fibroblasts, WI-38 Cells, to 20 Coded Chemicals. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program. Progress in Mutation Research*, Volume 1, New York, Elsevier/North Holland, pp. 33-48.

Rochkind-Dubinsky, M. L., G. S. Sayler and J. W. Blackburn (1987). Microbial *Decomposition of Chlorinated Aromatic Compounds*. Marcel Dekker Inc., New York, pp 48-65.

Rohm, M. and D. Werner (1985). Regulation of the ß-Ketoadipate Pathway in *Rhizobium japonicum* and Bacteroids by Succinate. *Archives of Microbiology* **140**: 375-379.

Rosssello-Mora, R. A., J. Lalucat and E. Garcia-Valdes (1994). Comparative Biochemical and Genetic Analysis of Naphthalene Degradation Among *Pseudomonas stutzeri* Strains. *Applied and Environmental Microbiology* **60**: 966-972.

Rotert, K. H., L. A. Cronkhite and P. J. J. Alvarez (1995). Enhancement of BTX Biodegradation by Benzoate. *In:* R. E. Hinchee, C. M. Vogel and F. J. Brockman (eds), *Microbial Processes for Bioremediation*. Battelle Press, Ohio, pp 161-168.

Rutherford, K. G. and M. S. Newman (1957). A New Synthesis and Some Reactions of 4-Phenanthrenecarboxylic Acid. *Journal of the American Chemist Society* **79**: 213-214.

Ryu, B. H., Y. K. Oh and J. H. Bin (1989). Biodegradation of Naphthalene by *Acinetobacter calcoaceticus* R-88. *Journal of the Korean Agriculture Chemist Society* **32**: 315-320.

Salamone, M. F., J. A. Heddle and M. Katz (1981). Mutagenic Activity of 41 Compounds in the In-Vivo Micronucleus Assay. *In*: F. J. de Serres and J. Ashby (eds), *Evaluation of Short Term Tests for Carcinogens. Report of the International Collaborative Program. Progress in Mutation Research,* Volume 1, New York, Elsevier/North Holland, pp. 686-697.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbor Press, USA.

Sanglard, D., M. S. A. Leisola and A. Fiechter (1986). Role of Extracellular Liginases in Biodegradation of Benzo[*a*]pyrene by *Phanerochaete chrysoporium*. *Enzyme Microbial Technology* **8**: 209-212.

Sasaki, J., J. Arey and W. P. Harger (1995). Formation of Mutagens from the Photooxidations of 2-4 Ring PAH. *Environmental Science and Technology* **29**: 1324-1335.

Savino, A. and M. N. Lollini (1977). Identification of Some Fermentation Products of Phenanthrene in Microorganisms of the Genus *Arthrobacter*. *Bulletin of the Society for Italian Biology* **53**: 916-921.

Sayler, G. S. (1991). Contribution of Molecular Biology to Bioremediation. *Journal of Hazardous Materials* **28**: 13-27.

Schleifer, K. H. and W. Ludwig (1989). Phylogenetic Relationship of Bacteria. *In*: B. Fernholm, K. Bremer and H. Jornvall (eds.), *The Hierarchy of Life*. Elsevier Science Publishers, Amsterdam, pp 103-117.

Schmidt, E., M. Hellwig and H. Knackmuss (1983). Degradation of Chlorophenols by a Defined Mixed Microbial Community. *Applied and Environmental Microbiology* **46**(5):1038-1044.

Schneider, J, R. Grosser, K. Jayasimhulu, W. Xue and D. Warshawsky (1996). Degradation of Pyrene, Benz[*a*]anthracene and Benzo[*a*]pyrene by *Mycobacterium sp.* Strain RJGII-135, Isolated from a Former Coal Gasification Site. *Applied and Environmental Microbiology* **62**: 13-19.

Schocken, M. J. and D. T. Gibson (1984). Bacterial Oxidation of the Polycyclic Aromatic Hydrocarbons Acenaphthene and Acenaphthylene. *Applied and Environemtnal Microbiology* **48**: 10-16.

Scordilis, G. E., H. Ree and T. G. Lessie (1987). Identification of Transposable Elements Which Activate Gene Expression in *Pseudomonas cepacia. Journal of Bacteriology* **169**: 8-13.

Scott, K. F., B. G. Rolfe and J. Shine (1981). Biological Nitrogen Fixation: Primary Structure of *Klebsiella pneumoniae* nifH and nifD Genes. *Journal of Molecular and Applied Genetics* 1: 71-81.

Selkirk, J. K., E. Huberman and C. Heidelberger (1971). An Epoxide is an Intermediate in the Microsomal Metabolism of the Chemical Carcinogen Dibenz[*a,h*]anthracene. *Biochemical and Biophysical Research Communications* **43**: 1010-1016.

Sexton, K., K. Liu, S. B. Hayward and J. D. Spengler (1985). Characterisation and Source Apportionment of Wintertime Aerosol in a Wood-Burning Community. *Atmospheric Environment* **19**: 1225-1236.

Shahin, M. M. and F. Fournier (1978). Suppression of Mutation Induction and failure to Detect Mutagenic Activity with Athabasca Tar Sand Fractions. *Mutation Research* **58**: 29-34.

Shiaris, M. P. (1986). Polycyclic Aromatic Hydrocarbons in Surficial Sediments of Boston Harbour, Massachusetts, USA. *Marine Pollution Bulletin* **17**: 469-472

Shiaris, M. P. (1989a). Phenanthrene Mineralisation Along a Natural Salinity Gradient in an Urban Estuary, Boston Harbor, Massachusetts. *Microbial Ecology* **18**: 135-146.

Shiaris, M. P. (1989b). Seasonal Biotransformation of Naphthalene, Phenanthrene and Benzo[*a*]Pyrene in Surficial Estuarine Sediments. *Applied and Environmental Microbiology* **55**(6):1391-1399.

Shiaris, M. P. and J. J. Cooney (1983). Replica Plating Method for Estimating Phenanthrene-Utilising and Phenanthrene-Cometabolising Microorganisms. *Applied and Environmental Microbiology* **45**:706-710.

Sims, P. (1970). Qualitative and Quantitative Studies on the Metabolism of a Series of Aromatic Hydrocarbons by Rat-Liver Preparations. *Biochemical Pharmacology* **19**: 795-818.

Sims, R. C. (1990). Soil Remediation Techniques at Uncontrolled Hazardous Waste Sites: A Critical Review. *Journal of the Air and Waste Management Association* **40**: 704-733.

Sims, R. C. and M. R. Overcash (1983). Fate of Polynuclear Aromatic Compounds (PNAs) in Soil-Plant Systems. *Residue Reviews* **88**: 1-68.

Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, Second Edition, The Willians and Wilkins Company, Baltimore.

Slaga, T. J., E. Huberman, J. K. Selkirk, R. G. Harvey and W. M. Bracken (1978). Carcinogenicity and Mutagenicity of Benz[*a*]anthracene Diols and Diol-Epoxides. *Cancer Research* **38**: 1699-1704.

Smith, R. V. and J. P. Rosazza (1974). Microbial Models of Mammalian Metabolism. Aromatic Hydroxylation. *Archives of Biochemistry and Biophysiology* **161**: 551-558.

Snell, K. C. and H. L. Stewart (1962). Pulmonary Adenomatosis Induced in DBA/2 Mice by Oral Administration of Dibenz[*a,h*]anthracene. *Journal of the National Cancer Institute* **28**: 1043

Spain, C. J., P. H. Pritchard and A. W. Bourquin (1980). Effects of Adaptation on Biodegradation Rates in Sediment/Water Cores from Estuarine and Freshwater Environments. *Applied and Environmental Microbiology* **40**(4):726-734.

Spain, J. C. and P. A. van Veld. (1983). Adaptation of Natural Microbial Communities to Degradation of Xenobiotic Compounds: Effects of Concentration, Exposure Time, Inoculum and Chemical Structure. *Applied and Environmental Microbiology* **45**:428-435.

Stackebrandt, E. and B. M. Goebel (1994). Taxonomic Note: A place for DNA-DNA Reassociation and 16SrRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic Bacteriology* **44**: 846-849.

Stanlake, G. J. and R. K. Finn (1982). Isolation and Characterisation of a Pentachlorophenol-Degrading Bacterium. *Applied and Environmental Microbiology* **44**: 1421-1427.

Stanton, M. F., E. Miller, C. Wrench and R. Blackwell (1972). Experimental Induction of Epidermoid Carcinoma in the Lung of Rats by Cigarette smoke Condensate. *Journal of the National Cancer Institute* **49**: 867-877.

Stringfellow, W. T. and Aitken, M. D. (1995) Competitive Metabolism of Naphthalene, Methylnaphthalenes and Fluorene by Phenanthrene-Degrading Pseudomonads. *Applied and Environmental Microbiology* **61**, 357-362.

Su, J. J. and D. Kafkewitz (1994). Utilisation of Toluene and Xylenes by a Nitrate-Reducing Strain of *Pseudomonas maltophilia* Under Low Oxygen and Anoxic Conditions. *FEMS Microbiology Ecology* **15**: 249-257.

Su, J. J. and D. Kafkewitz (1996). Toluene and Xylene Degradation by a Denitrifying Strain of *Xanthomonas maltophilia* With Limited or No Oxygen. *Chemosphere* **32**: 1843-1850.

Sutherland, J. B., J. P. Freeman, A. L. Selby, P. P. Fu, D, W. Miller and C. E Cerniglia (1990). Stereoselective Formation of a K-Region Dihydrodiol From Phenanthrene by *Streptomyces flavovirens*. *Archives of Microbiology* **154**: 260-266.

Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E. Evans and C. E. Cerniglia (1991). Metabolism of Phenanthrene by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **57**: 3310-3316.

Sutherland, J. B., A. L. Selby, J. P. Freeman, P. P. Fu, D. W. Miller and C. E. Cerniglia (1992). Identification of Xyloside Conjugates formed from Anthracene by *Rhizoctonia solani*. *Mycology Research* **96**: 509-517.

Swings, J., P. De Vos, M. Van den Mooter and J. De Ley (1983). Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. *International Journal of Systematic Bacteriology* **33**:409-413.

Symons, B. D. and R. C. Sims (1988). Assessing Detoxification of a Comples Hazardous Waste Using the Microtox Bioassay. *Archives of Environmental Contamination and Technology* **17**: 497-505.

Tagger, S., N. Truffaut and J. Le Petit (1990). Preliminary Study on the Relationships Among Strains Forming a Bacterial Community Selected on Naphthalene from Marine Sediment. *Canadian Journal of Microbiology* **36**: 676-681.

Taylor, J. (1962) The Estimation of Numbers of Bacteria by Ten Fold Dilution Series. *Journal of Applied Bacteriology* **25**: 54-61

Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing and B. Swaminathan (1995). Interpreting Chromosomal DNA Restriction Patterns Produced by Pulse Field Gel Electrophoresis: Criteria for Bacterial Strain Typing. *Journal of Clinical Microbiology* **33**: 2233-2239.

Thibault, S. L., M. Anderson and W. T. Frankenberger jr (1996). Influence of Surfactants on Pyrene Desorption and Degradation in Soils. *Applied and Environmental Microbiology* **62**: 283-287.

Thomas, J. M., M. D. Lee, M. J. Scott and C. H. Ward (1989). Microbial ecology of the Subsurface at an Abondoned Creosote Waste Site. *Journal of Industrial Microbiology* **4**: 109-120.

Tiehm, A. and C. Fritzsche (1995). Utilisation of Solubilised and Chrystalline Mixtures of Polycyclic Aromatic Hydrocarbons by a *Mycobacterium sp. Applied Microbiology and Biotechnology* **42**: 964-968.

Tongpim, S. and M. A. Pickard (1996). Growth of *Rhodococcus* S1 on Anthracene. *Canadian Journal of Microbiology* **42**: 289-294.

Torstensson, N. T. L., J. Stark and B. Goransson (1975). The Effect of Repeated Applications of 2,4-D and MCPA on Their Breakdown in *Soil. Weed Research* **15**: 159-164.

Treccani, V., N. Walker and G. H. Wiltshire (1954). The Metabolism of Naphthalene by Soil Bacteria. *Journal of General Microbiology* **11**: 341-348.

Trenz, S. P., K. H. Engesser, P. Fischer and H. J. Knackmuss (1994). Degradation of Fluorene by *Brevibacterium* sp. Strain DPO 1361: A Novel C-C Bond Cleavage Mechanism Via 1,10-Dihydro-1,10-Dihydroxyfluoren-9-One. *Journal of Bacteriology* **176**: 789-795.

Trower, M. K., F. S. Sariaslani and F. G. Kitson (1988). Xenobiotic Oxidation by Cytochrome P-450-Enriched Extracts of *Streptomyces griseus. Biochemical and Biophysical Research Communications* **3**: 1417-1422.

Trzesicka-Mlynarz, D. and O. P. Ward (1995). Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by a Mixed Culture and its Component Pure Cultures, Obtained from PAH-Contaminated Soil. *Canadian Journal of Microbiology* **41**: 470-476.

Trzesicka-Mlynarz, D. and O. P. Ward (1996). Degradation of Fluoranthene in a Soil Matrix by Indigenous and Introduced Bacteria. *Biotechnology Letters* **18**: 181-186.

Van deer Meer, J., W. M. de Vos, S. Harayama and A. J. B. Zehnder (1992). Molecular Mechanisms of Genetic Adaptation to Xenobiotic Compounds. *Microbiological Reviews* **56**: 677-694.

Van Duuren, B. L. and B. M. Goldschmidt (1976). Cocarcinogenic and Tumour-Promoting Agents in Tobacco Carcinogenesis. *Journal of the National Cancer Institute* **56**: 1237-1242.

Van Duuren, B. L., A. Sivak, B. M. Goldschmidt, C. Katz and S. Melchionne (1970). Initiating Activity of Aromatic Hydrocarbons in Two-Stage Carcinogenesis. *Journal of the National Cancer Institute* **44**: 1167

Van Duuren, B. L., A. Sivak, L. Langseth, B. M. Goldschmidt and A. Segal (1968). Initiators and Promotors in Tobacco Carcinogenesis. *National Cancer Institute Monographs* **28**: 173-180.

Van Zyl, E. and P. L. Stein (1992). Reinterpretation of the Taxonomic Position of *Xanthomonas maltophilia* and Taxonomic Criteria in the Genus. Request for an Opinion. *International Journal of Systematic Bacteriology* **42**:193-198.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters and J. Swings (1996). Polyphasic Taxonomy a Consensus Approach to Bacterial Systematics. *Microbiological Reviews* **60**: 407-438.

Vanneck, P., M. Beeckman, N. De Saeyer, S. D'Haene and W. Verstraete (1995). Biodegradation of Aromatic Hydrocarbons in a Two-Liquid-Phase System. *In*: R. E. Hinchee, R. E. Hoeppel and D. B. Anderson (eds), Bioremediation of Recalcitrant Organics. Battelle Press, Columbus, pp 55-62

Vashon, R. D., W. J. Jones and A. G. Payne (1982). The Effect of Water Hardness on Nitrilotriacetate Removal and Microbial Acclimation in Activated Sludge. *Water Research* **16**: 1429-1432.

Venosa, A. D., M. T. Suidan, J. R. Haines, B. A. Wrenn, K. L. Strohmeier, B. L. Eberhart, M. Kadkhodayan, E. Holder, D. King and B. Anderson (1995). Field Bioremediation Study: Spilled Crude Oil on Fowler Beach, Delaware. *In:* R. E. Hinchee, J. Fredrickson and B. C. Alleman (eds), *Bioaugmentation for Site Remediation*. Battelle Press, Ohio, pp 49-56.

Ventullo, R.M. and R.J Larson (1986). Adaptation of Aquatic Microbial Communities to Quaternary Ammonium Compounds. *Applied and Environmental Microbiology* **51**(2):356-361.

Wakeham, S. G., C. Schaffner and W. Giger (1980a). Polycyclic Aromatic Hydrocarbons in Recent Lake Sediments-I. Compounds Having Anthropogentic Origins. *Geochimica et Cosmochimica Acta* **44**: 403-413.

Wakeham, S. G., C. Schaffner and W. Giger (1980b). Polycyclic Aromatic Hydrocarbons in Recent Lake Sediments-II. Compounds Derived From Biogenic Precursors During Early Diagenesis. *Geochimica et Cosmochimica Acta* **44**: 415-429.

Walker, G. C. (1984). Mutagenesis and Inducible Responses to DNA Damage in *Escherichia coli. Microbiological Reviews* **48**: 60-93.

Walter, U., M. Beyer, J. Klein and H. J. Rehm (1991). Degradation of Pyrene by *Rhodococcus* sp. UW1. *Applied Microbiology and Biotechnology* **34**: 671-676.

Warshawsky, D., T. M. Keenan, R. Reilman, T. E. Cody and M. J. Radike (1990). Conjugation of Benzo[*a*]pyrene Metabolites by the Freshwater Green Alga *Selenastrum capricornutum. Chemical-Biological Interactions* **73**: 93-105.

Warshawsky, D., M. Radike, K. Jayasimhulu and T. Cody (1988). Metabolism of Benzo[*a*]pyrene by a Dioxygenase Enzyme System of the Freshwater Green Alga *Selenastrum capricornutum. Biochemical and Biophysical Research Communications.* **152**: 540-544.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, P. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr and H. G. Truper (1987). Report of the as hoc Committee on

Reconciliation of Approaches to Bacterial Systematics. *International Journal of Systematic Bacteriology* **37**: 463-464.

Weissenfels, W. D., M. Beyer and J. Klein (1989). Bakterieller Abbau von Phenanthren, Fluren und Fluoranthen. *Forum Mikrobiol* **1-2**: 104.

Weissenfels, W. D., M. Beyer and J. Klein (1990a). Degradation of Phenanthrene, Fluorene and Fluoranthene by Pure Bacterial Cultures. *Applied Microbiology and Biotechnology* **32**: 479-484.

Weissenfels, W. D., M. Beyer and J. Klein (1990b). Rapid Testing System for Assessing the Suitability of the Biological Reclamation for PAH-Contaminated Soil. In *Fifth European Congress on Biotechnology*, Copenhagen, 8-13 July, 1990, Vol **2**: 931-934.

Weissenfels, W. D., M. Beyer J. Klein and H. J. Rehm (1991). Microbial Metabolism of Fluoranthene: Isolation and Identification of Ring Fission Products. *Applied Microbiology and Biotechnology* **34**: 528-535.

Weissenfels, W. D., H. Klewer and J. Landhoff (1992). Adsorption of Polycyclic Aromatic Hydrocarbons (PAHs) by Soil Particles: Influence on Biodegradability and Biotoxicity. *Applied Microbiology and Biotechnology* **36**: 689-696.

West, P. A., G. C. Okpokwasili, P. R. Brayton, D. J. Grimes and R. R. Colwell (1984). Numerical Taxonomy of Phenanthrene-Degrading Bacteria Isolated from the Chesapeake Bay. *Applied and Environmental Microbiology* **48**(5):988-993.

Wiggins, B. A., S. H. Jones and M. Alexander (1987). Explanations of the Acclimation Period Preceeding the Mineralization of Organic Chemicals on Aquatic Environments. *Applied and Environmental Microbiology* **53**(4):791-796.

Williams, P. A., S. J. Assinder and L. E. Shaw (1990). Construction of Hybrid xylE Genes Between the Two Duplicate Homologous Genes from TOL Plasmid pWW53: Comparison of the Kinetic Properites of the Gene Products. *Journal of General Microbiology* **136**: 1583-1589.

Wilson, S. C. and K. C. Jones (1993). Bioremediation of Soils Contaminated With Polynuclear Aromatic Hydrocarbons (PAHs): A Review. *Environmental Pollution* **88**: 229-249.

Wiseman, A. L. and F. J. Woods (1979). Benzo[*a*]pyrene Metabolites formed by the Action of the Yeast Cytochrome P-450/P-448. *Journal of Chemical Technology and Biotechnology* **29**: 320-324.

Wodzinski, R. S. and D. Bertolini (1972). Physical State in which Naphthalene and Bibenzyl are Utilised by Bacteria. *Applied Microbiology* **23**(6):1077-1081.

Woese, C. R. (1987). Bacterial Evolution. *Bacteriological Review* **51**:221-271.

Wood, A. W., R. L. Chang, W. Levin, D. E. Ryan, P. E. Thomas, D. H. Mah, J. M. Karle, H. Yagi, D. M. Jerina and A. H. Conney (1979). Mutagenicity and Tumourigenicity of Phenanthrene and Chrysene Epoxides and Diol Epoxides. *Cancer Research* **39**: 4069-4077.

Wood, A. W., W. Levin, A. Y. H. Lu, H. Yagi, O. Hernandez, D. M. Jerina and A. H. Cooney (1976). Metabolism of Benzo[*a*]pyrene and Benzo[*a*]pyrene Derivatives to Mutagenic Products by Highly Purified Hepatic Microsomal Enzymes. *Journal of Biological Chemistry* **251**: 4882-4890.

Wood, M. S., C. Lory and T. G. Lessie (1990). Activation of the lac Gene of Tn951 by Insertion Sequences from *Pseudomonas cepacia*. *Journal of Bacteriology* **172**: 1719-1724.

Wu, J. and L. K. Wong (1981). Microbial Transformations of 7,12-Dimethylbenz[*a*]anthracene. *Applied and Environmental Microbiology* **41**(3):843-845.

Wunder, T., S. Kremer, O. Sterner and H. Anke (1994). Metabolism of the Polycyclic Aromatic Hydrocarbon Pyrene by *Aspergillus niger* SK9317. *Applied Microbiology and Biotechnology* **42**: 636-641.

Wyder, E. L. and D. Hoffmann (1959). A Study of Tobacco Carcinogenesis VII. The Role of Higher Polycyclic Hydrocarbons. *Cancer* **12**: 1079-1086.

Yamamoto, S., S. Shinoda, M. Kawaguchi, K. Wakamatsu and M. Makita (1983). Polyamine Distribution in *Vibrionaceae*: Norspemidine as a General Constituent of *Vibrio* Species. *Canadian Journal of Microbiology* **29**: 724-728.

Yang, Y., R. F. Chen and M. P. Shirais (1994). Metabolism of Naphthalene, Fluorene and Phenanthrene: Preliminary Characterisation of a Cloned Gene Cluster from *Pseudomonas putida* NCIB9816. *Journal of Bacteriology* **176**: 2158-2164.

Ye, B., M. A. Siddiqi, A. E. Maccubbin, S. Kumar and H. C. Sikka (1996). Degradation of Polynuclear Aromatic Hydrocarbons by *Sphingomonas paucimobilis*. *Environmental Science and Technology* **30**: 136-142.

Youngblood, W. W. and M. Blumer (1975). Polycyclic Aromatic Hydrocarbons in the Environment: Homologous Series in Soils and Recent Marine Sediments. *Geochimica et Cosmochimica Acta* **39**: 1303-1314.

Zeiger, E., R. S. Chhabra and B. H. Margolin (1979). Effects of the Hepatic S9 Fraction from Aroclor-1254-Treated Rats on the Mutagenicity of Benzo[*a*]pyrene and 2-Aminoanthracene in the Salmonella/Microsome Assay. *Mutation Research* **64**: 379-389.

Zuniga, M. C., D. R. Durham and R. A. Welch (1981). Plasmid- and Chromosome-Mediated Dissimilation of Naphthalene and Salicylate in *Pseudomonas putida* PMD-1. *Journal of Bacteriology* 147: 836-843.