

**BIODEGRADATION OF POLYCYCLIC AROMATIC
HYDROCARBONS: APPLICATION OF FUNGAL-BACTERIAL
CO-CULTURES AND SURFACTANTS**

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by

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ABBREVIATION

A ₇₅₀	Absorbance at 750 nm
ANGIS	Australian National Genomic Information Service
BSA	Bovine serum albumin
BSM	Basal salt medium
BSMG	Basal salt medium plus glucose
°C	Degree Celsius
CHO	Chinese-hamster ovary
CMC	Critical micelle concentration
CTAB	Cetyltrimethylammoniumbromide
d	Day
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FID	Flame ionization detector
g	Gram
GC	Gas chromatography
GEMs	Genetically engineered microorganisms
h	Hour
HPLC	High performance liquid chromatography
K _{ow}	Octanol/water partition coefficient
l	Litre
LiPs	Lignin peroxidases
M	Molar
MGP	Manufactured gas plants
MW	Molecular weight
μm	Micrometer
μmols/ml	Micromoles per millilitre
MIC	Minimum inhibition concentration
min	Minute
mM	Millimolar

ml	Milliliter
MnPs	Manganese peroxidases
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
PAHs	Polycyclic aromatic hydrocarbons
PCPs	Pentachlorophenol
PCR	Polymerase chain reaction
Rnase	Ribonuclease
rpm	round per minute
S9	Post mitochondrial supernatant
SCE	Sister-chromatid exchanges
SDS	Sodium dodecyl sulphate
SD	Standard deviation
SOSIP	SOS-induction potency
sal	Salicylic acid
sec	Second
TER	Tergitol NP-10
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
USEPA	United State Environmental Protection Agency
v	Volume
v/v	Volume per volume
w/v	Weight per vol
wt	Weight

SUMMARY

There has been a considerable amount of literature published over the last 15 years describing the isolation of various microorganism which are capable of degrading polycyclic aromatic hydrocarbons (PAHs). The bioaugmentation of PAH-polluted soils using these PAH degraders has, in most cases, resulted in the more rapid removal of some PAH compounds compared to the indigenous processes. Typically, however, such treatment currently falls short of removing the environmental and health risk posed by the site since it fails to remove a majority of the high molecular weight PAHs, especially the carcinogenic PAHs containing five or more benzene rings. In response to this, a major focus of current research in this area is on the isolation of microorganisms which can totally degrade high molecular weight PAHs. This has met with limited success since the isolation of a microbial culture which can mineralize and grow on five benzene ring PAHs, as a sole carbon and energy source, remains elusive. The primary objective of the work in this thesis was to isolate and characterize microorganisms that can grow on high molecular weight PAHs as a sole carbon and energy source and, in addition, totally degrade these compounds to carbon dioxide and water.

PAH-degrading microorganisms were enriched and isolated from PAH-contaminated soils. Two bacterial consortia (designated as VUN 10,008 and VUN 10,009) were enriched on pyrene as a sole carbon and energy source. Four pure bacterial strains were isolated from these enriched cultures and identified, based on 16S rRNA analysis, as *Stenotrophomonas maltophilia* VUN 10,010, *S. maltophilia* VUN 10,012, *Pseudomonas fluorescens* VUN 10,011 and *Burkholderia* sp. VUN 10,013. A pure culture of fungus was also isolated and identified as *Penicillium janthinellum* VUN 10,201. PAH-degrading consortia and the pure bacterial isolates were able to use pyrene, phenanthrene or fluorene as a sole carbon and energy source and also demonstrated an ability to degrade other PAH compounds. Although *P. janthinellum* VUN 10,201 could not utilize any PAHs as its source of carbon and energy, it could degrade most high molecular weight PAH compounds.

Further investigations into the biodegradation and mineralization of high molecular weight PAHs by the bacteria (*S. maltophilia* VUN 10,010 and bacterial consortium VUN 10,009) and the fungus (*P. janthinellum* VUO 10,201) were carried out in liquid and soil matrices. Chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene were degraded by axenic cultures of these isolates in a basal salts medium (BSM) containing a single PAH. However, these PAHs were slowly degraded and such conditions could not support the growth of the microorganisms nor PAH mineralization. Co-metabolic mineralization of benzo[a]pyrene by pure cultures of the bacterial consortium VUN 10,009 or strain VUN 10,010 was observed in BSM containing both benzo[a]pyrene and either pyrene or a PAH mixture (comprising three- to seven-benzene ring PAHs) as the growth substrate.

It was found that the fungus and bacterial consortium VUN 10,009 could be enriched from soils on benzo[a]pyrene if they were incubated together, however, neither organism could be enriched on this PAH if they were incubated separately. This phenomenon was further investigated by the deliberate incubation of these two organisms, and other bacterial isolates, in fungal-bacterial co-cultures. Co-cultures comprising *P. janthinellum* VUO 10,201 and either bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010, could degrade and grow on pyrene, chrysene, benz[a]anthracene, benzo[a]pyrene or dibenz[a,h]anthracene as the sole carbon and energy source in BSM. Benzo[a]pyrene as a sole carbon and energy source was mineralized to $^{14}\text{CO}_2$ by these co-cultures and a substantial amount of radioactivity was recovered from washed cell biomass suggesting the use of carbon from benzo[a]pyrene for the construction of cellular material. The nature of the cooperation between the bacteria and the fungus during benzo[a]pyrene degradation was investigated by culturing a high biomass population of one organism (either the bacteria or the fungus) on benzo[a]pyrene in BSM, then removing the biomass and inoculating the resulting culture broth with the other organism. It was found that the majority of benzo[a]pyrene was mineralized when *S. maltophilia* VUN 10,010 was incubated in a culture broth generated by axenic *P. janthinellum* VUO 10,201 cultures in BSM-benzo[a]pyrene. This suggests that in the co-culture

systems, *P. janthinellum* VUO 10,201 may play an important role in the initial breakdown of the benzo[a]pyrene molecule leading to the production of intermediate compounds that are further degraded to CO₂ and water by the bacterial population. Introduction of these co-cultures to PAH-spiked soil or PAH-contaminated soil resulted in superior high molecular weight PAH degradation and benzo[a]pyrene mineralization compared to the indigenous microbes or soil amended with axenic inocula. The recovery of initial radioactivity, added as [¹⁴C]benzo[a]pyrene, as ¹⁴CO₂ from soils inoculated with a co-culture was 37-48% from PAH-spiked soil and 37-53% from PAH-contaminated soil, both after 100 days incubation.

A feature of the high molecular weight PAHs which adds to their recalcitrance is their high hydrophobicity and low aqueous solubility. This limits their bioavailability which ultimately becomes one of the controlling factors responsible for the low biodegradation rates of high molecular weight PAHs. Increased PAH dissolution into the aqueous phase due to surfactant addition has been shown to increase the biodegradation rate of low molecular weight PAHs. Given the success of surfactant addition in increasing the biodegradation rates of low molecular weight PAHs, another objective of this thesis was to determine the effect of surfactants on the degradation rate of high molecular weight PAHs. *S. maltophilia* VUN 10,010, bacterial consortia VUN 10,009 and *P. janthinellum* VUO 10,201 were used for this investigation and some basic criteria for the selection of surfactants for PAH biodegradation were first evaluated. Anionic and cationic surfactants were highly toxic to the bacterial and fungal strains and the Tween series could be used as a bacterial growth substrate. Five nonionic surfactants (Brij 35, Igepal CA-630, Triton X-100, Tergitol NP-10, and Tyloxapol) were not utilized by, and were less toxic to, the tested organisms. MSR and log K_m values were determined for fluoranthene, pyrene, and benzo[a]pyrene in the presence of these nonionic surfactants. PAH apparent solubility was found to increase by over 250-fold in the presence of 10 g l⁻¹ of all surfactants. The effects of this increase in PAH apparent solubility due to surfactant addition on PAH degradation was extensively examined using *S. maltophilia* VUN 10,010. The

pyrene degradation rate by strain VUN 10,010 was enhanced by the addition of four of the nonionic surfactants (5-10 g l⁻¹), however, 5 g l⁻¹ Igepal CA-630 inhibited pyrene degradation and microbial growth. The specific growth rate of VUN 10,010 on pyrene was increased by 67% in the presence of 10 g l⁻¹ Brij 35 or Tergitol NP-10. The addition of Brij 35 and Tergitol NP-10 to BSM containing a single high molecular weight PAH (comprising four- and five-benzene rings) as the sole carbon source increased the maximum specific PAH degradation rate and decreased the lag period normally seen before the onset of PAH degradation. The addition of Tergitol NP-10 (5 g l⁻¹) to VUN 10,010 cultures which contained a PAH mixture (containing three- to seven-benzene ring components) substantially improved the overall degradation rate of each PAH and increased the specific growth rate of VUN 10,010 by 30%. Tergitol NP-10 was selected, based on the results obtained using liquid cultures, for further investigation of PAH degradation in soil. The addition of Tergitol NP-10 stimulated the rate and extent of pyrene and benzo[a]pyrene desorption from a soil matrix. In addition, it also stimulated the rate of high molecular weight PAH degradation and benzo[a]pyrene mineralization in soils. Approximately 70% of the labeled carbon evolved as ¹⁴CO₂ from the [¹⁴C]benzo[a]pyrene added to the PAH-contaminated soil amended with 2% surfactant and inoculated with a *P. janthinellum* and *S. maltophilia* co-culture compared to the 50% ¹⁴CO₂ evolution observed from a parallel treatment containing no added surfactant.

The biodegradation of high molecular weight PAHs by the PAH-degraders isolated in this thesis was also shown to result in a considerable detoxification of the polluted liquid cultures or soils. According to the Ames mutagenicity test, the mutagenic potential was substantially reduced by the introduction of co-culture inocula (42%) to PAH-contaminated soil compared to soil amended with axenic bacterial (6-11%) or fungal (36%) inocula or the indigenous microbes (5%). In the presence of surfactant, the reduction in mutagenic potential of these soils was even greater.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Human exploitation of fossil fuel reserves and the production of novel synthetic compounds or xenobiotics have introduced many pollutants into the environment. Many of these compounds are toxic to living systems and their presence in aquatic and terrestrial habitats often has serious ecological consequences, including major kills of indigenous biota. The disposal and accidental spillage of these compounds has created serious modern environmental pollution problems, particularly in circumstances where indigenous microbial biodegradation activities fail to remove these pollutants before the onset of environmental damage.

In recent years there has been an increasing awareness and concern over the contamination of the environment by polycyclic aromatic hydrocarbons (PAHs). PAHs are components of coal tar, creosote and crude oil, and are formed by the incomplete combustion of organic materials and as by-products of industrial processing (Cerniglia, 1992; Wilson and Jones, 1993; Cutright and Lee, 1994a). PAHs enter the biosphere through various routes such as direct aerial fallout, chronic leakage of industrial or sewage effluents, and accidental discharges during the transport, use and disposal of petroleum products. Owing to their hydrophobic nature, most PAHs in aquatic and terrestrial ecosystems become associated with, and bind to, particulate matter in soils and sediments which increases their environmental persistence (Cerniglia and Heitkamp, 1989). This has raised considerable concern since PAHs in the environment are problematic not only because they are a human health risk, but also due to their influence in altering native ecological communities. Many low molecular weight PAHs (containing up to three benzene rings) are acutely toxic, with some having effects on the reproduction and mortality rates of aquatic animals (Moore *et al.*, 1989).

Most high molecular weight PAHs (containing four or more benzene rings) have mutagenic, teratogenic and potential carcinogenic effects (Pahlman and Pelkonen, 1987). Because of their toxicity, carcinogenicity, recalcitrance and ubiquitous distribution, the U.S. Environmental Protection Agency has listed sixteen PAH compounds as priority pollutants (Keith and Telliard, 1979).

Several processes may be involved in the removal of PAHs from the environment and these include volatilization, photo-decomposition, chemical oxidation and biological transformation. Although physicochemical removal mechanisms can reduce the concentrations of some low molecular weight PAHs, microbial degradation is considered to be the predominant mechanism for PAH removal from soil and the aquatic environment (Cerniglia and Heitkamp, 1989). PAHs can be either totally biodegraded (mineralized) or partially transformed by a community of microorganisms or by a single microorganism (Cerniglia, 1992).

The current foci of PAH biodegradation research include (i) isolation of microorganisms that degrade highly condensed PAHs; (ii) innovative methods to detect PAH-degrading microorganisms and determine their biodegradative potential; (iii) studies on the genetics and regulation of PAH degradation, and; (iv) enhancing PAH degradation through various techniques, such as introducing allochthonous organisms, adding inorganic nutrients, adding surfactants, and utilizing engineered systems to aid in mass transfer and to optimize growth conditions (Cerniglia, 1993).

Successful use of such technologies requires the isolation and use of microorganisms capable of degrading PAH compounds. There has been considerable success in the isolation of microbial strains which are able to totally degrade low molecular weight PAHs, however, the recalcitrance of high molecular weight PAHs has made it especially difficult to isolate pure microbial cultures which can degrade these compounds. Two criteria in particular are important regarding the suitability of microbial isolates to be used for the bioremediation of high molecular weight PAH compounds. Firstly, the isolates should be capable of mineralizing the PAHs to carbon dioxide and water to minimize the accumulation

and distribution of toxic water-soluble intermediates. Secondly, the PAH-degraders must be able to catabolize and grow on these PAHs as a sole carbon source to ensure that their survival and dominance at PAH-contaminated sites is not compromised by the lack of a suitable carbon substrate in the contaminated soil. Despite a substantial research effort to date, there have been no reports on the isolation of microorganisms which are able to grow on and mineralize PAHs comprising more than four-fused aromatic rings as a sole carbon and energy source. This has important implications for the bioaugmentation of PAH-contaminated sites since many contain a high proportion of the carcinogenic and mutagenic five-benzene ring PAHs which, using the microbial strains currently available for PAH bioremediation, are either not degraded, or only partially degraded, during the remediation process. The issue of high molecular weight PAH biodegradation is further complicated by the very low solubility of these PAH compounds and their tendency to form associations with organic matter. This greatly decreases the bioavailability of high molecular weight PAHs which can severely restrict their biodegradation rate. This means that the successful remediation of PAH-contaminated sites may require, in addition to high molecular weight PAH degraders, special strategies to improve PAH bioavailability such that their degradation proceeds at a reasonable rate.

The general aim of this thesis was to address and resolve the above mentioned limitations associated with the bioremediation of high molecular weight PAHs. In Chapter 4, microorganisms are isolated from PAH-contaminated soils and then identified and characterized for their ability to degrade PAH compounds. A fungal-bacterial co-culture isolated in Chapter 4 is further characterized in Chapter 5 for its ability to mineralize and grow on high molecular weight PAHs and some preliminary investigations are conducted into the PAH catabolism of the co-culture. Chapter 6 examines the use of surfactants to improve the solubilization of high molecular weight PAHs and, consequently, their rate of biodegradation and mineralization by axenic cultures and fungal-bacterial co-cultures.

CHAPTER 2

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

An underlying theme of the research conducted in this thesis is evaluating the factors which are important for improving the bioremediation of PAH-contaminated soils. More specifically, this thesis aims to isolate high molecular weight PAH-degrading microorganisms, characterize their PAH degradation potential and mineralization capacity, and enhance their PAH biodegradation rate using mobilizing agents to increase substrate availability. The literature review in this chapter deals exclusively with unsubstituted PAHs in soil and provides an overview of the structures, sources and distribution of PAHs in the environment and their recalcitrant, toxic, carcinogenic and mutagenic properties. With regard to PAH bioremediation, microbial degradation provides an environmentally clean, versatile and efficient mode of PAH removal and researchers are currently trying to harness this decontamination mechanism for the remediation of PAH-polluted sites. Understanding PAH properties, their fates in the environment and the various factors which influence their biodegradation and mineralization in soil is important for the development of technologies for PAH decontamination. With this in mind, this chapter also reviews the mechanisms of microbial degradation and the underlying genetics, together with bioremediation performance evaluation methods based on the monitoring of site detoxification.

2.2 POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

2.2.1 Chemical and Physical Properties

PAHs are compounds consisting of two or more fused benzene rings in linear, angular or cluster arrangements (Blumer, 1976). For example, naphthalene

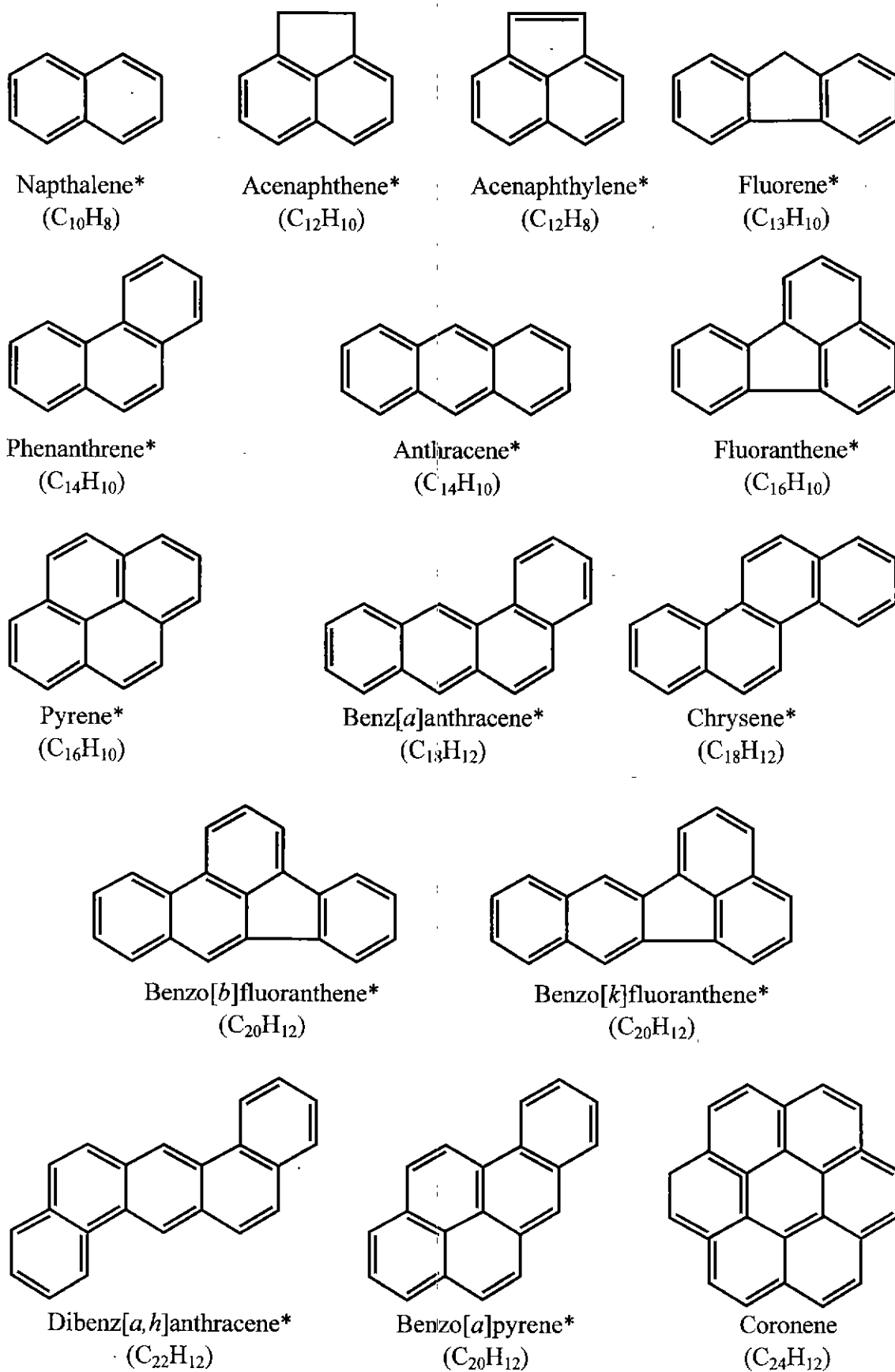


Figure 2.1 Chemical structures of some common polycyclic aromatic hydrocarbons.
*Denotes EPA priority Pollutants.

(C₁₀H₈) is composed of two fused benzene rings and is the simplest PAH molecule. By definition, the term hydrocarbon refers exclusively to compounds containing only carbon and hydrogen atoms. However, heterocyclic aromatic compounds can also be formed by substitution of nitrogen, sulfur, and oxygen for carbon atoms in the benzene rings. These compounds are commonly grouped with the true PAHs, since their chemical and functional properties resemble those of hydrocarbon-based PAHs (McElroy *et al.*, 1989).

PAHs with molecular weights ranging between naphthalene (C₁₀H₈, MW 128.16) to coronene (C₂₄H₁₂, MW 300.36) are of critical environmental concern and the chemical structures of some key PAHs are given in Figure 2.1. The number of rings and their orientation are important in determining their physical properties and hence their environmental importance: the aqueous solubility and volatility of PAHs decrease with increasing molecular weight (see Table 2.1 for the partition coefficients and aqueous solubilities of the 16 USEPA priority PAHs) (Sanseverino *et al.*, 1994). Most PAHs are recalcitrant to microbial degradation and demonstrate a high tendency to remain in nature by adsorbing strongly to soils and sediments. The partitioning of PAHs to soils and sediments correlates highly with the organic carbon content of soil and the contaminant's relative hydrophobicity, typically described by its octanol/water partition coefficient (K_{ow}). The equilibrium partitioning of PAHs into the soil organic fraction, however, is also influenced by physical parameters, of which temperature is a major contributor (Piatt *et al.*, 1996).

2.2.2 Toxic, Carcinogenic and Mutagenic Properties

PAHs are a group of hazardous organic substances of critical public health concern due to the following characteristics: (i) they exert acute toxic effects on living organisms, especially aquatic life, (ii) their high bioaccumulation potential in food chains, (iii) their presence can cause chronic health effects (*i.e.* carcinogenicity) and, (iv) their mutagenic potential (Southworth *et al.*, 1978; Moore *et al.*, 1989; Mueller *et al.*, 1991; Sasaki *et al.*, 1995). There is a general tendency for the low molecular weight, and relatively water-soluble, PAHs such as

Table 2.1 Chemical, physical and biological properties of the 16 USEPA priority PAHs (Sims and Overcash, 1983; Sanseverino *et al.*, 1994; Ashok and Saxena, 1995).

PAH	Number of rings	Log K_{ow} ^a	Aqueous solubility (mg l ⁻¹)	Acute toxicity ^b	Chronic toxicity	
					Carcinogenicity ^c	Mutagenicity ^d
Naphthalene	2	3.37	30.00	+	-	-
Acenaphthlene	3	4.43	3.47	?	?	?
Acenaphthylene	3	4.07	3.93	?	?	?
Anthracene	3	4.45	0.07	?	+	-
Phenanthrene	3	4.46	1.29	++	-	-
Fluorene	3	4.18	1.98	?	?	?
Fluoranthene	4	5.33	0.26	+	?	+
Benzo[a]anthracene	4	5.61	0.014	?	+++	+
Chrysene	4	5.61	0.002	?	++	+
Pyrene	4	5.32	0.14	?	-	-
Benzo[a]pyrene	5	6.04	0.0038	+++	+++	+
Benzo[b]fluoranthene	5	6.57	0.0012	?	++	?
Benzo[k]fluoranthene	5	6.84	0.00055	?	++	?
Dibenzo[a,h]anthracene	5	5.97	0.0005	?	+++	+
Benzo[g,h,i]perylene	6	7.23	0.00026	?	?	?
Indeno[1,2,3-cd]pyrene	6	7.66	0.062	?	++	?

^a Octanol/water partition coefficient.

^b Data obtained from standard toxicity testing with animals for acute LD50 (concentration at which 50% of the population died).

^c Data obtained from standard toxicity testing with animals for carcinogenicity.

^d Values are based on the standardized Ames/*Salmonella typhimurium*/mammalian microsomal assay.

+ Relative degree of effect on organism; - No effect, ? Unknown.

naphthalene and phenanthrene to exert acute toxic effects on their surroundings (Table 2.1) (Struble and Harman, 1983; Darvill and Wilhm, 1984; Aprill *et al.*, 1990; Ashok and Saxena, 1995). These low molecular weight PAHs are normally major components in PAH-contaminated soil and many laboratory studies have demonstrated that water eluents of PAH-contaminated soils inhibit light emission by *Photobacterium phosphoreum* i.e. are toxic, according to the Microtox™ assay (Symons and Sims, 1988; Weissenfel *et al.*, 1992; Belkin *et al.* 1994; Tiehm *et al.*, 1997). On the other hand, sub-acute effects such as genotoxicity, carcinogenicity and bioaccumulation tend to occur with the higher molecular weight, and less soluble, PAHs (Table 2.1).

Industrial disease due to contact with PAHs was first identified in 1775 in London, UK. Percival Pott noted a high incidence of scrotal cancer among chimney sweeps. In 1875, von Volkman showed that there was a prevalence of skin cancer among workers in the German coal tar industry. Causal links between PAHs and cancer were strengthened in 1933 when benzo[a]pyrene was isolated from chimney soot. Later, a notable contribution was made by Kennaways in 1947 by establishing a link between PAHs and lung cancer in workers in the coal tar and gas industries. This was extended in 1972 by Sir Richard Doll, who had made a study of mortality rates in a large group of workers in the same industry over a 12-year period, demonstrating that the incidence of death in manufactured gas workers due to cancers of the lung, bladder, skin and scrotum was far higher than the national rates (see review by White, Jr., 1986; Alloway and Ayres, 1993).

Evidence from extensive worldwide studies have concluded that PAHs are biotoxic, carcinogenic and mutagenic. The investigation of links between carcinogens and mutagens was carried out by McCann and co-workers in 1975 and subsequently in 1976. The authors conducted tests on the mutagenicity of 300 compounds which were recognized as either carcinogens or non-carcinogens, including 38 PAHs and their derivatives, using the Ames test. It was demonstrated that there was a high correlation between carcinogenicity and mutagenicity: 90% of carcinogens were mutagenic and, despite the severe

limitations inherent in defining non-carcinogenicity, few non-carcinogens were shown to be mutagens. Among carcinogenic PAHs, 100% (27/27) were determined to have mutagenic activities, and 27% (3/11) of non-carcinogenic PAHs proved to be mutagens. PAH compounds which demonstrated neither carcinogenic nor mutagenic activity in this test were naphthalene, anthracene and pyrene (McCann *et al*, 1975; McCann and Ames, 1976).

Most PAH compounds have been shown to induce mutagenic activities indirectly. Phillipson and Ioannides (1989) employed the Ames test, using activation systems prepared from various animal species including mouse, rat, hamster, pig and man, to investigate the mutagenic activity of six PAH compounds. Their results showed that benzo[*a*]pyrene, dibenzo[*a,i*]pyrene and benz[*a*]anthracene gave a positive mutagenic response only in the presence of activation systems (hepatic post-mitochondrial preparation, S9) derived from the hamster. With the exception of pigs, activation systems derived from all animal species could convert 3-methylcholanthrene to mutagens, with the hamster system being the most efficient. With the exception of rats and pigs, all animal species activated 7,12-dimethylbenz[*a*]anthracene to mutagens. On the other hand, dibenz[*a,h*]anthracene was not activated by any of the hepatic preparations in this study. It was concluded that, among the animal species studied, the hamster was generally the most efficient in activating PAHs to mutagens in the Ames test.

Several techniques have been adopted to evaluate the genotoxic activities of environmental pollutants, including PAHs. A useful approach was described by Nylund and co-workers (1992) who studied the composition of four samples of creosote, which was used broadly as a wood preservative in Finland, and the genotoxicities of these substances. The authors used three testing systems including the Ames mutagenicity test, the SOS chromotest, and the sister chromatid exchange (SCE) tests for the detection of genotoxins (see Section 2.8 for descriptions of these tests). The Ames test showed that mutagenic activities of the creosote samples were detected only in the presence of metabolic activation: all creosote samples were positive with *Salmonella typhimurium* strains TA98, YG1021 and YG1024 in the presence of S9 mix. Comparisons of the different

types of genotoxicity tests used in this study showed that the results of testing creosote with *S. typhimurium* strains TA98 and YG1024 were very similar to their SCE inducing effects, in terms of the relative strength of genotoxic potency. The results obtained from tests with strain TA100, however, seemed to correlate better with the SOS chromotest results. The mutagenic activities of the distillation fractions using strains TA98 and TA100 in the presence of the S9 preparation indicated that the mutagenic residues were in fractions with the highest boiling point ranges (>290°C). Results from the fractionation studies showed that, for all creosote samples, the PAHs found to be mutagenic were present in higher amounts in the fractions with the highest boiling point ranges compared to the remaining creosote fractions.

2.2.3 Sources and Distribution of PAHs

PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel production, during burning of vegetation in forest and bush fires and also by some plant and bacterial reactions (McElroy *et al.*, 1989). However, anthropogenic activities are regarded as the major sources of PAH contamination in the environment.

The first documented source of PAH contamination occurred in Germany during the early 1800s. At that time, increased production of dyes and organic chemicals developed from the coal tar industry led to 'PAH poisoning'. Approximately 50 years later, the primary source of PAH contamination stemmed from manufactured gas plant (MGP) operations (Cutright and Lee, 1994a). At that time, heating and lighting of both the industrial and residential communities came from coal gasification. To increase the heating value, light hydrocarbons were cracked with coal, producing a liquid byproduct rich in PAHs. As time and technology progressed, electricity replaced coal gasification and the MGPs were largely abandoned. Although MGPs are no longer in use, the operating sites are still heavily contaminated and many await remediation.

Table 2.2 Some sources of PAH input to natural ecosystems (Cerniglia, 1992; Wilson and Jones, 1993).

Natural constituents of unaltered fossil fuels
Direct biosynthesis
Incomplete combustion of fossil fuels
Heating and power production
Creosote and other wood preservative wastes
Coal gasification and liquification processes
Vehicular transport
Incineration and open burning
Forest and grass fires
Smoked, charcoal broiled, or pan fried food
Oil tanker operation: routine and accidents
Offshore oil production : routine and accidents
Natural oil seeps
Coke, carbon black, and other petroleum-derived products

PAHs mostly occur as a result of combustion of fossil fuels, other organic materials, and as by-products of industrial processing. Moreover, PAH contamination is also commonly associated with creosote oil, which has been used as a wood preservative. Creosotes are produced through the destructive distillation of coal-tar, derived from bituminous coal, and PAHs are a major component of creosote (approximately 85% by weight) (Wilson and Jones, 1993; Carriere and Mesania, 1995). PAHs enter the environment from a multiplicity of sources (Table 2.2) which include direct aerial fallout, chronic leakage of industrial or sewage effluents, and accidental discharges during the transport, use and disposal of petroleum products (Cerniglia, 1992; Wilson and Jones, 1993). The increasing occurrence of leaking underground storage tanks and accidental spills, such as that which occurred with the *Exxon Valdes* spill in Alaska (Mihelcec and Luthy, 1988; Atlas, 1991), also contribute to the amount of PAHs being released into the environment. To date, there are approximately 3,000-4,000 sites contaminated with PAHs from MGP sites and accidental spills in North America alone. In addition, it is estimated that over 30% of the 7 million underground storage tanks in the United States of America are leaking PAH-containing materials (Cutright and Lee, 1994a).

2.2.4 Fates of PAHs in the Environment

After entering the environment, PAHs can be transformed as a consequence of the following reactions which include physical, chemical and biological processes (Figure 2.2).

2.2.4.1 Sorption

Owing to their hydrophobic nature, most PAHs in aquatic and terrestrial ecosystems become associated with particulates and are adsorbed onto soils and sediments (Cerniglia and Heitkamp, 1989). This occurrence limits the availability of PAHs for other reactions such as volatilization, solubilization and biodegradation, and subsequently limits the mobilization of PAHs from the environment (Pignatello and Xing, 1996). Soil is a mixture of mineral and organic

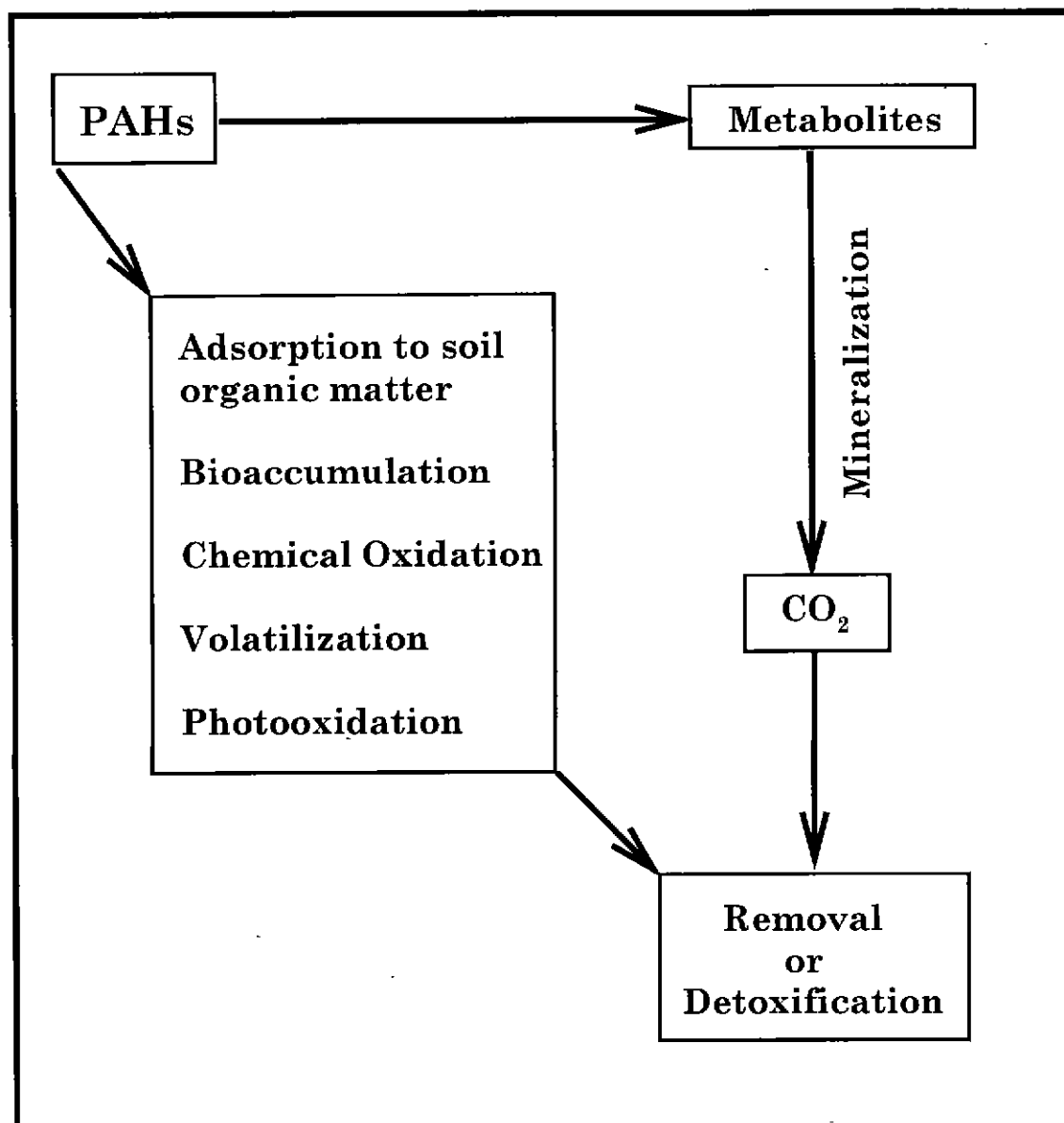


Figure 2.2 Fate of PAHs in the environment (from Cernilia, 1993).

materials and is an effective adsorbent for PAHs. The adsorption capacity is influenced by the nature of the soil, the soil moisture and other factors. Conceptually, the PAH-binding sites in soil could be classified into two groups: those on the mineral surface and those on organic matter. If the soil is wet, most of the mineral surface is occupied by water and PAHs would be mainly adsorbed to the organic matter. In dry soil, however, adsorption of PAHs to both mineral surfaces and organic matter can occur (Harayama, 1997). It has been demonstrated that a significant portion of adsorbed anthracene and other PAHs become non-extractable, especially when the water content of the soil is low. When the soil is dry, adsorbed PAHs are oligomerized and become non-available for biodegradation (Karimi-Lofabad *et al.*, 1996).

2.2.4.2 Volatilization

For most PAHs, volatilization occurs from the fraction that is in the water phase. However, in the case of low molecular weight PAHs, such as naphthalene, volatilization may also occur from a solid state (sublimation) (Ashok and Saxena, 1995). The rate of volatilization from aqueous environments is influenced by properties of the compound and those of the environment such as wind, water, turbulence and temperature (Sims and Overcash, 1983). In soil, this process is complicated due to the sorptive and diffusive capacity of the compounds. Owing to high sorption, very little PAH is available in the water-phase of contaminated soil for volatilization. Park *et al.* (1990) evaluated the interphase transfer potential of 14 PAHs, varying from two- to six-benzene ring compounds in artificially-contaminated soils, from the soil phase to the air phase. They reported that volatilization accounted for approximately 30% loss of naphthalene and 20% loss of 1-methylnaphthalene after 48 hours of incubation. For the remaining compounds, volatilization was negligible. The authors suggested that the interphase transfer potential appeared to be highly significant for low molecular weight PAHs, but not for high molecular weight PAH compounds.

2.2.4.3 Photochemical Oxidation

PAHs, in general, absorb solar radiation strongly and therefore undergo direct photolysis (Sims and Overcash, 1983). Photochemical degradation involving singlet oxygen is the most important form of chemical PAH oxidation in the environment (Ashok and Saxena, 1995). In water, the process is a linear function of light intensity as well as being oxygen and temperature dependent (Sims and Overcash, 1983). In contrast, little is known about the photosensitivity of sorbed hydrophobic compounds in soils and sediments. This process was reported to be potentially important for PAHs containing two- or three-benzene rings while for PAHs with greater than three-benzene rings this mechanism seemed to be invalid (Park *et al.*, 1990).

2.2.4.4 Microbial Transformation

Microorganisms possess a metabolic machinery of immense versatility, aiding the breakdown of various natural products as well as xenobiotics. In the course of evolution and with a rapid industrialization, microorganisms have been exposed to numerous chemicals, thereby causing adaptation and selection of the normal flora so that the necessary metabolic capacity has developed for degrading these compounds. However, biodegradation is not feasible if the compounds have structural features not found in natural compounds or if microorganisms have never encountered them during evolution. Microorganisms capable of degrading PAH compounds are common in soil and sediments previously contaminated with PAHs. Enrichment or selective procedures have been successfully used to isolate PAH-degrading microorganisms from contaminated soils (Heitkamp and Cerniglia, 1988; Cullen *et al.*, 1994; Kästner *et al.*, 1994; Grosser *et al.*, 1991; Trzesicka-Mlynarz and Ward., 1995; Juhasz *et al.*, 1997). A great deal of effort has been directed to the use of indigenous microorganisms to accomplish bioremediation of sites contaminated by such compounds. The use of specially selected microorganisms to enhance bioremediation efforts has proven effective in a number of applications (Dott *et al.*, 1989; Mueller *et al.*, 1989 and 1990; Møller and Ingvorsen., 1993; Otte *et al.*, 1994; Trzesicka-Mlynarz and Ward., 1996).

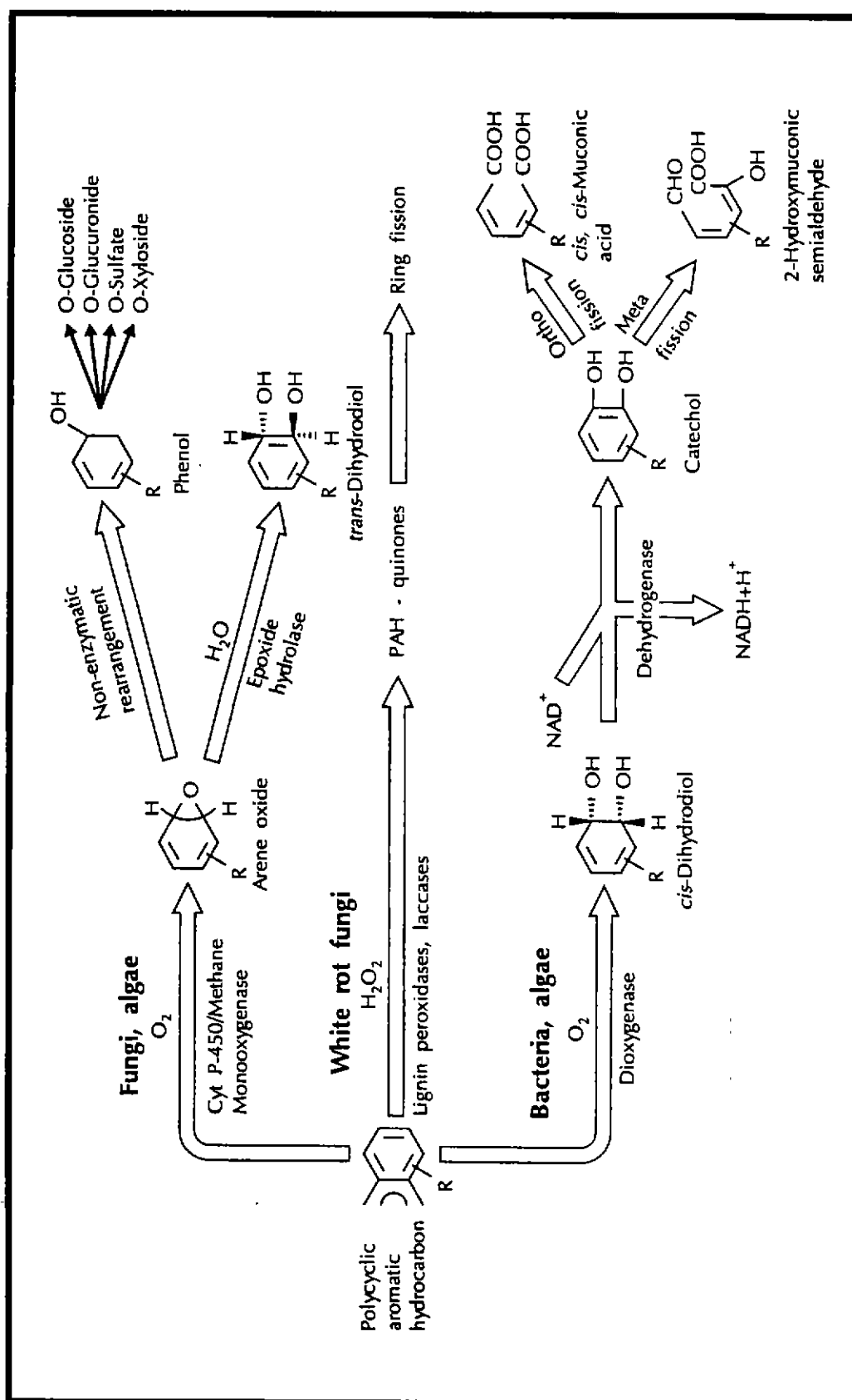


Figure 2.3 Pathways for the microbial catabolism of PAHs (Cerniglia, 1993; Pothuluri *et al.*, 1994).

Mechanisms involving the microbial metabolism of PAHs are reviewed in Section 2.3.

2.3 MICROBIAL METABOLISM OF PAHs

Bacteria, fungi, cyanobacteria and algae have the enzymatic systems to degrade a wide variety of structurally diverse PAHs (Cerniglia, 1993). However, there are significant differences in the mechanisms of PAH metabolism amongst prokaryotic and eukaryotic microorganisms (Figure 2.3).

2.3.1 Bacterial Metabolism of PAHs

The initial activation and oxidation of PAHs involve enzymes that catalyze oxygen-fixing reactions. The two groups of oxygenases are monooxygenases and dioxygenases. Bacteria characteristically produce dioxygenases, which incorporate two oxygen atoms into the aromatic ring to form *cis*-dihydrodiols (Figure 2.3). *Cis*-dihydrodiols are stereoselectively dehydrogenated by *cis*-dihydrodiols dehydrogenases, which rearomatize the benzene nucleus to form dihydroxylated intermediates (*eg.* catechol). Subsequently, aromatic ring fission of dihydroxyl products occurs by the catalytic activity of highly regio- and stereoselective dioxygenases (Cerniglia, 1984; Cutright and Lee, 1994b). As a prerequisite for this reaction, the aromatic ring must contain two hydroxyl groups placed either *ortho* or *para* to each other. If the hydroxyl moieties are *ortho* to each other, oxygenolytic ring cleavage can occur either between the two hydroxyl groups by *ortho* or intradiol cleaving dioxygenases, or adjacent to the two hydroxyl groups by *meta* or extradiol cleaving dioxygenases (Cerniglia, 1992). The initial ring oxidation is the rate-limiting step in the biodegradation of PAHs, after which the degradation proceeds relatively quickly with little or no accumulation of intermediates (Cerniglia, 1984). Although initial degradation reactions of specific PAHs differ to yield different initial oxidation products, only a few intermediate metabolites, namely catechol, protocatechuic acid and gentisic acids, are commonly produced (Ghosh and Mishra, 1983; Cerniglia, 1984; Yen and Serdar, 1988; Wilson and Jones, 1993; Williams and Sayers, 1994; Casellas *et al.*, 1997).

Table 2.3 High molecular weight PAHs oxidized by different species of bacteria.

Compound	Organisms	References
Fluoranthene	<i>Acinetobacter odorans</i> , <i>Alcaligenes denitrificans</i> , <i>Mycobacterium</i> sp., <i>Pseudomonas putida</i> , <i>Pseudomonas fluorescences</i> , <i>Pseudomonas aeruginosa</i> , <i>Rhodococcus</i> sp., <i>Sphingomonas</i> sp., <i>Sphingomonas paucimobilis</i>	Boldrin <i>et al.</i> , 1993; Dagher <i>et al.</i> , 1996; Kelly and Cerniglia, 1991; Kelly <i>et al.</i> , 1993; Lal & Khanna, 1996; Mueller <i>et al.</i> , 1990; Weissenfels <i>et al.</i> , 1990.
Pyrene	<i>Acinetobacter odorans</i> , <i>Mycobacterium</i> sp., <i>Mycobacterium flavescens</i> , <i>Rhodococcus</i> sp., <i>Sphingomonas paucimobilis</i>	Bouchez <i>et al.</i> , 1995; Bouchez <i>et al.</i> , 1997; Lal & Khanna, 1996; Schneider <i>et al.</i> , 1996; Ye <i>et al.</i> , 1996; Dean-Ross & Cerniglia, 1996
Chrysene	<i>Acinetobacter odorans</i> , <i>Rhodococcus</i> sp., <i>Sphingomonas paucimobilis</i>	Lal & Khanna, 1996; Ye <i>et al.</i> , 1996.
Benz[a]anthracene	<i>Mycobacterium</i> sp., <i>Pseudomonas putida</i> , <i>Pseudomonas fluorescences</i> , <i>Pseudomonas aeruginosa</i> , <i>Sphingomonas</i> sp., <i>Sphingomonas paucimobilis</i>	Dagher <i>et al.</i> , 1996; Schneider <i>et al.</i> , 1996; Ye <i>et al.</i> , 1996
Benzo[a]pyrene	<i>Mycobacterium</i> sp., <i>Sphingomonas paucimobilis</i>	Schneider <i>et al.</i> , 1996; Ye <i>et al.</i> , 1996.
Benzo[b]fluoranthene	<i>Sphingomonas paucimobilis</i>	Ye <i>et al.</i> , 1996.
Dibenz[a,h]anthracene	<i>Sphingomonas paucimobilis</i> , <i>Burkholderia cepacia</i> ^a	Ye <i>et al.</i> , 1996; Juhasz <i>et al.</i> , 1997

^a Recently identified by 16S rRNA sequencing as *Stenotrophomonas maltophilia* (Juhasz *et al.*, personal communication).

After aromatic ring fission, these metabolites are transformed to organic acids and aldehydes, all of which are utilized for cell-protein synthesis and energy production by microorganisms, alongside the production of carbon dioxide and water.

The bacterial catabolism of PAHs possessing three or fewer fused aromatic rings, such as naphthalene and phenanthrene, has been extensively studied. However, more interest has recently been focused on the isolation and characterization of bacteria which can degrade higher molecular weight compounds containing four or more aromatic rings. As listed in Table 2.3, many bacteria are capable of degrading high molecular weight PAHs (defined here as PAHs which contain more than three-aromatic rings). Weissenfels and co-workers (1991) isolated *Alcaligenes denitrificans* WW1, which has the ability to utilize fluoranthene as sole carbon source, from contaminated soil. This bacterium was also able to utilize naphthalene, 1- and 2-methyl-naphthalene, phenanthrene and anthracene as sole carbon sources and to co-metabolize fluorene, pyrene and benz[a]-anthracene. Two metabolites were observed during the growth of cells on fluoranthene, which were subsequently identified as acenaphthenone and 3-hydroxymethyl-4,5-benzocoumarine. The identification of these metabolites showed that the known dioxygenation mechanisms were initiating the degradation of fluoranthene. Fluoranthene-grown resting cells of strain WW 1 showed degradative activity towards 2,3-dihydroxybenzoic acid, 3-hydroxycatechol (pyrogallolivit), salicylic acid, and catechol. The enzymatic activities in extracts of fluoranthene-induced cells indicated that *meta* ring fission was involved in the degradation of fluoranthene. Ye *et al.* (1996) demonstrated the mineralization of chrysene and benzo[a]pyrene by *Sphingomonas paucimobilis* EPA 505, which indicated that this bacterium is able to metabolize these PAHs *via* ring cleavage. However this was found to only occur when a high density of EPA 505 resting cells was used; the resting cellsuspension being prepared from cells grown on nutrient agar. Strain EPA 505 could not, however, grow on benzo[a]pyrene as a sole carbon and energy source.

Interestingly, mycobacteria have been repeatedly isolated as PAH-degraders of degrading PAHs possessing four or more fused benzene rings. The hydrophobic cell surface may be advantageous for their adhesion to insoluble PAHs, thus facilitating mass transfer of the substrates into the cells (Harayama, 1997). A *Mycobacterium* strain PYR-I has been found to mineralize pyrene and fluoranthene more rapidly than naphthalene and phenanthrene (Heitkamp and Cerniglia, 1988). This strain can transform, but not mineralize, benzo[a]pyrene. Another *Mycobacterium* strain RJGII-135, which was isolated from an abandoned coal gasification site soil, has been found to co-metabolically oxidize pyrene and benzo[a]pyrene. A phylogenetic analysis based upon 16S rRNA gene sequence classified these two strains within the fast-growing group of mycobacteria (Govindaswami *et al.*, 1995). An analysis of the degradation products of pyrene, benz[a]anthracene and benzo[a]pyrene produced by these mycobacteria enabled the PAH degradation pathways to be proposed. Schneider and co-workers (1996) demonstrated that *Mycobacterium* sp. strain RJGII-135 degraded pyrene to form three metabolites, 4,5-phenanthrene-dicarboxylic acid, 4-phenanthrene-carboxylic acid, and 4,5-pyrene-dihydrodiol. At least two dihydrodiols (5,6-benz[a]-anthracene dihydrodiol and 10,11-benz[a]anthracene dihydrodiol) were also detected in spent cultures. The 7,8-benzo[a]pyrene dihydrodiol and three cleavage products of benzo[a]pyrene, including 4,5-chrysene-dicarboxylic acid and dihydro-pyrene-carboxylic acid, were isolated and identified as degradation products formed by *Mycobacterium* sp. strain RJGII-135. These latter results represent the first example of the isolation of benzo[a]pyrene ring fission products formed by bacteria. The proposed pathways for biodegradation of these compounds by this bacterium suggest that the dioxygenase enzymatic processes reported previously for other bacteria operate here. Another *Mycobacterium* sp. strain BB1, was reported to grow on phenanthrene, pyrene and fluoranthene (Boldrin *et al.*, 1993). Although mycobacteria are known for their slow growth, their growth on PAHs is faster than that of other microorganisms. For example, the growth rate of *Mycobacterium* sp. BB1 on pyrene was 0.056 h^{-1} , whereas that of *Rhodococcus* sp. strain UW1 was 0.023 h^{-1} (Heitkamp *et al.*, 1988).

Table 2.4 High molecular weight PAHs oxidized by co-metabolism by different species of fungi.

Compound	Organisms	References
Fluoranthene	<i>Cunninghamella elegans</i>	Pothuluri <i>et al.</i> , 1992
Pyrene	<i>Cunninghamella elegans</i> , <i>Phanerochaete chrysosporium</i> , <i>Penicillium janthinellum</i> , <i>Penicillium glabrum</i> , <i>Syncephalastrum</i> spp., <i>Aspergillus niger</i>	Launen <i>et al.</i> , 1995; Wunder <i>et al.</i> , 1994; Wunder <i>et al.</i> , 1997; Bezalel <i>et al.</i> , 1996
Chrysene	<i>Cunninghamella elegans</i> , <i>Penicillium janthinellum</i> , <i>Syncephalastrum racemosum</i>	Pothuluri <i>et al.</i> , 1994; Kiehlmann <i>et al.</i> , 1996
Benz[a]anthracene	<i>Cunninghamella elegans</i> , <i>Phanerochaete laevis</i>	Bogan and Lamar, 1996
Benzo[a]pyrene	<i>Phanerochaete chrysosporium</i> , <i>Bjerkandera adusta</i> , <i>Cunninghamella elegans</i> , <i>Pleurotus ostreatus</i> , <i>Pleurotus sapidus</i> , <i>Marasmiellus troyanus</i> , <i>Penicillium janthinellum</i> , <i>Trametes versicolor</i>	Wunch <i>et al.</i> , 1997; Bezalel <i>et al.</i> , 1996; Launen <i>et al.</i> , 1995 Collins <i>et al.</i> , 1996; Field <i>et al.</i> , 1992

2.3.2 Fungal Metabolism of PAHs

Whereas the pathway of PAH degradation by bacteria has been well studied, knowledge of similar activities in fungi remains limited. However, fungi may be as important as bacteria in the bioremediation of PAH-contaminated soil. Although there have not been any reports on the ability of fungi to utilize PAHs as their sole source of carbon and energy, several studies have shown that a wide taxonomic and phylogenetic spectrum of fungi (Table 2.4) have the enzymatic capacity to transform PAHs co-metabolically to detoxified chemical products when grown on an alternative carbon source.

A diverse group of lignolytic and non-lignolytic fungi are able to oxidize PAHs. There are two main groups of enzymes involved in the initial attack on PAHs by fungi which include the cytochrome P-450 monooxygenase and the lignin-degrading enzymatic systems. Both enzyme systems are relatively non-specific for the PAHs that they metabolize (Cerniglia, 1993). Fungi generally produce monooxygenases, which are utilized to incorporate one atom of oxygen into the aromatic nucleus and reduce the remaining atom to water. This is followed by the enzymatic addition of water to yield *trans*-dihydrodiols (Figure 2.3). The site of enzymatic attack, mechanisms, chemical intermediates and pathways for the metabolism of several PAHs by eukaryotic microorganisms have been extensively reviewed (Cerniglia, 1984; Pothuluri *et al.*, 1994).

The best studied non-lignolytic fungus is the Zygomycetous *Cunninghamella elegans*, which has been shown to metabolize PAHs in the range from naphthalene to benzo[a]pyrene (Cerniglia, 1984; Pothuluri *et al.*, 1992; Sutherland, 1992). The first step in fungal metabolism for an unsubstituted PAH is ring epoxidation by a monooxygenase enzyme complex. The product of epoxidation is an unstable arene oxide, such as naphthalene 1,2-oxide (Figure 2.3). The monooxygenase enzyme complex that catalyzes the formation of arene oxides generally contains an inducible, membrane-bound enzyme, cytochrome P-450 (Cerniglia, 1984), which probably occurs in most fungi. One fate of arene oxides is immediate hydration by epoxide hydrolase, which catalyzes the addition of a water molecule to an arene

oxide to form a *trans*-dihydrodiol. These reactions appear to be similar to those reported for mammalian enzyme systems (Davis, 1988). Alternatively, arene oxide can undergo non-enzymatic rearrangement to form a phenol, which can be conjugated with sulfate, glucuronic acid, xylose, or glucose (Figure 2.3). The arene oxides have not been isolated directly from fungal culture media but naphthalene 1,2-oxide has been detected indirectly in *C. elegans* cultures by isotopic labeling experiments. The toxicity of some arene oxides is less than that of the original PAHs; for example, synthetic benzo[*a*]pyrene 7,8-oxide is only about 20% as carcinogenic for mice as benzo[*a*]pyrene (Cerniglia, 1984).

The formation of sulfate conjugate metabolites from the metabolism of chrysene by *C. elegans* was elucidated by Pothuluri *et al.* (1994). This was the first report regarding chrysene degradation by fungi. In addition to chrysene, the transformation of defined PAH mixtures, which included acenaphthalene, phenanthrene, pyrene, fluoranthene, and benzo[*a*]pyrene, by this fungus was demonstrated. *C. elegans* grown in Sabouraud dextrose broth metabolized a mixture of PAHs completely to hydroxylated intermediates within 24 hours. Approximately 45% of the [5,6,11,12-¹⁴C]chrysene added to the cultures was metabolized during 144 hours of incubation. The two major metabolites of chrysene were separated and identified as sulfate conjugates of 2,8-dihydroxychrysene and 2-hydroxychrysene. The formation of sulfate conjugates of phenolic chrysene metabolites and hydroxylated products of PAH mixtures by this fungus were proposed as detoxification processes because these types of products are generally less toxic than the parent compounds (Müncnerová and Augustin, 1994).

Launen *et al.* (1995) isolated fungi from five different soils collected from a petroleum disposal site. PAH contents in these soil samples ranged from 0.8-80 µg g⁻¹ dry soil. A wide variety of soil fungi were obtained which were capable of oxidizing pyrene and benzo[*a*]pyrene when grown in malt-yeast extract-peptone-dextrose (MYPD) medium. Approximately 50% of the isolates in all samples were able to oxidize pyrene. The pyrene-oxidizing strains belonged to all fungal divisions except the basidiomycetes. The most common were *Penicillium* and

Syncephalastrum species which dominated in the more contaminated soils. *P. janthinellum* and *S. racemosum* exhibited the most rapid rates of pyrene oxidation. A high correlation was found between the ability to oxidize pyrene and benzo[a]pyrene, as 80% of pyrene-oxidizing strains were also able to metabolize benzo[a]pyrene. Further evaluation of the PAH-degrading ability of fungal isolates from the same site was undertaken by Kiehlmann and co-workers (1996). Among the fungal isolates screened, only four (*P. janthinellum*, *S. racemosum* and other two isolates of *Penicillium* spp.) showed an ability to oxidize chrysene to polar metabolites when grown in MYPD medium, however, the extent of chrysene degradation was low.

Recently, there has been much interest in the ability of the white-rot fungi to degrade a number of xenobiotic compounds (Paszczyński and Crawford, 1995). Therefore, there has been an intense effort to determine the capacity of white-rot fungi such as *Phanerochaete chrysosporium*, to metabolize PAHs, because lignolytic species have the potential to degrade PAHs completely to CO₂ (Bumpus, 1989). Bumpus (1989) demonstrated that the white rot fungus *P. chrysosporium* was able to degrade PAHs that were present in anthracene oil (a distillation product obtained from coal tar). After incubation in nitrogen-limited cultures of this fungus for 27 days, 70-100% of at least 22 PAHs, including all of the most abundant PAH components present in anthracene oil, disappeared. Mineralization of [¹⁴C]phenanthrene, which was the predominant component of this mixture, was also demonstrated. Furthermore, mass balance analysis and HPLC experiments showed that [¹⁴C]phenanthrene was also converted to more polar and water-soluble metabolites.

White-rot fungi produce extracellular lignin-degrading enzymes which have the potential to degrade high molecular weight and/or insoluble compounds that will not pass through bacterial cell walls (Hammel *et al.*, 1986). Fungal extracellular enzymes responsible for the degradation of lignin, lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases, are believed to be involved in the degradation of PAHs. Purified forms of two major ligninolytic enzymes, LiPs and MnPs, have been shown to be capable of oxidizing PAH compounds that have an

ionization potential of less than 7.55 eV (Hammel *et al.*, 1986). The lignin peroxidases rely on a supplementary enzyme system to supply the necessary hydrogen peroxide to start the oxidation of lignin. The extracellular lignin peroxidases initiate a free radical attack on PAHs, by a single electron transfer, to form quinones (Figure 2.3). Hammel *et al.* (1992) showed that in whole cultures of *P. chrysosporium*, LiPs catalyze the production of 9,10-anthraquinone from anthracene. The major product of the *in vitro* oxidation of anthracene by both LiPs and MnPs from *P. chrysosporium* was also anthraquinone (Field *et al.*, 1995). Hammel and co-workers (1992) reported that *P. chrysosporium* oxidized phenanthrene at its C₉ and C₁₀ positions to give 2,2'-diphenic acid as a ring cleavage product. Their results indicated that *P. chrysosporium* utilizes a different metabolic pathway from *C. elegans* and bacteria, since the major site of enzymatic attack on the phenanthrene molecule by most bacteria is at the its C₃ and C₄ positions. In addition, Sutherland *et al.* (1991) reported that under non-lignolytic conditions, *P. chrysosporium* metabolized phenanthrene to phenol and trans-dihydrodiol, which suggested a cytochrome P-450-mediated reaction. These observations indicated that several enzymatic mechanisms may occur in *P. chrysosporium* for the initial oxidation of PAHs.

The third major group of extracellular oxidative enzymes involved in the white-rot fungal lignin degradative process are laccases. These are extracellular copper-containing polyphenol oxidases which catalyze the reduction of one oxygen molecule to two molecules of water, simultaneously oxidizing aromatic substrates (Thurston, 1994). The *in vitro* oxidation of two PAHs, anthracene and benzo[*a*]pyrene, catalyzed by laccases from *Trametes versicolor* was demonstrated by Collins *et al.* (1996). Crude laccase preparations were able to oxidize both anthracene and the potent carcinogen benzo[*a*]pyrene. Oxidation of benzo[*a*]pyrene was enhanced by the addition of a co-oxidant, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), while an increased anthracene oxidizing ability was observed in the presence of the low molecular weight culture fluid ultrafiltrate. Two purified laccase isozymes from *T. versicolor* were found to have similar oxidative activities towards anthracene and benzo[*a*]pyrene. In all cases, anthraquinone was identified as the major end product of anthracene oxidation.

Other white rot fungi, such as *Bjerkandera* sp. (Field *et al.*, 1995), and *Pleurotus ostreatus* (Bezalel *et al.*, 1996), have also been shown to metabolize PAHs. Bezalel *et al.* (1996) reported that in the basidiomycetes rich medium (basal salts medium supplemented with glucose and peptone), *P. ostreatus* degrades phenanthrene, anthracene, pyrene, fluorene, and benzo[a]pyrene to CO₂ and water-soluble products. The activities of the enzymes considered to be part of the lignolytic systems, laccases and manganese peroxidase, were observed during fungal growth in the presence of various PAHs. Although laccases exhibit relaxed substrate specificity, they may be less important than LiPs and MnPs in the oxidation of PAHs (Thurston, 1994).

Current knowledge on the fungal degradation of PAHs has been reviewed and is summarized as follows (Cerniglia, 1993; Sutherland, 1992).

- (i) A wide variety of fungi have the ability to metabolize PAHs.
- (ii) Hydroxylation of unsubstituted PAHs always involves the incorporation of molecular oxygen.
- (iii) Fungal metabolism of PAHs is highly regio- and stereo-selective and in many cases similar to mammalian enzyme systems.
- (iv) White-rot fungi have the ability to cleave the aromatic ring and mineralize PAHs.
- (v) Although small amounts of mutagenic and carcinogenic metabolites are found during the metabolism of PAHs, most fungi hydroxylate PAHs as a prelude to detoxification, where as bacteria oxidize PAHs to dihydroxylated compounds as a prelude to ring fission and assimilation.

Table 2.5 PAHs oxidized by different species of cyanobacteria and algae.

Compound	Organisms	References
Naphthalene	<i>Oscillatoria</i> spp., <i>Dunaliella tertiolecta</i> , <i>Chlamydomonas angulosa</i> , <i>Chlorella</i> spp.	Narro <i>et al.</i> , 1992a
Phenanthrene	<i>Oscillatoria</i> sp. strain JCM, <i>Agmenellum quadruplicatum</i>	Narro <i>et al.</i> , 1992b
Benzo[a]pyrene	<i>Selenastrum capricornutum</i>	Warshawsky <i>et al.</i> , 1990

2.3.3 Cyanobacterial and Algal Metabolism of PAHs

It has been reported that cyanobacteria and eukaryotic algae have the capacity to metabolize PAHs (Table 2.5). As algae are widely distributed in aquatic environments and the photic zone, they may be a major source for transformation of PAHs and the ability of algae to oxidize these compounds could be important in their degradation by indigenous microflora. It has been shown that cyanobacteria as well as green, red, and brown algae oxidize naphthalene under photoautotrophic conditions, predominantly to 1-naphthol with a minor amount of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and 4-hydroxy-1-tetralone (Cerniglia *et al.*, 1980). A study by Narro *et al.* (1992a) indicates that the mechanism for 1-naphthol formation in the marine cyanobacterium, *Oscillatoria* sp. strain JCM, is *via* an arene oxide intermediate that isomerizes with a concomitant non-enzymatic rearrangement. These data suggest a monooxygenase-catalyzed reaction similar to those reported for fungal and mammalian enzymes is involved (Figure 2.3). Another marine cyanobacterium, *Agmenellum quadruplicatum* PR-6, metabolizes phenanthrene to form *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxyphenanthrene as major metabolites (Narro *et al.*, 1992b). These results indicate that marine cyanobacteria oxidize PAHs in a manner that is more similar to the reactions catalyzed by enzymes of mammals and fungi than to the reactions catalyzed by true bacteria.

In contrast, Warshawsky and co-workers (1990) have shown that the green alga, *Selenastrum capricornutum*, under photoautotrophic growth conditions, metabolizes benzo[a]pyrene predominantly to *cis*-11,12-dihydroxy-11,12-dihydrobenzo[a]pyrene. Other minor *cis*-dihydrodiols were also detected. The formation of *cis*-dihydrodiols suggests involvement of a dioxygenase-mediated reaction, similar to that found in heterotrophic prokaryotes, rather than a monooxygenase-catalyzed reaction as found in the cyanobacteria, fungi and mammals.

2.4 GENETICS OF PAH BIODEGRADATION

2.4.1 Genetics of PAH Degradation in Bacteria

The degradation of aromatic compounds by bacteria is generally plasmid encoded and is usually therefore transmissible (Cane and Williams, 1982; Schell, 1985; Bestetti and Galli, 1987; Yen and Serdar, 1988; Denome *et al.*, 1993; Menn *et al.*, 1993; Sanseverino *et al.*, 1993; Shields *et al.*, 1995). However, a few publications report that hydrocarbon degradative genes may also be chromosomally encoded (Sinclair *et al.*, 1986; Kiyohara *et al.*, 1994). The characterization of degradative plasmids has contributed knowledge to the study of genetics of PAH catabolism.

The NAH plasmid, which is involved in the degradation of naphthalene, has been isolated and extensively characterized. The catabolic pathway of naphthalene has been elucidated for *Ps. putida* G7, while for other PAHs this has been only partly done (Yen and Serdar, 1988; Cerniglia, 1992). Naphthalene catabolism in *Ps. putida* G7 involves hydroxylation of one aromatic ring to produce 1,2-dihydroxy-1,2 dihydronaphthlene by naphthalene dioxygenases. These multimeric enzymes are composed of a reductase (encoded by *nahAa*), a ferredoxin (encoded by *nahAb*), and two subunits of iron-sulfur protein (encoded by *nahAc* and *nahAd*). After a dehydrogenation of 1,2-dihydroxy-1,2 dihydronaphthlene to 1,2-dihydroxynaphthalene by a dehydrogenase (encoded by *nahB*), the hydroxylated ring is cleaved by the 1,2-dihydroxynaphthalene dioxygenase (encoded by *nahC*). Further reactions involving an isomerase (*nahD*), a hydratase-aldolase (*nahE*), and a dehydrogenase (*nahF*) leading to the formation of salicylate (Eaton and Chapman, 1995; Eaton, 1994). Salicylate is further transformed to catechol *via* salicylate hydroxylase. Catechol is further degraded by *ortho* cleavage to yield succinyl CoA and acetyl CoA, or by *meta* cleavage to yield pyruvate and acetaldehyde, depending on the bacterial strains; this is referred to as the lower pathway (Williams and Sayers, 1994). The catabolic pathway of naphthalene to salicylate, referred to as the upper pathway, also occurs with other PAHs. For example, it is involved in the transformation of phenanthrene and anthracene to 1-hydroxy-2-

naphthoic acid and 2-hydroxy-3-naphthoic acid, respectively, in *Ps. fluorescences* 5R (Menn *et al.*, 1993).

Genes encoding the lower and upper pathways in *Ps. putida* G7 are arranged in two respective polycistronic operons, *sal* and *nah*, located on the plasmid NAH7. Control of expression of the two operons is regulated by the *nahR* gene product. The activity of *nahR* depends on the intracellular concentration of salicylate (Schell, 1985). PAH-degradation genes have also been isolated from other pseudomonads. These genes are also arranged in a similar manner as a polycistronic operon, for example (i) the *nah* operon on the pDTG1 plasmid in *Ps. putida* NCIB 9816-4 (Harayama and Rekik, 1989; Simon *et al.*, 1993; Eaton, 1994); (ii) the *pah* operon located on the chromosome in *P. putida* OUS82 (Kiyohara *et al.*, 1994; Takizawa *et al.*, 1994); (iii) the *ndo* operon located on a plasmid in *Ps. putida* NCIB 9816 (Kurkela *et al.*, 1988); and (iv) the *dox* operon located on a plasmid in *Pseudomonas* strain C18 (Denome *et al.*, 1993). Nucleotide and the deduced amino acid sequences are highly homologous between the respective genes of the *nah*, *pah*, *ndo*, and *dox* operons. The gene order of the *nah* operon is *nahAa-nahAb-nahAc-nahAd-nahB-nahF-nahC-nahQ-nahE-nahD* (Eaton, 1994). This arrangement also occurs in the *pah*, *dox*, and *ndo* operons. These PAH-degrading genes are encountered in other *Pseudomonas* species. For example, strong hybridization signals were found between a *nah* probe and plasmids isolated from three *Pseudomonas* strains involved in the mineralization of naphthalene, phenanthrene, and anthracene (Sanseverino *et al.*, 1993). Similar observations were also made with strains of *Ps. stutzeri* involved in the degradation of naphthalene (Rossello-Mora *et al.*, 1994). The only published study of PAH catabolic genes from non-pseudomonads is for a strain of *Comamonas testosteroni*, which had the ability to degrade naphthalene, phenanthrene, and anthracene (Goyal and Zylstra, 1996). Although the PAH catabolic pathway is similar to the pseudomonads, Goyal and Zylstra (1996) showed that no hybridization signal was detected with the *nah* probe in these bacterial strains, and that the gene arrangement was different. These observations suggested that substantial differences exist at the gene level in *C. testosteroni* which may reflect a

different approach to PAH degradation at the level of gene regulation and enzymatic specificity.

2.4.2 Genetics of PAH degradation in Fungi

Regulation and expression of lignin-degrading enzyme systems have been extensively studied at the genetic level in white-rot fungi. The biochemistry and molecular genetics of *P. chrysosporium* lignolytic system are quite complex. Multiple LiP isozymes are produced in submerged culture, and the genome of *P. chrysosporium* contains at least ten structurally related *lip* genes, which are designated as *lipA* through *lipJ* (Gaskell *et al.*, 1994). The expression of ten *lip* genes in anthracene-transforming soil cultures of *P. chrysosporium* has been examined by mRNA extraction from soil and the quantification of *lip* mRNA by competitive reverse-transcriptase-PCR (RT-PCR) has been performed. The expression of ten *lip* genes was found to be regulated at different times during incubation of the fungus with a PAH. The oxidation of anthracene proceeded constantly throughout the 25-day course of the experiment, suggesting that this compound was oxidized by several LiP isozymes (Bogan *et al.*, 1996).

The structure and mechanism of MnPs, in addition to those of LiPs, have been studied extensively. MnP isozymes are encoded by several different genes in *P. chrysosporium*. Several cDNAs (MnP1, MnP2a, MnP2b and MP-1) (Pease *et al.* 1989; Pribnow *et al.*, 1989) and genomic DNAs (MnP1 and MnP2) (Godfrey *et al.*, 1990; Mayfield *et al.*, 1994) of MnP isozymes in *P. chrysosporium* have been isolated and characterized. The expression of three MnP isozyme genes in *P. chrysosporium* during a bench-scale PAH soil bioremediation experiment was investigated by Bogan and co-workers (1996) and mRNA was quantified by competitive RT-PCR with 'housekeeping' mRNAs as controls. The expression of these three MnP genes was coordinately regulated under these growth conditions. *P. chrysosporium* cultured in soil caused the transformation of fluorene and the disappearance of chrysene in the early phase of fungal colonization, these activities being correlated with the degree of expression of the MnP genes (Bogan *et al.*, 1996).

2.5 FACTORS AFFECTING PAH BIODEGRADATION

The rates and extents of PAH transformation and mineralization in soils are highly variable and depend on many extrinsic and intrinsic factors, including the physico-chemical properties of PAHs and soils, and PAH-degrading microflora. The topics reviewed in this section focus, in particular, on microorganisms responsible for PAH degradation and some phenomena which can initiate or stimulate the processes of PAH biodegradation.

2.5.1 Microbial Adaptation

Prior exposure of microbial communities to hydrocarbons is important in determining how rapidly subsequent hydrocarbon inputs can be degraded. This phenomenon is term 'adaptation'. There are basically three interrelated biological mechanisms where microbial adaptation to pollutants can take place:

- (i) Induction of necessary constitutive or adaptive enzymes.
- (ii) Alteration in the genetic coding, which results in the formation of new and/or more effective metabolic pathways.
- (iii) Selective enrichment of particular organisms capable of transforming the compound of interest.

The third type of change often follows one of the first two mechanisms (Spain *et al.*, 1980; Bandyopadhyay *et al.*, 1994).

Cross acclimation is an example of adaptation by microorganisms. This is a process wherein the microbial community is acclimatized to one compound, such as phenanthrene, and subsequently is able to degrade another compound of similar structure, such as naphthalene (Bauer and Capone, 1988). Shiaris (1989) reported an increase in the transformation rate of naphthalene, phenanthrene and benzo[a]pyrene added into sediments contaminated with high concentrations of

various PAHs. The highest PAH transformation rate was shown in a sediment which was the most heavily contaminated with PAH compounds. It has been observed that prior acclimation history significantly affects degradation patterns. Bauer and Capone (1988) observed that the degradation of specific polycyclic aromatic hydrocarbon compounds in marine sediment slurries may be stimulated by prior exposure to other polycyclic aromatic hydrocarbons and benzene. For example, anthracene, phenanthrene and benzene stimulated the mineralization of naphthalene significantly. However, naphthalene stimulated phenanthrene degradation but not anthracene degradation (Bauer and Capone, 1988). Pre-exposure to a low concentration of phenanthrene was found to increase the extent of [^{14}C]phenanthrene mineralization by soil microorganisms (Carmichael and Pfaender, 1997a).

2.5.2 Co-metabolism

It is evident that the individual constituents of hydrocarbon pollutants in soils are inherently degradable to varying degrees and will, therefore, be degraded at differing rates. The interactions between microorganisms and heterogeneous mixtures of PAHs in the natural environment are normally complicated. Basically, in the presence of readily degradable hydrocarbons, microorganisms will ignore other available molecules (Morgan and Watkinson, 1989). On the other hand, certain compounds may only be metabolized in the presence of others. These co-metabolic reactions are generally due to non-target molecules being attacked to some extent by degradative enzymes of low specificity.

'Co-metabolism' is defined as the condition where "non-growth hydrocarbons are oxidized when present as co-substrates in a medium in which one or more different hydrocarbons are furnished for growth" (Perry, 1979; Keck *et al*, 1989). This phenomenon has been particularly well characterized in the degradation of cycloalkanes, and it has been proposed that the partially-oxidized PAHs found in soil in which active biodegradation is occurring, may be products of co-metabolism (Perry, 1979). Co-metabolism is frequently cited as an important mechanism for the degradation of recalcitrant substrates in the soil environment (Sims and

Overcash, 1983; Keck *et al.*, 1989). Certain PAHs are degraded by co-metabolism, for example, recent studies of PAH mixtures have demonstrated the co-metabolic degradation of fluorene by *Mycobacterium* sp. in the presence of yeast extract and peptone or acetate as growth substrates (Tiehm and Fritzsche, 1995). Co-metabolism may be an important process in contaminated soils when larger, and often more recalcitrant, PAHs are present in mixtures of more readily degraded, smaller PAHs. This was noted by Bouchez and co-workers (1995) when a pure culture of a *Pseudomonas* sp. was unable to use fluoranthene as a sole carbon source, but was able to co-metabolize this compound in the presence of phenanthrene. Recently, Ye *et al.* (1996) investigated the ability of *Sphingomonas paucimobilis* strain EPA 505 to metabolize a variety of high molecular weight PAHs. This strain was previously isolated from a creosote-contaminated site and was capable of utilizing fluoranthene as the sole source of carbon and energy (Mueller *et al.*, 1990). Ye and co-workers (1996) found that when this bacterium was grown on fluoranthene, it was able to degrade a variety of PAHs, including pyrene, benz[a]anthracene, chrysene, benzo[a]pyrene, benzo[b]fluoranthene and dibenz[a,h]anthracene. The extent of degradation of the PAHs increased with an increase in cell density.

However, a particular PAH in a mixture of compounds may also have pronounced adverse effects on the biodegradation of other PAHs. Studies on the co-metabolism and inhibition phenomena of PAH biodegradation by Bouchez and co-workers (1995) demonstrated either stimulation or inhibition of PAH biodegradation. Six strains of bacteria were isolated from contaminated soil, with each strain being capable of using at least one of the following PAHs as a sole carbon and energy source: naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene. The interactions between these PAHs during their biodegradation were studied in experiments involving PAH pairs, with at least one PAH being used as a carbon source. All the strains were found to be capable of co-metabolic degradation of PAHs which varied among strains. Although synergistic interactions were detected, inhibition effects, which were sometimes drastic, were also seen. For example, naphthalene was toxic to all strains not isolated on this compound.

Sack and Günther (1993) demonstrated the potential of fungi of different ecological groups to metabolize PAHs, including fluorene, phenanthrene, anthracene, fluoranthene and pyrene, in the presence of glucose as a growth substrate. Among the species tested, *Laetiporus sulphureus* and *Penicillium* sp. MI showed the ability to co-metabolize PAHs with rates comparable to white-rot fungi. More recently, many fungi have been reported for their ability to transform high molecular weight PAHs when another carbon substrate which supports growth was present in the culture media (Kiehlmann *et al.*, 1996; Liao *et al.*, 1997; Sack *et al.*, 1997; Wunch *et al.*, 1997).

2.5.3 Augmentation

One interesting phenomenon is 'augmentation', which describes cases in which degradation of one compound is enhanced by the presence of another. The difference between augmentation and co-metabolism being that the degradation-enhancing compound does not have to be a growth-supporting substrate. Bouchez *et al.* (1995) found that fluorene augmented the degradation of phenanthrene in a pure culture of a *Rhodococcus* sp. Similar observations were made by Tiehm and Fritzche (1995), who found an increase in the degradation rate of pyrene in the presence of phenanthrene by a pure culture of a *Mycobacterium* sp. amended with surfactants. Without the increased solubility imparted by surfactants, augmentation did not occur, however, the increase in pyrene degradation was not described as co-metabolism since pyrene was degraded when present alone. This observation was attributed to increased growth of the bacteria on phenanthrene, and the increased biomass resulted in augmentation of the pyrene degradation rate. Mixture of PAHs, however, do not always produce these stimulation effects. Ye *et al.* (1996) reported that in the extent of benzo[a]pyrene degradation by *S. paucimobilis* EPA 505 was not affected by the presence of another PAH, including benz[a]anthracene, chrysene, dibenz[a,h]anthracene or fluoranthrene. Only one PAH compound, benzo[b]fluoranthrene, caused noticeable reduction in the degradation of benzo[a]pyrene. The authors suggested two explanations for their observations; these being the compounds fed did not compete for the active sites of

the enzyme responsible for benzo[a]pyrene degradation by *S. paucimobilis*, or there are different enzymes responsible for the degradation of the different PAHs.

2.6 METHODS FOR DETECTING OF PAH-DEGRADING MICROBIAL POPULATIONS IN THE ENVIRONMENT

It is hypothesized that the population density and activity of PAH-degrading microorganisms should be two of the key diagnostic parameters used in predicting field rates of PAH biodegradation and enhanced field bioremediation. Therefore, detection or monitoring of PAH-degrading microbes or their activity are of great interest. Traditional and molecular-based methods adopted for addressing this issue are briefly reviewed in this section.

2.6.1 Traditional Methods

Traditional methods for detecting PAH-degrading bacteria involve spraying a PAH solution on an agar plate. After a certain period of incubation, PAH degradation is shown by clear zones around colonies (Kiyohara *et al.*, 1982). The spray plate technique was later modified by Bogardt and Hammingsen (1992). The modification involves incorporating an environmental sample and a solubilized PAH into an agarose overlay and pouring this mixture over a mineral salt medium. By using this technique, technical problems of the spray plate method are avoided. This is a useful, safe and easy technique to visualize and enumerate PAH degraders. However, the traditional method for quantifying specific catabolic bacterial populations often underestimate the true total population count since laboratory media cannot accurately reproduce the physico-chemical environment encountered by the microbes in the field.

2.6.2 Molecular Methods

New methods based on molecular biological analysis of bacterial nucleic acids have been developed which enable detection of PAH-degrading bacteria that may not be detected using plate count methods. The accumulated molecular genetic

information and techniques from molecular biology research permit the development and application of a new generation of approaches for specific and quantitative diagnosis of the potential for microbial PAH degradation and activity in contaminated soils. These approaches, which are often used for tracking target microorganisms in the environment, include DNA gene probe technology, mRNA analysis, *lux* reporter systems and polymerase chain reaction (King *et al.*, 1990; Sayler and Layton, 1990; Picard *et al.*, 1992; Herrick *et al.*, 1993; Sanseverino *et al.*, 1993; Briglia *et al.*, 1996; Wang *et al.*, 1996; Matheson *et al.*, 1997).

2.6.2.1 DNA Probe Technology

DNA-DNA hybridization has been used to determine bacterial community diversity and structure (Smith and Tiedje, 1992), identify and quantify bacteria with specific genotypes and phenotypes (Erb and Wagner-Döbler, 1993; Collaghan *et al.*, 1994; Harmsen *et al.*, 1997; Matheson *et al.*, 1997), determine the fate of genetically engineered microorganisms (Jain *et al.*, 1987; Steffan and Atlas, 1988), and determine maintenance and transfer of specific DNA sequences in different environments (Jain *et al.*, 1987; Herrick *et al.*, 1997).

DNA probes prepared from the NAH7 plasmid have been successfully used to identify naphthalene degraders, which could potentially also degrade other PAHs, in microbial communities in aquatic and terrestrial environments (Sayler and Layton, 1990). Sanseverino *et al.* (1993) successfully quantified naphthalene degradative genes present in PAH-contaminated soil using DNA hybridization with *nahA* gene probe.

2.6.2.2 Polymerase Chain Reaction (PCR) Amplification

The PCR amplification of extracted DNA has been successfully employed as a rapid method to detect the *nahAc* (naphthalene dioxygenase) gene from both surface and subsurface samples (Picard *et al.*, 1992; Erb and Wagner-Döbler, 1993; Herrick *et al.*, 1993). Recently, Wang *et al.* (1996) employed PCR techniques based on 16S rRNA gene sequences to monitor the pyrene-degraders,

Mycobacterium sp. PYR-I and *Mycobacterium* sp. PAH135, in PAH-contaminated soil slurries. The method revealed high specificity towards the target species (PYR-I and PAH135). The authors also demonstrated the high sensitivity of this method, which was able to detect strains PAH135 and PYR-I in cell numbers as low as 20 and 200 cells ml⁻¹, respectively. Briglia *et al.* (1996) published a method for detection of *M. chlorophenolicum* PCP-1, the pentachlorophenol (PCP) degrader, in soil. The method was developed based on 16S rRNA gene-targeted PCR and oligonucleotide probing. Using this method, the authors described a high sensitivity in detecting strain PCP-1 in soil which showed a detection limit of 3×10^2 cells g⁻¹ soil.

2.6.2.3 mRNA Analysis

While the analysis of DNA extracted from soil gives information on the potential of soil bacteria to perform a specific function, no information can be inferred about the expression of the genes of interest. Direct extraction of mRNA and its characterization by hybridization provides evidence of *in situ* levels of bacterial activity at the time of sampling and allows corrections to be made between this and specific contaminant levels (Tsai and Olson, 1992; Flemming *et al.*, 1993). The analysis of mRNA provides direct evidence for specific operon induction *in situ* which implies availability of the substrate or a specific inducer of the catabolic operon. If the specific bacterial population is above the threshold level permitting mRNA isolation, a lack of specific catabolic gene transcripts implies either the lack of availability of inducing substrates, the presence of an inhibitor of gene expression or overall cell metabolism, or nutrient limitation (Sanseverino *et al.*, 1993). Molecular diagnostics of PAH biodegradation in MGP soils was carried out by Sanseverino and co-workers (1993) using direct RNA extraction and quantification of mRNA from soils in relationship to naphthalene mineralization. mRNA transcripts of *nahA* were isolated and quantified from four soil samples. The results indicated a high correlation between naphthalene mineralization and the amount of *nahA* gene transcripts present in the soils.

2.6.2.4 Reporter Systems

The development of naphthalene-*lux* reporter systems (King *et al.*, 1990; Burlage *et al.*, 1990) was the first environmental application of a catabolic operon linked with the promoterless genes for bioluminescence. The reporter system of King and co-workers (1990) was a *Ps. fluorescens* HK44 which carries a transcriptional *nahG-luxCDABE* fusion. The bioluminescent system was under the control of the *sal* or lower pathway promoter/operator. A quantitative relationship between bioluminescence and naphthalene was demonstrated in which bioluminescence was induced at a threshold of 45 ppb naphthalene in a laboratory assay system (Heitzer *et al.*, 1992). The *lux* bioreporter technique has an advantage of real time on-line monitoring of pollutant concentration and bioavailability, compared to conventional chemical methods (Cerniglia, 1993). Sanseverino *et al.* (1993) employed a naphthalene-*lux* reporter gene system to determine the bioavailability of naphthalene in PAH-contaminated soil. They found that the magnitude of the response varied from soil to soil and in some soil samples it was barely detectable. The authors suggested that several factors may affect the bioluminescent response, including bioavailability of substrate or inducer, inhibition by soil contaminants, soil interference and the physiological state of the reporter culture.

2.7 ENHANCEMENT OF PAH BIOREMEDIATION

This section reviews some of the factors which are important for improving PAH bioremediation *via* the introduction of potent PAH-degrading microbes.

2.7.1 Bioaugmentation

Although the presence of organic pollutants can select natural microbial populations capable of degrading the pollutants, the metabolic range of the natural soil microbiota may not be capable of degrading certain compounds or classes of compounds. Introducing allochthonous microorganisms into natural environments can increase the rate or extent, or both, of biodegradation of pollutants. Microorganisms with specialized degradative capacities may be

obtained by selective enrichment techniques and/or genetic manipulation. Such an approach has been successful for many decontamination schemes including chlorinated aromatics (Morgan and Watkinson, 1989) and *p*-nitrophenol (Zaidi *et al.*, 1988). Mixed bacterial populations have mostly been used in seeding operations. Otte and co-workers (1994) demonstrated the degradation of PCPs and PAHs by indigenous microbes of soil collected from an industrial site used for producing wood-preserving compounds. A portion of the contaminated soil was used as inoculum and cultured in a soil/slurry bioreactor to enhance the activity of indigenous biomass. The results indicated that by using activated soil as an inoculum, the time required for the degradation of PCPs and PAHs in contaminated soil was decreased, and the rate of PCPs and PAH mineralization was increased. Trzesicka-Mlynarz and Ward (1996) demonstrated that the degradation of fluoranthene in a soil matrix was enhanced by inoculation with a PAH-degrading mixed bacterial culture compared to uninoculated soil containing only an indigenous consortium.

Natural microbial strains which have been isolated on the basis of their degradative potential could be genetically manipulated to increase their biodegrading capacity. Considerable controversy, however, still surrounds the release of genetically engineered microorganisms (GEMs) into the environment. Therefore, the issues of safety, containment, and potential for ecological damage associated with these manipulated microbes need to be resolved before their application (Harker *et al.*, 1994).

The major concern of seeding microorganisms into soil is their activity and survival. The presence of indigenous microbial populations, which are highly adapted to the environment, may negatively influence the ability of seeded microbes to compete successfully and survive. There have been reports of improving the survival of allochthonous bacteria in contaminated soil by using alginate-encapsulated cells as the inoculum (Wiesel *et al.*, 1993; Weir *et al.*, 1995).

2.7.2 Addition of Nutrients

Addition of organic and inorganic nutrients have been found to increase the microbial catabolism of some target pollutants (Swindoll *et al.*, 1988; Manilal and Alexander, 1991), decrease the catabolism of other target pollutants (Manilal and Alexander, 1991; Carmichael and Pfaender, 1997a), shorten the adaptation period for certain compounds (Swindoll *et al.*, 1988; Steffensen and Alexander, 1995) or have no apparent effect on metabolism in other cases (Mihelcec and Luthy, 1991). Studies investigating the impact of nutrient addition on PAH bioremediation have been performed largely in connection with oil spill clean-up. Laboratory (Churchill *et al.*, 1995; Mueller *et al.*, 1992; Venosa *et al.*, 1992) and field (Pritchard and Costa, 1991; Venosa *et al.*, 1992; Venosa *et al.*, 1996) studies regarding the recovery of oil-contaminated areas have found that in general nutrient supplementation increases the rate of biodegradation over that of unamended controls. The effect of nutrient enhancement on the biodegradation of beach materials contaminated during the *Exxon Valdez* oil spill was investigated using three types of fertilizers: commercially available slow release formulations, an oleophilic fertilizer (oleophilic fertilizers are designed to concentrate nutrients at the oil/water interface, making them available to microorganisms at the oil surface [Atlas, 1995]), and water-soluble fertilizer applied as a solution (Pritchard and Costa, 1991). Two or three weeks after application of the oleophilic fertilizer, Inipol EAP 22, the oiled cobblestones on the beach were visibly cleaner than in control areas (Pritchard and Costa, 1991). However, the contribution of Inipol's oleophilic nature to bioremediation was not clear from the Prince William Sound field study (Pritchard and Costa, 1992). Churchill *et al.* (1995) found that Inipol EAP 22 possessed surface-active characteristics in their experiments with hexadecane, octadecane, toluene, and 2-methylnaphthalene and pure bacterial cultures. Their results suggested that the surfactant properties of Inipol increased the bioavailability of hydrocarbons and that Inipol's success in the *Exxon Valdez* clean-up effort may have been significantly influenced by its surface-active properties.

Hydrocarbon wastes are often deficient in inorganic nutrients essential to microorganisms (Morgan and Watkinson, 1989) and as a result, microbial metabolism of hydrocarbons in soils may be inorganic nutrient limited. However, in some cases, addition of inorganic supplements adversely affected on microbial metabolism of a target pollutant. More recently, Carmichael and Pfaender (1997a) demonstrated that the addition of inorganic nutrients, nitrogen (NH_4Cl) and/or phosphorus (Na_2HPO_4 or KH_2PO_4), decreased the mineralization of [^{14}C]pyrene in soil by approximately 50 to 90% compared to unamended soil. The addition of inorganic materials appears to produce an increase in the microbial metabolism of readily degradable carbon sources in preference to the PAH. Specific forms of nitrogen (NH_4^+ or NO_3^-) have also been found to have varied effects on the metabolism of organic compounds. This difference could be attributed to differential availability of the two forms of nitrogen; NH_4^+ tends to adsorb to clay minerals and may be less available than NO_3^- (Atlas, 1989). In addition to its use as a form of nitrogen for cellular maintenance, nitrate may also stimulate the metabolism of organic compounds when it is used as an alternative terminal electron acceptor (Leduc *et al.*, 1992).

Organic compounds may be used as supplements to either support co-metabolic transformation of PAHs, which is thought to be important in the metabolism of PAHs with three- or more aromatic rings (Keck *et al.*, 1989), or to generally stimulate microbial communities. Lower molecular weight PAHs have also been found to serve as co-metabolic substrates for PAH metabolism (Bouchez *et al.*, 1995; Juhasz *et al.*, 1997). Weir *et al.* (1995) investigated the effect of nutrient amendment on the survival of, and phenanthrene mineralization by, the bioluminescent *Pseudomonas* sp. UG14Lr in creosote-contaminated soil. They showed that the addition of skim milk to soil slurries doubled the extent of phenanthrene mineralization by UG14Lr as well as by the indigenous microorganisms compared to unamended soil. Carmichael and Pfaender (1997a) investigated the effect of a widely used non-specific carbon source, nutrient broth, and other PAHs (either phenanthrene or pyrene), on the mineralization of [^{14}C]phenanthrene and [^{14}C]pyrene. Their results showed that nutrient broth

consistently decreased [^{14}C]phenanthrene mineralization in soil. When soil was incubated with both pyrene and [^{14}C]phenanthrene, the extent of [^{14}C]phenanthrene mineralization decreased by approximately 20%, and if the soil was exposed to phenanthrene along with [^{14}C]pyrene, the mineralization of [^{14}C]pyrene also decreased, but only by around 5% of the extent mineralized in the absence of the PAH.

2.7.3 Addition of Inducers

Specific inducers of genetic operons can also be used to enhance the degradation of toxic compounds (Olson and Goldstein, 1988). This approach may provide two benefits to bioremediation: increased specificity of the target population to be stimulated and predictability of intermediates and end products since known degradative pathways are stimulated. Ogunseitan *et al.* (1991) determined the genetic response of six naphthalene-degrading pseudomonads to salicylate added to non-sterile PAH-contaminated soil samples. Results demonstrated that salicylate at a concentration of $160\ \mu\text{g g}^{-1}$ soil induced a significant increase in the number of naphthalene degraders and the levels of *nah*-related genes in the population. Further studies by Ogunseitan and Olson (1993) confirmed the role as well as the specificity of salicylic acid as an inducer of operons involved in the biodegradation of PAHs. The addition of salicylate to soil not only increased the population density of naphthalene degraders but also increased the extent of naphthalene mineralization. On the other hand, Sanseverino *et al.* (1993) have demonstrated that the addition of salicylic acid in four contaminated soils during a slurry treatment increased the *nahA* mRNA levels in only one soil. However, they suggested that this transcript was already induced in the other soils because of high concentration of naphthalene. Carmichael and Pfaender (1997a) found that salicylic acid did not influence the mineralization of [^{14}C]phenanthrene or [^{14}C]pyrene in soils, and in some cases decreased the extent of mineralization. In addition, cinnamic acid, an intermediate of pyrene metabolism by *Mycobacterium* sp. (Heitkamp *et al.*, 1988), was found to either increase, decrease or not affect [^{14}C]pyrene mineralization, depending upon soil types. A diverse effect seen with

these inducers may be attributed to differences in soil characteristics and their native microbial communities.

2.7.4 Application of Surfactants

Although various factors which could limit the biodegradation and removal of PAHs from the environment can be optimized, PAH degradation may be slow unless the bioavailability of PAH compounds for microbial metabolism is enhanced. A major question in the biodegradation of hydrocarbons is how the microbes actually contact the substrates, especially when the contaminants are in soil or sediments in which sorption of these hydrophobic compounds is recognized as the rate-limiting step controlling both the rate and extent of biodegradation. Microbial consumption of water-soluble substrates seems to present no problem, but how do microbes interact with materials that are as inherently insoluble as PAHs? Three mechanisms are often involved, and different bacterial species may use one or more of them: (i) interaction with the low levels of dissolved material, (ii) direct contact with drops or surfaces of the insoluble phase, and (iii) interaction with hydrocarbon solubilized by surfactants (Prince, 1993). Biosurfactants play an important role in the latter two mechanisms, both in terms of attaching to the cell surface to enhance contact of the organism with the substrate, and by dispersion the substrate into the medium to enhance solubility.

Biosurfactant-producing microorganisms, as well as several types of biosurfactants, have been isolated and characterized, where the latter includes glycolipids, fatty acids, peptidolipids and lipopolysaccharides (Banat, 1995). Biosurfactants are predominantly synthesized by hydrocarbon-degrading microorganisms and may be spontaneously released so that their function is often related to hydrocarbon uptake (Banat, 1995). Déziel and colleagues (1996) investigated the capacity of PAH-utilizing bacteria to produce biosurfactants. They found that there was some production of glycolipid molecules during the metabolism of naphthalene and phenanthrene by pseudomonad strains. However, some biosurfactants have been reported to be produced during growth on water-soluble compounds, such as glucose, sucrose, glycerol, or ethanol (Banat, 1995).

Recently, there has been an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil-pollution control, cleaning oil sludge from oil storage facilities, soil bioremediation and microbially-enhanced oil recovery (Banat, 1995; Scheibenbogen *et al.*, 1994). The efficacy of the natural biosurfactants has prompted a search for chemically synthesized, and therefore cheaper, surfactants that will stimulate the solubilization rate and thus biodegradation of recalcitrant compounds such as PAHs. One treatment option for decontaminating PAHs which are strongly adsorbed to soil particles, involves washing the soil to extract the PAHs into an aqueous phase, which is then treated using conventional waste water treatment approaches (Joshi and Lee, 1996).

2.7.4.1 Surfactant Characteristics

Surfactants are amphipathic molecules consisting of a hydrophilic polar head group and a hydrophobic non-polar tail group. The term "surfactant" is a contraction of 'SURFace ACTIVE Agent' and, as the term suggests, surfactants tend to migrate to surfaces and interfaces or create new molecular surfaces by forming aggregates (Aboul-Kassim and Simoneit, 1993; Rouse *et al.*, 1994). Surfactants can be categorized into four major groups based on the ionic charge associated with their polar moiety: anionic, cationic, nonionic and amphoteric (zwitterionic) surfactants (Zajic and Panchal, 1976; Aboul-Kassim and Simoneit, 1993).

- (1) Anionic surfactants usually have a large paraffin hydrocarbon chain or an aromatic ring as a hydrophobic group. The hydrophilic portion of the molecule is generally a sulfate, sulfonate, or a fatty acid condensation product with protein. Most synthetic soaps and detergents are anionic surfactants.
- (2) Cationic surfactants whose hydrocarbon residues usually contain a basic N, possess, almost without exception, a quaternary ammonium salt as the hydrophobic group. The negative ion is normally a chloride (Cl^-) or

bromide (Br^-). Cationic surfactants are of little use for wetting or washing purposes. However, they have an immense capacity for adsorption on most negatively charged interfaces and have a good adhesion capacity.

- (3) Nonionic surfactants have the same hydrophobic groups as those of the anionic and cationic surfactants, but differ in the hydrophilic moiety. The hydrophilic groups in this type of surfactants are generally phenols, amines or mercaptans. They often contain multiple hydroxyl groups.
- (4) Amphoteric surfactants ionize to produce either anions or cations, depending upon their immediate environment. Thus they are anionic in a basic medium and cationic in an acidic medium.

A fundamental property of surfactants is their ability to form micelles (colloidal-sized clusters) in solution. The concentration of surfactant at which the thermodynamics of the surfactant-solution system favor the micelle formation is called the critical micelle concentration (CMC) (Haigh, 1996). Micelles are capable of dissolving hydrophobic compounds in their hydrophobic core, which results in an increased apparent aqueous solubility of the compounds in the solution above its aqueous solubility limit and is referred to solubilization. However, solubilization of a particular compound depends on the type and dose of the surfactant, the hydrophobicity of the contaminant, the surfactant-soil interactions and the time that the contaminant has been in contact with soil (Joshi and Lee, 1996).

2.7.4.2 Surfactant Enhanced PAH Solubility and Bioavailability

Surfactants above their CMC can solubilize hydrophobic contaminants into their micelles. This process enhances the apparent solubility of contaminants such as hydrocarbons and, therefore, also their desorption from soils. Luthy and co-workers (1994) demonstrated that the addition of surfactants (nonionic Triton X-100) to soil-water systems effectively solubilizes PAH compounds, which included anthracene, phenanthrene and pyrene, by incorporation of these PAHs into the

surfactant micelles. Conceivably, in the absence of any inhibitory effects, such surfactants may enhance the biodegradation of the hydrocarbons. By increasing PAH solubility, surfactants increase the fraction of soluble compound and presumably the amount available for microbial uptake (Liu *et al.*, 1991; Guerin and Boyd, 1992; Crocker *et al.*, 1995; Guha and Jaffé, 1996a and 1996b).

The effect of a surfactant on the bioavailability of organic compounds may be explained by three main mechanisms (Edwards *et al.*, 1991 and 1992; Liu *et al.*, 1991; Volkerling *et al.*, 1995).

- (i) Dispersion of non-aqueous phase liquid hydrocarbon, leading to an increase in contact area, which is caused by a reduction in the interfacial tension between the aqueous phase and the non-aqueous phase.
- (ii) Increased apparent solubility of the pollutant, caused by the presence of micelles which may contain high concentrations of hydrophobic organic compounds (a mechanism which has been studied extensively).
- (iii) Facilitated transport of the pollutant from the solid phase to the aqueous phase, which can be caused by a number of phenomena, such as lowering of the surface tension of the pore water in soil particles, interaction of the surfactant with solid interfaces, and interaction of the pollutant with single surfactant molecules.

2.7.4.3 Effects of Surfactants on PAH Biodegradation

When surfactants have been used in attempts to accelerate the biodegradation of PAHs, variable impacts have been reported where some authors noted improved degradation rates (Grimberg *et al.*, 1996; Volkerling *et al.*, 1995) while others reported no impact (Foght *et al.*, 1989; Ghosh *et al.*, 1995) or inhibitory effects (Descehênes *et al.*, 1995; Laha and Luthy, 1991). However, these confounding results may stem from experimental conditions as well as various biotic and abiotic parameters. Thibault *et al.* (1996) demonstrated the effects of four

surfactants on pyrene desorption and degradation by two *Pseudomonas* strains. Under unsaturated but moist conditions, one surfactant, Witconcol SN70 (nonionic type), stimulated pyrene mineralization, whereas the same surfactant inhibit pyrene mineralization in soil slurries. The authors suggested that the suppression of pyrene mineralization under soil slurry condition may due the increased pyrene solubilization and subsequently increase in toxicity to the bacteria.

Mueller *et al* (1990) used the nonionic surfactant, Tween 80, to enhance the solubility and biodegradation of fluoranthene by *Ps. paucimobilis* (now call *Sphingomonas paucimobilis*) EPA505. This was the first report of the primary utilization of a PAH containing four or more rings by a pure microbial culture. Biodegradation of this poorly soluble PAH was enhanced by surfactant addition up to 2 g l⁻¹, which was above its CMC, without exhibiting toxic effects on the isolate.

Laha and Luthy (1991) investigated the effects of nonionic surfactants on the biodegradation of phenanthrene in soil-water systems. The surfactants used in the experiment included Brij 30 (dodecylethoxylate, C₁₂E₄), Tergitol NP-10 (nonylphenylethoxylate, C₉PE_{10.5}), and Triton X-100 (octylphenylethoxylate, C₈PE_{9.5}). The bacteria used were a mixture of PAH-degrading cultures. At surfactant concentrations below the CMC, no significant enhancement or inhibition of phenanthrene degradation was observed compared to the surfactant-free control, whereas at supra-CMC levels there was virtually complete inhibition of biodegradation. Inhibitory effects of surfactants at high concentration was also demonstrated by Deschênes *et al.* (1996). Adding the anionic surfactant, sodium dodecyl sulfate (SDS) and the *Ps. aeruginosa* UG2 biosurfactants at low concentrations did not affect the biodegradation of fluoranthene, pyrene, benz[a]anthracene and chrysene, which were aged PAH residues in weathered contaminated soil, however, by adding these two surfactants at high concentrations, the biodegradation of the four PAHs was significantly inhibited. Possible reasons for the inhibition were taken into consideration: (i) toxicity by surfactant or solubilized PAH at high concentrations, (ii) preferential metabolism

of the surfactant, (iii) lowering of the available substrate concentration in aqueous solution due to micellization, and (iv) interference of microbial membrane processes. The first two reasons were experimentally ruled out, and chemical modeling indicated that the third reason was not a significant concern (Laha and Luthy, 1991). Therefore, it was hypothesized that the inhibition might be due to interference with substrate transport into the cell or to reversible physical-chemical interference with the activity of enzymes and other membrane proteins involved in hydrocarbon degradation. Additional work was suggested to assess the specific mechanism(s) of surfactant interference on biodegradation.

There has been some debate as to whether PAHs accommodated in the micelle molecules are bioavailable. Since surfactant micelles represent a separate suspended-pseudophase, the potential exists for the formation of a non-biologically-available PAH fraction during surfactant-enhanced solubilization of compounds. Guha and Jaffé. (1996a and 1996b) studied the biodegradation kinetics of phenanthrene partitioned into micellar-phase nonionic surfactants. An effective bioavailable fraction of the micellar-phase phenanthrene was defined using four different surfactants, evaluated under various experimental conditions, and modeled mathematically. The results indicated that a fraction of micellar-phase phenanthrene was directly bioavailable, implying the direct mass transfer from the micelle to bacterial cells, and that this fraction decreased with an increase in micelle concentration. It has also been hypothesized that the fraction available is a function of the bacterial culture (hydrophobicity of cell surface) and the type of surfactant (length of the hydrophilic chain). Recently, studies by Zhang *et al.* (1997) showed that the addition of a rhamnolipid markedly increased the rate of phenanthrene dissolution, however, the rate of degradation of this PAH by *Ps. putida* strain CRE 7 was limited due to lack of substrate availability, the surfactant-PAH complex in this case not being directly available to the microorganisms.

Studies on degradation of PAHs by a PAH-degrading bacterial community in the presence of nonionic surfactants and an anionic surfactant, SDS, by Tiehm (1994) revealed that PAH degradation was inhibited by SDS due to this surfactant being

preferred as a growth substrate. Some nonionic surfactants were toxic when tested on the growth of a PAH-degrading *Mycobacterium* sp. isolated from the mixed bacterial population. However, the non-toxic surfactants enhanced the degradation of fluorene, phenanthrene, anthracene, fluoranthene and pyrene by mixed cultures. Growth of mixed bacterial cultures on phenanthrene and fluoranthene solubilized by a nonionic surfactant was exponential which indicated a high bioavailability of the solubilized PAHs.

Lui *et al.* (1995) quantified the bioavailability of micelle-solubilized naphthalene to naphthalene-degrading bacteria. Nonionic surfactants, Brij 30 and Triton X-100, were employed in this study. The results showed that surfactant concentrations above CMC were not toxic to naphthalene-degrading bacteria and that the presence of surfactant micelles did not inhibit naphthalene mineralization. It was concluded that naphthalene solubilized by micelles of Brij 30 or Triton X-100 in liquid media were bioavailable and degradable by the mixed culture of bacteria.

Volkerling *et al.* (1995) conducted batch growth experiments which demonstrated that rates of naphthalene and phenanthrene degradation by *Pseudomonas* sp. strain 8909N in the dissolution-limiting growth phase were increased by the addition of nonionic surfactants, Triton X-100, Tergitol NPX, Brij 35 or Igepal CA-720. No toxic effects of the surfactants were observed at concentrations up to 10 g l⁻¹. Naphthalene at high concentrations were toxic to bacteria, however, in the presence of surfactant a decrease in its toxic effects were observed. The authors also suggested that PAH present in the micellar phase were not readily available for degradation by the microorganisms, but micellar PAH act as a protected reservoir that may replenish aqueous-phase PAH when it is depleted by biodegradation. Grimberg *et al.* (1996) reported the ability of the nonionic surfactant, Tergitol NP-10, to increase the phenanthrene dissolution rate. This resulted in an overall increase in growth rate of *Ps. stutzeri* P16, in the presence of surfactant, although micellized phenanthrene did not appear to be directly available to the bacterium.

Carmichael and Pfaender (1997a) tested the impact of a nonionic surfactant, Triton X-100, and Inipol EAP-22, a compound with both surfactant and nutrient properties, on [^{14}C]phenanthrene mineralization in a PAH-contaminated soil. The authors found that Triton X-100 at concentrations above CMC decreased mineralization of [^{14}C]phenanthrene by soil microorganisms by 30-40% and the addition of Inipol EAP-22 decreased mineralization of [^{14}C]phenanthrene by greater than 90% of the amount mineralized in the absence of these supplements. The authors did not investigate the cause of the suppression of phenanthrene mineralization, however, they suggested that it may be due to the unavailability of phenanthrene partitioning in the micelles, preferential use of the surfactants as a growth substrate or toxicity of the surfactants.

Recently, Tiehm and colleagues (1997) showed the enhanced-mobilization and biodegradation of PAHs in soil from manufactured gas plant operations by using the nonionic surfactants Arkopal N-300 and Sapogenat T-300. In addition to the mobilization and biodegradation rates of PAHs, they also monitored the biodegradation of the surfactant. Both surfactants enhanced the mass transfer rate of sorbed PAHs into aqueous phase due to solubilization. Solubilized PAHs were found to be available for biodegradation. The authors demonstrated that Arkopal N-300 was also degraded rapidly, which resulted in a lack of oxygen and an inhibition of PAH biodegradation. Therefore, biodegradability of surfactants is one of the key issues for their practical use in PAH bioremediation.

2.8 METHODS FOR TOXICITY ASSESSMENT

The effectiveness of biological soil remediation is usually evaluated on the basis of removal of selected contaminants. In the case of PAH-contaminated environments, 16 EPA-PAHs are analyzed to demonstrate site restoration (Weissenfels *et al.*, 1992). It is very important, however, to correlate site restoration, *i.e.* the successful disappearance of parent materials, with a discernible decrease in the health hazards associated with biotreated materials. To address this issue, an evaluation of the potential of the contaminated soil system to accomplish detoxification and degradation of hazardous constituents

Table 2.6 Auxotrophic strains of *S. typhimurium* used in the Ames test (from Maron and Ames, 1983).

Histidine mutations				LPS	Repair	R-factor
hisD6610 his01242 (pAQ1) = TA88	hisD3052	hisG46	hisG428			
TA90	TA1538	TA1535	—	<i>rfa</i>	$\Delta uvrB$	—R
[TA97]	[TA98]	[TA100]	—	<i>rfa</i>	$\Delta uvrB$	+R
—	TA1978	TA1975	—	<i>rfa</i>	+	—R
TA110	TA94	TA92	—	+	+	+R
—	TA1534	TA1950	—	+	$\Delta uvrB$	—R
—	—	TA2410	—	+	$\Delta uvrB$	+R
TA89	TA1964	TA1530	—	Δgal	$\Delta uvrB$	—R
—	TA2641	TA2631	—	Δgal	$\Delta uvrB$	+R
—	—	—	[TA102]	<i>rfa</i>	+	+R

Tester strains in brackets are recommended for general mutagenic testing. All strains were originally derived from *S. typhimurium* LT2. Wild-type genes are indicated by a +. The deletion (Δ) through *uvrB* also includes the nitrate reductase (*chl*) and biotin (*bio*) genes. The Δgal strain and the *rfa. uvrB* strains have a single deletion through *gal chl bio uvrB*. The *rfa* repair⁺ strains have a mutation in *galE*. R = pKM101, a plasmid carrying the ampicillin-resistant gene.

present in the waste need to be performed. Bioassay response data are necessary to demonstrate an overall change in toxicity of soil contaminants. Several bioassays have been developed to evaluate the toxicity of pollutants in environmental systems. Among others, the Ames and Microtox™ test systems are widely used for the evaluation of mutagenic and acute toxic effects of particular compounds, respectively.

2.8.1 The Ames Test

The most generally used and validated bacterial reverse-mutation test for detection of genotoxins is the Ames test, which was devised by B.N. Ames and his colleagues in 1971 (Ames *et al.*, 1971) and the methodology was revised in 1983 (Maron and Ames, 1983). This assay is a measurement of the mutagenic potential of hazardous compounds and has been widely used to evaluate the mutagenicity of environmental samples (Bos *et al.*, 1984; McMillian *et al.*, 1988; Wang *et al.*, 1990; Nylund *et al.*, 1992; Rudd *et al.*, 1996). In this method, bacterial strains are used which carry base substitution or frameshift mutations in the operons coding for synthesis of specific amino acids. Therefore, unlike their wild-type prototrophic counterparts, these mutants cannot synthesize all their required amino acids, being auxotrophic for a specific amino acid. The assay determines whether the test chemical can reverse the effect of the pre-existing mutation by introducing a second mutation, either at the structural gene, or at another site on the bacterial chromosome. Mutant strains of *S. typhimurium* that require histidine for growth are used to test for mutagenicity.

The Ames test (also named the '*Salmonella*/mammalian microsome test') employs several mutant strains of *S. typhimurium*, each carrying a different type of mutation in the operon coding for histidine biosynthesis (Table 2.6). In addition to histidine mutation, the standard tester strains contain other mutations that greatly increase their ability to detect mutagens as follows:

- (i) *rfa* mutation, which causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large

molecules, such as benzo[a]pyrene, that do not penetrate the normal cell wall.

- (ii) *uvrB* mutation, which is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens. The deletion excising the *uvrB* gene extends through the *bio* gene and, as a consequence, these bacteria also require biotin for growth.
- (iii) resistant transfer factor (R-factor), in which the bacterial strains that carry the particular R-factor are more effective in detecting mutagens that are detected weakly or not at all in its absence.

These assays detect only point mutations and measure reverse mutation from amino acid auxotrophy to prototrophy. Unlike mammals, these bacteria lack the necessary oxidative enzyme systems for metabolizing foreign compounds to electrophilic metabolites capable of reacting with DNA. The bacteria are, therefore, treated with the test compound in the presence of a post-mitochondrial supernatant ('S9' or 'microsome fraction') prepared from the livers of mammals (usually rats). The metabolic activity of the S9 (predominantly mono-oxygenase activity) mediated *via* the cytochrome P-450/P-448 system (Davis, 1988) is enhanced by treating the rats with a potent inducer of drug-metabolizing enzymes before they are killed and their livers removed. A mixture of polychlorinated biphenyls, Aroclor 1254, is the most commonly used inducer, although other compounds and combinations of compounds are also used. The best alternative to Aroclor 1254 is the combination of phenobarbitone and β -naphthoflavone (Venitt *et al.*, 1984).

In this test, numbers of revertant colonies ('*his*⁺ revertants') are demonstrated as a result of the mutagenic potential of compounds tested. An increase in revertant colonies related to increasing dose of the tested compound indicates a mutagenic response. However, without the addition of test chemicals, back mutation occurs at a rate specific to each strain type (spontaneous reversion rate) which needs to

be taken into consideration in the determination of the mutagenic potential of a particular compound.

2.8.2 The SOS Chromotest

This assay, which is one of the test systems used to detect DNA-damaging agents (genotoxins), was developed by Quillardet and Hofnung (1985). It is based on the knowledge of SOS responses of *E. coli* which occur in response to DNA damage which can be caused chemically. In *E. coli*, some of the responses induced by DNA-damaging agents involve a set of functions known as the SOS responses (Walker, 1984) as well as 'damage-inducible' (*din*) genes (Kenyon and Walker, 1980). A simple system of colorimetric assay for genotoxins was devised by operon fusion, in which *lacZ* (the structural gene for β -galactosidase) was placed under the control of the *sfiA* gene (an SOS function involved in cell-division inhibition) (Quillardet *et al.*, 1982). The *E. coli* PQ37, which is normally used as the tester strain in the SOS chromotest (Quillardet *et al.*, 1982), carries a *sfiA::lacZ* fusion and has a deletion for the normal *lac* region, so that β -galactosidase activity is strictly dependent on *sfiA* expression. This bacterium also carries a *uvrA* mutation, which renders the strain deficient in excision repair and accordingly increases the response to certain DNA-damaging agents. Moreover, an *rfa* mutation renders the strain to be lipopolysaccharide deficient and allows better diffusion of certain chemicals into cells.

The assay consists of incubating the tester strain with increasing concentrations of the agent being examined. After a time for protein synthesis, β -galactosidase activity is assayed. A microsomal activation preparation, modified from that of Ames (1975), can be included in the incubation mixture. The response is obtained in a few hours. The test is based on kinetic measurements of β -galactosidase and alkaline phosphatase (a chromosomally-specified enzyme in *E. coli*) formation in the test cuvettes. Toxicity and inhibitory effects on protein synthesis are checked using measurements for alkaline phosphatase levels. Based on these measurements, the degree of induction of β -galactosidase relative to alkaline phosphatase for tests and controls is calculated as an index, which is indicative of

the DNA-damaging potential of the test chemical. The assay is quantitative and there is normally a linear relationship within the dose-response curves where the SOS-inducing potency (SOSIP) is the slope of the linear region. The SOSIP is thus a single parameter which represents the induction factor per mass unit or per nanomole of compound tested and this value reflects the ability of the compound to induce gene *sfiA* expression (Quillardet *et al.*, 1982).

2.8.3 Sister-Chromatid Exchange (SCE) Assay

Although the classical mutagenic test system such as the Ames test and SOS chromotests are widely used and provide sensitive results in the detection of genotoxins, they are not considered adequate when used alone because they are prokaryotic test systems from which results cannot readily be extrapolated to man. It is also important to apply test systems in addition to bacterial ones in evaluating risk to humans by using mammalian systems, which probably are related more closely to the human system. The induction of sister-chromatid exchanges (SCE) is a sensitive indicator of mutagenic carcinogens (Hytönen *et al.*, 1983), and is a widely used test system for the screening of such compounds. Carrano *et al.* (1980) demonstrated the sensitivity of the SCE assay in human lymphocytes and supports the use of this test as a measure of environmental stress on man. In addition, this assay was shown to be sensitive to the genotoxic effects of organic-extractable components of urban airborne particles (Lockard *et al.*, 1981) and for comparing the mutagenic activities of extracts from ambient and forest-fire polluted air (Viau *et al.*, 1982).

The SCE assay is normally conducted using lymphocytes or Chinese hamster ovary (CHO) cells. A dose-response curve is performed for all samples in the presence and absence of S9. Under certain circumstances, exchange between sister chromatids of the chromosomes in cells is induced, such an increase in sister chromatid exchanges indicate chromosomal damage. The toxicity of the samples limits the dose range which can be tested: obvious toxic effects, like cell death, mitotic delay and chromosome aberrations and fragmentation, can be observed. After exposing to the test chemicals, cells are fixed and stained by the

fluorescence-plus-Giemsa (FPG) technique to provide permanently stained harlequin chromosomes. SCE-inducing activity is expressed as the number of induction of SCEs per cell (Hytönen *et al.*, 1983).

2.8.4 The Microtox™ Test

The measurement of the emission of light from luminescent bacteria has been used to develop a sensitive test for the quick assessment of aquatic toxicants. This bacterial bioassay is based on the reduction of light emitted by a non-pathogenic strain of luminescent marine bacteria upon exposure to a toxic sample. Several species of light emitting bacteria have been tested and proposed as biological systems suitable for toxicity testing. These organisms emit light under normal life conditions, as a consequence of the series of metabolic reactions which liberate energy in the form of visible light. On exposure to toxic substances, the light output is reduced and this reduction is proportional to the toxicity of the sample. Therefore, a toxicity bioassay may be based on the light emission of these bacteria, as a measurement of their metabolic activity. The most extensively used strain is *P. phosphoreum* (now called *Vibrio phosphoreum*), in which cultures, conditions and the test procedures as well as the accuracy and sensitivity have been developed and standardized. The system was developed commercially in 1982 under the trademark Microtox™, by Beckman Instruments, Inc (cited in Ribo, 1987).

Microtox™ has been utilized to provide an indication of the acute toxicity of the water soluble fractions of wastes, waste-amended soils from the incubation studies, and of leachates from polluted soils (Elnabarawy *et al.*, 1988; Symons and Sims, 1988; Weissenfels *et al.*, 1992; Belkin *et al.*, 1994; Tiehm *et al.*, 1997). Bioluminescence of the test organism depends on a complex chain of biochemical reactions. Chemical inhibition of any of the biochemical reactions causes a reduction in bacterial luminescence. Therefore, the Microtox™ test considers the physiological effect of a toxicant and not just mortality. Normally, the Microtox™ assay has been used in the acute toxicity assessment of polluted water (Aprill *et al.*, 1990; McTernan *et al.*, 1983), applications of the bioassay have been recently

extended to assessment of the treatability of hazardous organic compounds in soils (Microbics Corporation: the microtox solid-phase test).

In the assay, suspensions containing approximately one-million luminescent bacteria are challenged by addition of serial dilutions of an aqueous sample. A temperature-controlled photometric device, the Microtox™ analyzer, quantitatively measures the light output of each suspension before and after addition of the sample. A dose response curve is determined, using data from the Microtox™ analyzer for each dilution of the sample. The relative toxicity of a set of samples is generally expressed as the value of the median effective concentration (EC_{50}), which is specifically defined as the sample concentration resulting in a 50 percent loss of light emission compared to the light emission from a toxicant-free control. High EC_{50} values indicate lower toxicity than lower values. Therefore, as the toxicity of an aqueous solution decreases, the EC_{50} value will increase (Symons and Sims, 1988).

2.8.5 Other Methods

Other methods which have been used to assess the toxicity and genotoxicity of compounds include such methods as analysis of alkylated DNA by high pressure liquid chromatography, detection of DNA repair in cultured mammalian cells, mutation tests with the fruit fly *Drosophila melanogaster*, detection of gene mutation in cultured mammalian cells and cytogenetic tests in mammals (Venitt and Perry, 1984).

2.9 CONCLUDING REMARKS

Since the early 1970's, a considerable amount of research has been conducted on the microbial degradation of PAHs and its application for the bioremediation of PAH-contaminated sites. Such work has been stimulated by the vast number of sites polluted with these compounds and by the accumulation of data which shows PAHs to be highly toxic, carcinogenic and mutagenic.

There have been some significant advances made in this area and many reports now claim the successful removal of PAHs containing up to four-benzene rings from PAH-contaminated sites. However, such treatment still falls short of achieving a number of bioremediation goals since in most cases the carcinogenic five-benzene ring PAHs, such as benzo[a]pyrene and dibenz[a,h]anthracene, remain in the contaminated soil. The importance of removing these PAHs during bioremediation is reflected in the high amount of research activity which currently aims to discover microbial strains, or a process, that can effectively remove these PAHs from contaminated soils and sediments.

The research focus on five-benzene ring PAH degradation has, up to date, met with few successes. No microorganisms, or microbial consortia, have yet been isolated which, in pure culture, can grow on five-benzene ring PAHs as a sole carbon and energy source. Only a small number of workers have thus far reported on the microbial degradation of benzo[a]pyrene in pure culture and even fewer reports describe the mineralization of this compound by microorganisms. Most of these reports describe benzo[a]pyrene biotransformation which involves one or two catabolic steps, but further degradation of the resulting products does not occur. Benzo[a]pyrene mineralization in soils and sediments has been reported on a number of occasions but such investigations have failed to isolate pure cultures capable of mineralizing this compound. A recent report describes the promising result of benzo[a]pyrene mineralization by *S. paucimobilis* strain EPA 505 as a sole carbon and energy source (Ye *et al.*, 1996). However, the EPA 505 was unable to grow on benzo[a]pyrene as a sole carbon and energy source and high cell numbers were required for mineralization to occur. Some white-rot fungi have also been shown to mineralize benzo[a]pyrene in pure culture, but again such cultures require a growth substrate before the fungi can mineralize and grow in the presence of benzo[a]pyrene (Barclay *et al.*, 1995; Bezalel *et al.*, 1996; Bogan and Larmar, 1996). The importance of discovering microorganisms that can grow on and mineralize benzo[a]pyrene as a sole carbon and energy source lies in the ability to prepare inocula of such organisms which, when used for the bioaugmentation of PAH-contaminated sites, are not dependent on a particular

organic compound, or other nutrients, in the contaminated soil for growth of the PAH degraders and benzo[*a*]pyrene mineralization to occur.

Aside from the search for high molecular weight PAH degraders, researchers have also focused on developing technologies that can improve the degradability of these recalcitrant compounds. The use of surfactants to improve PAH degradation rates is of particular importance for the degradation of high molecular weight PAHs due to their low water solubility and hydrophobic nature. While there has been mixed results regarding the success of using surfactants to improve PAH degradation rates, the more recent literature generally reports improved degradation rates when surfactants are used. The common emerging theme emerging is that a number of factors can affect the successful application of surfactants for improving PAH degradation, so use of surfactants must first be trailed for any particular system before being used for bioremediation purposes. Nearly all of the previous work with surfactants involves low molecular weight PAHs, however, in recent times the improved degradation rate of some four-benzene ring PAH has also been achieved by surfactant application. Considering the low aqueous solubility of five benzene-ring PAHs and their partitioning into hydrophobic regions in soil, it would seem appropriate to evaluate the effectiveness of using surfactants to improve the bioavailability of such PAHs for microbial degradation.

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CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 SOIL SAMPLES

3.1.1.1 PAH-Contaminated Soils Used for Enrichment and Isolation of PAH-degrading Microorganisms

Two samples of contaminated soils, used for enrichment and isolation of the PAH-degrading microorganisms studied in this thesis, were provided by Albert Juhasz, Centre for Bioprocessing and Food Technology (CBFT), Victoria University of Technology (VUT), Australia. One soil, designated as soil A, was collected from an abandoned factory site located near Port Melbourne, Victoria, Australia. The site had previously housed a gas manufacturing plant operation and recently was used as a defence facility site. Another soil sample, designated as soil B, was collected from a creosote-contaminated site in Warracknabeal, Victoria, Australia.

3.1.1.2 Soils Used for PAH Degradation Studies

(a) Soil with No Known PAH Contamination

A sample of soil which had no known history of PAH contamination was provided by Albert Juhasz, CBFT, VUT, and this came from St Albans, Melbourne, Victoria. This soil was used to prepare the PAH-spiked soil (Section 3.2.4.5) for the investigations into PAH degradation in soils. The physical and chemical characteristics of this soil were analyzed by National Laboratories Pty. Ltd., Melbourne, Australia (Table 3.1).

Table 3.1 Characteristics of the soil with no known PAH contamination which was used to prepare PAH-spiked soil^a.

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
Calcium, 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
Nickel, ppm	19
Zinc, ppm	32
Cation exchange capacity	29

^a Analysis using GC-FID could not detect PAH compounds in the soil.

Table 3.2 Physical and chemical characteristics of the PAH-contaminated soil.

Parameter	Value
Chemical characteristics:	
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:	
C6-C9, ppm	66
C10-C14, ppm	350
C15-C28, ppm	6,700
C29-C36, ppm	1,300
BTEX:	
Benzene, ppm	0.07
Toluene, ppm	0.12
Ethylbenzene, ppm	0.03
Xylene, ppm	0.28
Total PCBs, ppm	< 0.1
Chlorinated hydrocarbons:	
Trichloromethane, ppm	0.007
1,2-Dichloroethylene, ppm	0.006
Polycyclic aromatic hydrocarbons (PAHs):	
Naphthalene, ppm	186.2
Acenaphthalene, ppm	42.7
Fluorene, ppm	86.9
Phenanthrene, ppm	156.0
Anthracene, ppm	53.3
Fluoranthene, ppm	136.6
Pyrene, ppm	98.6
Benz[a]anthracene, ppm	32.7
Benzo[a]pyrene, ppm	15.1
Dibenz[a,h]anthracene, ppm	12.4

(b) PAH-contaminated Soil

A PAH-contaminated soil, used to test the biodegradation capacity of microbes isolated from soils A and B was supplied by Dr Brent Davey, Australian Defence Industry, Environmental Services, Melbourne, Australia. This soil was obtained from a petroleum-contaminated site in Sydney, Australia. The physical and chemical characteristics of this soil were analyzed by National Laboratories Pty. Ltd., Melbourne, Australia (Table 3.2).

3.1.2 MICROORGANISMS

The microorganisms used in this thesis are listed in Table 3.3.

Table 3.3 Microorganisms used in this thesis.

Organism	Strain ^a	Trait
Bacterial consortium	VUN 10,008	
Bacterial consortium	VUN 10,009	
<i>Stenotrophomonas maltophilia</i>	VUN 10,010	PAH degraders; natural isolates ^b
<i>Stenotrophomonas maltophilia</i>	VUN 10,012	
<i>Pseudomonas fluorescens</i>	VUN 10,011	
<i>Burkholderia</i> sp.	VUN 10,013	
<i>Penicillium janthinellum</i>	VUN 10,201	
<i>Salmonella typhimurium</i> TA100	VUN 0041	Tester strain for the Ames test ^c

^a Victoria University (VU) Numbering System.

^b All PAH-degrading microorganisms used in this thesis were isolated from PAH-contaminated soils as described in Section 3.2.2.2.

^c See Chapter 2, Table 2.6.

3.1.3 CHEMICALS

3.1.3.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were purchased from Sigma Chemical Company (St Louis, MO, USA); fluorene, chrysene and coronene were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). All PAHs were high purity grade. Reagent-grade [4,5,9,10-¹⁴C]pyrene (58.7 mCi/mmol, radiochemical purity > 98%) and [7-¹⁴C]benzo[*a*]pyrene (26.6 mCi/mmol, radiochemical purity > 98%) were purchased from Sigma Chemical Company.

3.1.3.2 Surfactants

All synthetic surfactants used in this thesis, *viz.* nonionic surfactants, Brij 35 Igepal CA-630, Tergitol NP-10, Triton X-100, Tyloxapol, and the Tween series (Tween 20, Tween 40, Tween 60 and Tween 80); anionic surfactant, sodium dedocyl sulfate (SDS); and cationic surfactant, cetyltrimethyl-ammoniumbromide (CTAB), were obtained in laboratory grade from Sigma Chemical Company (St Louis, MO).

3.1.3.3 Solvents

Dichloromethane (DCM), dimethylformamide (DMF), dimethylsulfoxide (DMSO), methanol and other solvents, except where specified, were obtained in analytical grade from BDH Laboratory Supplies (Poole, England).

3.1.4 BACTERIOLOGICAL MEDIA

Bacteriological media, including nutrient agar, nutrient broth, yeast extract, beef extract, peptone and agar were purchased from Oxoid (Unipath Ltd, Hampshire, England). Potato dextrose agar and malt extract were obtained from Difco

Laboratories (Difco Laboratories, Detroit, MI). The preparation of some media used in this thesis is described in Section 3.2.1.

3.2 METHODOLOGY

3.2.1 MEDIA PREPARATION

Unless specified in this section, the preparation of media used in this thesis is described in appendices A, B and D.

3.2.1.1 Basal Salt Medium (BSM)

This medium used for enrichment of PAH-degrading microorganisms and PAH degradation studies.

Formula per litre:

Basal salt solution ^a	985	ml
Trace element solution ^b	5	ml
Vitamin solution ^c	5	ml
Mg/Ca solution ^d	5	ml

Trace element, vitamin, and magnesium/calcium solutions were filter sterilized (0.22 µm, Sartorius AG, Germany) and added to the basal salt solution after autoclaving. To prepare BSM agar, 15 g of bacteriological agar was added to BSM solution before sterilization.

^a**Basal salt solution** (*formula per litre*)

K ₂ HPO ₄	0.4	g
KH ₂ PO ₄	0.4	g
(NH ₄) ₂ SO ₄	0.4	g
NaCl	0.3	g

^bTrace element solution (formula per litre)

FeSO ₄ ·7H ₂ O	200 mg
ZnSO ₄ ·7H ₂ O	10 mg
MnCl ₂ ·4H ₂ O	3 mg
CoCl ₂ ·6H ₂ O	20 mg
CuCl ₂ ·2H ₂ O	1 mg
NiCl ₂ ·6H ₂ O	2 mg
NaMoO ₂ ·2H ₂ O	500 mg
H ₃ BO ₃ (boric acid)	30 mg

^cVitamin solution (formula per 100 ml)

Biotin	2 mg
Folic acid	2 mg
Thiamine HCl (B ₁)	5 mg
D-calcium pantothenate	5 mg
Vitamin B ₁₂	5 mg
Riboflavin (B ₂)	5 mg
Niacin (nicotinic acid)	20 mg
Pyridoxal HCl	3 mg
<i>p</i> -aminobenzoic acid	2 mg

^dMg/Ca solution (formula per 1 litre)

MgSO ₄ ·7H ₂ O	0.4 g
CaCl ₂ ·2H ₂ O	0.4 g

3.2.1.2 Basal Salts Medium Supplemented With Glucose (BSMG)

This medium was used in the investigations on co-metabolic PAH degradation by fungi. Glucose from a 10% sterile (0.2 µm filtered) stock solution was introduced to sterile BSM to obtained final concentrations of 0.1%, 1%, 1.5% and 2%.

3.2.1.3 Malt-Yeast Extract Peptone Dextrose Medium (MYPD)

This medium was used in the investigations on PAH degradation by fungi.

Formula per litre:

Malt extract	3	g
Yeast extract	3	g
Peptone	5	g
Dextrose	10	g
Distilled water	1	l

The ingredients were dissolved in distilled water, the pH adjusted to 6.0 and this was sterilized by autoclaving for 15 min at 121°C.

3.2.1.4 Nutrient Broth (NB)

This medium used for the enumeration of bacterial cells (MPN method).

Formula per litre:

Nutrient broth powder (Oxoid)	13	g
Distilled water	1	l

Nutrient broth powder was dissolved in 1 litre of distilled water and sterilized by autoclaving for 15 min at 121°C. To prepare agar medium (NA), 15 g of bacteriological agar was added to the broth solution before sterilization.

3.2.1.5 Peptone Water (0.1%)

Formula per litre of distilled water:

Bacteriological Peptone	1.0	g
NaCl	0.5	g

3.2.1.6 Peptone-Glucose Medium

This medium was used for the determination of surfactant toxicity by the agar diffusion method.

Formula per litre:

Peptone	5	g
Glucose	5	g
NaCl	0.5	g
Beef extract	3	g
Agar	20	g

The ingredients were dissolved by boiling, the pH adjusted to 7.0 and sterilized by autoclaving for 15 min at 121°C.

3.2.1.7 Potato Dextrose Agar (PDA)

This medium was used for growing fungal culture and the production of fungal spores. It was also used for the maintenance of fungal stock cultures for routine work.

Formula per litre:

Potato dextrose agar powder (Difco)	39	g
Distilled water	1	l

PDA powder was dissolved in distilled water and autoclaved for 15 min at 121°C.

3.2.1.8 Ringer's Solution

One Ringer's solution tablet (BDH Laboratory Supplies, Poole, England) was dissolved in 500 ml of distilled water; this yields a quarter strength Ringer's solution. This solution was sterilized by autoclaved for 15 min at 121°C.

3.2.2 MICROBIOLOGICAL METHODS

3.2.2.1 Enrichment of PAH-degrading Microorganisms

Enrichments using soils A and B and the isolation of PAH-degrading bacteria was carried out using, as the selective pressure, single PAH compounds (containing from three- to seven-ring compounds) supplied as a sole carbon and energy source. Firstly, a microbial consortium was enriched by shaking 20 g (wet weight) of PAH-contaminated soil overnight in a 100 ml volume of Ringer's solution at 30°C and 175 rpm, then transferring 5 ml of the supernatant into 45 ml of BSM containing a PAH (100 mg l⁻¹ for fluorene, phenanthrene and pyrene; 50 mg l⁻¹ for chrysene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]-anthracene; and 25 mg l⁻¹ for coronene). Cultures were incubated at 30°C, 175 rpm, until microbial growth was visible, then a portion of the culture was transferred to fresh PAH-containing BSM using an inoculum size of 10 % (v/v). Cultures were subjected to several successive transfers (four successive transfers) to ensure that the microbial growth did not arise due to the carry over of soil nutrients.

3.2.2.2 Isolation of PAH-degrading Bacteria

After several successive subcultures, pure cultures were isolated by the spray plate method (Kiyohara *et al.*, 1982). A series of ten-fold dilutions of pyrene-enriched culture were prepared in Ringer's solution and spread on BSM agar (BSM plus 1.5% bacteriological agar). The plates were then sprayed with a 2 % ethereal solution of pyrene. After the ether had evaporated, the plates were sealed with paraffin and incubated at 30°C. Pyrene-degrading cultures were visualized by a distinct clear zone surrounding individual colonies. Single colonies were purified and selected isolates were stored in pyrene-glycerol medium at -20°C after assigning a Victoria University culture collection number.

3.2.2.3 Isolation of PAH-degrading Fungi

The benzo[*a*]pyrene-enriched culture of soil B was ten-fold serially diluted and 0.1 ml was spread on PDA plates supplemented with filter-sterilized penicillin G (60 µg ml⁻¹) and streptomycin sulphate (100 µg ml⁻¹) plus benzo[*a*]pyrene (50 µg ml⁻¹). All plates were incubated at room temperature. After various time periods for growth (up to several weeks), fungal colonies were selected and re-plated onto the same medium until a pure culture was obtained. A pure single colony of representative fungi was selected for storage.

3.2.2.4 Storage of Microbial Isolates

All isolated organisms were maintained in the Victoria University culture collection. PAH-degrading bacterial strains were maintained in glycerol medium containing 50% glycerol in BSM supplemented with 100 mg l⁻¹ of pyrene, and stored at -20°C and -80°C. Stock cultures of the fungi were maintained on a slant of potato dextrose agar supplemented with benzo[*a*]pyrene (50 mg l⁻¹) and stored at 4°C; this was sub-cultured every two months. Freeze-dried stock cultures of bacteria and the fungus were also prepared (Romano, 1995) and maintained at -80°C. *S. typhimurium* strain TA100 (VUN 0041), used in the Ames mutagenicity studies, was obtained from the Victoria University culture collection. The stock cultures of the *Salmonella* strain was maintained in nutrient broth (Oxoid) containing DMSO (for each 1 ml of culture, added 0.09 ml of DMSO) (Maron and Ames, 1983).

3.2.2.5 Identification of PAH-degrading Microorganisms

(a) Bacteria

Pure isolates of PAH-degrading bacteria were identified based on the standard biochemical tests (see Appendix A for media and tests) and 16S rRNA gene analyses (Section 3.2.8).

(b) Fungal Isolates

A single species of fungus was isolated in this work and this was identified by Associate Professor Anne Lawrie, Department of Applied Biology and Biotechnology, The Royal Melbourne Institute of Technology University.

3.2.2.6 Preparation of Inocula

(a) Bacterial Inocula

PAH-degrading bacteria were grown in a 1-litre Erlenmeyer flask containing 500 ml BSM supplemented with pyrene (250 mg l⁻¹). Incubation was performed at 30°C/175 rpm until microbial growth reached the late exponential phase (4-5 days). If the experimental design required a concentrated cell inoculum, bacteria were cultured in a 15-litre fermenter (Applikon®, Dependable Instruments, Holland) using the same growth media and culture conditions. Cells were then harvested by centrifugation, washed with sterile BSM, and resuspended with BSM to concentrate the biomass by ten fold.

(b) Fungal Inocula

The fungal inoculum was prepared by growing *P. janthinellum* VUO 10,201 on PDA medium at 30°C for 7–10 days. On the 7th day, 25 ml of sterile MYPD was added to the plate with gentle agitation to suspend the spores. The total 10-ml volume of this suspension was used to inoculate a 500-ml flask containing 250 ml sterile MYPD broth. Incubation was performed for 48 hours in a shaking incubator at 30°C/175 rpm. After 48 hours, the tiny mycelial pellets were filtered through a Whatman no.1 filter paper, and then washed twice with sterile phosphate buffer (pH 7.0). If spore inocula were required, the fungal spore suspension was prepared by resuspending spores from a seven-day old fungal culture on PDA with 20 ml of sterile 0.1% peptone water or BSM.

(c) Killed Inocula

Unless stated otherwise, killed inocula served as controls in all PAH-degradation experiments conducted in this thesis and were prepared from high cell populations poisoned with HgCl_2 . Pyrene-grown cells were harvested and concentrated in BSM. Killed-bacterial cells were prepared by poisoning bacterial cultures with 0.2% HgCl_2 for 24 hours (30°C/175 rpm), and this was used to inoculate media or soils to achieve an initial cell density of approximately 10^6 cells ml^{-1} (or per g soil). To prepare killed fungal inocula, fungal mycelia grown in MYPD (2-day-old) were collected by filtration (Whatman No.1) and washed twice with sterile phosphate buffer (pH 7.0). These mycelia were then resuspended in sterile BSM containing 0.7% HgCl_2 and incubated for 24 hours (30°C/175 rpm).

- This was used to inoculate into media or soils to obtain an initial inoculum size of 0.04 g mycelia (wet wt) ml^{-1} (or per g soil).

3.2.2.7 Bacterial Growth on Various Carbon Substrates

Growth of PAH-degrading bacteria, which utilize pyrene as a sole carbon and energy source, was tested on a variety of carbon sources. Various compounds as listed in Table 3.3 were added into BSM as a sole carbon and energy source at a final concentration of 50 mg l^{-1} . Volatile compounds were supplied to the cultures in the vapor phase, *i.e.* a small test tube containing the compound solution was placed in the flask containing inoculated media. Pyrene-grown cultures (100 ml) from exponential phase was harvested by centrifuging at 4,000 rpm for 5 min, and washed twice with sterile BSM. The cell pellet was then resuspended in BSM to concentrate the biomass by ten fold and used to inoculate (1 ml) respective media to achieve an initial cell population of approximately 10^3 cells ml^{-1} . Uninoculated BSM containing a respective test compound and inoculated BSM without a test compound served as controls. All cultures were incubated at 30°C/175 rpm for up to 14 days, and growth was determined by visual monitoring as well as protein determination (Section 3.2.3.2) of samples from the first and last days of incubation.

3.2.2.8 Microbial Growth on Surfactants

Microbial growth on anionic, cationic and nonionic surfactants was conducted in BSM (10 ml) containing each surfactant as a sole carbon source (0.1 g l^{-1}). Starter cultures of bacteria or fungal spores were prepared as described in Section 3.2.2.6. Bacterial growth was monitored by observing changes in turbidity and samples were taken to determine the protein concentration (Section 3.2.3.2). Fungal growth was determined by the evidence of spore germination and/or the further development of mycelia.

3.2.2.9 Surfactant Toxicity Tests

The agar diffusion method was used to determine the effect of surfactants on bacterial growth by observing zones of inhibition on peptone-glucose agar (Section 3.2.1.6). Bacterial cultures were mixed with the molten medium (25 ml) at $10^8 \text{ cells ml}^{-1}$ and this was poured into a Petri dish. Once solidified, 7 mm diameter wells were made and 150 μl of aqueous surfactant solution was added ($0.1\text{-}10.0 \text{ g l}^{-1}$). Growth inhibition zones were observed after 48-72 hours at 30°C . The minimum inhibitory concentration (MIC) was taken to be the lowest surfactant concentration which resulted in a zone clear of bacterial growth around the wells containing the surfactant solution.

The effect of surfactant on the bacterial growth on pyrene was also determined by first incubating BSM (9 ml) containing pyrene (250 mg l^{-1}) overnight ($30^\circ\text{C}/175 \text{ rpm}$) to obtain a saturated aqueous pyrene concentration. Surfactant (0.1 g l^{-1}) was then added to this medium which was subsequently inoculated with pyrene-grown cells (*ca.* $10^3 \text{ cells ml}^{-1}$). Growth was monitored by measuring protein concentration and viable cell numbers at regular intervals up to ten days. Viable cell numbers were determined by most probable number (MPN) estimates (Alexander, 1965; Collins *et al.*, 1989).

The toxicity of surfactants on fungal growth was performed in MYPD medium supplemented with surfactant at concentrations between 0.1-20 g l⁻¹. This medium (50 ml) was inoculated with fungal spores (approximately 10⁶ spores ml⁻¹) and incubated at 30°C/175 rpm up to 14 days. In addition to MYPD broth, surfactant toxicity on the fungus was also tested on MYPD agar containing a surfactant (0.1-20 g l⁻¹). In the latter case, a small mycelial mat (seven-day old culture) was inoculated onto the agar medium and incubation was performed at 30°C for 14 days. In both cases, media without surfactant addition served as a control. Effects of surfactants on fungal growth was determined by comparing the dry biomass or colony size obtained from cultures containing surfactants relative to that observed with the controls.

3.2.2.10 Most Probable Number (MPN) Estimates

Most probable number estimates for bacteria were conducted in 96-well microtitre plates. This method was used to enumerate bacterial numbers from either liquid cultures or soils. Culture samples were ten-fold serially diluted in 0.1% peptone water. For soil samples, 1 g of soil was suspended in Ringer's solution (9 ml) and mixed by vortexing. After settling of the solid soil particles, 1 ml of supernatant was removed for preparing a series of ten-fold dilutions. A volume (100 µl) from each dilution was inoculated into each of five replicate wells containing 100 µl of double strength nutrient broth. All cultures were incubated at 30°C for 2-7 days and growth was visualized by the appearance of turbidity compared to the controls (uninoculated medium and peptone-water inoculated medium). The number of positive wells in each set of three or five were tabulated and the MPN of bacteria estimated from the MPN table (Appendix E, Table E.1).

3.2.2.11 Determination of Fungal Biomass

Dry weight was used to determine the fungal biomass. Cultures were filtered using Whatman No.1 filter paper which pre-dried to a constant weight at 105°C. The mycelia were washed with approximately 200 ml of deionized water and then

dried at 105°C until a constant weight was achieved. To allow for additional weight from organic and inorganic components in the media, including possible bacterial biomass in the case of co-culture experiments, a respective PAH medium (not containing the fungus) was similarly filtered and its dry weight subtracted from the fungal dry weight measurements.

3.2.3 BIOCHEMICAL METHODS

3.2.3.1 Determination of Dioxygenase Activity

Dioxygenase activity in bacteria is indicated by the formation of indigo from indole in bacterial cultures. The method used to detect the activity of dioxygenase enzymes was based on those described by Ensley *et al.* (1983). Bacteria were grown at 30°C/175 rpm in BSM containing fluorene (100 mg l⁻¹), phenanthrene (250 mg l⁻¹) or pyrene (250 mg l⁻¹). Cultures were grown until late exponential phase where cells were harvested by centrifugation and resuspended in fresh PAH-medium containing 2 mM indole. Cultures were incubated at 30°C on a rotary shaker at 175 rpm. The time required for the appearance of blue indigo was noted and the synthesis of indigo was monitored by removing 1 ml of the culture fluid at various time intervals and extracting twice with equal volumes of ethyl acetate. The organic phases were combined and the absorbance of the ethyl acetate solution at 600 nm was determined. The concentration of indigo were taken from a linear standard curve ($R^2 > 0.985$) for synthetic indigo (Sigma) (concentration range of 0-10 µg ml⁻¹).

3.2.3.2 Protein Concentration Assay

The growth of bacteria was evaluated by protein determination according to the methods described by Lowry and co-workers (1951). All buffers and reagents used for the protein concentration assay are described in Appendix C. Bacterial cells were harvested from duplicate cultures by centrifugation for 10 min at 4,000 rpm/4°C, and the cell pellet was resuspended in 1 ml of 4.6 M NaOH and held

overnight. The NaOH cell suspension was heated at 90°C for 10 min, and 50 µl of heated sample was ten-fold diluted in 0.45 M NaH₂PO₄ buffer (450 µl), and then mixed briefly by vortexing for 10 sec. Bovine serum albumin (BSA), which was used as a protein standard, was prepared at various concentrations (0-100 µg ml⁻¹) in either distilled water or BSM solution, and simultaneously submitted to the reaction assay. To 0.5 ml of protein sample, 2.5 ml of the alkaline-copper reagent (Lowry C reagent, see Appendix C for the preparation of buffers and reagents for the Lowry assay) was added, followed by vortexing and then standing at room temperature for 10 min. After adding 0.25 ml of diluted Folin-Ciocalteu reagent (2:3 in deionized water), the contents were mixed thoroughly and allowed to stand at room temperature for another 30 min. The absorbance at wavelength of 750 nm was read for each sample. The concentrations of protein were taken from a linear standard curve ($R^2 > 0.99$) for BSA (concentration range of 0-100 µg ml⁻¹).

A slight modification of the method for the determination of cell protein in medium containing surfactant was conducted based on the procedures described by Lowry *et al.* (1951), as well as Wang and Smith (1975). Cells were prepared and the assay procedures were carried out as described above. After 2.5 ml of the Lowry C reagent was added, the mixture was mixed briefly on a vortex mixer and allowed to remain at room temperature for 10 min. Subsequently, 0.25 ml of 10% SDS solution was added and the contents of the tube were mixed thoroughly on a vortex mixer. Finally, 0.25 ml of diluted Folin-Ciocalteu reagent (1:1 in deionized water) was introduced and mixed as above. The reactions were permitted to stand for 30 min prior to spectrophotometric analysis at 750 nm.

3.2.4 PHYSICO-CHEMICAL METHODS

3.2.4.1 Preparation of PAH-stock Solutions

Stock solutions of each PAH compound and PAH mixture were prepared in dimethylformamide (DMF). In enrichment procedures, the stock solution of each PAH compound was introduced into BSM to achieve final concentrations of 100

mg l⁻¹ for fluorene, phenanthrene, pyrene and fluoranthene; 50 mg l⁻¹ for benz[a]anthracene, chrysene, benzo[a]pyrene and dibenz[a,h]anthracene; 20 mg l⁻¹ for coronene. In PAH degradation experiments containing a single PAH, the stock solutions of individual PAHs were added to BSM to achieve a final concentration of 250 mg l⁻¹ for phenanthrene and pyrene; 100 mg l⁻¹ for fluorene; 50 mg l⁻¹ for fluoranthene, benz[a]anthracene, chrysene, benzo[a]pyrene and dibenz[a,h]anthracene; 20 mg l⁻¹ for coronene. In experiments containing a PAH mixture, BSM was supplemented with a mixed-PAH stock solution to attain a final concentration of 100 mg l⁻¹ for phenanthrene and pyrene; 20 mg l⁻¹ for fluorene; 10 mg l⁻¹ for fluoranthene, benz[a]anthracene, chrysene, benzo[a]pyrene and dibenz[a,h]-anthracene; and 5 mg l⁻¹ for coronene.

3.2.4.2 Preparation of Surfactant-stock Solutions

Surfactant stock solutions were prepared in either deionized water or BSM at a concentration of 20 g l⁻¹. The solutions were sterilized by autoclaving at 121°C for 20 min and the sterile solutions were allowed to equilibrate at ambient temperature for 48 hours before their use in any further experiments.

3.2.4.3 Determination of Soil Water Holding Capacity

Portions of uncontaminated soil (Section 3.1.1.2) and PAH-contaminated soil were dried at 150°C until a constant weight was obtained and 100 g of dried soil was removed and placed in a dry container. Water was gradually added to the soil sample; with thoroughly mixing until water could not be further accommodated by the soil particles. The water holding capacity of each soil sample was evaluated by either weighing the soil (containing the water) or by registering the volume of added water (Albert Juhasz, personal communication).

3.2.4.4 Adjustment of Soil Moisture

For all soil experiments, unless stated otherwise, the water content of all samples was adjusted to approximately 65% of the soil water holding capacity.

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For all soil experiments, unless stated otherwise, the water content of all samples was adjusted to approximately 65% of the soil water holding capacity.

3.2.4.5 Preparation of PAH-Spiked Soil

PAH-spiked soil was prepared by spiking the uncontaminated soil (Section 3.1.1.2) with a stock solution of a defined PAH mixture, prepared in DCM, to attain a final concentration of 100 mg kg⁻¹ soil for fluorene; 250 mg kg⁻¹ soil for phenanthrene and pyrene and 50 mg kg⁻¹ soil for fluoranthrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene, and 25 mg kg⁻¹ soil for coronene. The soil was well-mixed to form a homogenous mixture. The homogeneity of the mixture was confirmed before commencing experiments by testing the PAH concentration of five random portions of the soil. The relative standard deviation obtained between the PAH concentrations in five samples was less than 2%.

3.2.4.6 Critical Micelle Concentration (CMC) Determination

Experiments to evaluate surfactant CMC values were conducted at 30°C using a duNouy ring tensiometer (Cambridge Instrument Company Limited, London, England). Various concentrations of surfactant solutions were prepared in deionized water and BSM. Surfactant solutions were allowed to equilibrate for approximately 48 hours before measurements were made. All glassware was cleaned with chromic acid solution and the tensiometer ring was cleaned with acetone and heated to redness in a gas flame. Measurements were an average of five readings (SD < 0.3%). The CMC value was taken from the plot of surface tension versus log surfactant concentration as described by Kile and Chiou (1989).

3.2.4.7 Determination of PAH Solubilization in Liquid Media

PAHs (approximately 1 g l⁻¹) were added to a 10 ml solution of BSM plus surfactant in a 30-ml glass reaction vial, which was sealed with an aluminum cap

fitted with a septum. Surfactant concentrations corresponded to levels above, below, or at the CMC and replicates were set up to allow sampling in duplicate at different time intervals up to 72 hours at 30°C/175 rpm. The entire vial contents was collected by syringe and expressed through a preconditioned membrane filter (0.22 μm) to remove solid-phase crystalline PAH while allowing solubilized PAH in the aqueous phase and micellar pseudophase to pass into sterile vials. Filter preconditioning involved purging the filter with a few milliliters of sample solution to allow sorption saturation of the internal surfaces of the syringe, filter and needle. The filtrates were extracted with dichloromethane (DCM) and the concentration of solubilized PAH was determined by GC-FID (Section 3.2.6.2).

3.2.4.8 Desorption of PAHs from Soils

Pyrene and benzo[a]pyrene were used as model compounds in soil-water systems. Firstly, an equilibrium slurry was prepared by using 250-ml Erlenmeyer flasks containing 250 mg kg^{-1} soil of pyrene or 50 mg kg^{-1} soil of benzo[a]pyrene, 500,000 dpm labeled PAH compound, and 25 g of air-dried, sterile, sieved soil. The flasks were sealed with rubber stoppers and incubated in the dark at 30°C on a rotary shaker operating at 45 rpm. Preliminary experiments showed that equilibrium between the solid and solution phases was attained after approximately 48-72 hours.

To determine the effect of surfactants on the rates of desorption, 10 ml of surfactant solution, that had been sterilized by passage through 0.2- μm pore-size membrane filter units, was added aseptically in duplicate to 30-ml reaction vials. After the slurry equilibrium was reached, an amount of particulate matter corresponding to 1 g of air-dried soil was aseptically removed from the soil slurries and added to each of the flasks containing surfactant solution. These flasks were then incubated in the dark at 30°C on a rotary shaker operating at 45 rpm. After 0.5 hours and specified intervals thereafter, 2-ml aliquots were removed and filtered (through 0.2- μm membrane filter) to remove soil particulates. Filtration rather than centrifugation was used to avoid the possibility of rapid re-adsorption of PAHs to soil materials. Fresh filter-sterilized surfactant solution at the

appropriate concentration was introduced to replace the withdrawn sample. The filtrate (1 ml) was mixed with 9 ml of CytoScint™ liquid scintillation cocktail in a 10-ml scintillation vial, and the radioactivity was determined using a liquid scintillation counter (LSC). The counts were obtained in disintegration per minute (DPM) which was related to the proportion of the total mass of PAH in the sample. The results were expressed as percentage desorption of the PAH, *i.e.* the fraction of the total PAH in the aqueous phase:

$$\text{Desorption (\%)} = \frac{(\text{Measured sample DPM} - \text{Sample blank DPM})}{\text{Sample volume}} \times \frac{V_1 \times 100}{\text{Total DPM}}$$

where; 'sample volume' is the counted volume of the aqueous surfactant phase; V_1 is the total volume of the aqueous phase which was 10 ml, and the 'total DPM' refers to the total radioactivity applied to the sealed vials. The amount of PAH adsorbed on the soil was the difference between total added PAH and the desorbed PAH; losses through volatilization and sampling were negligible, as verified in a separate test which showed no significant changes during treatment.

3.2.4.9 PAH Extraction

The efficiency of the PAH extraction method was evaluated by comparing the amount of PAH and internal standard (2,3-benzo[b]fluorene) recovered from liquid cultures or PAH-spiked soil to the known amount of these compounds which was added to the media before extraction; these tests were performed in the presence and absence of killed-microbial inocula. Sequential DCM extraction using three successive repeats were conducted under certain circumstances (as detailed in the following subsections) and PAH concentrations were measured after each extraction. Based on the high recovery and reproducibility of the data obtained, this thesis employed a vigorous shaking procedure for PAH extraction from bacterial cultures in BSM, ultrasonication extraction for bacterial culture containing a surfactant and three successive sequential ultrasonicated extractions

for PAH extraction from fungal cultures and soils. The efficiency of the extraction method used in this thesis showed a high percentage of PAH recovery (see Chapter 5, Section 5.2 and Chapter 6, Sections 6.2 and 6.8.1 for the results of PAH extraction efficiency).

(a) Extraction of PAHs from Liquid Cultures

After adding 2,3-benzo[b]fluorene (2,3-BF) as an internal standard (100 μl of a stock solution containing 1 mg ml^{-1} in DCM) and DCM (1 ml), bacterial cultures (originally 10 ml volume) were vigorously shaken for 20 sec and allowed to stand at room temperature 1-2 hours before freezing overnight at -20°C . Ultrasonication was performed for bacterial cultures containing surfactant: experimental samples were placed on ice and sonicated (output control of seven and a duty cycle of 50%) for 30 seconds using a 1/8" tapered microtip attached to a 1/2" horn (Branson Sonifer 450); the total sonication time for each sample was 3 min (6 x 30 sec). The sonicated samples were shaken vigorously and allowed to settle at room temperature for 1-2 hours after which time two layers were visible; an upper water phase and the lower organic phase. At this stage, a large emulsion layer formed between phases, which made phase separation impossible. This was circumvented by freezing the biphasic solution overnight at -20°C . After thawing, the vial was inverted and approximately 500-800 μl of the heavier organic phase was withdrawn from the bottom using a 1 ml glass syringe (SGE, Australia).

To extract PAHs from samples containing fungal inocula, sequential extraction with ultrasonication was employed. After adding the internal standard, cultures were extracted with an equal volume of DCM using ultrasonication as described above for the extraction of surfactant-containing sample. The extract was separated from the mycelial debris by filtration through glass wool. Mycelial extraction was repeated twice, the extracts were filtered and pooled in a separating funnel. This was subsequently shaken vigorously and the phases allowed to separate. The DCM phase was collected and dried over anhydrous sodium sulfate. The DCM extract was evaporated on a rotary evaporator to

reduce the volume to approximately 1 ml. PAH concentrations in the extract were measured using gas chromatography with flame ionization (GC-FID).

(b) Extraction of PAHs from Soil

Ultrasonication Extraction

PAHs were recovered from soil using the method 3550A, ultrasonic extraction of nonvolatile and semi-volatile organics from solids, described by the EPA (1992). The PAHs in the soil were extracted three times in succession using 4.5 ml total volume of DCM. Anhydrous sodium sulfate (1 g) was added to soil samples (1 g) and mixed thoroughly. After adding an internal standard (2,3-benzo[b]fluorene; 100 μ l of 1 mg ml⁻¹ in DCM) and DCM (2 ml), samples were placed on ice and extracted ultrasonically in the same manner as described in section 3.2.4.9 (a). Samples were extracted for a total sonication time of 3 min per extraction (6 x 30 sec). After the organic phase was removed, the extraction was repeated twice more. Extracts were mixed and cleaned by passing through columns containing glass wool or Whatman No.41 filter paper, and then dried over anhydrous sodium sulfate. PAH concentrations were determined by GC-FID.

Soxhlet Extraction

The standard Soxhlet extraction method was adopted to compare its PAH recovery efficiency with that obtained by the ultrasonic extraction method used for the extraction of PAHs in PAH-spiked soil. Soil was prepared for Soxhlet extraction according to the EPA method 3540B (EPA, 1992). The excess water was drained. Soil was weighed and mixed with anhydrous sodium sulfate in a 1:1 ratio and was then used for Soxhlet extraction. A sample quantity of 10 g was extracted with 300 ml of DCM for 16 hours. The extraction cycles around the thimble were approximately 6-8 cycles per hour. The extract was evaporated in a rotary evaporator. The residual PAH content was determined using GC-FID.

3.2.4.10 Extraction of PAH Intermediate Products

At various time intervals, triplicate samples from PAH degradation experiments were sacrificed for metabolite extraction. In the case of cultures containing fungal inocula, samples were subjected to ultrasonication prior to extraction. The contents from each reaction vial (10 ml) were pooled in 100 ml-separating funnels and extracted twice with an equal volume of DCM. Subsequently, the pH of the contents were adjusted to 2.5 by adding a few drops of concentrated HCl and further extracted twice more with an equal volume of DCM. The organic extracts were pooled and dried over anhydrous Na_2SO_4 . The DCM phase was evaporated to partial dryness with a rotary evaporator and then dried completely under nitrogen gas. Dried samples were re-dissolved in methanol for HPLC analysis (see Section 3.2.6.3) or in DMSO for the Ames test (see Section 3.2.7).

3.2.5 PHYSIOLOGICAL STUDIES

3.2.5.1 Incubation Conditions

Unless stated otherwise, all experiments described in this section were incubated in the dark at 30°C and 175 rpm (for liquid samples) or at 25°C without shaking (for soil samples).

3.2.5.2 Biodegradation of Single PAHs and Defined PAH mixtures in Liquid Media

PAH degradation experiments using axenic bacterial or fungal cultures, and fungal-bacterial co-cultures, were performed in reaction vials (30 ml) containing 10 ml of medium. Before inoculation, 0.1 ml PAH of stock solution (either a single PAH or mixed PAH stock as described in Section 3.2.4.1) was introduced into the medium. This medium was then inoculated with microorganisms to produce either axenic cultures or co-cultures. Axenic cultures were inoculated with either bacteria (1 ml) to provide an initial cell population of approximately 10^4 cells ml^{-1} , or mycelial pellets (0.25 g wet weight) in 10 ml of BSM. For the co-culture

experiments, BSM (10 ml) contained bacteria (10^4 cells ml^{-1}) and 0.25 g wet weight of mycelial pellets. All cultures were aseptically flushed with filtered (0.22 μm) air (0.2 litres per min) for 5 sec on the day of inoculation and every seven days thereafter. Abiotic controls were sterile medium containing only PAH(s). The entire culture (10 ml) was sacrificed for either the PAH-extraction procedure or biomass determination.

3.2.5.3 Co-metabolism Studies

(a) Co-metabolic Degradation of PAHs by Bacteria

Investigations into the co-metabolic degradation of PAHs using bacteria were performed in BSM supplemented with pyrene (250 mg l^{-1}) and another high molecular weight PAH. The bacterial inoculum was grown in BSM containing pyrene for 5 days. The volume of the inoculum was such as to give starting populations of approximately 10^3 cells ml^{-1} . Typically, the experimental cultures were conducted in triplicate, while abiotic and killed-cell controls were run in duplicate. Growth of bacteria was determined from MPN estimates.

(b) Co-metabolic Degradation of PAHs by a Fungus

Experiments were conducted to investigate PAH degradation by *P. janthinellum* VUO 10,201 in BSM supplemented with various concentrations of glucose (0.1%, 1%, 1.5%, and 2%), BSM plus 1% glucose and in MYPD medium using 0.25 g (wet wt) of 2-day-old mycelial pellets as inocula. PAHs tested were pyrene (250 mg l^{-1}), chrysene (50 mg l^{-1}), benz[a]anthracene (50 mg l^{-1}), benzo[a]pyrene 50 mg l^{-1}) and dibenz[a,h]anthracene (50 mg l^{-1}). PAHs were also incubated with killed-fungus controls (duplicate). All cultures were aseptically flushed with filtered (0.22 μm) air for 5 sec at 0.2 litres per min on the day of inoculation and every seven days thereafter. At designated time intervals, samples were sacrificed for analysis. The entire culture was utilized for the PAH-extraction procedure or biomass determinations. Fungal growth was determined by dry weight measurement.

3.2.5.4 Biodegradation of Crystalline and Surfactant-solubilized Pyrene

Crystalline or DMF-solubilized pyrene (250 mg l⁻¹ final concentration) was added to sterile BSM (9 ml) with or without addition of Tergitol NP-10 (10 g l⁻¹), then these broths were inoculated with approximately 10³ cells ml⁻¹ of VUN 10,010 from a pyrene-BSM parent culture. Three types of controls were used; cultures containing no surfactant, uninoculated broth and killed-cell cultures (0.2% HgCl₂).

3.2.5.5 Biodegradation of PAHs in the Presence of Nonionic Surfactants

- Evaluation of fluoranthene, benz[a]anthracene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene and coronene degradation by strain VUN 10,010 was performed in 30 ml reaction vials containing 10 ml of culture medium. An appropriate amount of selected surfactant was added to each vial containing BSM with the appropriate volume of PAH stock solution prepared as previously described (Sections 3.2.4.1 and 3.2.4.2). The experiments were started by inoculating each vial with 1 ml of washed pyrene-grown cells, to give initial cell populations of approximately 2 x 10⁶ cells ml⁻¹ (for testing single PAH degradation) or 9 x 10³ cells ml⁻¹ (for testing mixed-PAH degradation). Cultures without surfactant, uninoculated cultures (with and without surfactant) and killed cell cultures (with and without surfactant) were used as controls. The experimental cultures were conducted in triplicate and the abiotic controls were run in duplicate. Samples were sacrificed for analysis at designated time intervals during a 49 day incubation period.

3.2.5.6 PAH Degradation in PAH-spiked Soil

The PAH-spiked soil (Section 3.2.4.5) was distributed to sterile 1.5 litre jars, each jar receiving amounts equal to 200 g dry weight of soil. Bacteria were inoculated into this soil to give an initial bacterial population of approximately 10⁷ cells g⁻¹

soil. In the case of fungal-inoculated soil, mycelia were inoculated to attain an initial biomass of 25 g (wet wt.) kg^{-1} soil. Co-culture inoculated soils received 10^7 bacterial cells g^{-1} soil and 25 g (mycelial wet wt.) kg^{-1} soil. Killed-cell controls were soils inoculated with a respective killed inoculum. Abiotic controls were uninoculated soil containing a PAH mixture. Inoculated and uninoculated soil samples were supplemented with sterile BSM solution to approximately 65% of the soil water holding capacity. At designated time intervals, triplicate samples soil (1 g) from each jar were removed for analysis of PAH concentration and duplicate soil samples (1 g each) were sacrificed for MPN estimates.

3.2.5.7 PAH Mineralization

(a) PAH Mineralization in Liquid Media

PAH mineralization was measured by quantification of $[^{14}\text{C}]\text{CO}_2$ in duplicate biometers. One microlitre of $[^{14}\text{C}]\text{pyrene}$ (specific radioactivity of 1 mCi ml^{-1}) or $[^{14}\text{C}]\text{benzo}[a]\text{pyrene}$ (specific radioactivity of 1 mCi ml^{-1}) and unlabeled pyrene or benzo[*a*]pyrene (final concentration was 250 and 50 mg l^{-1} , respectively) were added to sterile BSM (100 ml) in a 250-ml biometric flask, which had a 50-ml fused side-armed flask containing 5 ml of 0.5 M NaOH (Figure 3.1). The side tube was sealed with a rubber stopper pierced by a 15-gauge needle; 15 cm long, which was used to withdraw samples for measuring the production of radiolabeled CO_2 . The top of the needle was sealed with a rubber cap which was removed only during sampling. Bacteria were then inoculated to start the incubation and the biometers were sealed tightly with rubber stoppers. Pyrene-grown cells of bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010 (*ca.* 10^3 cells ml^{-1}) were used as inocula. When the influence of surfactants was being evaluated, cultures containing no surfactant as well as abiotic medium and killed cell cultures (0.2% HgCl_2) served as controls. Mineralization of respective PAHs by *P. janthinellum* VUO 10,201 in BSM, BSM plus 1% glucose (BSMG) or MYPD was conducted in a manner similar to that described above. In this case, 2.5 g (wet wt.) of 2-day-old mycelia was used to inoculate 100 ml of medium. Co-culture

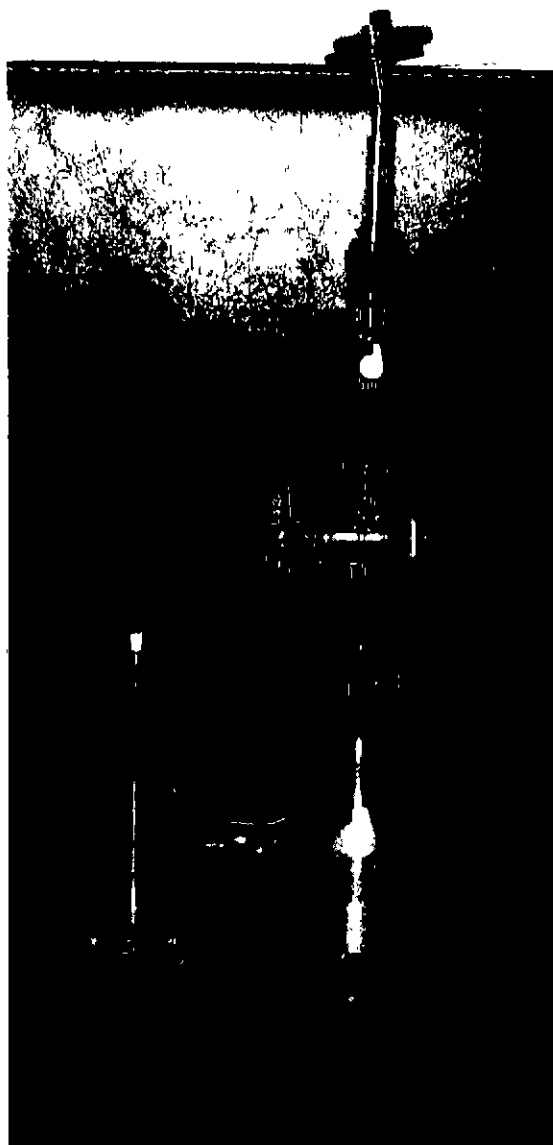


Figure 3.1 The biometric flask used for monitoring the evolution of carbon dioxide in PAH mineralization experiments.

mineralization experiments were conducted using an inoculum containing around 10^3 cells ml⁻¹ bacteria and 2.5 g (wet wt) of 2-day-old mycelia in 100 ml medium.

The evolved $^{14}\text{CO}_2$ was trapped in the NaOH solution. At regular time intervals, the whole alkali content in the side-armed tube was taken for $^{14}\text{CO}_2$ analysis, and the NaOH was replaced with the fresh alkali solution. $^{14}\text{CO}_2$ was measured according to the procedure described for monitoring mineralization of ^{14}C -labeled compounds in aqueous samples (Fedorak *et al.*, 1982). The removed NaOH (1 ml) was mixed with 9 ml of CytoScint™ scintillation cocktail (ICN, Research Product Division, Costa Mesa, CA) in 20-ml scintillation vials (Crown Scientific Pty., Ltd., Australia), and the radioactivity was determined as DPM using a liquid scintillation counter (LSC; Wallac 1410, Pharmacia).

(b) PAH Mineralization in Soils

Soil was moistened with sterile BSM to adjust the soil water content to around 65% of water holding capacity. Each flask received 10 g of soil spiked with 2.5 mg of pyrene, and 1 μl of [^{14}C]pyrene (1.0 mCi/ml) or 0.5 mg of benzo[*a*]pyrene and 1 μl of [^{14}C]benzo[*a*]pyrene (1.0 mCi/ml). The unlabeled compounds were added to the flasks in a DCM solution, and the DCM was allowed to evaporate before the addition of soil. The inoculum, prepared as described above (Section 3.2.2.6), was inoculated (approximately 0.1 ml) directly into the soil samples to obtain an initial bacterial population density of 10^7 cells g⁻¹ of air-dried soil. In flasks containing a fungal inoculum, soil was inoculated with the fungal mycelia (25 g [wet wt.] kg⁻¹ soil). The controls consisted of an uninoculated sample which relied on the indigenous microbial population to degrade pyrene or benzo[*a*]pyrene. For soil experiments involving surfactant addition, surfactant was applied at a final concentration of 2 mg g⁻¹ of air-dried soil, and the contents entirely mixed with a sterile glass rod. All biometer flasks and side-arms, which accommodated 5 ml of alkali solution, were then sealed with rubber stoppers and incubated in the dark at an ambient temperature for 30 and 100 days for pyrene and benzo[*a*]pyrene analysis, respectively. The evolved $^{14}\text{CO}_2$ which was trapped in the alkali

solution was measured as described above. In all cases, the percentage of label recovered as $^{14}\text{CO}_2$ was presented in terms of the cumulative CO_2 relative to the original amount of the radioactivity added.

(c) Mass Balance Analysis

Mass balance determinations for $[^{14}\text{C}]$ pyrene and $[^{14}\text{C}]$ benzo[*a*]pyrene was quantified as the percentage recovery of radioactivity in alkali solution, biomass and DCM-extractable fractions relative to the initial radioactivity supplied. $^{14}\text{CO}_2$ was measured according to the procedure described previously in (i) above. At the end of the experiment, cultures were acidified with a few drops of concentrated HCl to drive off the dissolved CO_2 from the culture media; this amount of CO_2 was trapped in the alkali solution and then was taken for the last $^{14}\text{CO}_2$ measurement. The percentage conversion of radiolabeled PAH to bacterial biomass was determined by pelleting cells followed by resuspension in water. To remove PAHs adsorbed to the biomass, the resuspended bacterial cells were extracted with DCM. After separation of the two phases by centrifugation (4,000 rpm/5 min), the radioactivity in the organic extract was quantified by LSC. Then an aliquot of the extracted, resuspended cell solution was mixed with CytoScintTM scintillation cocktail and quantified by LSC. The cell-free supernatant obtained after the initial pelleting of bacterial cells was quantified by LSC to determine the percentage of PAH converted into aqueous soluble products. This aqueous phase was also extracted with DCM, and the organic soluble ^{14}C -labelled products were quantified by LSC.

Fungal cultures were treated in a slightly different manner to the bacteria since the fungal mycelia was sonicated, to break up the mycelial clumps, for 30 sec over a 3 min sonication period using an ultrasonicator. The sonicated material was then extracted three times with 10 ml DCM portions; these three portions were subsequently pooled. Following DCM extraction, the mycelial debris was separated from the aqueous fraction by filtration through glass wool. CytoScintTM scintillation cocktail (9 ml) was then added to 1 ml portions of the DCM extract, the aqueous fractions and the entire mycelial fraction. Radioactivity was

quantified by LSC. Mass balance analysis was not performed for soil samples, only $^{14}\text{CO}_2$ evolution was determined.

3.2.5.8 PAH Degradation by Sequential Incubation with Fungi and Bacteria

Experiments were conducted to identify the catabolic role of the bacteria and the fungus during the co-culture degradation of high molecular weight PAHs. Figure 3.2 presents the schematic diagram of the experimental design used for the sequential degradation of benzo[a]pyrene. Axenic cultures of *S. maltophilia* VUN 10,010 (8×10^6 cells ml^{-1}) and *P. janthinellum* VUO 10,201 (0.04 g [wet wt.] ml^{-1}) were incubated separately in BSM containing radiolabeled benzo[a]pyrene (1.0 μCi) and non-labeled benzo[a]pyrene (50 mg l^{-1}) as a sole carbon and energy source. Parallel experiments were conducted in the same manner but only non-labeled benzo[a]pyrene was added to the inoculated BSM; these cultures were used for the extraction and HPLC analysis for benzo[a]pyrene metabolites. Killed cell inocula used in this experiment comprised of an autoclaved high cell population of either the bacteria (8×10^6 cells ml^{-1}) or the fungus (0.04 g [wet wt.] ml^{-1}). After 35 days of incubation, the evolution of $^{14}\text{CO}_2$ from these cultures was monitored (see Section 3.2.5.7) before the contents of the cultures were extracted with DCM and dried completely under nitrogen (see Section 3.2.4.11 for extraction method). HPLC analyses of the DCM extracts was performed according to the method described in the following section (Section 3.2.6.3). The dry extract of each culture was resuspended in DMF and added to a fresh BSM, and then inoculated with the opposite organism to that with which it was originally incubated (Figure 3.2), *eg.* the preparation of bacterial culture DCM extract was inoculated with *P. janthinellum* VUO 10,201 culture and the preparation of fungal culture DCM extract was inoculated with *S. maltophilia* VUN 10,010 culture. After 14 days, the distribution of radiolabeled carbon was examined for $^{14}\text{CO}_2$ evolution, ^{14}C incorporation into cellular biomass, polar metabolites in the aqueous phase, and non-polar products and non-degraded benzo[a]pyrene in the DCM phase (see Section 3.2.5.7 for methods).

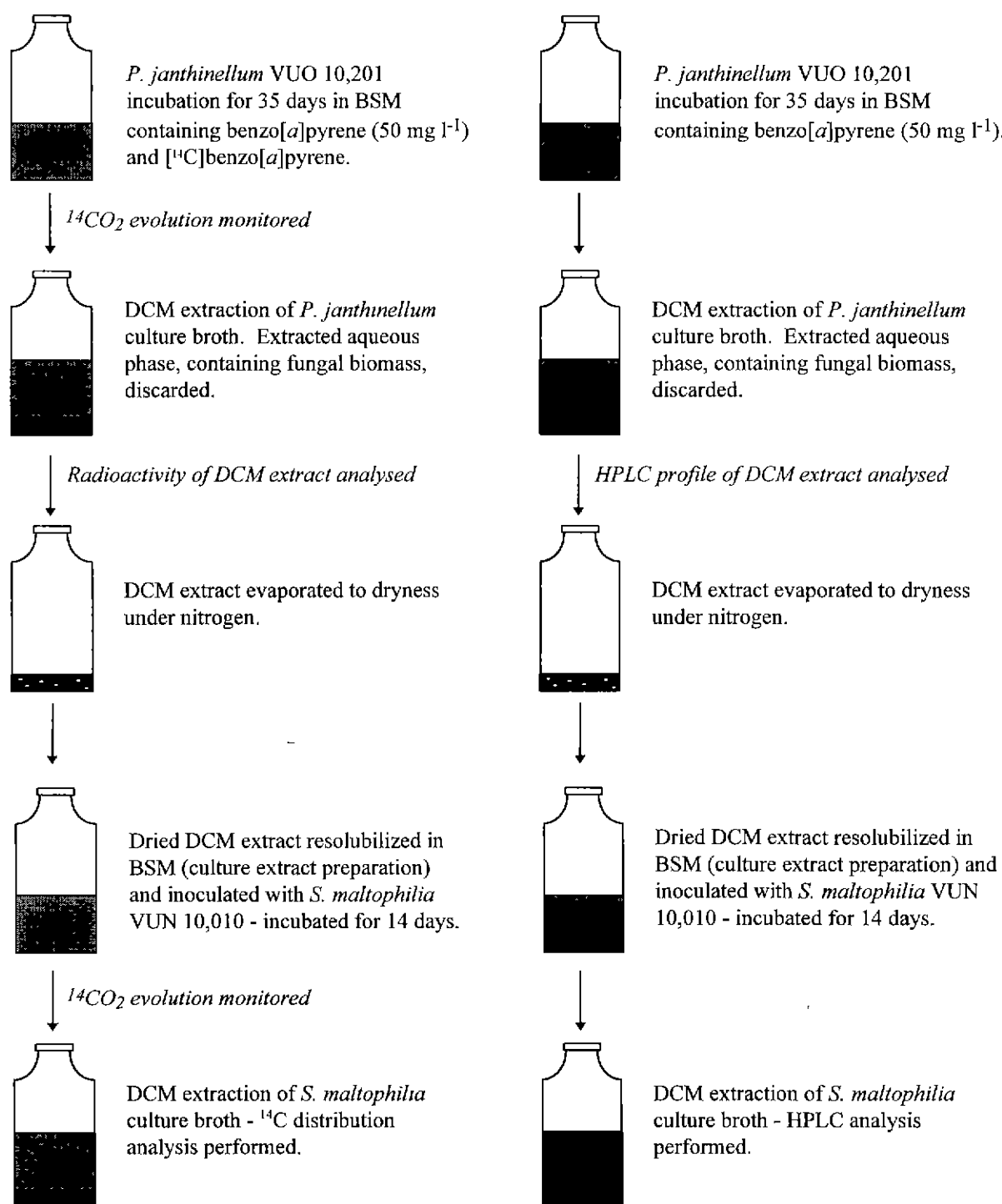


Figure 3.2 Schematic diagram of the protocol used for the sequential degradation of benzo[a]pyrene. The first incubation used *P. janthinellum* VUO 10,201 and the resulting culture extract preparation was subsequently inoculated with *S. maltophilia* VUN 10,010. Parallel incubations were conducted to measure ¹⁴C distribution from [¹⁴C]benzo[a]pyrene degradation (left hand sequence) and benzo[a]pyrene concentration (right hand sequence). The same protocol was used for another experiment in which the first incubation was conducted with *S. maltophilia* VUN 10,010 and the subsequent incubation used *P. janthinellum* VUO 10,201.

3.2.6 ANALYTICAL METHODS

3.2.6.1 Quantitative Determination of Phenolic Compounds

Folin-Ciocalteu phenol reagent was used to determine phenolic intermediate concentrations which accumulated during PAH degradation according to the method described by Box (1983). To 10 ml of sample, 1.5 ml of sodium carbonate solution (200 g l^{-1}) and 0.5 ml of Folin-Ciocalteu phenol reagent were added in succession. The reaction mixture was then incubated at 20°C for 60 min before the absorbance at 750 nm was measured. Readings for tests were corrected for color changes arising from BSM components. Resorcinol (BDH) was used as the standard. The concentration of phenolic intermediates were taken from a linear standard curve ($R^2 > 0.99$) for resorcinol (concentration range of $0\text{--}10 \text{ } \mu\text{g ml}^{-1}$) and expressed as microgram resorcinol equivalents per ml.

3.2.6.2 Gas Chromatographic (GC) Analysis of PAHs

Analysis of PAH concentrations in the extracts and of PAH standards was performed on a Varian Star 3400 gas chromatograph equipped with a flame ionization detector, using a BPX-5 capillary column ($25 \text{ m} \times 0.22 \text{ mm}$). The nitrogen carrier flow rate was 0.8 ml min^{-1} with a pressure of 20 psi under split conditions (95:1). The oven temperature was programmed at 200°C for 1 min, followed by a linear increase of $10^{\circ}\text{C min}^{-1}$ to 320°C , then this temperature was held for 10 min. Injector and detector temperatures were maintained at 300°C . Based on these conditions, the injection variation (duplicate injections of one sample) was shown to be less than 2%.

The peak areas of both internal standard and PAH were used to calculate peak area ratios. Ratios obtained for each sample as well as controls were compared to those of PAH standards. For the PAH dissolution experiments (Section 3.2.4.7), the standard curves were linear in the concentration range of 0.1 to 200 mg l^{-1} for fluoranthene and pyrene, and 0.05 to 50 mg l^{-1} for benzo[a]pyrene. For the

biodegradation experiments (Sections 3.2.5.2-3.2.5.6), the standard curves were linear in the concentration range of 0.5 to 100 mg l⁻¹ for fluorene; 0.5 to 250 mg l⁻¹ for phenanthrene and pyrene; 0.5 to 50 mg l⁻¹ for fluoranthene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene; 0.5 to 25 mg l⁻¹ for coronene.

3.2.6.3 High Performance Liquid Chromatographic (HPLC) Analysis of PAH-catabolic Products

Reverse-phase HPLC was performed on a Varian Star Liquid Chromatograph System comprising a solvent delivery system 9012, an autosampler 9100, and a variable wavelength UV-VIS detector 9050; this was controlled by Varian Star chromatographic software (Version 4.0). Dried organic extracts prepared from experimental samples, including abiotic and killed-cell controls, were appropriately diluted with methanol and injected (70 µl) onto a Spherex 5 C₁₈ column (250 mm x 4.6 mm id; Phenomenex, California, USA). The solvent consisted of water and methanol using the following gradient: 0 min, 50:50; 0 to 30 min, ramp to 0:100; 30 to 50 min, isocratic at 0:100. The flow rate was 0.7 ml min⁻¹. Compounds in the eluate were detected using a UV wavelength of 254 nm.

3.2.7 TOXICOLOGICAL ASSESMENT: AMES MUTAGENIC ASSAY

The mutagenicity assays were conducted according to the revised methods of Maron and Ames (1983) using *S. typhimurium* TA100 as a tester strain. TA100 was one of tester strains recommended for general mutagenicity testing (Maron and Ames, 1983). The bacterial mutagenicity assays were performed on the PAH compounds and crude DCM extracts of metabolites formed during the degradation of those PAHs from the time course incubation described previously (Section 3.2.4.10). In addition, soil DCM extracts were also subjected to testing for their mutagenic potential. All the tested samples were dissolved in dimethyl sulfide (DMSO). To determine the direct mutagenic activity, a 0.1 ml aliquot of the tested substance was mixed with 0.1 ml of a culture of the tester strain and 2.5 ml of molten top agar (50 µM L-histidine, 50 µM d-biotin, 0.5% NaCl, 0.6% agar) and

poured onto minimal glucose plates (Vogel-Bonner medium E containing 2.0% glucose and 1.5% agar). Assays were also performed in the presence of hepatic post-mitochondrial supernatant (S9) prepared from male Sprague-Dawley rats pretreated with Aroclor 1254 (Moltox™ post-mitochondrial supernatant, Molecular Toxicology, Inc., Monsanto, USA). Dose response concentrations for chrysene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene were performed on TA100 in the presence and absence of S9 (data presented in Table 3.4). In assays including S9, 0.1 ml of the test substance, 0.1 ml of the tester strain, 0.5 ml of an S9 mix (0.2 ml of S9, 1.0 ml of MgCl₂-KCl salt, 0.25 ml of 1 M glucose-6-phosphate, 2.0 ml of 0.1 M NADP, 25 ml of 0.2 M sodium phosphate buffer, pH 7.4, adjusted to total volume of 50 ml) and 2.0 ml of molten top agar were mixed and poured onto plates containing Vogel's minimal glucose agar. Controls used in this test included plates with no addition of tests, solvent control with DMSO added, and positive control with a known mutagen (10 ppm aflatoxins) added. All assays were performed in triplicate. The numbers of revertant colonies were counted after 48 hours of incubation at 37°C. The number of spontaneous revertants was used for correcting the mutagenic response arising from the tested substances (see Chapter 5, Section 5.9).

3.2.8 MOLECULAR BIOLOGICAL METHODS

3.2.8.1 Total Genomic DNA Isolation

The method used to extract total genomic DNA from PAH-degrading bacteria was a modification of the method described by Sambrook and Maniatis (1989). Broth cultures (50 ml) of bacterial strains were grown overnight in Luria broth (Appendix D) at 30°C and harvested by centrifugation (7,000 rpm, 4°C, 10 min). The resulting pellet was washed with 10 ml of TES buffer (25% sucrose in TE buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged for 10 min at 7000 rpm, 4°C. The pellet was resuspended in lysis buffer (5 ml TES buffer, 0.4 ml of 0.25 M EDTA, pH 8.0 and 0.2 ml of 5 mg/ml lysozyme). The mixture was vortexed at once and incubated at 37°C for 15 min to complete cell lysis. Proteinase K (stock solution 20 mg/ml) was added to a final concentration of 0.2 mg/ml, and the

mixture was incubated at 37°C for 1 hour. Phenol extraction followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction were performed. After centrifugation (15,000 rpm, 4°C, 20 min), DNA in the aqueous phase was collected and further precipitated with 2 volumes of ice-cold absolute ethanol and 1/10 volume of 3.0 M sodium acetate, pH 4.8. The white DNA precipitate formed upon mixing, and these solutions were stored at 20°C, overnight. After centrifugation at 10,000 rpm at 4°C for 30 min, the DNA pellet was washed with 70% ice-cold ethanol. The DNA pellet was dried and resuspended in 300 µl of sterile Tris-EDTA (TE) buffer. DNA solutions were dialyzed at 4°C in three changes of 1 x TE buffer and then stored at -20°C.

3.2.8.2 Estimation of DNA Concentration

DNA concentrations were determined using the spectrophotometric and ethidium bromide/agarose plate method as described by Sambrook and Maniatis (1989). Amounts of synthesized oligonucleotides were determined using the spectrophotometric method. Isolated nucleic acid samples and synthesized oligonucleotides were diluted 1:50 (for DNA) or 1:100 (for oligonucleotides) in distilled water and absorbance readings were taken at wavelengths of 260 nm and 280 nm, and the A_{260}/A_{280} ratio provided an estimation of the purity of samples. Nucleic acid concentration was determined according to the equation: 1 A_{260} unit (1 cm light path) = 50 µg/ml solution for double-stranded DNA and 33 µg/ml solution for single-stranded oligonucleotides (Sambrook and Maniatis, 1989).

3.2.8.3 Agarose Gel Electrophoresis

Electrophoresis of DNA was usually performed with horizontal 0.8% (w/w) agarose slab gels measuring 10.5 x 8 cm (Gel Electrophoresis Apparatus GNA-100, Pharmacia, Sweden). For good separation of small DNA fragments, different concentrations (1.0%, 1.5%, or 2.0%) of agarose gels were used. Large gels (20 x 20 cm, GNA-200, Pharmacia, Sweden) were used for digestion of genomic DNA. Electrophoresis was carried out using 1 x Tris-acetate-EDTA (TAE) buffer running normally at 80 volts, for 1-2 hour or until the dye ran near the bottom of

the gel, whilst 20-25 volts was used for large gels in 1 x TAE buffer, overnight. Ethidium bromide staining solution was added to the agarose gel (0.5 µg/ml) before electrophoresis. The DNA bands were visualized under a UV transilluminator ($\lambda = 302$ nm) (LKB 2011 Macrovue transilluminator). *Eco* RI and *Hind* III digested lambda DNA (Progen) containing 11 fragments (21.2, 5.1, 5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.9, 0.8, 0.6 and 0.1 kb) was used as a molecular marker for the digested genomic DNA. Alternatively, a 100 bp DNA ladder (Promega) containing 11 fragments (1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp) or Probase 50™ (Progen) containing 16 fragments (3,147, 2,647, 900, 800, 700, 600, 500, 450, 400, 350, 300, 250, 200, 150, 100 and 50 bp) were used as size markers for PCR products or digested PCR products. Approximately, 0.5-1.0 µg of size markers was used.

3.2.8.4 Photography

A record of the distribution of DNA, as seen under UV light, was obtained using a Polaroid MR 4 land camera assembly loaded with black and white film (Polaroid 665 or 667), using a yellow filter.

3.2.8.5 Polymerase Chain Reaction (PCR) Amplification

(a) Amplification of 16S rRNA Genes (1.5 kb)

A long template PCR kit was used for which two master mixtures were prepared as followed: Master mix I contained deoxynucleoside triphosphates (final concentration 200 µM of each dNTP), PCR primers (final concentration 0.6 µM each), 10 ng of DNA template; water was added to make the volume up to 25 µl. Master mix II consisted of Long template PCR buffer (1x) and 2.5 units of DNA polymerase mix (Expand™ Long Template PCR System; Boehringer Mannheim) and water was added to make the volume up to 25 µl. The two master mixtures were each mixed in a 0.6 ml PCR tube. Amplification was performed in the automated thermal cycler (PTC-200, Perkin-Elmer-Cetus) using the protocol described by the manufacturer (was designated in this thesis as the 16S protocol).

The mixture was initially denatured at 94°C for 2 min followed by nine cycles of the following temperature profile: denaturation at 94°C for 10 sec; annealing at 55°C for 30 sec; extension at 68°C for 1.30 min. A further 19 cycles were performed under the following conditions: denaturation at 94°C for 10 sec; annealing at 55°C for 30 sec; extension at 68°C for 1.30 min; plus cycle elongation, for a greater yield, of 20 sec for each cycle. A final extension of 2 min was performed at 68°C. Following PCR, 5 µl of the amplified product was electrophoresed in a 1% agarose gel containing ethidium bromide (0.5 mg ml⁻¹) with 1 x TAE buffer and viewed on an ultraviolet transilluminator.

(b) Amplification of Internal Regions of 16S rRNA Genes

- Purified 1.5 kb products were used as templates to amplify their internal regions. The PCR reaction mixture contained per 100 µl: PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), deoxynucleoside triphosphates (final concentration 200 µM of each dNTP), 1.5 mM MgCl₂ solution, PCR primers (final concentration 0.5 µM each), 50 ng of DNA template, and 2.5 units of *AmpliTaq* DNA polymerase. All of the reagents except *Taq* polymerase were mixed gently in a 1.0 ml PCR tube. Amplification was performed in the automated thermal cycler (PTC-200, Perkin-Elmer-Cetus) using the protocol (was designated in this thesis as the BCEP protocol) described as follows. The mixture was initially denatured at 97°C for 7 min and then the *Taq* polymerase was added. Thirty-five cycles were performed as follows: denaturation at 95°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 2 min. A final extension of 5 min was performed at 72°C. Following PCR, 5 µl of the amplified product was electrophoresed on a 1% agarose gel containing ethidium bromide (0.5 mg ml⁻¹) and viewed on an ultraviolet transilluminator.

3.2.8.6 Purification of PCR Products

PCR products were purified by using Wizard™ PCR Preps DNA Purification System (Promega) following the manufacturer's instructions. For each complete

PCR reaction, the entire content (approximately 95 μ l) was added to a 1.5 ml microcentrifuge tube containing 100 μ l of Direct Purification buffer, and then vortexed briefly to mix. Subsequently, 1 ml of PCR Preps DNA Purification Resin was added and the samples were then vortexed briefly 3 times over a one minute period. One Wizard™ Minicolumn was prepared for each PCR reaction. The 3-ml disposable syringe barrel was attached to the Luer-Lok® extension of each minicolumn while the syringe plunger was set aside. The DNA/resin mixture was applied into the syringe barrel and then the syringe plunger was inserted slowly and gently to push the DNA slurry into the minicolumn. Subsequently, the column was washed with 2 ml of 80% isopropanol. The column was centrifuged for 20 seconds at 12,000 x g to dry the resin. To remove DNA, 50 μ l of sterile distilled water was added to the column, which was left for 2 min. The column was centrifuged for 20 seconds at 12,000 x g to elute the bound DNA fragment, and the purified DNA solution was stored at -20°C.

3.2.8.7 Cycle Sequencing

Sequencing reactions were prepared using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Each reaction mixture contained 8.0 μ l of Terminator Ready Reaction Mix, 3 μ l of DNA template (10 μ g μ l⁻¹), primer 3.2 pmole, and water was added to obtain a total volume of 20 μ l. Cycle sequencing was performed in a thermal cycler (PT-200). The thermal cycling was performed for 25 cycles under the following conditions: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 min. The extension products were then purified using ethanol precipitation. A 1.5-ml microcentrifuge tube containing 2.0 μ l of 3 M sodium acetate, pH 4.6 and 50 μ l of 95% ethanol, was prepared for each reaction. The entire 20 μ l contents of the reaction was transferred into the microcentrifuge tube containing ethanol solution. The tube was then vortexed and placed on ice for 10 min before being centrifuged at maximum speed (14,000 x g) for 30 min. The ethanol solution was carefully aspirated using a micropipettor. The pellet was rinsed by adding 250 μ l of 70% ethanol and centrifuged at maximum speed for 15 min. The ethanol solution was carefully removed and the pellet was dried in a vacuum centrifuge. The DNA

Table 3.5 Restriction endonucleases used in the 16S DNA genotyping.

Endonuclease ^a	Restriction site (5'.....3')	Conc. (U) ^b	Buffer	Incubation condition	Heat inactivation
<i>BssK</i> I	↓CCNGG	10	NEBuffer 3, BSA (100 µg ml ⁻¹)	37°C, 2.5 h	–
<i>Dpn</i> II	↓GATA	10	NEBuffer 2	37°C, 2.5 h	65°C 20 min
<i>Eco</i> 0109 I (progen)	G↓GNCC	10	Buffer blue, BSA (100 µg ml ⁻¹)	37°C, 2.5 h	65°C 10 min
<i>Fnu</i> 4H I	GC↓NGC	10	NEBuffer 4	37°C, 2.5 h	65°C 20 min
<i>Hae</i> III	GG↓CC	10	NEBuffer 2	37°C, 2.5 h	–
<i>Hin</i> f I	G↓ANTC	10	NEBuffer 2	37°C, 2.5 h	–
<i>Hpa</i> II	C↓CGG	10	NEBuffer 1	37°C, 2.5 h	65°C 20 min
<i>Rsa</i> I	GT↓AC	10	NEBuffer 1	37°C, 2.5 h	65°C 20 min
<i>Mse</i> I	T↓TAA	10	NEBuffer 2, BSA (100 µg ml ⁻¹)	37°C, 2.5 h	65°C 20 min
<i>Sma</i> I	CCC↓GGG	10	NEBuffer 4	25°C, 2.5 h	65°C 20 min

^a Unless otherwise specified, all restriction endonucleases were purchased from New England Laboratory, England.

^b Concentrations presented as units of enzymes used per reaction.

– No heat inactivation required.

sequences were determined on the ABI 373-Automated DNA Sequencer (Perkin-Elmer) at Department of Microbiology, Monash University.

3.2.8.8 Analysis of 16S rRNA Gene Sequences

The 16S rRNA gene sequences were compared with all currently accessible 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) databases using the program BLASTN *via* Australian National Genomic Information Service (ANGIS).

3.2.8.9 Restriction Fragment Length Polymorphism (RFLP) Studies

RFLP of the 16S rRNA genes of PAH-degrading bacterial strains was performed using 1 µg of DNA (1.5 kb PCR product, Section 3.2.8.5) digested with a restriction endonuclease. Restriction digestion profiles of 16S rRNA genes of each bacterial strain were obtained from the digestion of with ten restriction enzymes: *BssK* I, *Dpn* II, *Eco0109* I, *Fnu4H* I, *Hae* III, *Hinf* I, *Hpa* II, *Mse* I, *Rsa* I, and *Sma* I. Table 3.5 shows the suppliers, specificity, compatible buffers, and digestion conditions for a respective enzyme. The 500 ng aliquots of digested DNA were electrophoresed in a 2% agarose gel containing ethidium bromide (0.5 mg ml⁻¹) with 1 x TAE buffer and viewed on an ultraviolet transilluminator.

3.2.8.10 Southern Blot Hybridization

(a) Genomic DNA Digestion with Restriction Endonucleases

Genomic DNA from four strains of PAH-degrading bacteria were digested with *Eco0109* I and *Sma* I. The buffer and incubation conditions for the enzymes are shown in Table 3.5. The 500 ng of digested DNA samples were run overnight on agarose gels (0.8%) with 1 x TAE buffer at a low voltage (20 volts).

(b) DNA Transfer to Membrane

After electrophoresis, agarose gels were blotted with nitrocellulose membranes (Hybond; Amersham, Buckinghamshire, UK) (Sambrook and Maniatis, 1989) as follows. Gels were transferred into denaturing solution (1.5 M NaCl, 0.5 M NaOH) and incubated at room temperature with gentle shaking for 45 min. Subsequently, gels were rinsed with water and transferred to neutralizing solution (1.0 M Ammonium acetate, 0.02 M NaOH) and incubated in the same manner as above for 30 min. Neutralized gels were placed carefully on a perspex support and the nitrocellulose membrane was aligned on the gel. Three filter papers (Whatman) were placed on the membrane. A stack of paper towel was placed on the top of the filter papers. A weight (0.5 kg) was put on the top, and the set was left overnight for DNA transfer. After complete transfer had occurred, the membrane was removed from the gel and washed with 2 x SSC solution (20 x SSC contains 3.0 M NaCl, 0.3 M tri-sodium citrate) for 5 min. The membrane was then wrapped and exposed to UV light for 5 min. The membrane was sealed in a plastic bag and stored at room temperature.

(c) α [^{32}P]CTP Labelling Probe

Probes for southern blot hybridization were 16S rRNA genes (1.5 kb) obtained from the PCR reaction (see Section 3.2.8.5). DNA was denatured at 95°C for 10 min before labelling. The reaction contained 25 ng of denatured DNA, 4 μl of High Prime solution (Boehringer), 20 μCi of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$, and the total volume was adjusted to 25 μl with sterile distilled water. The reaction mixture was spun for 5 sec and then incubated for 10 min at 37°C. The reaction was stopped by adding 2.0 μl of 0.2 M EDTA (pH 8.0). Transfer RNA (tRNA, stock 5 mg/ml) and 75 μl of 1x TE buffer were added to the reaction mixture, spun for 5 sec, and then added to the Sephadex G-50 column saturated with TE buffer. The column was spun for 30 seconds, and the labeled DNA solution was collected in a sterile Eppendorf microcentrifuge tube. TE buffer (200 μl of 1 x) was added to the labeled solution, which was then denatured at 95°C for 10 min and placed on ice until required.

(d) Hybridization of Membrane-bound Nucleic Acids to Labeled Probes

Membranes were pre-hybridized at 65°C for 8-12 hours in a plastic bag using 25 ml hybridization buffer (6 x SSC, 5 x Denhardt's reagent [50 x Denhardt's reagent contains 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA and 500 ml sterile H₂O], 0.5% (w/v) SDS) and 1.25 ml denatured salmon sperm DNA (5 mg ml⁻¹). For the subsequent hybridization, labeled probe (25 ng of denatured DNA, 4 µl of High Prime solution (Boehringer), and 20 µCi of [α -³²P]dCTP) as well as 15 µl of labelled marker (Lambda DNA digested with *Hind* III and *Eco* RI) were added to the bag and incubated at 65°C, overnight. After hybridization, the membranes were washed twice at room temperature with high stringency washing buffer (2 x SSC, 0.1% SDS) followed by washing at 65°C with a low stringency washing buffer (0.2 x SSC, 0.1% SDS) for an additional two times. Membranes were checked for radioactive counts using a Geiger counter.

(e) Detection of Hybridization Signals

When the membranes appeared to be satisfactorily washed, they were air-dried for 5 min, wrapped with plastic wrap and taped to the back of an X-ray cassette. In the dark, an X-ray film was placed on the top of the membrane, and the assembly was covered with an intensifying screen. The cassette was placed at -80°C for 6-24 hours depending on the strength of signals. The film was developed in the dark, under safety lights, by emersion in developer for 2-5 min, 1 min in acetic acid solution and 4 min in the fixer solution. The developed film was washed thoroughly in running tap water for 5-10 min, then air-dried.

CHAPTER 4

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PAH-DEGRADING MICROORGANISMS

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CHAPTER 4

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PAH-DEGRADING MICROORGANISMS

4.1 INTRODUCTION

The results reported in this chapter are concerned with screening the microflora from two different contaminated soil sites in Victoria, Australia, for their ability to degrade high molecular weight PAHs. The source of pollution at the sites were from coal gasification works and creosote contamination, both of which are well known as a rich source of PAHs. This chapter describes the isolation of PAH-degrading bacteria and fungi with an emphasis on characterization of the PAH-degrading bacteria. Comparative studies at a taxonomic level as well as determining PAH degradability are reported. Identification of PAH-degrading bacteria in current times needs to go further than the classical biochemical methods. Molecular perspectives must be taken into consideration for bacterial classification and phylogenetic analysis. The principle of using ribosomal RNA gene sequences to characterize microorganisms has now gained wide acceptance, since it has proven the most useful method for identifying and establishing relationships between bacteria. The larger rRNA molecules (16S and 23S rRNA) are useful as a measure of phylogenetic relationships, since they provide all the prerequisites of phylogenetic marker molecules, *i.e.* universal distribution, structural and functional conservation, and sufficient size (*i.e.* high information content). Several reports have now been published on the identification of bacterial species and phylogenetic relationship analysis by 16S rRNA gene sequencing (Collins *et al.*, 1991; Canard *et al.*, 1992; van Kuppeveld *et al.*, 1992; Duncan *et al.*, 1993; Lawson *et al.*, 1993; Wang *et al.*, 1995).

The general aim of the work in this chapter was to isolate PAH-degrading microorganisms from contaminated soils. More detailed studies were conducted on PAH-degrading bacteria with the following major objectives:

Table 4.1 Growth of microbial communities enriched from PAH-contaminated soils.

PAH ^b	Growth from inoculum ^a :	
	Soil A	Soil B
Fluorene ^c	+ ^d	+ /+ ^e
Phenanthrene ^c	+	+ /+
Fluoranthene ^f	—	+ /+
Pyrene ^c	+	+ /+
Chrysene ^f	—	+ /+
Benz[<i>a</i>]anthracene ^f	—	+ /+
Benzo[<i>a</i>]pyrene ^f	—	+ /+
Dibenz[<i>a,h</i>]anthracene ^f	—	+ /+
Coronene ^f	—	— /—

^a Soil A and Soil B were contaminated by coal gasification operations and creosote, respectively.

^b A single PAH was supplied to BSM at a concentration of 100 mg l⁻¹ for fluorene, phenanthrene, and pyrene; 50 mg l⁻¹ for fluoranthene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene; and 25 mg l⁻¹ for coronene.

^c Microbial growth was determined after two weeks of incubation.

^d Bacterial growth (+) or no growth (—) was observed as indicated by medium turbidity compared to the uninoculated control.

^e Growth of bacterial and fungal mycelia (+ /+) or no microbial growth (— /—) was evident after two weeks of incubation.

^f Microbial growth on such PAHs was determined after four weeks of incubation.

- (1) to verify the physiology of PAH-degrading bacteria in terms of substrate specificity, dioxygenase activity and versatility in degrading various PAH compounds; and
- (2) to identify, characterize and differentiate PAH-degrading bacterial species using ribotyping, genotyping and DNA-DNA hybridization techniques.

4.2 ENRICHMENT OF PAH-DEGRADING MICROORGANISMS

Enrichment techniques were used to select microorganisms capable of degrading PAH compounds. After several successive transfers, growth of bacteria in enriched cultures of soil A (PAH-contaminated soil from an abandoned coal gasification site, Port Melbourne) and soil B (creosote-contaminated soil from Warracknabeal, Victoria) was observed in BSM containing only fluorene, phenanthrene, or pyrene (Table 4.1). These PAHs served as carbon and energy sources for the microbial consortia. Evidence of bacterial growth and PAH utilization was recognized by an increase in turbidity, the disappearance of PAH crystals, and a change in color of the media from white and cloudy (undissolved PAHs) to yellow or brown compared to uninoculated controls. Using soil A as inoculum, microbial growth was observed on fluorene, phenanthrene and pyrene. However, no microbial growth originating from soil A was observed in enrichment media containing fluoranthene, benz[a]anthracene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene, or coronene. In contrast, growth of fungal mycelia alongside bacteria was observed in soil B-enriched cultures for a majority of PAHs tested, the one exception being coronene (Table 4.1). Microbial growth was confirmed by sequential transfer in the same enrichment medium.

4.3 ISOLATION OF PAH-DEGRADING MICROORGANISMS

PAH-degrading bacteria from soil A enrichments were isolated on pyrene as a sole carbon and energy source using the spray-plate technique. Although soil A bacteria could be enriched on fluorene and phenanthrene, growth on the higher molecular weight compound, pyrene, was of great interest because of the relative

scarcity of bacteria which can degrade this compound. Following enrichment of soil A microflora on pyrene, three distinct pyrene-degrading bacterial isolates (initially named PYR11, PYR12 and PYR13) were recognized by the formation of clear zones around colonies on BSM agar sprayed with 2% pyrene and these were initially differentiated on the basis of colony shape and color.

On the other hand, the soil B-benzo[a]pyrene-enriched culture was chosen for the isolation of benzo[a]pyrene degraders. This decision was based on the rapid growth of bacteria and fungi in the enriched culture and because of the importance of benzo[a]pyrene as an environmental health hazard. Although it was anticipated that microbes able to use benzo[a]pyrene as sole carbon source would be isolated, neither growth of fungi or bacteria nor the formation of clear zones on BSM plates sprayed with 0.5% etheral solution of benzo[a]pyrene were detected. It was decided to select the fungi on PDA supplemented with benzo[a]pyrene (50 mg l⁻¹), penicillin G (60 µg ml⁻¹) and streptomycin sulfate (100 µg ml⁻¹). A single type of fungal colony and mycelial growth was observed, and a colony of this fungus was selected for further work. A bacterial consortium from the same enriched medium as used for the isolation of the fungus was also isolated by transferring a sample from the benzo[a]pyrene-enriched culture into to BSM containing pyrene (100 mg l⁻¹) and cyclohexamide (0.1 g l⁻¹). Bacterial growth was observed along with the disappearance of pyrene crystals and a color change from cloudy white to brown in the medium. Pure cultures of pyrene-degrading bacteria were isolated as described above. All colonies seen on BSM plates sprayed with 2% etheral solution of pyrene were morphologically similar and one representative isolate was stored (initially named as PYR41).

4.4 IDENTIFICATION OF PAH-DEGRADING MICROORGANISMS

4.4.1 Identification of PAH-Degrading Bacteria

The four isolates of pyrene-degrading bacteria (PYR11, PYR12, PYR13 and PYR41) were subjected to identification procedures based on biochemical tests.

All isolates were rod-shaped, aerobic, motile, Gram-negative bacteria. Colonies were smooth and glistening with a pale yellow color. Their biochemical characteristics and ability to utilize various carbohydrates as their growth substrate are listed in Tables 4.2 and 4.3, respectively. None of these bacterial isolates utilized erythritol, rhamnose or starch as their growth substrate. These bacteria possess the cytochrome oxidase enzyme and all isolates demonstrated growth on MacConkey agar. Oxidation-fermentation (O-F) tests showed that these isolates metabolize carbohydrate by oxidation. Growth at 41°C was not observed. Fluorescent pigments were not produced by most isolates, however, one exception being PYR12 when grown on Fluorescein medium. On the basis of these characteristics, these bacterial isolates were tentatively classified as belonging to the genus *Pseudomonas*. However, a more accurate identification of these bacterial isolates was conducted by sequencing and analysis of their 16S rRNA genes (see Section 4.7).

4.4.2 Identification of the Fungal Isolate

The fungal isolate was kindly identified as *Penicillium janthinellum* by Associate Professor Anne Lawrie, Department of Applied Biology and Biotechnology, The Royal Melbourne Institute of Technology University.

4.5 CULTURE COLLECTION NUMBERS

A mixed bacterial population obtained by pyrene enrichment of soil A and its pure isolates were assigned a Victoria University of Technology culture collection number: VUN 10,008, the bacterial consortium; VUN 10,010, PYR11; VUN 10,011, PYR12 and VUN 10,012, PYR13.

The mixed bacterial population from soil B benzo[a]pyrene- and then pyrene-enriched culture and its pure isolate were assigned the following numbers: VUN 10,009, the bacterial consortium and strain VUN 10,013, PYR41. The fungal isolate (*P. janthinellum*) from soil B-benzo[a]pyrene enriched culture was designated strain VUN 10,201.

Table 4.2 Biochemical characteristics of four PAH-degrading isolates.

Test	PYR11 ^a	PYR12 ^a	PYR13 ^a	PYR41 ^a
Motility	+	+	+	+
O-F test	Ox	Ox	Ox	Ox
Oxidase	+	+	+	+
Indol production	–	–	–	–
Urease	–	–	–	+
Phenylalanine deaminase	–	–	–	–
Lysine decarboxylase	+	–	+	+
Arginine dihydrolysis	–	+	–	–
Aesculin	+	–	+	+/-
Nitrate reduction	–	+/-	–	–
Fluorescent pigment	–	+	–	–
Gelatin liquefaction	+	+	+	+
Starch hydrolysis	–	–	–	–
Growth at 4°C	–	+/-	–	–
42°C	–	–	–	+/-
Growth on McConkey agar	+	+	+	+

^a PYR11, PYR12, PYR13 and PYR41 were subsequently given names according to the culture collection number of Victoria University of Technology as VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013, respectively.

Table 4.3 Carbohydrate utilization^a as a growth substrate by bacterial isolates.

Carbohydrate ^b	VUN 10,010	VUN 10,011	VUN 10,012	VUN 10,012
Adonitol	+	–	+	+
Arabinose	+	+	+	+
Cellobiose	+	–	+	+
Erythritol	–	–	–	–
Fructose	+	+	+	+
Galactose	–	+	–	+
Glucose	+	+	+	+
Glycerol	+	+	+	+
Inulin	+	–	+	–
Lactose	+/-	+/-	+/-	+/-
Maltose	+	–	+	+
Mannitol	–	+	–	+
Mannose	+	+	+	+
Rhamnose	–	–	–	–
Sorbitol	+	+	+	+
Starch	–	–	–	–
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	+/-	+	+/-	+

^a Growth of bacteria on a tested substrate is signified as growth (+), poor growth (+/-) or no growth (–).

^b Carbon substrate was supplied to basal medium at the final concentration of 0.2% (Collins *et al.*, 1989).

Table 4.4 Characteristics of the four PAH-degrading bacterial strains.

Bacterial strain/ Characteristic	Fluorene ^c		Phenanthrene ^c		Pyrene ^c	
	No sal	Sal	No Sal	Sal	No Sal	Sal
Specificity^a						
VUN 10,010	++++/y	Yes (**)	++++/y	Yes (*)	+++/br	No
VUN 10,011	++++/y	Yes (**)	++/y	Yes (**)	++/br	No
VUN 10,012	++++/y	Yes (*)	++++/y	Yes (*)	+++/br	No
VUN 10,013	++++/y	Yes (**)	++++/y	Yes (**)	+/br	No
Indole^b						
VUN 10,010	++++/y	Yes (*)	++++/y	Yes (*)	+++/br	No
VUN 10,011	++++/y	Yes (*)	++/y	Yes (**)	++/br	No
VUN 10,012	++++/y	Yes (*)	++++/y	Yes (*)	+++/br	No
VUN 10,013	++++/y	Yes (*)	++++/y	Yes (*)	+/br	No

^a Specificity was determined by the appearance of clear zones and color of colonies on BSM agar plates sprayed with ethereal solutions of 5% (w/v) fluorene or phenanthrene, or 2% (w/v) pyrene. Relative lag periods in the appearance of zones is presented as (+) 8-10 days; (+ +) 7 days; (+ + +) 5 days; and (+ + + +) 3 days. Color of colonies were yellow (y) or brown (br). Effect of salicylic acid (Sal) on the time required for the formation of clear zones was carried out on BSM agar supplemented with salicylic acid (100 µg ml⁻¹); pH was adjusted to 6.9-7.0. Results are expressed as enhancement (Yes; decreased the lag period by [*] 1 day or [**] 2 days) or no enhancement (No).

^b Appearance of blue indigo color for cultures transferred into BSM containing a respective PAH (100 mg l⁻¹) and 0.2 mM indole. Relative lag periods in the appearance of blue indigo is given as (+) 3-4 days; (+ +) 2 days; (+ + +) 1 day; and (+ + + +) < 1 day (6-8 hours). Effect of salicylic acid (Sal) on the time required for the formation of blue indigo was carried out in BSM-PAH-indole supplemented with salicylic acid (100 µg ml⁻¹); pH was adjusted to 6.9-7.0. Results are expressed as enhancement (Yes; decreased the lag period of blue indigo appearance by [*] 4-6 hours or [**] > 6 hours up to 12 hours) or no effect (No).

4.6 PHYSIOLOGICAL CHARACTERISTICS OF PAH-DEGRADING BACTERIA

This section mainly describes the comparative physiology of the four isolates of PAH-degrading bacteria: VUN 10,010, VUN 10,011, VUN 10,012, and VUN 10,013. The major parameters studied were:

- (i) specificity and versatility of PAH utilization, including preferred substrate determination,
- (ii) growth on various carbon substrates,
- (iii) ability to degrade various PAH compounds,
- (iv) the presence of aromatic ring dioxygenase activity, such as naphthalene dioxygenase, and
- (v) the effect of salicylate on the time required for the appearance of clearing zones on PAH growth substrates and the transformation of indole to indigo.

4.6.1 Substrate Versatility of the PAH-Degrading Bacteria

The screening procedure had inherently selected bacteria or bacterial consortia that were able to grow on one particular PAH compound. Therefore, these PAH-degrading bacteria were further evaluated for their ability to grow on other PAHs. It was found that all isolates demonstrated a broad spectrum of substrate specificity, since they were able to grow, and form clear zones on PAH-BSM agar, on more than one PAH substrate (Table 4.4). In addition to pyrene, strains VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013 were able to grow on and utilize fluorene and phenanthrene as sole carbon and energy source. None of these isolates were able to grow on other high molecular weight PAH compounds, such as fluoranthene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene, dibenz[*a,h*]-anthracene and coronene, when supplied as a sole carbon and energy source.

The most promising PAH-degrading isolate was strain VUN 10,010. This strain grew quite well on pyrene, phenanthrene and fluorene, with concomitant formation of a yellow to brown coloration and the disappearance of non-solubilized PAH crystals when supplied as a sole carbon and energy source (Table 4.4). Analysis by gas chromatography showed that fluorene, phenanthrene and pyrene were degraded to undetectable levels after seven days incubation at 30°C in BSM containing the respective PAH as the sole substrate. On BSM agar plates, this strain formed clear zones faster than the other bacterial strains (VUN 10,011, VUN 10,012, and VUN 10,013). The times required for VUN 10,010 to produce clear zones on fluorene, phenanthrene and pyrene were two, three and six days, respectively. The other strains required a longer period to form clear zones, which appeared at the following times: three to five days for fluorene, three to seven days for phenanthrene, and three to more than seven days for pyrene (Table 4.4). Figure 4.1 shows the ability of the four bacterial strains to grow on and form clear zones on BSM agar plates sprayed with pyrene.

4.6.2 Conversion of Indole to Indigo

The ability of bacteria to oxidize indole to indigo indicates that they possess a dioxygenase enzymatic system (Ensley *et al.*, 1983). Dioxygenase is one of the enzymes responsible for PAH degradation in many PAH-degraders. To pursue whether the PAH-degrading bacteria isolated in this study degrade PAH compounds *via* dioxygenase activity, transformation of indole to indigo in culture media was investigated. The four strains of PAH-degrading bacteria tested in this experiment demonstrated the ability to synthesize indigo from an indole substrate supplemented in the growth medium (BSM plus fluorene, phenanthrene or pyrene). Therefore, the ability of these bacteria to form indigo from indole indicates that they possess dioxygenase enzymes. There were differences among the bacterial species and strains in their ability to synthesize indigo. The biotransformation of indole to indigo was subjected to a lag period of less than one day and up to four days in BSM supplemented with fluorene, phenanthrene or pyrene and 0.2 mM indole. The appearance of blue indigo in media containing three-ring PAHs (*i.e.* fluorene or phenanthrene) was more rapid than seen for

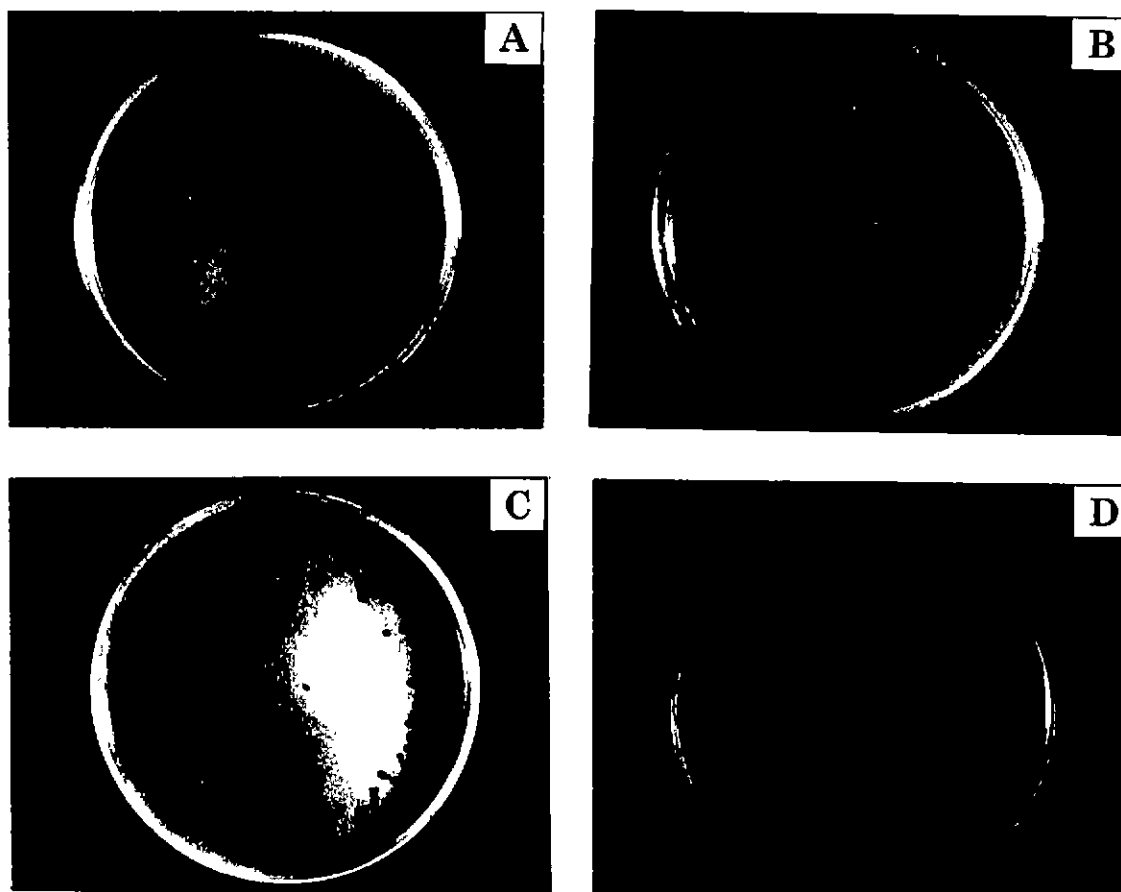


Figure 4.1 Formation of clear zones on BSM agar plates sprayed with pyrene (2% w/v, in ether) by VUN 10,010 (A), VUN 10,011 (B), VUN 10,012 (C), and VUN 10,013 (D). Incubation was performed at 30°C for 10 days.

media containing the four-ring pyrene, with indigo appearing at least 12 hours and up to four days earlier. As shown in Table 4.4, strains VUN 10,010 and VUN 10,012 demonstrated the ability to initiate the transformation of indole to indigo more rapidly than the other strains.

4.6.3 Effect of Salicylic Acid on Indole Transformation and PAH degradation

Salicylic acid is known to be an inducer of the expression of genes encoding the upper and lower pathways of naphthalene catabolism in the NAH7 plasmid (Schell, 1985). In addition to naphthalene, salicylic acid is also able to induce the catabolism of other PAHs, such as phenanthrene, fluoranthrene and benz[a]anthracene (Ogunseitan *et al.*, 1991; Dagher *et al.*, 1996). Therefore, experiments were conducted to investigate whether salicylic acid has such an effect on the degradation of fluorene, phenanthrene or pyrene in cultures of VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013. It was observed that the formation of clear zones on fluorene and phenanthrene spray plates by all bacterial strains appeared more rapidly (by about one or two days) when BSM agar contained salicylic acid ($100\ \mu\text{g ml}^{-1}$) (Table 4.4). However, for all bacterial strains tested the effect of salicylic acid in enhancing the formation of clear zones was not observed for pyrene. This corresponded with the results observed for dioxygenase activity, where salicylic acid enhanced the rate of blue indigo formation in BSM containing fluorene or phenanthrene, and 0.2 mM indole, but not for BSM containing pyrene (Table 4.4). For example, the time required for the transformation of indole by strain VUN 10,010 was, in general, 6-8 hours in BSM containing fluorene or phenanthrene. However, this was reduced to 2-3 hours when salicylic acid was present in the medium. No such effect was observed for the transformation of indole to indigo in pyrene-BSM cultures supplemented with salicylic acid.

Table 4.5 Growth of PAH-degrading bacteria on a variety of carbon sources.

C-source ^b	Growth ^a			
	VUN 10,010	VUN 10,011	VUN 10,012	VUN 10,013
Succinic acid	+	+	+	+
<i>p</i> -hydroxybenzoic acid	+	+	+	+
Maleic acid	+	+	+	+
Pyruvate	+	+	+	+
Protocatechuic acid	+	+	+	+
Catechol	+	+	+	+
D-panthothenic acid	+	+	+	+
Gentisic acid	+	+	+	+
Cinnamic acid	+	—	—	—
Benzoic acid	+	+	+	+
Salicylic acid	+	+	+	+
Phthalic acid	+	+	+	+
Tween 20	+	+	+	+
Tween 80	+	+	+	+
2,5-dichlorophenol	+	+	+	+
2,4,5-trichlorophenol	+	+	+	+
2,3,5,6-tetrachlorophenol	+	+	+	+
Pentachlorophenol	+	—	+	—
4-nitrophenol	—	—	—	—
4-chlorophenol	+	—	+	+
Benzene ^c	+	+	+	+
Toluene ^c	+	+	+	—
η -hexadecane ^c	+	+	+	+
η -octane ^c	+	+	+	+
Nitrobenzene ^c	+	—	+	—

^a Growth was determined by visual observation of turbidity of culture media compared to the uninoculated control.

^b Substrate concentration supplied to media was 50 mg l⁻¹.

^c Substrates were supplemented in vapor phase.

4.6.4 Bacterial Growth on Various Carbon Substrates

The substrate utilization tests showed that isolates which had been selected on the same primary substrate (*i.e.* pyrene) also demonstrated a highly similar utilization pattern with other substrates (Table 4.5). Some of these compounds are known to be intermediate products of PAH degradative pathways, for instance, salicylic acid, cinnamic acid, phthalic acid, and protocatechuic acid. All strains grew on the following compounds as the sole carbon and energy source supplied at 50 mg l⁻¹: succinic acid, salicylic acid, pyruvate, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, Tween 20, Tween 40, Tween 60, Tween 80, catechol, phthalic acid, 4-chlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, and 2,3,5,6-tetrachlorophenol. Moreover, all of bacterial strains grew on a vaporized phase of benzene, *n*-hexadecane and *n*-octane. Growth patterns of the four strains were different on cinnamic acid, pentachlorophenol, 4-chlorophenol, toluene and nitrobenzene (Table 4.5). None of these bacteria grew on 4-nitrophenol.

4.6.5 PAH Biodegradation

Although the four bacterial strains were unable to grow on some high molecular weight PAHs as a sole carbon and energy source, investigations were conducted to determine whether they were still able to degrade these compounds when supplied to culture media as a sole source of carbon and energy. Unwashed pyrene-grown cells of each bacterial strain were (VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013) used as inocula at low cell density (approximately 10⁴ cell ml⁻¹).

The degradation of various PAHs by the individual isolates as well as by parent communities is summarized in Table 4.6. Low molecular weight PAHs seem to be more readily degraded by these bacteria: all four bacterial strains degraded fluorene and phenanthrene to undetectable levels within seven days. In contrast, high molecular weight PAHs containing four-, five-, or seven-benzene rings were more resistant to bacterial attack. With the exception of pyrene, all of the

Table 4.6 Degradation of PAHs by mixed and pure bacterial cultures.

PAH ^b	% PAH removal ^{a,c}						Time (d)
	VUN 10,008	VUN10,010	VUN10,011	VUN10,012	VUN 10,009	VUN10,013	
Fluorene	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	7
Phenanthrene	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	7
Fluoranthene	5.5 ± 0.2	5.1 ± 0.2	2.4 ± 0.2	4.3 ± 0.2	4.4 ± 1.2	3.3 ± 0.9	56
Pyrene	100.0 ± 0.0	100.0 ± 0.0	95.6 ± 1.8	100.0 ± 0.0	100.0 ± 0.0	93.3 ± 1.6	7
Chrysene	19.2 ± 1.7	18.3 ± 0.6	9.4 ± 0.6	15.3 ± 0.5	10.1 ± 0.8	8.2 ± 0.1	56
Benz[<i>a</i>]anthracene	9.5 ± 0.7	8.8 ± 0.1	6.9 ± 0.2	8.4 ± 0.8	8.8 ± 1.3	6.2 ± 0.8	56
Benzo[<i>a</i>]pyrene	20.2 ± 1.1	18.2 ± 0.3	14.5 ± 0.3	18.0 ± 1.3	6.7 ± 0.4	11.3 ± 1.2	56
Dibenz[<i>a,h</i>]anthracene	13.7 ± 0.5	12.4 ± 1.0	8.9 ± 0.4	12.6 ± 0.8	5.9 ± 0.7	5.9 ± 0.1	56
Coronene	4.1 ± 0.7	3.7 ± 0.2	1.8 ± 0.2	3.7 ± 0.4	2.8 ± 0.6	2.2 ± 0.5	56

^a %PAH removal calculated relative to the amount of PAH that disappeared from the uninoculated control; averages are from triplicate experiments.

^b PAH was supplied as a sole carbon and energy source at the following concentrations: 250 mg ml⁻¹ for phenanthrene and pyrene; 100 mg ml⁻¹ for fluorene, fluoranthene, and benz[*a*]anthracene; 50 mg ml⁻¹ for chrysene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene; and 25 mg ml⁻¹ for coronene.

^c Inocula were grown in BSM containing pyrene (250 mg l⁻¹) and unwashed cells transferred into BSM containing a single PAH to give an initial cell density of approximately 10⁴ cells ml⁻¹. Incubations was performed at 30°C/175 rpm.

Table 4.7 List of primers used in the amplification of 16S rRNA genes of PAH-degrading bacteria.

Primer ^{a,b,c,e}	Sequence (5'.....3')	mer	T _m (°C)
MAS2F	TCC TGG CTC AGA TTG AAC GCT	21	64
MAS1R	CTT GTT ACG ACT TCA CCC CAG	21	64
MZ1	GGC GAC GAT CCG TAG CTG GTC	21	70
MZ2	TAC GCC CAG TAA TTC CGA GTA	21	62
MZ3	GTA CCC AAA GAA TAA GCA CCG	21	62
MZ4	GTA CTC CCC AGG CGG CGA ACT	21	70
MZ5	CGC CCT AAA CGA TGC GAA CTG	21	66
MZ6	AGA GTT CCC ACC ATT ACG TGC	21	64
Psf1	GAG GAT GAT CAG TCA CAC ACT	21	62
Psf2	AGG TGG TCG CCT TCG CCA CTG	21	70
Psf3	GTT AAT CGG AAT TAC TGG GCG	21	62
Psf4	GGC CCC CGT CAA TTC ATT TGA	21	64
Psf5	GTG CGA AAG CGT GGG GAG CAA	21	68
Psf6	TTA CGT GCT GGT AAC TAA GGA	21	60
F1	ATT GAA CGC TGG CGG CAT GCC	21	68
R1	AAT TCC CCA CTG CTG CCT CCC	21	68
F2	GTG AAA TCC CCG GGC TCA ACC	21	68
R2	TGA AGC CCT ACC CAT AAG GGC	21	66
F3	TGG TCT GAG AGG ACG ACC AGC	21	68
R3	CGC ATT TCA CTG CTA CAC GTG	21	64
F4	CTA ACT ACG TGC CAG CAG CCG	21	68
R4	CCA CAT CAT CCA CCG CTT GTG	21	66

^a Primers MAS2F and MAS1R were used to amplify the 16S rRNA genes for the four bacterial strains.

^b Internal primers (MZ1 through MZ6) for amplification and sequencing the internal regions of 16S rRNA genes for VUN 10,010 and VUN 10,012.

^c Internal primers (Psf1 through Psf6) for amplification and sequencing the internal regions of 16S rRNA genes for VUN 10,011.

^d Internal primers (F1 and R1 through F4 and R4) for amplification and sequencing the internal regions of 16S rRNA genes for VUN 10,013.

bacterial isolates displayed limitations in degrading PAHs containing four- or more benzene ring compounds when supplied as a sole source of carbon and energy. Over 56 days, the disappearance of fluoranthrene, chrysene, benz[a]anthracene, benzo[a]pyrene, dibenz[a,h]anthracene and coronene from cultures of each bacterial strain was approximately 2.4–5.5%, 8.2–19.2%, 6.2–9.5%, 6.7–20.2%, 5.9–13.7%, and 1.8–4.1%, respectively. In particular, the extent of coronene degradation by all isolates and communities was very low. Strain VUN 10,010, VUN 10,011 and VUN 10,012 were isolated from the same site (soil A) and they demonstrated similar PAH-degradation patterns. Strain VUN 10,013, which originated from a different site (soil B), also showed a similar pattern of PAH degradation to the isolates of soil A.

4.7 CHARACTERIZATION OF PAH-DEGRADING BACTERIA BASED ON 16S rRNA GENE ANALYSIS: CONFIRMATION AND DIFFERENTIATION OF BACTERIAL SPECIES

This section describes the identification and differentiation of PAH-degrading bacteria based on molecular analysis. In the previous section (Section 4.4.1), all four bacterial strains were tentatively classified as belonging to the same genus, which was *Pseudomonas*, based on the selected determinative criteria (see Table 4.2). However, due to a recent reclassification of bacterial genera and species, it was decided to confirm if the bacteria belong to the genus *Pseudomonas*, as determined by biochemical tests. In the modern taxonomy of bacteria, 16S rRNA gene sequence analysis has become a standard method for bacterial identification and investigation of their phylogenetic relationships. To differentiate among species, DNA-DNA hybridization is required. Recently, polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) have also been used for differentiation amongst species (Urakawa *et al.*, 1997).

4.7.1 Construction of Primers

Primers for the amplification of 16S rRNA genes of the four bacterial strains, as listed in Table 4.7, were designed by Dr. Maria D. E. Serafica, Centre for

Table 4.8 DNA fragments obtained after PCR with primers specific to 16S rRNA genes on total genomic DNA isolated from four PAH-degrading bacterial strains.

Primers	Predicted DNA fragment length (bp)	PCR protocol ^a	VUN 10,010	VUN 10,011	VUN 10,012	VUN 10,013
MAS2F-MAS1R	1,500 ^b	16S	+	+	+	+
F1-R1	333	BCEP	–	–	–	+
FR-R2	619	BCEP	–	–	–	+
F3-R3	309	BCEP	–	–	–	+
F4-R4	447	BCEP	–	–	–	+
F1-MAS1R	1,465	16S	–	–	–	+
MZ1-MZ2	298	BCEP	+	–	+	–
MZ3-MZ4	410	BCEP	+	–	+	–
MZ5-MZ6	345	BCEP	+	–	+	–
Psf1-Psf2	442	BCEP	–	+	–	–
Psf3-Psf4	382	BCEP	–	+	–	–
Psf5-Psf6	378	BCEP	–	+	–	–
Psf1-MAS1R	840	16S	–	+	–	–

^a See Chapter 3, Materials and Methods (Section 3.2.8.5).

^b Approximate length.

– indicates that a particular pair of primers was used to amplify the 16S rRNA gene or a part of the gene of a respective strain.

– indicates that a particular pair of primers was not used to amplify the 16S rRNA gene or a parts of the gene of a respective strain.

Bioprocessing and Food Technology, Victoria University of Technology (personal communication). The primers MAS2F and MAS1R were designed based on a multiple sequence alignment of 16S rRNA gene sequences of *Pseudomonas* spp., *Stenotrophomonas maltophilia* and *Burkholderia cepacia* obtained from National Centre for Biotechnology Information (NCBI) database. This was performed using a multiple comparison program (eclustalw) and then a clear picture of the conserved region among these sequences was generated using a prettybox program. All primers were used as PCR primers as well as sequencing primers. MAS2F and MAS1R (Table 4.7 and 4.8) were used as forward and reverse primers, respectively, to amplify the 1.5 kb 16S rRNA genes for all bacterial strains tested. To complete the whole sequence of the amplified gene, internal primers for the 16S rRNA gene of each bacterial strain were subsequently designed (Table 4.7 and 4.8) for PCR amplification and sequencing based on the available sequence of each individual strain (see Section 4.7.3).

4.7.2 PCR Amplification of 16S rRNA Genes

Genomic DNAs of all four strains tested were amplified with the ribosomal primers used in this study (MAS2F-MAS1R; Tables 4.7 and 4.8). All the strains produce a single band of approximately 1.5 kb PCR product. This size corresponded to the predicted size of the 16S rRNA genes based on the primer pair used (Figure 4.2).

4.7.3 16S rRNA Gene Sequence Analysis (Ribotyping)

Although the 16S rRNA gene of approximately 1.5 kb was amplified from each bacterial strain using MAS2F and MAS1R (Figure 4.2), good sequence data can only be obtained for 300-500 bp due to instrument limitations, quality of the DNA template, primers and reagents. Therefore, internal primers based on the available 16S rRNA gene sequences of each bacterial strain obtained from MAS2F and MAS1R primers were constructed for amplification of the internal regions of the gene and, consequently, their sequencing (Tables 4.7 and 4.8). All sequences were analyzed for their overlap portions and then assembled. The PCR and

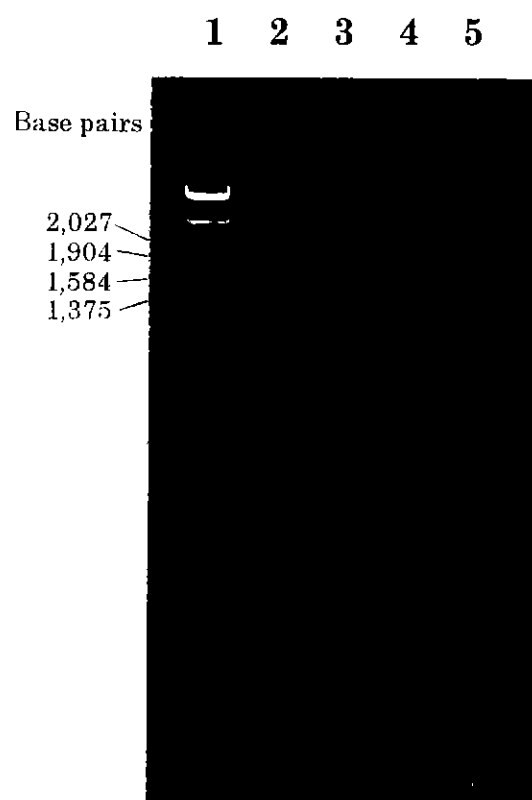


Figure 4.2 PCR amplification of 16S rRNA genes from four PAH-degrading bacteria using primers MAS2F and MAS1R (Table 4.7 and 4.8). Lanes 1, lambda DNA digested with *Eco* RI and *Hind* III (molecular size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

sequencing strategy used generated sequence information for a DNA segment comprising approximately 90-92% of the 16S rRNA gene for each strain.

4.7.3.1 Bacterial Strains VUN 10,010 and VUN 10,012

PCR-amplified 16S rRNA genes, as well as their internal regions, of strains VUN 10,010 and VUN 10,012 are shown in Figures 4.3 and 4.6, respectively. Sequences obtained for strains VUN 10,010 were from the positions 1 to 341 and 987 to 1,380 using MAS2F and MAS1R respectively, as a sequencing primer (Figure 4.4). With the same set of primers, sequences from positions 1 to 344 and 980 to 1382 were obtained for VUN 10,012 (Figure 4.7). Sequences of these two strains based on MAS2F and MAS1R showed a very high similarity in their base compositions. Therefore, the same sets of internal primers (MZ1, MZ2, MZ3, MZ4, MZ5, and MZ6) were designed for these two strains. Internal sequences of the VUN 10,010 16S rRNA gene obtained from these primers were from positions 214 to 498 (MZ1/MZ2), 437 to 813 (MZ4/MZ5) and 753 to 1,074 (MZ5/MZ6) (Figure 4.4). In the same manner, 16S rRNA gene sequences of VUN 10,012 obtained from internal primers were from positions 217 to 499 (MZ1/MZ2), 448 to 810 (MZ4/MZ5) and 753 to 1,076 (MZ5/MZ6) (Figure 4.7). After sequence assembly, the complete sequence of the 16S rRNA gene of strain VUN 10,010 (1,380 bp) and VUN 10,012 (1,382 bp) were found to present approximately 90% of the whole gene (compared to the length of the completed *E. coli* 16S rRNA gene sequence [Brosius *et al.*, 1978]) (Figures 4.5 and 4.8). Sequence comparisons with the Genetics Computer Group (GCG) program demonstrated 100% similarity between strains VUN 10,010 and VUN 10,012 (Figure 4.9). A sequence similarity search based on the NCBI database was performed *via* Australian National Genomic Information Service (ANGIS) using BLASTN (nucleotide sequence vs. nucleotide sequence database) identified these two strains as most closely resembling *Stenotrophomonas maltophilia*, where the level of similarity of 16S rRNA gene sequences of strains VUN 10,010 and VUN 10,012 to *S. maltophilia* was greater than 99.9%. Table 4.9 shows the similarity levels between strains VUN 10,010 and VUN 10,012, and several strains of *Stenotrophomonas* spp. obtained from the NCBI database. Figure 4.10 shows the multiple sequence

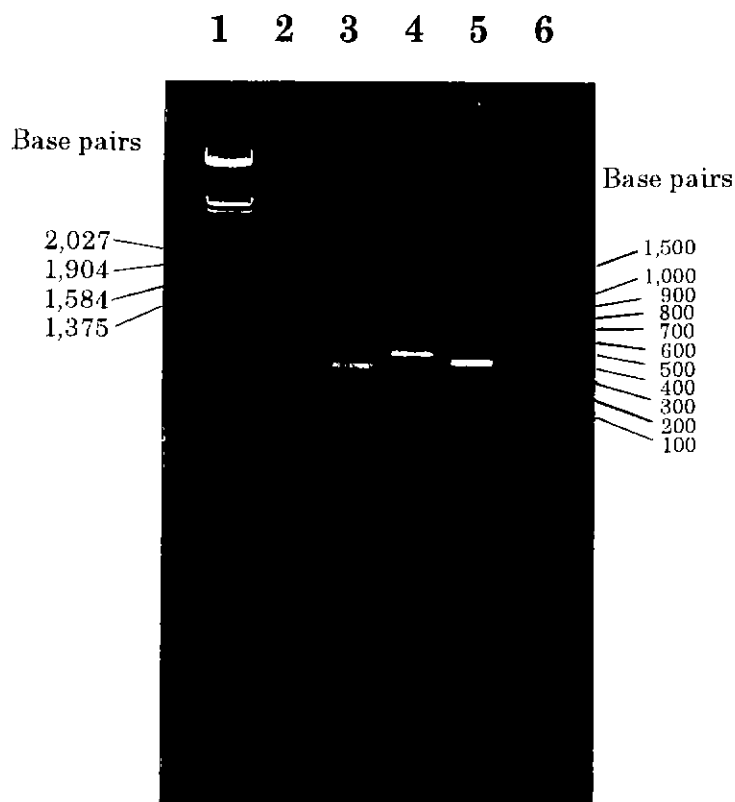


Figure 4.3 PCR amplification of the 16S rRNA gene, and the gene's internal regions, of strain VUN 10,010. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III; lane 2, MAS2F/MAS1R PCR product; lane 3, MZ1/MZ2 PCR product; lane 4, MZ3/MZ4 PCR product; lane 5, MZ5/MZ6 PCR product; and lane 6, 100 bp DNA ladder.

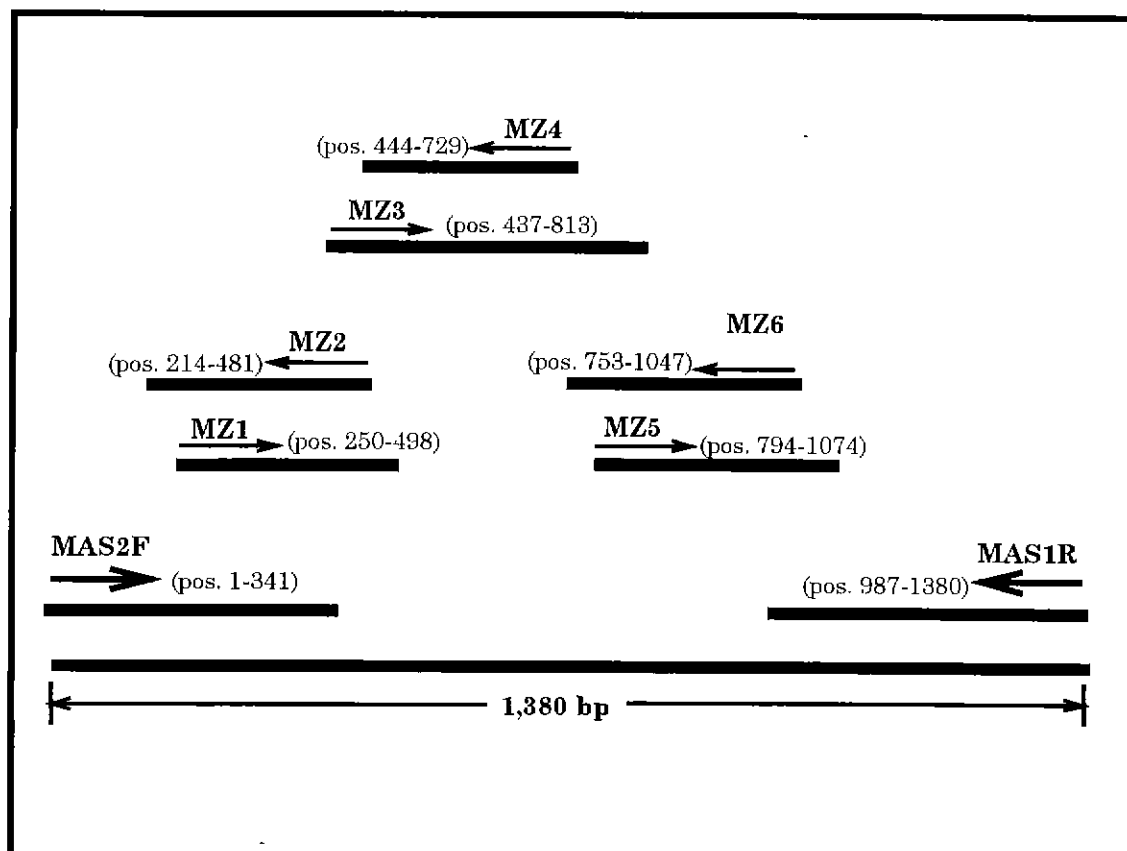


Figure 4.4 Schematic illustration of 16S rRNA gene sequence assembly for strain VUN 10,010. Lists of primers and completed sequence of 16S rRNA gene for VUN 10,010 are shown in Table 4.7 and Figure 4.5, respectively.

1	GGCAGCACAG	GAGAGCTTGC	TCTCTGGGTG	GCGAGTGGCG	GACGGGTGAG
51	GAATACATCG	GAATCTACTT	TTTCGTGGGG	GATAACGTAG	GGAAACTTAC
101	GCTAATACCG	CATACGACCT	ACGGGTGAAA	GCAGGGGATC	TTCGGACCTT
151	GCGCGATTGA	ATGAGCCGAT	GTCGGATTAG	CTAGTTGGCG	GGGTAAAGGC
201	CCACCAAGGC	GACGATCCGT	AGCTGGTCTG	AGAGGATGAT	CAGCCACACT
251	GGAACTGAGA	CACGGTCCAG	ACTCCTACGG	GAGGCAGCAG	TGGGGAATAT
301	TGGACAATGG	GCGCAAGCCT	GATCCAGCCA	TACCGCGTGG	GTGAAGAAGG
351	CC'TCGGGTT	GTAAAGCCCT	TTTGTGTTGG	AAGAAATCCA	GCTGGCTAAT
401	ACCCGGTTGG	GATGACGGTA	CCCAAAGAAT	AAGCACCGGC	TAAC'TCGTG
451	CCAGCAGCCG	CGGTAATACG	AAGGGTGCAA	GCGTTACTCG	GAATTATCGG
501	GCGTAAAGCG	TGCGTAGGTG	GTCGTTTAAG	TCCGTTGTGA	AAGCCCTGGG
551	CTCAACCTGG	GAAGTGCAGT	GGATACTGGG	CGACTAGAGT	GTGGTAGAGG
601	GTAGCGGAAT	TCCTGGTGTA	GCAGTGAAAT	GCGTAGAGAT	CAGGAGGAAC
651	ATCCATGGCG	AAGGCAGCTA	CCTGGACCAA	CACTGACACT	GAGGCACGAA
701	AGCGTGGGGA	GCAAACAGGA	TTAGATACCC	TGGTAGTCCA	CGCCCTAAAC
751	GATGCGAACT	GGATGTTGGG	TGCAATTTGG	CACGCAGTAT	CGAAAGCTAA
801	CGCGTTAAGT	TCGCCGCCTG	GGGAGTACGG	TCGCAAGACT	GAAACTCAAA
851	GGAATTGACG	GGGGCCCGCA	CAAGCGGTGG	AGTATGTGGT	TTAATTCGAT
901	GCAACGCGAA	GAACCTTACC	TGGCCTTGAC	ATGTCGAGAA	CTTTCCAGAG
951	ATGGATTGGT	GCCTTCGGGA	ACTCGAACAC	AGGTGCTGCA	TGGCTGTCTG
1001	CAGCTCGTGT	CGTGAGATGT	TGGGTAAAGT	CCCGCAACGA	GCGCAACCCT
1051	TGTCCTTAGT	TGCCAGCACG	TAATGGTGGG	AACTCTAAGG	AGACCGCCGG
1101	TGACAAACCG	GAGGAAGGTG	GGGATGACGT	CAAGTCATCA	TGGCCCTTAC
1151	GGCCAGGGCT	ACACACGTAC	TACAATGGTA	GGGACAGAGG	GCTGCAAGCC
1201	GGCGACGGTA	AGCCAATCCC	AGAAACCCTA	TCTCAGTCCG	GATTGGAGTC
1251	TGCAACTCGA	CTCCATGAAG	TCGGAATCGC	TAGTAATCGC	AGATCAGCAT
1301	TGCTGCGGGT	GAATACGTTT	CCGGGCCTTG	TACACACCGC	CCGTCACACC
1351	ATGGGAGTTT	GTTGCACCAG	AAGCAGGTAG		

Figure 4.5 The nucleotide sequence of the 16S rRNA gene of strain VUN 10,010, based on the designed primers (MAS2F, MAS1R, MZ1, MZ2, MZ3, MZ4, MZ5, and MZ6). 1,380 bp of the whole gene were sequenced.

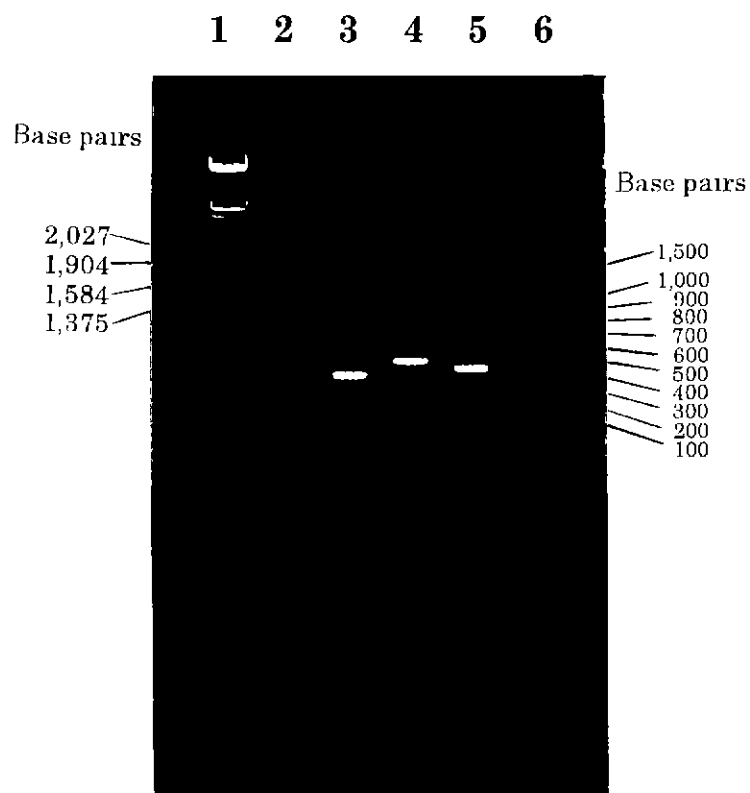


Figure 4.6 PCR amplification of the 16S rRNA gene, and the gene's internal regions, of strain VUN 10,012. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III; lane 2, MAS2F/MAS1R PCR product; lane 3, MZ1/MZ2 PCR product; lane 5, MZ3/MZ4 PCR product; lane 5, MZ5/MZ6 PCR product; and lane 6, 100 bp DNA ladder.

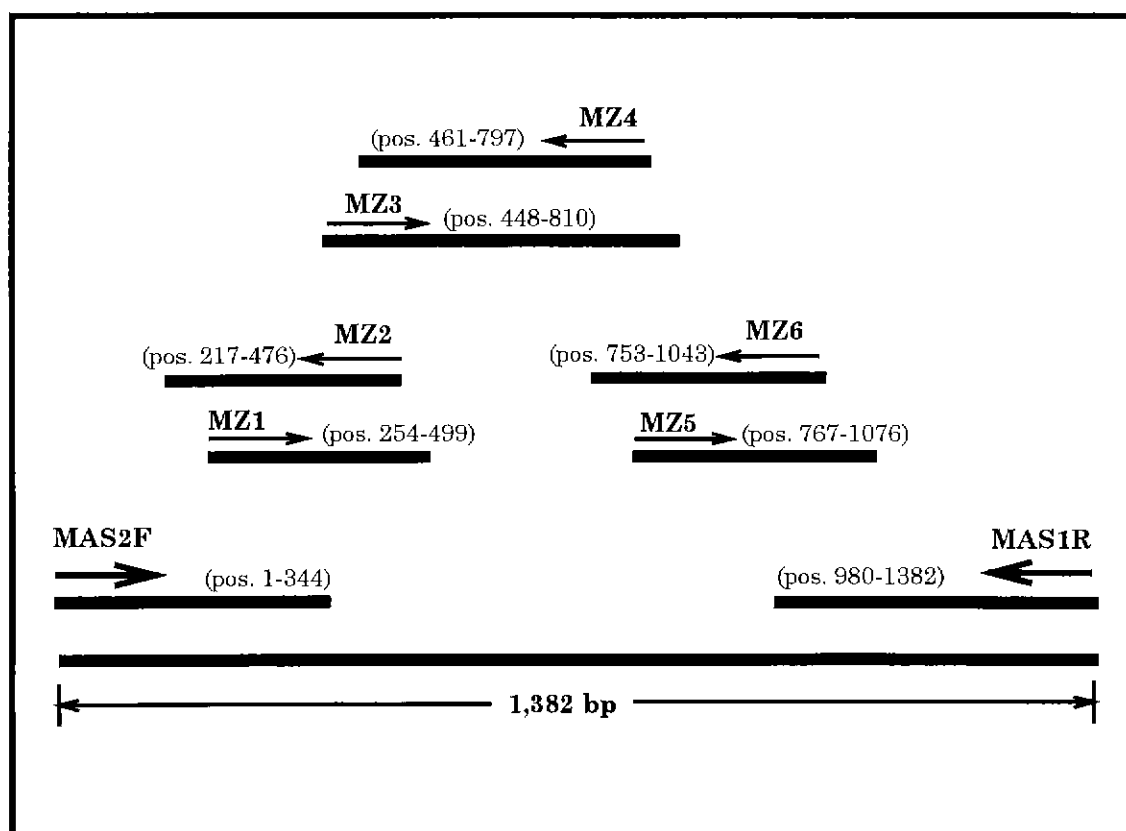


Figure 4.7 Schematic illustration of 16S rRNA gene sequence assembly for strain VUN 10,012. Lists of primers and completed sequence of 16S rRNA gene for VUN 10,012 are shown in Table 4.7 and Figure 4.8, respectively.

1	AACGGCAGCA	CAGGAGAGCT	TGCTCTCTGG	GTGGCGAGTG	GCGGACGGGT
51	GAGGAATACA	TCGGAATCTA	CTTTTTCTGT	GGGGATAACG	TAGGGAAACT
101	TACGCTAATA	CCGCATACGA	CCTACGGGTG	AAAGCAGGGG	ATCTTCGGAC
151	CTTGCGCGAT	TGAATGAGCC	GATGTCGGAT	TAGCTAGTTG	GCGGGGTAAA
201	GGCCCACCAA	GGCGACGATC	CGTAGCTGGT	CTGAGAGGAT	GATCAGCCAC
251	ACTGGAAGTG	AGACACGGTC	CAGACTCCTA	CGGGAGGCAG	CAGTGGGGAA
301	TATTGGACAA	TGGGCGCAAG	CCTGATCCAG	CCATACCGCG	TGGGTGAAGA
351	AGGCCTTCGG	GTTGTAAAGC	CCTTTTGTGT	GGAAAGAAAT	CCAGCTGGCT
401	AATACCCGGT	TGGGATGACG	GTACCCAAAG	AATAAGCACC	GGCTAACTTC
451	GTGCCAGCAG	CCGCGGTAAT	ACGAAGGGTG	CAAGCGTTAC	TCGGAATTAC
501	TGGGCGTAAA	GCGTGCGTAG	GTGGTCGTTT	AAGTCCGTTG	TGAAAGCCCT
551	GGGCTCAACC	TGGGAACTGC	AGTGGATACT	GGGCGACTAG	AGTGTGGTAG
601	AGGGTAGCGG	AATTCCTGGT	GTAGCAGTGA	AATGCGTAGA	GATCAGGAGG
651	AACATCCATG	GCGAAGGCAG	CTACCTGGAC	CAACACTGAC	ACTGAGGCAC
701	GAAAGCGTGG	GGAGCAAACA	GGATTAGATA	CCCTGGTAGT	CCACGCCCTA
751	AACGATGCGA	ACTGGATGTT	GGGTGCAATT	TGGCACGCAG	TATCGAAGCT
801	AACGCGTTAA	GTTCCGCCGC	TGGGGAGTAC	GGTCGCAAGA	CTGAAACTCA
851	AAGGAATTGA	CGGGGGCCCG	CACAAGCGGT	GGAGTATGTG	GTTTAATTCC
901	ATGCAACGCG	AAGAACCCTA	CCTGGCCTTG	ACATGTCGAG	AACTTTCCAG
951	AGATGGATTG	GTGCCTTCGG	GAAACTCGAA	CACAGGTGCT	GCATGGCTGT
1001	CGTCAGCTCG	TGTCGTGAGA	TGTTGGGTTA	AGTCCCGCAA	CGAGCGCAAC
1051	CCTTGTCTTT	AGTTGCCAGC	ACGTAATGGT	GGGAACTCTA	AGGAGACCGC
1101	CGGTGACAAA	CCGGAGGAAG	GTGGGGATGA	CGTCAAGTCA	TCATGGCCCT
1151	TACGGCCAGG	GNTACACACG	TACTACAATG	GTAGGGACAG	AGGGCTGCAA
1201	GCCGGCGACG	GTAAGCCAAT	CCCAGAAACC	CTATCTCAGT	CCGGATTGGA
1251	GTCTGCAACT	CGACTCCATG	AAGTCGGAAT	CGCTAGTAAT	CGCAGATCAG
1301	CATTGCTGCG	GTGAATACGT	TCCCGGGCCT	TGTACACACC	GCCCGTCACA
1351	CCATGGGAGT	TTGTTGCACC	AGAAGCAGGT	AG	

Figure 4.8 The nucleotide sequence of the 16S rRNA of strain VUN 10,012, based on the designed primers (MAS2F, MAS1R, MZ1, MZ2, MZ3, MZ4, MZ5, and MZ6). 1,382 bp of the whole gene were sequenced.

```

1  GGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAG 50
   |||
4  GGCAGCACAGGAGAGCTTGCTCTCTGGGTGGCGAGTGGCGGACGGGTGAG 53

51 GAATACATCGGAATCTACTTTTTTCGTGGGGGATAACGTAGGGAACTTAC 100
   |||
54 GAATACATCGGAATCTACTTTTTTCGTGGGGGATAACGTAGGGAACTTAC 103

101 GCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTT 150
     |||
104 GCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTT 153

151 GCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGC 200
     |||
154 GCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGC 203

201 CCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACT 250
     |||
204 CCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACT 253

251 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT 300
     |||
254 GGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT 303

301 TGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGG 350
     |||
304 TGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGG 353

351 CCTTCGGGTGTAAAGCCCTTTTGTGGGAAAGAAATCCAGCTGGCTAAT 400
     |||
354 CCTTCGGGTGTAAAGCCCTTTTGTGGGAAAGAAATCCAGCTGGCTAAT 403

401 ACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTG 450
     |||
404 ACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTG 453

451 CCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGG 500
     |||
454 CCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGG 503

501 GCGTAAAGCGTGCGTAGGTGGTCGTTTAAAGTCCGTTGTGAAAGCCCTGGG 550
     |||
504 GCGTAAAGCGTGCGTAGGTGGTCGTTTAAAGTCCGTTGTGAAAGCCCTGGG 553

551 CTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGG 600
     |||
554 CTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGG 603

601 GTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAAC 650
     |||
604 GTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAAC 653

```

Figure 4.9 Comparison between the 16S rRNA gene sequences for strains VUN 10,010 (from positions 1-1,380) and VUN 10,012 (from positions 4-1,382) using similarity comparison (Bestfit) program *via* ANGIS. Similarity of the 16S rRNA genes between the two strains based on the sequences compared is 100%.

```

651 ATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAA 700
    |||
654 ATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAA 703
    |||
701 AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC 750
    |||
704 AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAAC 753
    |||
751 GATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAAGCTAA 800
    |||
754 GATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCG .AAGCTAA 802
    |||
801 CGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA 850
    |||
803 CGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA 852
    |||
851 GGAATTGACGGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGAT 900
    |||
853 GGAATTGACGGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGAT 902
    |||
901 GCAACGCGAAGAACCCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAG 950
    |||
903 GCAACGCGAAGAACCCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAG 952
    |||
951 ATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGT 1000
    |||
953 ATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGT 1002
    |||
1001 CAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCT 1050
    |||
1003 CAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCT 1052
    |||
1051 TGTCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCCGG 1100
    |||
1053 TGTCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCCGG 1102
    |||
1101 TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC 1150
    |||
1103 TGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC 1152
    |||
1151 GGCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCC 1200
    |||
1153 GGCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCC 1202
    |||
1201 GGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTC 1250
    |||
1203 GGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTC 1252
    |||
1251 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCAT 1300
    |||
1253 TGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCAT 1302
    |||
1301 TGCTGCGGGTGAATACGTTCCCGGGCCTTGACACACCGCCCGTCACACC 1350
    |||
1303 TGCTGCGGGTGAATACGTTCCCGGGCCTTGACACACCGCCCGTCACACC 1352
    |||
1351 ATGGGAGTTTGTGTCACCAGAAGCAGGTAG 1380
    |||
1353 ATGGGAGTTTGTGTCACCAGAAGCAGGTAG 1382
    |||

```

Figure 4.9 (continued).

Table 4.9 Comparison of the similarity of the 16S rRNA genes of strains VUN 10,010 and VUN 10,012 with *Stenotrophomonas* spp. on the NCBI database.

Organism	Accession number	%Similarity to strain:	
		VUN 10,010	VUN 10,012
<i>Stenotrophomonas maltophilia</i> LMG 958-T	X95923	99.927	99.928
<i>Stenotrophomonas maltophilia</i> ATCC 13736T	AB008509	99.855	99.855
<i>Stenotrophomonas</i> sp. isolate S3	AJ002814	99.492	99.493
<i>Stenotrophomonas maltophilia</i> LMG 11087	X95924	99.057	99.059
<i>Stenotrophomonas maltophilia</i> N4-15	AF017749	98.838	98.841

	10	20	30	40	50	60
AB008509.dna	AGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAGCACAG	-GAGAGCTTG				
AJ002814.dna	AGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAGCACAG	TAAGAGCTTG				
VUN10010.seq	-----GGCAGCACAG	-GAGAGCTTG				
VUN10012.seq	-----AACGGCAGCACAG	-GAGAGCTTG				
X95923.dna	AGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAGCACAG	-GAGAGCTTG				
X95924.dna	AGTGAACGCTGGCGGTAGGCCTAACACATGCAAGTCGAACGGCAGCACAG	TAAGAGCTTG				
		*****	*****			
AB008509.dna	CTCTC-TGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
AJ002814.dna	CTCTTACGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
VUN10010.seq	CTCTC-TGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
VUN10012.seq	CTCTC-TGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
X95923.dna	CTCTC-TGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
X95924.dna	CTCTTATGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG					
	****	*****	*****			
AB008509.dna	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
AJ002814.dna	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
VUN10010.seq	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
VUN10012.seq	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
X95923.dna	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
X95924.dna	GGGATAACGTAGGGAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGA					
	*****	*****	*****			
AB008509.dna	TCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
AJ002814.dna	TCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
VUN10010.seq	TCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
VUN10012.seq	TCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
X95923.dna	TCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
X95924.dna	CCTTCGGGCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAG					
	*****	*****	*****			
AB008509.dna	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
AJ002814.dna	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
VUN10010.seq	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
VUN10012.seq	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
X95923.dna	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
X95924.dna	CCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGA					
	*****	*****	*****			
AB008509.dna	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
AJ002814.dna	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
VUN10010.seq	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
VUN10012.seq	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
X95923.dna	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
X95924.dna	GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGC					
	*****	*****	*****			

Figure 4.10 Alignment of VUN 10,010 and VUN 10,012 16S rRNA gene sequences and 16S rRNA gene sequences from *Stenotrophomonas maltophilia* ATCC 13736T (accession number AB008509), *Stenotrophomonas* sp. isolate S3 (accession number AJ002814), *S. maltophilia* LMG 958-T (accession number X95923) and *S. maltophilia* LMG 11087 (accession number X95924). A dash indicates a gap at a particular position on the sequence compared to other sequences. An asterisk indicates that at a particular position all of sequences aligned possess the same nucleotide.

```

AB008509.dna      CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
AJ002814.dna      CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
VUN10010.seq      CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
VUN10012.seq      CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
X95923.dna        CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
X95924.dna        CTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTGG
*****

AB008509.dna      GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
AJ002814.dna      GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
VUN10010.seq      GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
VUN10012.seq      GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
X95923.dna        GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
X95924.dna        GAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCG
*****

AB008509.dna      GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
AJ002814.dna      GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
VUN10010.seq      GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
VUN10012.seq      GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
X95923.dna        GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
X95924.dna        GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACT
*****

AB008509.dna      GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
AJ002814.dna      GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
VUN10010.seq      GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
VUN10012.seq      GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
X95923.dna        GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
X95924.dna        GGGCGTAAAGCGTGCGTAGGTGGTTCGTTTAAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
*****

AB008509.dna      GGGAACTGCAGTGGATACTGGACGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
AJ002814.dna      GGGAACTGCAGTGGAACTGGACGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
VUN10010.seq      GGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
VUN10012.seq      GGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
X95923.dna        GGGAACTGCAGTGGATACTGGNCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
X95924.dna        GGGAACTGCAGTGGAACTGGACAACACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTG
*****

AB008509.dna      TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
AJ002814.dna      TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
VUN10010.seq      TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
VUN10012.seq      TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
X95923.dna        TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
X95924.dna        TAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
*****

AB008509.dna      AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
AJ002814.dna      AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
VUN10010.seq      AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
VUN10012.seq      AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
X95923.dna        AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
X95924.dna        AACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCCTGGTAGTC
*****

AB008509.dna      CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAA-GCT
AJ002814.dna      CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAA-GCT
VUN10010.seq      CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAAGCT
VUN10012.seq      CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAA-GCT
X95923.dna        CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAA-GCT
X95924.dna        CACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAA-GCT
*****

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Figure 4.10 (continued).

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AB008509.dna      AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
AJ002814.dna      AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
VUN10010.seq      AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
VUN10012.seq      AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
X95923.dna        AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
X95924.dna        AACGCGTTAAGTTCGCCGCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
*****

AB008509.dna      CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
AJ002814.dna      CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
VUN10010.seq      CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
VUN10012.seq      CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
X95923.dna        CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
X95924.dna        CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
*****

AB008509.dna      CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
AJ002814.dna      CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
VUN10010.seq      CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
VUN10012.seq      CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
X95923.dna        CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
X95924.dna        CCTGGCCTTGACATGTCGAGAACCTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAAC
*****

AB008509.dna      ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
AJ002814.dna      ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
VUN10010.seq      ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
VUN10012.seq      ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
X95923.dna        ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
X95924.dna        ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAAC
*****

AB008509.dna      GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
AJ002814.dna      GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
VUN10010.seq      GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
VUN10012.seq      GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
X95923.dna        GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
X95924.dna        GAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACCTAAGGAGACCGCC
*****

AB008509.dna      GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
AJ002814.dna      GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
VUN10010.seq      GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
VUN10012.seq      GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
X95923.dna        GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
X95924.dna        GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGG
*****

AB008509.dna      CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
AJ002814.dna      CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
VUN10010.seq      CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
VUN10012.seq      CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
X95923.dna        CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
X95924.dna        CTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATC
*****

AB008509.dna      CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
AJ002814.dna      CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
VUN10010.seq      CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
VUN10012.seq      CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
X95923.dna        CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
X95924.dna        CCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATC
*****

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Figure 4.10 (continued).


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AB008509.dna      GCTAGTAATCGCAGATCAGCATTGCTGCGG-TGAATACGTTCCCGGGCCTTGTACACACC
AJ002814.dna      GCTAGTAATCGCAGATCAGCATTGCTGCGG-TGAATACGTTCCCGGGCCTTGTACACACC
VUN10010.seq      GCTAGTAATCGCAGATCAGCATTGCTGCGGGTGAATACGTTCCCGGGCCTTGTACACACC
VUN10012.seq      GCTAGTAATCGCAGATCAGCATTGCTGCGGGTGAATACGTTCCCGGGCCTTGTACACACC
X95923.dna        GCTAGTAATCGCAGATCAGCATTGCTGCGG-TGAATACGTTCCCGGGCCTTGTACACACC
X95924.dna        GCTAGTAATCGCAGATCAGCATTGCTGCGG-TGAATACGTTCCCGGGCCTTGTACACACC
*****

AB008509.dna      GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGC
AJ002814.dna      GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGC
VUN10010.seq      GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAG-----
VUN10012.seq      GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAG-----
X95923.dna        GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGC
X95924.dna        GCCCGTCACACCATGGGAGTTTGTGTCACCAGAAGCAGGTAGCTTAACCTTCGGGAGGGC
*****

AB008509.dna      GCTTGCCACGGTGTGGCCGATGACTGGGGTG-----
AJ002814.dna      GCTTGCCACGGTGTGGCCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAG
VUN10010.seq      -----
VUN10012.seq      -----
X95923.dna        GCTTGCCACGGTGTGGCCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAG
X95924.dna        GCTTGCCACGGTGTGGCCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAG

AB008509.dna      ----
AJ002814.dna      GTGC
VUN10010.seq      ----
VUN10012.seq      ----
X95923.dna        GTGC
X95924.dna        GTGC

```

Figure 4.10 (continued).

alignment of the 16S rRNA gene sequences of strains VUN 10,010, VUN 10,012 with some other strains of *Stenotrophomonas* spp., which was performed by using a multiple comparison program (eclustalw).

4.7.3.2 Strain VUN 10,011

PCR amplification products of the 16S rRNA gene as well as the internal regions for strain VUN 10,011 are shown in Figure 4.11. Sequences were obtained for strain VUN 10,011 from positions 1 to 348 and 771 to 1,399 using MAS2F and MAS1R, respectively, as sequencing primers. Based on these portions, internal primers (Psf1, Psf2, Psf3, and Psf4; Tables 4.7 and 4.8) were constructed. Internal sequences obtained from these primers were from positions 246 to 660 (Psf1/Psf2) and from positions 507 to 854 (Psf3/Psf4). The same approach was used for Psf5 and Psf6 to complete sequences from positions 691 to 1,061 (Figure 4.12). After sequence assembly (Figure 4.13), the completed sequence of the 16S rRNA gene of VUN 10,011 (1,399 bp) was found to represent approximately 91% of the whole gene (compared to the length of the completed *E. coli* 16S rRNA gene sequence [Brosius *et al.*, 1978]). A sequence similarity search based on the NCBI database was performed *via* ANGIS using BLASTN identified this strain as most closely resembling *Pseudomonas fluorescens* (the level of similarity was greater than 98.5%). Table 4.10 shows the similarity levels between strains VUN 10,011 and several strains of *Pseudomonas* spp. obtained from the NCBI database. Figure 4.14 shows the multiple sequence alignment of the 16S rRNA gene sequences of strain VUN 10,011 with some other strains of *Pseudomonas* spp., which was performed by using a multiple comparison program (eclustalw).

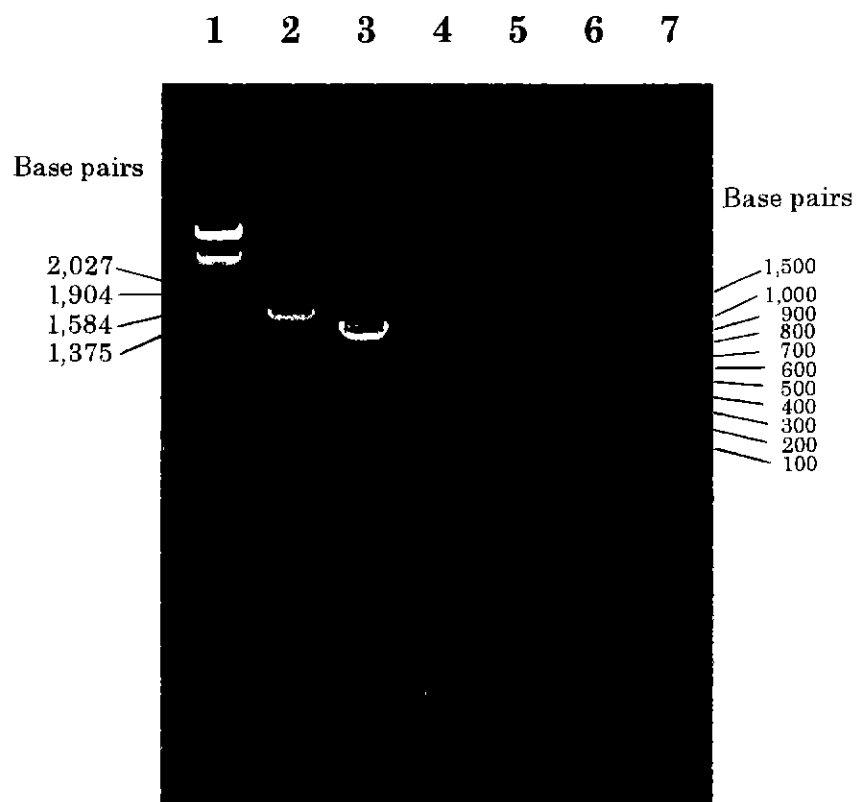


Figure 4.11 PCR amplification of the 16S rRNA gene, and the gene's internal regions, of strain VUN 10,011. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III; lane 2, MAS2F/MAS1R PCR product; lane 3, Psf1/MAS 1R PCR product; lane 4, Psf1/Psf2 PCR product; lane 5, Psf3/Psf4 PCR product; lane 6, Psf5/Psf6 PCR product; and lane 7, 100 bp DNA ladder.

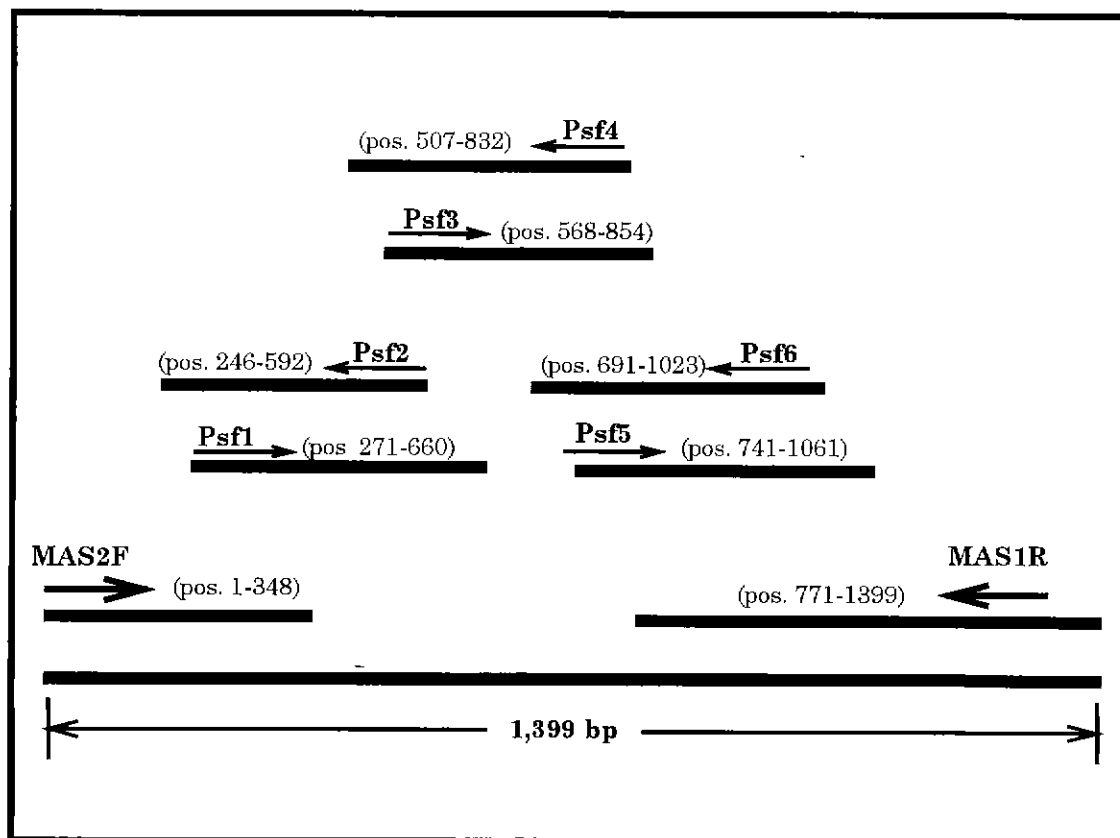


Figure 4.12 Schematic illustration of 16S rRNA gene sequence assembly for strain VUN 10,011. Lists of primers and completed sequence of 16S rRNA gene for VUN 10,011 are shown in Table 4.7 and Figure 4.13, respectively.

1	GTCGAGCGGT	AAGAGAGANC	TTGCTTCTCT	TGTAGAGCGG	CGGTACGGGT
51	GAGTAAAGCC	NAGGAATCTG	CCTGGTAGTG	GGGGATAACG	TTCGGAAACG
101	GACGCTAATA	CCGCATACGT	CCTACGGGAG	AAAGCAGGGG	ACCTTCGGGC
151	CTTGCGCTAT	CANATGAGCC	TANGTCGGAT	TAGCTAGTTG	GTGAGGTAAT
201	GGCTCACCAA	GGCGACGATC	CGTAACTGGT	CTGAGAGGAT	GATCAGTCAC
251	ACTGGAAGTG	AGACACGGTC	CAGACTCCTA	CGGGAGGCAG	CAGTGGGGAA
301	TATTGGACAA	TGGGCGAAAG	CCTGATCCAG	CCATGCCGCG	TGTGTTAAGA
351	AGGTCTTCGG	ATTGTAAAGC	ACTTTAAGTT	GGGAGGAAGG	GCATTAACCT
401	AATACGTTAG	TGTTTTGACG	TTACCGACAG	AATAAGCACC	GGCTAACTCT
451	GTGCCAGCAG	CCGCGGTAAT	ACAGAGGGTG	CAAGCGTTAA	TCGGAATTAC
501	TGGGCGTAAA	GCGCGCGTAG	GTGGTTTGTT	AAGTTGGATG	TGAAATCCCC
551	GGGCTCAACC	TGGGAACTGC	ATTCAAAACT	GATTGACTAG	AGTATGGTAG
601	AGGGTGGTGG	AATTTCTGT	GTAGCGGTGA	AATGCGTAGA	TATAGGAAGG
651	AACACCAGTG	GCGAAGGCGA	CCACCTGGAC	TAATACTGAC	ACTGAGGTGC
701	GAAAGCGTGG	GGAGCAAACA	GGATTAGATA	CCCTGGTAGT	CCACGCCGTA
751	AACGATGTCA	ACTAGCCGTT	GGGAGCCTTG	AGCTCTTAGT	GGCGCAGCTA
801	ACGCATTAAG	TTGACCGCCA	GGGGAGTACG	GCCGCAAGGT	TAAAAC TCAA
851	ATGAATTGAC	GGGGGCCCGC	ACAAGCGGTG	GAGCATGTGG	TTTAATT CGA
901	AGCAACGCGA	AGAGCCTTAC	CAGGCCTTGA	CATCCAATGA	ACTTTCTAGA
951	GATAGATTGG	TGCCTTCGGG	AACATTGAGA	CAGGTGCTGC	ATGGCTGT CG
1001	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG	TCCCGTAACG	AGCGCAACCC
1051	TTGTCCCTTAG	TTACCAGCAC	GTAATGGTGG	GCACTCTAAG	GAGACTGCCG
1101	GTGACAAACC	GGAGGAAGGT	GGGGATGACG	TCAAGTCATC	ATGGCCCTTA
1151	CGGCCTGGGN	TACACACGTG	CTACAATGGT	CGGTACAGAG	GGTTGCCAAG
1201	CCGCGAGGTG	GAGCTAATCC	CACAAAACCG	ATCGTAGTCC	GGATCGCAGT
1251	CTGCAACTCG	ACTGCGTGAA	GTCGGAATCG	CTAGTAATCG	CGAATCAGAA
1301	TGTCGCGGTG	AATACGTTCC	CGGGCCTTGT	ACACACCGCC	CGTCACACCA
1351	TGGGAGTGGG	TTGCACCAGA	AGTAGCTAGT	CTAACNTTCG	GGAGANCGG

Figure 4.13 The nucleotide sequence of the 16S rRNA gene of strain VUN 10,011, based on the designed primers (MAS2F, MAS1R, Psf1, Psf2, Psf3, Psf4, Psf5, and Psf6). 1,399 bp of the whole gene were sequenced.

Table 4.10 Comparison of the similarity of the 16S rRNA genes of strain VUN 10,011 with *Pseudomonas* spp. on the NCBI database.

Organism	Accession number	%Similarity to strain VUN 10,011
<i>Pseudomonas fluorescens</i> strain IAM 12022	D84013	99.141
<i>Pseudomonas fluorescens</i> strain DSM 50090T	Z76662	98.576
<i>Pseudomonas</i> sp. strain A177	U85870	98.354
<i>Pseudomonas</i> sp. strain IpA-2	X96788	97.781
<i>Pseudomonas putida</i> strain IFO 14164	D37923	96.782

```

          10          20          30          40          50          60
D37923.dna -----TGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
D84013.dna AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
U85870.dna -----TTGANCNTGNCGGNAGGCCTAACACATGCAAGTCGAGCGG
VUN10011.seq -----GTCGAGCGG
X96788.dna -----ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
Z76662.dna -----ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGG
                      *****

D37923.dna ATGAGAAGAGCTTGCT-CTTCGATTCAGCGGCGG-ACGGGTGAGTAATGCCTAGGAATCT
D84013.dna TAGAGAGAAGCTTGCTTCTCTTG-AGAGAGGCGG-ACGGGTGAGTAAAGCCTAGGAATCT
U85870.dna TAGAGAGAAGCTTGCTTCTCTTG-AGAGCGGCGG-ACGGGTGAGTAATGCCTAGGAATCT
VUN10011.seq TAAGAGAGANCTTGCTTCTCTTG TAGAGCGGCGGTACGGGTGAGTAAAGCCNAGGAATCT
X96788.dna ATGAAGAGAGCTTGCT-CTCTGATTCAGCGGCGG-ACGGGTGAGTAATGCCTAGGAATCT
Z76662.dna TAGAGAGAAGCTTGCTTCTCTTG-AGAGCGGCGG-ACGGGTGAGTAAAGCCTAGGAATCT
                      * ***** **

D37923.dna GCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGA
D84013.dna GCCTGGTAGTGGGGGATAACGTTTCGAAACGGACGCTAATACCGCATACGTCCTACGGGA
U85870.dna GCCTGGTAGTGGGGGATAACGTTTCGAAACGAACGCTAATACCGCATACGTCCTACGGGA
VUN10011.seq GCCTGGTAGTGGGGGATAACGTTTCGAAACGGACGCTAATACCGCATACGTCCTACGGGA
X96788.dna GCCTGGTAGTGGGGGACAACGTTTCGAAAGGGACGCTAATACCGCATACGTCCTACGGGA
Z76662.dna GCCTGGTAGTGGGGGATAACGTTTCGAAACGGACGCTAATACCGCATACGTCCTACGGGA
                      *****

D37923.dna GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTT
D84013.dna GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTT
U85870.dna GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTT
VUN10011.seq GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCANATGAGCCTANGTCGGATTAGCTAGTT
X96788.dna GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTT
Z76662.dna GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTTCGGATTAGCTAGTT
                      *****

D37923.dna GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
D84013.dna GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
U85870.dna GGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
VUN10011.seq GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
X96788.dna GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
Z76662.dna GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAAC TGGTCTGAGAGGATGATCAGTCA
                      ****

D37923.dna CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
D84013.dna CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
U85870.dna CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
VUN10011.seq CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
X96788.dna CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
Z76662.dna CACTGGAACTGAGACTCGGTCCANACNCCTACGGNNGCNGCAGTGGGGAATATTGGACA
                      *****

```

Figure 4.14 Alignment of VUN 10,011 16S rRNA gene sequence and the 16S rRNA gene sequences from *Pseudomonas fluorescens* strain IAM 12022 (accession number D84013), *Ps. fluorescens* strain DSM 50090T (accession number Z76662), *Pseudomonas* sp. strain A177 (accession number U85870), *Psuedomonas* sp. strain IpA-2 (accession number X96788) and *Ps. putida* strain IFO 14164 (accession number D37923). A dash indicates a gap at a particular position on the sequence compared to others. An asterisk indicates that at a particular position all of sequences aligned possess the same nucleotide.

```

D37923.dna      ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
D84013.dna      ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGGTTGTAAAG
U85870.dna      ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
VUN10011.seq    ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
X96788.dna      ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
Z76662.dna      ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
*****

D37923.dna      CACTTTAAGTTGGGAGGAAGGGCATTAAACCTAATACGTTAGTGTGTTTGACGTTACCGACA
D84013.dna      CACTTTAAGTTGGGAGGAAGGGCATTAAACCTAATACGTTAGTGTGTTTGACGTTACCGACA
U85870.dna      CACTTTAAGTTGGGAGGAAGGGCATTAAACCTAATACGTTAGTGTGTTTGACGTTACCGACA
VUN10011.seq    CACTTTAAGTTGGGAGGAAGGGCATTAAACCTAATACGTTAGTGTGTTTGACGTTACCGACA
X96788.dna      CACTTTAAGTTGGGAGGAAGGGTNGTANATTAATACNTGTCNATTTGACGTTACCGACA
Z76662.dna      CACTTTANGTTGGGAGGAAGGGCATTAAACCTAATACGTTAGTGTGTTTGACGTTACCGACA
*****

D37923.dna      GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
D84013.dna      GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
U85870.dna      GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
VUN10011.seq    GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
X96788.dna      GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
Z76662.dna      GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
*****

D37923.dna      ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAAGCCC
D84013.dna      ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAATCCC
U85870.dna      ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAATCCC
VUN10011.seq    ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAATCCC
X96788.dna      ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAAGCCC
Z76662.dna      ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTGTTAAGTTGGATGTGAAATCCC
*****

D37923.dna      CGGGCTCAACCTGGGAAGTGCATCCAAACTGCGCAAGCTAGAGTACGGTAGAGGGTGGTG
D84013.dna      CGGGCTCAACCTGGGAAGTGCATCCAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
U85870.dna      CGGGCTCAACCTGGGAAGTGCATCCAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
VUN10011.seq    CGGGCTCAACCTGGGAAGTGCATCCAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
X96788.dna      CGGGCTCAACCTGGGAAGTGCATCCAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTG
Z76662.dna      CGGGCTCAACCTGGGAAGTGCATCCAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
*****

D37923.dna      GAATTTCCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCG
D84013.dna      GAATTTCCCTGTGTAGCGGTGAAATGCGCAGATATAGGAAGGAACACCAAGTGGCGAAGGCG
U85870.dna      GAATTTCCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCG
VUN10011.seq    GAATTTCCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCG
X96788.dna      GAATTTCCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCG
Z76662.dna      GAATTTCCCTGTGTAGNGGTGAAATGCGTTGATATAGGAAGGAACACCAAGTGGTGAAGGCG
*****

D37923.dna      ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
D84013.dna      ACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
U85870.dna      ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
VUN10011.seq    ACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
X96788.dna      ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
Z76662.dna      ACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
*****

D37923.dna      ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAG
D84013.dna      ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAG
U85870.dna      ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAG
VUN10011.seq    ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAG
X96788.dna      ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAG
Z76662.dna      ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGTGCCTTGAGCTCTTAG
*****

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Figure 4.14 (continued).


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D37923.dna      TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
D84013.dna      TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
U85870.dna      TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
VUN10011.seq    TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
X96788.dna      TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
Z76662.dna      TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
*****

D37923.dna      AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
D84013.dna      AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
U85870.dna      AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
VUN10011.seq    AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
X96788.dna      AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
Z76662.dna      AATGAATTGACGGGGGCCCCGACACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
*****

D37923.dna      AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
D84013.dna      AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
U85870.dna      AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
VUN10011.seq    AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
X96788.dna      AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
Z76662.dna      AAGAACCTTACCAGGCCTTGACATGCAGAGAACTTCCAGAGATGGATTGGTGCCTTCGG
****

D37923.dna      GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
D84013.dna      GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
U85870.dna      GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
VUN10011.seq    GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
X96788.dna      GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
Z76662.dna      GAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAA
****

D37923.dna      GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
D84013.dna      GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
U85870.dna      GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
VUN10011.seq    GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
X96788.dna      GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
Z76662.dna      GTCCCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
*****

D37923.dna      GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
D84013.dna      GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
U85870.dna      GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
VUN10011.seq    GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
X96788.dna      GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
Z76662.dna      GGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
*****

D37923.dna      ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
D84013.dna      ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
U85870.dna      ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
VUN10011.seq    ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
X96788.dna      ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
Z76662.dna      ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGT
*****

D37923.dna      GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
D84013.dna      GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
U85870.dna      GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
VUN10011.seq    GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
X96788.dna      GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
Z76662.dna      GGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGA
*****

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Figure 4.14 (continued).

```

D37923.dna      AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCG-----
D84013.dna      AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
U85870.dna      AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
VUN10011.seq    AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
X96788.dna      AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
Z76662.dna      AGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTG
*****

D37923.dna      -----
D84013.dna      TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC
U85870.dna      TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC
VUN10011.seq    TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACNTTC
X96788.dna      TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC
Z76662.dna      TACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC

D37923.dna      -----
D84013.dna      GGGAGGGCGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
U85870.dna      GGGAGGACGGTTACCANGGTGTGATTCATGACTGGGGTG-----
VUN10011.seq    GGGAGNACGG-----
X96788.dna      GGGAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTANCCGT
Z76662.dna      GGGAGGACGGTTACCACGGTGTGATTCATGACTGGAGTGAAGTCGTAACAAGGTAGCCGT

D37923.dna      -----
D84013.dna      AGGGGAACCTGCGGCTGGATCACCTCCTT
U85870.dna      -----
VUN10011.seq    -----
X96788.dna      AGGGGAACCTGC-----
Z76662.dna      AGGGGAACCTGCGGCTGGATCACCTCC--

```

Figure 4.14 (continued).

4.7.3.3 Strain VUN 10,013

PCR amplification products of the 16S rRNA gene as well as the gene internal regions for strain VUN 10,013 are shown in Figures 4.15. Sequences obtained for strain VUN 10,013 were from positions 13 to 274 and 962 to 1,395 using MAS2F and MAS1R, respectively, as sequencing primers. Internal sequences obtained from internal primers were as follows: positions 1 to 298 (F1/R1), positions 575 to 1141 (F2/R2), positions 282 to 604 (F3/R3) and positions 467 to 868 (F4/R4) (Figure 4.16). After sequence assembly, the completed sequence of the 16S rRNA gene of VUN 110,013 (1,395 bp; Figure 4.17) accounted for approximately 91% of the whole gene (compared to the length of the completed *E. coli* 16S rRNA gene sequence [Brosius *et al.*, 1978]). A sequence similarity search based on the NCBI database was performed *via* ANGIS using BLASTN identified this strain as most closely resembling *Burkholderia* sp. (the level of similarity was greater than 97%). Table 4.11 shows the similarity levels between strains VUN 10,013 and several strains of *Burkholderia* spp. obtained from the NCBI database. Figure 4.18 shows the multiple sequence alignment of the 16S rRNA gene sequences of strain VUN 10,013 with some other strains of *Burkholderia* spp., which was performed by using a multiple comparison program (eclustalw).

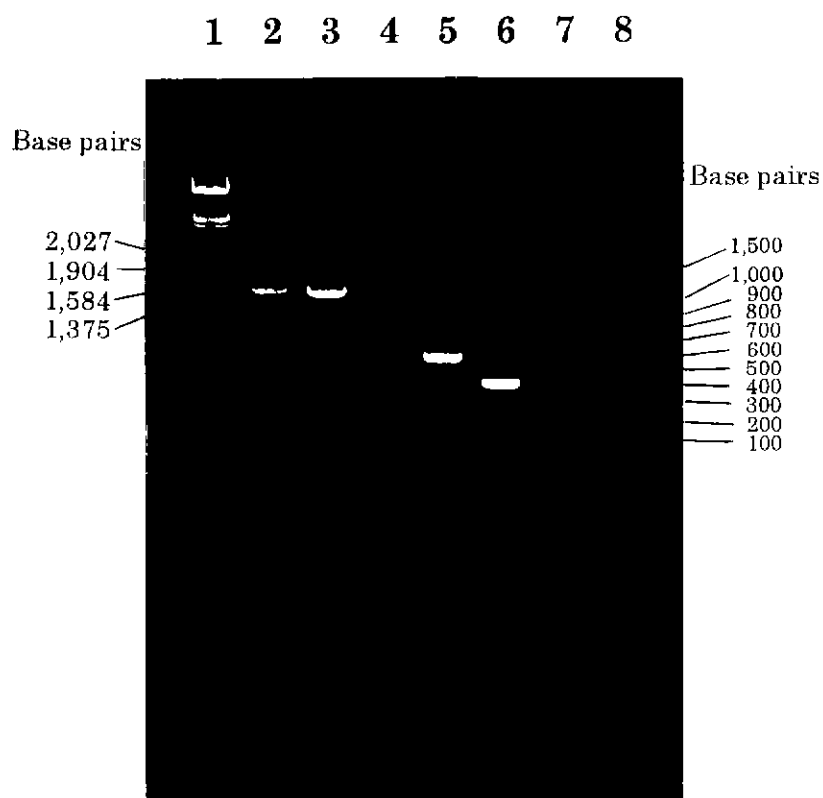


Figure 4.15 PCR amplification of the 16S rRNA gene, and the genes' internal regions, of strain VUN 10,013. Lane 1, lambda DNA digested with *Eco* RI and *Hind* III (molecular marker); lane 2, MAS2F/MAS1R PCR product; lane 3, F1/MAS 1R PCR product; lane 4, F1/R1 PCR product; lane 5, F2/R2 PCR product; lane 6, F3/R3 PCR product; lane 7, F4/R4 PCR product; and lane 8, 100 bp DNA ladder.

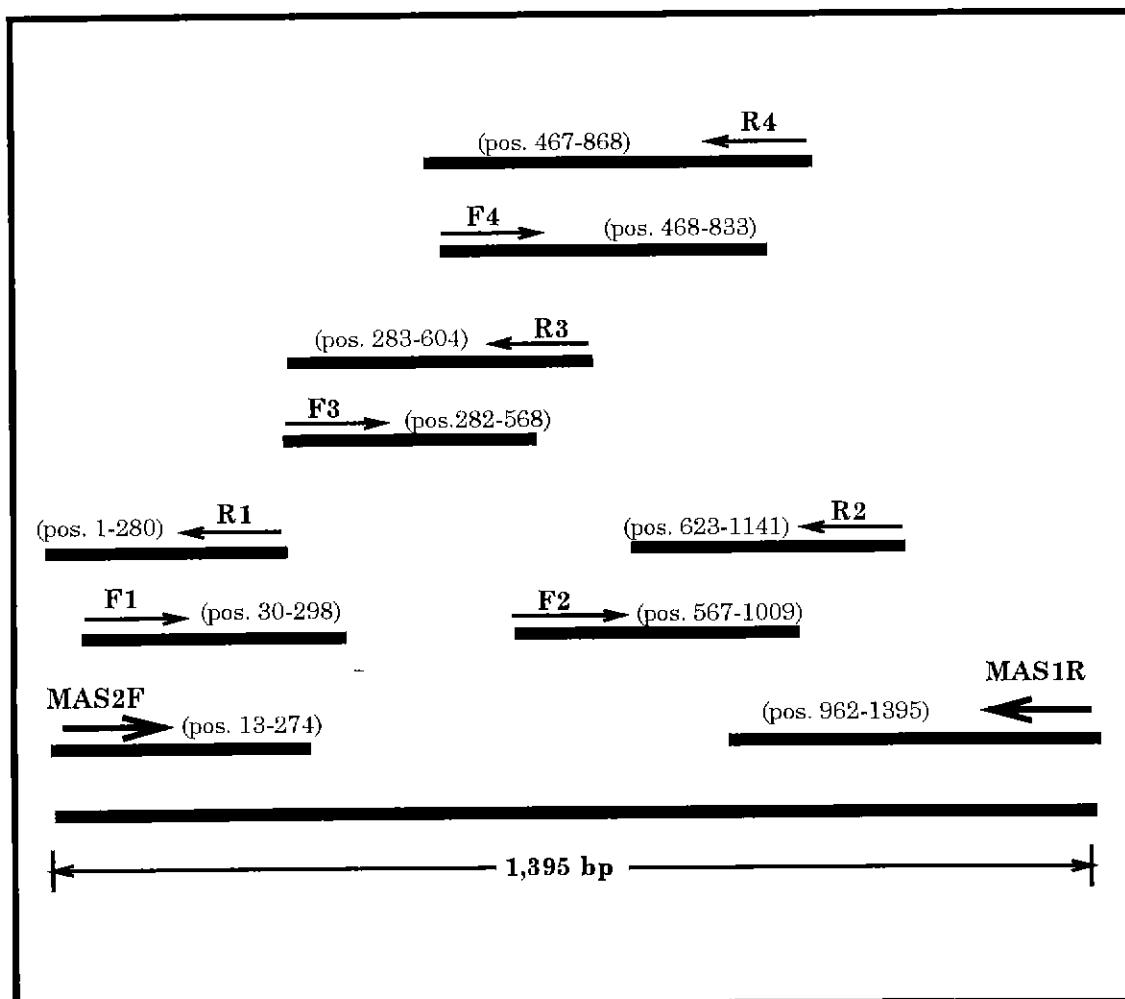


Figure 4.16 Schematic illustration of 16S rRNA gene sequence assembly for strain VUN 10,013. Lists of primers and completed sequence of 16S rRNA gene for VUN 10,013 are shown in Table 4.7 and Figure 4.17, respectively.

1	ACATGCAAGT	CGAACGGCAG	CACGGGAGCA	ATCCTGGTGG	CGAGTGGCGA
51	ACGGGTGAGT	AATACATCGG	AACGTGTCCT	GTAGTGGGGG	ATAGCCCGGC
101	GAAAGCCGGA	TTAATACCGC	ATACGATCTG	TGGATGAAAG	CGGGGGATCT
151	TAGGACTCTG	CGCTACAGGG	GCGGCCGATG	GCAGATTAGC	TAGTTGGTGG
201	GGTAAAGGCC	TACCAAGGCG	ACGATCTGTA	GCTGGTCTGA	GAGGACGACC
251	AGCCACACTG	GGACTGAGAC	ACGGCCCAGA	CTCCTACGGG	AGGCAGCAGT
301	GGGGAATTTT	GGACAATGGG	CGCAAGCCTG	ATCCAGCAAT	GCCGCGTGTG
351	TGAAGAAGGC	CTTCGGGTTG	TAAAGCACTT	TTGTCCGGAA	AGAAAACCTC
401	TGGGTTAATA	CCCTGGGGGG	ATGACGGTAC	CGGAAGAATA	AGCACCGGCT
451	AACTACGTGC	CAGCAGCCGC	GGTAATACGT	AGGGTGCAAG	CGTTAATCGG
501	AATTACTGGG	CGTAAAGCGT	GCGCAGGCGG	TTCGCTAAGA	CAGATGTGAA
551	ATCCCCGGGC	TTAACCTGGG	AACTGCATTT	GTGACTGGTG	GGCTAGAGTA
601	TGGCAGAGGG	GGGTAGAATT	CCACGTGTAG	CAGTGAAATG	CGTAGAGATG
651	TGGAGGAATA	CCGATGGCGA	AGGCAGCCCC	CTGGGCCAAT	ACTGACGCTC
701	ATGCACGAAA	GCGTGGGGAG	CAAACAGGAT	TAGATACCCT	GGTAGTCCAC
751	GCCCTAAACG	ATGTCAACTA	GTTGTGCGGT	CTTCATTGAC	TTGGTAACGT
801	AGCTAACGCG	TGAAGTTGAC	CGCCTGGGGA	GTACGGTCGC	AAGATTAAAA
851	CTCAAAGGAA	TTGACGGGGA	CCCGCACAAAG	CGGTGGATGA	TGTGGATTAA
901	TTGGATGCAA	CGCGAAAAAC	CTTACCTACC	CTTGACTAGT	ATGGAAAACCT
951	GCTGAGAGGT	CGGGGTGCCC	GAAAGGGAGC	CATAACACAG	GTGCTGCATG
1001	GCTGTGCTCA	GCTCGTGTCTG	TGAGATGTTG	GGTTAAGTCC	CGCAACGAGC
1051	GCAACCCTTG	TCCCTAGTTG	CTACGCAAGA	GCACTCTAGG	GAGACTGCCG
1101	GTGACAAACC	GGAGGAAGGT	GGGGATGACG	TCAAGTCATC	ATGGCCCTTA
1151	TGGGTAGGGC	TTACACGTC	ATACAATGGT	CGGAACAGAG	GGTCGCCAAC
1201	CCGCGAGGGG	GAGCCAATCC	CAGAACACCG	ATGGTAGTCC	GGATCGCATT
1251	CTGCAATTCTG	AGTGCGTGAA	GCTGGAATCG	TTAGTAATCG	CGGATCAGCA
1301	TGCCGCGGTG	AATACGTTCC	CGGGTCTTGT	ACACACCGCC	CGTCACACCA
1351	TGGGAGTGGG	TTTTACCAGA	AGTGGCTAGG	CCCACCGCAA	GGAGG

Figure 4.17 The nucleotide sequence of the 16S rRNA of strain VUN 10,013, based on the designed primers (MAS2F, MAS1R, F1, R1, F2, R2, F3, R3, F4, R4). 1,395 bp of the whole gene were sequenced.

Table 4.11 Comparison of the similarity of the 16S rRNA genes of strain VUN 10,013 with *Burkholderia* spp. on the NCBI database.

Organism	Accession number	%Similarity to strain VUN 10,013
<i>Burkholderia</i> sp. isolate N3P2	U37344	98.065
<i>Burkholderia</i> sp. isolate N2P5	U37342	97.204
<i>Burkholderia caryophylli</i> strain MCII-8	U91570	97.035
<i>Burkholderia cepacia</i> strain DSM 50181	X87275	94.763

	10	20	30	40	50	60
U37342.seq	-----TTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGC					
U37344.dna	GATCCCGACAGAGTTTGTATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGC					
U91570.dna	-----GAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGC					
VUN10013.seq	-----ACATGC					
X87275.dna	-TAAACTGAAGAGTTTGTATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTAACACATGC					

U37342.seq	AAGTCGAACGGCAGCACGGGGGC--AACCTGGTGGCGAGTGGCGAACGGGTGAGTAATA					
U37344.dna	AAGTCGAACGGCAGCACGGGAGT--AATCCTGGTGTGCGAGTGGCGAACGGGTGAGTAATA					
U91570.dna	AAGTCGAACGGCAGCACGGGAGC--AATCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA					
VUN10013.seq	AAGTCGAACGGCAGCACGGGAGC--AATCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA					
X87275.dna	AAGTCGAACGGCAGCACGGGTGCTTGACCTGGTGGCGAGTGGCGAACGGGTGAGTAATA					

U37342.seq	CATCGGAACGTGTCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATAC					
U37344.dna	CATCGGAACGTGTCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATAC					
U91570.dna	CATCGGAACGTGTCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATAC					
VUN10013.seq	CATCGGAACGTGTCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATAC					
X87275.dna	CATCGGAACATGTCTCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATAC					

U37342.seq	GCTCTACGGAGGAAAGGGGGGATCTT--AGGACCTCCGCTACAGGGGCGGCCGATGGC					
U37344.dna	GATCTGTGGATGAAAGCGGGGATCTT--AGGACCTCGCGCTACAGGGGCGGCCGATGGC					
U91570.dna	GCTCTGCGGAGGAAAGCGGGGATCTTTCGGGACCTCGCGCTACAGGGGCGGCCGATGGC					
VUN10013.seq	GATCTGTGGATGAAAGCGGGGATCTT--AGGACTCTGCGCTACAGGGGCGGCCGATGGC					
X87275.dna	GATCTACGGATGAAAGCGGGGACCTT--CGGGCCTCGCGCTATAGGGTTGGC--GATGGC					
	* * * * *					
U37342.seq	AGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGA					
U37344.dna	AGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGA					
U91570.dna	AGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGA					
VUN10013.seq	AGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGA					
X87275.dna	TGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGA					

U37342.seq	GGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGG					
U37344.dna	GGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGG					
U91570.dna	GGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGG					
VUN10013.seq	GGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGG					
X87275.dna	GGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGG					

U37342.seq	GGAATTTTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCT					
U37344.dna	GGAATTTTGGACAATGGGGGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCT					
U91570.dna	GGAATTTTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCT					
VUN10013.seq	GGAATTTTGGACAATGGGGGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCT					
X87275.dna	GGAATTTTGGACAATGGGGGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCT					

Figure 4.18 Alignment of VUN 10,013 16S rRNA gene sequence and the 16S rRNA gene sequences from *Burkholderia* sp. isolate N3P2 (accession number U37344), *Burkholderia* sp. isolate N2P5 (accession number U37342), *B. caryophylli* strain MCII-8 strain (accession number U91570) and *B. cepacia* strain DSM 50181 (accession number X87275). A dash indicates a gap at a particular position on the sequence compared to others. An asterisk indicates that at a particular position all of sequences aligned possess the same nucleotide.


```

U37342.seq      TCGGGTTGTAAAGCACTTTTGTCCGGAAGAAAACGCCGTGGTTAATACCCGTGGCGGAT
U37344.dna      TCGGGTTGTAAAGCACTTTTGTCCGGAAGAAAACCTCCGTCTTAATACGGTGGGGGGAT
U91570.dna      TCGGGTTGTAAAGCACTTTTGTCCGGAAGAAAACGCCGTGGCTAATATCCGGGCGGGAT
VUN10013.seq    TCGGGTTGTAAAGCACTTTTGTCCGGAAGAAAACCTCTGGGTTAATACCCGTGGGGGAT
X87275.dna      TCGGGTTGTAAAGCACTTTTGTCCGGAAGAAAATCCCTGGCTCTAATACAGTCGGGGGAT
*****

U37342.seq      GACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
U37344.dna      GACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
U91570.dna      GACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
VUN10013.seq    GACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
X87275.dna      GACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
*****

U37342.seq      GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTCCGCTAAGACA
U37344.dna      GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGCTAAGACA
U91570.dna      GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGCTAAGACA
VUN10013.seq    GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGCTAAGACA
X87275.dna      GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGCTAAGACC
*****

U37342.seq      GATGTGAAATCCCCGGGCTTAACCTGGGAAGTGCATTGTGACTGGCGGGCTAGAGTATG
U37344.dna      GATGTGAAATCCCCGGGCTTAACCTGGGAAGTGCATTGTGACTGGCGGGCTAGAGTATG
U91570.dna      GATGTGAAATCCCCGGGCTTAACCTGGGAAGTGCATTGTGACTGGCGGGCTAGAGTATG
VUN10013.seq    GATGTGAAATCCCCGGGCTTAACCTGGGAAGTGCATTGTGACTGGTGGGCTAGAGTATG
X87275.dna      GATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATTGGTGACTGGCAGGCTAGAGTATG
*****

U37342.seq      GCAGAGGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACC
U37344.dna      GCAGAGGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACC
U91570.dna      GCAGAGGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACC
VUN10013.seq    GCAGAGGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACC
X87275.dna      GCAGAGGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACC
*****

U37342.seq      GATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCA
U37344.dna      GATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCA
U91570.dna      GATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCA
VUN10013.seq    GATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCA
X87275.dna      GATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCA
*****

U37342.seq      AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCTGGGTCT
U37344.dna      AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCTGGGTCT
U91570.dna      AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCTGGGTCT
VUN10013.seq    AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCTGGGTCT
X87275.dna      AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTCTGGGGAT
*****

U37342.seq      TCATTGACTTGGTAAACGTACGTAAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAA
U37344.dna      TCATTGACTTGGTAAACGTACGTAAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAA
U91570.dna      TCATTGCTTGGTAAACGTACGTAAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAA
VUN10013.seq    TCATTGACTTGGTAAACGTACGTAAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAA
X87275.dna      TCATTTCCTTAGTAAACGTACGTAAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAA
*****

U37342.seq      GATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
U37344.dna      GATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
U91570.dna      GATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
VUN10013.seq    GATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
X87275.dna      GATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATT
*****

U37342.seq      CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTATGGAATCCTGCTGAGAGGTGG
U37344.dna      CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTATGGAATCCTGCTGAGAGGTGG
U91570.dna      CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTATGGAATCCTGCTGAGAGGTGG
VUN10013.seq    GGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTATGGAATCCTGCTGAGAGGTGG
X87275.dna      CGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGTCGGAATCCTGCTGAGAGGTGG
*****

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Figure 4.18 (continued).

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U37342.seq      GAGTGCCCGAAAGGGAGCCATAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTG
U37344.dna      GAGTGCCCGAAAGGGAGCCATAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTG
U91570.dna      ATGTGCCCGANAGG-AGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTG
VUN10013.seq    GGGTGCCCGAAAGGGAGCCATAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTG
X87275.dna      GAGTGCTCGAAAGAGAACC CGCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTG
                ****  * * * * *

U37342.seq      AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGC
U37344.dna      AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGC
U91570.dna      AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGC
VUN10013.seq    AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGC
X87275.dna      AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGAGC
                *****

U37342.seq      ACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCAAGTCCTCAT
U37344.dna      ACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCAAGTCCTCAT
U91570.dna      ACTCCAGG-AGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCAAGTCCTCAT
VUN10013.seq    ACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCAAGTCCTCAT
X87275.dna      ACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAGTCAAGTCCTCAT
                *****

U37342.seq      GGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAACCC
U37344.dna      GGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAACCC
U91570.dna      GGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAAGCC
VUN10013.seq    GGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAACCC
X87275.dna      GGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGTCGCCAACCC
                *****

U37342.seq      GCGAGGGGGAGCCAATCCCAGAAAACCGATCGTAGTCCGGATCGCACTCTGCAACTCGAG
U37344.dna      GCGAGGGGGAGCCAATCCCAGAAAACCGATCGTAGTCCGGATCGCACTCTGCAACTCGAG
U91570.dna      GCGAGGTGGAGCCAATCCCAGAAAACCGATCGTAGTCCGGATCGCACTCTGCAACTCGAG
VUN10013.seq    GCGAGGGGGAGCCAATCCCAGAACACCGATGGTAGTCCGGATCGCATTCTGCAATTCGAG
X87275.dna      GCGAGGGGGAGCTAATCCCAGAAAACCCATCGTAGTCCGGATTCGCACTCTGCAACTCGAG
                *****

U37342.seq      TCGGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCCGCGTGAATACGTTCCCG
U37344.dna      TCGGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCCGCGTGAATACGTTCCCG
U91570.dna      TCGGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCCGCGTGAATACGTTCCCG
VUN10013.seq    TCGGTGAAGCTGGAATCGTTAGTAATCGCGGATCAGCATGCCCGCGTGAATACGTTCCCG
X87275.dna      TGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCCGCGTGAATACGTTCCCG
                ***

U37342.seq      GGTCTTGTAACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCT
U37344.dna      GGTCTTGTAACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCT
U91570.dna      GGTCTTGTAACACACCGCCCGTCACACCAT-----
VUN10013.seq    GGTCTTGTAACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGGCC
X87275.dna      GGTCTTGTAACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTGGCTAGTCT
                *****

U37342.seq      AACC GCAAGGAGGACGGTCACCACGGTAGGATTCATGACTGGGG-----
U37344.dna      AACC GCAAGGAGGACGGTCACCACGGTAGGATTCATGACTGGGGTGAAGTCGTAACAAGG
U91570.dna      -----
VUN10013.seq    CACCGCAAGGAGG-----
X87275.dna      AACC GCAAGGAGGACGGTCACCACGGTAGGATTCATGACTGGGGTGAAGTCGTAACAAGG

U37342.seq      -----
U37344.dna      TAGGCGNGGNTCAGTTNCCC-----
U91570.dna      -----
VUN10013.seq    -----
X87275.dna      TAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCT

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Figure 4.18 (continued).

Table 4.12 Accession numbers for the 16S rRNA gene sequences of the four bacterial strains submitted to GeneBank.

Organism	Accession number
<i>Stenotrophomonas maltophilia</i> VUN 10,010	AF068008
<i>Stenotrophomonas maltophilia</i> VUN 10,012	AF068009
<i>Pseudomonas fluorescens</i> VUN 10,011	AF068010
<i>Burkholderia</i> sp. VUN 10,013	AF068011

4.7.4 Submission of 16S rRNA Gene Sequences to GeneBank

The 16S rRNA gene sequences (partial sequences) of *S. maltophilia* VUN 10,010, *S. maltophilia* VUN 10,012, *Ps. fluorescens* VUN 10,011 and *Burkholderia* sp. VUN 10,013 were deposited into GeneBank. These sequences were assigned accession numbers as shown in Table 4.12.

4.7.5 Restriction Fragment Length Polymorphism (RFLP)

Analysis (16S rDNA Genotyping)

Differentiation of bacterial strains by RFLP was also performed in parallel with the analysis of 16S rRNA gene sequencing. Ten endonucleases (*BssK* I, *Dpn* II, *Eco0109* I, *Fnu4H* I, *Hae* III, *Hinf* I, *Hpa* II, *Mse* I, *Rsa* I and *Sma* I) were used to digest the 16S rRNA genes. Among the ten enzymes used, four enzymes (*BssK* I, *Fnu4H* I, *Nla* III and *Dpn* II) were omitted because of their ambiguous results (too many fragments which were not clearly distinct). Identification of restriction site of the six endonucleases, *Eco0109* I, *Hinf* I, *Hpa* II, *Mse* I, *Rsa* I and *Sma* I, on the 16S rRNA genes of strains VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013 was performed by sequence analysis as shown in Figure 4.19. These restriction enzymes gave different pattern types for three to six clear and distinct bands (Figures 4.20-4.22). Three different 16S rDNA genotypes were selected from the restriction patterns seen (Table 4.13). It was evident that the 16S rDNA genotype of strains VUN 10,010 and VUN 10,012 were the same, and that these were different from strains VUN 10,011 and VUN 10,013. No difference was observed between strains VUN 10,010 and VUN 10,012, which is not unexpected as they otherwise showed similar traits and were most likely the same strain.

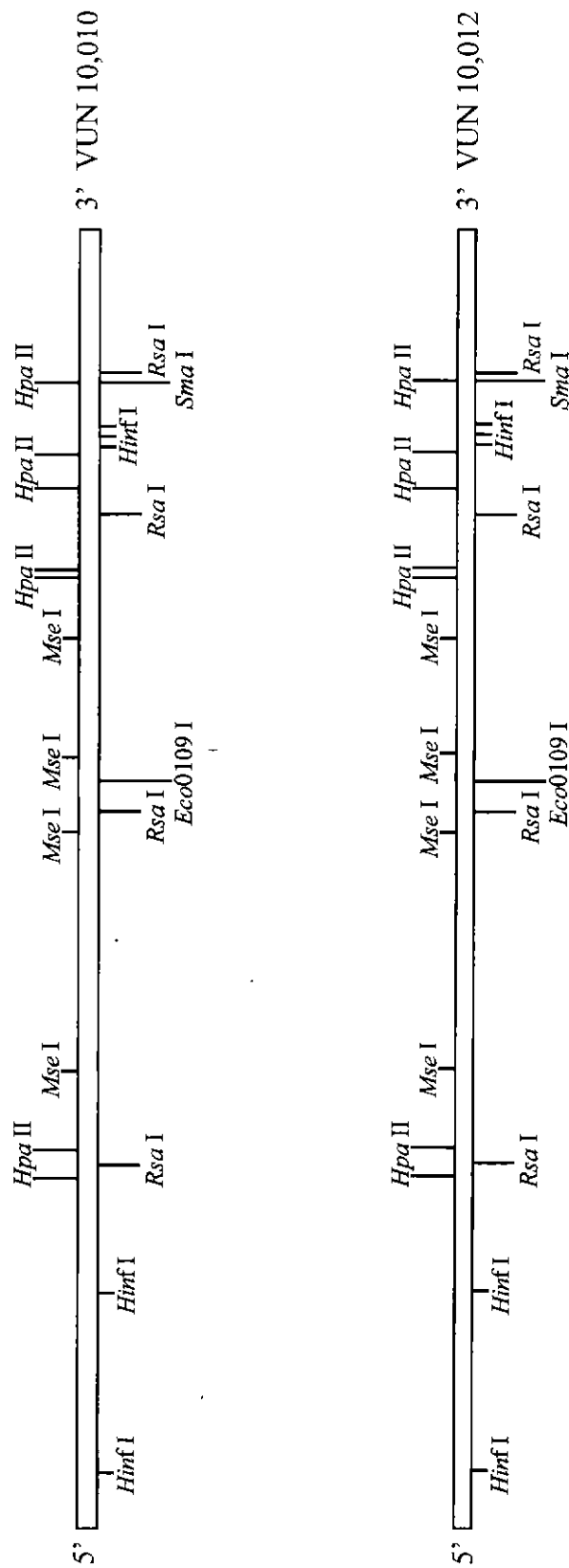


Figure 4.19a Restriction map of the 16S rRNA genes of *S. maltophilia* strains VUN 10,010 and VUN 10,012 with the six endonucleases used in the restriction fragment length polymorphism studies (*Eco*0109 I, *Hinf* I, *Hpa* II, *Mse* I, *Rsa* I and *Sma* I).

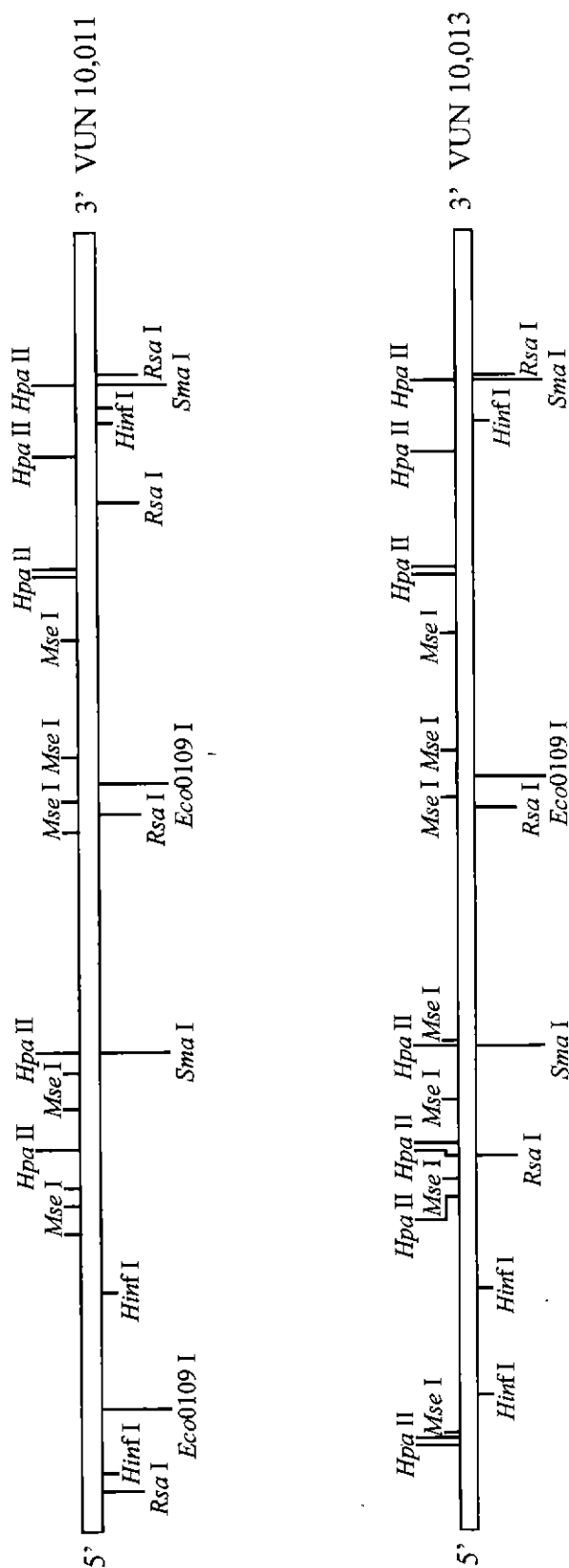


Figure 4.19b Restriction map of the 16S rRNA genes of *Pseudomonas fluorescens* VUN 10,011 and *Burkholderia* VUN 10,013 with the six endonucleases used in the restriction fragment length polymorphism studies (*Eco*0109 I, *Hinf* I, *Hpa* II, *Mse* I, *Rsa* I and *Sma* I).

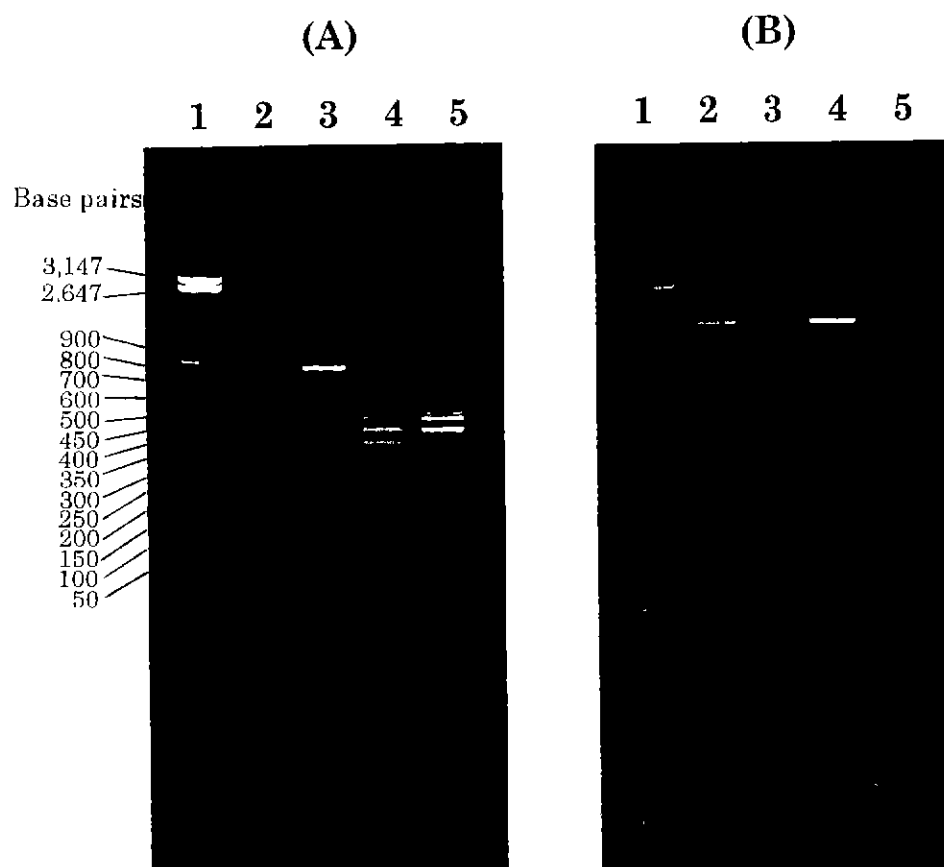


Figure 4.20 Restriction patterns of the 16S rRNA genes digested with *Rsa* I (A) and *Sma* I (B). Lane 1, Probase 50 (size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

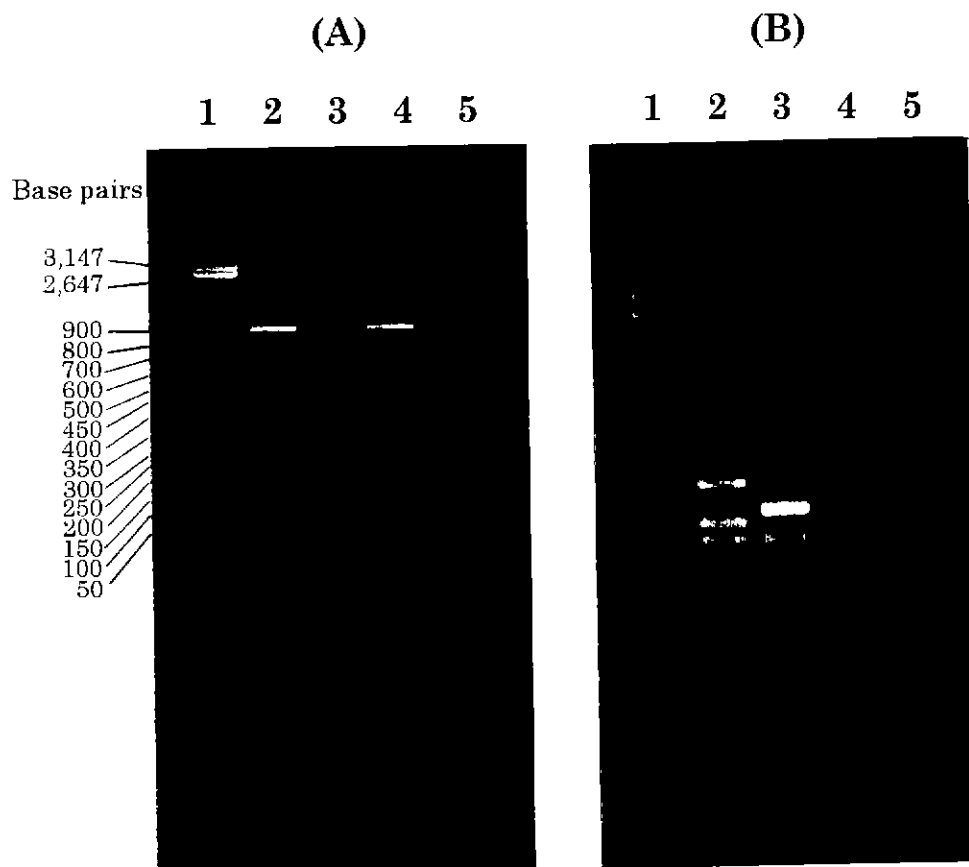


Figure 4.21 Restriction patterns of the 16S rRNA genes digested with *Eco0109* I (A) and *Mse* I (B). Lane 1, Probase 50 (size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

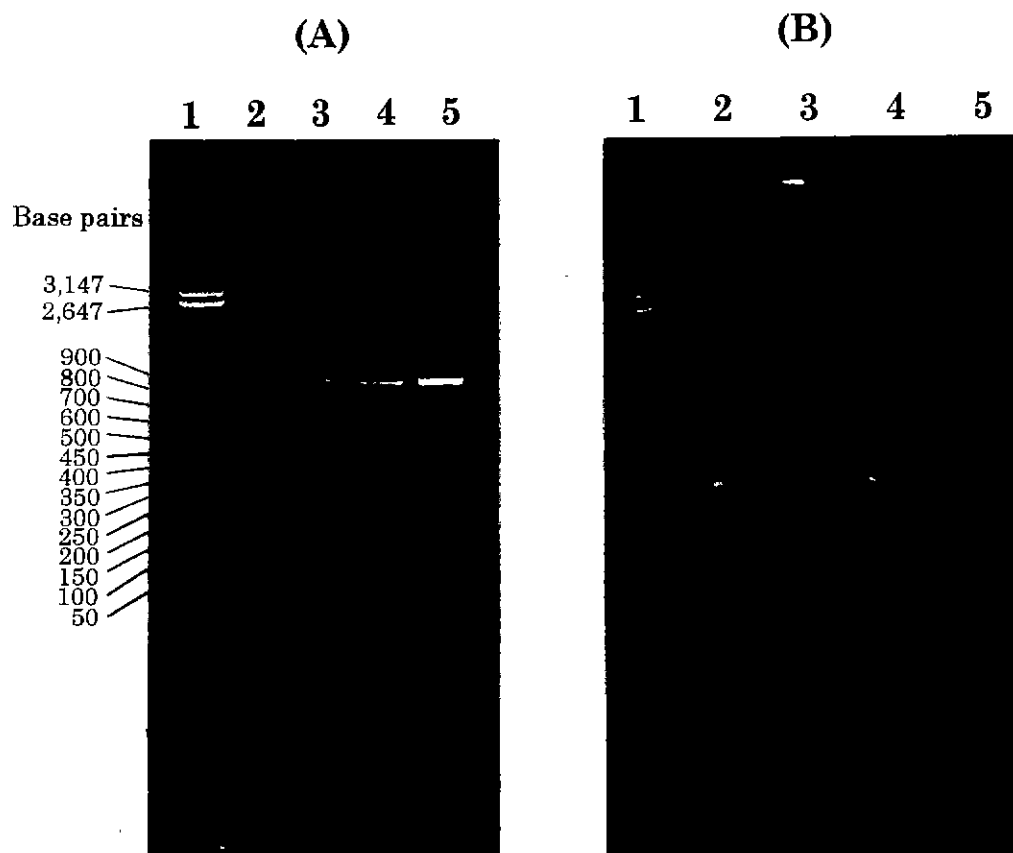


Figure 4.22 Restriction patterns of 16S rRNA genes digested with *Hinf* I (A) and *Hpa* II (B). Lane 1, Probase 50 (size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

Table 4.13 Restriction patterns type obtained from the digestion of 16S rRNA genes.

Bacterial strain	16Sr DNA genotype ^b	Restriction pattern type ^a of 16S rRNA gene digested with					
		<i>Eco</i> 0109I [G↓GNCC]	<i>Hinf</i> I [G↓ANTC]	<i>Hpa</i> II [C↓CCG]	<i>Rsa</i> I [GT↓AC]	<i>Mse</i> I [T↓TAA]	<i>Sma</i> I [CCC↓GGG]
VUN 10,010	a	1Ec	1Hf	1Hp	1Rs	1Ms	1Sm
VUN 10,011	b	2Ec	2Hf	2Hp	2Rs	2Ms	2Sm
VUN 10,012	a	1Ec	1Hf	1Hp	1Rs	1Ms	1Sm
VUN 10,013	c	3Ec	3Hf	3Hp	3Rs	3Ms	3Sm

^a Patterns obtained from the digestion of the 16S rRNA gene of four bacterial strains by each restriction enzyme are indicated by numbering. Different numbers were given to different patterns observed within a particular enzyme used.

^b The 16S rDNA genotypes for each strain of bacteria, which are designated as a, b, and c, represent the combination of restriction pattern types obtained with the six endonucleases.

4.7.6 Southern Blot Hybridization

DNA-DNA hybridization has routinely been used for differentiation among species. Genomic DNA of each bacterial strain was digested with restriction endonucleases, transferred to a nylon membrane and probed with a 1.5 kb 16S rRNA gene. Figures 4.23-4.24 show results of Southern blot hybridization of genomic DNA of the four bacterial strains digested with *Sma* I and *Eco*0109 I and probed with the 16S rRNA gene of strain VUN 10,010 or VUN 10,012. Three patterns were evident from the hybridization signals for each enzyme used, demonstrating the homology between strain VUN 10,010 and VUN 10,012 (100% homology). The level of similarity between the DNA of *S. maltophilia* (VUN 10,010 and VUN 10,012) and *Burkholderia* sp. (VUN 10,013) was approximately 75% homology. In contrast, none of the fragments of digested genomic DNA probed with the 16S rRNA gene of VUN 10,010 or VUN 10,012 was found to be homologous to the DNA of *Ps. fluorescens* VUN 10,011. The results obtained in this study, which corresponded to those results of 16S rRNA analysis (Section 4.7.3) and RFLP (Section 4.7.5), led to the conclusion that VUN 10,010 and VUN 10,012, are most likely the same strain.

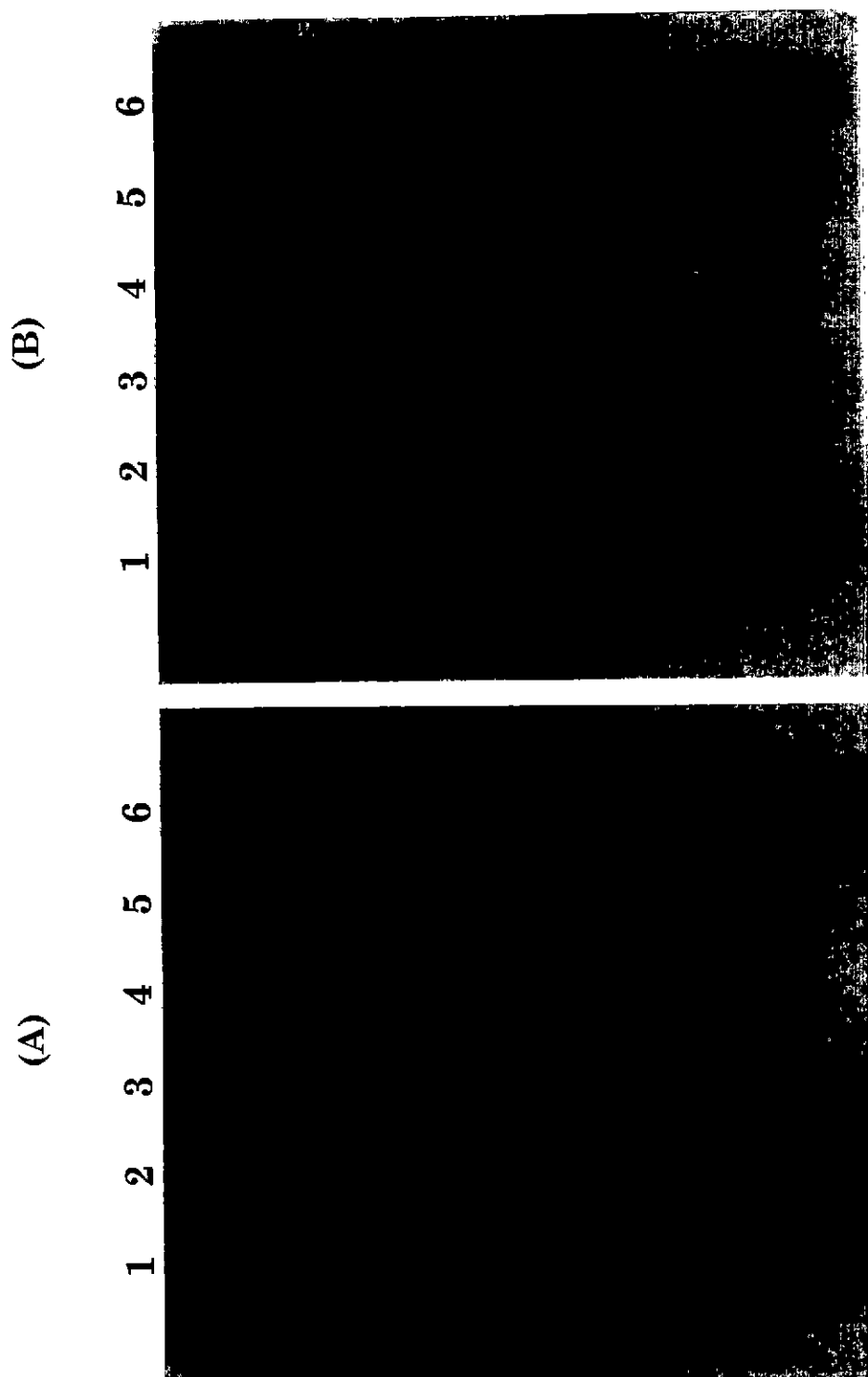


Figure 4.23 Southern blot hybridization of *Sma* I digested genomic DNA probed with the 16S rRNA gene (MAS2F/MAS1R PCR product) of VUN 10,010 (A) or VUN 10,012 (B). Lanes 1 and 6, lambda DNA digested with *Eco* RI and *Hind* III (size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

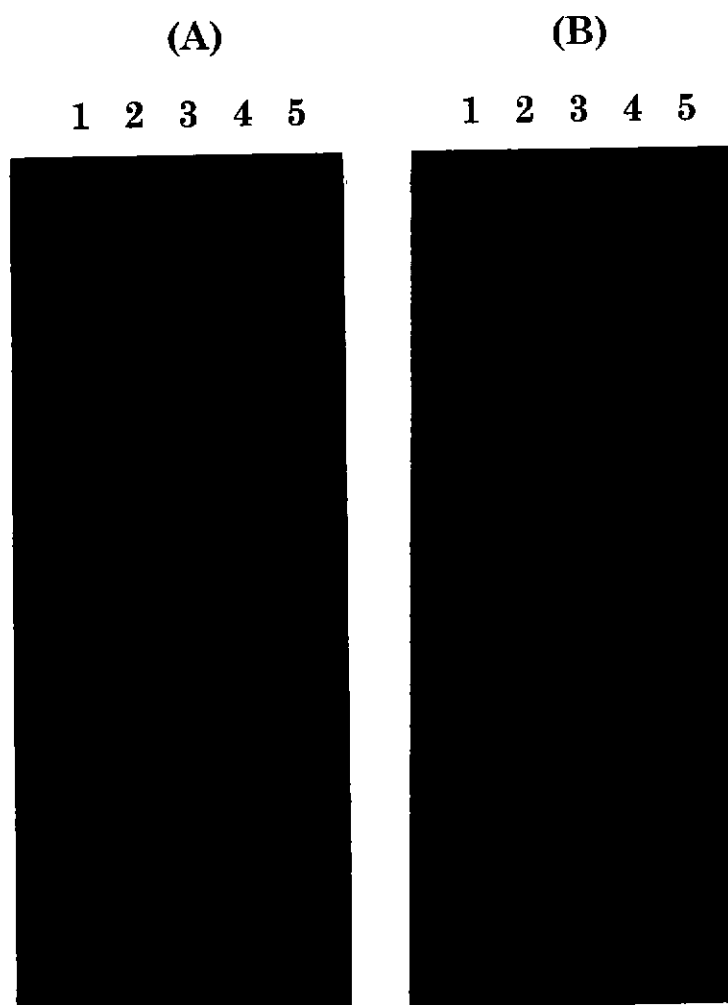


Figure 4.24 Southern blot hybridization of *Eco*0109 I digested genomic DNA probed with the 16S rRNA gene (MAS2F/MAS1R PCR product) of VUN 10,010 (A) and VUN 10,012 (B). Lane 1, lambda DNA digested with *Eco* RI and *Hind* III (size marker); lane 2, VUN 10,010; lane 3, VUN 10,011; lane 4, VUN 10,012; and lane 5, VUN 10,013.

4.8 DISCUSSION

4.8.1 Isolation of High Molecular Weight PAH-Degrading Bacteria

While the isolation of high molecular weight PAH degraders reported in the literature has been rare, many studies have the reported isolation of bacteria capable of degrading two- and three-aromatic ring compounds. Until recently, most high molecular weight PAHs have been considered as recalcitrant to microbial degradation, and only a few reports have demonstrated the growth of selected enrichment isolates on high molecular weight PAH compounds as a sole carbon and energy source (Mueller *et al.*, 1990; Walter *et al.*, 1991, Boldrin *et al.*, 1993; Kästner *et al.*, 1994; Juhasz *et al.*, 1997). PAH-contaminated soils or sediments have proven to be a rich source of PAH-degrading bacteria (Walter *et al.*, 1991; Weissenfels *et al.*, 1990 and 1991; Heitkamp and Cerniglia, 1988). As demonstrated in this study, pyrene-metabolizing bacteria were successfully isolated from soil contaminated with PAHs using enrichment procedures. In addition to pyrene, these isolates were also able to utilize fluorene and phenanthrene as a sole source of carbon and energy.

Although over the years the number of isolated PAH-degrading bacteria has increased, not all of them use PAH substrates as sole carbon sources. As reported by Shiaris and Cooney (1983), slightly more than 50% of the isolated fluoranthene-degrading species use this substrate as sole source of carbon and energy, while the remainder utilized phenanthrene in the presence of yeast extract, peptone, glucose, benzoate, and kerosene oil. Trzesicka-Mlynarz and Ward (1995) isolated a mixed bacterial population from PAH-contaminated soil which demonstrated the ability to grow on and degrade fluoranthene in aqueous media supplemented with glucose (1 g l^{-1}), yeast extract (1 g l^{-1}) and peptone (1 g l^{-1}). Four bacterial strains were isolated from this mixed culture, and three of the isolates were identified as *Ps. putida*, *Flavobacterium sp.* and *Ps. aeruginosa*.

To date, there are several reports which demonstrate bacterial growth on tetracyclic PAHs such as fluoranthene, pyrene, or chrysene as a sole carbon source

Table 4.14 Comparison of the PAH-degradation patterns for different bacterial strains able to grow on four-ring PAHs.

Isolated species	Growth on ^a								Reference
	NAP	FLU	PHEN	ANT	FA	PYR	BAA	CHRY	BAP
<i>Mycobacterium</i> sp. PYR-1	+	*	+	*	+	+	*	*	-
<i>Alcaligenes denitrificans</i> ww1	+	-	+	+	+	-	-	-	-
<i>Sphingomonas paucimobilis</i> EPA 505	-	-	+	+	+	-	*	-	-
<i>Rhodococcus</i> sp. UW1	-	-	+	+	+	+	-	+	-
<i>Mycobacterium</i> sp. BB1	*	+	+	*	+	+	*	*	*
<i>Mycobacterium</i> sp. VF1	+	*	+	+	+	+	-	*	-
<i>Gordonia</i> sp. BP9	+	*	+	-	+	+	-	-	-
<i>Stenotrophomonas maltophilia</i> VUN 10 003	+	+	+	-	-	+	-	-	-
<i>Stenotrophomonas maltophilia</i> VUN 10 010	+	+	+	-	-	+	-	-	-
<i>Pseudomonas fluorescens</i> VUN 10 011	+	+	+	-	-	+	-	-	-
<i>Stenotrophomonas maltophilia</i> VUN 10 012	+	+	+	-	-	+	-	-	-
<i>Burkholderia</i> sp. VUN 10 013	+	+	+	-	-	+	-	-	-

^a The ability to utilize a PAH as growth substrate is indicated by growth (+) or no growth (-) on a tested compound. Abbreviations: NAP, naphthalene; FLU, fluorene; PHEN, phenanthrene; ANT, anthracene; FA, fluoranthene; PYR, pyrene; BAA, benz[a]anthracene; CHRY, chrysene; and BAP, benzo[a]pyrene.

* Not tested or information was not provided by the authors.

(Table 4.14). Heitkamp *et al.* (1988) and Boldrin *et al.* (1993) have reported the isolation of *Mycobacterium* sp. capable of degrading PAHs such as phenanthrene and pyrene as a sole carbon and energy source. Using enrichment techniques, Weissenfels *et al.* (1990) were able to isolate three pure strains of bacteria from mixed cultures capable of degrading either phenanthrene, fluorene, or fluoranthene as a sole carbon and energy source. These bacteria were identified, based on biochemical tests, as *Alcaligenes* sp. and two different species of *Pseudomonas*. Walter *et al.* (1991) reported isolating a *Rhodococcus* sp. which was capable of utilizing pyrene and chrysene as a sole carbon and energy source. This bacterial strain also showed the ability to degrade other PAH compounds such as benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene, however, this only occur *via* co-metabolism. Grosser and co-workers (1991) reported the mineralization of PAHs including anthracene, phenanthrene, pyrene, benz[a]anthracene, benzo[a]pyrene and the *N*-heterocyclic aromatic hydrocarbon, carbazole, in soil by the indigenous population. In this case, the authors used a *Mycobacterium* sp. capable of degrading phenanthrene, anthracene and pyrene and reintroduced it into the PAH-contaminated soil, resulting in the enhancement of PAH mineralization above that of the indigenous soil microbial population. Recently, Juhasz and co-workers (1996 and 1997) isolated strains of *S. maltophilia* (previously described as *B. cepacia*) which were able to utilize pyrene as a sole source of carbon and energy. This strain also possessed the ability to degrade other high molecular weight PAHs *via* co-metabolism or when high cell populations were used.

To date, no microorganisms have been isolated which utilize PAHs consisting of five- or more benzene rings as sole carbon and energy source in pure culture. In this chapter, although bacterial and fungal growth on five-ring PAHs supplied as a sole source of carbon and energy was observed in soil B-enriched cultures, pure cultures were not isolated of either the bacteria or fungi which could utilize these compounds as a sole carbon substrate. Further investigations on the growth and degradation of high molecular weight PAHs containing four- and five-benzene rings by these bacteria and fungi are described in Chapter 5 and will not be discussed further here.

4.8.2 PAH Utilization and Dioxygenase Activity

PAH degradation by the four bacterial strains isolated from the contaminated soil in this work was compared. The hierarchy of the most effective strains in terms of (i) substrate specificity, (ii) rapidness in of forming clear zones around colonies when sprayed by PAH solutions, and (iii) bacterial growth on, and degradation of, different PAHs as sole source of carbon and energy, was found to be according to the following order (from best to worst): VUN 10,010, followed by VUN 10,012, VUN 10,011 and VUN 10,013. The PAH-degrading bacteria tested in this study demonstrated dioxygenase activity during the degradation of some PAHs (*i.e.* fluorene, phenanthrene and pyrene). Indole transformation rates in these isolates seemed to be related to the PAH degradation rates. This may due to dioxygenase enzymes being responsible for both the initial ring oxidation of PAH molecules and the oxidation of indole. Therefore, once the bacteria begin to degrade PAHs, the expressed dioxygenases also attacked the indole substrate present in the cultures at the same time. Addition of salicylic acid accelerated the formation of clearing zones on BSM agar plates sprayed with fluorene or phenanthrene by all strains. It also accelerated the transformation of indole to indigo. No such effects were observed in media containing pyrene. However, salicylic acid did not broaden the range of PAH specificity for VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013. Results obtained from this study suggest that gene regulation for fluorene and phenanthrene *via* salicylic acid might occur in *S. maltophilia* VUN 10,010, *S. maltophilia* VUN 10,012, *Ps. fluorescens* VUN 10,011, and *Burkholderia* sp. VUN 10,013. Dioxygenase activity was shown to be involved in pyrene degradation by these bacteria, however, the pyrene catabolism might not involved salicylic acid since there was no enhancement by the presence of salicylic acid in the time taken for the appearance of a clearing zone and dioxygenase activity on this PAH. Conclusions drawn from the results regarding effects of salicylic acid are as follows.

- (i) salicylic acid enhanced dioxygenase activity and the degradation rate of fluorene and phenanthrene. This implies that the degradation pathways for fluorene and phenanthrene are most likely closely linked to the

naphthalene degradative pathway in which salicylic acid is an intermediate product; its presence inducing or enhancing degradation of the parent compound. The genes encoding for naphthalene degradation are most likely not involved in the pyrene catabolism since salicylic acid appears not to be an intermediate product of pyrene degradation by the four PAH-degrading bacteria. Other gene regulation systems may be responsible for pyrene degradation by these bacteria or they may use other pathways to metabolize pyrene. Pyrene degradation has been previously reported to occur *via* a dioxygenase enzyme system in which cinnamic acid and phthalic acid were detected as ring fission intermediates (Heitkamp *et al.*, 1988). Rather than salicylic acid, these metabolites (cinnamic acid and/or phthalic acid) might be an inducer for pyrene metabolism;

- (ii) it is possible that bacteria may use different pathways to metabolize different PAHs. This study demonstrated that the metabolism among the bacterial strains tested was different for three-ring PAHs and the four-ring compounds. Also, one PAH compound could be metabolized by different mechanisms for any particular bacterial strain. As reported by Heitkamp *et al.* (1988), multiple pathways were involved in the pyrene metabolism of *Mycobacterium* sp., which included mono-oxygenase and dioxygenase oxidation;
- (iii) the role of salicylic acid in the degradation of the PAHs tested is unclear based on the results of this study. More investigation in this area may provide a clearer picture. Such studies may involve the identification of the PAH metabolic pathways in these bacteria on different PAHs.

4.8.3 Identification and Differentiation of Bacteria

Based on Molecular Analyses

A fundamental advance has been the identification and tracking of PAH degraders based on molecular biological analysis of their nucleic acids. Studies reported in this Chapter and the following discussion are mainly concerned with

the analysis of 16S rRNA genes which allow bacterial identification and differentiation among species. 16S rRNA (or ribosomal DNA [rDNA] gene) sequencing is one of the most powerful and precise methods for determining the interrelationships between bacteria (Woese, 1987). It has been established that the base sequences of this molecule (or gene) can be used to determine distant, as well as close (eg. intrageneric) genealogical relationships (Woese, 1987). 16S rRNA sequence characterization is being widely used in evolutionary, taxonomic, and ecological studies not only to define taxa but also to characterize which taxa are present. The PAH-degrading bacteria isolated in this study were assumed to be very closely related based on their physiological and biochemical characteristics. It was demonstrated that the 16S rRNA gene sequences obtained in this study were able to differentiate and specify the genera and/or species of the isolated PAH-degrading bacteria. It has been reported that the sequence data from known bacterial small sub-unit rRNA genes are largely incomplete as many investigators focus their efforts on partial sequences (cited in Urakawa *et al.*, 1997). For the four strains investigated in the present study, partial 16S rRNA gene sequences (> 90%) were determined for strains VUN 10,010, VUN 10,011, VUN 10,012 and VUN 10,013.

Polymerase chain reaction, using a primer pair annealing at the 5'- and 3'-termini of the 16S rRNA genes, followed by using several pairs of internal primers, enabled the amplification and sequence determination of 1,380 or more base pairs (approximately 90% of the entire gene, as estimated by comparison with the completed 16S rRNA gene sequence of *E. coli* [length of 1,541 bp] [Brosius *et al.*, 1978]). The partial 16S rRNA sequence of more than 1,350 nucleotides were determined for *S. maltophilia* VUN 10,010 (1,380 bp), *S. maltophilia* VUN 10,012 (1,382 bp), *Ps. fluorescens* VUN 10,011 (1,399 bp), and *Burkholderia* sp. VUN 10,013 (1,395 bp) by sequencing the PCR-amplified DNAs. The resulting 16S rRNA gene sequences of strains VUN 10,010 and VUN 10,012 were highly homologous to the 16S rRNA gene sequence of *S. maltophilia*. *S. maltophilia* was originally considered as a member of the genus *Pseudomonas* and subsequently *Xanthomonas*. It was recently reclassified to the new genus, *Stenotrophomonas*, mainly due to phylogenetic reasons (van den Mooter and Swings, 1990; van Zyl

and Steyn, 1992; Palleroni and Bradbury, 1993). Firstly, *Ps. maltophilia* was reclassified to being a member of the genus *Xanthomonas* (as *X. maltophilia*) due to similarity at the level of their rRNA gene sequences (Palleroni *et al.*, 1973; Swing *et al.*, 1983). However, there are many phenotypic differences between these two taxa such as flagellation, pigment production, pathogenicity, resistance to antibiotics and a substantial numbers of miscellaneous properties (Palleroni and Bradbury, 1993). Aside from similarities in the sequences of rRNA genes, Palleroni and Bradbury (1993) considered that the differences between *X. maltophilia* and *Xanthomonas* spp. are greater than their resemblance. Therefore, the author proposed a new genus, *Stenotrophomonas*, for the species *X. maltophilia* which better represents the characteristics of the organism.

Analysis using 16S rRNA gene sequencing identified strains VUN 10,011 and VUN 10,013 as *Ps. fluorescens* and *Burkholderia* sp., respectively. The 16S rRNA sequences from *S. maltophilia* strains VUN 10,010 and VUN 10,012 were almost identical but there were exceptions, as shown below. Although there were three additional bases (position 1 to 3) in VUN 10,012 relative to the sequence of VUN 10,010 and there was also one gap at position 793 (VUN 10,010 numbering) (Figure 4.9), but these cannot be regarded as significant differences. A DNA probe could not distinguish the two strains since the available sequence from PCR-amplification products were similar. It is possible that there are additional sequences which may differentiate *S. maltophilia* VUN 10,010 and VUN 10,012, since the remaining short unsequenced region encompassed one of the more variable sections of the 16S rRNA molecule. These parts of the gene sequences can be obtained in future studies, in which the 16S rRNA gene could be cloned into a plasmid vector and sequenced. The extremely high level of similarity of these sequences must be considered in the context of possible uncertainties associated with 16S rRNA gene sequencing. These uncertainties include sequencing error, amplification error, and the possibility of microheterogeneity that is due to the presence of multiple rRNA cistrons (Fox *et al.*, 1992).

It has been recognized that 16S rRNA sequence comparisons are very promising for identifying and determining intergeneric relationships across the taxonomic

spectrum at the uppermost levels of sequence similarities (*i.e.* 99% or higher). As for the studies presented in this chapter on the identification of the four PAH-degrading bacteria, biochemical tests alone determined that all of these strains were of the same genus (*Pseudomonas*). The correction of their taxonomic positions was made using the 16S rRNA gene sequence analysis. However, the resolving power of 16S rRNA to reflect the phylogenetic relationships may not be adequate for guaranteeing that two organisms are the same species. In terms of extremely closely related strains, the key issue is the relationship between 16S rRNA sequence similarity and DNA-DNA hybridization results. It is apparent that when the level of 16S rRNA identity is 99% or greater, DNA-DNA hybridization may or may not document the existence of species identity (Fox *et al.*, 1992). It has been reported previously wherein species of a certain genus are clearly distinguishable by their DNA-DNA similarities being below the accepted molecular criterion of 70% (Wayne *et al.*, 1987), yet these same two organisms possess no, or very few, nucleotide differences in their 16S rRNA gene primary sequences (Fox *et al.*, 1992; Martinez-Murcia *et al.*, 1992; Bennasa *et al.*, 1996). Such observations have important implications for the use of 16S rRNA sequence data in bacterial taxonomy. These implications have been expressed previously by Fox *et al.* (1992), and subsequently by Stackebrandt and Goebel (1994), who have pointed out certain limitations in the use of 16S rRNA sequence data for defining bacterial identities. However, this study showed that there were three different DNA-DNA hybridization profiles obtained for the four bacterial strains in which one pattern was the same for *S. maltophilia* VUN 10,010 and VUN 10,012 (Figures 4.23 and 4.24). Another two different patterns were unique for *Ps. fluorescens* VUN 10,011 and *Burkholderia* sp. VUN 10,013. The level of DNA-DNA homology between VUN 10,010 and VUN 10,012 was 100%. Therefore, these strains were confirmed to constitute the same DNA-DNA hybridization group. Furthermore, the results derived from restriction fragment length polymorphism (RFLP) also showed the effectively identical 16S rRNA sequences of VUN 10,010 and VUN 10,012. From the results based on these molecular analyses, strain VUN 10,010 is considered to be identical to strain VUN 10,012. However, differences among these two strains was illustrated in some physiological characteristics, *eg.* strain VUN 10,010 utilized cinnamic acid as a

growth substrate but strain VUN 10,012 was unable to utilize this compound as a growth substrate.

4.8.4 Concluding Remarks

The ubiquitous presence of a potent and versatile degrading microflora in PAH-contaminated soils and the ability to easily increase the number of PAH-degrading bacteria by enrichment procedures support previous findings that showed soil bioaugmentation might be sufficient in many cases to initiate a biological remediation of contaminated sites (Wang *et al.*, 1990; Mueller *et al.*, 1992). However, if one compares the PAH degradation patterns of all bacterial strains that are able to degrade high molecular weight PAHs, either isolated in this work or as published elsewhere (Table 4.14), it can be seen that the degradation of high molecular weight PAHs is still limited to very few microorganisms. No microorganisms could be found that were able to degrade and utilize PAHs with five-benzene rings or more as a sole carbon and energy source. The implementation of an optimized laboratory-grown culture for biological remediation could therefore improve bioremediation productivity at sites where it is necessary to speed up the PAH turnover or to degrade recalcitrant compounds such as high molecular weight PAHs, which could not be degraded by autochthonous microflora.

The significance of the work presented in this chapter is on the isolation and characterization of PAH-degrading bacteria. Taxonomic positions of these bacteria were identified the using 16S rRNA analysis. Further studies in more detail regarding degradation, mineralization and detoxification of high molecular weight PAH compounds using the selected strains is described in Chapters 5 and 6. These studies include the application of PAH-degrading microbes in a more representative bioremediation systems, *i.e.* PAH-contaminated soils, and the application of surface-active agents to enhance the rate and extent of PAH biodegradation.

CHAPTER 5

BIODEGRADATION OF HIGH MOLECULAR WEIGHT PAHs BY AXENIC AND CO-CULTURES OF BACTERIA AND A FUNGUS

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CHAPTER 5

BIODEGRADATION OF HIGH MOLECULAR WEIGHT PAHs BY AXENIC AND CO-CULTURES OF BACTERIA AND A FUNGUS

5.1 INTRODUCTION

A common failing among PAH-degraders is an inability to fully degrade high molecular weight PAH compounds, resulting in the production of toxic intermediates. Furthermore, when only partial degradation of these compounds occurs it is often the case that a microbial culture is unable to grow on PAHs as its sole carbon and energy source. If such organisms were used to augment a PAH-contaminated site, then their lack of growth would make them uncompetitive with the soil microflora and large amounts of inocula would be required to ensure an adequate microbial population was present for PAH degradation to occur at a reasonable rate. This chapter continues on from Chapter 4 by trialing the most promising PAH-degrading isolates described in that chapter for their ability to degrade, grow on and detoxify high molecular weight PAHs in liquid and soil media.

Although axenic cultures of the PAH-degrading isolates are examined in this chapter, an unconventional approach to PAH biodegradation is also adopted by using inocula comprising a combination of fungal and bacterial cultures, otherwise known as a co-culture. The reasons for testing the PAH-degrading ability of fungal-bacterial co-cultures were mainly based on two observations. The first, and major, reason for using bacterial-fungal co-cultures was based on the observation that *P. janthinellum* VUO 10,201 and bacterial consortium VUN 10,009 could always be enriched on benzo[a]pyrene as a sole carbon and energy source when incubated together, but neither could grow separately on benzo[a]pyrene when cultured separately. The second, and lesser reason, was based on the general observation that many reports describe the mineralization of ¹⁴C-benzo[a]pyrene when added to soil and sediments, but such reports also

describe the subsequent failure to isolate axenic cultures from the soils that can mineralize this PAH (Grosser *et al.*, 1991, 1995). Some of these studies also described an overall increase in microbial population, especially of fungal and bacterial biomass (Kanaly *et al.*, 1997), in soils to which high molecular weight PAHs had been added; such observations suggest that complete degradation of high molecular weight PAHs in nature largely depends upon the cooperative metabolic activities of mixed microbial populations which may include fungi and other organisms.

The general aim of this chapter was to examine the ability of bacterial consortium VUN 10,009, *S. maltophilia* strain VUN 10,010 and *P. janthinellum* strain VUO 10,201 to degrade and grow on high molecular weight PAHs and to detoxify liquid and soil media contaminated with these compounds. Three different cultivation approaches were trialed in order to better understand the remediation performance of these isolates, these being to use:

- (1) axenic cultures of the above isolates with a single high molecular weight PAH as a sole carbon and energy source;
- (2) axenic cultures under co-metabolic conditions; and
- (3) fungal-bacterial co-cultures.

5.2 EFFICIENCY OF THE PAH-EXTRACTION METHODS

The high recovery of PAHs present in liquid and soil media during extraction is an important aspect of this study. The efficiency of the extraction procedure in recovering PAHs from the microbial culture used in the chapter was at risk of being compromised due to PAH adsorption to biomass, especially the fungus, and to organic materials in soil. The effect of such interactions on PAH extraction efficiency needed to be investigated and, if found to affect recovery, modifications to the extraction method would have been needed to overcome the deficiency in PAH recovery.

Table 5.1 Efficiency of PAH extraction methods.

PAH ^b	% PAH Recovery ^a				
	BSM ^c		PAH-spiked soil ^d		
	A	B	C	D	E
Fluorene	99.7 ± 0.2	99.6 ± 0.2	99.9 ± 0.2	99.6 ± 0.2	99.6 ± 0.6
Phenanthrene	99.9 ± 0.1	99.3 ± 0.6	100 ± 0.2	100 ± 0.4	98.4 ± 1.5
Fluoranthrene	99.7 ± 0.4	98.9 ± 0.7	99.6 ± 0.2	99.7 ± 0.3	98.2 ± 0.9
Pyrene	100.0 ± 0.1	99.5 ± 0.6	99.6 ± 0.0	99.5 ± 0.4	98.7 ± 1.1
Benz[<i>a</i>]anthracene	99.3 ± 0.7	98.4 ± 0.8	99.2 ± 0.5	99.1 ± 0.7	99.0 ± 0.8
Chrysene	98.7 ± 2.2	97.7 ± 1.7	98.6 ± 0.2	98.5 ± 1.5	98.9 ± 1.3
Benzo[<i>a</i>]pyrene	99.8 ± 0.4	98.2 ± 1.2	99.3 ± 0.3	99.7 ± 0.3	97.3 ± 2.0
Dibenz[<i>a,h</i>]anthracene	99.7 ± 0.4	98.1 ± 2.1	99.4 ± 0.2	98.3 ± 1.5	99.0 ± 0.5
Coronene	99.4 ± 0.5	96.9 ± 2.2	98.3 ± 0.5	97.9 ± 0.2	98.3 ± 1.8

^a %PAH recovery represents an average and standard deviation of each PAH compound recovered from triplicate samples, where calculation was based on the known amount of each PAH added to a particular medium.

^b PAH concentrations in the conditions tested were 100 mg l⁻¹ for fluorene; 250 mg l⁻¹ for phenanthrene and pyrene; 50 mg l⁻¹ for fluoranthrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene; and 25 mg l⁻¹ for coronene.

^c PAHs were recovered from BSM solution containing killed bacterial culture (ca. 10⁶ cells ml⁻¹) (A) by the simple DCM extraction method (one step vigorous shaking). Sequential (three successive) extraction by ultrasonication was used to recover PAHs from BSM containing killed fungal culture (0.04 g mycelia [wet wt.] ml⁻¹) (B).

^d PAHs were recovered, after 48 hours of the PAH spiking, from PAH-spiked soil using the Soxhlet extraction (C) and sequential (three successive) extraction using ultrasonication (D). The sequential (three successive) extraction using ultrasonication was used to extract PAH-spiked soil inoculated with a killed bacterial-fungal co-culture (ca. 10⁶ bacterial cells g⁻¹ soil and 0.04 g mycelia [wet wt.] g⁻¹ soil) (E).

5.2.1 PAH Extraction Efficiency for Bacterial Cultures

PAH extraction efficiency was evaluated by comparing the amount of PAH and internal standard (2,3-benzo[*b*]fluorene) recovered from standard solutions of BSM, using the DCM extraction method, to the known amount of these compounds added to the BSM prior to extraction; these tests were performed in the presence and absence of killed-bacterial inocula (*ca.* 10^6 cells ml⁻¹). Sequential extractions (three successive extractions) were conducted and the PAH concentration measured after each extraction; vigorous shaking by hand (see Chapter 3, Section 3.2.4.9) was used to transfer PAHs (three- to seven-benzene rings) and internal standard into the DCM phase. According to this procedure, greater than 99% of the added PAH, for all PAHs, was recovered from the first extraction when bacteria were present in BSM containing PAHs (Table 5.1).

5.2.2 Effect of Fungal Inoculum on PAH Extraction Efficiency

The procedures employed for PAH extraction from bacterial cultures could not be used when cultures contained a fungal inoculum. The extraction of PAHs from fungal cultures in BSM was found to be affected by the presence of fungal mycelia due to the high adsorption of these compounds to cell biomass. Three successive sequential extractions using ultrasonication (see Chapter 3, Section 3.2.4.9), followed by vigorous shaking, was required to achieve a total recovery of each PAH at approximately 97% to greater than 99% compared to the known PAH standard concentration.

5.2.3 Efficiency of Extraction of PAHs from PAH-Spiked Soil

The method adopted for the extraction of PAHs from soils must be highly efficient if accurate representation of the PAH concentration in soil samples are to be obtained. The composition of soil could place additional pressure on PAH recovery from this matrix, therefore, the efficiency of the PAH extraction method using PAH contaminated soils was examined. The efficiency of the recovery was based on the following criteria. Firstly, the extraction method must be effective and

particularly reproducible for the residual PAH concentration to be followed over time. Also, the presence of introduced inoculum and subsequent biomass must not interfere with the extraction method. Moreover, the distribution of PAHs in the soil at the beginning of the experiment must be homogenous in order for soil samples to accurately reflect the PAH concentration in the entire culture matrix.

5.2.3.1 A Comparison of Methods Used for the Extraction of PAHs from Soils

The sequential ultrasonification-DCM-extraction method (EPA, 1992a) and the Soxhlet methods (EPA, 1992b) were compared on the basis of their efficiency in recovering PAHs from soil. Nine PAHs were added to sterile uncontaminated soil to a known concentration and the above two methods were then used to extract these PAHs from the soil after 48 hours following PAH addition. In comparison to the standard Soxhlet extraction method, the use of sequential ultrasonication procedures to extract PAHs from soil recovered a relatively high percentage of each compound (approximately 97.9% to greater than 99%), and the variation amongst replicates of individual PAHs was acceptable, *i.e.* standard deviations were between 0.2-1.5% (Table 5.1).

In this chapter, the degradation experiments in PAH-spiked soil were conducted for an incubation period of 100 days. During this period, it is conceivable that some of the PAHs may have become tightly bound to soil particulate matter, subsequently affecting their recovery during extraction. This was not found to be the case since the amount of PAHs recovered from the controls (uninoculated PAH-spiked soil and PAH-spiked soil inoculated with killed cells) in these experiments using the ultrasonic extraction method demonstrated that there was no significant decrease (*ca.* 1.6-2.1%) in PAH recovery over the 100 day period (Section 5.8.1).

5.2.3.2 Effects of Inoculum on the Extraction Efficiency of PAHs from Soils

After three successive ultrasonication extractions, approximately 97% to greater than 99% of individual PAHs was recovered from PAH-spiked soil inoculated with fungal and or bacterial biomass (Table 5.1). The results also indicated a good extraction reproducibility. For most of the PAHs studied, the results showed that the standard deviations between the analysis of triplicate samples was quite low (see Section 5.2.3.4). Therefore, the sequential ultrasonication method was considered suitable for PAH extraction from small quantities of soil samples and was adopted for soil extraction in all related experiments. It should be noted that the appropriate controls (spiked-uninoculated, spiked-inoculated with killed cells) were also used in the soil experiments to monitor changes in extraction efficiency over time.

5.2.3.3 PAH Homogeneity in Soil Cultures

Characterization of PAH-spiked soil was necessary in order to confirm that there was a uniform distribution of PAHs throughout the soil profile. The homogeneity of the soil mixture was confirmed by testing several random portions of a test batch for PAH concentration. The relative standard deviation between the concentrations of each PAH studied from five samples of each preparation was less than 1.5%. This value was considered to be acceptable taking into account that such a standard deviation could be attributed to the extraction error. Thus, the soil used was assumed to be homogenous within replicate treatments.

5.2.3.4 Reproducibility

No significant differences were measured among replicate samples obtained from bacterial cultures according to a variance analysis at 95% confidence level. Results obtained from sequential extraction of each PAH from fungal cultures and soils by the methodology employed also indicated a good extraction reproducibility. For most of the PAHs investigated, results showed that the

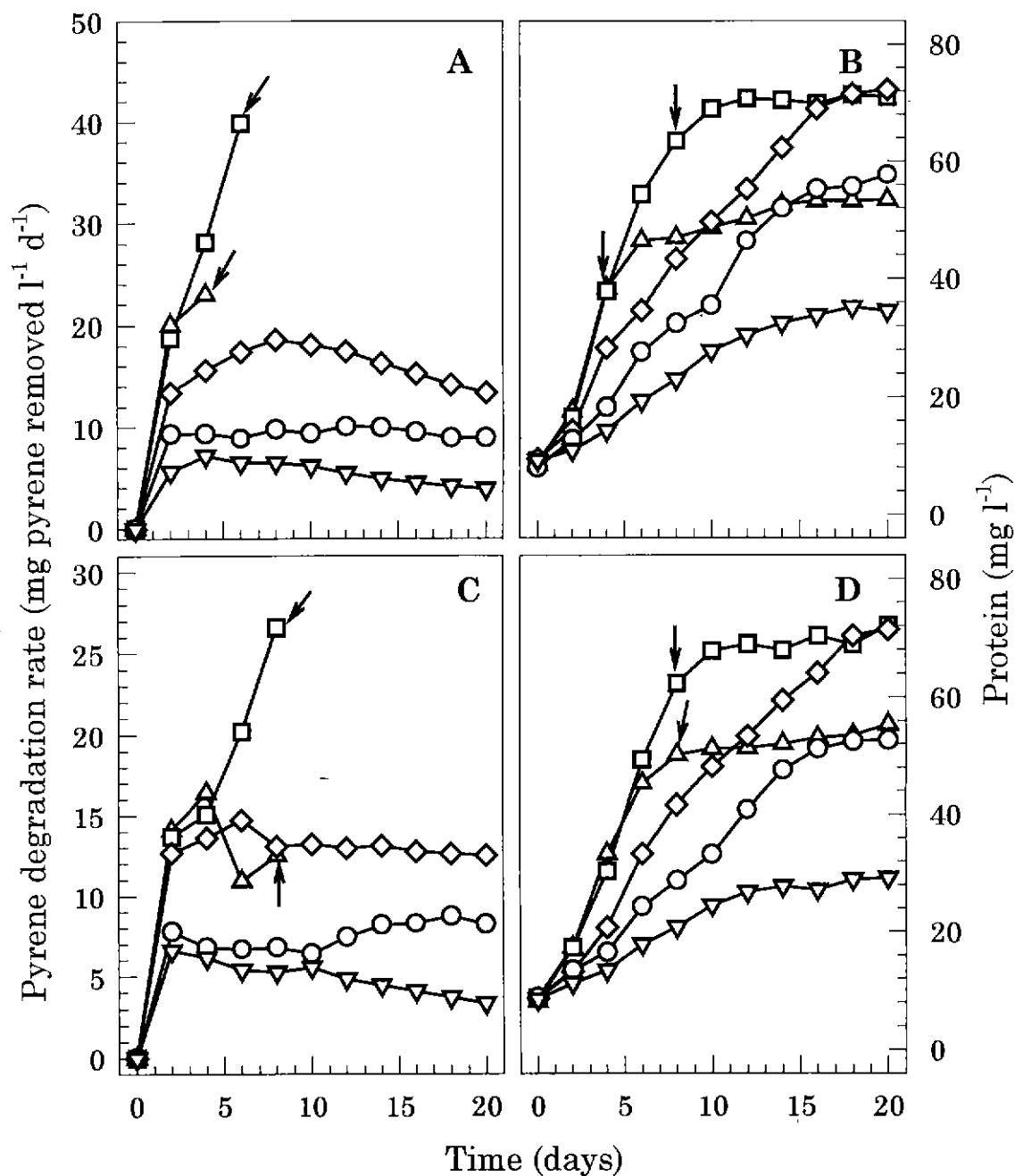


Figure 5.1 Pyrene degradation by *S. maltophilia* VUN 10,010 (A) and bacterial consortium VUN 10,009 (C) in cultures containing various initial pyrene concentrations. Pyrene was added to BSM to obtain final concentrations of 100 (Δ), 250 (◻), 400 (◊), 500 (○) and 750 mg l⁻¹ (▽). Pyrene degradation by both cultures is shown as the rate of pyrene removal. Bacterial growth on a respective pyrene concentration is presented as protein biomass for VUN 10,010 (B) and VUN 10,009 (D). The incubations were performed at 30°C/175 rpm. Arrows indicate the stages at which all pyrene had been totally degraded.

standard deviations between analyses of triplicate samples were quite low: 0.2-0.6% for fluorene, 0.1-1.5% for phenanthrene, 0.2-0.9% for fluoranthene, 0.1-1.1% for pyrene, 0.5-0.8% for benz[a]anthracene, 0.2-2.2% for chrysene, 0.3-2.0% for benzo[a]pyrene, 0.2-1.5% for dibenz[a,h]anthracene and 0.2-2.2% for coronene.

5.3 PAH DEGRADATION BY AXENIC MICROBIAL CULTURES

PAH-degrading isolates, namely *S. maltophilia* VUN 10,010, bacterial consortium VUN 10,009 and *P. janthinellum* VUO 10,201, obtained from Chapter 4 are examined in this section for their ability to degrade and grow on high molecular weight PAHs either as a sole carbon and energy source or *via* co-metabolic processes. Pyrene was used as a model PAH substrate in this work and its degradation by the isolates is covered in considerable detail prior to experiments on the degradation of other PAHs by the isolates.

5.3.1 *S. maltophilia* VUN 10,010 and Bacterial Consortium VUN 10,009

S. maltophilia VUN 10,010 and bacterial consortium VUN 10,009 were isolated from pyrene- and benzo[a]pyrene-enriched populations, respectively (Chapter 4, Section 4.2 and 4.3). However, a culture bacterial consortium VUN 10,009 was unable to grow on benzo[a]pyrene as a sole carbon source, therefore, this consortium was subsequently isolated and sub-cultured in medium containing pyrene as a growth substrate. These pyrene degraders were tested for their growth and degradation capability on various pyrene concentrations (100, 250, 400, 500 and 750 mg l⁻¹) as a sole carbon source in BSM. The results obtained from both bacterial strains demonstrated an ability to utilize and grow on pyrene at concentrations up to 750 mg l⁻¹ (Figure 5.1), however, microbial growth rates and pyrene degradation rates decreased when the initial pyrene concentrations added into the medium exceeded 250 mg l⁻¹.

Since many of the known pyrene metabolites are hydroxylated aromatic compounds, a separate experiment was performed in which the supernatant fluids

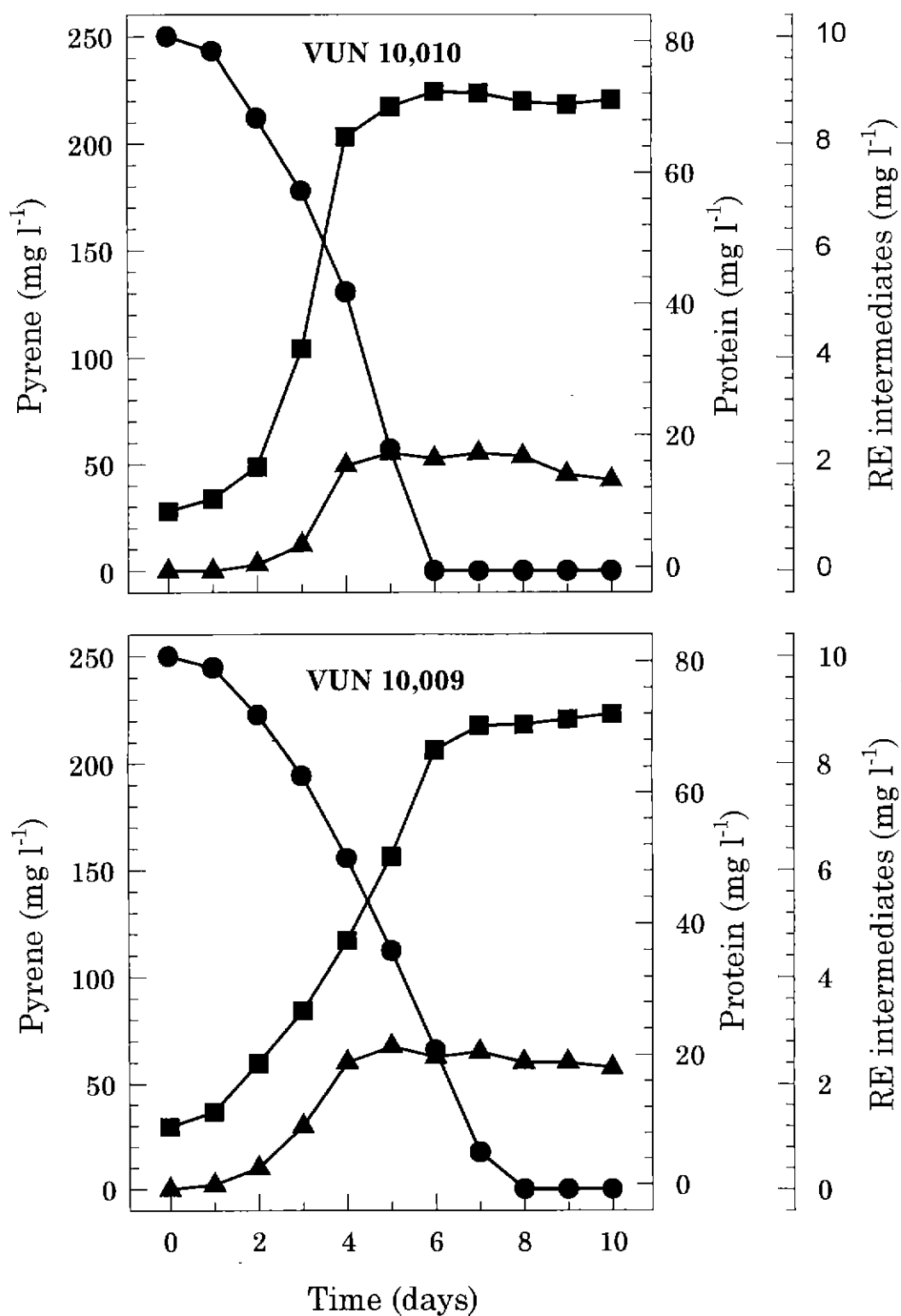


Figure 5.2 Time course for pyrene degradation by *S. maltophilia* VUN 10,010 and bacterial consortium VUN 10,009. The inoculum was added to BSM containing pyrene (250 mg l⁻¹) as the sole source of carbon and energy and incubated at 30°C/175 rpm in the dark. Pyrene (●), protein (■) and resocinol equivalent (RE) intermediate (▲) concentrations are shown.

of bacterial cultures, initially containing pyrene (250 mg l^{-1}) in BSM, were measured for the presence of phenolic compounds, otherwise known as resocinol equivalent (RE) intermediates. Pyrene degradation by VUN 10,010 (250 mg l^{-1} degraded in six days) was more rapid than that observed by VUN 10,009 (250 mg l^{-1} degraded in eight days) which reflected the faster growth rate of the former ($16 \text{ mg protein l}^{-1} \text{ day}^{-1}$) compared to the latter ($11.2 \text{ mg protein l}^{-1} \text{ day}^{-1}$) (Figure 5.2). The concentration of RE intermediates was not significantly different between the two cultures, with the concentration reaching a plateau after four days and remaining constant at around 2 mg l^{-1} for the remainder of the incubation period.

Preliminary results indicated that these PAH-degrading bacterial isolates were able to remove a variety of high molecular weight PAHs from cultures when supplied as a sole carbon source (Chapter 4, Section 4.6.5). However, the rate of PAH removal by these cultures was low (see Chapter 4, Table 4.6). Besides fluorene, phenanthrene and pyrene, none of the bacterial isolates demonstrated the ability to grow on the high molecular weight PAHs tested in the experiment. The low degradation rate of the high molecular weight PAHs is not surprising given that the bacteria could not grow on these compounds as a sole carbon source, meaning that the size of the bacterial population was very low during the incubation period. In order to stimulate the degradation rate of these PAHs, pyrene (250 mg l^{-1}) was added to BSM containing one other high molecular weight PAH. The degradation of the high molecular weight PAH in such cultures would be *via* co-metabolism, with pyrene serving as the growth substrate.

The results (Table 5.2) showed improved degradation of the more recalcitrant PAH compounds by VUN 10,009 and VUN 10,010 *via* co-metabolism. The co-metabolic removal of high molecular weight PAHs during bacterial growth on pyrene was twice as high as that observed in cultures without added pyrene. The amount of chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]-anthracene removed co-metabolically by strain VUN 10,010 were approximately 28%, 20%, 32% and 26%, respectively, after 28 days of incubation; in the absence of pyrene, and over a 56 day incubation period, the amount of these PAHs removed was 9%, 8%, 12% and 8%, respectively (Table 5.2). Consortium VUN

Table 5.2 Degradation of high molecular weight PAHs by bacterial isolates.

PAH ^b	Bacterial consortium VUN 10,009 ^a				<i>S. maltophilia</i> VUN 10,010 ^a			
	BSM		BSM + Pyrene		BSM		BSM + Pyrene	
	% PAH removal ^c	Protein (mg l ⁻¹) ^e	% PAH removal ^d	Protein (mg l ⁻¹)	% PAH removal ^c	Protein (mg l ⁻¹)	% PAH removal ^d	Protein (mg l ⁻¹)
Pyrene	100.0 ± 0.0	72.3 ± 3.2	NT	73.7 ± 1.7	100.0 ± 0.0	70.2 ± 1.3	NT	71.5 ± 1.5
Chrysene	8.2 ± 0.5	12.3 ± 0.8	12.1 ± 0.4	72.8 ± 1.2	9.1 ± 1.2	13.2 ± 2.2	28.3 ± 1.2	70.4 ± 4.1
Benz[<i>a</i>]anthracene	7.8 ± 0.3	10.7 ± 1.1	9.6 ± 0.7	74.1 ± 3.5	8.2 ± 1.6	12.4 ± 3.1	19.8 ± 0.3	69.3 ± 5.2
Benzo[<i>a</i>]pyrene	6.7 ± 0.9	13.6 ± 2.4	23.3 ± 0.5	74.0 ± 1.6	12.3 ± 1.0	14.8 ± 2.7	32.2 ± 1.2	70.1 ± 2.6
Dibenz[<i>a,h</i>]anthracene	5.9 ± 0.5	12.1 ± 0.5	13.8 ± 0.9	71.8 ± 2.9	7.8 ± 0.6	11.7 ± 2.6	26.7 ± 0.7	73.4 ± 3.0

^a Inocula were grown in BSM containing pyrene (250 mg l⁻¹). Washed cells were used to inoculate BSM containing a PAH or BSM containing pyrene (250 mg l⁻¹) and a respective PAH. Protein concentrations of the initial cell biomass were approximately 8.3-8.7 mg l⁻¹. % degradation was calculated relative to the amount of PAH removed from abiotic and killed-cell controls; averages are from triplicate experiments.

^b Initial PAH concentrations were 250 mg l⁻¹ for pyrene and 50 mg l⁻¹ for chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene.

^c Data represents PAH degradation in 56 days.

^d Data represents PAH degradation in 28 days.

^e Bacterial growth is represented as protein biomass. NT, not tested.

Table 5.3 Degradation of high molecular weight PAHs by *P. janthinellum* VUO 10,201.

PAH ^b	PAH removal (%) ^a		
	BSM ^c	BSMG ^d	MYPD ^d
Pyrene	3.3 ± 0.3	80.3 ± 2.8	93.6 ± 2.8
Chrysene	12.2 ± 0.4	25.6 ± 1.9	31.7 ± 3.7
Benz[<i>a</i>]anthracene	9.1 ± 1.4	18.1 ± 2.3	23.1 ± 1.2
Benzo[<i>a</i>]pyrene	16.9 ± 0.2	56.5 ± 2.4	72.2 ± 3.2
Dibenz[<i>a,h</i>]anthracene	12.9 ± 0.3	24.4 ± 2.7	36.7 ± 1.7

^a %degradation calculated relative to the amount of PAH remaining in the abiotic and killed cell controls; averages are from triplicate experiments. Inocula consisted of 0.25 g (wet weight) of 2-day old mycelia grown in MYPD. This was inoculated into 10 ml of medium. All incubations were performed at 30°C/175 rpm in the dark.

^b Initial PAH concentrations were 250 mg l⁻¹ for pyrene and 50 mg l⁻¹ for chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene.

^c Data represents PAH degradation in 56 days.

^d Data represents PAH degradation in 28 days.

10,009 grown in BSM supplemented with pyrene (250 mg l⁻¹) co-metabolically removed chrysene (12%), benz[*a*]anthracene (10%), benzo[*a*]pyrene (23%) and dibenz[*a,h*]anthracene (14%) over 28 days to a much greater extent than their removal by this consortium in the absence of pyrene, *i.e.* 8%, 8%, 7% and 6%, respectively over 56 days.

Bacterial growth, determined as increased protein concentration, was observed in cultures supplemented with pyrene (Table 5.2). No growth was observed in BSM cultures containing a high molecular weight PAH in the absence of pyrene. From a comparative perspective, strain VUN 10,010 was superior in the co-metabolic degradation of four- and five-ring PAHs compared to bacterial consortium VUN 10,009.

5.3.2 *P. janthinellum* VUO 10,201

P. janthinellum VUO 10,201 was unable to degrade pyrene in BSM when this was supplied as a sole source of carbon and energy. No significant amount of pyrene (3.3%) was removed from the culture after a 56 day incubation period, relative to the amount that disappeared from the killed cell control (Table 5.3). This suggested that *P. janthinellum* VUO 10,201 is unable to utilize pyrene as a growth substrate nor can it degrade this compound when present as a sole carbon source. Therefore, an experiment was designed to investigate whether this fungus could degrade pyrene in the presence of another carbon substrate that would support fungal growth, *i.e.* co-metabolic degradation. MYPD (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% dextrose), BSMG (BSM supplemented with various concentrations of glucose; 0.1%, 1%, 1.5% and 2%) and BSMGY (BSM plus 1% glucose and 0.5% yeast extract) were used as culture media. Pyrene was added to these media at 250 mg l⁻¹.

Pyrene was rapidly removed from MYPD and BSMG inoculated with *P. janthinellum* VUO 10,201 in comparison to the BSM culture (Figure 5.3). No significant amount of pyrene was removed from BSM as previously described. When 0.1% of glucose was added to the BSM culture after 3 days of incubation, a

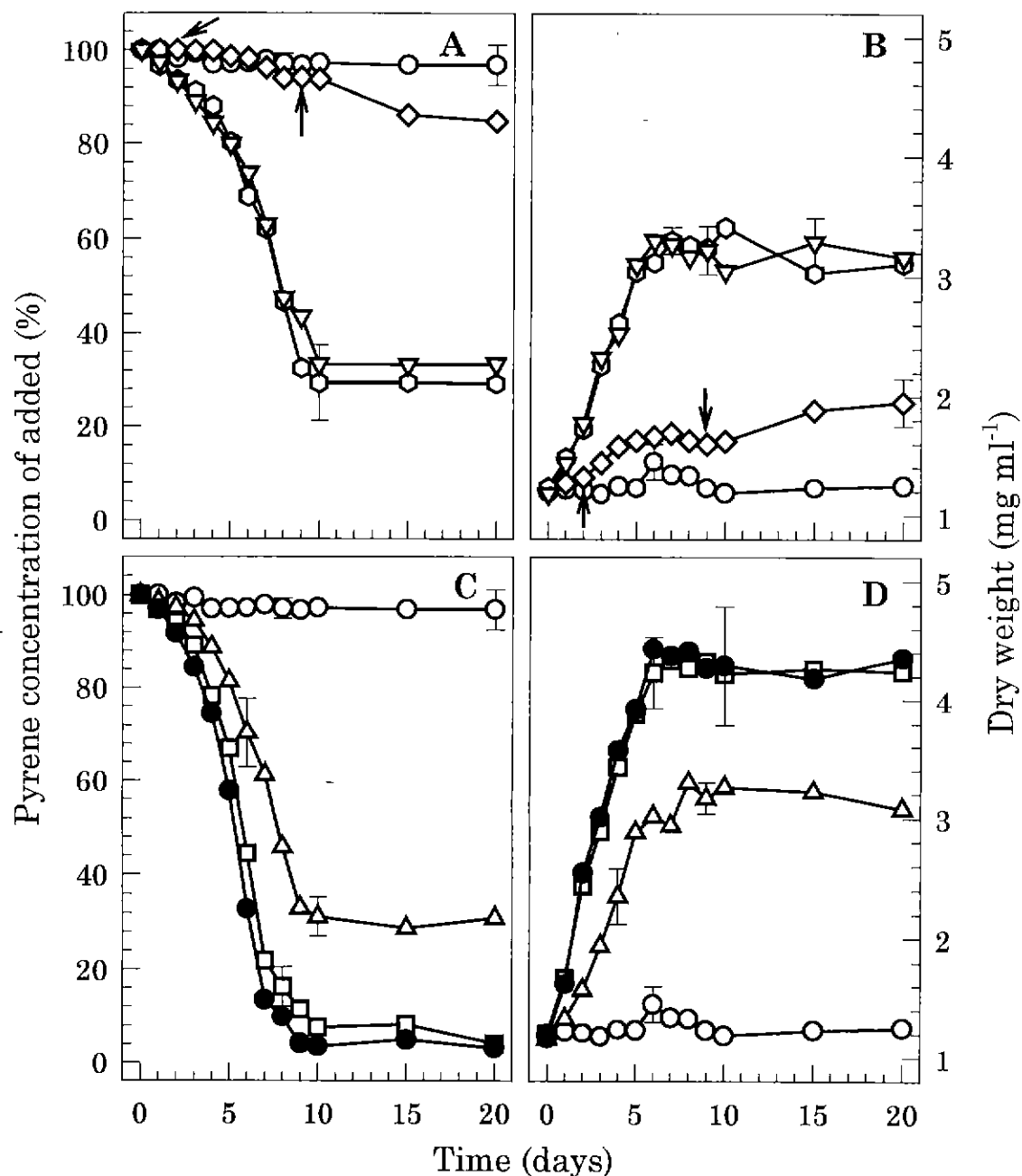


Figure 5.3 Degradation of pyrene by *P. janthinellum* VUO 10,201 added to different media: BSM (○), BSM plus 0.1% glucose (◇), BSM plus 1.0% glucose (◐), BSM plus 1.5% glucose (▽), BSM plus 2.0% glucose (△), BSM plus 1.0% glucose and 0.5% yeast extract (◻) and MYPD (●). Pyrene concentrations in the absence and presence of 0.1, 1.5 and 2.0% glucose (A); biomass production for A (B); pyrene concentrations in BSM plus 1.0% glucose, BSM plus 1% glucose and 0.5% yeast extract and MYPD (C); and biomass production for C (D). Arrows indicate the supplementation of 0.1% glucose (final concentration) to BSM. The incubations were conducted at 30°C/175 rpm in the dark.

slight increase in the amount of pyrene removed was observed until the 10th day of incubation. A second addition of 0.1% glucose to this culture was performed at day 10 and this significantly improved the pyrene removal rate. Parallel incubations in BSM containing various increasing glucose concentrations demonstrated increasing pyrene removal rates by *P. janthinellum* VUO 10,201 up to a glucose concentration of 1%; the extent of pyrene removal was not improved for glucose concentrations greater than 1% (Figure 5.3). However, the removal of pyrene by *P. janthinellum* VUO 10,201 was further improved by adding additional nutrients to the medium *i.e.* using BSMGY and MYPD (Figure 5.3). Approximately 85-90% of initial pyrene was removed from these culture media. No significant difference was observed in pyrene degradation between cultures grown in BSMGY and MYPD. The pyrene degradation results correspond with the production of fungal biomass. No growth of *P. janthinellum* VUO 10,201 was observed in BSM containing pyrene while fungal growth was evident in BSM plus glucose as well as in the nutrient-rich media (MYPD and BSMGY) (Figure 5.3). The increased fungal biomass seems to correlate with the increased PAH removal by this fungus. Based on these results, BSMG (with 1% glucose), MYPD and BSM were selected as media for further studies on the degradation of other high molecular weight PAHs by *P. janthinellum* VUO 10,201.

P. janthinellum VUO 10,201 growth and its degradation of high molecular weight PAHs, including chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]-anthracene was initially investigated in BSM only. There was no growth of *P. janthinellum* VUO 10,201 in BSM containing a respective PAH as a sole carbon source. Time-course experiments revealed that PAH disappearance from BSM began during the first week of incubation (Figures 5.4 and 5.5). However, after 14-28 days, there was no further substantial degradation of these PAHs by this fungus.

The disappearance of high molecular weight PAHs from BSMG and MYPD media due to *P. janthinellum* VUO 10,201 was significantly higher compared to those observed in BSM. Table 5.4 shows the removal of five PAHs from three different media incubated with *P. janthinellum* VUN 10,201, over a 28 day period, relative

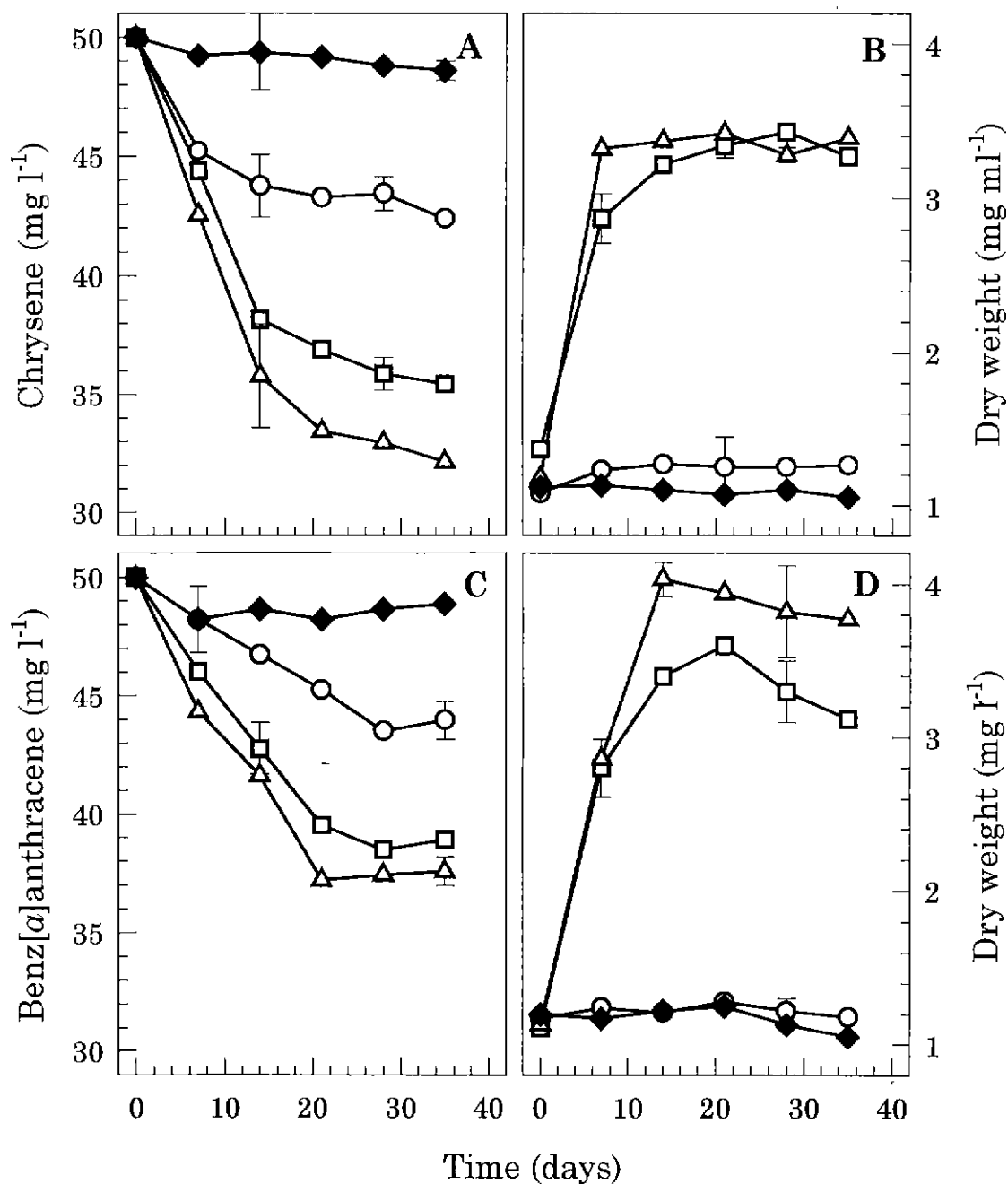


Figure 5.4 PAH degradation (A and C) by *P. janthinellum* VUO 10,201 in three different media: BSM (○), BSMG (□) and MYPD (△). Growth of the fungus in the respective medium is shown as dry weight (B and D). Killed fungal mycelia in MYPD (◆) is also shown. The incubations were performed at 30°C/175 rpm in the dark.

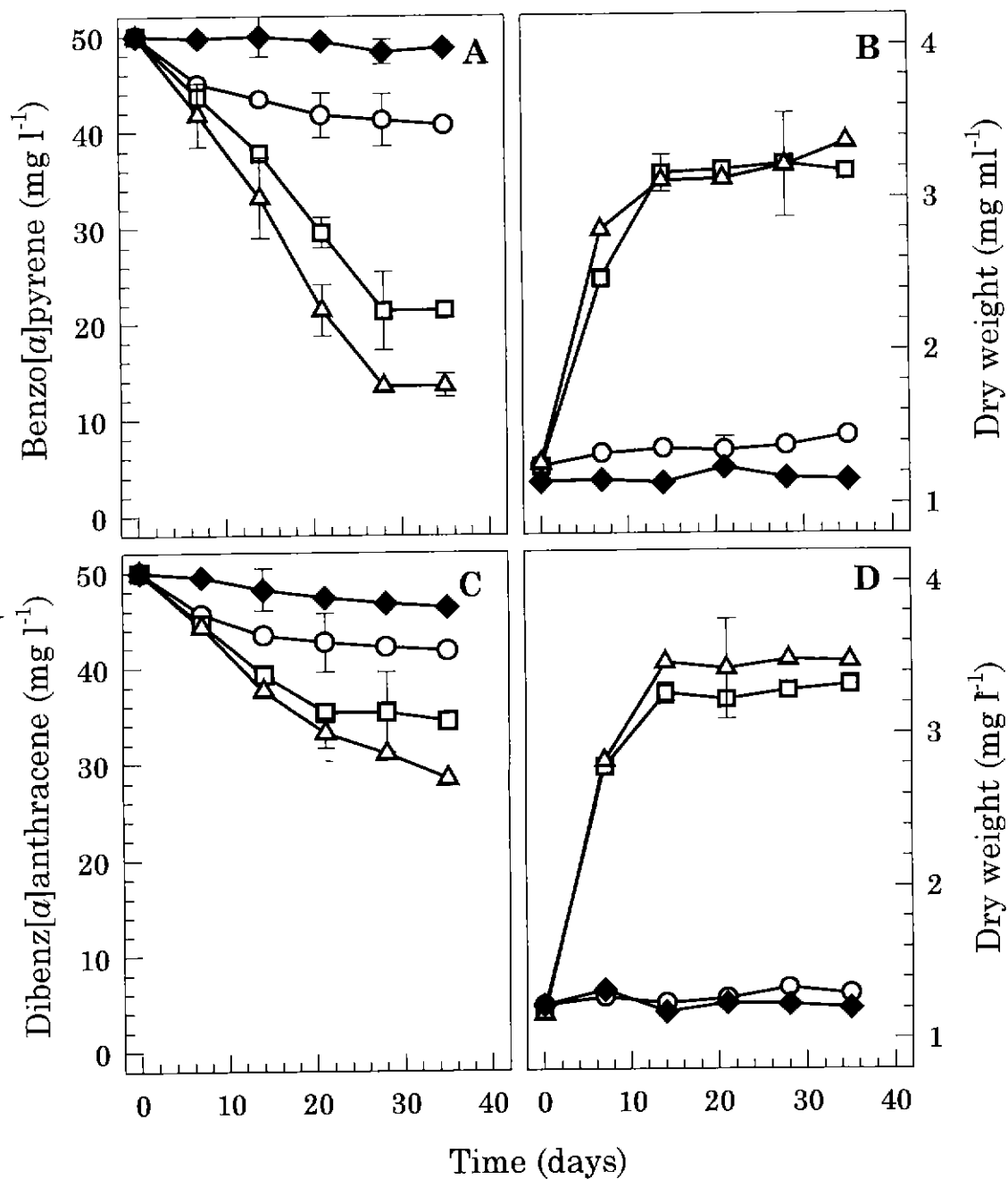


Figure 5.5 PAH degradation (A and C) by *P. janthinellum* VUO 10,201 in three different media: BSM (○), BSMG (□) and MYPD (△). Growth of the fungus in the respective medium is shown as dry weight (B and D). Killed fungal mycelia in MYPD (◆) is also shown. The incubations were performed at 30°C/175 rpm in the dark.

to the amount of PAH lost from killed cell controls. This fungus is able to degrade PAHs containing four- and five-benzene rings when sufficient nutrients are provided in the culture medium. As previously observed, the amount of chrysene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene removed by VUO 10,201 in BSM was low. However, as was the case for pyrene, the disappearance of these PAHs was substantially improved in BSMG and particularly in MYPD. For example, only 12%, 9%, 17% and 13% of chrysene, benz[*a*]anthracene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene, respectively, was removed by the fungus in BSM after 56 days. In contrast, the removal of these PAHs was significantly enhanced by incubating the fungus in MYPD such that approximately 32%, 23%, 72 and 37% of a respective PAHs disappeared after 28 days. Although the amount of each PAH removed from BSMG cultures was lower than those of MYPD cultures, it was significantly higher than that removed in the BSM cultures (Table 5.3). PAH disappearance was confirmed as biodegradation since PAH disappearance from abiotic and HgCl₂-killed cell controls did not exceed $5.0 \pm 1.2\%$ throughout the experiments. The greater PAH removal in MYPD and BSMG compared to BSM is probably due to the fact that *P. janthinellum* VUO 10,201 can grow, thus producing more biomass to degrade such PAHs (under co-metabolic conditions).

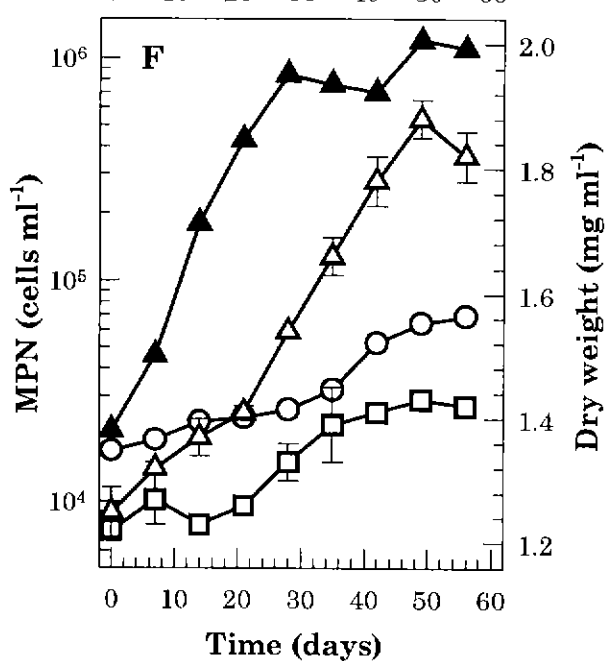
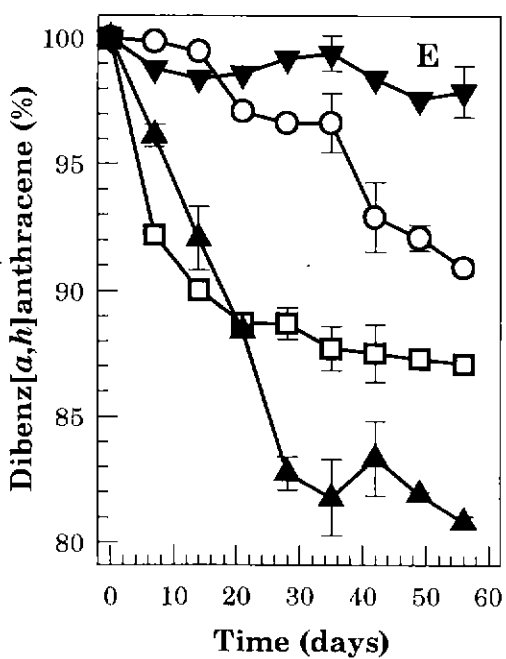
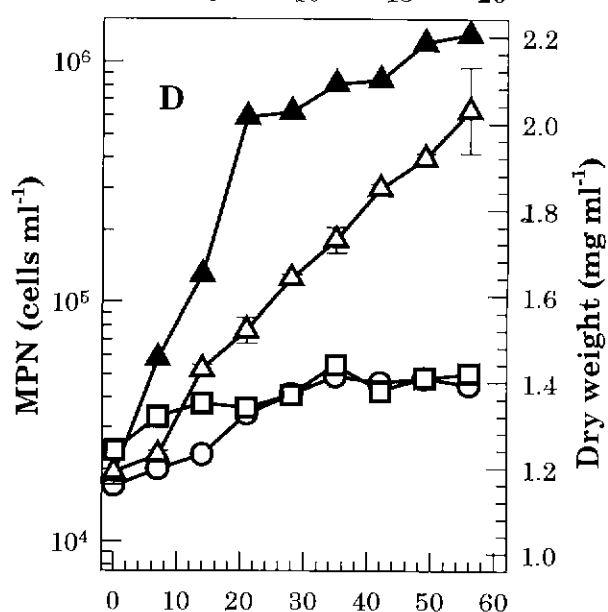
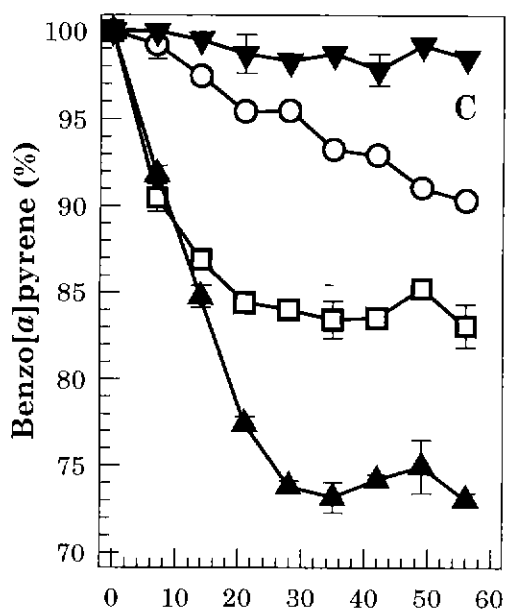
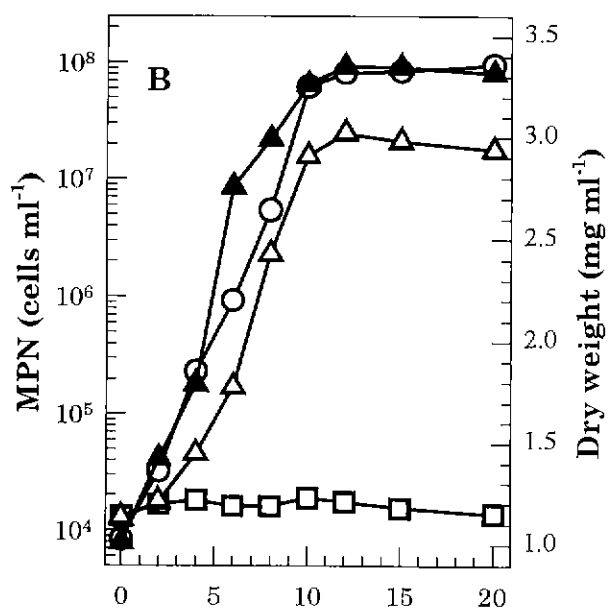
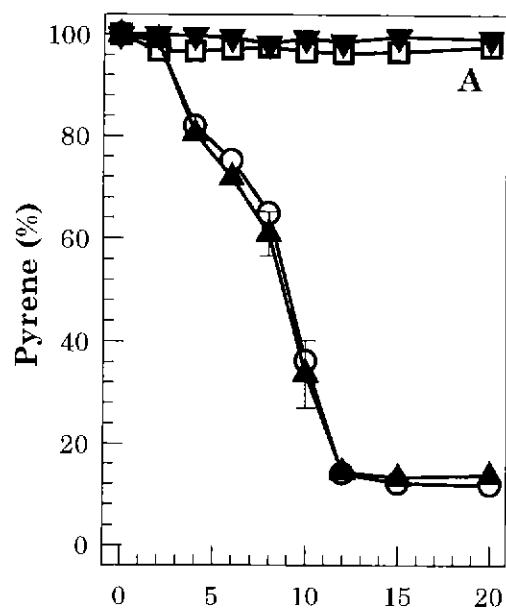
5.4 PAH DEGRADATION BY FUNGAL-BACTERIAL CO-CULTURES

The assemblage of different PAH-degraders in one culture would, in principle, broaden enzymatic capability and possibly promote an increased capacity by the culture to completely degrade or mineralize PAHs generally considered to be recalcitrant to microbial degradation. Evidence that fungal-bacterial co-cultures are superior to axenic microbial cultures in high molecular weight PAH degradation is presented in the remaining sections of this chapter.

5.4.1 *P. janthinellum* VUO 10,201 and Bacterial Consortium VUN 10,009

The first co-culture investigated combined *P. janthinellum* VUO 10,201 and bacterial consortium VUN 10,009, since these organisms were enriched together on benzo[a]pyrene from the same soil sample. An experiment was conducted to investigate the growth of bacterial consortium VUN 10,009 and *P. janthinellum* VUO 10,201 strains when combined as a co-culture (designated as co-culture A) in BSM containing a single high molecular weight PAH as the only source of carbon and energy. Both *P. janthinellum* VUO 10,201 and the bacterial consortium VUN 10,009 grew on benzo[a]pyrene and dibenz[a,h]anthracene as a sole carbon and energy source when incubated in co-culture (Figure 5.6); no significant growth on these PAHs was observed in axenic cultures of these organisms. The bacterial population in the co-culture increased by at least two log scale (from 10^4 to 10^6 cells ml^{-1}) and fungal dry weight of the same co-culture increased by around 50-70% over a 56 day period. Co-culture A also degraded a significantly greater amount of benzo[a]pyrene (26%) and dibenz[a,h]anthracene (19%) compared to axenic cultures of VUN 10,009 (9.7% benzo[a]pyrene, 9.2% dibenz[a,h]anthracene) and VUO 10,201 (17% benzo[a]pyrene, 13% dibenz[a,h]anthracene). Similar results were obtained for chrysene and benz[a]anthracene (Figure 5.7), in which 25% and 17% of the respective compounds was degraded by co-culture A. Degradation of chrysene and benz[a]anthracene by axenic cultures of VUN 10,009 were 9% and 6%, respectively, while 12% and 9% of the respective PAHs was degraded by axenic cultures of VUO 10,201. Pyrene degradation by co-culture A and the axenic bacterial culture was similar with the only difference being that *P. janthinellum* VUO 10,201 could grow in BSM containing pyrene when the bacterial consortium VUN 10,009 was also present (Figure 5.6); suggesting that VUO 10,201 is feeding on pyrene breakdown intermediates produced by the consortium. This is in contrast to the results of Section 5.3.2 where *P. janthinellum* VUO 10,201 could only grow on pyrene when other nutrients were available in the medium. According to the above results as shown in Figures 5.6 and 5.7, PAH degradation and microbial growth were significantly promoted in

Figure 5.6 Degradation of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene in BSM by *P. janthinellum* VUO 10,201 (◻), bacterial consortium VUN 10,009 (○) or co-culture A (▲). PAH-BSM media inoculated with a killed co-culture A (▼) is also shown. Culture media consisted of BSM and a single PAH. Bacterial growth is shown as an estimate of cell population determined in axenic cultures (○) and co-culture A (▲). Fungal dry weight is shown for axenic cultures (◻) and co-culture A (Δ). Incubations were performed at 30°C/175 rpm in the dark.



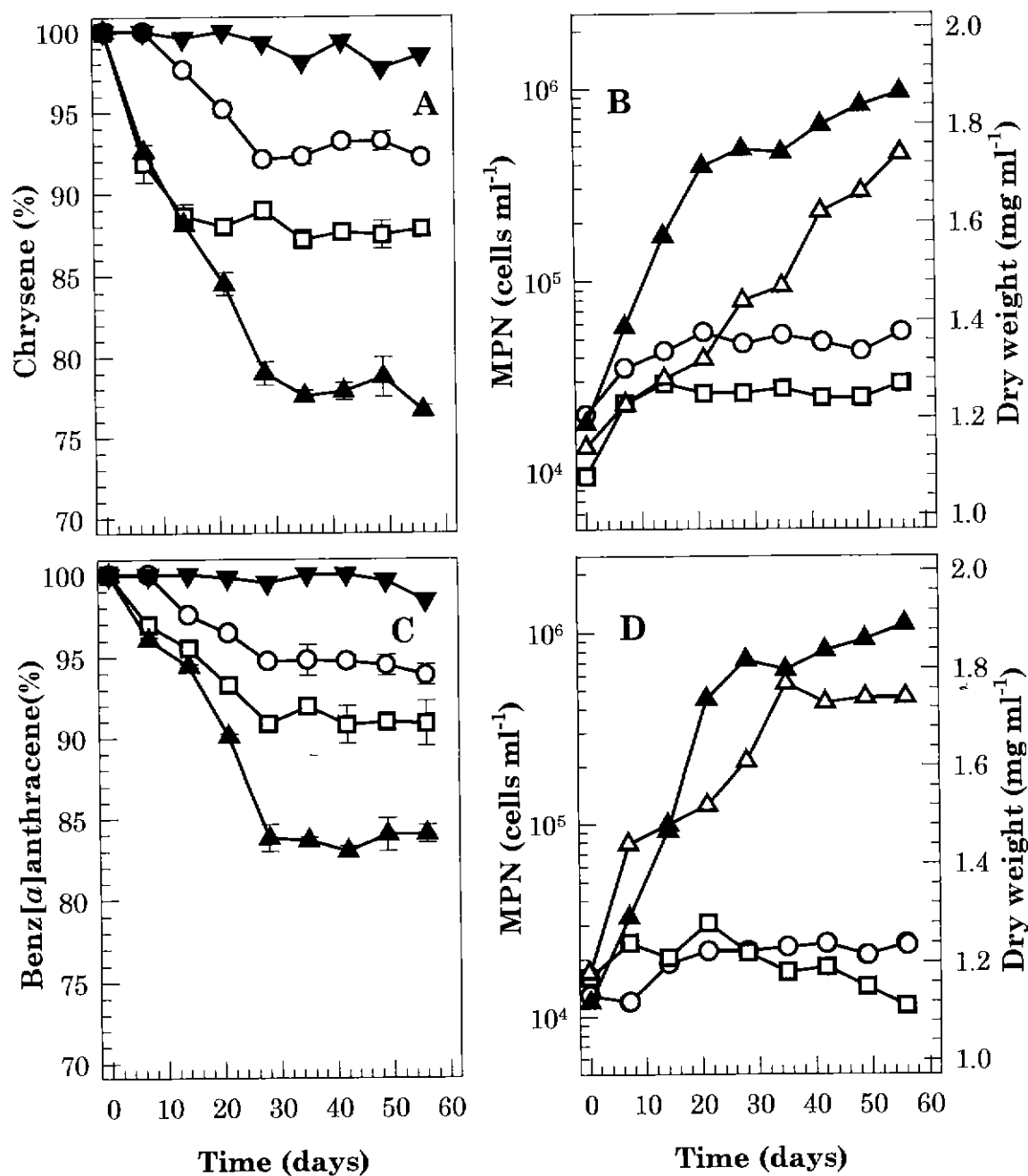


Figure 5.7 Degradation of chrysene and benz[a]anthracene in BSM by bacterial consortium VUN 10,009 (○), *P. janthinellum* VUO 10,201 (□) and co-culture A (▲). PAH-BSM media inoculated with a killed co-culture A (▼) is also shown. Culture media comprised BSM and a single PAH. Bacterial growth is shown as an estimated cell number determined in axenic cultures (○) and co-culture A (▲). Fungal dry weight is shown for axenic cultures (□) and co-culture A (Δ). Incubations were performed at 30°C/175 rpm in the dark.

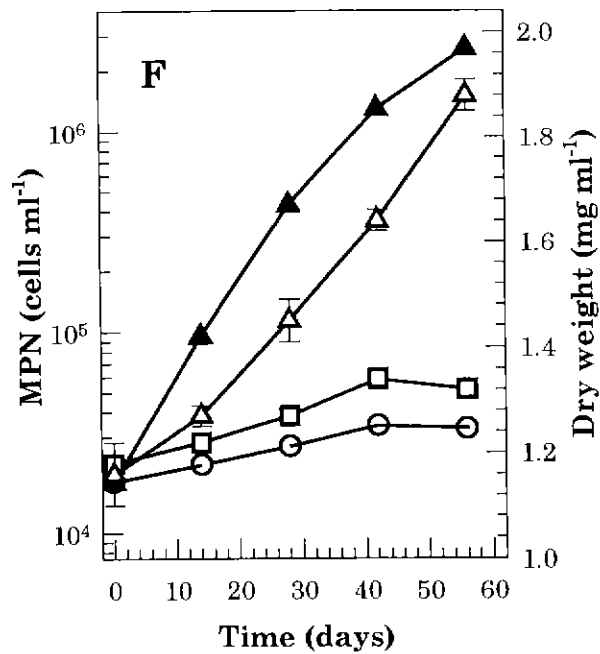
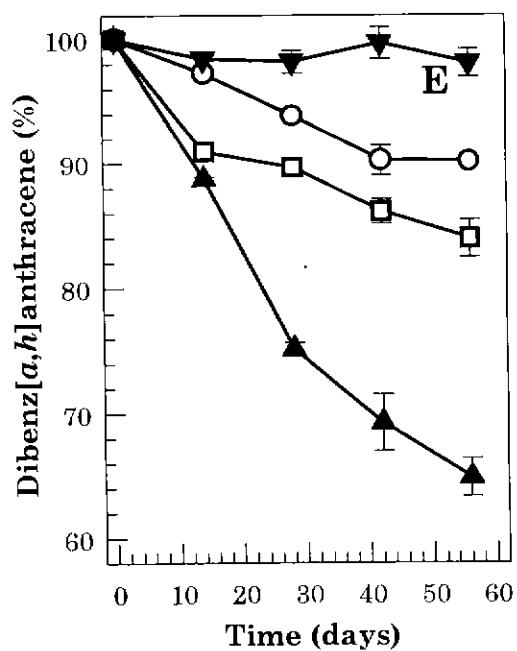
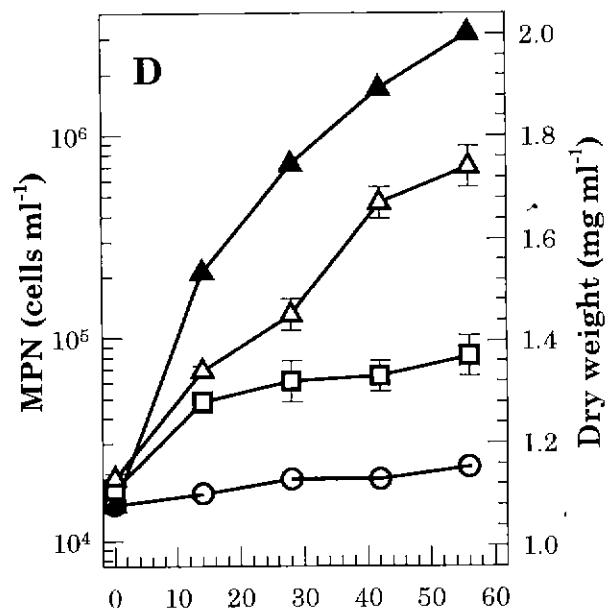
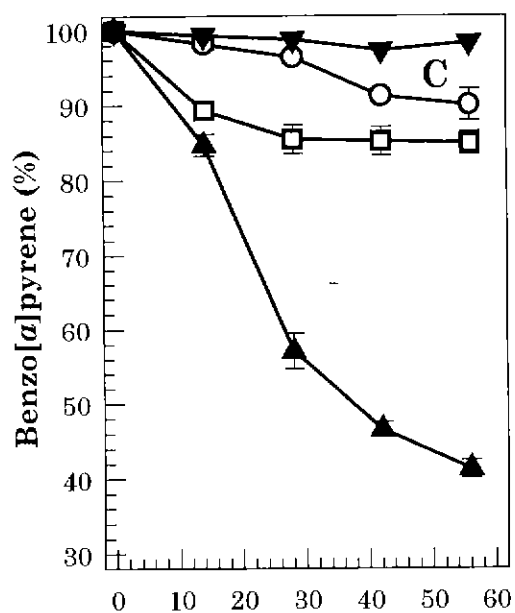
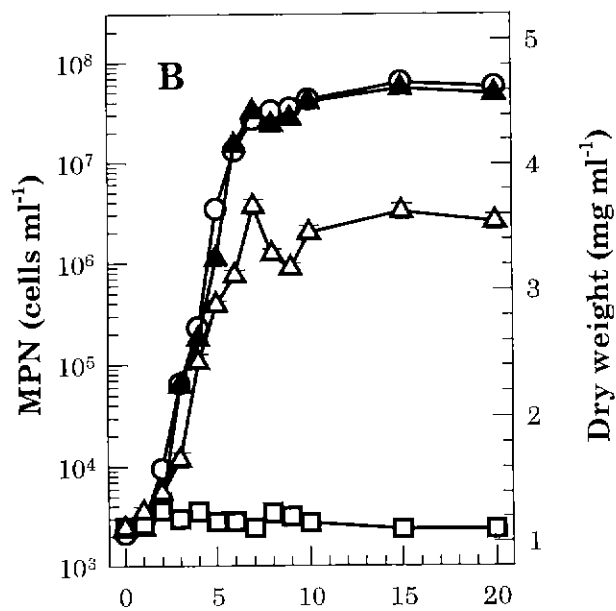
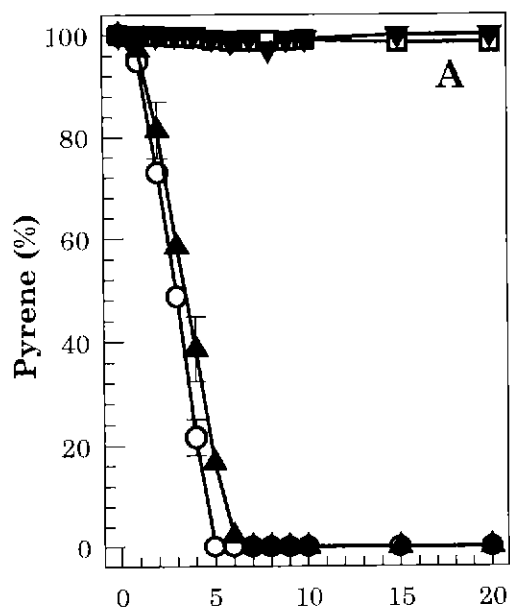
the co-culture (all data points in these figures have error bars but in some cases they are smaller than the symbols used in the graphs).

5.4.2 *P. janthinellum* VUO 10,201 and *S. maltophilia* VUN 10,010

The results of Section 5.3.2 demonstrated that *P. janthinellum* VUO 10,201 has a greater ability to degrade the five-benzene ring PAHs compared to the bacterial cultures. It was also shown previously that of the two bacterial isolates, *S. maltophilia* VUN 10,010 was able to degrade five-benzene ring PAHs to a greater extent than bacterial consortium VUN 10,009 and it also had a superior growth rate on pyrene as a sole carbon and energy source (Section 5.3.1). With this in mind, it was decided to combine *P. janthinellum* VUO 10,201 and *S. maltophilia* VUN 10,010 as a co-culture with the aim of determining whether this improved PAH degradation rates relative to co-culture A. One potential problem with this fungal-bacterial combination is that the two organisms were isolated from soils obtained from different sites, and hence different selective pressures. It was reasonable to assume that these two organisms may have an antagonistic, rather than synergistic, relationship when introduced to each other. This possibility was investigated before PAH degradation experiments were conducted with this co-culture combination.

Preliminary investigation showed that there was no antagonistic effect between *P. janthinellum* VUN 10,201 and *S. maltophilia* VUN 10,010 when dual cultures were performed on PDA and NA plates. Moreover, fungal culture supernatant as well as chloroform extracts from fungal mycelia did not exert an inhibitory effect on the growth of VUN 10,010 on NA plates. In addition, the fungus did not appear to interfere with bacterial growth and pyrene degradation by strain VUN 10,010 when these two organisms were combined in BSM containing pyrene (Figure 5.8A and B). In the absence of any observable antagonistic effects, *P. janthinellum* VUN 10,201 and *S. maltophilia* VUN 10,010 were combined to form another co-culture, which was designated co-culture B.

Figure 5.8 Degradation of pyrene, benzo[*a*]pyrene and dibenz[*a,h*]anthracene in BSM by *P. janthinellum* VUO 10,201 (○), *S. maltophilia* VUN 10,010 (□) or co-culture B (▲). PAH-BSM media inoculated with a killed co-culture B (▼) is also shown. Culture media consisted of BSM and a single PAH. Bacterial growth is shown as an estimate of cell population determined in axenic cultures (○) and co-culture B (▲). Fungal dry weight is shown for axenic cultures (□) and co-culture B (Δ). Incubations were performed at 30°C/175 rpm in the dark.



Co-culture B was inoculated into BSM containing a single high molecular weight PAH as the sole carbon and energy source. *P. janthinellum* VUO 10,201 and *S. maltophilia* VUN 10,010 both grew on benzo[a]pyrene or dibenz[a,h]anthracene when these two organisms were incubated together (Figure 5.8); growth on these PAHs was very poor in axenic cultures of these organisms. The rate of five-benzene ring PAH degradation by co-culture B was substantially higher than that observed by the axenic cultures and by co-culture A. After 56 days, co-culture B degraded 59% (29.5 mg l⁻¹) benzo[a]pyrene and 35% (17.5 mg l⁻¹) dibenz[a,h]-anthracene compared to 27% (13.5 mg l⁻¹) benzo[a]pyrene and 19% (9.5 mg l⁻¹) dibenz[a,h]anthracene degraded by co-culture A over the same time period (Figures 5.6 and 5.9). In BSM containing pyrene, the pyrene degradation rate and fungal biomass yield was greater using co-culture B (ca. 41.7 mg l⁻¹ day⁻¹, 3.6 mg ml⁻¹ dry weight) compared to co-culture A (ca. 18.3 mg l⁻¹ day⁻¹, 3.1 mg ml⁻¹ dry weight). The improved pyrene degradation rate was probably due to the superior pyrene-degrading ability of VUN 10,010 compared to the bacterial consortium rather than the co-culture *per se*.

5.5 HIGH MOLECULAR WEIGHT PAH MINERALIZATION

The previous sections have described the amount and type of PAHs degraded by the axenic cultures and their co-culture combinations, but the information obtained could not be used to determine the extent to which the PAHs were degraded. In many cases of microbial PAH degradation, the PAHs are often only partially degraded forming byproducts which are sometimes more toxic and more mobile than their parent compounds. This is especially true for the high molecular weight PAHs (Müncnerová and Augustin, 1994). Ideally, microbial degradation of PAHs should lead to complete breakdown of these compounds to form biomass, CO₂ and water, which is otherwise known as PAH mineralization. In the results reported in this section, the mineralization of pyrene and benzo[a]pyrene by the PAH-degrading isolates as axenic culture and co-cultures was investigated.

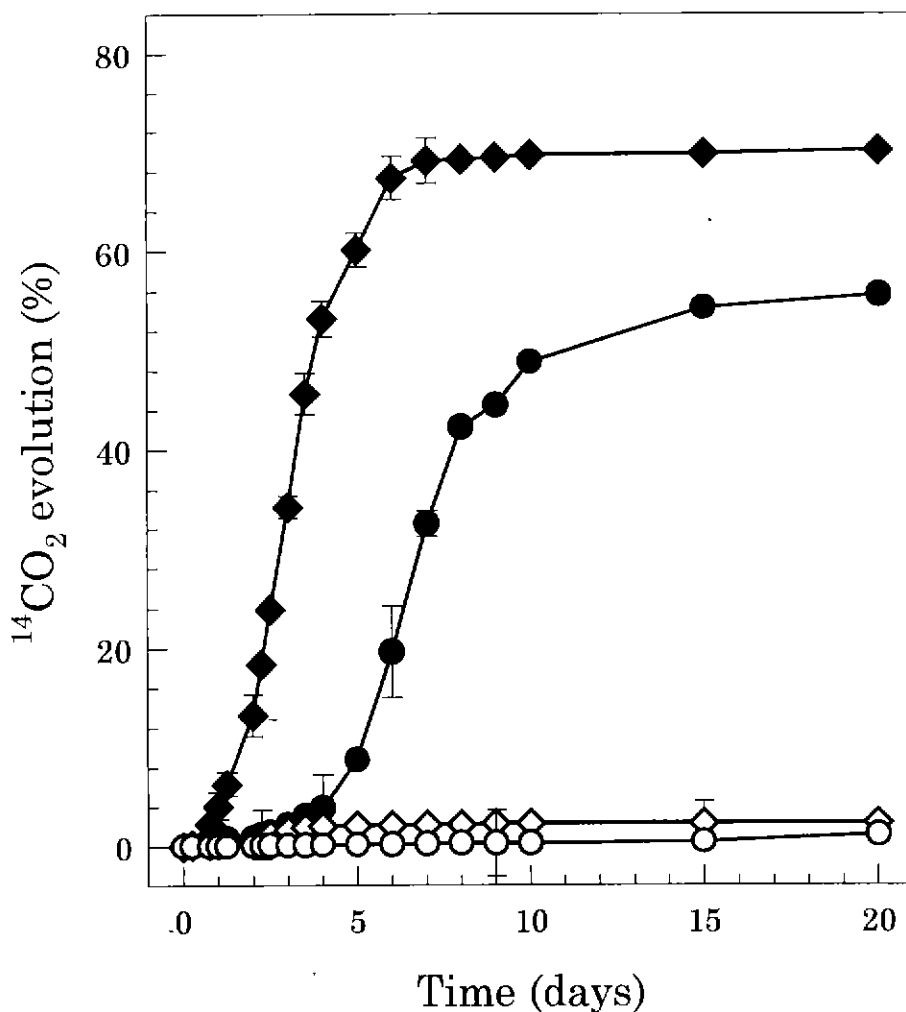


Figure 5.9 Mineralization of [^{14}C]pyrene by PAH-degrading bacteria. Labeled pyrene was added to BSM containing pyrene (250 mg l^{-1}) and inoculated with *S. maltophilia* VUN 10,010 (◆) or bacterial consortium VUN 10,009 (●) to obtain the initial populations of approximately $10^3 \text{ cells ml}^{-1}$. A respective HgCl_2 -killed cell control (◇, ○) contained a high cell density (*ca.* $10^6 \text{ cells ml}^{-1}$). All incubations were performed at $30^\circ\text{C}/175 \text{ rpm}$ in the dark.

5.5.1 High Molecular Weight PAH Mineralization by Axenic Microbial Cultures

Since pyrene was utilized by *S. maltophilia* VUN 10,010 and the bacterial consortium VUN 10,009 as a growth substrate (Section 5.3.1), and *P. janthinellum* VUO 10,201 was able to co-metabolically degrade this compound (Section 5.3.2), the amount of pyrene mineralized by these organisms as a sole carbon and energy source in BSM was evaluated first.

Bacterial consortium VUN 10,009 began to mineralize pyrene after a four day lag phase, with an appreciable amount of $^{14}\text{CO}_2$ evolving from the culture in the following six days; the $^{14}\text{CO}_2$ evolved accounted for approximately 50% of the initial labeled carbon added to the medium (Figure 5.9). Incubation of the culture continued for an additional ten days during which the cumulative amount of $^{14}\text{CO}_2$ obtained on the final day was approximately 55% of the initial radioactivity added to the culture. The rate and extent of pyrene mineralization by *S. maltophilia* VUN 10,010 was considerably greater than VUN 10,009. Approximately 70% of initial radioactivity added was detected as $^{14}\text{CO}_2$ in the VUN 10,010 culture after 10 days of incubation. There was virtually no lag period in $^{14}\text{CO}_2$ evolution in the *S. maltophilia* VUN 10,010 culture. In contrast, *P. janthinellum* VUO 10,201 did not degrade ^{14}C -pyrene to $^{14}\text{CO}_2$ in BSM as a sole carbon and energy source nor did this occur in BSMG or MYPD medium (Table 5.4), in which this fungus previously demonstrated substantial pyrene degradation.

A mass balance on the added radiolabeled carbon was performed to determine the fate of pyrene degradation products in each of the microbial cultures. The data, represented in Table 5.4, provide some answers to the observations made in the pyrene degradation experiments performed in Section 5.3. Both of the axenic bacterial cultures were able to mineralize pyrene to $^{14}\text{CO}_2$, but higher yields were secured by *S. maltophilia* VUN 10,010 (70%) compared to the bacterial consortium VUN 10,009 (55.6%). For VUN 10,010, the remaining labeled carbon was mostly

Table 5.4 Distribution of ^{14}C -residues after incubation of PAH-degrading organisms with $[^{14}\text{C}]$ pyrene.

Microorganism	Radioactivity in the following fractions:				% ^{14}C recovery
	$^{14}\text{CO}_2$	Biomass	Aqueous phase	DCM phase	
VUN 10,009 VUN 10,010 VUO 10,201	BSM containing pyrene (250 mg l ⁻¹)				99.8 98.9 97.8
	55.6	18.7	21.7	3.8	
	70.1	18.3	10.5	NA	
	NA	NA	1.2	96.6	
VUO 10,201	BSMG containing pyrene (250 mg l ⁻¹)				98.9
	NA	NA	76.8	22.1	
VUO 10,201	MYPD containing pyrene (250 mg l ⁻¹)				99.0
	NA	NA	88.7	10.3	

NA, Not appreciable.

Table 5.5 Distribution of ^{14}C -residues after incubation of [^{14}C]benzo[*a*]pyrene with PAH-degrading organisms.

Microorganism	$^{14}\text{CO}_2$	Biomass	Aqueous phase	DCM phase	% ^{14}C recovery
VUN 10,009 VUN 10,010 VUO 10,201	BSM containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹)				100.3 100.1 99.1
	NA	NA	1.2	99.1	
	NA	NA	1.6	98.5	
	NA	NA	13.7	85.4	
VUO 10,201	BSMG containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹)				97.5
	NA	NA	48.3	49.2	
VUO 10,201	MYPD containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹)				96.9
	NA	NA	60.7	30.2	
VUN 10,009 VUN 10,010 VUO 10,201	BSM containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹) and pyrene (250 mg l ⁻¹)				100.1 99.9 98.6
	12.8	0.7	2.3	84.3	
	32.4	NA	2.7	64.8	
	NA	NA	14.5	84.1	

NA, not appreciable.

found in biomass (18.3%) and in water soluble metabolites (10.5%). For VUN 10,009, most of the remaining labeled carbon was found in the aqueous phase (21.7%) with the remaining portion being in the biomass (18.7%). The more complete breakdown of pyrene by VUN 10,010 compared to VUN 10,009 is consistent with its higher pyrene degradation rate and growth rate on pyrene compared to the latter (Section 5.3.1). The mass balance for labeled pyrene added to *P. janthinellum* VUN 10,201 cultures reveals a total absence of labeled carbon in the form of $^{14}\text{CO}_2$ or as fungal biomass. Such results are consistent with the inability of *P. janthinellum* VUN 10,201 to grow on pyrene as a sole carbon and energy source with the logical conclusion being that it is unable to breakdown this PAH sufficiently to procure carbon for anabolic processes. This is further supported by the large amount of labeled carbon recorded in the aqueous phase, which represents water soluble intermediates arising from the partial breakdown of pyrene by the fungus.

The five-benzene ring benzo[a]pyrene was examined for mineralization because of its significance in issues regarding environmental and human health due to its recalcitrant, carcinogenic and mutagenic properties. The [^{14}C]benzo[a]pyrene used in this investigation was labeled at the C₇ position in the molecule. Enzymatic attack of the ring containing the labeled carbon at either the 7-8 or 9-10 positions are some of the most energetically favorable in benzo[a]pyrene (Loew *et al.*, 1978).

There was no mineralization of ^{14}C -benzo[a]pyrene by axenic cultures of either bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010 when this PAH was added to BSM as a sole source of carbon and energy (Table 5.5). However, axenic cultures of VUN 10,010 and VUN 10,009 co-metabolically mineralized benzo[a]pyrene (50 mg l⁻¹) in the presence of pyrene (250 mg l⁻¹) (Figure 5.10). The co-metabolic benzo[a]pyrene mineralization rate by VUN 10,010 was substantially higher than VUN 10,009 with $^{14}\text{CO}_2$ evolution after 56 days being 32% and 13%, respectively. The remaining labeled carbon was found mainly in the DCM extract (65-84%), most likely in the form of undegraded benzo[a]pyrene

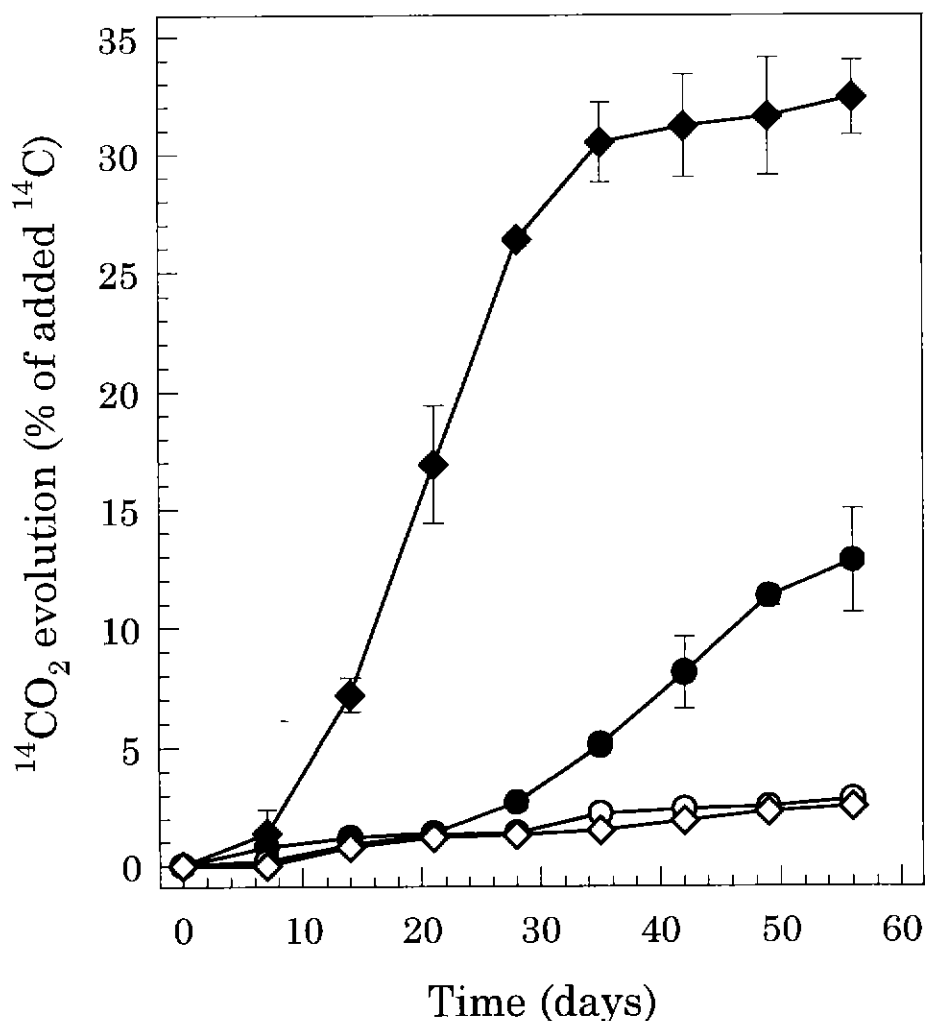


Figure 5.10 Mineralization of [^{14}C]benzo[*a*]pyrene in BSM containing pyrene (250 mg l^{-1}) and benzo[*a*]pyrene (50 mg l^{-1}). $^{14}\text{CO}_2$ data are the cumulative amount recovered from medium incubated with either bacterial consortium VUN 10,009 (●), *S. maltophilia* VUN 10,010 (◆), or the respective HgCl_2 -killed cell controls (○, ◇). The initial cell populations were approximately $10^3 \text{ cells ml}^{-1}$ (or $10^6 \text{ cells ml}^{-1}$ in killed cell controls). All incubations were performed at $30^\circ\text{C}/175 \text{ rpm}$ in the dark.

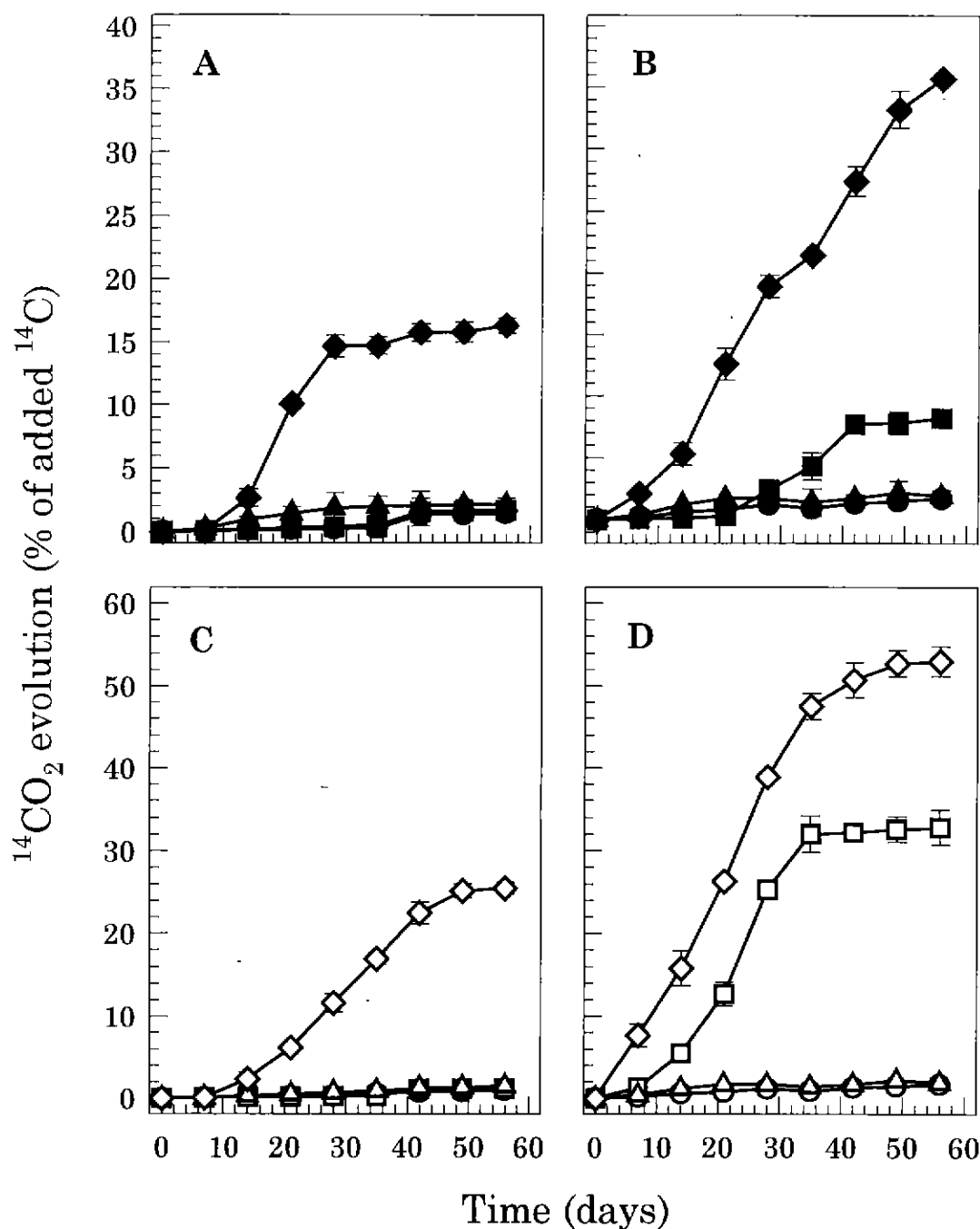


Figure 5.11 Mineralization of [^{14}C]benzo[a]pyrene in BSM. Radiolabeled benzo[a]pyrene was added to BSM containing only benzo[a]pyrene (A and C) and to BSM containing a PAH mixture (B and D). The upper panels represent media inoculated with axenic cultures of *P. janthinellum* VUO 10,201 ($\blacktriangle$), bacterial consortium VUN 10,009 ($\blacksquare$) or co-culture A ($\blacklozenge$). The lower panels represent media inoculated with axenic cultures of *P. janthinellum* VUO 10,201 ($\triangle$), *S. maltophilia* VUN 10,010 ($\square$) or co-culture B ($\diamond$). Media inoculated with HgCl_2 -killed cells of either co-culture A ($\bullet$) or co-culture B ($\circ$) are also shown. Incubations were performed at $30^\circ\text{C}/175$ rpm in the dark.

and probably non-polar degradation products (Table 5.5). No $^{14}\text{CO}_2$ was liberated from [^{14}C]benzo[*a*]pyrene when added to axenic *P. janthinellum* VUN 10,201 cultures comprising benzo[*a*]pyrene and pyrene in BSM, benzo[*a*]pyrene in MYPD or benzo[*a*]pyrene in BSMG (Table 5.5). A high amount of the ^{14}C was recovered in the aqueous phase (15-64%), probably as polar products generated from the partial degradation of benzo[*a*]pyrene and in the DCM phase (36-84%) which can be regarded as non-polar products as well as non-degraded benzo[*a*]pyrene.

5.5.2 Benzo[*a*]pyrene Mineralization by Co-cultures

It was shown earlier (Section 5.4) that fungal-bacterial co-cultures were able to significantly degrade and grow on dibenz[*a,h*]anthracene or benzo[*a*]pyrene when provided in BSM as a sole carbon and energy source. In this section, the extent of benzo[*a*]pyrene degradation by these co-cultures was examined by analyzing the distribution of labeled carbon arising from the breakdown of [^{14}C]benzo[*a*]pyrene added to these cultures. The evolution of $^{14}\text{CO}_2$ was monitored in fungal-bacterial co-cultures comprising BSM and benzo[*a*]pyrene as a sole carbon and energy source (Figure 5.11). No significant $^{14}\text{CO}_2$ evolution was detected in the axenic cultures or the killed-cell controls. The evolution of $^{14}\text{CO}_2$ from the co-cultures started after a lag period of approximately seven days. After 56 days, approximately 16.3% and 25.5% of the initial ^{14}C radioactivity was liberated as $^{14}\text{CO}_2$ by co-culture A and co-culture B, respectively (Figure 5.11A and 5.11C, Table 5.6). A ^{14}C balance analysis at the end of experiment identified less than 3% of radiolabeled carbon in the aqueous phase with the majority (approximately 63-73%) being recovered in the DCM extract (Table 5.6). A relatively high amount of ^{14}C was recovered in the co-culture biomass (7-9%) indicating its incorporation into cellular material which is consistent with its ability to grow on benzo[*a*]pyrene as a sole carbon and energy source. Co-culture A and co-culture B also mineralized substantial amounts of [^{14}C]benzo[*a*]pyrene in BSM containing pyrene or a PAH mixture (consisting of three- to five-ring PAHs) with the amount of initial ^{14}C recovered as $^{14}\text{CO}_2$ being 36-37% and 53-58%, respectively, after 56 days of incubation (Figure 5.11B and 5.11D, Table 5.6). Mass balance analysis of

Table 5.6 Distribution of ^{14}C -residues after incubation of [^{14}C]benzo[*a*]pyrene with PAH-degrading organisms.

Microorganism	$^{14}\text{CO}_2$	Biomass	Aqueous phase	DCM phase	% ^{14}C recovery
VUN 10,009 ^a VUN 10,010 ^a VUO 10,201 ^a Co-culture A Co-culture B	BSM containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹)				100.3 100.1 99.1 99.1 99.4
	NA	NA	1.2	99.1	
	NA	NA	1.6	98.5	
	NA	NA	13.7	85.4	
	16.3	7.2	2.2	73.4	
	25.5	9.3	1.7	62.9	
VUO 10,201	BSMG containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹) ^a				97.5
	NA	NA	48.3	49.2	
VUO 10,201	MYPD containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹) ^a				96.9 ^a
	NA	NA	60.7	30.2	
VUN 10,009 ^a VUN 10,010 ^a VUO 10,201 ^a Co-culture A Co-culture B	BSM containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹) and pyrene (250 mg l ⁻¹)				100.1 99.9 98.6 99.3 97.1
	12.8	0.7	2.3	84.3	
	32.4	NA	2.7	64.8	
	NA	NA	14.5	84.1	
	37.2	7.3	1.1	53.7	
	58.1	8.1	NA	30.9	
VUN 10,009 VUN 10,010 VUO 10,201 Co-culture A Co-culture B	BSM containing benzo[<i>a</i>]pyrene (50 mg l ⁻¹) and PAH mixture				99.2 99.4 98.9 99.3 97.9
	8.2	1.2	2.1	87.7	
	32.8	1.7	2.3	62.6	
	NA	NA	13.2	85.7	
	35.7	6.9	1.4	55.3	
	53.0	8.5	0.6	35.8	

^a Data taken from Table 5.5 for comparison.

NA, not appreciable.

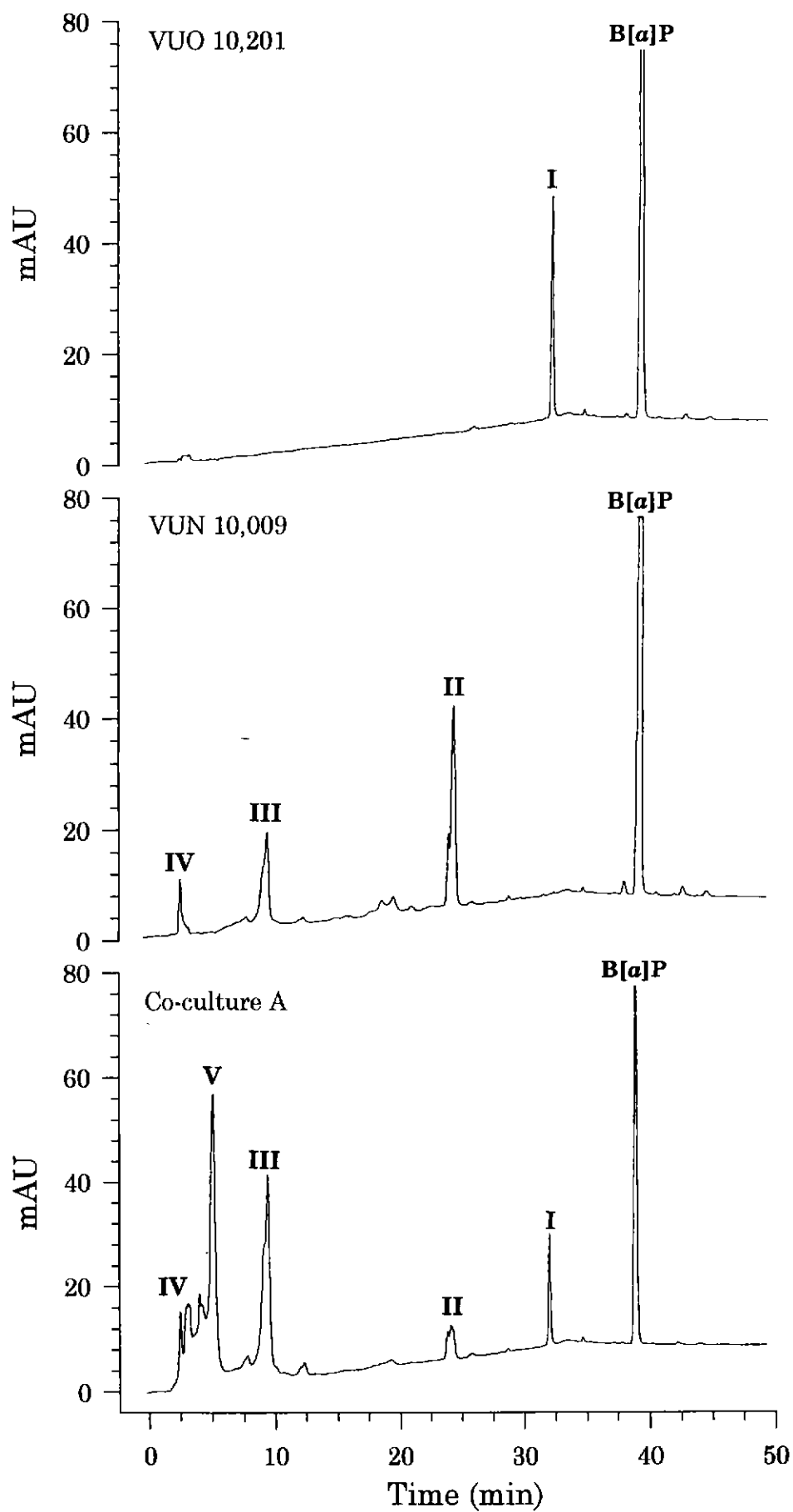
the co-cultures showed that in addition to $^{14}\text{CO}_2$, less than 2% of the radioactivity was detected as polar metabolites in the aqueous phase, and approximately 31-55% of the initial radioactivity was recovered by DCM extraction, most of which was probably non-degraded benzo[a]pyrene (Table 5.6). Approximately 8-13% and 32-33% of ^{14}C was recovered as $^{14}\text{CO}_2$ (via co-metabolism) from the respective axenic bacterial cultures over 56 days. The distributions of ^{14}C from axenic cultures of *P. janthinellum* were unaffected by the BSM carbon source. Benzo[a]pyrene degradation by these fungal cultures in BSM, containing either benzo[a]pyrene and pyrene, benzo[a]pyrene in a PAH mixture or benzo[a]pyrene only, consistently resulted in around 13-15% labeled carbon in the aqueous phase with the remainder being in the DCM extract (Table 5.6).

5.6 ANALYSIS OF PAH DEGRADATION PRODUCTS

The dependency of benzo[a]pyrene mineralization on the cooperative catabolism between *P. janthinellum* and either of the bacterial isolates is an unprecedented result in PAH biodegradation and as a consequence there is scant information on the shared metabolic pathways by which this occurs. A preliminary investigation was undertaken in this section aimed at identifying differences in the HPLC profile of DCM extracts taken from co-cultures A and B and axenic cultures of the complementary microbial strains.

DCM extracts from co-cultures, and axenic bacterial and fungal cultures, comprising BSM with benzo[a]pyrene as a sole carbon and energy source were analyzed for PAH degradation products by HPLC. One degradation compound (nominated compound I) was observed to accumulate in axenic *P. janthinellum* VUO 10,201 cultures over the 56 day incubation (Figures 5.12a and 5.12b). Compound I was not present in axenic cultures of bacterial consortium VUN 10,009, however, three additional degradation compounds (nominated compounds II, III and IV) were detected (Figures 5.12a and 5.12b). Compound II was the first to appear in axenic bacterial consortium cultures and its concentration initially increased but then decreased to a constant level during the remainder of the 56 day incubation; compounds III and IV first appeared in the 28 day sample and

Figure 5.12a Production of unidentified compounds by *P. janthinellum* VUO 10,201, bacterial consortium VUN 10,009 and co-culture A during incubation in BSM containing benzo[*a*]pyrene at 30°C/175 rpm in the dark. HPLC chromatograms of DCM extracts of each respective culture are shown for day 28 of the incubation period. The production of compound (I) is shown for the fungal culture. Three major compounds (II, III and IV) are shown for the bacterial culture. In addition to the compounds (I-IV) observed for axenic cultures of bacteria and the fungus, at least one different product (compound V) was observed in the co-culture. The corresponding peak areas of the individual compounds are shown in Figure 5.12b.



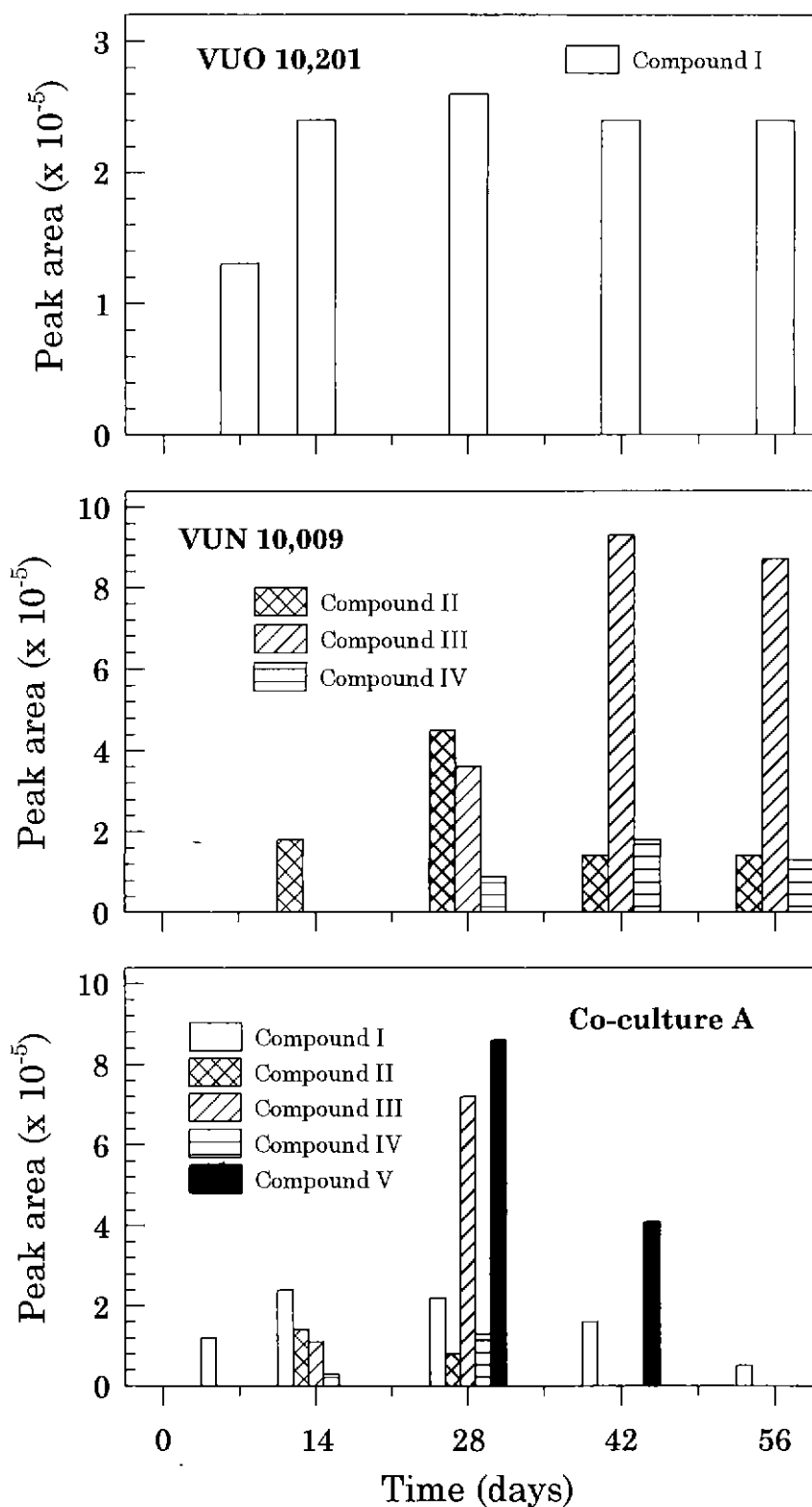


Figure 5.12b Production of compounds formed during benzo[a]pyrene degradation by axenic cultures of *P. janthinellum* VUO 10,201, bacterial consortium VUN 10,009 and co-culture A over a 56 day period. Culture medium was BSM containing benzo[a]pyrene as a sole source of carbon and energy. The data represent the peak areas of degradation compounds as determined by HPLC analysis.

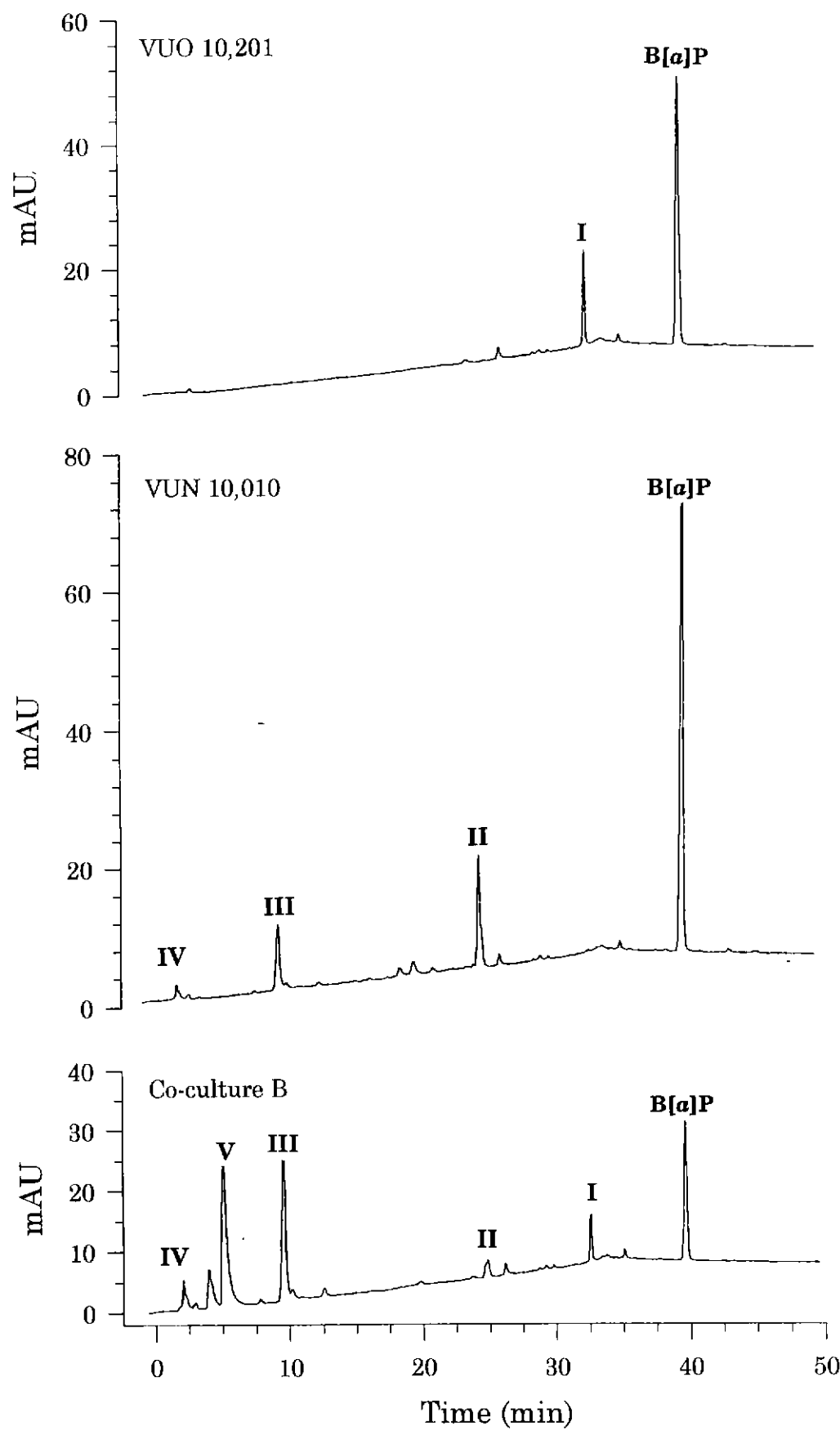
their concentrations initially increased but then were constant during the remainder of the 56 day incubation.

In co-culture A, compound I was the first peak to appear but, unlike in axenic *P. janthinellum* VUO 10,201 cultures, the concentration of this compound decreased in the later stages of the incubation. Compounds II, III and IV were also present in the 14 and 28 day samples of co-culture A, but these compounds were seemingly degraded to other products by the co-culture since they were not detected in the 56 day sample (Figures 5.12a and 5.12b). At least one degradation compound (designated as compound V) appeared in the co-culture which was not detected in the axenic cultures. The concentration of compound V increased up to 28 days, then it decreased and was not detected in the 56 day sample. Similar HPLC profiles were observed for axenic VUN 10,010 cultures and VUN 10,009 cultures (Figures 5.12 and 5.13). Co-culture A and B also had similar (Figures 5.12 and 5.13).

5.7 CATABOLIC RÔLES OF FUNGI AND BACTERIA IN PAH DEGRADATION BY CO-CULTURES

In this section, initial investigations were conducted to identify the role of the the fungus and bacteria in the co-culture degradation of high molecular weight PAHs. Axenic cultures of *S. maltophilia* VUN 10,010 and *P. janthinellum* VUO 10,201 were incubated separately in BSM containing radiolabeled benzo[a]pyrene (1.0 μ Ci) and non-labeled benzo[a]pyrene (50 mg l⁻¹) as a sole carbon and energy source. Figure 5.14 presents the schematic diagram of experimental design used for the sequential degradation of benzo[a]pyrene. After 35 days of incubation, the evolution of ¹⁴CO₂ from these cultures was monitored before the contents of the cultures were extracted with DCM and dried completely under nitrogen. At this stage, no ¹⁴CO₂ had evolved from either of these cultures. HPLC analyses of the DCM extracts revealed all of the peaks observed in Section 5.6, *i.e.* compound I from fungal cultures (Figure 5.15a) as well as compounds II, III and IV from the bacterial cultures (Figure 5.16a) along with the parent compound, benzo[a]-pyrene. The dry extract of each culture was resuspended in DMF and added to

Figure 5.13a Production of unidentified compounds by *P. janthinellum* VUO 10,201, *S. maltophilia* VUN 10,010 and co-culture B during incubation in BSM containing benzo[a]pyrene at 30°C/175 rpm in the dark. HPLC chromatograms of DCM extracts of each respective culture are shown for day 28 of the incubation period. The production of compound (I) is shown for the fungal culture. Three major compounds (II, III and IV) are shown for the bacterial culture. In addition to the compounds (I-IV) observed for axenic cultures of bacteria and the fungus, at least one different product (compound V) was observed in the co-culture. The corresponding peak areas of the individual compounds are shown in Figure 5.13b.



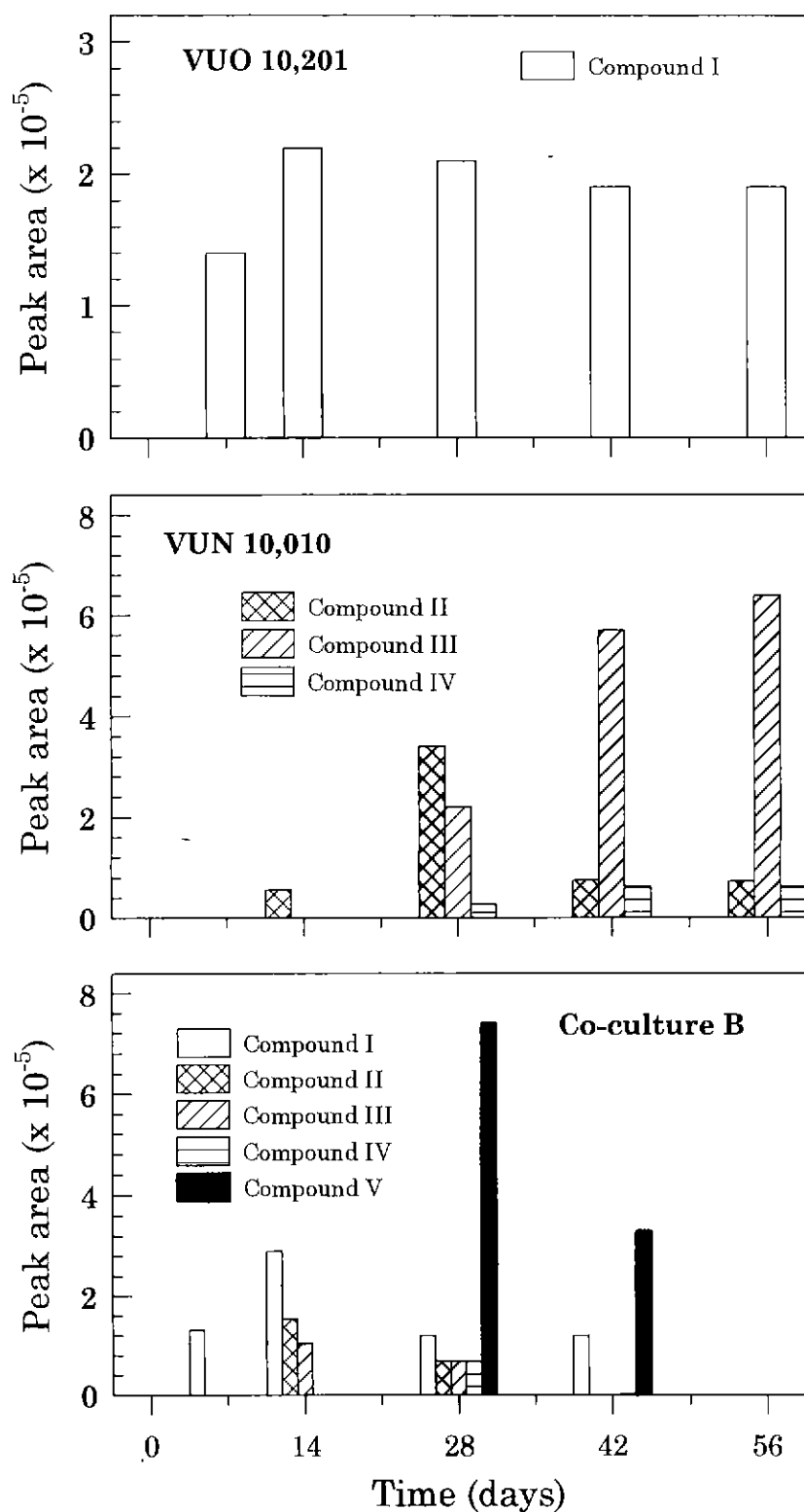


Figure 5.13b Production of compounds formed during benzo[a]pyrene degradation by axenic cultures of *P. janthinellum* VUO 10,201, *S. maltophilia* VUN 10,010 and co-culture B over a 56 day period. Culture medium was BSM containing benzo[a]pyrene as a sole source of carbon and energy. The data represent the peak areas of degradation compounds as determined by HPLC analysis.

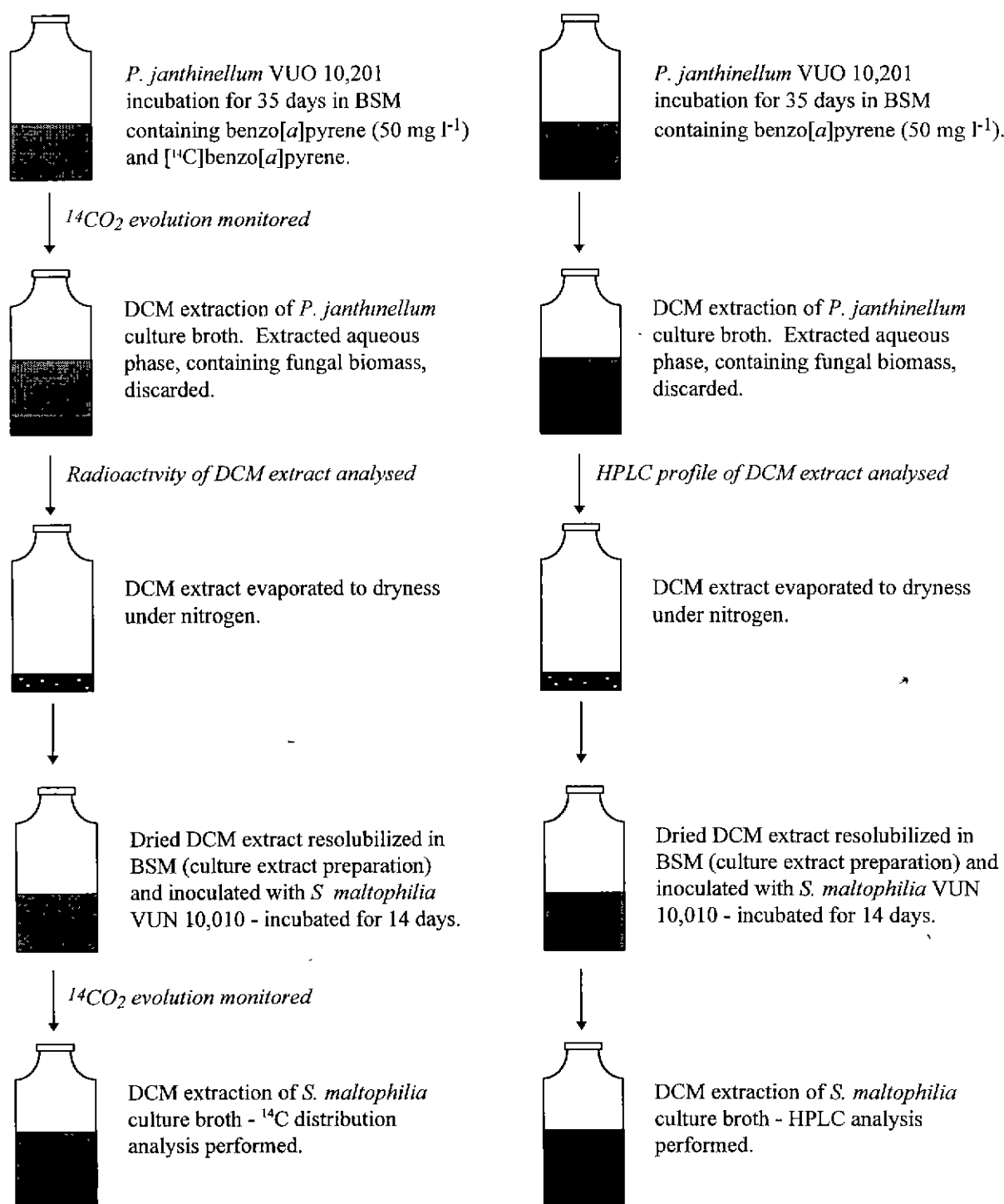


Figure 5.14 Schematic diagram of the protocol used for the sequential degradation of benzo[a]pyrene. The first incubation used *P. janthinellum* VUO 10,201 and the resulting culture extract preparation was subsequently inoculated with *S. maltophilia* VUN 10,010. Parallel incubations were conducted to measure ¹⁴C distribution from [¹⁴C]benzo[a]pyrene degradation (left hand sequence) and benzo[a]pyrene concentration (right hand sequence). The same protocol was used for another experiment in which the first incubation was conducted with *S. maltophilia* VUN 10,010 and the subsequent incubation used *P. janthinellum* VUO 10,201.

fresh BSM, and then inoculated with the organism opposite to that with which it was originally incubated (Figure 5.14), e.g. the preparation of bacterial extract was inoculated with *P. janthinellum* VUO 10,201 culture and the preparation of fungal extract was inoculated with *S. maltophilia* VUN 10,010 culture. After 14 days, the distribution of radiolabeled carbon was examined for $^{14}\text{CO}_2$ evolution, ^{14}C incorporation into cellular biomass, polar metabolites in the aqueous phase, and non-polar products and non-degraded benzo[a]pyrene in DCM phase (Table 5.7).

5.7.1 Fungal Culture Extract Preparations Inoculated with *S. maltophilia* VUN 10,010

At the time of inoculation with *S. maltophilia*, the fungal culture extract preparations contained one peak, in addition to the benzo[a]pyrene peak, when analyzed by the HPLC (Figure 5.15a); this peak has previously been observed and designated as compound I (Figure 5.13a). After 14 days of incubation with a high cell density of *S. maltophilia* VUN 10,010 culture (ca. 8×10^6 cells ml^{-1}), compound I was no longer detectable in the medium (Figure 5.15b), but a different peak was detectable which, based on the retention times, appeared to be compound II as identified by HPLC analysis of the axenic *S. maltophilia* cultures in Figure 5.13a. Furthermore, a significant amount of $^{14}\text{CO}_2$ evolved from this culture during the 14 day incubation period with *S. maltophilia* VUN 10,010 and a ^{14}C mass balance analysis identified a low, but significant, amount of radioactivity in the bacterial biomass (Table 5.7).

Compound I was not present in the extract preparations prepared from BSM-benzo[a]pyrene cultures incubated with killed (autoclaved) *P. janthinellum* VUO 10,201; only the benzo[a]pyrene peak was detectable (Figure 5.15a). When these preparations were inoculated with *S. maltophilia* VUN 10,010, one small peak other than benzo[a]pyrene was detected by the HPLC (Figure 5.15b) and, based on the retention times, this appeared to be compound II from the axenic bacterial culture HPLC chromatograms in Figure 5.13a. More importantly, this culture did

Figure 5.15a HPLC profiles of DCM extracts prepared from *P. janthinellum* VUO 10,201 cultures after 35 days incubation in BSM containing benzo[*a*]pyrene (50 mg l⁻¹). An extract from benzo[*a*]pyrene incubated with killed (autoclaved) cells of *P. janthinellum* VUO 10,201 is also shown. All incubations were performed at 30°C/175 rpm in the dark for 35 days before culture extract preparation (see Chapter 3, Section 3.2.5.8 for methods). Two VUO 10,201 culture extracts (#1 and #2) were prepared from duplicate cultures which were subsequently amended with different inocula (see Figure 5.15b).

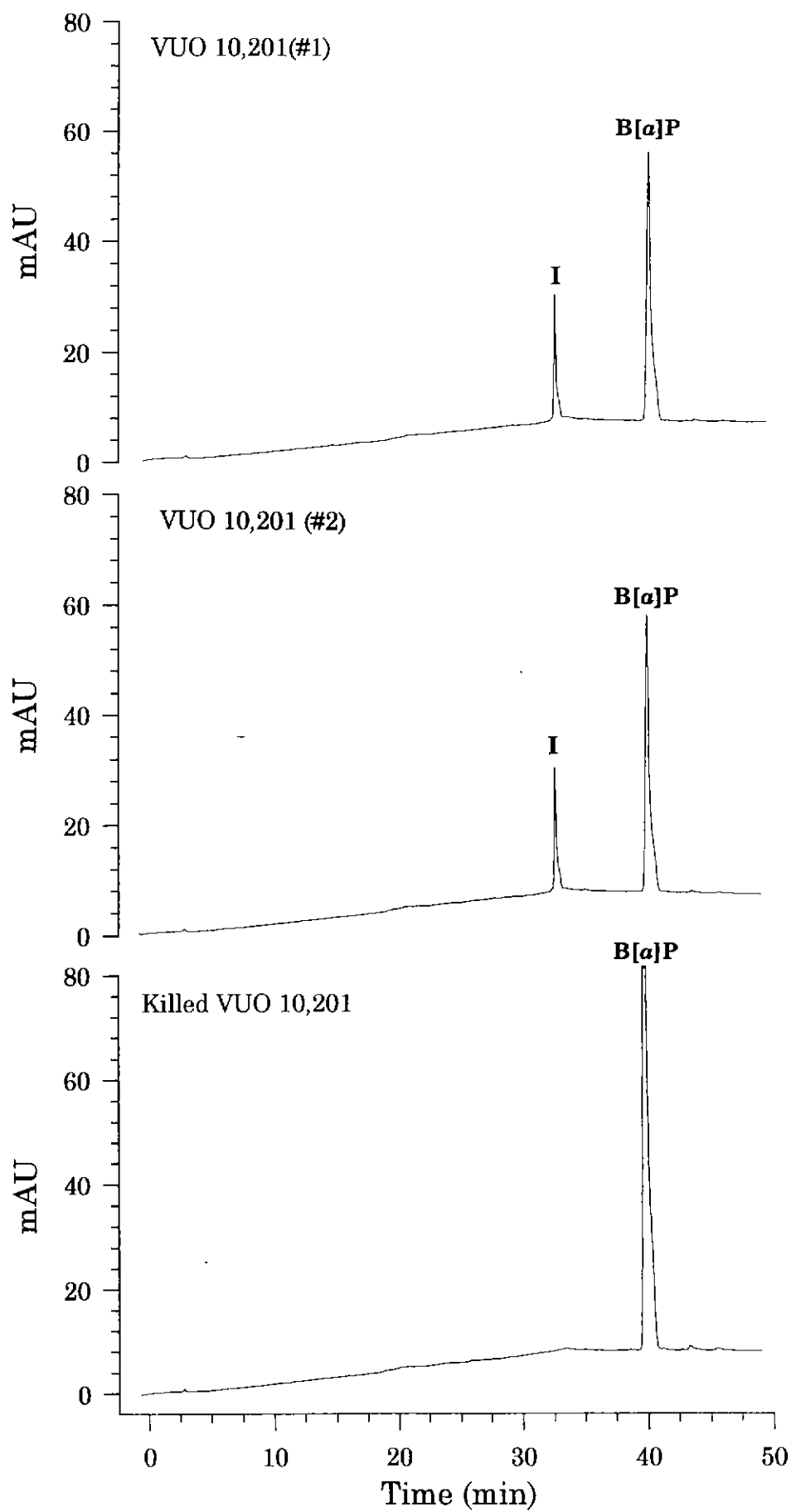


Figure 5.15b HPLC profiles of extract preparation from *P. janthinellum* VUO 10,201 culture, as shown in Figure 5.15a, inoculated with *S. maltophilia* VUN 10,010 or killed (autoclaved) *S. maltophilia* VUN 10,010 cells; the HPLC analysis was performed after 14 days incubation. Incubations were performed at 30°C/175 rpm in the dark (see Chapter 3, Section 3.2.5.8 for methods).

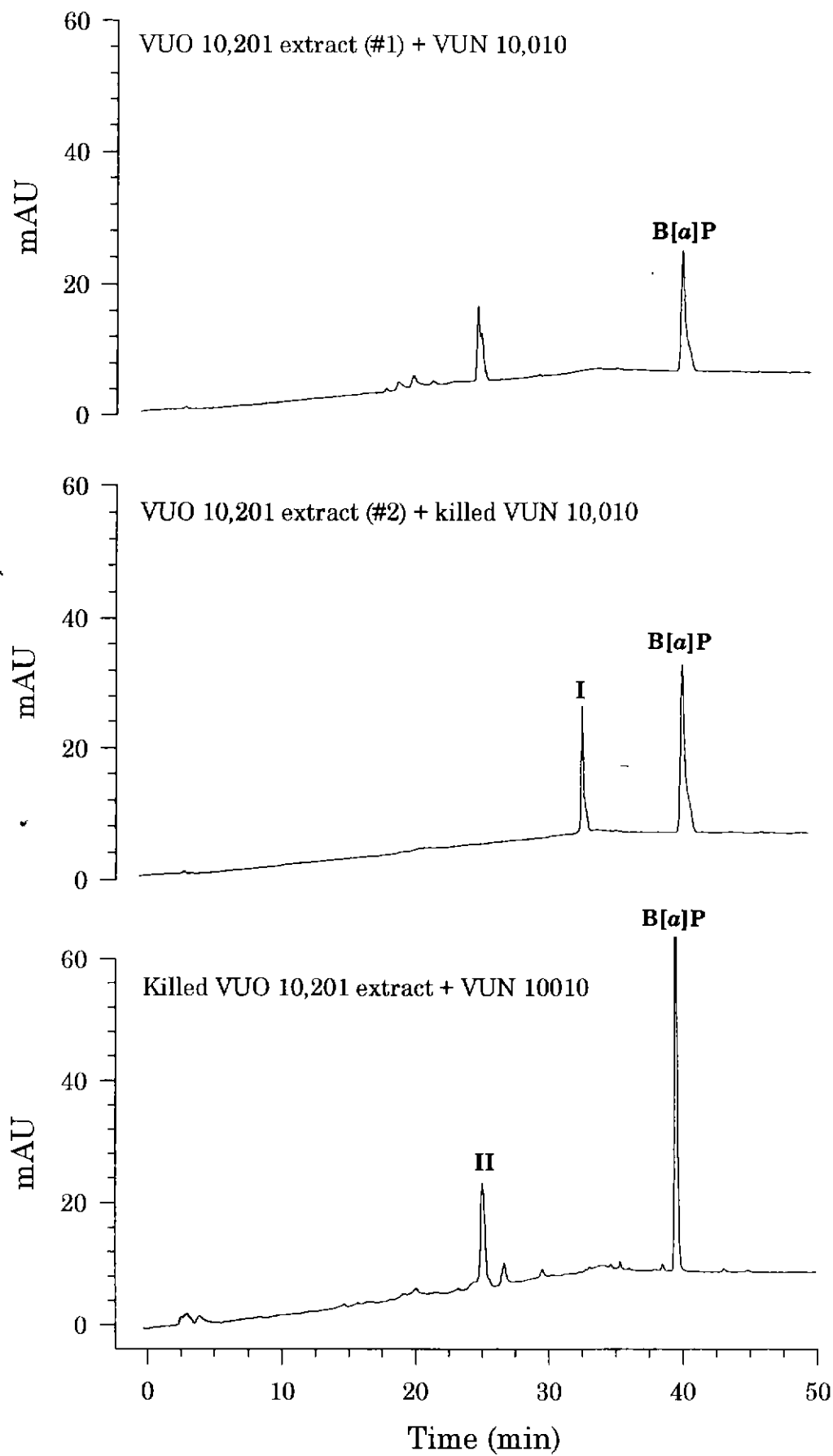


Table 5.7 Fate of [¹⁴C]benzo[*a*]pyrene in axenic bacterial and fungal cultures.

Culture	% Radioactivity recovered ^c from:				% ¹⁴ C recovery
	¹⁴ CO ₂	Biomass	Aqueous phase	DCM phase	
Preparation of <i>P. janthinellum</i> VUO 10,201 extract ^a inoculated with <i>S. maltophilia</i> VUN 10,010 culture	9.82	3.77	1.69	83.22	98.48
Preparation of killed <i>P. janthinellum</i> VUO 10,201 extract ^b inoculated with <i>S. maltophilia</i> VUN 10,010 culture	0.97	NA	2.27	95.21	98.50
Preparation of <i>P. janthinellum</i> VUO 10,201 extract ^a inoculated with killed <i>S. maltophilia</i> VUN 10,010 culture	NA	NA	8.62	91.17	99.79
Preparation of <i>S. maltophilia</i> VUN 10,010 extract ^a inoculated with <i>P. janthinellum</i> VUO 10,201 culture	2.16	2.03	7.81	85.20	97.20
Preparation of killed <i>S. maltophilia</i> VUN 10,010 extract ^b inoculated with <i>P. janthinellum</i> VUO 10,201 culture	0.12	0.61	9.17	88.74	98.64
Preparation of <i>S. maltophilia</i> VUN 10,010 extract ^a inoculated with killed <i>P. janthinellum</i> VUO 10,201 culture	1.1	NA	3.31	93.30	97.70

^a Extracts prepared from cultures of either *S. maltophilia* VUN 10,010 or *P. janthinellum* VUO 10,201 incubated with [¹⁴C]benzo[*a*]pyrene (1.0 µCi) and non-labeled benzo[*a*]pyrene (50 mg l⁻¹) in BSM for 35 days at 30°C/175 rpm in the dark. A high cell density of bacteria (8 x 10⁶ cells ml⁻¹) and fungus (0.04 g [wet wt.] ml⁻¹) was used as an initial inoculum for a respective culture.

^b Extracts prepared from killed (autoclaved) cultures of either *S. maltophilia* VUN 10,010 or *P. janthinellum* VUO 10,201 incubated with [¹⁴C]benzo[*a*]pyrene (1.0 µCi) and non-labeled benzo[*a*]pyrene (50 mg l⁻¹) for 35 days at 30°C/175 rpm in the dark. A high cell density of autoclaved bacteria (8 x 10⁶ cells ml⁻¹) or autoclaved fungus (0.04 g [wet wt.] ml⁻¹) was used as an initial inoculum for a respective culture.

^c Mass balance analysis was performed after 14 days of re-inoculating the respective extract in BSM with an opposite inoculum and incubated at 30°C/175 rpm in the dark.

NA, not appreciable.

not produce significant amounts of $^{14}\text{CO}_2$ during the 14 day incubation period (Table 5.7). When killed *S. maltophilia* VUN 10,010 cells were inoculated into *P. janthinellum* VUO 10,201 extract preparations, only compound I (which was present at the time of inoculation) was detected after 14 days (Figure 5.15b). There was no $^{14}\text{CO}_2$ evolution from this culture and most of the radioactivity was found in the DCM phase (Table 5.8) with a small portion in the aqueous phase (8.62%).

5.7.2 *S. maltophilia* Culture Extract Preparations Inoculated with *P. janthinellum* VUO 10,201

At the time of inoculation with *P. janthinellum* VUO 10,201, the *S. maltophilia* VUN 10,010 culture extract preparations contained three peaks, in addition to the benzo[a]pyrene peak, when analyzed by the HPLC (Figure 5.16a); these peaks had previously been observed in axenic bacterial cultures and designated as compounds II, III and IV (Figure 5.13a). After 14 days of incubation with a high biomass concentration of *P. janthinellum* VUO 10,201 (ca. 0.04 g [wet wt] ml⁻¹), compounds II, III and IV were no longer detectable in the medium (Figure 5.15b), but a different peak was detectable which, based on the retention times, appeared to be compound I identified by HPLC analysis of the axenic *P. janthinellum* VUO 10,201 cultures in Figure 5.13a. A small amount of $^{14}\text{CO}_2$ evolved from this culture during the 14 day incubation period and a ^{14}C mass balance analysis identified a low amount of radioactivity in the fungal biomass (Table 5.7).

Compounds II, III and IV were not present in the extracts prepared from BSM-benzo[a]pyrene cultures incubated with killed (autoclaved) *S. maltophilia* VUN 10,010 (Figure 5.16a); only the benzo[a]pyrene peak was detectable. When these preparations were inoculated with *P. janthinellum* VUO 10,201, one small peak other than benzo[a]pyrene was detected by HPLC (Figure 5.16b) after 14 days and, based on the retention times, this appeared to be compound I from the axenic fungal culture HPLC chromatograms in Figure 5.13a. More importantly, this culture did not produce significant amounts of $^{14}\text{CO}_2$ during the 14 day incubation

Figure 5.16a HPLC profiles of DCM extracts prepared from *S. maltophilia* VUN 10,010 cultures in BSM containing benzo[a]pyrene (50 mg l⁻¹) after 35 days incubation. An extract from benzo[a]pyrene incubated with killed (autoclaved) cells of *S. maltophilia* VUN 10,010 is also shown. All incubations were performed at 30°C/175 rpm in the dark for 35 days before culture extract preparation (see Chapter 3, Section 3.2.5.8 for methods). Two VUN 10,010 culture extracts (#1 and #2) were prepared from duplicate cultures which were subsequently amended with different inocula (see Figure 5.16b).

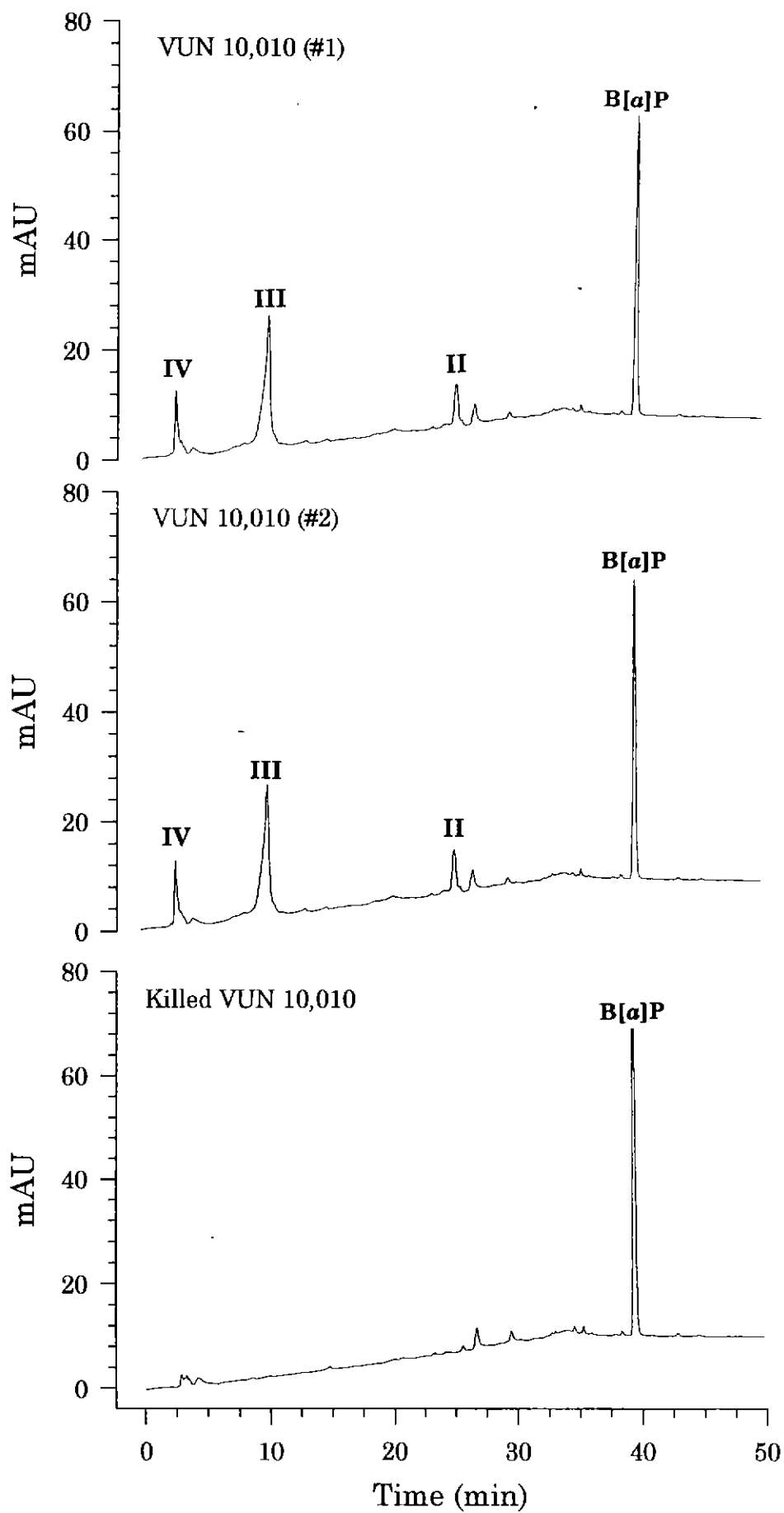
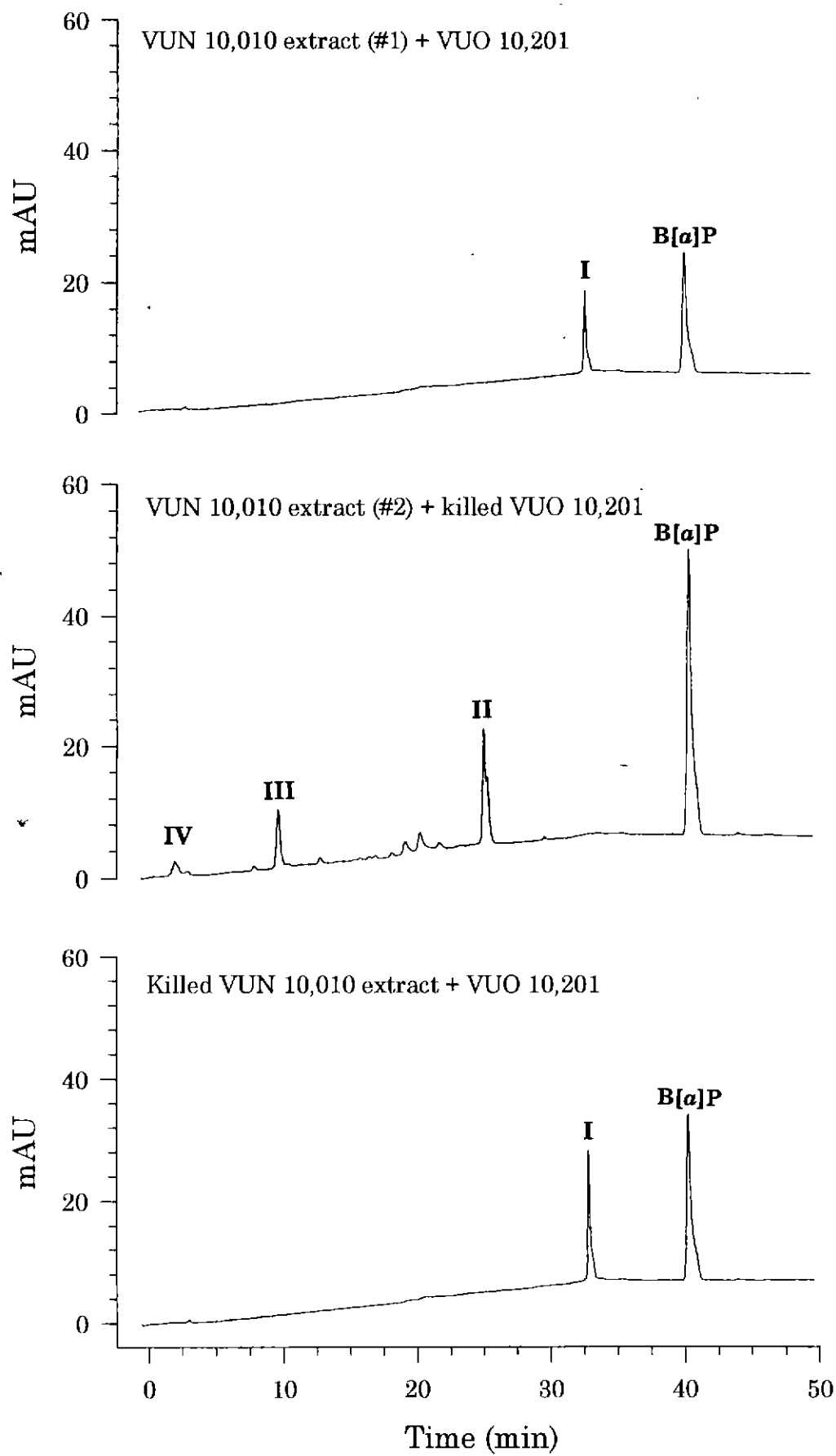


Figure 5.16b HPLC profiles of extract preparation from *S. maltophilia* VUN 10,010 culture, as shown in Figure 5.16a, inoculated with *P. janthinellum* VUO 10,201 or killed (autoclaved) *P. janthinellum* VUO 10,201 cells; the HPLC analysis was conducted after 14 days incubation. Incubations were performed at 30°C/175 rpm in the dark (see Chapter 3, Section 3.2.5.8 for methods).



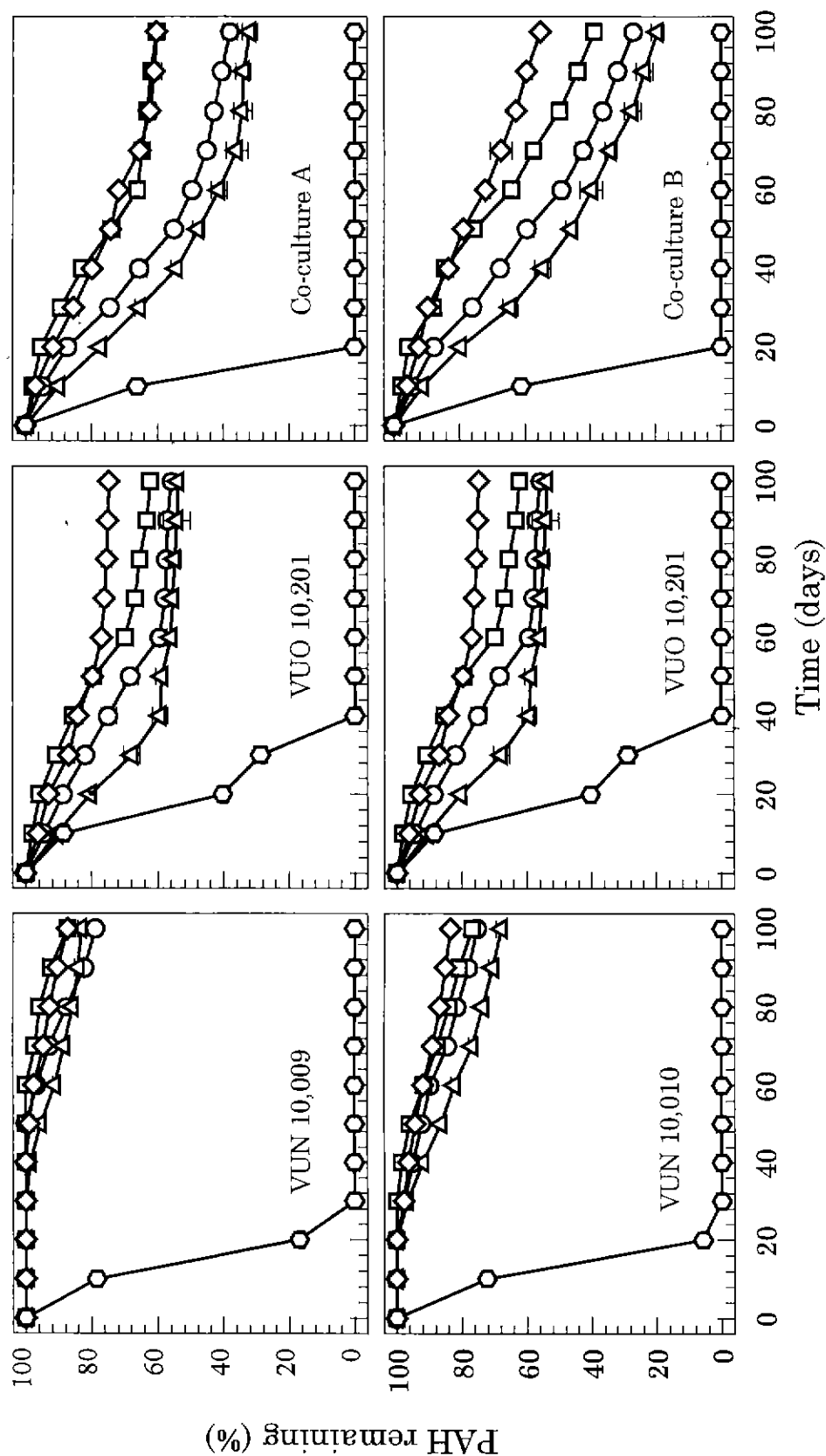


FIGURE 5.17 PAH concentrations in PAH-spiked soil containing a mixture of PAHs. PAHs represented are pyrene (○), chrysene (◻), benz[a]anthracene (Δ), benz[a,h]anthracene (◊). High initial cell populations of axenic cultures of bacteria ($\approx 10^6$ cells g^{-1} soil) or the fungus (25 mg [mycelial wet wt.] g^{-1} soil) or the co-cultures comprising bacteria and the fungus (10^6 cells g^{-1} soil, 25 mg [mycelial wet wt.] g^{-1} soil) were used to inoculated the soil. All incubations were performed at 25°C in the dark. Concentrations of PAHs represent the amounts recovered from inoculated soil relative to the amounts of such PAHs recovered from soil containing killed inocula.

period (Table 5.7). When killed *P. janthinellum* VUO 10,201 biomass was inoculated into *P. janthinellum* VUO 10,201 extract preparations, only compounds II, III and IV (which were present at the time of inoculation) were detected after 14 days (Figure 5.16b). There was no $^{14}\text{CO}_2$ evolution from this culture and most of the radioactivity was found in the DCM phase (Table 5.7) with a small portion in the aqueous phase (3.31%).

5.8 HIGHT MOLECULAR WEIGHT PAH DEGRADATION IN SOILS

In Sections 5.4 and 5.5, fungal-bacterial co-cultures were shown to have considerable potential for the bioremediation of high molecular weight PAHs due to their unprecedented ability to degrade and grow on high molecular weight PAHs, some of which were previously recognized as recalcitrant, and to mineralize benzo[a]pyrene as a sole carbon and energy source in BSM. In practice, however, such organisms will be expected to mineralize these PAHs in foreign, contaminated soils. This section investigates the degradation and mineralization of PAHs in soils inoculated with fungal-bacterial co-cultures.

5.8.1 PAH-spiked Soil

PAH degradation by co-cultures and axenic cultures of strains VUO 10,201, VUN 10,010 and the bacterial consortium VUN 10,009 was initially tested in soil which had no history of PAH contamination. Chemical analyses of this soil revealed that PAHs were present. The soil contained substantial amounts of trace elements and small quantities of some heavy metals (see Chapter 3, Table 3.1). This soil was spiked with a PAH mixture consisting of three- to seven-benzene ring compounds (designated PAH-spiked soil).

The fate of five PAH compounds (pyrene, chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene) in the PAH-spiked soil was monitored over 100 days (Figure 5.17). Pyrene was rapidly degraded in soil inoculated with *S. maltophilia* VUN 10,010 or the bacterial consortium with all the pyrene (250

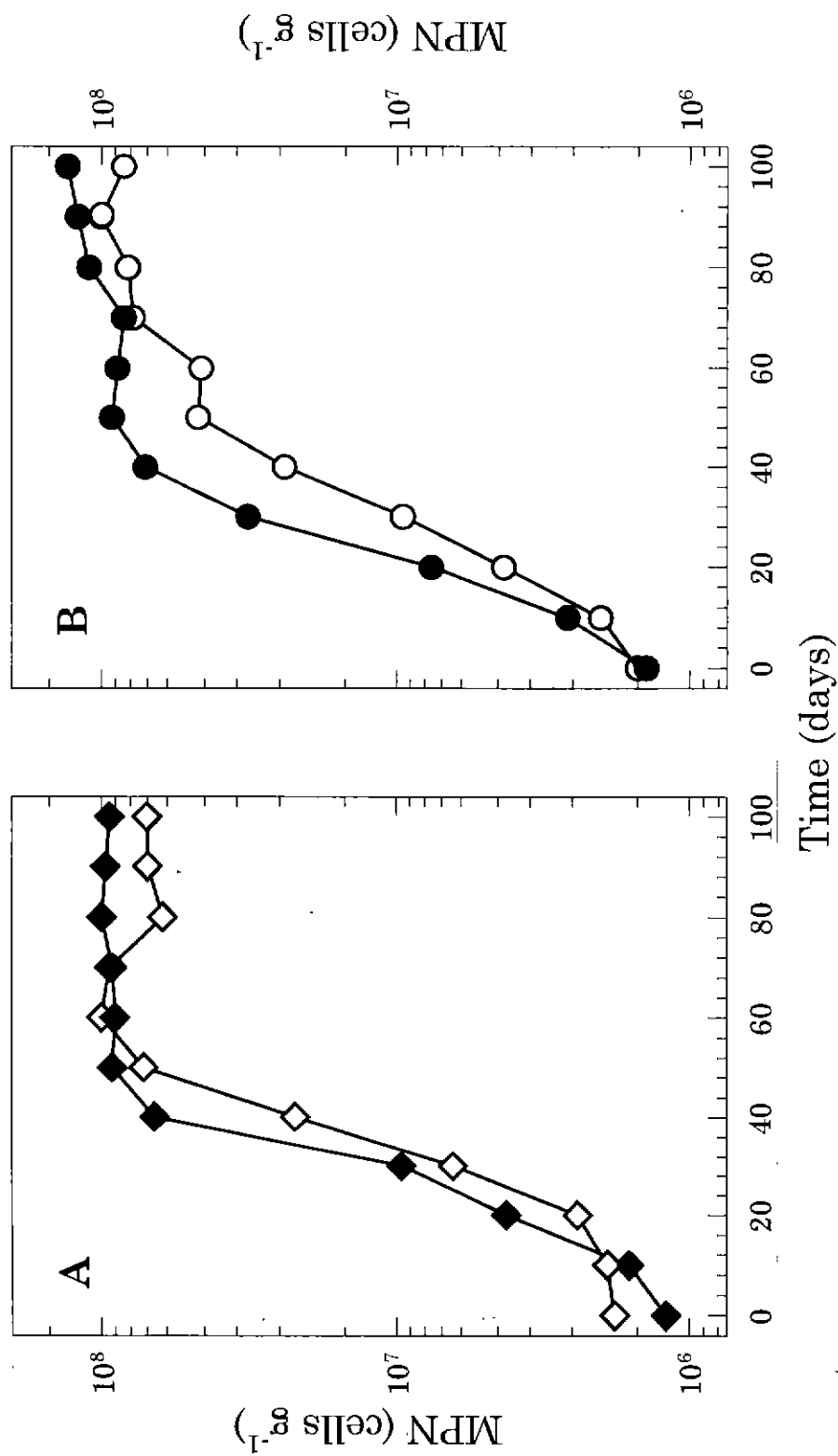


Figure 5.18 Bacterial growth in PAH-spiked soil. PAH-spiked soil inoculated with bacterial consortium VUN 10,009 (○) or co-culture A (●) (A) and PAH-spiked soil inoculated with *S. maltophilia* VUN 10,010 (○) or co-culture B (●) (B). PAH degradation in these soil cultures is shown in Figure 5.17. Incubations were performed at 25°C in the dark.

mg kg⁻¹ soil) being degraded in around 16-18 days. The degradation rate of chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene was slow in soil amended with only the bacterial consortium or VUN 10,010. The amount of these PAHs degraded over 100 days was in the range of 16-32% (VUN 10,010) and 12-22% (VUN 10,009) and long degradation lag periods of around 30 days (VUN 10,010) and 40-60 days (VUN 10,009) were observed. On the other hand, there was no detectable degradation lag period in soils inoculated with *P. janthinellum* VUO 10,201 and the PAH degradation rate was relatively high in the first 30-40 days compared to soils inoculated with only the bacteria. The amount of chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene degraded by VUO 10,201 after 100 days was in the range 24-48%, however, the degradation of each PAH all but ceased after 60 days.

There was a substantial improvement in PAH degradation when the PAH-spiked soil was inoculated with either co-culture A or co-culture B. The amount of chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene degraded after 100 days was in the range 40-68% (co-culture A) and 44-80% (co-culture B). Furthermore, soils inoculated with the co-cultures did not have any apparent degradation lag period and PAH degradation occurred during the entire incubation period. Bacterial populations showed similar growth profiles (bacterial populations increased 100 fold) in soils amended with either axenic bacterial inocula or co-cultures A and B (Figure 5.18). Although fungal growth was not determined in the soil cultures, viable fungal biomass was still present in the soil after 100 days for both the axenic cultures and the co-cultures.

5.8.2 Benzo[a]pyrene Mineralization

Benzo[a]pyrene mineralization by co-cultures A and B was tested in PAH-spiked soil and PAH-contaminated soil obtained from a petroleum-polluted site (designated PAH-contaminated soil). For the PAH-spiked soil, no ¹⁴CO₂ evolved during the first 14 days of incubation, but over the next 86 days 38% and 45% of the initial radioactivity was recovered as ¹⁴CO₂ from soil inoculated with co-cultures A and B, respectively (Figures 5.19A and 5.19C, Table 5.8).

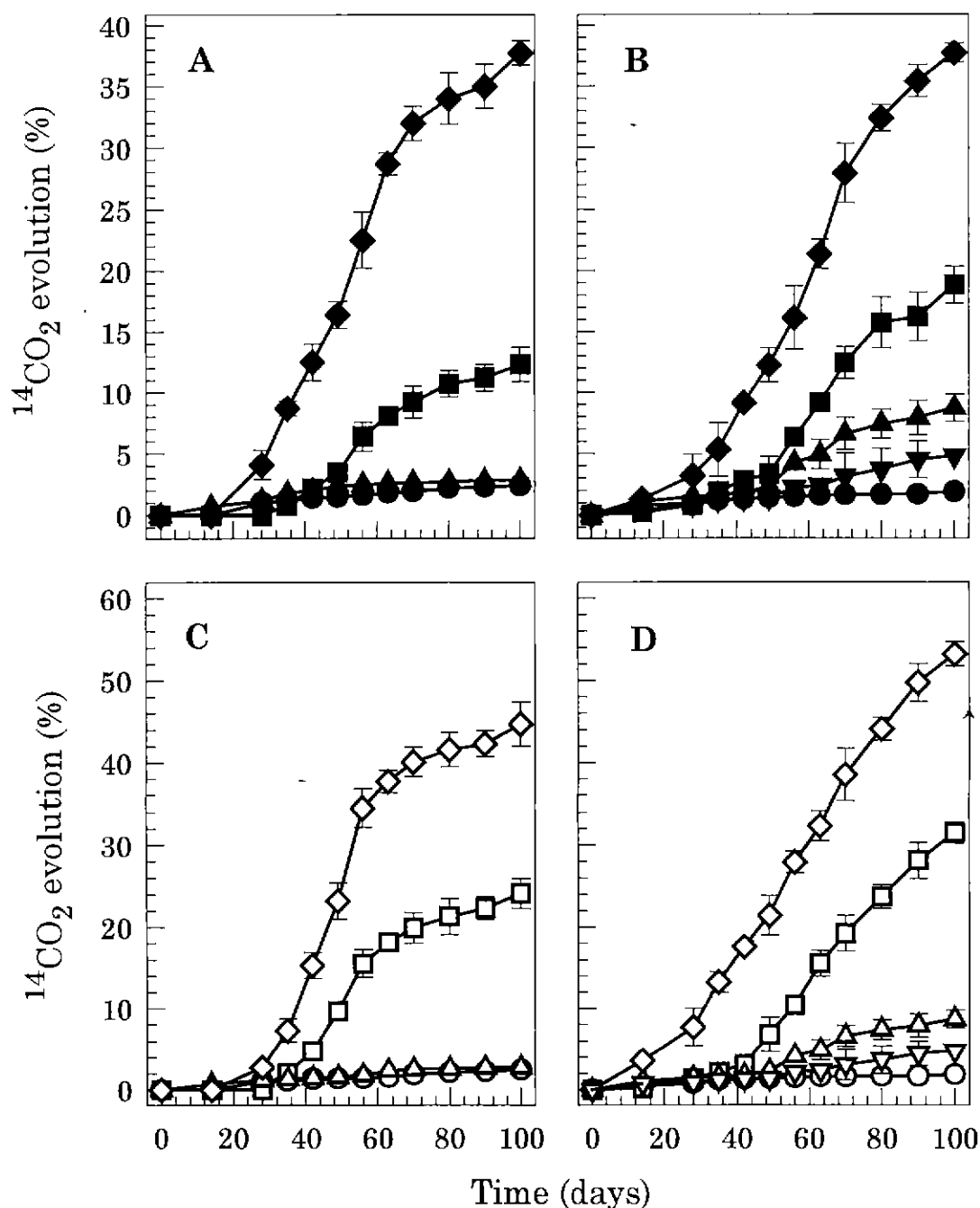


Figure 5.19 Mineralization of $[^{14}\text{C}]$ benzo[a]pyrene in soils. Labeled benzo[a]pyrene was added to PAH-spiked soil (A and C) and PAH-contaminated soil (B and D). The upper panels represent soils inoculated with axenic cultures of *P. janthinellum* VUO 10,201 (▲), bacterial consortium VUN 10,009 (■) or co-culture A (◆). The lower panels represent soils inoculated with axenic cultures of *P. janthinellum* VUO 10,201 (Δ), *S. maltophilia* VUN 10,010 (□) or co-culture B (◇). Soils inoculated with HgCl₂-killed cells of either co-culture A (●) or co-culture B (○) are also shown. $^{14}\text{CO}_2$ evolution due to indigenous microbial activities in uninoculated PAH-contaminated soil (▼, ▽) is also shown. The incubations were performed at 25°C in the dark.

Mineralization of benzo[*a*]pyrene by the co-cultures was superior; *i.e.* greater than the sum of the two alone, to that observed in soil amended with axenic bacterial inocula in which the amount of $^{14}\text{CO}_2$ that evolved was approximately 12% (VUN 10,009) and 24% (VUN 10,010); no $^{14}\text{CO}_2$ evolution was recorded for soils amended only with *P. janthinellum* VUO 10,201 or for uninoculated soils.

The PAH-contaminated soil used in this study contained high concentrations of petroleum hydrocarbons, especially long chain hydrocarbons (see Chapter 3, Table 3.2). The PAH composition of the PAH-contaminated soil varied from low molecular weight (two- and three-benzene rings) to high molecular weight (four- and five-benzene rings) compounds. In addition, this soil was also contaminated with a variety of heavy metals such as zinc, lead and copper. All of these parameters could adversely affect, or destabilize, the introduced inocula. However, similar results to those observed with the PAH-spiked soil were obtained for the PAH-contaminated soil: benzo[*a*]pyrene mineralization by the co-cultures was apparently unaffected by the potentially adverse soil environment (Figures 5.19B and 5.19D). Co-culture B mineralized benzo[*a*]pyrene to a greater extent (53% of initial ^{14}C recovered as $^{14}\text{CO}_2$ after 100 days), and without an apparent lag period, compared to its performance in the PAH-spiked soil. This may be due to the indigenous microflora cooperatively mineralizing benzo[*a*]pyrene with the co-cultures. Evidence of this is seen with the soil amended with axenic *P. janthinellum* VUN 10,201 inocula in which 8.7% of the initial radioactivity was recovered as $^{14}\text{CO}_2$; this did not occur in PAH-spiked soil in which there was no measurable indigenous microbial population activity. The indigenous microbial population alone mineralized only 5% of the added [^{14}C]benzo[*a*]pyrene to $^{14}\text{CO}_2$ over 100 days. Introduction of allochthonous microorganisms to PAH-contaminated soil significantly accelerated the natural attenuation of [^{14}C]benzo[*a*]pyrene mineralization by the indigenous community. The balance of ^{14}C added to the soils which was not recovered as $^{14}\text{CO}_2$ was found in the DCM extract (Table 5.8).

Table 5.8 Distribution of ^{14}C -residues after incubation of [^{14}C]benzo[a]pyrene with PAH-degrading organisms.

Microorganism	$^{14}\text{CO}_2$	Biomass	Aqueous phase	DCM phase	% ^{14}C recovery
BSM containing benzo[a]pyrene (50 mg l $^{-1}$) ^a					
VUN 10,009	NA	NA	1.2	99.1	100.3
VUN 10,010	NA	NA	1.6	98.5	100.1
VUO 10,201	NA	NA	13.7	85.4	99.1
Co-culture A	16.3	7.2	2.2	73.4	99.1
Co-culture B	25.5	9.3	1.7	62.9	99.4
BSMG containing benzo[a]pyrene (50 mg l $^{-1}$) ^a					
VUO 10,201	NA	NA	48.3	49.2	97.5
MYPD containing benzo[a]pyrene (50 mg l $^{-1}$) ^a					
VUO 10,201	NA	NA	60.7	30.2	96.9
BSM containing benzo[a]pyrene (50 mg l $^{-1}$) and pyrene (250 mg l $^{-1}$) ^a					
VUN 10,009	12.8	0.7	2.3	84.3	100.1
VUN 10,010	32.4	NA	2.7	64.8	99.9 [*]
VUO 10,201	NA	NA	14.5	84.1	98.6
Co-culture A	37.2	7.3	1.1	53.7	99.3
Co-culture B	58.1	8.1	NA	30.9	97.1
BSM containing benzo[a]pyrene (50 mg l $^{-1}$) and PAH mixture ^a					
VUN 10,009	8.2	1.2	2.1	87.7	99.2
VUN 10,010	32.8	1.7	2.3	62.6	99.4
VUO 10,201	NA	NA	13.2	85.7	98.9
Co-culture A	35.7	6.9	1.4	55.3	99.3
Co-culture B	53.0	8.5	0.6	35.8	97.9
PAH-spiked soil					
VUN 10,009	12.3	ND	ND	84.8	97.1
VUN 10,010	24.1	ND	ND	73.9	98.0
VUO 10,201	NA	ND	ND	97.7	97.7
Co-culture A	37.7	ND	ND	61.4	99.1
Co-culture B	44.7	ND	ND	53.6	98.3
PAH-contaminated soil					
Indigenous	4.8	ND	ND	93.1	97.9
VUN 10,009	18.8	ND	ND	80.6	99.4
VUN 10,010	31.5	ND	ND	67.3	98.8
VUO 10,201	8.7	ND	ND	89.1	97.8
Co-culture A	37.7	ND	ND	60.8	98.5
Co-culture B	53.2	ND	ND	44.2	97.4

^a Data taken from Table 5.6 for comparison.

NA, not appreciable.

ND, not determined.

5.8 MUTAGENICITY ASSESSMENT

The microbial degradation of PAHs can result in either a reduction or an increase in the mutagenic potential of a PAH-contaminated medium, depending upon the extent of PAH degradation, the catabolic pathways of the PAH-degraders and mutagenicity of the degradation products. In order to determine whether PAH degradation by the microbial isolates reduces the mutagenic potential associated with the parent PAHs, the mutagenicity of DCM extracts from BSM containing added PAHs, PAH-spiked soil and PAH-contaminated soil was assessed following incubation with either a co-culture or axenic bacterial and fungal cultures. The DCM extracts of the liquid culture medium and soils were tested for mutagenic activity using *S. typhimurium* TA 100 with the S9 metabolic system. This bacterial strain is one of the recommended tester strains for the Ames test (Maron and Ames, 1989). Results of the Ames assay were used to calculate a mutagenic ratio, *i.e.* the number of mutant colonies growing in the presence of a test sample compared to the number of spontaneous mutant colonies growing in the absence of a test sample. When spontaneous reversion rates (without test sample) were within the acceptable range proposed by DeSerres and Shelby (1979), a positive mutagenic response was defined as a mutagenic ratio (MR) greater than or equal to 2.5, where:

$$\text{MR} = \frac{\text{Number of colonies with sample}}{\text{Number of colonies without sample}}$$

A test compound was considered to have a slight mutagenic response if the MR was greater than 2.0 but less than 2.5.

Most of the axenic cultures significantly reduced the mutagenic potential of BSM containing either chrysene, benz[a]anthracene, benzo[a]pyrene or dibenz[a,h]-anthracene as the sole carbon and energy source, however, in many cases these liquid cultures were still highly mutagenic at the end of the incubation period. For example, after 56 days incubation with VUN 10,009, VUN 10,010 or VUO 10,201, the mutagenic ratio of BSM-benzo[a]pyrene cultures were 5.30, 5.10 and

2.73, respectively; for the BSM-dibenz[*a,h*]anthracene cultures the mutagenic ratio's were 3.13, 3.10 and 3.08, respectively (Table 5.9a and 5.9b). Both co-culture A and co-culture B were more effective at reducing the mutagenicity of the above culture such that in all cases the mutagenic ratio indicated only a slight mutagenic potential. For example, after 56 days incubation with either co-culture A or co-culture B, the mutagenic ratio of BSM-benzo[*a*]pyrene cultures were 2.19 and 2.04, respectively; for the BSM-dibenz[*a,h*]anthracene cultures, the mutagenic ratios were 2.35 and 2.23, respectively. Such results for the co-culture incubations represented decreases in the mutagenic potential of the samples of 60-63% for BSM-benzo[*a*]pyrene cultures and 35-40% for the BSM-dibenz[*a,h*]anthracene cultures.

The mutagenic potential of DCM extracts from PAH-spiked soil and PAH-contaminated soil was substantially reduced over 100 days following amendment with PAH-degrading inocula (Tables 5.10a and 5.10b). Amongst axenic inocula, amendment of these soils with *P. janthinellum* inocula resulted in a higher reduction in the mutagenicity of the DCM extracts (47% for PAH-spiked soil and 35% for PAH-contaminated soil) compared to either VUN 10,009 (mutagenic potential reduced by 15% for PAH-spiked soil and 7% for PAH-contaminated soil) or VUN 10,010 (mutagenic potential reduced by 30% for PAH-spiked soil and 10% for PAH-contaminated soil). The mutagenic potential of DCM extracts from these soils was reduced even further by the co-cultures being 58-62% (PAH-spiked soil) and 42-43% (PAH-contaminated soil); the difference in mutagenicity reduction by co-culture A and co-culture B was not significant. PAH-spiked and PAH-contaminated soils inoculated with axenic cultures registered mutagenic ratio's greater than 2.5 at the end of the incubation period, indicating a high mutagenic potential. PAH-spiked and PAH-contaminated soils inoculated with the co-cultures registered mutagenic ratios greater than 2.5 for PAH-contaminated soil, but the mutagenic ratios for the PAH-spiked soils was in the range 2.0-2.5, suggesting only a slight mutagenic potential. In all cases, the mutagenic ratio of the co-cultures was less than that recorded for all the axenic cultures.

Table 5.9a The number of mutant colonies arising from exposure to PAH extracts from liquid cultures.

Culture extracts ^b	Number of revertants per plate ^a											
	Chrysene			Benz[a]anthracene			Benzo[a]pyrene			Dibenz[a,h]anthracene		
	Day 0	Day 28	Day 56	Day 0	Day 28	Day 56	Day 0	Day 28	Day 56	Day 0	Day 28	Day 56
Abiotic control	389 ± 4	384 ± 1	385 ± 4	201 ± 6	207 ± 4	209 ± 3	687 ± 6	704 ± 8	701 ± 6	444 ± 5	438 ± 6	447 ± 7
VUN 10,009	394 ± 1	362 ± 6	355 ± 4	198 ± 7	184 ± 1	181 ± 6	696 ± 8	688 ± 9	657 ± 6	437 ± 7	411 ± 6	388 ± 1
VUN 10,010	392 ± 8	357 ± 5	331 ± 9	202 ± 7	176 ± 3	154 ± 8	682 ± 3	651 ± 8	632 ± 13	441 ± 4	407 ± 5	384 ± 2
VUO 10,201	402 ± 11	259 ± 14	254 ± 3	204 ± 4	169 ± 1	162 ± 1	673 ± 10	337 ± 13	339 ± 4	452 ± 7	392 ± 3	382 ± 8
Co-culture A	386 ± 12	266 ± 7	211 ± 4	201 ± 6	137 ± 1	144 ± 1	682 ± 6	306 ± 11	272 ± 7	449 ± 10	296 ± 8	291 ± 4
Co-culture B	391 ± 7	256 ± 6	204 ± 10	197 ± 5	132 ± 8	127 ± 13	687 ± 14	294 ± 5	253 ± 9	460 ± 16	277 ± 10	276 ± 3

^a Assay was performed using *S. typhimurium* TA100 with the post mitochondrial activation system (S9). DMSO served as the solvent control for the spontaneous mutation culture, for which the number of revertants per plate was 124 ± 3.

^b Culture comprised BSM with a respective PAH as a sole carbon and energy source.

Table 5.9b Mutagenic ratio and % reduction in mutagenic response for PAH-containing liquid cultures following the incubation with pure and co-cultures.

Culture extracts	Mutagenic ratio (MR) ^a										
	Chrysene			Benz[<i>a</i>]anthracene			Benzo[<i>a</i>]pyrene			Dibenz[<i>a,h</i>]anthracene	
	Day 0	Day 28	Day 56	Day 0	Day 28	Day 56	Day 0	Day 28	Day 56	Day 0	Day 28
Abiotic control	3.14	3.09 (-1.6%)	3.10 (-1.3%)	1.62	1.67 (+3.1%)	1.69 (+4.3%)	5.54	5.68 (+2.5%)	5.65 (+2.0%)	3.58	3.53 (-1.4%)
VUN 10,009	3.17	2.92 (-7.9%)	2.86 (-9.8%)	1.60	1.48 (-7.5%)	1.46 (-9.9%)	5.61	5.55 (-1.1%)	5.30 (-5.5%)	3.52	3.31 (-6.0%)
VUN 10,010	3.16	2.88 (-8.9%)	2.67 (-15.5%)	1.63	1.42 (-12.9%)	1.24 (-23.9%)	5.50	5.25 (-4.5%)	5.10 (-7.3%)	3.56	3.28 (-7.9%)
VUO 10,201	3.24	2.09 (-35.5%)	2.05 (-36.7%)	1.65	1.36 (-17.6%)	1.31 (-20.6%)	5.43	2.72 (-49.9%)	2.73 (-49.7%)	3.65	3.16 (-13.4%)
Co-culture A	3.11	2.15 (-30.9%)	1.70 (-45.3%)	1.62	1.10 (-32.1%)	1.16 (-28.4%)	5.50	2.47 (-55.1%)	2.19 (-60.2%)	3.62	3.39 (-6.4%)
Co-culture B	3.15	2.06 (-34.6%)	1.65 (47.6%)	1.59	1.06 (-33.3%)	1.02 (-35.8%)	5.54	2.37 (-57.2%)	2.04 (-63.2%)	3.71	2.23 (-39.9%)

^a Data calculated from the results presented in Table 5.9a. The mutagenic ratio is the number of revertants found for tested samples relative to the number of spontaneous revertants. The percentages shown in parentheses are the % change in mutagenic response to the sample, based on MR values, compared to the same sample at day 0; a negative percentage change indicates a decrease in mutagenicity.

Table 5.10a The number of mutant colonies arising from exposure to PAH extracts from soils.

Extracts	Number of revertants per plate ^a					
	PAH-spiked soil			PAH-contaminated soil		
	Day 0	Day 56	Day 100	Day 0	Day 56	Day 100
Uninoculated controls	689 ± 11	671 ± 14	693 ± 6	718 ± 4	711 ± 8	689 ± 4
VUN 10,009	702 ± 14	686 ± 9	594 ± 7	724 ± 9	703 ± 11	677 ± 12
VUN 10,010	695 ± 7	623 ± 10	501 ± 16	722 ± 20	697 ± 6	648 ± 4
VUO 10,201	697 ± 5	477 ± 12	367 ± 8	716 ± 7	634 ± 3	461 ± 8
Co-culture A	705 ± 3	392 ± 10	295 ± 11	722 ± 8	617 ± 13	420 ± 6
Co-culture B	698 ± 7	367 ± 15	263 ± 6	719 ± 4	576 ± 6	409 ± 4

^a Assay was performed using *S. typhimurium* TA100 with the post mitochondrial activation system (S9). DMSO served as the solvent control for the spontaneous mutation culture, for which the number of revertants per plate was 124 ± 3 .

Table 5.10b Mutagenic ratio and % reduction in mutagenic response of PAH extracts from soils.

Extracts	Mutagenic ratio ^a					
	PAH-spiked soil			PAH-contaminated soil		
	Day 0	Day 56	Day 100	Day 0	Day 56	Day 100
Uninoculated controls	5.56	5.41 (12.7%)	5.59 (+0.5%)	5.79	5.73 (-1.0%)	5.56 (-4.0%)
VUN 10,009	5.66	5.53 (-2.3%)	4.79 (-15.4%)	5.84	5.67 (-2.9%)	5.46 (-6.5%)
VUN 10,010	5.60	5.02 (-10.4%)	4.04 (-27.9%)	5.82	5.62 (-3.4%)	5.23 (-10.1%)
VUO 10,201	5.62	3.85 (-31.5%)	2.96 (-47.3%)	5.77	5.11 (-11.4%)	3.72 (-35.5%)
Co-culture A	5.69	3.16 (-44.5%)	2.38 (-58.2%)	5.82	4.98 (-14.4%)	3.39 (-41.8%)
Co-culture B	5.63	2.96 (-47.4%)	2.12 (-62.3%)	5.80	4.65 (-19.8%)	3.30 (-43.1%)

^a Data calculated from the results presented in Table 5.10a. The mutagenic ratio is the number of revertants found for tested samples relative to the number of spontaneous revertants. The percentages shown in parentheses are the % change in mutagenic response to the sample, based on MR values, compared to the same sample at day 0; a negative percentage change indicates a decrease in mutagenicity.

5.10 DISCUSSION

5.10.1 High Molecular Weight PAH Degradation by Bacterial Cultures

The focus of PAH research in recent years has been on the degradation of high molecular weight PAHs, due to their environmental persistence and threat to community health. This has resulted in the isolation of a number of microorganisms that can mineralize and grow on four-benzene ring PAHs as a sole carbon and energy source. Some of these isolates have been used to identify the biochemical pathways involved in the catabolism of four-benzene ring PAHs (Heitkamp *et al.*, 1988; Schneider *et al.*, 1996). The work in this thesis has demonstrated that bacterial consortium VUN 10,009 and *S. maltophilia* VUN 10,010 can degrade and grow on pyrene as sole carbon and energy source. Furthermore, these bacteria can substantially mineralize this PAH with 55-70% of labeled carbon recovered as $^{14}\text{CO}_2$ and incorporation into biomass accounting for a further 18% of the added labeled carbon. These bacteria were able to degrade pyrene concentrations up to 750 mg l^{-1} , however, the degradation rate was adversely affected by pyrene concentrations greater than 250 mg l^{-1} . *P. janthinellum* VUO 10,201 demonstrated little degradative activity toward pyrene when supplied as a sole carbon and energy source in BSM. Pyrene degradation by *P. janthinellum* VUO 10,201 was stimulated significantly by the addition of glucose or yeast extract to BSM, or by replacing BSM with MYPD, but a mass balance analysis of labeled carbon, added to these cultures as radiolabeled pyrene, identified only a partial degradation of pyrene to polar metabolites. Pyrene mineralization by axenic *P. janthinellum* VUO 10,201 cultures was not observed under any conditions.

Microorganisms capable of degrading five-benzene ring PAHs, or recalcitrant four-benzene ring PAHs such as chrysene and benz[a]anthracene, are not widely reported, however, there are some reports on bacteria and fungi which are able to co-metabolically degrade these compounds. For bacteria, there are a few reports that describe the isolation of various strains which can degrade benz[a]anthracene

(Ye *et al.*, 1996; Schneider *et al.*, 1996; Juhasz *et al.*, 1997), dibenz[*a,h*]anthracene (Ye *et al.*, 1996; Juhasz *et al.*, 1997), benzo[*a*]pyrene (Ye *et al.*, 1996; Juhasz *et al.*, 1996; Schneider *et al.*, 1996) or coronene (Juhasz *et al.*, 1996), but these reports only describe the partial degradation of these compounds, either as sole carbon and energy source or in media containing a growth-supporting substrate, by the isolates in pure culture. The degradation of benz[*a*]anthracene, chrysene, dibenz[*a,h*]anthracene, or benzo[*a*]pyrene as a sole carbon and energy source by bacterial consortium VUN 10,009 and *S. maltophilia* VUN 10,010 was poor with the amounts degraded being in the range 6-12% over 56 days. Although small, the amounts degraded were significant since these results were based on the amount degraded taking into account abiotic losses in the control cultures. Further evidence of degradation was provided by the mass balance data for the radiolabeled benzo[*a*]pyrene which, although not mineralized by the bacteria as a sole carbon and energy source, demonstrated a small amount of labeled carbon in the aqueous phase representing partial breakdown of this molecule to polar metabolites. More convincing evidence to support the partial degradation of benzo[*a*]pyrene, as a sole carbon and energy source, by the bacteria was found by analyzing the DCM extracts of the cultures for degradation products. HPLC analysis identified the production of three compounds in inoculated cultures containing benzo[*a*]pyrene which were not detected in the control cultures, in fact, only the benzo[*a*]pyrene peak was identified in the control cultures during 56 days of incubation. The appearance of compounds (II), (III) and (IV) suggests that VUN 10,010 and VUN 10,009 were at least transforming benzo[*a*]pyrene into other compounds, compound (II) was itself transformed in the later stages of the incubation. The small amount of benzo[*a*]pyrene, chrysene, dibenz[*a,h*]anthracene and benzo[*a*]pyrene degraded by the low inoculum size bacterial cultures was probably due to the small bacterial population in the culture over the incubation period arising from the inability of these PAHs to support the growth of these bacteria. In the case of benzo[*a*]pyrene, a mass balance of labeled carbon identified that the partial transformation of this compound was insufficient to provide metabolites which could serve as substrates for anabolic processes *i.e.* producing bacterial biomass.

The lack of bacterial growth on benz[a]anthracene, chrysene, dibenz[a,h]-anthracene and benzo[a]pyrene as a sole carbon and energy source lead to experiments in which a growth substrate, such as pyrene, was added to BSM containing one of these PAHs. Under these co-metabolic conditions, bacterial consortium VUN 10,009 and *S. maltophilia* VUN 10,010 could degrade substantial amounts of these PAHs. By using radiolabeled benzo[a]pyrene, it was shown that the co-metabolic degradation of benzo[a]pyrene led to 8-13% and 32-33% of the carbon catabolized to $^{14}\text{CO}_2$ for VUN 10,009 and VUN 10,010, respectively. These results are significant, since they demonstrate that benzo[a]pyrene was more than partially transformed, rather, they showed that the benzo[a]pyrene was mineralized under co-metabolic conditions. This is a unique result since, although bacterial co-metabolism has been shown to degrade benzo[a]pyrene partially (Schneider *et al.*, 1996), pure cultures of bacteria have not previously been shown to co-metabolically mineralize benzo[a]pyrene. It was suggested in a recent report (Ye *et al.*, 1996) that pure cultures of *S. paucimobilis* EPA505 could mineralize benzo[a]pyrene. This strain could degrade a variety of high molecular weight PAHs in the presence of a growth-supporting PAH, fluoranthene, however, the mineralization of benzo[a]pyrene by *S. paucimobilis* EPA505 was not tested under co-metabolic conditions.

The co-metabolic degradation of at least a portion of the benzo[a]pyrene to CO_2 by VUN 10,010 and the bacterial consortium points to the existence of a catabolic pathway in the bacteria by which they may possibly obtain carbon and generate energy from this PAH. The reason why benzo[a]pyrene alone cannot induce its mineralization by, or support the growth of, VUN 10,010 and VUN 10,009 is not clear but it may be due to the poor stimulation of lower PAH-catabolic pathway enzymes by benzo[a]pyrene. Evidence for this poor enzyme stimulation is provided in this thesis by the HPLC analysis of DCM extracts from axenic bacterial cultures in benzo[a]pyrene-BSM. The HPLC data demonstrated the production of catabolic byproducts which accumulated during incubation but were not further degraded, suggesting that the lower catabolic pathway(s) for the degradation of these compounds was not 'active'. Although not verified in this project, it is possible that these byproducts can be further degraded by the pyrene

catabolism. In cultures containing both pyrene and benzo[a]pyrene, the poor induction of lower PAH-catabolic pathway enzymes by benzo[a]pyrene may be compensated for by pyrene induction of its 'lower' pathway enzymes. Under these conditions, a complete benzo[a]pyrene catabolic pathway is available thus leading to its total breakdown *i.e.* mineralization. There is also evidence in this thesis which shows that the benzo[a]pyrene byproducts, produced from axenic bacterial cultures, can be mineralised if the appropriate catabolic pathway is present. Such evidence is provided by the mineralization of *S. maltophilia* VUN 10,010 benzo[a]pyrene catabolic byproducts by *P. janthinellum* VUO 10,201 (this is further discussed in Section 5.10.4). In this case, rather than the bacterial pyrene catabolism completing the mineralization of benzo[a]pyrene byproducts, the catabolic pathway is instead provided by the fungal metabolism. Evidence to support this hypothesis has been published in other work. For example, Heitkamp and Cerniglia (1988) demonstrated that pyrene is capable of inducing the degradative enzyme(s) responsible for the microbial degradation of PAHs having two to five aromatic rings. Recent studies with *S. paucimobilis* (Ye *et. al.*, 1996) have shown that fluoranthene also induces enzyme(s) that can metabolize a variety of high molecular weight PAHs.

5.10.2 High Molecular Weight PAH Degradation by Fungal Culture

In contrast to bacteria, fungi do not utilize PAHs as their sole source of carbon and energy but transform these compounds co-metabolically to detoxified products (Cerniglia, 1993). The fungal species isolated in this work, *P. janthinellum* VUO 10,201, was able to degrade significant amounts of chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene as a sole carbon and energy source in BSM. The reason why PAH degradation in BSM by *P. janthinellum* VUO 10,201 ceased after 14-28 days of incubation is probably due to this fungus being unable to grow on the tested PAH as a sole source of carbon and energy. PAH degradation by the fungus in BSM during the initial period of incubation may have been sourced from the fungal carbon reserve. This was confirmed when BSMG and MYPD were used as culture media, in which this fungus showed the ability to grow and degrade PAHs to a greater extent. Evidence of benzo[a]pyrene

degradation by the fungus in BSM was obtained by HPLC analysis of DCM extracts of the cultures. It was found that in addition to the benzo[a]pyrene peak, another obvious peak appeared which was assumed to be a benzo[a]pyrene degradation product (compound I) since this peak was not present in abiotic controls. A significant feature of this peak is that it appeared after seven days incubation, but was not further degraded over the remainder of the 56 day incubation period. As in other studies with PAH degradation by fungi, the recalcitrance of this peak suggested that VUN 10,201 could only partially degrade benzo[a]pyrene.

The partial degradation of benzo[a]pyrene by *P. janthinellum* VUO 10,201 was verified by mass balance analysis of radiolabeled benzo[a]pyrene added to fungal cultures. The results of this work showed that none of the ^{14}C -benzo[a]pyrene degraded by VUO 10,201, as a sole carbon and energy source, was used to generate biomass nor was it mineralized to $^{14}\text{CO}_2$. The only degradation products were found in the aqueous phase (as polar metabolites) and the DCM phase (as non-polar products and non-degraded benzo[a]pyrene). It is reasonable to assume that the observed degradation of other high molecular weight PAHs as a sole carbon and energy source by this organism would also result in the formation of polar products, but not in biomass or CO_2 . However, this assumption would need to be verified.

There was a marked contrast between the results obtained for the fungus and bacteria, since *P. janthinellum* VUO 10,201 could not co-metabolically mineralize benzo[a]pyrene even though in MYPD medium the degradation of this PAH by VUO 10,201 was substantially stimulated. Further evidence of the differences between the bacteria and the fungus in the co-metabolic utilization of benzo[a]pyrene is given by the complete absence of labeled carbon from this PAH in the fungal biomass. Under co-metabolic conditions in a PAH mixture, the bacteria used a small portion of the benzo[a]pyrene to build biomass, however, the fungus could not degrade this PAH sufficiently to be used for anabolic processes. The degradation of benzo[a]pyrene as well as other high molecular weight PAHs by non-basidiomycete fungi has been shown to result only in the oxidation of the

substrate (Pothuluri *et al.*, 1994; Kiehmman *et al.*, 1996), analogous to the phase I metabolism of xenobiotics by mammals (Cerniglia, 1992). These fungi oxidize PAH compounds to dead-end intermediate products, which cannot be transformed further by fungi. There has been one exception to the general rule on PAH degradation by fungi reported in the literature. Several species of white rot fungi such as *Phanerochaete* spp., have been reported to mineralize benzo[a]pyrene in a medium containing another growth-supporting carbon substrate (Barclay *et al.*, 1995; Bogan and Lamar, 1996). This is probably due to the unique feature of these fungi in producing lignin-degrading enzymatic systems.

5.10.3 High Molecular Weight PAH Degradation by Fungal-bacterial Co-cultures

The use of fungal-bacterial co-cultures to degrade PAHs has not yet been reported in the literature. The results in this thesis have demonstrated that a *P. janthinellum* VUO 10,201 and bacterial consortium VUN 10,009 co-culture was able to mineralize and grow on benzo[a]pyrene as a sole carbon and energy source. Higher benzo[a]pyrene mineralization and degradation rates were achieved when *P. janthinellum* VUO 10,201 was co-cultured with *S. maltophilia* VUN 10,010. This result is significant since VUN 10,010 was isolated from a different site to VUO 10,201, indicating that fungal-bacterial cooperative mineralization of benzo[a]pyrene is not restricted to species isolated from the same site, and hence selective pressures.

Aside from the novelty of using fungal-bacterial combinations to degrade PAHs, the results arising from the co-culture experiments have broken new ground in several areas of research on PAHs. Firstly, the general message arising from the many unsuccessful attempts to isolate pure cultures that can grow on five-benzene ring PAHs as a sole carbon and energy source, is that it is not possible for microorganisms to degrade these compounds and obtain sufficient net energy from the process to support growth. This work has shown that while a single organism cannot grow on these PAHs as a sole carbon and energy source, a shared PAH catabolism between a fungus and bacteria in the one culture can generate

sufficient energy for both the bacteria and fungus to grow on these PAHs and overcome the metabolic bottlenecks associated with axenic cultures of these organisms. The mass balance analysis of [^{14}C]benzo[*a*]pyrene added to these co-cultures accounted for 7-9% of the labeled carbon being incorporated into the culture biomass.

Secondly, the ability of a pure culture to mineralize and grow on benzo[*a*]pyrene as a sole carbon and energy source has not yet been reported. There are, however, a few reports of benzo[*a*]pyrene mineralization in pure cultures. Some of these reports refer to the co-metabolic mineralization of benzo[*a*]pyrene by *Phanerochaete* spp. (Barclay *et al.*, 1995; Bogan and Lamar, 1996), but this fungus could not mineralize or grow on benzo[*a*]pyrene as a sole carbon and energy source. The only other report in this area demonstrated benzo[*a*]pyrene mineralization by a high population of resting *S. paucimobilis* EPA505 cells in a phosphate buffer (Ye *et al.*, 1996). Such culture conditions were required since EPA505 could not grow on benzo[*a*]pyrene as a sole carbon and energy source. The authors did not try to account for the reasons why this bacterial strain could not utilize benzo[*a*]pyrene as a sole carbon source for biomass production, despite it being able to mineralize benzo[*a*]pyrene under resting cell conditions.

Finally, microbial growth on dibenz[*a,h*]anthracene as a sole carbon and energy source has not previously been reported, however, the fungal-bacterial co-cultures described in this thesis were able to grow on this compound as a sole carbon source in BSM. Microbial growth of the bacteria and the fungus also occurred in co-cultures containing chrysene and benz[*a*]anthracene as a sole carbon and energy source. Microbial degradation and growth on these recalcitrant PAHs as a sole carbon and energy source by the co-culture indicates that such microbial cultures have considerable diversity in the type of PAHs that they can degrade. Such growth also suggests that these co-cultures may also be able to mineralize these PAHs, but this would be need to be verified.

While the use of fungal and bacterial combinations to degrade xenobiotics is novel, the use of co-cultures in biodegradation studies is not without precedence,

although the only reports to date involve the use of different bacterial species rather than different groups of microbes as presented in this work. Trzesicka-Mlynarz and Ward (1995) demonstrated that PAH degradation by a fluoranthene-degrading mixed culture, obtained from creosote-contaminated soil, involved at least four bacterial isolates viz. *Ps. putida*, *Ps. aeruginosa*, *Flavobacterium* sp. and one unknown species. In minimal medium supplemented with yeast extract, axenic cultures of these isolates were able to degrade acenaphthalene, fluorene, phenanthrene and fluoranthene, but their ability to degrade anthracene, pyrene and benzo[a]pyrene was poor compared to the original mixed culture and a co-culture containing all four isolates. The performance of the bacterial co-culture in PAH degradation was demonstrated to be similar to that of original mixed culture. Outside the field of PAH degradation, bacterial co-cultures have been used to degrade sulfonated aromatics. The degradation of 4-aminobenzenesulfonate as a sole source of carbon, nitrogen, sulfur and energy was accomplished by co-cultures of *Hydrogenophaga palleronii* strain S1 and *Agrobacterium radiobacter* strain S2 (Dangmann *et al.*, 1996). Under these conditions, there was no growth in axenic cultures of either strain, but when combined in co-culture, both strains were able to grow.

5.10.4 The Catabolic Mechanism of Fungal-bacterial Co-cultures During PAH Degradation

The finding that neither *P. janthinellum*, *S. maltophilia* or the bacterial consortium can independently mineralize or grow on benzo[a]pyrene as a sole carbon and energy source suggests that a mutually dependent relationship exists between these organisms during co-culture PAH degradation. HPLC data showed the accumulation of different benzo[a]pyrene degradation products in axenic *P. janthinellum* (compound I) and bacterial (compounds II, III and IV) cultures which were further substantially or totally degraded only in the co-cultures. The cooperative catabolic relationship between the fungus and the bacteria was authenticated by the appearance and disappearance of a degradation compound (compound V) which was not detected in the axenic cultures. These degradation products were not identified, however, many identified benzo[a]pyrene

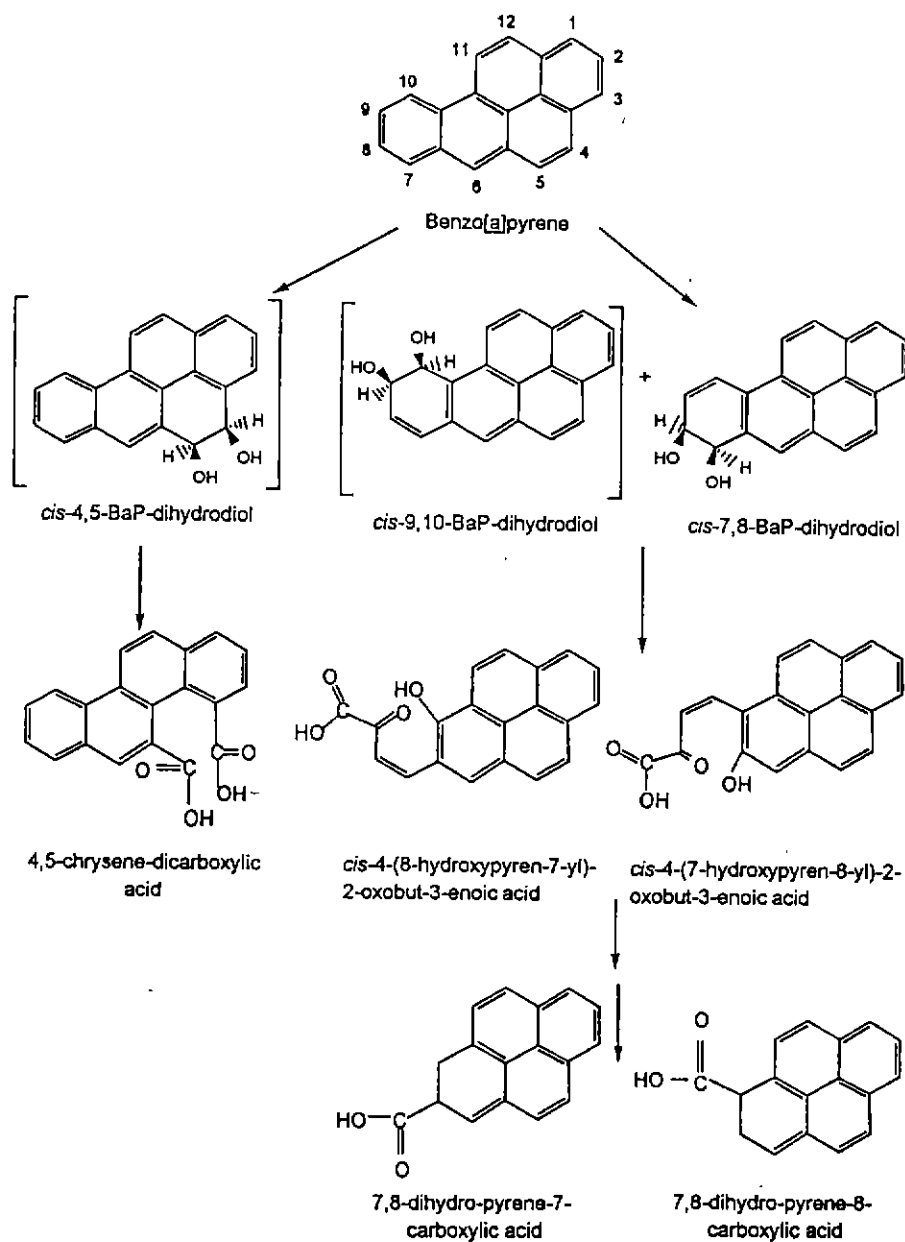


Figure 5.20 Proposed pathways for the degradation of benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135 based on isolated metabolites. Structures in brackets are hypothetical intermediates. Arrows between metabolites indicate multiple steps, not single reactions (from Schneider *et al.*, 1996).

metabolites have been generated by benzo[a]pyrene oxidation at the 4-5, 7-8 or 9-10 positions by either bacteria or fungi (Cerniglia and Gibson, 1980, 1979; Pothuluri *et al.*, 1994; Schneider *et al.*, 1996; Launen *et al.*, 1995; Bogan and Lamar, 1996) and these oxidation products generally are not further metabolized and do not result in ring cleavage (Schneider *et al.*, 1996). Fungi have previously been reported to oxidize benzo[a]pyrene to *trans*-dihydrodiols at the 4,5, 7,8 or 9,10 positions (Cerniglia and Gibson, 1980, 1979; Launen *et al.*, 1995) and a *P. janthinellum* strain has been reported to oxidize benzo[a]pyrene to 9-hydroxybenzo[a]pyrene. White-rot fungi can also oxidize benzo[a]pyrene to quinones at the 1,6; 3,6; or 6,12 positions (Haemmerli *et al.*, 1986).

For bacteria, Schneider *et al.* (1996) recently reported the isolation of ring cleavage products of benzo[a]pyrene, produced by resting cells of a *Mycobacterium* sp. strain RJGII-135, which were not metabolized further. One of the products, 7,8-dihydro-pyrene-carboxylic acid, is proposed to be derived from the initial hydroxylation of benzo[a]pyrene at the C₇ and C₈ positions, resulting in the removal of carbon 7 from the benzo[a]pyrene molecule. The other isolated product, 4,5-chrysene dicarboxylic acid, would result from initial hydroxylation of benzo[a]pyrene at C₄ and C₅ positions (Figure 5.20).

It appears from the above reports on benzo[a]pyrene degradation that either the bacteria or the fungus could be responsible for the initial steps in benzo[a]pyrene degradation by the co-culture. Indeed, all the HPLC peaks that appeared in the axenic bacterial (compound II, III and IV) and fungal (compound I) cultures, were also present in the co-cultures and all of these compounds were further degraded by the co-cultures. Further information on the nature of the cooperative PAH catabolism in the co-cultures was obtained by preparing culture extract preparations from a 35 day culture of one co-culture organism (either the fungus or bacteria), and then inoculating this culture extract preparation with the other organism belonging to the co-culture association. The results of this investigation verified that degradation products from one organism (*S. maltophilia* VUN 10,010 or *P. janthinellum* VUO 10,201) could be degraded by its co-culture partner, *i.e.* the fungal degradative product (compound I) could be degraded by bacteria or, *vice*

versa, the bacterial degradative products (compound II, III and IV) could be degraded by the fungal culture. The most significant result from these experiments was the considerable $^{14}\text{CO}_2$ evolution from *P. janthinellum* culture extract preparations which were subsequently inoculated with *S. maltophilia*. Such $^{14}\text{CO}_2$ emissions occurred only in culture extract preparations prepared from live *S. maltophilia* inocula. Use of either killed *P. janthinellum* or *S. maltophilia* cells did not result in $^{14}\text{CO}_2$ emissions. Furthermore, a small but significant proportion of the labeled carbon was recovered in the bacterial biomass, signaling its involvement in anabolic metabolism. *S. maltophilia* culture extract preparations which were subsequently inoculated with *P. janthinellum* cell also resulted in $^{14}\text{CO}_2$ evolution, but this was 4.5-fold lower than that observed in the alternative incubation i.e. *P. janthinellum* culture extract preparations inoculated with *S. maltophilia*.

These results suggest the following regarding the fungal-bacterial co-culture PAH catabolism:

- (i) *S. maltophilia* VUN 10,010 alone can mineralize catabolic products formed during benzo[a]pyrene degradation by *P. janthinellum* VUO 10,201 as a sole carbon and energy source.
- (ii) *S. maltophilia* VUN 10,010 catabolism of the fungal benzo[a]pyrene degradation products results in carbon incorporation into bacterial biomass.
- (iii) *P. janthinellum* VUO 10,201 alone can mineralize catabolic products formed during benzo[a]pyrene degradation by *S. maltophilia* VUN 10,010 as a sole carbon and energy source, although this mineralization rate is considerably slower than that observed in (i) above.

These results suggest that while the *P. janthinellum* benzo[a]pyrene degradation products represent a catabolic end-point for axenic cultures of this organism, some

of these products are not recalcitrant to the *S. maltophilia* catabolism which can mineralize them to CO₂. The reverse situation is also true, but to a lesser extent *i.e.* the mineralization of *S. maltophilia* benzo[a]pyrene degradation products by *P. janthinellum*. The above data suggest that *P. janthinellum* may play a major role in the initial degradation of benzo[a]pyrene by fungal-bacterial co-cultures (with the degradation products subsequently mineralized by *S. maltophilia*), however, at this stage it cannot confidently be deduced whether the fungus, bacteria or both play a dominant role in the initial degradation and subsequent mineralization of benzo[a]pyrene by the co-cultures.

While the cooperative catabolic route by which benzo[a]pyrene is completely degraded (mineralized) in the co-cultures is not clear at this stage, a number of previous reports have suggested that PAH degradation in nature is a consequence of the sequential breakdown by fungi and bacteria, with the fungi performing the initial PAH oxidation step (Meulenberg *et al.*, 1997; Sack *et al.*, 1997). The initial oxidation of the PAH by fungi can stimulate PAH degradation *via* a number of mechanisms. Heitkamp *et al.* (1988) demonstrated that mono- or dihydroxylated PAHs can serve as substrates for bacteria. The addition of such substrates to bacterial cultures was shown to stimulate PAH degradation by the bacteria. It was postulated that fungal oxidation of PAHs may be an important factor for stimulation PAH degradation in soils where the rate-limiting step for indigenous bacteria is the initial ring oxidation. The oxidation of PAHs by fungi is known to produce more water-soluble and bioavailable products (Meulenberg *et al.*, 1997). For example, oxidation of anthracene by *P. chrysosporium* increases the water solubility of the pollutant from 0.07 to 7000 mg l⁻¹ (Cerniglia, 1992; de Morsier *et al.*, 1987). Oxidation of phenanthrene to diphenic acid increases the water solubility nearly 1,000 fold (Cerniglia, 1992). The increased solubility of the transformed PAH compound due to oxidation by fungal activity may be important for increasing the bioavailability of the compound to other microbial groups, such as bacteria, which can subsequently mineralize the compound (Meulenberg *et al.*, 1997). This is especially true for soil environments, where the low water solubility of PAHs causes their migration to hydrophobic regions of the soil where they are less available to the soil microflora.

Some of the data in this chapter, other than that already presented, is consistent with the hypothesis that the fungi perform a major role in the initial oxidation of benzo[a]pyrene. For example, benzo[a]pyrene degradation and mineralization in soil and BSM (containing benzo[a]pyrene and pyrene) by axenic bacterial cultures commences only after considerable lag periods; such degradation lag periods are absent in axenic *P. janthinellum* cultures in soil. These degradation and mineralization lag periods are also absent in co-cultures, which would not be expected if bacteria are responsible for initiating benzo[a]pyrene oxidation. Also, a *P. janthinellum* VUO 10,201 degradation product compound I was the first degradation product to appear in co-cultures containing benzo[a]pyrene as the sole carbon and energy source (noting that there could be some other compounds also formed that may not be detected by the method used). As mentioned earlier, compound I is most likely a mono- or dihydroxylated PAH which are proposed to be more easily degraded substrates for bacteria compared to the parent PAH (Heitkamp *et al.*, 1988). One possible role for this compound in fungal-bacterial co-cultures is as a bacterial growth substrate which may be used as the sole carbon source for the bacteria, thus relying on the fungus alone to initially oxidize benzo[a]pyrene. Alternatively, the bacteria may use the compound as a substrate for co-metabolic mineralization of benzo[a]pyrene in a similar way to the bacterial use of pyrene for co-metabolic benzo[a]pyrene mineralization as described earlier; initial benzo[a]pyrene oxidation in the latter case may be performed by either the fungus or the bacteria. *P. janthinellum* VUO 10,201 also clearly profits from its relationship with the bacteria, since it is able to grow in BSM containing benzo[a]pyrene which does not occur if the bacteria are absent. Growth of the fungus is most likely attributable to a bacterial metabolic product which can serve as a fungal growth substrate. This is supported by the inability of axenic *P. janthinellum* cultures to degrade or grow on pyrene as a sole carbon source, yet fungal growth occurs in the presence of pyrene when pyrene-degrading bacteria are added to the culture.

It is recognized that identification of the catabolic intermediate compounds I-V is important for determining the benzo[a]pyrene catabolic pathway used by the co-cultures. Such identification work would require the incubation of large culture

volumes to enable the production of sufficient quantities of intermediate compounds for fractionation and subsequent analysis. Such work was beyond the scope of this PhD project, however, in Chapter 7 it is recommended that this metabolite identification is performed as follow-up work to this thesis.

5.10.5 High Molecular Weight PAH Degradation in Soils

The potential drawback with using co-cultures in a soil environment, being a heterogeneous mix of organics, inorganics and microbes, is that it contains many external influences such as interactions with indigenous microbes, the influence of other organic substrates and the effects of inhibitory compounds *e.g.* heavy metals. Such influences may be transitory or may have a more chronic effect on the co-culture with a possible outcome being co-culture destabilization. The consequences of co-culture instability on PAH degradation, especially high molecular weight PAHs, are unknown but could be expected to increase the lag periods for growth and PAH degradation, PAH degradation rate, the range of PAHs degraded and the extent of degradation *i.e.* mineralization versus partial degradation, and/or the production of toxic, water soluble intermediates. Therefore, fungal-bacterial co-cultures could potentially fail to remediate PAH-contaminated soils. The results in this thesis suggest otherwise, since they demonstrate faster and more extensive degradation of low and high molecular weight PAHs, especially benzo[a]pyrene mineralization, in PAH-spiked soil and PAH-contaminated soil using a combined *Penicillium* and *Stenotrophomonas* inoculum compared to single organism inocula and the indigenous microflora. A small amount of benzo[a]pyrene was mineralized in the authentic PAH-contaminated soil inoculated with only *P. janthinellum* VUO 10,201. Since it has been demonstrated that the fungus alone cannot mineralize this PAH, it is suspected that the indigenous soil bacteria and *P. janthinellum* VUO 10,201 were cooperatively mineralizing benzo[a]pyrene. This is consistent with the observation that benzo[a]pyrene mineralization was not detected in sterile PAH-spiked soil (containing no indigenous microflora) inoculated only with *P. janthinellum* VUO 10,201. Sack and Fritsche (1997) showed that the production of degradable PAH metabolites by wood-decaying fungi inoculated into non-sterile,

artificially-contaminated soil enhanced the mineralization of pyrene. It is reasonable to expect that ring cleavage intermediates, produced by the fungi, would be metabolized further in a soil ecosystem containing a variety of microbes acting as a consortium.

Co-metabolic degradation by either bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010 also contributed to the mineralization of benzo[a]pyrene in PAH-spiked soil and PAH-contaminated soil. This was evident by the substantial amount of $^{14}\text{CO}_2$ that evolved from soil inoculated with axenic inocula of these bacteria. Chemical analysis of the PAH-contaminated soil revealed that there were substantial quantities of pyrene and low molecular weight PAHs in this soil to support co-metabolic mineralization of benzo[a]pyrene by bacteria. It is worth noting that co-metabolic benzo[a]pyrene mineralization would also have occurred in the soils inoculated with the co-culture, however, mineralization of this PAH also occurred as a result of co-culture activity in such soils since there was an absence of the mineralization lag period typical of soils inoculated only the bacteria, and because substantially greater yields of $^{14}\text{CO}_2$ were achieved in the co-culture soils compared to the axenic bacteria-inoculated soils. A small amount of benzo[a]pyrene was mineralized by the indigenous soil microflora in the PAH-contaminated soil. The slow mineralization of benzo[a]pyrene by the indigenous microbes can be accredited to their lack of ability to degrade and mineralize benzo[a]pyrene at a significant rate, rather than a due to a shortage of non-carbon nutrients. This can be assumed since a nutrient shortage in the PAH-contaminated soil would also be expected to have an effect on the co-culture and bacterial co-metabolic mineralization rates, but this was not observed.

5.10.6 PAH Mutagenicity

Depending on the metabolic pathway, microbial degradation of a PAH may result in the formation of products that are more toxic or less toxic than the parent compound. The removal of the original PAHs does not necessarily indicate a successful operation. Belkin *et al.* (1994) showed that in several cases of their treatment of PAH-contaminated soil, genotoxicity actually increased along with

the decrease in the PAH concentration. In the literature published so far, although some mutagenic compounds have been identified as minor metabolites of some PAHs, in general most PAH transformations performed by fungi reduce the mutagenicity, and thus detoxify a PAH-contaminated environment (Sutherland, 1992; Münchnerová and Augustin, 1994).

Although in this work the products resulting from the microbial degradation of PAHs were not identified, the data demonstrated that *P. janthinellum* VUO 10,201, *S. maltophilia* VUN 10,010 and bacterial consortium VUN 10,009 degrade high molecular weight PAHs to less genotoxic products. The mutagenic assay of DCM extracts from liquid cultures and soils inoculated with VUN 10,009, VUN 10,010, VUO 10,201, co-culture A and co-culture B demonstrated detoxification.

As with the liquid culture experiments, there was an exceptional decrease in the mutagenic potential of the contaminated soil inoculated with a co-culture compared to single organism inocula. This is probably due to the higher degradation rates and the formation of less toxic degradation byproducts, e.g. CO₂, by the co-culture compared to cultures inoculated with a single organism. The decrease in mutagenic potential of soil incubated with strain VUN 10,010 was less than for soil incubated with *P. janthinellum* VUO 10,201, even though the former strain co-metabolically mineralized the potent mutagen benzo[a]pyrene to a far higher extent. This is probably due to the ability of the fungus to transform this compound, and other high molecular weight PAHs, to more biodegradable and non-toxic products, consequently reducing the amount of parent mutagenic PAH compound in the soil.

5.10.7 Concluding Remarks

The introduction, or bioaugmentation, of microorganisms has been considered a valuable tool for increasing the rate and extent of pollutant biodegradation in contaminated sites. Although increased biodegradation of recalcitrant compounds by such treatments has been demonstrated in several studies (Brodkorb and Legge, 1992; Grosser *et al.*, 1991; Guthrie and Pfaender, 1998), it is often viewed negatively by those who have had cautionary experiences (Harkness *et al.*, 1993;

Liu *et al.*, 1990). The advantage of bioaugmentation is not generally accepted since inoculation experiments in soil have shown ambiguous results in comparison to the degradation by indigenous microorganisms (Dott *et al.*, 1989; Leahy and Colwell, 1990). Moreover, studies have reported the failure of introduced microorganisms to perform degradative activities or simply to survive in soil environments (Kästner *et al.*, 1994; 1998).

The results in this chapter demonstrate the unprecedented ability of fungal-bacterial co-cultures to grow on five-benzene ring PAHs, and mineralize benzo[a]pyrene, as a sole carbon and energy source. Inoculation of these co-cultures into authentic PAH-contaminated soil demonstrated their superiority in the degradation and mineralization of high molecular weight PAHs, and consequent reduction in mutagenicity, compared to axenic inocula and the indigenous microflora. These results support further investigation into the use of fungal-bacterial inocula for the complete bioremediation of PAH-contaminated sites.

CHAPTER 6

SURFACATANT-ENHANCED BIODEGRADATION AND MINERALIZATION OF HIGH MOLECULAR WEIGHT PAHs

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CHAPTER 6

SURFACTANT-ENHANCED BIODEGRADATION AND MINERALIZATION OF HIGH MOLECULAR WEIGHT PAHs

6.1 INTRODUCTION

The approaches used in Chapters 4 and 5 to improve the removal of high molecular weight PAHs from PAH-contaminated soils were to enrich, isolate and characterize microorganisms capable of degrading such PAHs. This strategy resulted in the successful isolation of PAH degraders which were able to degrade and mineralize high molecular weight PAHs in liquid and soil environments. However, PAHs are recognized for their high hydrophobicity and low water solubility, which are known to adversely affect their biodegradation rate. In an aqueous environment, such physical properties limit PAH bioavailability due to low aqueous PAH concentrations and slow dissolution rates into the aqueous phase. This is further complicated in soils by the binding of PAHs to soil organic carbons and PAH migration into hydrophobic regions in the soil matrix (Mihelcec and Luthy, 1991; Carmichael and Pfaender, 1997b). With regard to the biodegradation experiments in the preceding chapters, such characteristics of PAHs would almost certainly have restricted their biodegradation rate by the fungal and bacterial isolates and their co-cultures.

One approach that has been proposed for increasing the PAH biodegradation rate is to use surfactants for increasing the PAH dissolution rate into the aqueous phase. In recent years, much research has been conducted on surfactant-aided bioremediation of PAHs and other poorly-soluble organic pollutants (Kile and Chiou, 1989; Lui *et al.*, 1991, Jahan *et al.*, 1997; Tiehm *et al.*, 1997). Surfactants have been shown to increase the solubility and dispersion of poorly-soluble hydrocarbons and oils, thereby enhancing desorption from soil particles and increasing bioavailability. Research to date on the use of surfactants to enhance microbial PAH degradation has mostly been concerned with low molecular weight

PAHs. Such research has reported variable impacts on the use of surfactants to accelerate the biodegradation of PAHs, where some authors noted improved degradation rates (Grimberg *et al.*, 1996; Volkering *et al.*, 1995), while others reported no impact (Foght *et al.*, 1989; Ghosh *et al.*, 1995) or inhibitory effects (Deschênes *et al.*, 1995; Laha and Luthy, 1991). These data suggest that successful application of surfactants for improving PAH degradation may need to be optimized for any particular microbial-PAH system to account for the variety of factors which influence biodegradation, some of which include surfactant type and concentration, the substrate mixture and the microbial species involved (Haigh, 1996; Joshi and Lee, 1996; Jahan *et al.*, 1997). The variable success of preceding work on improving PAH biodegradation rates using surfactant-enhanced biodegradation of low molecular weight PAHs provides no assurance that surfactants will improve the high molecular weight PAH degradation rate by bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010 or *P. janthinellum* VUO 10,201.

The general aim of the research reported in this chapter was to investigate the use of various surfactants for improving the degradation rate of high molecular weight PAHs by the above isolates and their co-culture combinations. In particular, this chapter reports results on:

- (1) evaluating different surfactants for their microbial compatibility and utility in increasing the solubility of high molecular weight PAHs in aqueous solutions;
- (2) trialing various surfactant types and concentrations in PAH-containing liquid cultures to optimize high molecular weight PAH degradation rates by the PAH-degraders described in Chapter 4; and
- (3) using the data obtained from the above work to improve the degradation and mineralization rates of high molecular weight PAHs in soils inoculated with axenic bacterial and fungal cultures, and fungal-bacterial co-cultures.

Table 6.1 Efficiency of the PAH extraction method used for the recovery of PAHs from liquid medium in the presence and absence of surfactant.

PAH	% PAH Recovery ^a	
	Without surfactant ^b	With surfactant ^c
Fluorene	99.7 ± 0.2	99.7 ± 0.1
Phenanthrene	99.9 ± 0.1	99.8 ± 0.2
Fluoranthrene	99.7 ± 0.4	99.6 ± 0.2
Pyrene	100.0 ± 0.1	99.9 ± 0.1
Benz[<i>a</i>]anthracene	99.3 ± 0.7	99.3 ± 0.6
Chrysene	98.7 ± 2.2	99.5 ± 0.5
Benzo[<i>a</i>]pyrene	99.8 ± 0.4	99.4 ± 0.3
Dibenz[<i>a,h</i>]anthracene	99.7 ± 0.4	99.4 ± 0.2
Coronene	99.4 ± 0.5	99.5 ± 0.7

^a %PAH recovery represents the average amount of each PAH recovered from triplicate samples compared to the known amount of PAH added to the standard solution, expressed as a percentage. The standard deviation between the amount of PAH recovered from each triplicate sample is also shown.

^b PAHs were recovered from BSM solution by simple DCM extraction method (one step vigorous shaking).

^c PAHs were recovered from BSM solution containing 10 g l⁻¹ Tergitol NP-10 by one step ultrasonication-DCM extraction method.

6.2 EFFICIENCY OF THE PAH-EXTRACTION METHODS

The DCM extraction method used to measure residual PAH concentration in biodegradation experiments must be reliable in terms of PAH recovery and reproducibility. These criteria were satisfied in the experiments in preceding chapters but the effect of surfactants in the cultures on DCM extraction efficiency was unknown. This section examines the effects of the presence of surfactant on the DCM extraction of PAHs and develops a method for maximizing PAH recovery and reproducibility in the presence of surfactants.

6.2.1 PAH Extraction Efficiency for Surfactant-containing Media

PAH extraction efficiency was evaluated by comparing the amount of PAH and internal standard (2,3-benzo[*b*]fluorene) recovered from standard solutions of BSM, using the DCM extraction method, to the known amount of these compounds added to the BSM prior to extraction; these tests were performed in the presence and absence of surfactant. Sequential extractions (three successive extractions) were conducted and the PAH concentration measured after each extraction; vigorous shaking and sonication was used to improve the mass transfer of PAHs (three- to seven-benzene rings) and internal standard into the DCM.

In the absence of surfactant, greater than 98.7% of the added PAH, for all PAHs, was recovered from the first extraction with no sonication being required (Table 6.1). PAH extraction from BSM was found to be slightly affected by the presence of surfactant (*ca.* 90% recovery on the first extraction). PAH extraction efficiency in the presence of surfactant was appreciably improved by the combination of ultrasonication and vigorous shaking with the amount of PAH recovered from the first extraction being greater than 99% (Table 6.1). In the light of these results, ultrasonication and vigorous shaking was adopted for all extractions performed in the experiments in this chapter.

Table 6.2 Chemical and physical properties of the surfactants used in this study^a.

Surfactant	MW	Formula	CMC ^b (mM)	HLB ^c
Cationic: Sodium dodecyl sulphate (SDS)	288	$C_{12}OSO_3^-Na^+$	8.2	40
Anionic: Cetyltrimethylammoniumbromide (CTAB)	364	$C_{16}N(CH_3)^+Br^-$	0.9	NA
Nonionic: Brij 35	1,200	$C_{12}H_{25}(CH_2CH_2O)_nOH$ with average $n = 23$	0.06	16.9
Igepal CA-630	603	tert-octylphenol poly (oxyethylene) ethanol	0.08	13.0
Tergitol NP-10	652	$C_9H_{19}C_6H_4O(CH_2CH_2O)_nH$ with average $n = 10.5$	0.064	13.1
Triton X-100	625	$C_8H_{17}C_6H_4O(CH_2CH_2O)_nH$ with average $n = 9.5$	0.24	13.5
Tween 20	NA	Minolaurate polyoxyethylenesorbitan	NA	NA
Tween 40	1277	Monopalmitate polyoxyethylenesorbitan	0.02	15.6
Tween 60	NA	Monosterate polyoxyethylenesorbitan	NA	NA
Tween 80	NA	Monooleate polyoxyethylenesorbitan	NA	NA
Tyloxapol	4,500	Polymer of alkyl aryl polyether alcohol	0.018	12.9

^a Data obtained from the manufacturer.^b Critical micelle concentration (CMC).^c Hydrophilic-lipophilic balance (HLB).

NA signifies that data could not be obtained from supplier or the source cited by the supplier.

6.2.2 Reproducibility

The results obtained from sequential PAH extraction showed a good extraction reproducibility. For most of the PAHs studied, the standard deviation between the analysis of triplicate samples from liquid medium containing surfactant were quite low: 0.1% for fluorene and pyrene, 0.2% for phenanthrene, fluoranthene and dibenz[*a,h*]anthracene, 0.3% for benzo[*a*]pyrene, 0.5% for benz[*a*]anthracene and 0.7% for coronene.

6.3 MICROBIAL GROWTH ON SURFACTANTS

Surfactants to be used for enhancing the biodegradation of hydrophobic pollutants should not themselves serve as growth substrates since this would cause a decrease in surfactant concentration, and hence pollutant-mobilizing capacity, and their preferential use as a carbon source may result in the loss of, or reduction in, pollutant-degrading activity. Therefore, *S. maltophilia* VUN 10,010, bacterial consortium VUN 10,009 and *P. janthinellum* VUO 10,201 were tested for their ability to grow on a variety of anionic, cationic and nonionic surfactants supplied as a sole carbon and energy source. Table 6.2 lists the types and chemical properties of surfactants employed in this investigation. These surfactants were chosen to provide a mix of anionic, cationic and nonionic surfactants. Of the surfactants tested, only the Tween series (Tween 20, 40, 60, and 80) could be utilized by the bacteria as a growth substrate. Brij 35 and the Tween series promoted the germination of fungal spores, however, no substantial growth or development of mycelia was observed on these surfactants (Table 6.3).

6.4 SURFACTANT TOXICITY

Another criterion for surfactants which are used to enhance pollutant biodegradation is low microbial toxicity. Therefore, the surfactants described in Section 6.3 were tested for evidence of toxic effects on the growth of *S. maltophilia* VUN 10,010, bacterial consortium VUN 10,009 and *P. janthinellum* VUO 10,201. The Tween series was excluded from these tests since their use as microbial

Table 6.3 Effects of surfactants on microbial growth.

Surfactant	Bacterial growth on surfactant ^a		MIC (g l ⁻¹) ^b		<i>P. janthinellum</i> growth on surfactant ^a		MIC (g l ⁻¹) ^c
	VUN 10,009	VUN 10,010	VUN 10,009	VUN 10,010	Spores	Mycelia	
Tween 20	+	+	ND	ND	+	-	NA
Tween 40	+	+	ND	ND	+	-	NA
Tween 60	+	+	ND	ND	+	-	NA
Tween 80	+	+	ND	ND	+	-	NA
Brij 35	-	-	20	20	+	-	NA
Igepal CA-630	-	-	5	5	-	-	NA
Tergitol NP-10	-	-	20	20	-	-	NA
Triton X-100	-	-	20	20	-	-	NA
Tyloxapol	-	-	20	20	-	-	NA
SDS	-	-	0.2	0.2	-	-	1.0
CTAB	-	-	0.01	0.05	-	-	1.0

^a All growth tests were performed in BSM supplemented with a respective surfactant (0.1 g l⁻¹) as a sole source of carbon and energy. Bacterial growth was determined by visual observation of medium turbidity and protein concentration as compared to the uninoculated control. Fungal growth was determined by the evidence of spore germination and/or further development of mycelia. Growth (+) or no growth (-) is indicated.

^b Tests were performed using the agar diffusion method. MIC represents the minimum concentration of a surfactant which inhibited bacterial growth as observed by the production of clear zones. ND, not determined.

^c Tests were performed in MYPD agar and broth. MIC represents the concentration of a surfactant which inhibited spore germination. The maximum concentration tested was 20 g l⁻¹. No inhibitory effect was observed (NA), except for SDS and CTAB (1.0 g l⁻¹), both of which inhibited spore germination in MYPD broth and also inhibited mycelial growth on MYPD agar.

growth substrates invalidated their inclusion in the PAH biodegradation experiments presented later in this chapter. The sensitivity of bacterial growth to surfactant concentrations was tested by the agar diffusion method.

The cationic surfactant, CTAB, was inhibitory at relatively low concentrations ($0.01\text{--}0.05\text{ g l}^{-1}$) and the anionic surfactant, SDS, had a minimum inhibitory concentration (MIC) of 0.2 g l^{-1} (Table 6.3). In contrast, the nonionic surfactants exerted little or no effect on the growth of *S. maltophilia* VUN 10,010 or bacterial consortium VUN 10,009 at concentrations up to 10 g l^{-1} . Igepal CA-630 caused some growth inhibition at concentrations of 5 and 10 g l^{-1} ; inhibition of bacterial growth by all surfactants was observed at 20 g l^{-1} . Surfactant toxicity on the growth of *P. janthinellum* VUO 10,201 was tested in MYPD medium. CTAB and SDS exerted an inhibitory effect on fungal spore germination at a concentration of 1 g l^{-1} . Although spore germination occurred at a lower concentration (0.1 g l^{-1}) of these two surfactants, the germination period was longer than that observed in controls containing no surfactant, and there was no subsequent growth or development of mycelia. In contrast, nonionic surfactants did not exert a toxic effect on the growth of *P. janthinellum* up to concentrations of 20 g l^{-1} . Given these results, no tests were performed at surfactant concentrations above 20 g l^{-1} .

6.5 SURFACTANT CRITICAL MICELLE CONCENTRATION (CMC)

A unique property of surfactants is their solubilizing power for water-insoluble compounds such as PAHs; this is attributed to the formation of pseudophase micelles *via* self-association of surfactant molecules as they reach the CMC. The CMC values for any surfactant may be obtained using a conventional plot of the surface tension versus the logarithm of surfactant concentration (Figure 6.1). The surfactant CMC is taken as the surfactant concentration at which an intersection is formed between two linear portions of a curve showing variation in surface tension as a function of the logarithm of surfactant concentrations (Edwards *et al.*, 1991). The nonionic surfactants listed in Table 6.2, except for the Tween series, were selected for determination of their CMC values. CMC measurements for

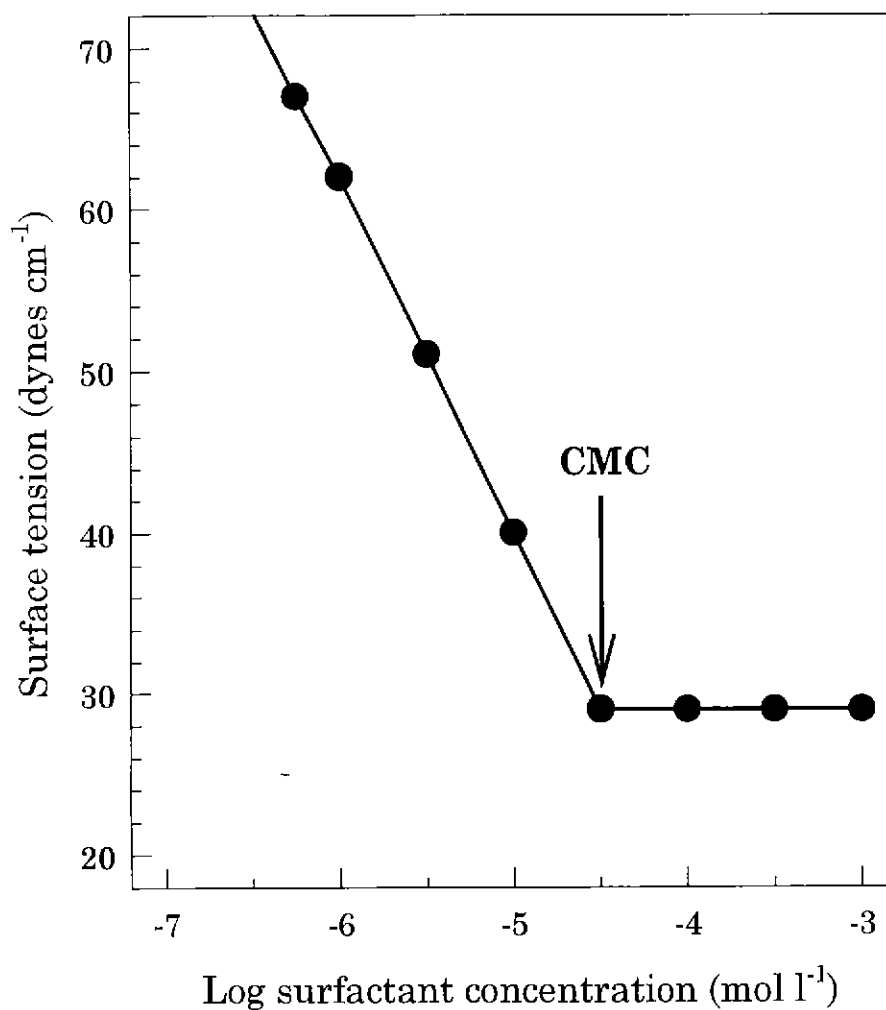


Figure 6.1 Determination of surfactant critical micelle concentration (CMC) using surface tension measurements. The CMC value is taken from the intersection of the two linear portions of a curve showing variation in surface tension as a function of the logarithm of surfactant concentration (Edwards *et al.*, 1991).

each surfactant were performed in sterile BSM, since this is the medium used in the PAH degradation experiments, as well as sterile deionized water at 30°C.

Figures 6.2 and 6.3 show the plot of the surface tension of surfactant solutions in deionized water and BSM, respectively, versus the logarithm of surfactant concentration. Using this plot, the CMC of a respective surfactant was determined, using a ruler and graph paper, as the concentration at which a pronounced break occurred in the slope. Slightly different values were obtained between BSM and distilled water; Brij 35, Igepal CA-630 and Tergitol NP-10 gave higher CMC values in BSM, while Triton X-100 and Tyloxapol gave higher CMC values in distilled water (Table 6.4). The surfactant CMC values in BSM were taken into account for all further related degradation experiments.

6.6 HIGH MOLECULAR WEIGHT PAH SOLUBILIZATION BY NONIONIC SURFACTANTS

Due to the lack of information in the literature on surfactant-enhanced solubilization of high molecular weight PAHs, the apparent solubility of fluoranthene, pyrene and benzo[*a*]pyrene was measured in the presence of the nonionic surfactants tested in Section 6.5 at concentrations above, below and at the surfactant CMC. The chemical and physical properties of these PAHs are given in Table 6.5. An attempt was also made to determine the solubility of dibenz[*a,h*]anthracene and coronene in the presence of the nonionic surfactants, but their concentrations in the aqueous phase at surfactant CMC were below the detection limit of the gas chromatograph. Preliminary experiments determined that PAH concentrations in the aqueous phase equilibrated, for all surfactant solutions tested, after approximately 42 hours for fluoranthene and pyrene, and 72 hours for benzo[*a*]pyrene (Figure 6.4). Consequently, the PAH concentrations in further experiments in this chapter were measured after 42 hours for fluoranthene and pyrene, and 72 hours for benzo[*a*]pyrene to ensure that equilibrium had been reached.

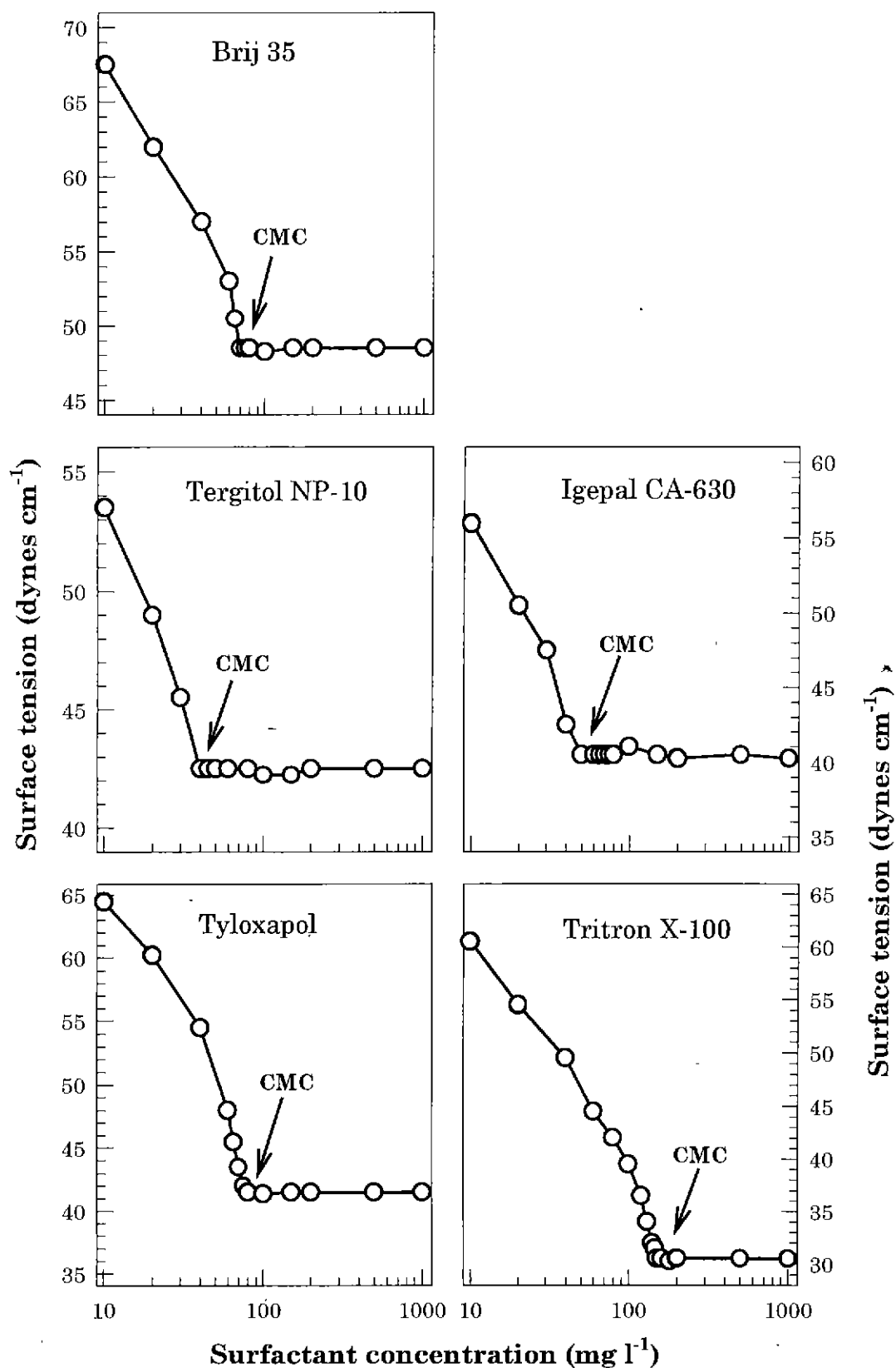


Figure 6.2 Critical micelle concentration (CMC) determination of five nonionic surfactants in deionized water. Measurements were performed at 30°C. The arrow indicates the CMC value for a respective surfactant.

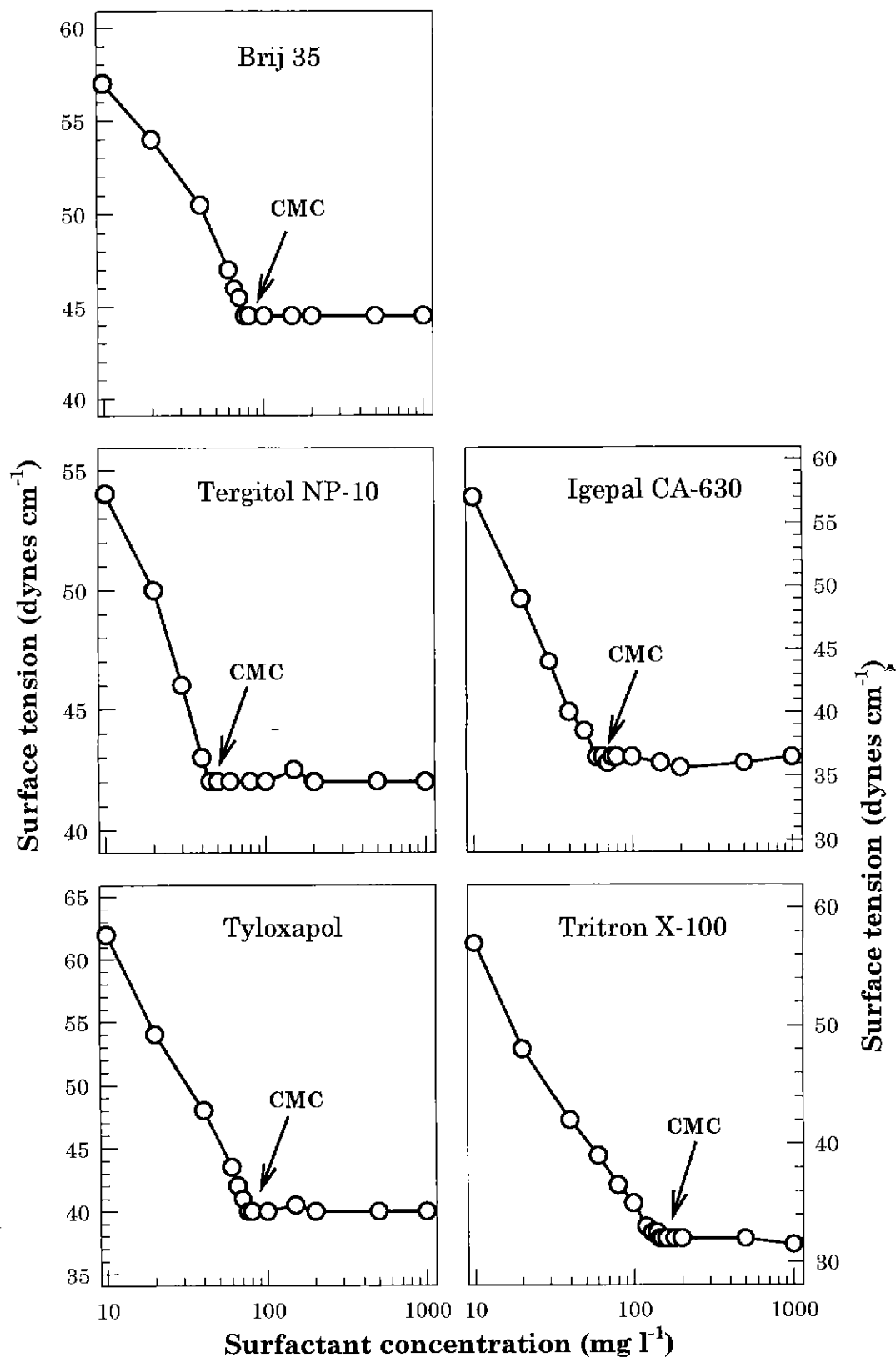


Figure 6.3 Critical micelle concentration (CMC) determination of five nonionic surfactants in BSM solutions. Measurements were performed at 30°C. The arrow indicates the CMC value for a respective surfactant.

Table 6.4 Critical micelle concentration of selected nonionic surfactants.

Surfactant	CMC (mg l ⁻¹) ^a	
	Deionized water	BSM
Brij 35	70 (0.058) ^b	75 (0.063)
Igepal CA-630	50 (0.083)	65 (0.108)
Tergitol NP-10	40 (0.061)	45 (0.069)
Triton X-100	150 (0.240)	145 (0.232)
Tyloxapol	80 (0.018)	75 (0.017)

^a Determination was performed at 30°C.

^b Numbers in parenthesis are CMC values (mM) on a molar basis.

The solubility of crystalized pyrene, fluoranthene and benzo[a]pyrene incubated in BSM in the presence of the five nonionic surfactants slightly increased with increasing surfactant concentration below the CMC, however, surfactant concentrations above CMC proportionally increased the apparent solubility of pyrene, fluoranthene and benzo[a]pyrene (Figures 6.5 and 6.6). The apparent PAH solubility, or concentration, as defined here, is the PAH concentration which reflects the total concentration of PAH in both the aqueous phase and in the micelles. Clearly, it is only used to described PAH concentrations in media containing surfactants at concentrations above CMC. According to the results obtained in this experiment, the liquid phase concentrations of pyrene, fluoranthene and benzo[a]pyrene were 0.48-0.56 mM (700-fold increase), 0.73-0.81 mM (500-fold increase) and 0.16-0.18 mM (10^4 -fold increase), respectively, when the surfactant concentrations were 10.0 g l^{-1} (the aqueous solubilities of pyrene, fluoranthene and benzo[a]pyrene at 30°C in the absence of surfactant were approximately $6.97 \times 10^{-4} \text{ mM}$, $1.37 \times 10^{-3} \text{ mM}$ and $1.59 \times 10^{-5} \text{ mM}$, respectively). Only Tyloxapol failed to give a 700-fold increase in apparent pyrene concentration; the pyrene aqueous phase concentration being 0.182 mM (260-fold increase) at 10 g l^{-1} Tyloxapol.

The effectiveness of a particular surfactant in solubilizing a given PAH can be assessed by determining the molar solubilization ratio (MSR) and micelle/water partition coefficient (K_m). The MSR is defined as the number of moles of organic compound solubilized per mole of surfactant added to solution. The MSR for solubilization of PAH compounds can be calculated as follows:

$$\text{MSR} = \frac{(S_{\text{PAH, CMC}} - S_{\text{PAH, CMC}})}{(C_{\text{SURF}} - \text{CMC})}$$

where $S_{\text{PAH,CMC}}$ is the apparent solubility of a PAH compound in moles per litre at the CMC; $S_{\text{PAH,MIC}}$ is the total apparent solubility of the PAH compound in moles per litre in micellar solution at a particular surfactant concentration greater than

Table 6.5 Formulae and properties of PAHs (from Sanseverino *et al.*, 1994; Ashok and Saxena, 1995).

PAH	Molecular formula	MW (average)	Solubility ^a (mg l ⁻¹)	Log K _{ow} ^b
Fluoranthene	C ₁₆ H ₁₀	202	0.260	5.33
Pyrene	C ₁₆ H ₁₀	202	0.135	5.32
Benzo[<i>a</i>]pyrene	C ₂₀ H ₁₂	252	0.004	6.04

^a Solubility of PAHs in water

^b Log octanol/water coefficient.

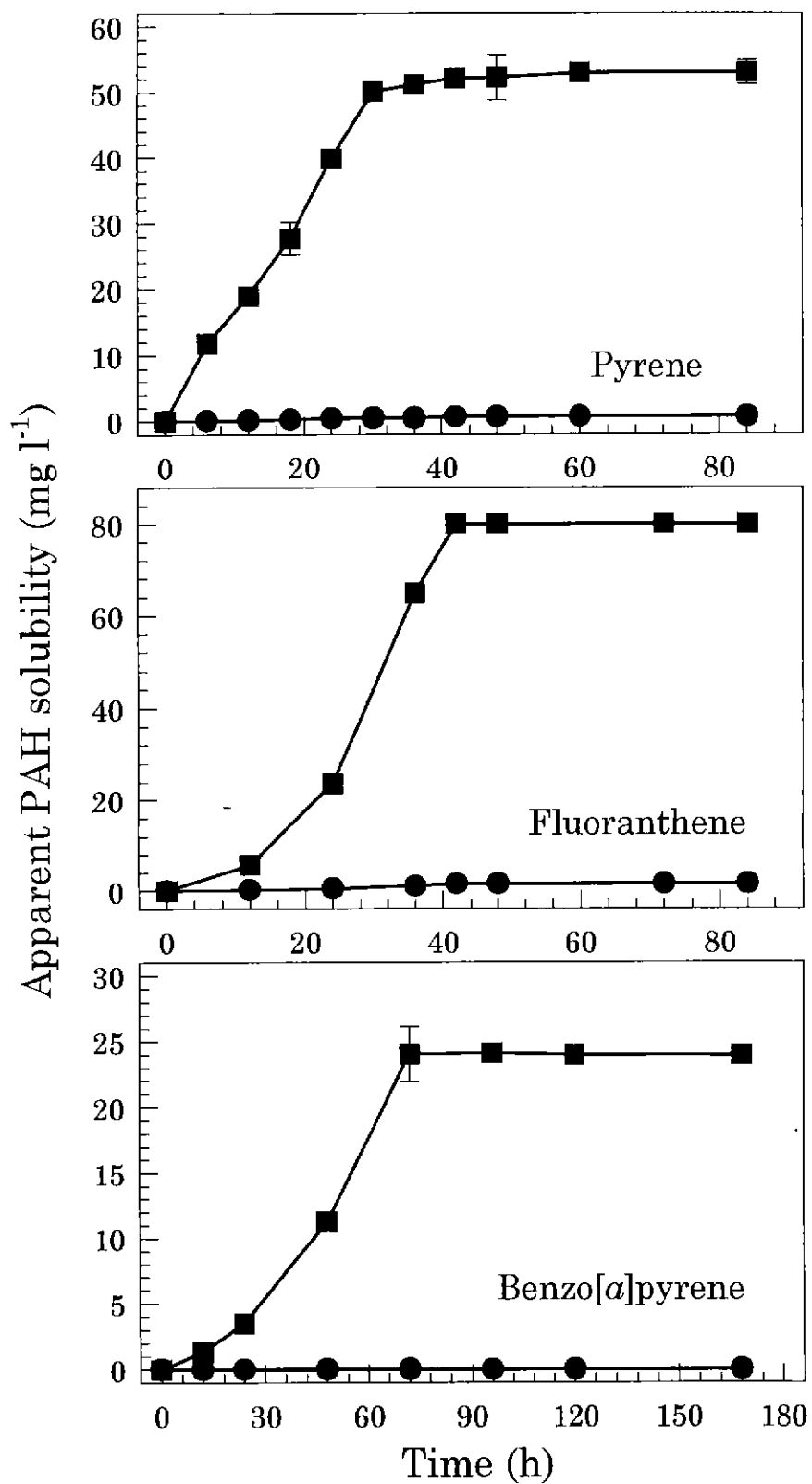


Figure 6.4 PAH solubilization by Tergitol NP-10 at the CMC (●, 0.045 g l⁻¹) and above CMC (■, 5.0 g l⁻¹). Experiments were performed in BSM at 30°C.

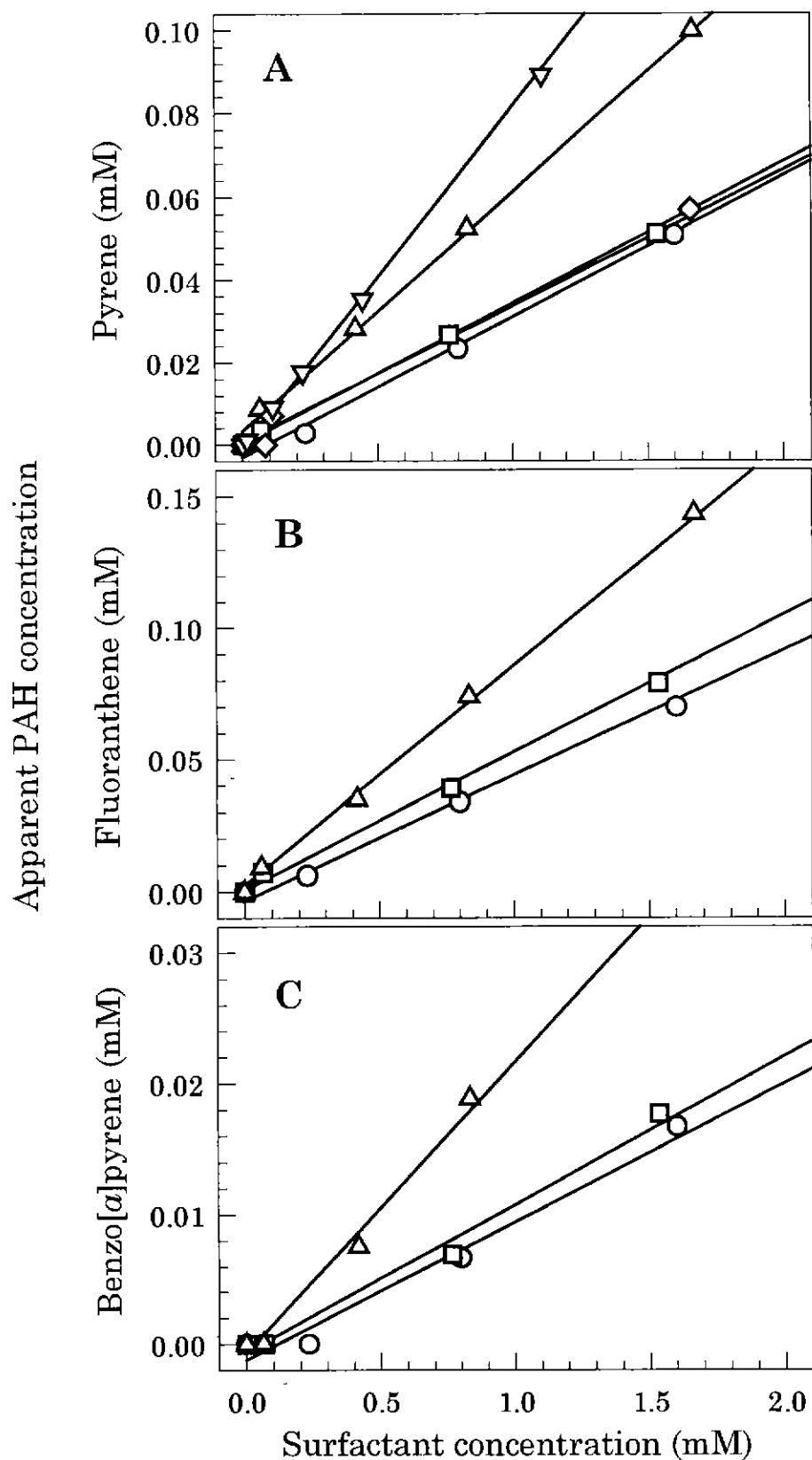


FIGURE 6.6 Solubilization of pyrene (A), fluoranthene (B) and benzo[a]pyrene (C) by nonionic surfactants. PAH solubility was measured in BSM at 30°C. Symbols: Δ , Brij 35; $\diamond$, Igepal CA-630; $\square$, Tergitol NP-10; $\circ$, Triton X-100; and ∇ , Tyloxapol. From Figure 6.5.

the CMC; and C_{SURF} is the surfactant concentration at which $S_{\text{PAH,MIC}}$ is evaluated (Edwards *et al.*, 1991).

The concentration of added PAHs in this study were well in excess of aqueous saturation concentrations, *i.e.* not limiting, therefore, the MSR value of each compound can be obtained from the slope of the curve generated from a plot of PAH concentration versus surfactant concentration for surfactant concentrations above CMC (Edwards *et al.*, 1991). There is a high correlation factor for all the curves shown in Figure 6.5 (greater than 0.999, 0.998 and 0.995 for pyrene, fluoranthene and benzo[*a*]pyrene respectively) and the corresponding MSR values for pyrene, fluoranthene and benzo[*a*]pyrene are shown in Table 6.6. Fluoranthene had the highest MSR value and the lowest MSR value was measured for benzo[*a*]pyrene.

An alternative approach in quantifying surfactant solubilized PAH is the determination of the partitioning of PAH between micelles and monomeric solution with a mole fraction micelle phase/aqueous phase coefficient (K_m). The K_m refers to the ratio of the mole fraction of the compound in the micelle pseudophase to the mole fraction of the compound in the aqueous phase (Edwards *et al.*, 1991). As presented in this experiment, K_m values were determined from the measured MSR values according to the following equation (Edwards *et al.*, 1991):

$$K_m = \frac{1}{V_w S_{\text{PAH, CMC}}} \left(\frac{\text{MSR}}{1 + \text{MSR}} \right)$$

$S_{\text{PAH,CMC}}$ is the apparent solubility of the PAH (mol l^{-1}) at surfactant CMC. V_w is the molar volume of water at 30°C *i.e.* $0.018015 \text{ mol l}^{-1}$ (Rogers and Mayhew, 1981). Log K_m values were determined for fluoranthene, pyrene and benzo[*a*]pyrene in the presence of each nonionic surfactant using the above equation (Table 6.6). The comparative log K_m values for each PAH varied

Table 6.6 Surfactant solubilization properties for pyrene, fluoranthene and benzo[*a*]pyrene.

Surfactant	MSR ^a			log K _m ^a		
	PYR	FA	BAP	PYR	FA	BAP
Brij 35	0.0570	0.0871	0.0198	5.54	5.68	7.78
Igepal CA-630	0.0326	–	–	5.39	–	–
Tergitol NP-10	0.0336	0.0502	0.0118	5.71	5.56	7.43
Triton X-100	0.0352	0.0508	0.0113	5.84	5.64	7.49
Tyloxapol	0.0820	–	–	6.60	–	–

^aThe molar solubilization ratio (MSR) and micelle-phase/aqueous-phase partition coefficient (K_m) were determined for pyrene (PYR), fluoranthene (FA) and benzo[*a*]pyrene (BAP) in BSM at 30°C.

– Not tested.

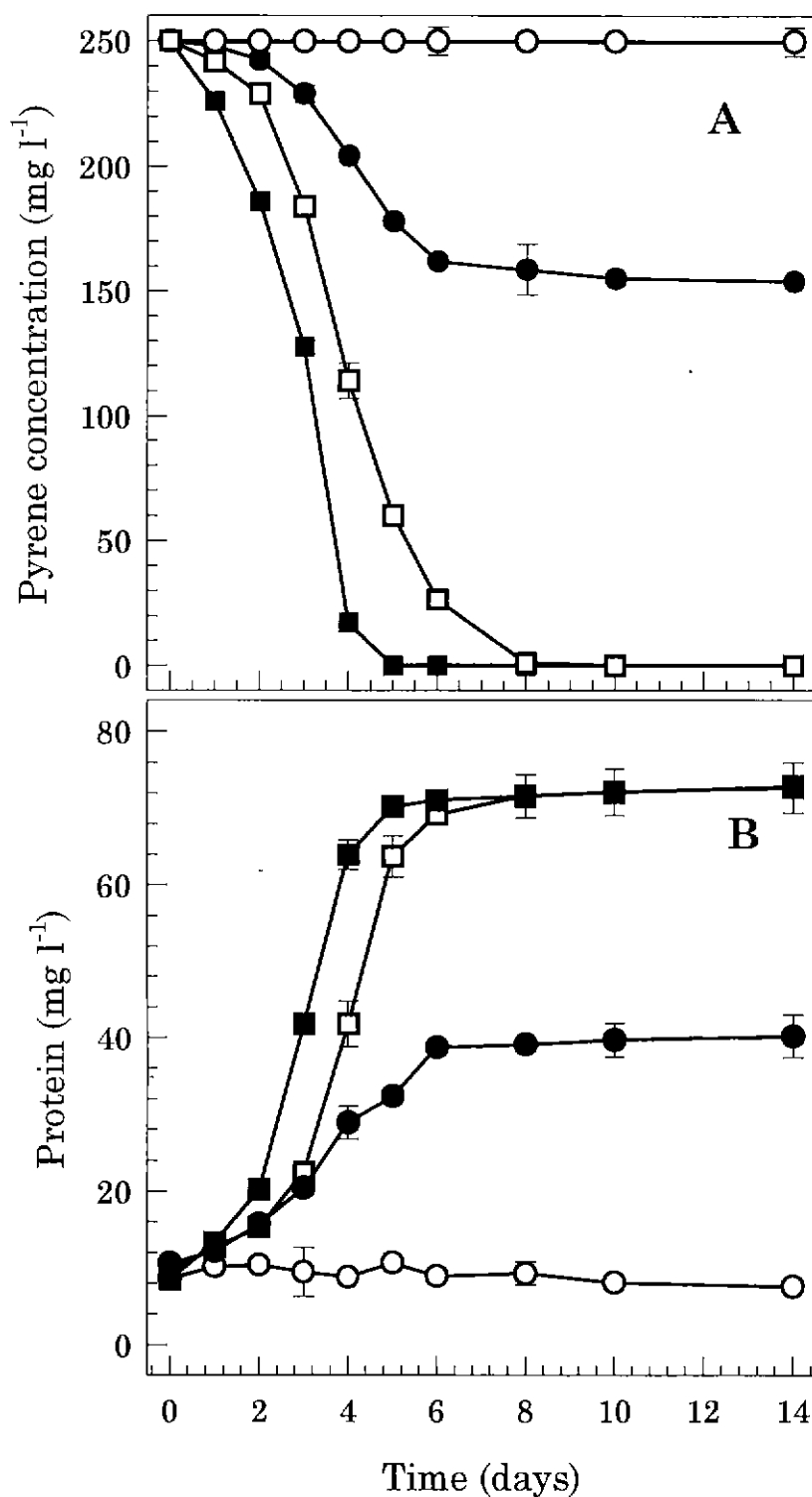


FIGURE 6.7 Pyrene degradation (A) by *S. maltophilia* VUN 10,010. Pyrene was added to the medium in one of two ways; pyrene crystals or presolubilized in DMF. The cultures contained the following: pyrene crystals in the presence (●) or absence (○) of Tergitol NP-10 (10 g l⁻¹); DMF-solubilized pyrene in the presence (■) or absence (□) of Tergitol NP-10 (10 g l⁻¹). The incubations were performed in BSM at 30°C using low initial cell populations (*ca.* 10⁴ cells ml⁻¹). Growth of VUN 10,010 (B) was determined as an increase in protein biomass.

depending on the surfactant with the only clear trend being the significantly higher values obtained for benzo[*a*]pyrene compared to fluoranthene and pyrene.

6.7 EFFECTS OF SURFACTANTS ON THE BIODEGRADATION OF HIGH MOLECULAR WEIGHT PAHs

6.7.1 Biodegradation of Crystalline and Surfactant-solubilized Pyrene

PAHs may be added to cultures either as relatively large crystals (referred to as 'PAH crystals') as provided by the supplier or first solubilized in DMF (referred to as DMF-solubilized PAHs) and then added to the incubation vessel; in the latter case, very fine and uniform PAH crystals are formed when added to BSM. Both forms of PAHs have been used in the literature regarding PAH biodegradation. A decision was required on which form to use for the experiments in this chapter, therefore, a preliminary experiment was conducted to examine the effect of PAH crystal size on the PAH biodegradation rate. In this experiment, pyrene was added to BSM in the form of large PAH crystals or presolubilized in DMF; *S. maltophilia* VUN 10,010 was then inoculated into these media (Figure 6.7). The degradation of pyrene crystals by strain VUN 10,010 was extremely slow in the absence of surfactant, however, the degradation rate of crystalline pyrene was substantially improved in the presence of Tergitol NP-10 (10 g l⁻¹). The degradation rate of DMF-solubilized pyrene by VUN 10,010 was significantly greater than the pyrene crystals whether the latter was in the presence or absence of surfactant, presumably due to the much higher surface area to mass ratio of the DMF-solubilized pyrene and hence increased bioavailability. The highest pyrene degradation rate occurred when Tergitol NP-10 (10 g l⁻¹) was added to cultures containing DMF-solubilized pyrene. In view of the effect of crystal surface area on PAH degradation rate, PAHs solubilized in DMF were used in all further experiments to ensure that consistent PAH crystal surface area to mass ratios were maintained within and between experiments.

6.7.2 High Molecular Weight PAH Biodegradation

The microbial degradation of high molecular weight PAHs in the presence of surfactants was first investigated by examining the effect of various surfactant types and concentrations on pyrene degradation. Once optimum surfactant conditions were determined for maximizing the pyrene degradation rate, these conditions were then used to test the effect of surfactants on the biodegradation of other high molecular weight PAHs. Unless otherwise stated, *S. maltophilia* VUN 10,010 was used in all these experiments.

In general, the degradation rate of DMF-solubilized pyrene was significantly improved by the addition of nonionic surfactants (5-10 g l⁻¹) (Figure 6.8). The time required for VUN 10,010 to degrade 250 mg l⁻¹ of pyrene was reduced by 50% in the presence of Brij 35, Tergitol NP-10 and Tyloxapol, but at the same concentrations (5-10 g l⁻¹) Igepal CA-630 was inhibitory to pyrene degradation. The growth rate, as determined by protein analysis, of VUN 10,010 on pyrene was significantly improved by Brij 35, Tergitol NP-10 and Tyloxapol, but inhibited by Igepal CA-630 (5-10 g l⁻¹).

As demonstrated previously, *S. maltophilia* VUN 10,010 mineralized [¹⁴C]pyrene to ¹⁴CO₂ when pyrene was supplied as a sole carbon and energy source in BSM (see Chapter 5, Section 5.5.1). The effect of surfactant on pyrene mineralization by *S. maltophilia* VUN 10,010 was evaluated by the addition of [¹⁴C]pyrene to cultures containing unlabeled pyrene (250 mg l⁻¹) and surfactant. The addition of 5 g l⁻¹ Brij 35 or Tergitol NP-10 significantly improved the pyrene mineralization rate (Figure 6.9). In all cultures, the total recovery of added ¹⁴C was close to 100%; this being comprised of 70-74% ¹⁴CO₂, the remainder being recovered as cell biomass (9-11%), in the aqueous phase (15-18%) and in the DCM phase after it was used for culture extraction (Table 6.7). The significantly improved pyrene-degrading performance of VUN 10,010 due to Brij 35 and Tergitol NP-10 prompted further investigation into the effect of these two surfactants on the degradation of other high molecular weight PAHs by this strain.

Figure 6.8 Pyrene degradation by *Stenotrophomonas maltophilia* VUN 10,010 in the presence of various surfactants. Apparent pyrene concentration (left hand panels) and cellular protein concentration (right hand panels) are shown for each culture. Each surfactant was added to the medium to achieve the following concentrations; 0.5 g l⁻¹ (▲), 5.0 g l⁻¹ (●) and 10.0 g l⁻¹ (■). The control culture (◇) contained no added surfactant. The incubations were performed in BSM at 30°C/175 rpm in the dark (the initial cell population was approximately 10⁴ cells ml⁻¹).

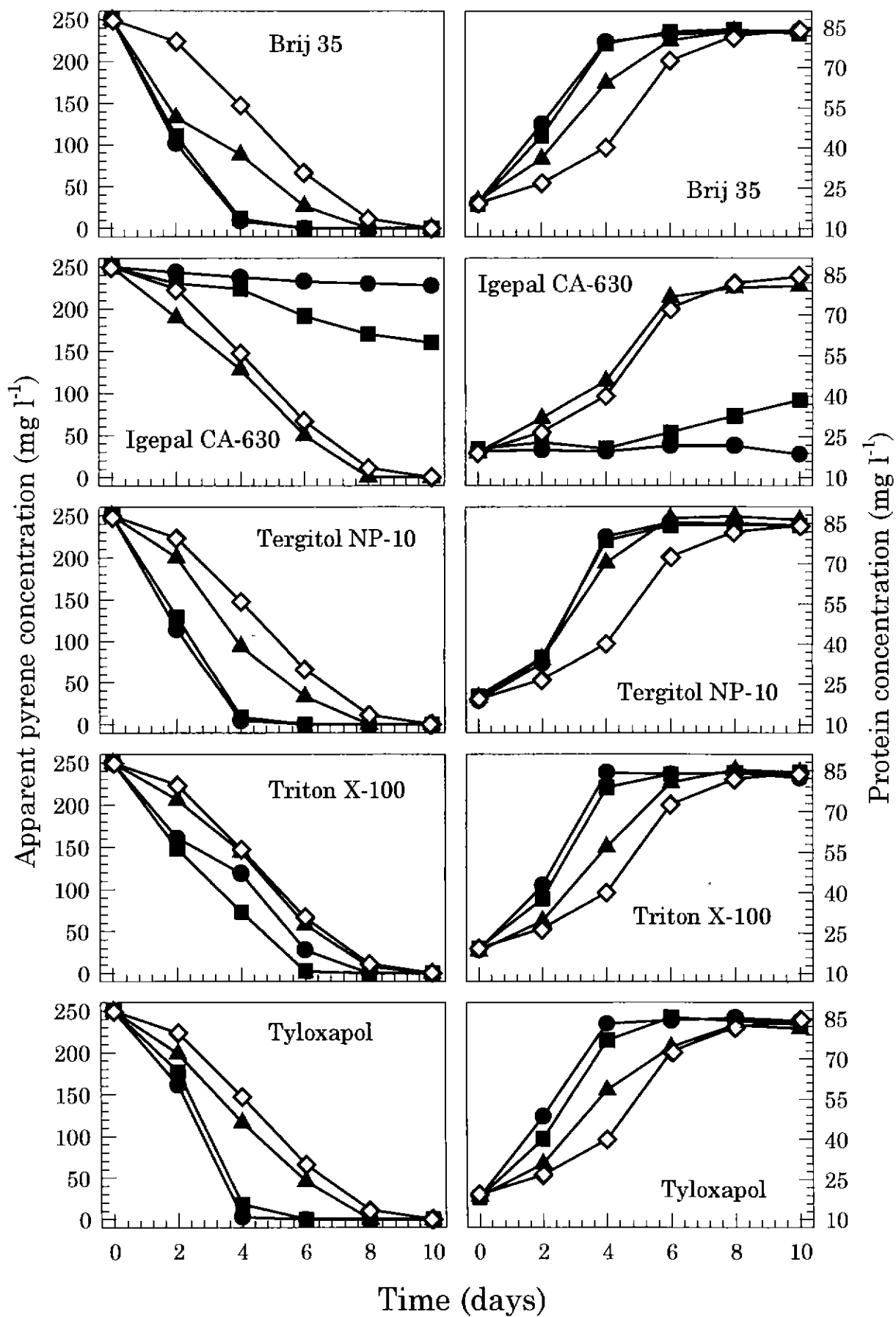


Table 6.7 Distribution of ^{14}C -residues after incubation of *S. maltophilia* VUN 10,010 with [^{14}C]pyrene in BSM at 30°C/175 rpm.

Condition	% radioactivity in the following fractions:				Total recovery (%)
	$^{14}\text{CO}_2$	Biomass	Aqueous phase	DCM phase	
HgCl ₂ control	2.6 ± 1.3	NA	NA	97.1 ± 2.3	99.7
No surfactant	70.2 ± 1.8	10.7 ± 3.0	16.8 ± 2.0	2.2 ± 0.6	99.9
Brij 35	73.2 ± 0.7	10.4 ± 1.6	15.4 ± 1.0	0.8 ± 0.4	99.8
Tergitol NP-10	72.8 ± 1.1	9.3 ± 1.6	17.6 ± 3.7	NA	99.7

NA, not appreciable.

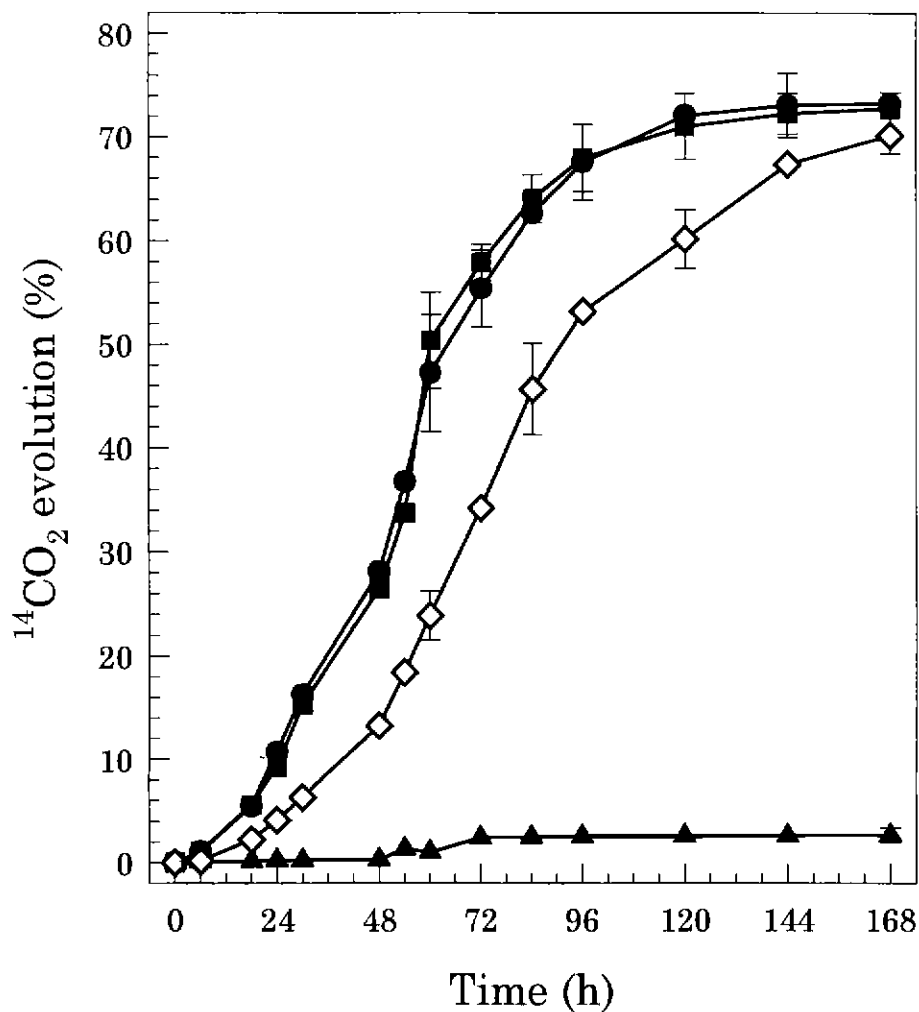


FIGURE 6.9 Pyrene mineralization by *S. maltophilia* VUN 10,010 in the presence of 5.0 g l⁻¹ Tergitol NP-10 (■), 5.0 g l⁻¹ Brij 35 (●) or in the absence of surfactant (◇). An inoculated control containing 2% HgCl₂ (▲) is also shown. The incubations were performed in BSM at 30°C/175 rpm in the dark using low cell populations (ca. 10⁴ cells ml⁻¹).

Brij 35 or Tergitol NP-10 (5 and 10 g l⁻¹) was added separately to BSM containing a single high molecular weight PAH (Figures 6.10 and 6.11). A high initial cell population (*ca.* 2 x 10⁶ cells ml⁻¹), prepared from washed inocula, was used since there was no substantial growth of strain VUN 10,010 on high molecular weight PAH compounds in the PAH degradation tests as described previously (Chapter 5, Section 5.3.1). The disappearance of most PAHs was substantially improved in the presence of Brij 35 or Tergitol NP-10. For a majority of the cultures, this was the result of both an increase in the maximum specific degradation rate (Table 6.8) and a decrease in the time taken for PAH degradation to commence (Figures 6.10 and 6.11). Benzo[*a*]pyrene was the exception, since the increase in the amount of this PAH degraded over the incubation period was largely due to a significant decrease in the degradation lag period. For all PAHs, an increase in Tergitol NP-10 concentration from 5 g l⁻¹ to 10 g l⁻¹ did not significantly improve the PAH degradation profile. Similar results were obtained when Brij 35 (5 g l⁻¹ and 10 g l⁻¹) was used (Table 6.8). Chrysene and coronene were the only PAHs which did not show a significant increase in their rate of disappearance when incubated in the presence of surfactants. PAH concentrations over the time course period were not significantly different between the uninoculated controls and the inoculated controls containing both HgCl₂ and surfactant.

6.7.3 Biodegradation of a PAH Mixture

PAH-contaminated soils usually contain a mixture of different PAHs which, in many cases, changes the degradation profile of each PAH from that observed when they are degraded as single PAH compounds. This is usually due to preferential substrate utilization and inhibitory effects on cell viability and growth caused by the greater toxicity of some PAHs. The potential of surfactants for the biodegradation of PAH-contaminated sites was further evaluated by investigating the effect of Tergitol NP-10 (5 g l⁻¹) on the degradation rate of a PAH mixture (Figure 6.12); a low initial cell population of around 9 x 10³ cells ml⁻¹ was used since strain VUN 10,010 has previously been shown to be able to grow on pyrene and/or phenanthrene as a growth substrate (Section 5.3.1). Although the specific growth rate of VUN 10,010 in this experiment was found to increase from 0.010 h⁻¹ (in the absence of

Figure 6.10 Degradation of high molecular weight PAHs by high initial cell populations ($ca. 2 \times 10^6$ cells ml^{-1}) of *S. maltophilia* VUN 10,010 in the absence of surfactant (○) and in the presence of 5.0 g l^{-1} (▲) or 10.0 g l^{-1} (■) Tergitol NP-10. Inoculated controls containing 0.2% $HgCl_2$ and 5 g l^{-1} Tergitol NP-10 (◆) are also shown. Incubations contained a single PAH in BSM at 30°C and 175 rpm in the dark. The average PAH concentration remaining was calculated as a percentage relative to the PAH recovered from the uninoculated control. Averages for the test culture are from triplicate experiments.

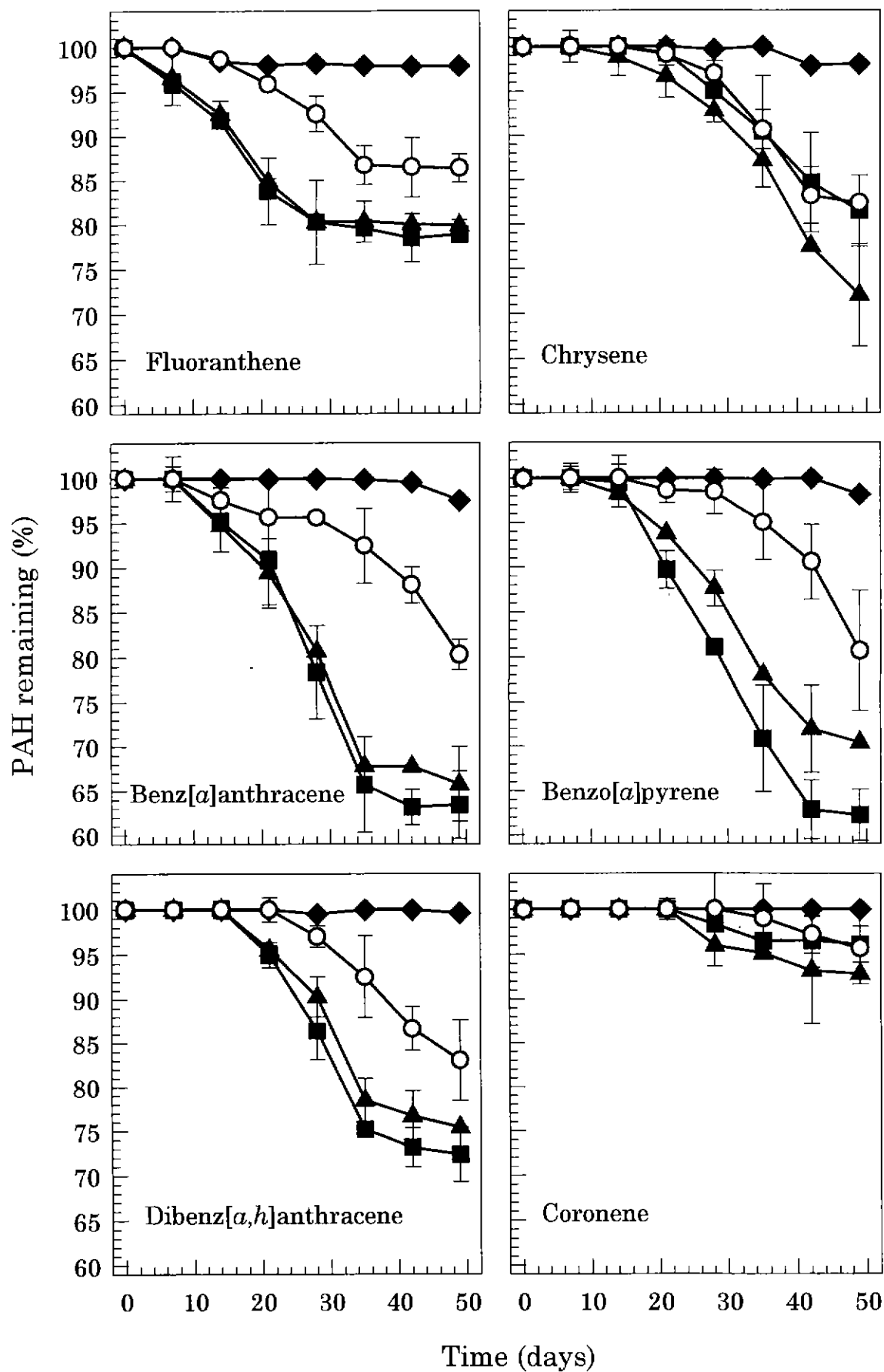


Figure 6.11 Degradation of high molecular weight PAHs by high initial cell populations ($ca. 2 \times 10^6$ cells ml^{-1}) of *S. maltophilia* VUN 10,010 in the absence of surfactant (○) and in the presence of 5.0 g l^{-1} (▲) or 10.0 g l^{-1} (■) Brij 35. Inoculated controls containing 0.2% $HgCl_2$ and 5 g l^{-1} Brij 35 (◆) are also shown. Incubations contained a single PAH in BSM at 30°C/175 rpm in the dark. The average PAH concentration remaining was calculated as a percentage relative to the PAH recovered from the uninoculated control. Averages for the test culture are from triplicate experiments.

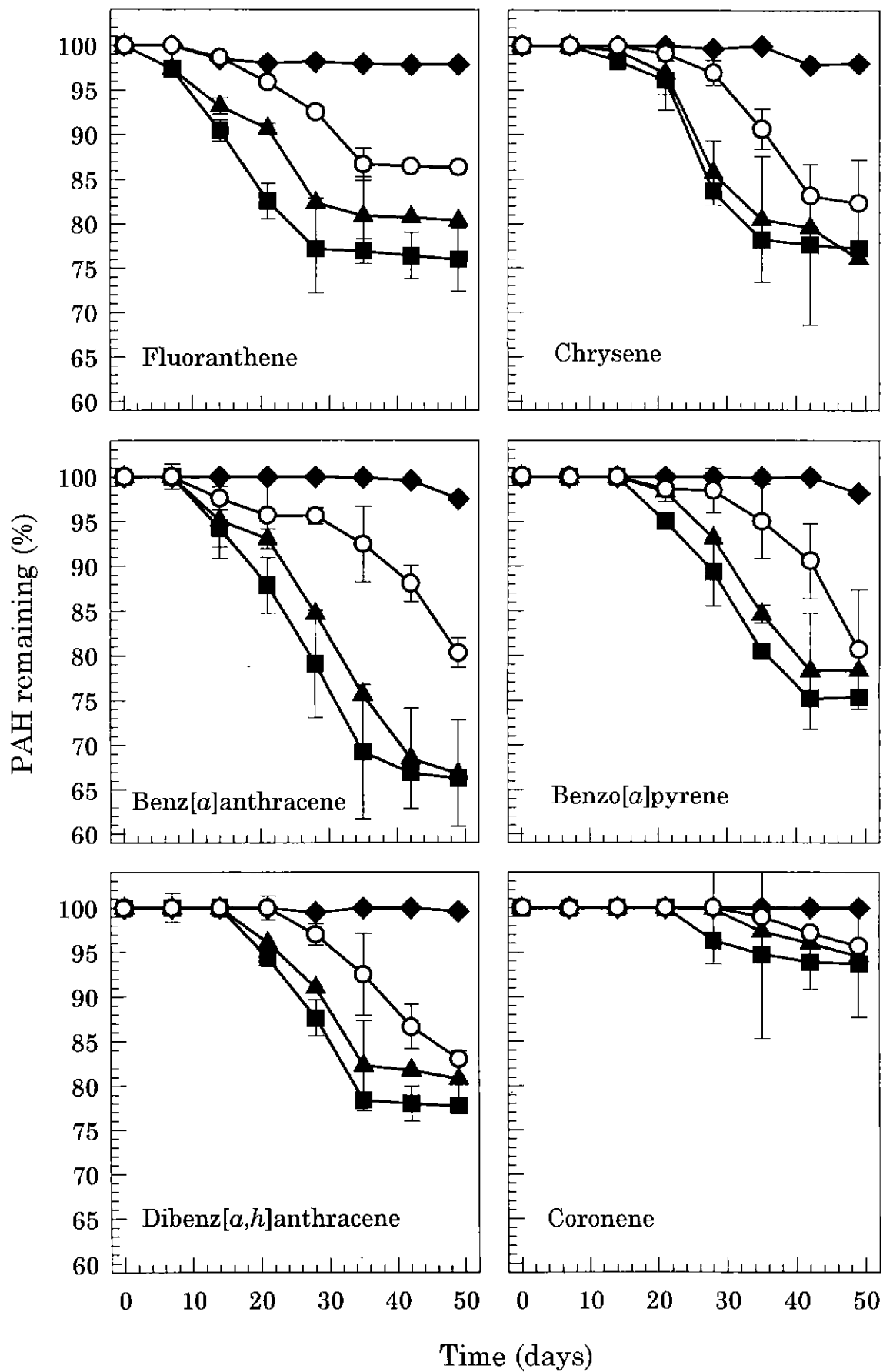


Table 6.8 The influence of surfactants on maximum specific degradation rates of high molecular weight PAHs.

PAH	Maximum Specific Degradation Rate (mg PAH degraded mg ⁻¹ protein day ⁻¹) ^a						
	No Surfactant		Tergitol NP-10 ^b			Brij 35 ^b	
	Single ^c	Mixed ^d	5 g l ⁻¹		10 g l ⁻¹	5 g l ⁻¹	10 g l ⁻¹
			Single ^c	Mixed ^d	Single ^c	Single ^e	Single ^e
Fluoranthene	2.90	1.02	4.54 (+57%)	1.11 (+9%)	4.59 (+58%)	3.71 (+28%)	12.11 (+318%)
Chrysene	4.49	1.42	6.44 (+43%)	2.76 (+94%)	4.16 (-7%)	7.35 (+64%)	7.93 (+77%)
Benzanthracene	4.41	1.71	9.66 (+119%)	3.25 (+90%)	11.14 (+153%)	7.18 (+63%)	7.37 (+67%)
Benzo[a]pyrene	6.21	1.43	6.73 (+8%)	4.30 (+201%)	7.90 (27%)	5.84 (-6%)	5.47 (-12%)
Dibenz[α, h]anthracene	4.17	1.20	7.32 (+76%)	2.89 (+140%)	8.74 (110%)	6.26 (+50%)	7.20 (+73%)
Coronene	0.75	--	0.98 (+31%)	—	0.79 (+5%)	0.79 (+5%)	1.19 (+59%)

^a *S. maltophilia* VUN 10,010 was used for all experiments.^b The percentage change in the PAH maximum specific degradation rate due to surfactant addition is shown in parenthesis.^c These figures were calculated from the data collected from the experiment shown in Figure 6.10.^d These figures were calculated from the data collected from the experiment shown in Figure 6.12.^e These figures were calculated from the data collected from the experiment shown in Figure 6.11.

-- Not appreciable.

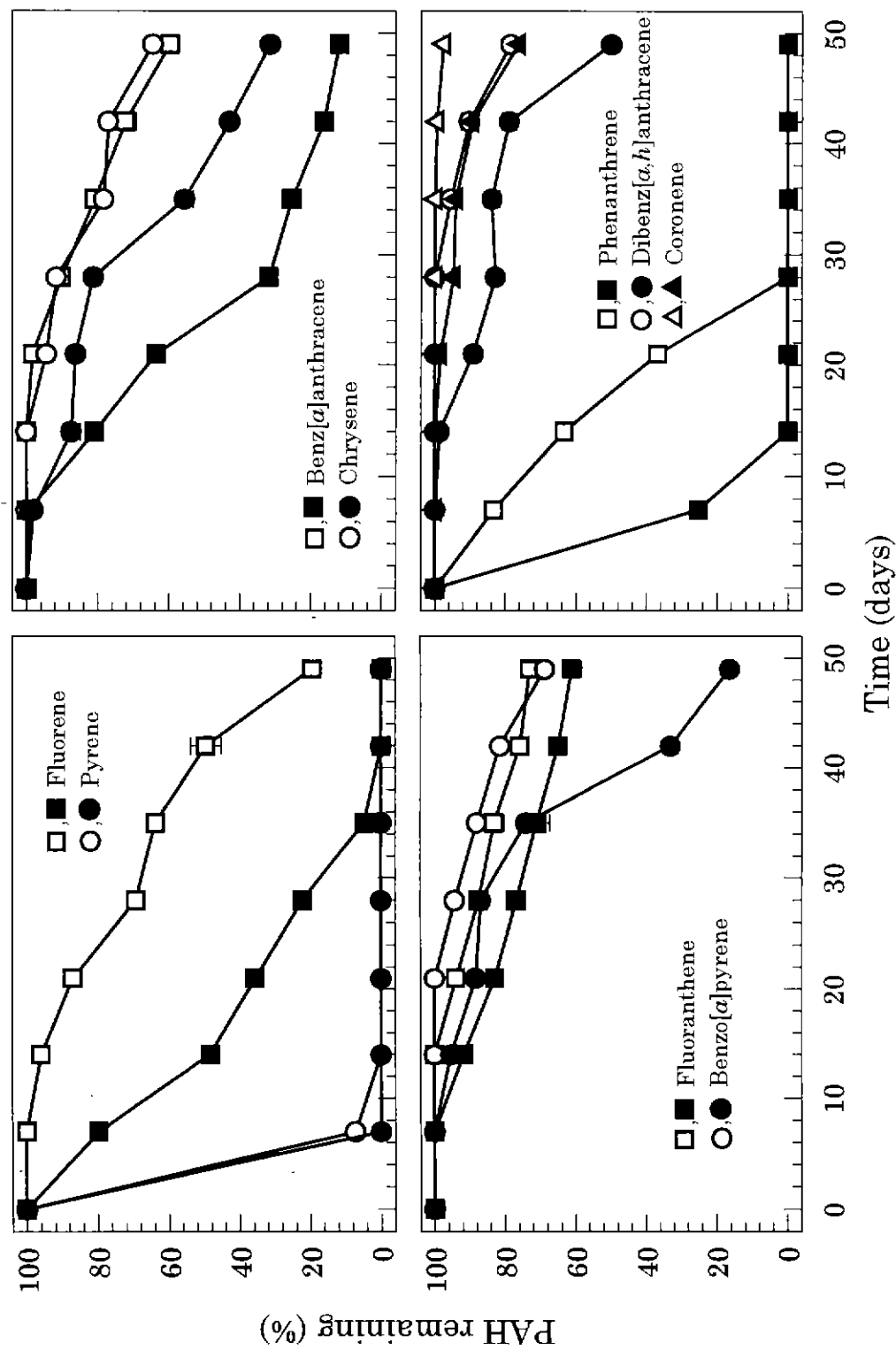


FIGURE 6.12 PAH degradation by *S. maltophilia* VUN 10,010 in a PAH mixture and either 5 g l⁻¹ Tergitol NP-10 (closed symbols) or no added surfactant (open symbols). The incubations were conducted in BSM at 30°C using a low initial cell population. The PAH remaining was calculated as a percentage relative to the PAH recovered from the uninoculated control.

surfactant) to 0.013 h^{-1} (in the presence of Tergitol NP-10), the final biomass yield was not affected by the surfactant. The overall degradation rate of each PAH in the PAH cocktail was substantially improved by the addition of Tergitol NP-10 and, for most of the PAHs, this was attributable to a decrease in the degradation lag period and an increase in the maximum specific degradation rate (Figure 6.12, Table 6.8); the lag period and specific degradation rate for pyrene could not be measured because there were few collected data points due to its rapid consumption. Strain VUN 10,010 was able to degrade the PAHs concurrently however the maximum specific degradation rate of each PAH was less than that observed when these compounds were present in BSM as a single compound (Table 6.8).

6.8 INFLUENCE OF SURFACTANT AND INOCULUM ADDITION ON THE BIODEGRADATION AND MINERALIZATION OF HIGH MOLECULAR WEIGHT PAHs IN SOILS

The results in Section 6.7 demonstrated that the degradation and mineralization rate of high molecular weight PAHs by *S. maltophilia* VUN 10,010 can be significantly stimulated by nonionic surfactants. Although this result is important for our understanding of the rate limiting steps in microbial PAH degradation, any predictions based on the results of the previous section regarding the effectiveness of surfactants in soil bioremediation programs must take into account the considerable differences between liquid and soil cultures. The effect of surfactants on PAH biodegradation in soils can be affected by many influences which were not present in the liquid culture work, such as the binding of PAHs to humic compounds, PAH partitioning into hydrophobic compartments in the soil and the effects of indigenous microorganisms and soil components on surfactant concentration, to name a few. With this in mind, it was considered important that the investigations of the previous section be extended to test the effects of surfactants on the biodegradation rate of high molecular weight PAHs in soil matrices.

Tergitol NP-10 was the only surfactant used in the soil experiments due to its proven superiority in stimulating the degradation rate of high molecular weight

Table 6.9 Efficiency of PAH extraction methods in the recovery of PAHs from soils.

PAH ^a	% PAH Recovery ^b			
	Soxhlet extraction ^{c*}	Sequential ultrasonication ^d		
		A*	B	C
Fluorene	99.9 ± 0.2	99.6 ± 0.2	99.5 ± 0.2	99.6 ± 0.1
Phenanthrene	100 ± 0.2	100 ± 0.4	99.9 ± 0.1	99.6 ± 0.4
Fluoranthrene	99.6 ± 0.2	99.7 ± 0.3	99.2 ± 0.8	99.5 ± 0.3
Pyrene	99.6 ± 0.0	99.5 ± 0.4	99.5 ± 0.2	99.8 ± 0.2
Benz[a]anthracene	99.2 ± 0.5	99.1 ± 0.7	98.9 ± 0.7	99.2 ± 0.9
Chrysene	98.6 ± 0.2	98.5 ± 1.5	98.5 ± 1.2	98.8 ± 0.6
Benzo[a]pyrene	99.3 ± 0.3	99.7 ± 0.3	99.4 ± 0.3	99.4 ± 0.4
Dibenz[a,h]anthracene	99.4 ± 0.2	98.3 ± 1.5	99.1 ± 0.5	98.9 ± 1.1
Coronene	98.3 ± 0.5	97.9 ± 0.2	97.0 ± 1.0	97.8 ± 0.9

^a Component of PAH-spiked soil.

^b %PAH recovery represents the average of each PAH compound recovered from triplicate samples, expressed as a percentage based on the known amount of each PAH added to the soil. The standard deviation between triplicate samples is also given.

^c PAHs were recovered from soil by Soxhlet extraction method (16 hours extraction with DCM) (see Chapter 3, Section 3.2.4.9).

^d PAHs were recovered from unamended PAH-spiked soil (A), PAH-spiked soil amended with 2% (w/w) Tergitol NP-10 (B), and PAH-spiked soil receiving 2%(w/w) Tergitol NP-10 and autoclaved fungal-bacterial co-culture (C) by the sequential ultrasonication extraction method. Data represent the total recovery of three successive extractions.

* Data taken from Table 5.1 (Chapter 5, Section 5. 2) for comparison.

PAHs (Section 6.7). Two types of PAH-containing soils were used in this study (see Section 3.1.1); a sterile uncontaminated soil artificially spiked with a PAH mixture (designated as 'PAH-spiked soil') and a non-sterile soil obtained from a PAH-contaminated site (designated as 'PAH-contaminated soil'). The PAH-spiked soil contained the following PAHs per kilogram of soil: fluorene (100 mg), phenanthrene (250 mg), fluoranthene (50 mg), pyrene (250 mg), benz[a]-anthracene (50 mg), chrysene (50 mg), benzo[a]pyrene (50 mg), and dibenz[a,h]-anthracene (50 mg), and coronene (25 mg).

6.8.1 PAH Extraction Efficiency for Soils Amended With Surfactant and Inoculum

The method adopted for the extraction of PAHs from soils must be highly efficient if accurate representation of the PAH concentration in soil samples are to be obtained. The addition of surfactants to the liquid cultures in Section 6.2 resulted in a decrease in the recovery of PAHs from the medium using a one step DCM-extraction procedure. To overcome this fall in recovery, a sequential ultrasonication DCM extraction method was developed which recovered greater than 99% of the PAHs from the liquid medium. This method was also successfully used to recover PAHs from PAH-spiked soil as described earlier in Chapter 5 (Section 5.2.3).

As shown in Table 6.9, by comparison to the standard Soxhlet extraction method, the use of sequential ultrasonication procedures to extract PAHs from soil containing surfactant and/or killed inocula resulted in the recovery of relatively high percentages of each PAH compound (approximately 97% to greater than 99%). PAH extraction efficiency was test on soils which had been spiked with PAHs for 48 hours. Statistical analysis (Student's t-test at 95% confidential level) of the data obtained from the recovery of PAHs from soils using the sequential DCM-extraction technique failed to recognize a significant effect on PAH recovery arising from the addition of either a microbial inoculum (fungal and bacterial biomass) or surfactant to soil (Table 6.9). The recovery of PAHs from soil inoculated with a killed co-culture of high population size and in the presence of

Tergitol NP-10 (2% w/w) was similar to those obtained from abiotic controls containing no surfactant. The presence of a microbial inoculum and surfactant in the soil was considered to have a negligible effect on the recovery of PAHs from these soil using the sequential DCM-extraction method.

6.8.2 Desorption of PAHs from Soil

It could not be assumed that the optimum surfactant concentration used in the liquid culture experiments (Section 6.7) would also be the optimum concentration, on a ppm basis, for the soil experiments due to surfactant absorption to soil particles. A higher surfactant concentration was most likely needed in order to overcome the loss of available surfactant for PAH mobilization. Tests were therefore conducted to optimize surfactant concentration for the desorption of pyrene and benzo[*a*]pyrene from soil slurries. Tergitol NP-10 was the mobilizing agent used in these experiments due to its superiority in stimulating PAH biodegradation in liquid cultures (Section 6.7.2). The experiments were conducted with a soil slurry prepared from soil containing a radiolabeled PAH compound (either ^{14}C -pyrene or ^{14}C -benzo[*a*]pyrene) and to which was added either 0.5, 1.0, 2.0 or 5.0% (w/v) Tergitol NP-10 (see Chapter 3, Section 3.2.4.8). The aqueous phase of the slurry was sampled and measured for radioactivity as a measure of the amount of PAH desorbed from the soil into aqueous phase.

The results show that the addition of 0.5% (w/v) Tergitol NP-10 to the soil resulted in only small quantities of PAH being desorbed from the soil matrix, benzo[*a*]pyrene in particular (Figures 6.13 and 6.14). Increasing the Tergitol NP-10 concentrations to 1, 2 and 5% (w/v) appreciably increased the desorption of pyrene and benzo[*a*]pyrene from the soil compared to soil which contained no surfactant (Figures 6.13 and 6.14). The rate and yield of PAH desorbed from the soil increased with increasing surfactant concentration. At 5% (w/v) Tergitol NP-10, approximately 22% and 18% of the initial radiolabeled carbon in [^{14}C]pyrene was obtained in the aqueous phase from PAH-spiked soil and PAH-contaminated soil, respectively (Figure 6.13). The same concentration of Tergitol NP-10 (5%, w/v) solubilized into the aqueous phase approximately 9% and 5% of initial

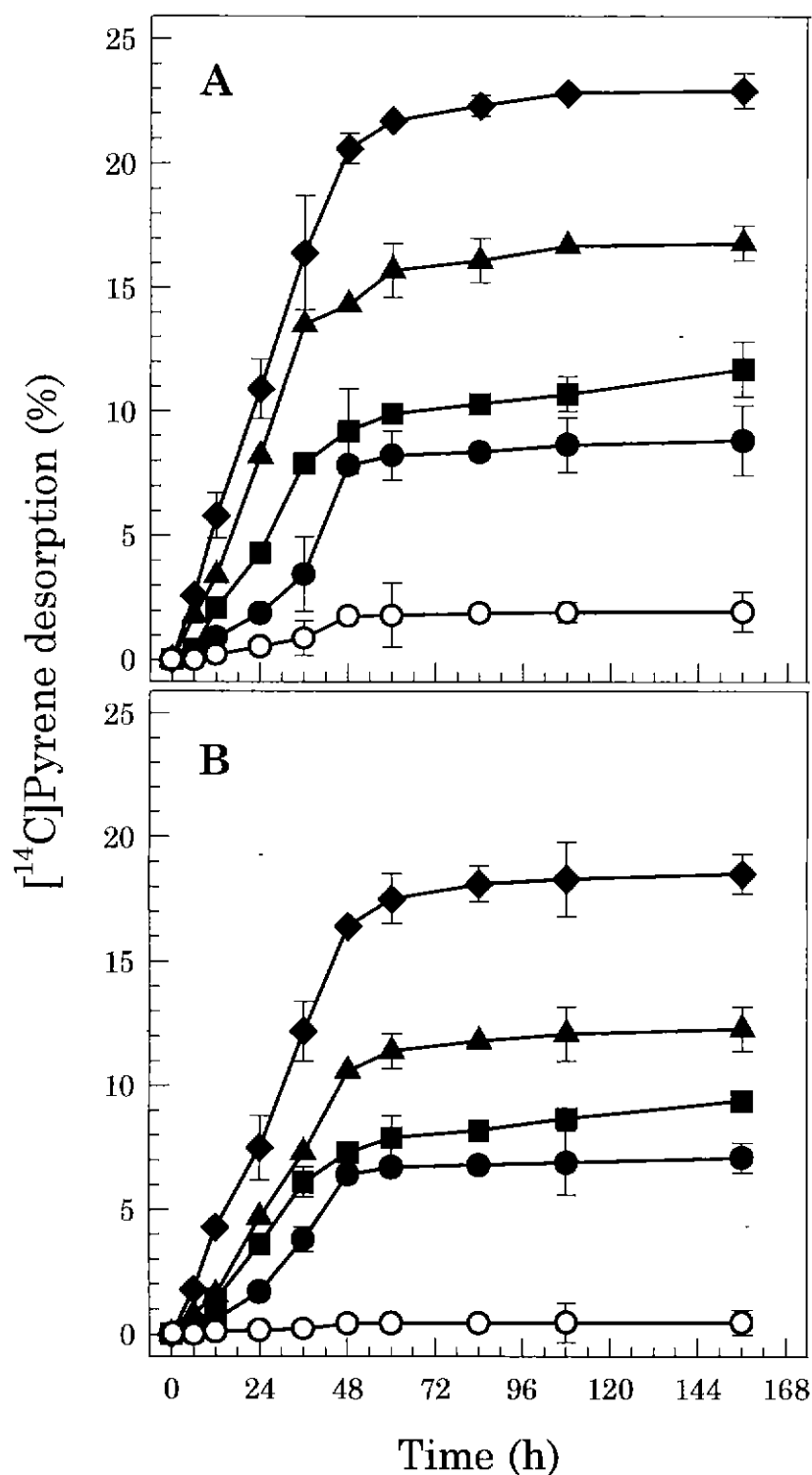


FIGURE 6.13 Desorption of $[^{14}\text{C}]$ pyrene from PAH-spiked soil (A) and PAH-contaminated soil (B) amended with Tergitol NP-10 at a concentration (w/v) of 0.5% (●), 1.0% (■), 2.0% (▲) and 5.0% (◆). Controls for the respective soils contained no surfactant (○). The data represents $[^{14}\text{C}]$ pyrene concentrations in the aqueous phase of the soil slurries (see Chapter 3, Section 3.2.4.8). Pyrene desorption is given as the percentage of $[^{14}\text{C}]$ pyrene in the aqueous phase relative to added ^{14}C .

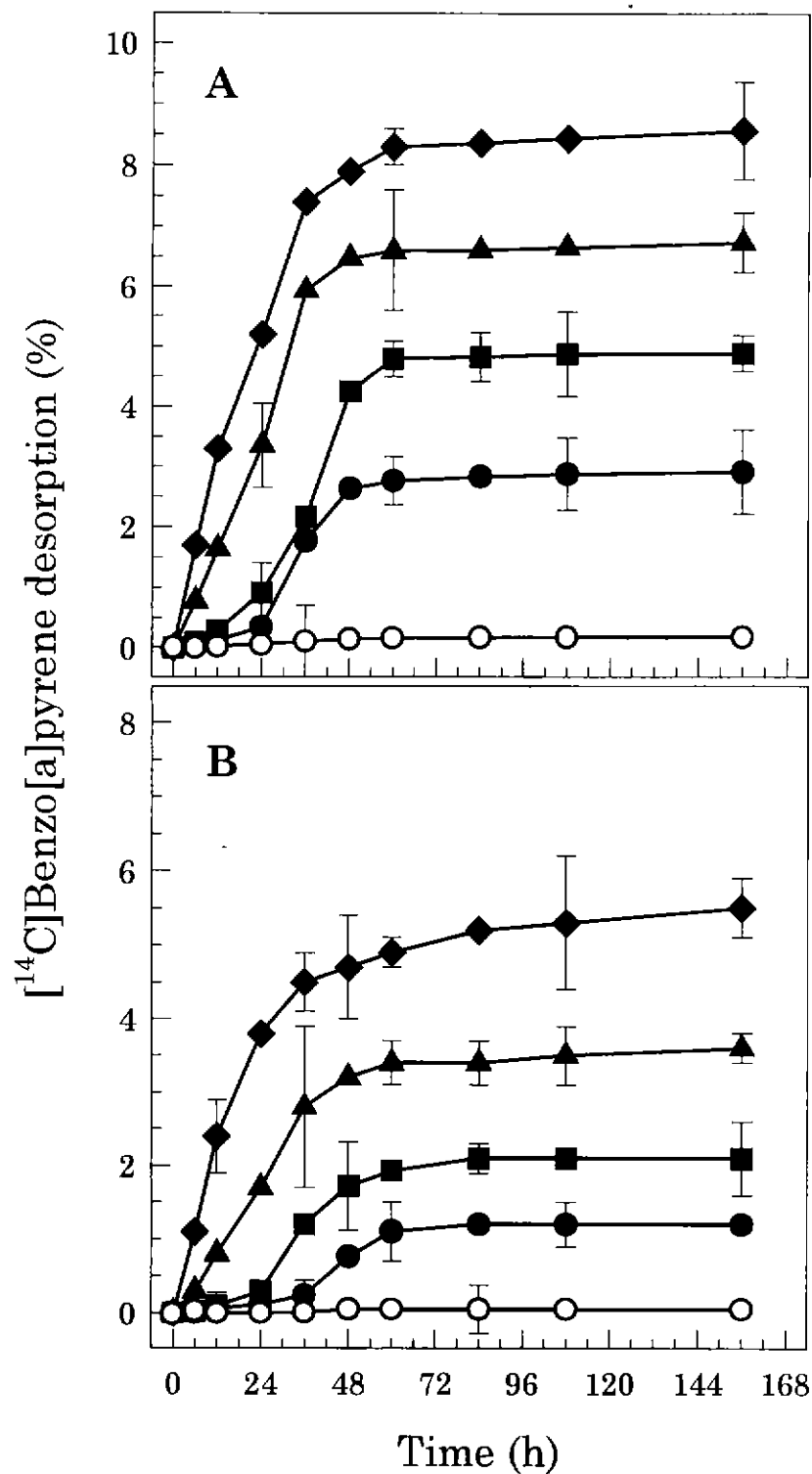


FIGURE 6.14 Desorption of [^{14}C]benzo[*a*]pyrene from PAH-spiked soil (A) and PAH-contaminated soil (B) amended with Tergitol NP-10 at a concentration (w/v) of 0.5% (●), 1.0% (■), 2.0% (▲) and 5.0% (◆). Controls for the respective soils contained no surfactant (○). The data represents [^{14}C]benzo[*a*]pyrene concentrations in the aqueous phase of the soil slurries (see Chapter 3, Section 3.2.4.8). Pyrene desorption is given as the percentage of [^{14}C]pyrene in the aqueous phase relative to added ^{14}C .

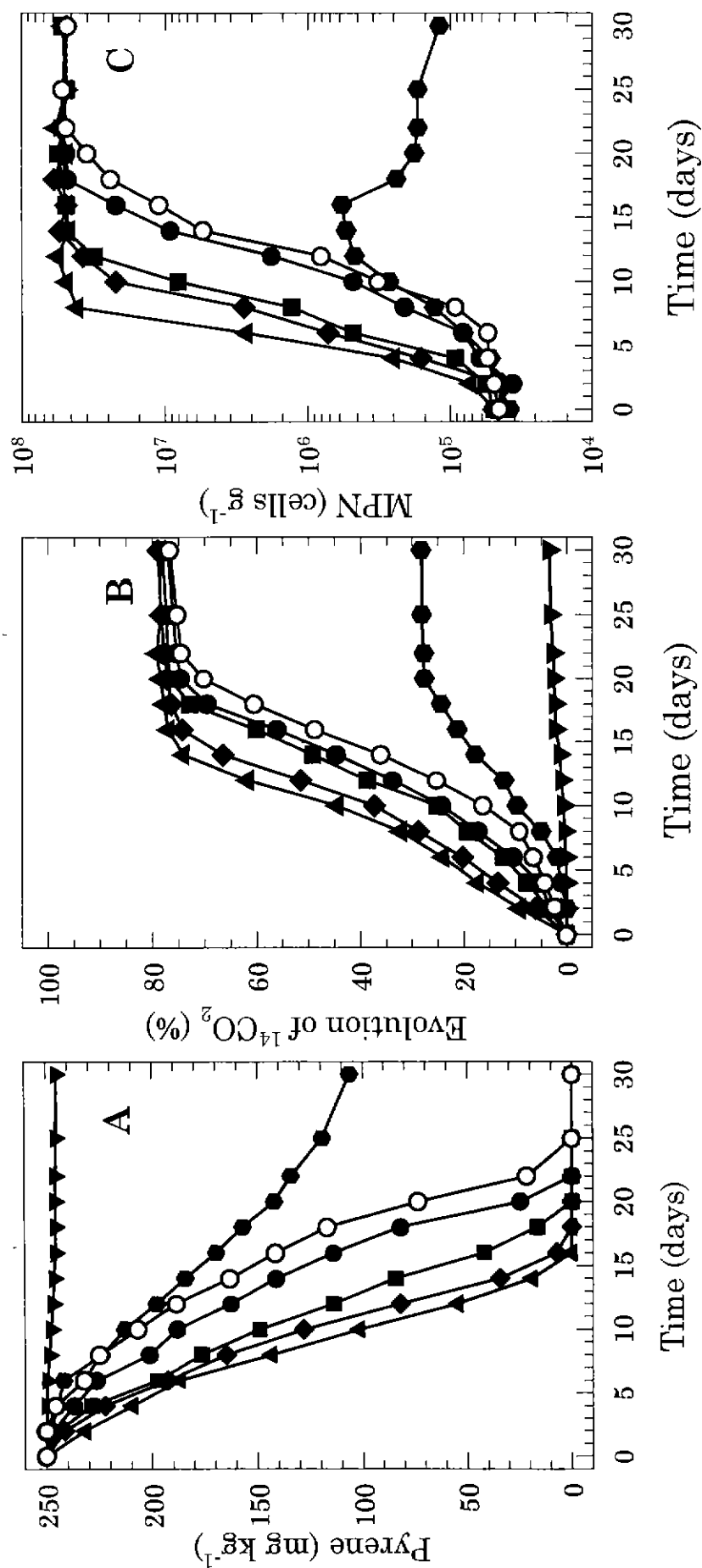


FIGURE 6.15 The effect of Tergitol NP-10 on pyrene degradation (A) and mineralization (B) by *S. maltophilia* VUN 10,010 in PAH-spiked soil. Bacterial growth (C) was monitored over 30 days. Tergitol NP-10 was added at the following concentrations (w/w): 0.5% (●), 1.0% (■), 2.0% (▲), 5.0% (◆) and 10.0% (○). A control without added surfactant (○) was also used. Inoculated controls containing 0.7% HgCl₂ (▼) are also shown. Incubations were performed at 25°C in the dark.

radiolabeled carbon in [^{14}C]benzo[*a*]pyrene from PAH-spiked soil and PAH-contaminated soil, respectively (Figure 6.14). This lower result was most likely due to the greater hydrophobicity and lower aqueous solubility of benzo[*a*]pyrene compared to pyrene. The amount of ^{14}C recovered in the aqueous phase was always lower in the PAH-contaminated soil compared to the PAH-spiked soil for both PAHs and for all surfactant concentrations used.

6.8.3 Surfactant Toxicity in Soils

The concentrations of Tergitol NP-10 found to be effective at enhancing PAH desorption from soils were much higher than those used in liquid media. Such high surfactant concentrations could potentially have an adverse effect on the viability of introduced and indigenous soil microbes. Therefore, an experiment was conducted to evaluate the effects of Tergitol NP-10 at various concentrations on viability and pyrene-degradative ability of *S. maltophilia* VUN 10,010, bacterial consortium VUN 10,009, *P. janthinellum* VUO 10,201 and the indigenous microorganisms in PAH-contaminated soil and PAH-spiked soil. The rate and extent of pyrene degradation and mineralization by strain VUN 10,010 and bacterial consortium VUN 10,009 and their population over time were monitored in the PAH-spiked soil; the rate and extent of pyrene degradation only by *P. janthinellum* VUO 10,201 was also evaluated in the PAH-spiked soil. The effect of Tergitol NP-10 on the activity of soil indigenous microorganisms was evaluated in the PAH-contaminated soil by monitoring pyrene mineralization.

The addition of Tergitol NP-10 at concentrations in the range 0.5-5.0% (w/w) to soils stimulated the rate of pyrene degradation and mineralization by VUN 10,010 (Figure 6.15), bacterial consortium VUN 10,009 (Figure 6.16) and the indigenous microbes (Figure 6.18) in PAH-contaminated soil. The growth rate of VUN 10,010 and VUN 10,009 also increased proportionally with surfactant concentrations in the range 0.5-5.0% (w/w). PAH-spiked soil inoculated with *P. janthinellum* VUO 10,201 had a similar increase in pyrene degradation when the surfactant concentration was increased (Figure 6.17). The degradation of pyrene in soil by axenic *P. janthinellum* inocula was unexpected given that it could not degrade this

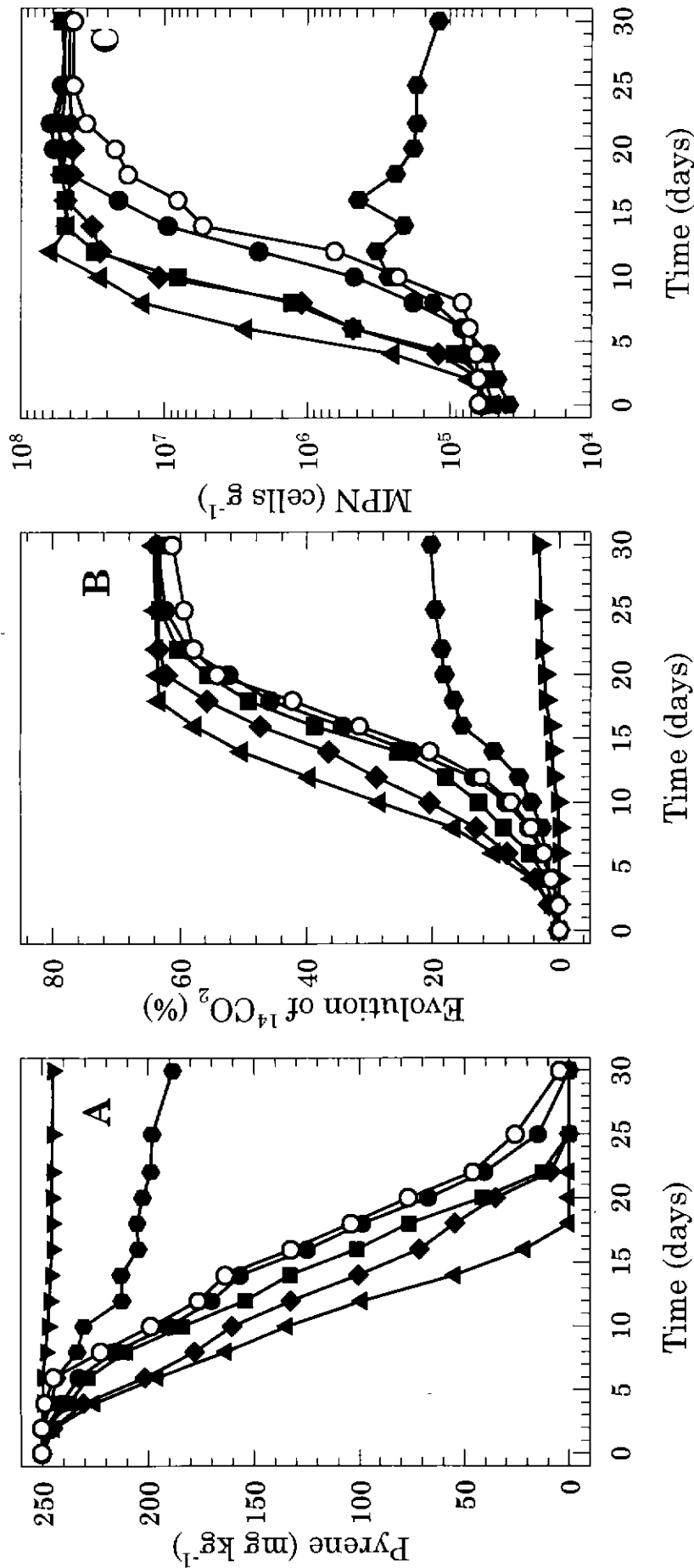


FIGURE 6.16 The effect of Tergitol NP-10 on pyrene degradation (A) and mineralization (B) by bacterial consortium VUN 10,009 in PAH-spiked soil. Bacterial growth (C) was monitored over 30 days. Tergitol NP-10 was added at the following concentrations (% w/w): 0.5% ($\bullet$), 1.0% ($\blacksquare$), 2.0% ($\blacktriangle$), 5.0% ($\blacklozenge$) and 10.0% ($\circ$). A control without added surfactant ($\circ$) was also used. Inoculated controls containing 0.7% HgCl_2 ($\blacktriangledown$) are also shown. Incubations were performed at 25°C in the dark.

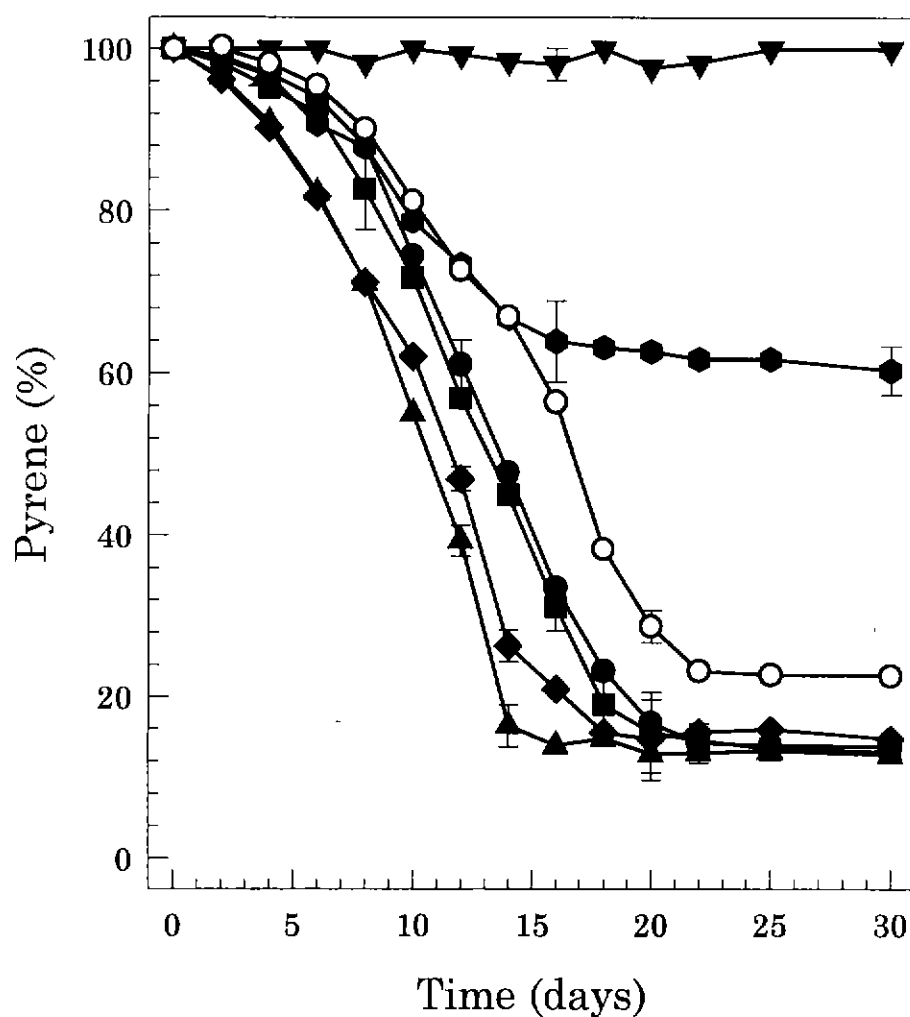


FIGURE 6.17 Effect of Tergitol NP-10 on pyrene degradation by *P. janthinellium* VUO 10,201 in PAH-spiked soil. The concentrations of Tergitol NP-10 employed were (w/w); 0.5% (●), 1% (■), 2% (▲), 5% (◆) and 10% (⬢). Killed-cell controls (▼) comprised 0.7% HgCl₂-killed fungi inoculated into PAH-spiked soil. Controls containing no added surfactant (○) was also used. The soil cultures were incubated at 25°C in the dark. Pyrene remaining is given as a percentage of the initial pyrene concentration.

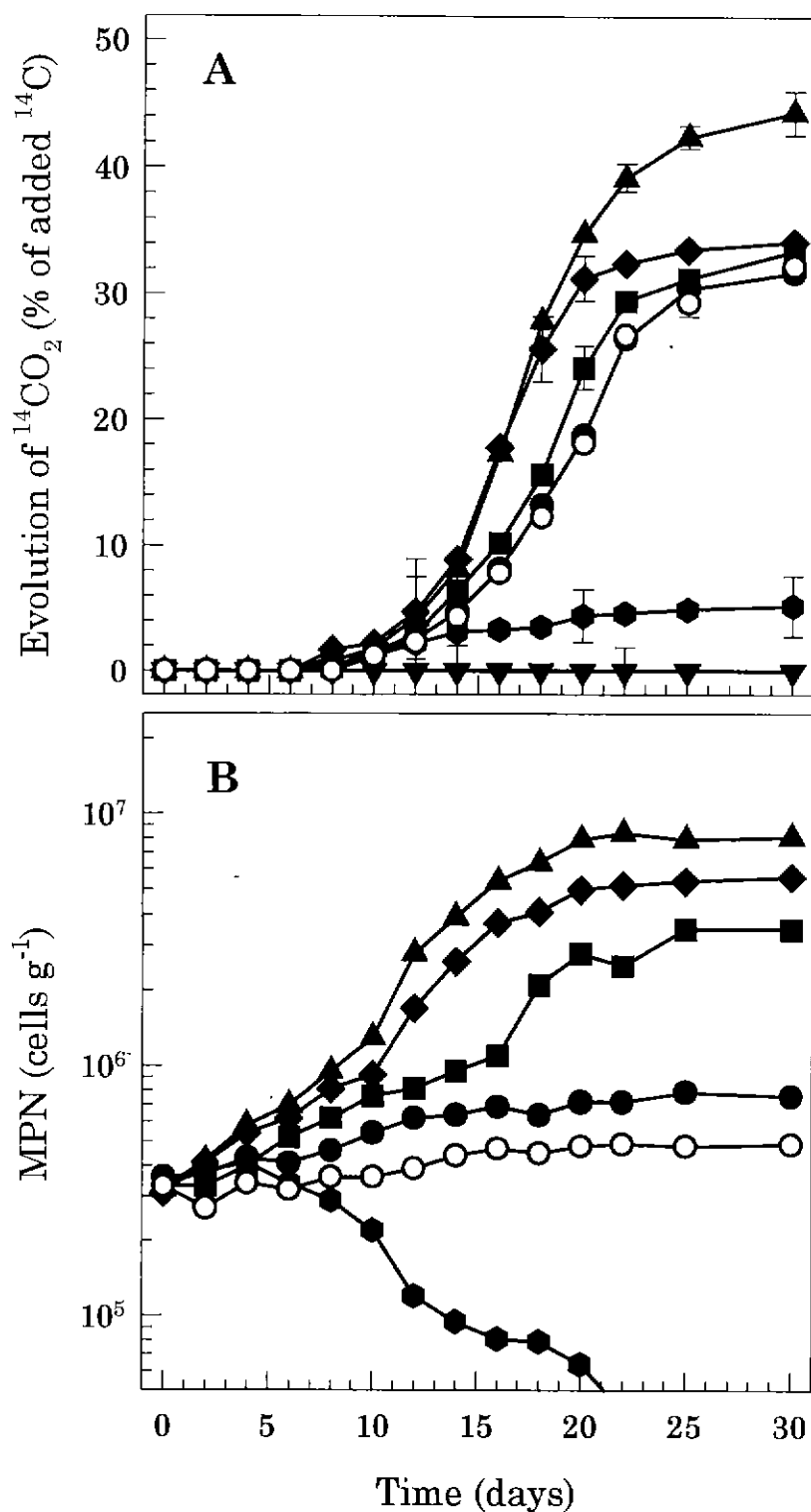


FIGURE 6.18 Effect of Tergitol NP-10 on pyrene mineralization (A) by indigenous microbes in PAH-contaminated soil. Estimated numbers of soil indigenous bacterial population is also shown (B). The concentrations of Tergitol NP-10 employed were (w/w); 0.5% (●), 1% (■), 2% (▲), 5% (◆) and 10% (⬤). The killed-cell control (▼) was PAH-contaminated soil containing 0.7% HgCl_2 . Controls containing no added surfactant (O) were also used. The soil cultures were incubated at 25°C in the dark.

compound in BSM (see Chapter 5, Section 5.3.2). However, it was able to degrade pyrene in MYPD and BSMG cultures, suggesting that pyrene degradation in the PAH-spiked soil was most likely co-metabolic with soil organic material. In the case of PAH-contaminated soil, increasing the surfactant concentration up to 5% (w/w) not only enhanced the pyrene mineralization rate but also the amount of pyrene mineralized by the indigenous microbes (Figure 6.18). A parallel set of soil cultures amended with the same surfactant concentrations demonstrated a greater than ten fold increase in indigenous bacterial population from approximately 3.2×10^5 to 8.1×10^6 cells g^{-1} soil compared to a control containing no surfactant with a population of $3.3\text{--}4.9 \times 10^5$ cell g^{-1} soil after 30 days of incubation (Figure 6.18).

Tergitol NP-10 at 2% (w/w) and 5% (w/w) significantly enhanced the microbial growth rate and pyrene degradation compared to soil without added surfactant, however, 10% (w/w) Tergitol NP-10 had an adverse effect on microbial growth and pyrene degradation. The addition of 10% (w/w) Tergitol NP-10 to PAH-spiked soil inhibited the growth of strain VUN 10,010 (Figures 6.15) and bacterial consortium VUN 10,009 (Figure 6.16). The population of indigenous bacteria in PAH-contaminated soil also decreased from an initial population of 3×10^5 cells g^{-1} soil to approximately 8×10^3 cells g^{-1} soil after a 30 day incubation period (Figure 6.18). Furthermore, pyrene degradation by either introduced microorganisms or the indigenous microbes was also adversely affected by 10% (w/w) Tergitol NP-10. Conclusions drawn from this data was that 2% and 5% (w/w) Tergitol NP-10 are optimum concentrations for the stimulation of pyrene biodegradation in soil. At these concentrations no toxic or inhibitory effects were observed on microbial growth or pyrene degradation. The results for 2% and 5% (w/w) Tergitol NP-10 were not significantly different, however, in all cases the trend was for better growth and pyrene degradation in the presence of 2% (w/w) of Tergitol NP-10. With this in mind, 2% (w/w) Tergitol NP-10 was chosen as the optimum concentration for further surfactant-related biodegradation experiments in soil.

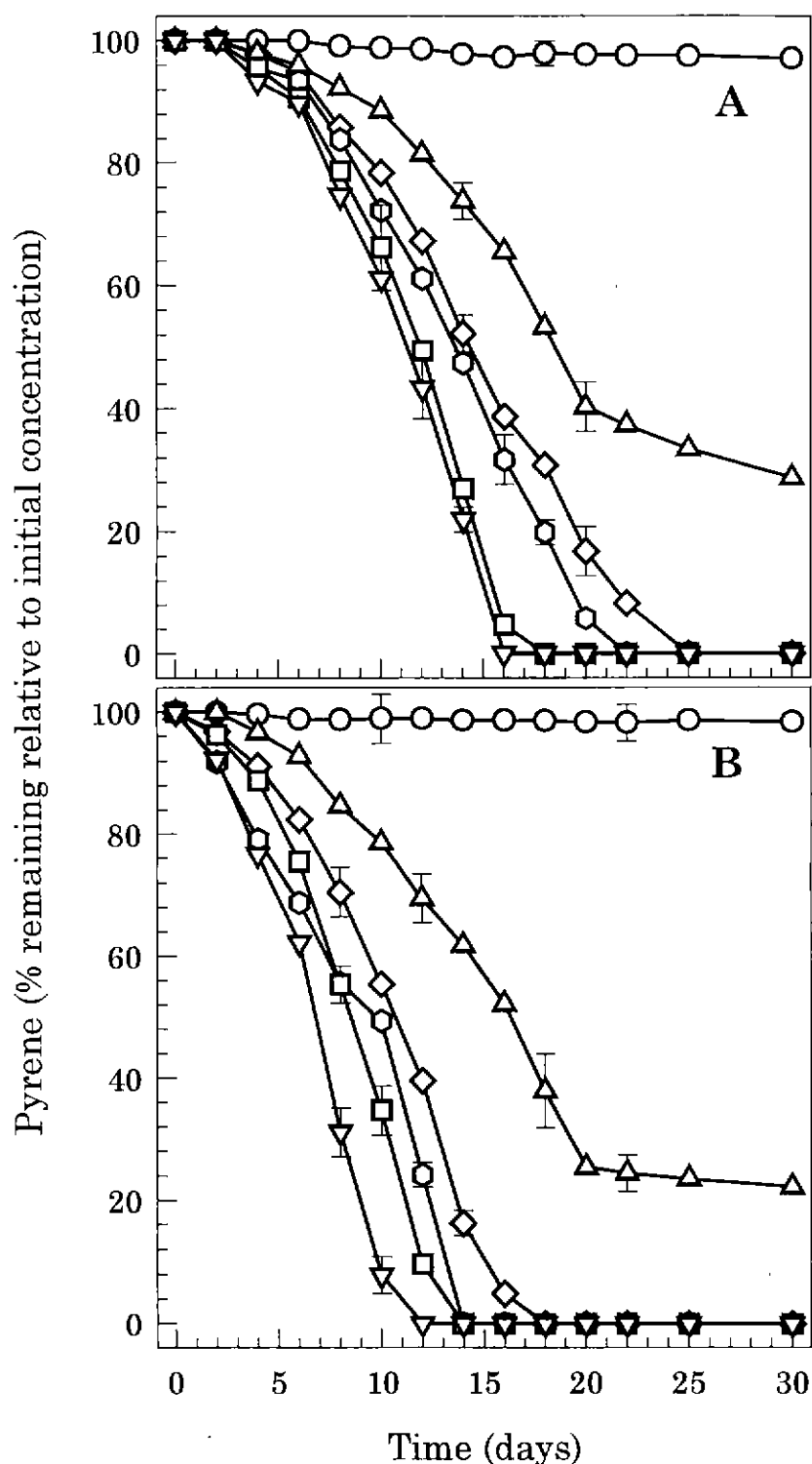


FIGURE 6.19 Fate of pyrene in PAH-spiked soil in the absence (A) and presence (B) of Tergitol NP-10 (2%, w/w). Soil was inoculated with killed cells (O), bacterial consortium VUN 10,009 (◇), *S. maltophilia* VUN 10,010 (◐), *P. janthinellum* VUO 10,201 (Δ), co-culture A (◻) or co-culture B (▽). The soils were incubated at 25°C in the dark. The data presented in this figure complements the experiments described in Figures 6.20 and 6.21; it is presented here to provide more detail on pyrene degradation in a PAH mixture during the first 30 days.

6.8.4 Biodegradation of High Molecular Weight PAHs in PAH-Spiked Soil

The fate of several high molecular weight PAHs was investigated in sterile PAH-spiked soil inoculated with either bacteria (VUN 10,009 or VUN 10,010), a fungus VUO 10,201 or fungal-bacterial co-cultures (co-culture A or co-culture B). These PAH degradation experiments were conducted in PAH-spiked soil in the absence and presence of Tergitol NP-10 (2% w/w) for a 100 day incubation period. The PAH mixture in the soil contained fluorene (100 mg kg⁻¹ soil), phenanthrene and pyrene (250 mg kg⁻¹ soil), benz[a]anthracene, chrysene, benzo[a]pyrene, and dibenz[a,h]anthracene (50 mg kg⁻¹ soil), and coronene (25 mg kg⁻¹ soil). In the absence of surfactant, as shown in Chapter 5 (Section 5.8.1), the degradation rate of pyrene as a single substrate and other high molecular weight PAHs in a PAH mixture was greater in PAH-spiked soil inoculated with the co-cultures than soil inoculated with either *P. janthinellum* VUO 10,201, bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010 (Table 6.10, Figures 6.19-6.21). With no added surfactant, the degradation rate of pyrene in PAH-spiked soil was higher using bacterial inocula compared to the fungus (Figure 6.19), but the fungus degraded all other high molecular weight PAHs more quickly than an inoculum composed of either bacterial strains (Table 6.11, Figure 6.20 and 6.21).

The addition of Tergitol NP-10 (2% w/w) to PAH-spiked soil significantly increased the degradation rate of all PAHs by inocula containing either the bacteria, fungus or co-cultures (Tables 6.10 and 6.11, Figures 6.20 and 6.21). One exception was the marginal increase in the pyrene degradation rate by axenic culture of *P. janthinellum* inocula (Table 6.11) which implies that pyrene bioavailability was not a rate limiting step in the degradation of this compound by the fungus. For other high molecular weight PAHs, the removal rate in the absence or presence of surfactant by the microbial inocula proceeded according to the following order from the highest to the lowest: co-culture B > co-culture A > *P. janthinellum* VUO 10,201 > *S. maltophilia* VUN 10,010 > bacterial consortium VUN 10,009 (Table 6.11). For example, approximately 81% of the added benzo[a]pyrene was removed from PAH-spiked soil inoculated with co-culture B after 100 days compared to the

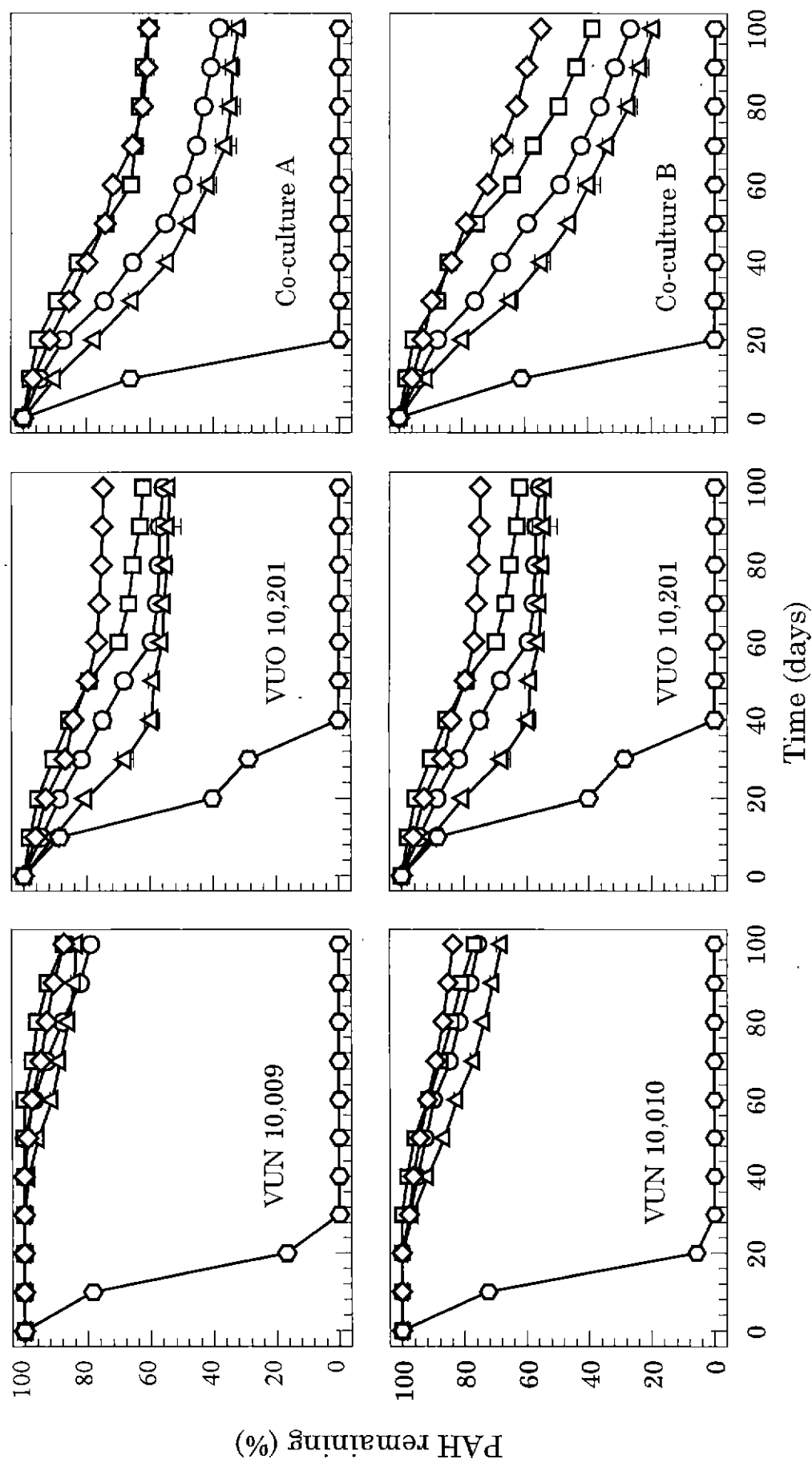


FIGURE 6.20 PAH concentrations in PAH-spiked soil containing a mixture of PAHs and no added surfactant. PAHs represented are pyrene (○), chrysene (○), benzo[a]anthracene (□), benzo[a]pyrene (△) and dibenz[a,h]anthracene (◇). High initial cell populations of either axenic culture of bacteria (approximately 10^6 cell g^{-1} soil) or the fungus (25 mg [mycelial wet wt.] per g soil) or the co-cultures comprising bacteria and the fungus were used to inoculate the soil. All incubations were performed at 25°C in the dark.

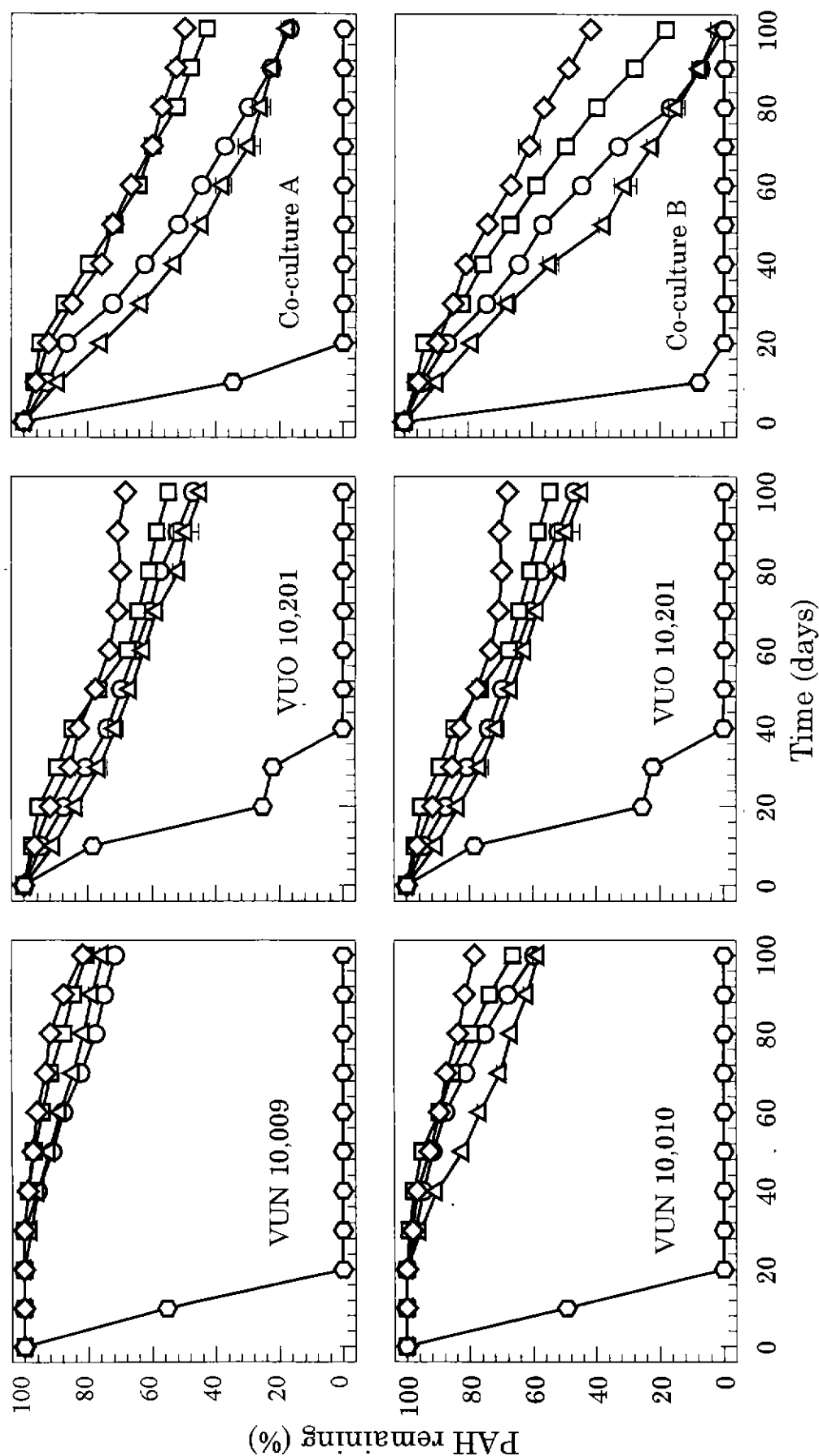


FIGURE 6.21 PAH concentrations in PAH-spiked soil containing a mixture of PAHs and Tergitol NP-10. PAHs represented are pyrene (○), chrysene (□), benz[a]anthracene (Δ), and dibenz[a,h]anthracene (◇). Soils were amended with 2% (w/w) Tergitol NP-10 and high initial cell populations of axenic cultures of either bacteria (approximately 10^6 cell g^{-1} soil) or the fungus (25 mg [wet wt. of mycelia] per g soil) or the co-cultures comprising bacteria and the fungus were used to inoculate the soil. All incubations were performed at 25°C in the dark.

Table 6.10 Disappearance of high molecular weight PAHs in PAH-spiked soil in the presence and absence of surfactant.

PAH	% Removal ^a									
	VUN 10,009		VUN 10,010		VUO 10,201		Co-culture A ^b		Co-culture B ^c	
	No TER	TER	No TER	TER	No TER	TER	No TER	TER	No TER	TER
Pyrene	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Chrysene	21.2 ± 0.7	28.4 ± 1.2	24.7 ± 0.5	40.3 ± 0.9	44.2 ± 1.2	52.9 ± 1.5	62.1 ± 2.2	83.2 ± 1.2	73.4 ± 1.2	100 ± 0.0
Benz[<i>a</i>]anthracene	12.7 ± 0.8	19.4 ± 0.9	23.2 ± 0.3	33.6 ± 0.6	37.8 ± 2.4	45.1 ± 0.3	39.7 ± 0.9	57.2 ± 3.3	61.3 ± 1.3	81.8 ± 1.5
Benzo[<i>a</i>]pyrene	16.7 ± 1.0	24.5 ± 0.7	32.1 ± 1.7	41.7 ± 1.6	46.2 ± 1.0	55.5 ± 1.6	68.1 ± 2.2	82.3 ± 1.8	80.7 ± 1.9	97.7 ± 2.7
Dibenz[<i>a,h</i>]anthracene	12.7 ± 0.4	18.3 ± 0.7	16.4 ± 1.1	21.5 ± 1.2	25.3 ± 1.0	31.8 ± 1.4	39.7 ± 0.6	50.2 ± 1.3	44.9 ± 0.8	58.4 ± 2.1

^a Means ± standard deviations of triplicate samples based on PAH loss from killed cell controls after 100 days of incubation (25°C in the dark). Experiments were conducted in sterile soil containing a PAH mixture, amended with 2% (w/w) Tergitol NP-10 (TER) or without surfactant addition (No TER). Initial PAH concentrations (per kg soil) were: 250 mg for pyrene and 50 mg for chrysene, benz[*a*]anthracene, benzo[*a*]pyrene, and dibenz[*a,h*]anthracene.

^b VUN 10,009 and VUO 10,201.

^c VUN 10,010 and VUO 10,201.

Table 6.11 Rate of high molecular weight PAH degradation in PAH-spiked soil in the presence of surfactant.

PAH	PAH degradation rate ($\text{mg kg}^{-1} \text{d}^{-1}$) ^a				
	VUN 10,009	VUN 10,010	VUO 10,201	Co-culture A	Co-culture B
Pyrene	14.95 (+ 75.1%)	17.86 (+ 90.2%)	6.82 (+ 47.5%)	17.86 (+ 36.9%)	17.86 (+ 28.0%)
Chrysene	0.14 (+ 27.3%)	0.20 (+ 66.7%)	0.26 (+ 18.2%)	0.42 (+ 35.5%)	0.50 (+ 35.1%)
Benz[<i>a</i>]anthracene	0.10 (+ 66.7%)	0.17 (+ 41.7%)	0.23 (+ 21.1%)	0.29 (+ 45.0%)	0.41 (+ 32.3%)
Benzo[<i>a</i>]pyrene	0.12 (+ 50.0%)	0.21 (+ 31.3%)	0.28 (+ 21.7%)	0.41 (+ 20.6%)	0.49 (+ 22.5%)
Dibenz[<i>a,h</i>]anthracene	0.10 (+ 66.7%)	0.11 (+ 37.5%)	0.16 (+ 23.1%)	0.25 (+ 25.0%)	0.30 (+ 36.4%)

^a PAH degradation rate calculated from the data collected from the experiments shown in Figure 6.20, 6.21 and Table 6.10. The number in brackets represent the percentage increase in PAH degradation rate in soil amended with Tergitol NP-10 (2% w/w) compared to unamended soil inoculated with the respective culture.

amount removed by co-culture A (68%), *P. janthinellum* VUO 10,201 (46%), VUN 10,010 (32%) and bacterial consortium VUN 10,009 (17%) over the same incubation period in the absence of surfactant (Table 6.11, Figure 6.20).

Higher rates of PAH degradation in PAH-spiked soil was observed in the presence of 2% (w/w) Tergitol NP-10 (Figure 6.21). After 100 days of incubation with co-culture B, chrysene was degraded to undetectable levels in the presence of surfactant while approximately 27% of this compound remained in PAH-spiked soil unamended with surfactant. PAH degradation in soils that received Tergitol NP-10 (2% w/w) was significantly more rapid than PAH degradation in the control soils receiving no surfactant according to the Student's t-test at 95% confidence levels. The control without inocula and killed-cell control were observed to have negligible PAH losses by volatilization or adsorption to microbial cells, thereby confirming that abiotic and adsorption processes were insignificant in these experiments.

6.8.5 Mineralization of [^{14}C]Pyrene and [^{14}C]Benzo[*a*]pyrene in PAH-Spiked Soil and PAH-Contaminated Soil

The mineralization of [^{14}C]pyrene and [^{14}C]benzo[*a*]pyrene by axenic and co-culture inocula was examined in PAH-spiked soil and PAH-contaminated soil in the presence and absence of Tergitol NP-10 (2% w/w). [^{14}C]pyrene or [^{14}C]benzo[*a*]pyrene was added to PAH-spiked soil, containing a PAH mixture, and to PAH-contaminated soil and the evolution of $^{14}\text{CO}_2$ was monitored.

6.8.5.1 Pyrene Mineralization in Soils

In the absence of surfactant, pyrene mineralization in the PAH-spiked soil showed the established trend with co-culture B mineralizing pyrene at a faster rate than co-culture A and the bacterial isolates; pyrene mineralization did not occur in PAH-spiked soil inoculated only with *P. janthinellum* VUO 10,201 (Figure 6.22). A four- to ten-day lag period (depending on inocula used) was observed before pyrene mineralization commenced. After 12 days, the percentage of radioactivity

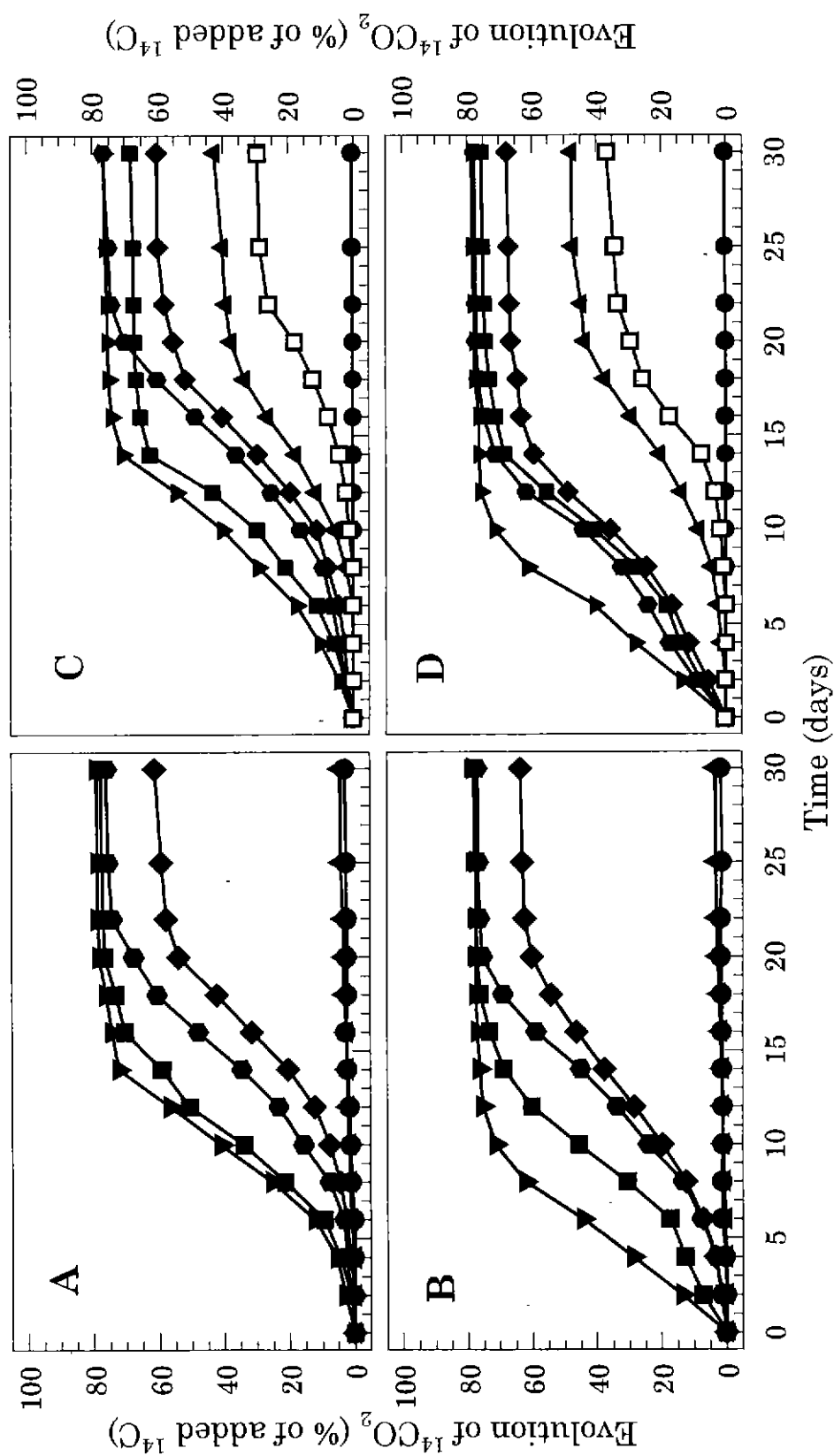


FIGURE 6.22 Mineralization of [^{14}C]pyrene in PAH-spiked soil (A, B) and PAH-contaminated soil (C, D). Soils without added surfactant (A, C) and soils amended with 2% (w/w) Tergitol NP-10 (B, D) were incubated with either bacterial consortium VUN 10,009 (◆), *S. multophila* VUN 10,010 (●), *P. janthinellum* VUN 10,201 (▲), co-culture A (■) or co-culture B (▼). Mineralization of [^{14}C]pyrene by indigenous microorganisms (□) in PAH-contaminated soil was also monitored. Sterile controls comprised cells killed with 0.7% HgCl_2 (●). All incubations were performed at 25°C in the dark.

added to the soil which evolved as $^{14}\text{CO}_2$ was 12%, 23%, 51% and 57% in PAH-spiked soil inoculated with either bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010, co-culture A or co-culture B, respectively (Figure 6.22). The addition of Tergitol NP-10 (2% w/w) to PAH-spiked soil slightly increased the pyrene mineralization rate such that the percentage of radioactivity added to the soil which evolved as $^{14}\text{CO}_2$ after 12 days was 14%, 34%, 60% and 75% when inoculated with either bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010, co-culture A or co-culture B, respectively (Figure 6.22). One reason for the more rapid mineralization of pyrene in surfactant-amended soil was the absence of a mineralization lag period. No $^{14}\text{CO}_2$ evolution was detected in PAH-spiked soil inoculated with *P. janthinellum* VUO 10,201 or HgCl_2 -killed cells.

A similar trend to that observed above in pyrene mineralization by the axenic and co-culture inocula was observed when these organisms were added to PAH-contaminated soil. In the absence of added Tergitol NP-10, the percentage of radioactivity added to the soil which evolved as $^{14}\text{CO}_2$ was 19%, 25%, 43% and 54% in PAH-contaminated soil inoculated with either the bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010, co-culture A or co-culture B, respectively after 12 days (Figure 6.22). The addition of Tergitol NP-10 (2% w/w) to PAH-contaminated soil substantially increased the pyrene mineralization rate such that the percentage of radioactivity added to the soil which evolved as $^{14}\text{CO}_2$ after 12 days was 49%, 62%, 55% and 76% when inoculated with either the bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010, co-culture A or co-culture B, respectively (Figure 6.22). The improvement in pyrene mineralization by these inocula due to the addition of Tergitol NP-10 was considerably greater in the PAH-contaminated soil compared to PAH-spiked soil. The indigenous microflora in the PAH-contaminated soil was able to mineralize around 30% of the initial radioactivity to $^{14}\text{CO}_2$ over 30 days in the absence of Tergitol NP-20. The addition of Tergitol NP-10 (2% w/w) increased the amount of radioactivity mineralized to $^{14}\text{CO}_2$ by the indigenous microflora to 37% over the same time period. Given that *P. janthinellum* VUO 10,201 alone is unable to mineralize pyrene in liquid or sterile soil cultures (Chapter 5, Section 5.5.1), the pyrene mineralization in PAH-

Table 6.12 Lag period and PAH mineralization rates in contaminated soils inoculated with axenic or co-culture inoculum^a.

Condition	¹⁴ C-Pyrene ^b			¹⁴ C-Benz[a]pyrene ^c		
	Lag period (days)		Mineralization rate (% ¹⁴ CO ₂ evolved g ⁻¹ soil d ⁻¹)	Lag period (days)		Mineralization rate (% ¹⁴ CO ₂ evolved g ⁻¹ soil d ⁻¹)
	No	TER	No TER	TER	No TER	TER
PAH-spiked soil						
VUN 10,009	10	6	0.014	0.025	NT	NT
VUN 10,010	8	4	0.025	0.029	35	0.0036
VUN 10,201	NA	NA	NA	NA	NA	NA
Co-culture A	4	2	0.043	0.05	NT	NT
Co-culture B	4	1	0.054	0.063	28	0.0064
						0.009
PAH-contaminated soil						
Indigenous microbes	14	12	0.002	0.003	60	< 0.001
VUN 10,009	6	1-2	0.021	0.042	NT	NT
VUN 10,010	6	1-2	0.028	0.053	42	0.001
VUN 10,201	10	8	0.013	0.013	60	0.003
Co-culture A	4	1-2	0.046	0.048	NT	NT
Co-culture B	2	1	0.055	0.065	14	0.006
						0.001
						NT
						0.002
						0.005
						NT
						0.008

^a Data represents lag period and rate of PAH mineralization in either PAH-spiked soil or PAH-contaminated soil in the absence (No TER) or presence (TER) of 2% (w/w) Tergitol NP-10.

^b These figures were calculated from the data collected from the experiment shown in Figure 6.22.

^c These figures were calculated from the data collected from the experiment shown in Figure 6.23.

NA, not appreciable; lag period and/or PAH mineralization was not observed.
NT, not tested.

contaminated soil inoculated with axenic *P. janthinellum* culture was probably due to the cooperative mineralization of this compound by the fungus and the indigenous microbes in a similar fashion to that observed with co-cultures A and B. This 'fungal-indigenous microbial co-culture' was able to mineralize pyrene to a higher extent (50% of initial radioactivity recovered as $^{14}\text{CO}_2$) in the presence of Tergitol NP-10 (2% w/w) compared to the amount mineralized in the absence of this surfactant (40% of initial radioactivity recovered as $^{14}\text{CO}_2$) after 30 days. It is also clear that the addition of the fungus to the PAH-contaminated soil reduced the mineralization lag period from 10 days for the indigenous microflora to around 5 days in the fungal-inoculated PAH-contaminated soil (Table 6.12).

6.8.5.2 Benzo[a]pyrene Mineralization in Soils

The mineralization of benzo[a]pyrene in PAH-spiked soil and PAH-contaminated soil by *S. maltophilia* VUN 10,010, *P. janthinellum* VUO 10,201 and co-culture B was investigated in the absence and presence of Tergitol NP-10 (2% w/w). Benzo[a]pyrene mineralization in these soils was not investigated using inocula containing bacterial consortium VUN 10,009 or co-culture A because of the superior result achieved thus far with VUN 10,010 and co-culture B.

The same trends were observed for the mineralization of benzo[a]pyrene in soils by the inocula and indigenous microflora as those observed for pyrene mineralization described in Section 6.8.5.1. In the absence of surfactant, the percentage of radioactivity recovered as $^{14}\text{CO}_2$ was 24% and 45% for PAH-spiked soils inoculated with VUN 10,010 and co-culture B, respectively, over 100 days (Figure 6.23). The addition of Tergitol NP-10 (2% w/w) increased the benzo[a]pyrene mineralization rate such that the percentage of radioactivity recovered as $^{14}\text{CO}_2$ was 37% and 57% for PAH-spiked soils inoculated with VUN 10,010 and co-culture B, respectively, over 100 days. Benzo[a]pyrene was not mineralized in PAH-spiked soil inoculated with only *P. janthinellum* VUO 10,201 either in the absence or presence of surfactant (Figure 6.23).

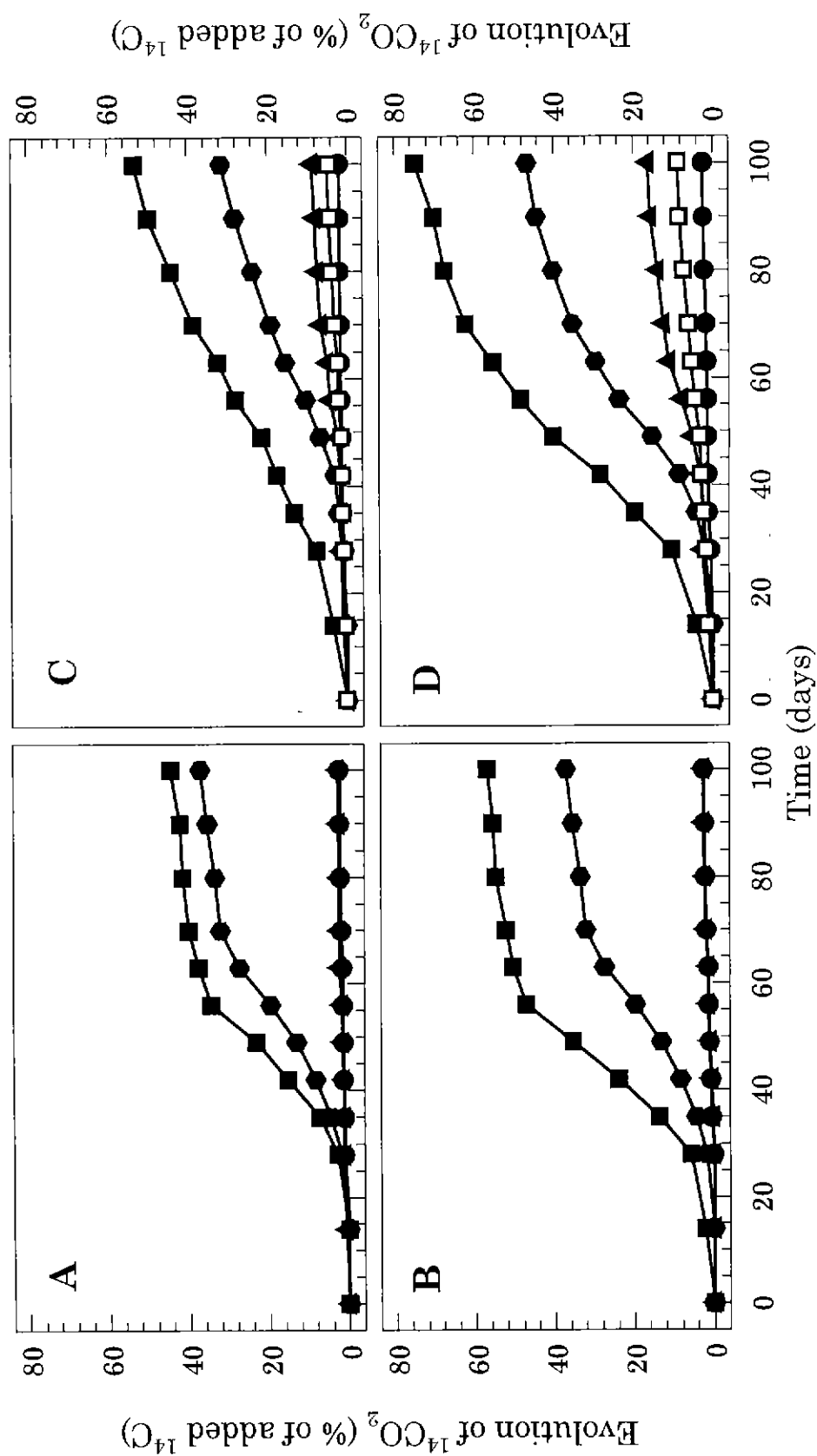


FIGURE 6.23 Mineralization of ^{14}C benzo[a]pyrene in PAH-spiked soil (A and B) and PAH-contaminated soil (C and D). Unamended soils (A and C) and soil amended with 2% (w/w) Tergitol NP-10 (B and D) were incubated with *S. maltophilia* VUN 10,010 (●), *P. janthinellum* VUO 10,201 (▲) or co-culture B (■). Mineralization of ^{14}C benzo[a]pyrene by indigenous microorganisms (□) was also monitored in PAH-contaminated soil. Sterile controls comprised cells killed with 0.7% HgCl_2 (●). All incubations were performed at 25°C in the dark.

The benzo[a]pyrene mineralization rate by introduced and indigenous microorganisms in PAH-contaminated soil was also increased by the addition of 2% (w/w) Tergitol NP-10. In the absence of surfactant, the percentage of radioactivity recovered as $^{14}\text{CO}_2$ was 32% and 53% for *S. maltophilia* VUN 10,010 and co-culture B, respectively, after 100 days (Figure 6.22). The addition of Tergitol NP-10 to these cultures increased the benzo[a]pyrene mineralization rate such that the percentage of recovered radioactivity as $^{14}\text{CO}_2$ was 47% and 75% for *S. maltophilia* VUN 10,010 and co-culture B, respectively, after 100 days (Figure 6.23). The indigenous microbial population in the PAH-contaminated soil was able to mineralize around 5% of the initial radioactivity to $^{14}\text{CO}_2$ in 100 days and this was increased to around 9% by the addition of Tergitol NP-10 to the uninoculated soil. Benzo[a]pyrene mineralization was also observed in soils containing axenic *P. janthinellum* VUO 10,201 inocula which is assumed to be due to cooperative benzo[a]pyrene degradation with the indigenous microorganism, as observed with pyrene mineralization in PAH-contaminated soil (Section 6.8.5.1). The percentage recovery of radioactivity as $^{14}\text{CO}_2$ from soil inoculated with *P. janthinellum* VUO 10,201 was increased from 9% to 16% by the addition of Tergitol NP-10. The increase in $^{14}\text{CO}_2$ evolution rates from soil cultures, containing added [^{14}C]benzo[a]pyrene, due to the addition of surfactant were significant according to the Student's t-test at 95% confidence level. Although the mineralization of both pyrene and benzo[a]pyrene was significantly improved by the addition of Tergitol NP-10 (2% w/w) to the soil, a relatively greater increase in the benzo[a]pyrene mineralization rate resulted from surfactant addition. The lower aqueous solubility of benzo[a]pyrene compared to pyrene may account for this observation.

6.9 SOIL DETOXIFICATION

The toxicity of PAH-contaminated soil and PAH-spiked soil was assessed using the Ames mutagenicity assay. Soil samples were sacrificed at regular time intervals for extraction with DCM. Extracts were evaporated to partial dryness and dried completely under nitrogen gas. The dry extracts were redissolved in DMSO and subjected to the assay according to the method described in Chapter 3

(Section 3.2.7). The tests were performed with *S. typhimurium* TA100 and rat liver homogenate (S9) activation system. The number of revertants after exposure to the soil extract was recorded and subtracted from spontaneous revertants obtained from the solvent (DMSO) control.

All inoculated PAH-spiked soils containing either axenic or co-cultures of bacteria and the fungus showed an appreciable reduction in mutagenic activity, which corresponded to the period of incubation and the amount of PAH removed from the soil (See corresponding Section 6.8.4). The highest reduction rate and decrease in extent of mutagenic potential in both soils was evident with the co-culture inocula. In the absence of surfactant, the PAH-spiked soil inoculated with either co-culture A or co-culture B had a decrease in mutagenic potential of greater than 50%. The soil mutagenicity was further reduced to approximately 65-70% in the surfactant-amended PAH-spiked soil incubated with the respective co-cultures (Figure 6.24, Table 6.13). Compared to inoculated soils, the uninoculated soils, either in the absence or presence of Tergitol NP-10, showed no substantial reduction in mutagenicity over the time course study. In the absence of surfactant, the axenic fungal inoculum also reduced the mutagenicity of the PAH-spiked soil by approximately 47% over 100 days, while bacterial inocula of either VUN 10,009 or VUN 10,010 reduced the soil mutagenicity by 15-28% over the same time period. However, the application of surfactant to the soil cultures resulted in an even greater decrease in soil mutagenicity. This may be due to the mobilization of PAHs by surfactant which led to a greater decrease in PAH concentration.

PAH-contaminated soil had a higher mutagenicity compared to PAH-spiked soil (Figure 6.25). Although the extent of mutagenic potential reduction in this soil over the same incubation period (100 days) was lower than that observed in PAH-spiked soil, a similar trend in mutagenicity reduction was observed due to the addition of Tergitol NP-10 (Table 6.13, Figure 6.25); the reduction in mutagenicity obeyed the following order of: co-cultures > *P. janthinellum* VUO 10,201 > bacteria (either VUN 10,009 or VUN 10,010). A slight decrease in mutagenicity of

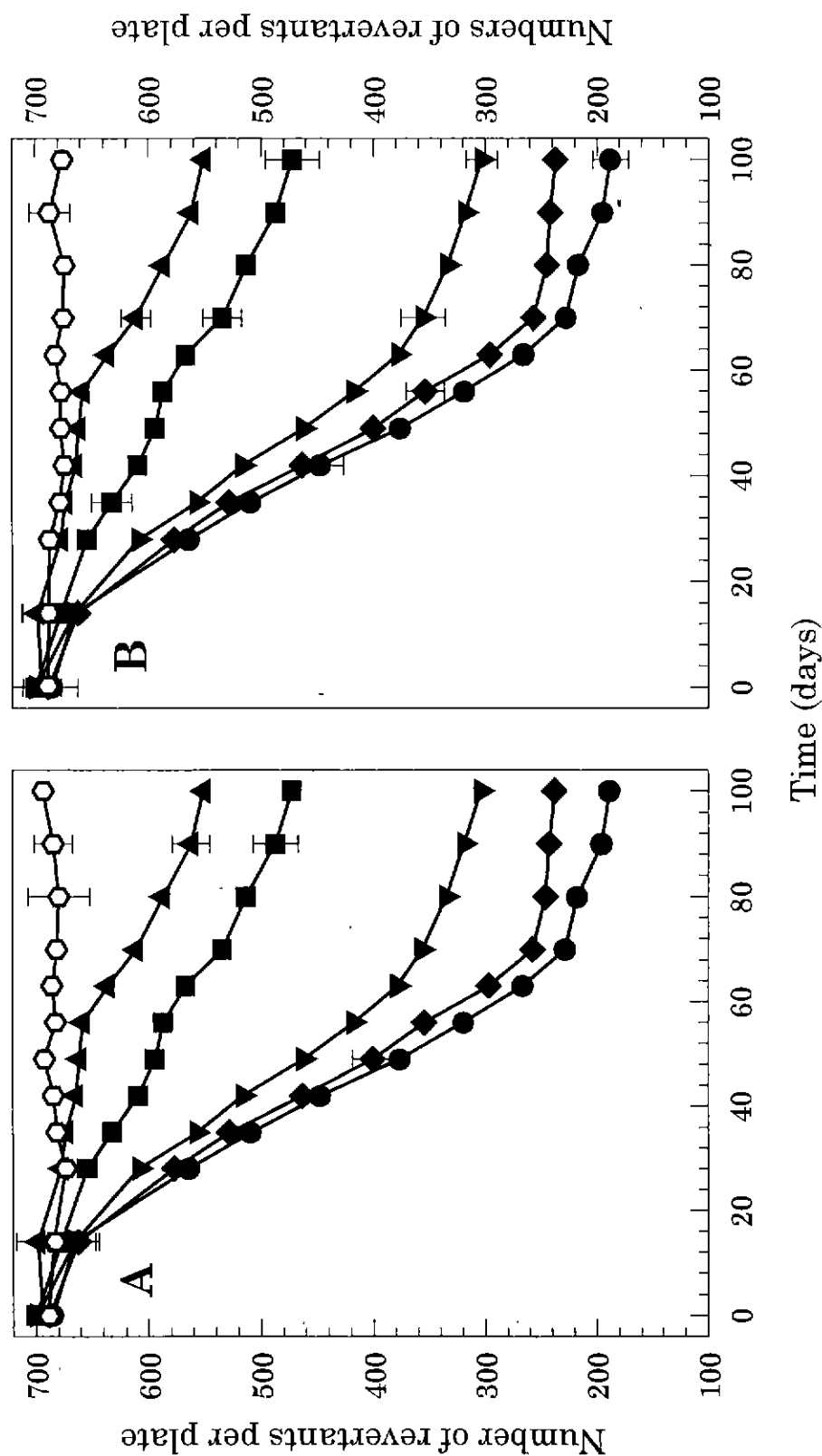


FIGURE 6.24 Mutagenicity of PAH-spiked soil in the absence (A) or presence (B) of Tergitol NP-10 (2%, w/w). Soils were inoculated with bacterial consortium VUN 10,009 (▲), *S. maltophilia* VUN 10,010 (■), *P. janthinellum* VUN 10,201 (▼), co-culture A (◆) or co-culture B (●). Mutagenic activity of the uninoculated soil (○) is also shown. Number of spontaneous revertants of this experiment was 109 ± 12 . Incubations were performed at 25°C in the dark.

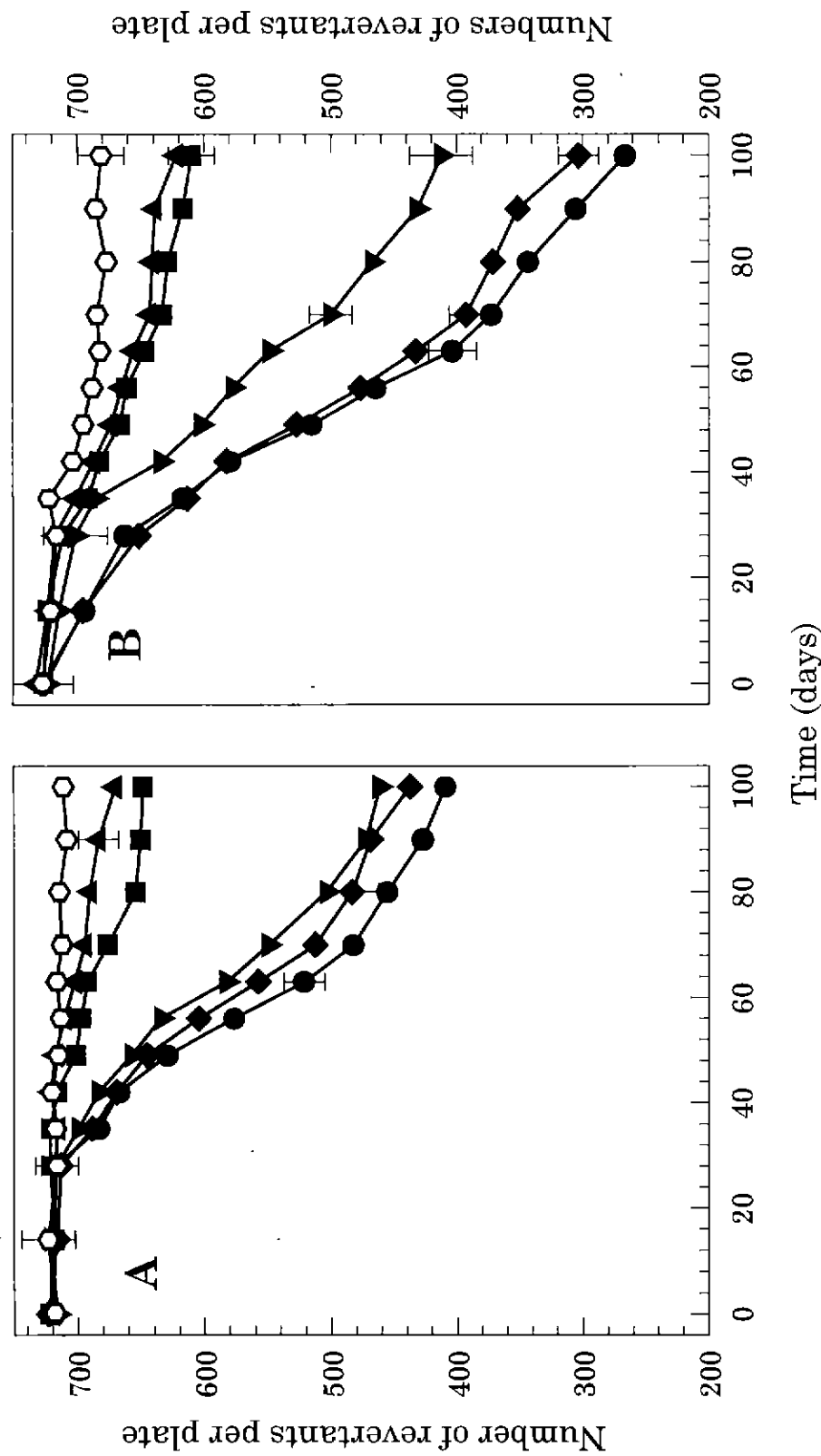


FIGURE 6.25 Mutagenicity of PAH-contaminated soil in the absence (A) or presence (B) of Tergitol NP-10 (2%, w/w). Soils were inoculated with bacterial consortium VUN 10,009 (▲), *S. maltophilia* VUN 10,010 (■), *P. janthinellum* VUN 10,201 (▼), co-culture A (◆) or co-culture B (●). Mutagenic activity of uninoculated soils containing indigenous microbes (○) is also shown. Number of the spontaneous revertants for this experiment was 109 ± 12 . Incubations were performed at 25°C in the dark.

Table 6.13 Influence of surfactant on the reduction in mutagenic activity of PAH containing soils.

Condition ^b	Mutagenic ratio (MR) ^a			
	PAH-spiked soil		PAH-contaminated soil	
	No TER	TER	No TER	TER
Indigenous microbes ^c	–	–	6.3 (–4.5%)	5.9 (–11.9%)
Abiotic control ^d	6.4 (NA)	6.2 (–3.1%)	–	–
VUN 10,009	5.4 (–15.6%)	5.1 (–20.3%)	6.2 (–6.1%)	5.7 (–14.9%)
VUN 10,010	4.6 (–28.1%)	4.3 (–32.8%)	5.9 (–10.6%)	5.6 (–16.4%)
VUN 10,201	3.4 (–46.9%)	2.8 (–56.3%)	4.2 (–36.4%)	3.8 (–43.3%)
Co-culture A	2.7 (–57.8%)	2.2 (–65.6%)	4.3 (–34.8%)	2.8 (–58.2%)
Co-culture B	2.4 (–62.5%)	1.7 (–73.4%)	3.8 (–42.4%)	2.4 (–64.2%)

^a Mutagenic ratio calculated from the number of revertants from each sample to the number of spontaneous revertants (see Chapter 5, Section 5.8). % Reduction in mutagenic potential is shown in brackets which was calculated based on the mutagenic potential of 100 day samples incubated with a respective culture in the absence (No TER) or presence (TER) of 2% (w/w) Tergitol NP-10, compared to the mutagenic potential of the respective sample at day 0. These figures were calculated relative to the mutagenic activity of soil before inoculation.

^b Soils, either PAH-spiked soil or PAH-contaminated soil, were inoculated with a respective culture and incubated at 25°C for 100 days.

^c Uninoculated PAH-contaminated soil contained only indigenous microorganisms.

^d Abiotic control comprised sterile PAH-spiked soil with no added inoculum.

– Not tested.

NA signifies the reduction in the percentage of mutagenic potential is not appreciable.

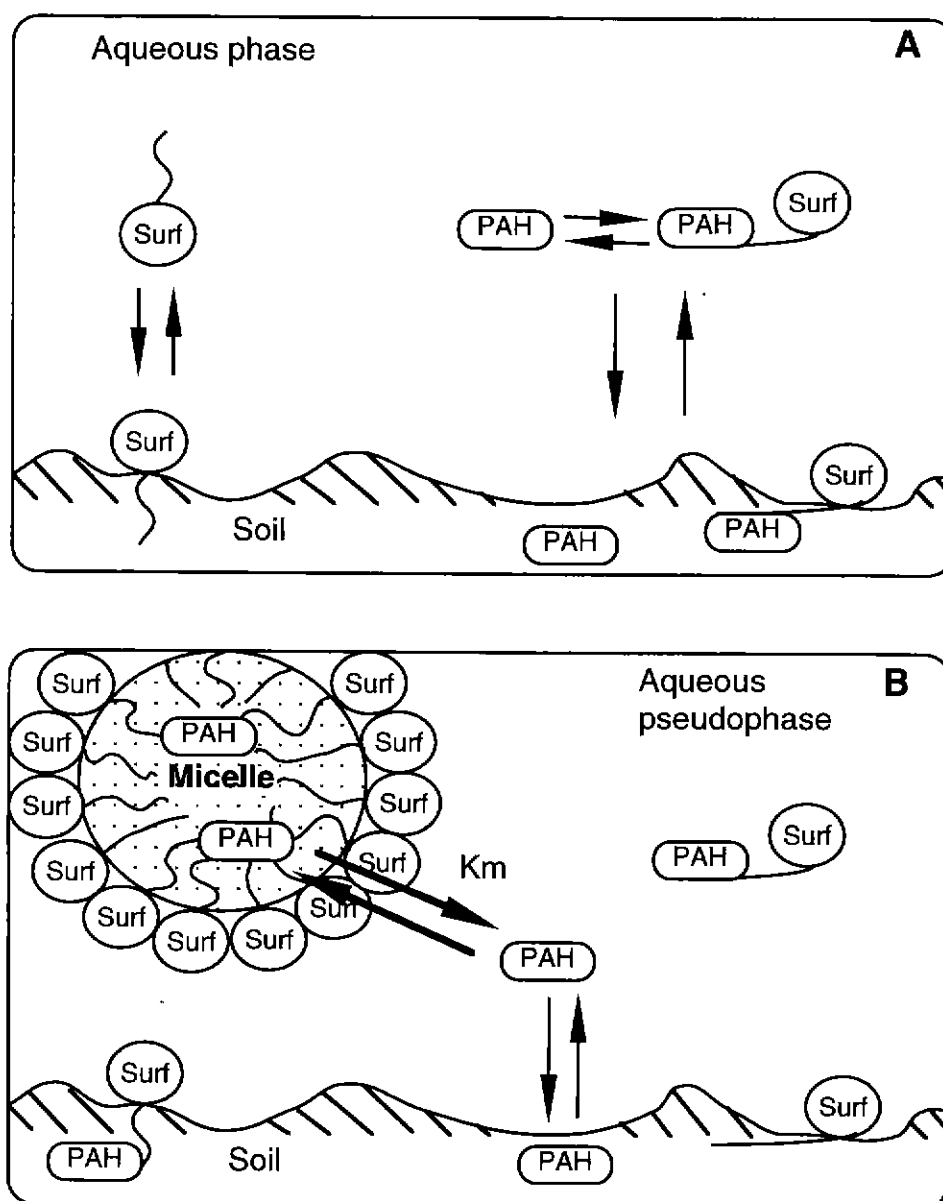


Figure 6.26 Schematic illustrating interactions between nonionic surfactant (surf), polycyclic aromatic hydrocarbon (PAH) and soil in soil-aqueous system under (A) sub-CMC and (B) supra-CMC conditions. Figure A illustrates a soil-aqueous system for which nonionic surfactant is present at a concentration less than that required for surfactant CMC formation. The PAH in such a system as shown in (A) is distributed in four different states, *i.e.*, (i) freely dissolved in water, (ii) sorbed onto soil, (iii) associated with nonionic surfactant monomers in the aqueous phase, and (iv) associated with nonionic surfactant monomers that are sorbed on the soil. Figure B illustrates the enhanced solubilization of PAH that may occur in soil-aqueous systems if the surfactant concentration exceeds the bulk solution surfactant CMC. In this case, the PAH is distributed between the interiors of the nonionic surfactant micelles, and the water and the soil phases, with the distribution of PAH being dependent on dissolved and sorbed nonionic surfactant molecules (from Edwards *et al.*, 1992).

uninoculated PAH-contaminated soil was observed in the absence of surfactant and this was improved when Tergitol NP-10 was present in the soil.

6.10 DISCUSSION

6.10.1 Surfactant Solubilization Theory

The addition of surfactants to environments contaminated with PAHs is a possible means of increasing the bioavailability of these compounds for the purpose of facilitating their biodegradation. A primary characteristic of surfactants is their ability to form colloidal aggregates called micelles. Micelles are an arrangement of surfactant molecules formed by the hydrophobic tail groups coming together to create a thermodynamically-favored hydrocarbon pseudophase with a hydrophilic exterior (Figure 6.26). According to the conventional phase-separation model for surfactants in aqueous solution (Edwards *et al.*, 1992), surfactants are assumed to exist predominantly in a monomeric form at a concentration below the CMC. The monomeric concentration remains constant at CMC. At surfactant concentrations greater than CMC, a constant monomer concentration is maintained while the excess surfactant molecules form pseudophase micelles. The CMC value of surfactants varies depending upon various parameters such as molecular structure of the surfactant, temperature, ionic strength and other factors (Edwards *et al.*, 1991). The findings in this work recognized that the CMC value of a particular surfactant in BSM solution and deionized water were slightly different. However, there was no consistency in these CMC differences between BSM and deionized water. Similar observations have been reported by Roch and Alexander (1995) who measured the CMC of several surfactants including Triton X-100, Alfonic 810-60, Alfonic 1412-40, Neodol 25-35, Novel 1412-56, Novel 1012-62, LXS-814 in either deionized water or in a low-salt solution; the values of CMC for surfactants in the deionized water were occasionally lower and sometimes higher than the CMC obtained for surfactants in the inorganic solution. The CMC values obtained for the surfactants tested in this study are either similar or different to some other reports published elsewhere (Table 6.14).

Table 6.14 Comparison of surfactant critical micelle concentration (CMC) obtained in this thesis with other publications.

Surfactant	CMC		Condition	Reference
	mM	mg l ⁻¹		
Brij 35	–	40	deionized water	Guha and Jaffé, 1996
	–	190	NA	Jahan <i>et al.</i> , 1997
	–	74	deionized water	Kile and Chiou, 1989
	0.058	70	deionized water, 30°C	This work
	0.063	75	BSM, 30°C	This work
Igepal CA-630	0.083	50	deionized water, 30°C	This work
	0.108	65	BSM, 30°C	This work
Tergitol NP-10	0.054	–	deionized water, 25°C	Edwards <i>et al.</i> , 1991
	–	52	deionized water	Guha and Jaffé, 1996
	0.061	40	deionized water, 30°C	This work
	0.069	45	BSM, 30°C	This work
Triton X-100	–	130	deionized water	Kile and Chiou., 1989
	0.017	–	deionized water, 25°C	Edwards <i>et al.</i> , 1991
	–	195	distilled water	Roch and Alexander, 1995
	–	180	low-salt solution	
	–	43	deionized water	Guha and Jaffé, 1996
	0.240	150	deionized water, 30°C	This work
	0.232	145	BSM, 30°C	This work
Tyloxapol	0.018	80	deionized water, 30°C	This work
	0.017	75	BSM, 30°C	This work

–, indicates that the CMC value given by the authors was not specified in either molar or weight basis.

NA, signifies that the conditions used for surfactant CMC determination was not specified by the authors.

The formation of micelles allows for the partitioning of hydrocarbons into the hydrophobic pseudophase of the micellar core. This phenomenon can greatly enhance the total concentration of a compound in solution above its aqueous solubility limit and is referred to as solubilization. Solubilization may be envisioned as the dissolution of a substance by reversible interaction with surfactant micelles in a solvent to form a thermodynamically stable isotropic solution (Liu *et al.*, 1991). The amount of material solubilized is small until a critical concentration of the surfactant is reached *i.e.* CMC. The apparent solubility of slightly soluble hydrophobic organic compounds such as PAHs may be dramatically enhanced in surfactant solutions at concentrations greater than the CMC. The hydrophobic core of each micelle can accommodate a certain amount of lipophilic organic compound as a solubilize. Solubilization of hydrophobic substances commences at the CMC and in general is a linear function of surfactant concentration over a wide range of surfactant concentrations greater than the CMC. The amount of organic compound that is solubilized depends on surfactant structure, aggregation number, micelle geometry, ionic strength and chemistry, temperature, solubilize chemistry and solubilize size (Edwards *et al.*, 1991).

6.10.2 Selection of Surfactants for PAH Bioremediation

The selection of surfactants for bioremediation purposes is based on several criteria. Cationic surfactants are not normally used for soil washing because of their germicidal property (Ganeshalingam *et al.*, 1994). This is particularly important if the wash solution has to eventually undergo biodegradation, where the microorganisms responsible for PAH degradation would be inhibited by this type of surfactant. Furthermore, since the primary purpose of the work in this chapter was to apply surfactants to soil, interaction between soils and surfactant needed to be considered. Most soils are negatively charged and attract cationic surfactants to their surfaces. This reduces the amount of surfactant available in solution with a consequent reduction in the amount of PAH solubilized. Moreover, the adsorbed surfactant molecule could bind with PAHs, thereby further reducing PAH mobility (Ganeshalingam *et al.*, 1994). Anionic surfactants, owing to the

presence of negative charges, are repelled by most soils and therefore are usually the least retained in soil compare to cationic and nonionic surfactants. Nonionic surfactants may show a higher degree of sorption than anionic surfactants, depending upon soil composition (Ganeshalingam *et al.*, 1994). In fact, nonionic surfactants are generally more preferred for bioremediation applications of poorly soluble compounds such as PAHs because this class of surfactants are characterized by their greater hydrocarbon solubilizing power, weaker sorption to charged sites, lower toxicity toward bacteria and have less foaming properties (Aronstein and Alexander, 1992; Rouse *et al.*, 1994; Churchill *et al.*, 1995; Volkerling *et al.*, 1995; Jahan *et al.*, 1997; Madsen and Kristensen, 1997).

In keeping with other published results, it was found in this work that the nonionic surfactants were less toxic to VUN 10,010, bacterial consortium VUN 10,009 and *P. janthinellum* VUO 10,201 than cationic or anionic surfactants. These bacterial strains were also able to use the Tween series of surfactants as growth substrates, which is undesirable for the purpose of stimulating biodegradation since it lowers the surfactant concentration and, if used as a preferred carbon source, could result in the repression of PAH degradation by the isolate. The promotion of fungal spore germination by Tween series and Brij 35 may favor PAH degradation by this fungus but no conclusions can be made on this from the available data. These results necessarily excluded the use of the cationic, anionic and the Tween series of the nonionic surfactants in the PAH biodegradation experiments.

The hydrophile-lipophile balance (HLB) describes the balance between the hydrophilic and hydrophobic moieties of the surfactant molecule (Rouse *et al.*, 1994). The HLB number, which is often determined experimentally, is basically a function of the ratio of the formula weight of the hydrophilic moiety to the entire molecule. Therefore, the higher the HLB value, the more hydrophilic the molecule (Zajic and Panchal, 1976). Unlike CMC, the HLB relates the characteristics of a particular surfactant in combination with a specific organic compound. Furthermore, a HLB number may be calculated for mixtures, and thus a surfactant system can be tailored for a specific application (Vigon and Rubin,

1989). HLB is a useful parameter for the primary selection of surfactants to be used for solubilization work, however, differences in surfactant structures have been observed to result in a wide performance variation for surfactants despite having similar HLB numbers (Vigon and Rubin, 1989). This was evident in the recent study of Jahan and co-workers (1997) which demonstrated a variation in PAH solubilization performance of surfactants that have close HLB values (13-17) and this was believed to be responsible for the observed differences in PAH degradation rates.

No correlation could be drawn from the results in this study between surfactant HLB values and the apparent PAH solubility. Tiehm (1994) studied the degradation of PAHs *Mycobacterium* sp. in the presence of surfactants with HLB numbers ranging from 13 to 40. The author suggested that surfactant toxicity is related to their HLB values. This hypothesis was based on the observation that low HLB (high lipophilicity) surfactants inhibited growth of *Mycobacterium* sp. on PAHs and also inhibited the degradation of fluorene and pyrene by a mixed bacterial population. Tiehm (1994) also suggested that the more hydrophilic surfactants were better able to enhance the degradation of PAHs. Tiehm's latter conclusion is not widely supported in the literature since it has previously been observed that the apparent solubility of phenanthrene was greater when more lipophilic (lower HLB) surfactants were used (Guerin and Jones, 1988a; 1988b). The results derived from toxicity tests in this study revealed a high microbial toxicity by SDS which has a HLB value of 40. Nonionic surfactants, which have much lower HLB values (13-17; Table 6.2), possessed a lower toxicity toward the PAH-degrading strains used in this work. Although the nonionic surfactants have close HLB numbers, a superior ability to enhance PAH degradation was observed with Tergitol NP-10 and Brij 35 compared to the other nonionic surfactants. Based on this study, the relationship between surfactant HLB and PAH biodegradation could not be correlated. This is in keeping with the results of Guerin and Jones (1988a and 1988b) who could not relate the order of enhancement of phenanthrene biodegradation by various Tween surfactants to their HLB numbers, indicating that one single parameter cannot be a determining factor in surfactant selection.

6.10.3 Surfactant Effects on PAH Solubility

A proportional increase in PAH solubility was observed in this work when surfactant concentrations were increased above CMC. The apparent solubility of pyrene and fluoranthene in BSM was increased by over 250-fold and the apparent solubility of benzo[*a*]pyrene was increased by a factor of 10^3 - 10^4 at a surfactant concentration of 10 g l^{-1} compared to their solubility at CMC. A measure of the effectiveness of a particular surfactant in solubilizing a given hydrophobic compound is known as the molar solubilization ratio (MSR). The MSR is defined as the number of moles of organic compound solubilized per mole of surfactant added to solution. The increase in solubilize concentration per unit increase in micellar surfactant concentration is equivalent to the MSR (Edwards *et al.*, 1991). Another approach for quantifying surfactant solubilization consists of characterizing the partitioning of the PAH between micelles and monomeric solution with a mole fraction micelle-phase/aqueous-phase partition coefficient (Edwards *et al.*, 1991). The micelle-phase/aqueous-phase partition coefficient (K_m) is the ratio of the mole fraction of the compound in the micellar pseudophase to the mole fraction of the compound in the aqueous pseudophase (Figure 6.26). The value of K_m is dependent on surfactant chemistry, solubilize chemistry and temperature.

Lower MSR and higher $\log K_m$ values were observed for benzo[*a*]pyrene relative to those measured for fluoranthene and pyrene. Compared to the latter two PAHs, this indicates that, on a molar basis, less benzo[*a*]pyrene was incorporated into the surfactant micelles from the nonpolar pseudophase and that a greater proportion of the total amount of benzo[*a*]pyrene in the micelle/aqueous pseudophases was embodied in the micelles. This result can be attributed to the much higher hydrophobicity of benzo[*a*]pyrene compared to that of fluoranthene and pyrene. The MSR and $\log K_m$ values obtained in this work for pyrene using Tergitol NP-10 and Triton X-100 are comparable to those obtained by Edwards, *et al.* (1991), the slight differences most likely being attributable to the experiments in this work being performed in an ionic solution at a higher temperature (*viz.*

Table 6.15 Comparison of MSR and Log K_m of surfactant-solubilized PAHs.

PAH	Surfactant	Condition	MSR	Log K_m	Reference
Fluoranthene	Brij 35	BSM, 30°C	8.7×10^{-2}	5.68	This work
	Tergitol NP-10		5.0×10^{-2}	5.56	
	Triton X-100		5.1×10^{-2}	5.64	
Pyrene	Brij 30	Deionized water, 25°C	7.2×10^{-2}	6.53	Edwards <i>et al.</i> , 1991
	Igepal CA-720		4.3×10^{-2}	6.01	
	Tergitol NP-10		5.8×10^{-2}	6.41	
	Triton X-100		3.5×10^{-2}	6.03	
	Triton X-100	NA	3.5×10^{-2}	6.02	Ghosh <i>et al.</i> , 1995
	Brij 35	BSM, 30°C	5.7×10^{-2}	5.54	This work
	Igepal CA-630		3.3×10^{-2}	5.39	
	Tergitol NP-10		3.4×10^{-2}	5.71	
	Triton X-100		3.5×10^{-2}	5.84	
Benzo[a]pyrene	Tyloxapol		8.2×10^{-2}	6.60	
	Triton X-100	NA	1.5×10^{-2}	7.56	Ghosh <i>et al.</i> , 1995
	Brij 35	BSM, 30°C	2.0×10^{-2}	7.78	This work
	Tergitol NP-10		1.2×10^{-2}	7.43	
	Triton X-100		1.1×10^{-2}	7.49	

NA signifies that the conditions of the test were not specified by the authors.

30°C) compared to 25°C. Ghosh *et al.* (1995) reported MSR and $\log K_m$ values for benzo[a]pyrene in the presence of Triton X-100 to be 1.5×10^{-2} and 7.56, respectively (Table 6.15). These results for benzo[a]pyrene are slightly different to the values obtained in this thesis but this may also be due to a difference in solubilization medium and temperature, noting that Ghosh *et al.* (1995) did not specify either of these parameters. Besides the work of Ghosh and co-workers (1995), there are no other reports which determine the molar MSR and $\log K_m$ values for fluoranthene and benzo[a]pyrene in the presence of the nonionic surfactants used in this study. These values are important for the proper modeling of PAH partitioning in contaminated soils following surfactant addition (Aronstein *et al.*, 1991; Edwards *et al.*, 1991). Modeling soil-water-surfactant systems is necessary since the surfactant dose required to achieve PAH solubility is usually in considerable excess of the value found to effectively solubilize PAHs in liquids only.

6.10.4 The Effect of Surfactant Addition on High Molecular Weight PAH Degradation

There has been only a limited amount of research into the use of surfactants for improving the biodegradation of high molecular weight PAHs which is most likely due to the scarcity of microbial isolates that can degrade these compounds. The isolation of the high molecular weight PAH-degrading microorganisms as described in Chapter 4 provided a means of investigating the effect of surfactants on the microbial degradation of this class of PAH compounds.

The results in this thesis show that microbial growth and pyrene degradation by *S. maltophilia* VUN 10,010 was enhanced by the addition of Brij 35, Tergitol NP-10, Triton X-100 and Tyloxapol at concentrations up to 10 g l^{-1} . The specific growth rate on pyrene in the presence of Brij 35 or Tergitol NP-10 at 10 g l^{-1} (0.015 h^{-1}) was 67% greater than the growth rate in the absence (0.009 h^{-1}) of these two nonionic surfactants. Igepal CA-630 at 5 g l^{-1} was inhibitory to pyrene degradation which correlates with its growth-inhibitory effect at this concentration on glucose-grown VUN 10,010. Enhanced PAH degradation was

also observed when Brij 35 and Tergitol NP-10 were used in *S. maltophilia* VUN 10,010 cultures containing high numbers of washed cells and a single high molecular weight PAH, or cultures inoculated with low numbers of washed cells and containing mixed PAHs. The maximum specific PAH degradation rate for most PAH compounds supplied as single or mixed PAH substrates was increased by surfactant addition. The disappearance of coronene was an exception, since no significant difference in its concentration was detected between the control and experimental cultures in the single PAH experiments, indicating that VUN 10,010 is unable to degrade this compound as a sole carbon and energy source under the conditions tested. However, significant amounts of coronene disappeared in the mixed PAH substrate cultures suggesting co-metabolic transformation of this compound may occur with VUN 10,010.

Surfactants have previously been observed to reduce or inhibit biodegradation. Where negative effects of surfactants on microbial degradation of PAHs have been found, explanations vary from the surfactant being used as preferential substrates (Thiem, 1994) to surfactant toxicity (Aronstein *et al.*, 1991; Thiem, 1994) and increased PAH toxicity due to its higher apparent solubility (Thibault *et al.*, 1996). It has also been suggested that reduced biodegradation could be caused by micelled shielding of PAHs from microbial attack (Putcha and Domach, 1993). However, in the case of Putcha and Domach (1993) there was no definitive demonstration that their observed effects were not a reflection of surfactant toxicity, or preferential degradation of surfactant at the relatively high concentrations required to achieve the CMC. Another explanation was proposed by Laha and Luthy (1991) to describe the inhibition of phenanthrene mineralization by nonionic surfactants. These authors suggested that reduced degradation may be due to micellization and a resulting decrease in free phenanthrene available to the degrading microbes. It should be noted that these authors used limiting PAH concentrations such that the PAHs which partitioned into the micelles were not replaced in the aqueous phase from a solid phase PAH supply. If high PAH concentrations were used in the matrix, it is expected that the PAHs absorbed into the micelles from the aqueous phase would be replaced by the dissolution of PAH from the solid phase. However, the above explanations do not

apply to the results in this thesis, since the inhibition of pyrene degradation and mineralization at high concentrations of Tergitol NP-10 were due to surfactant toxicity and/or toxicity of high concentration of solubilized PAHs. Also, since the microbes used in this thesis do not utilize Tergitol NP-10, the use of this surfactant as a preferential growth substrate can be dismissed.

Tiehm (1994) observed that biomass production was dependent on the amount of surfactant added to *Mycobacterium* cultures containing a mixture of three- and four-ring PAHs; the biomass yield changed from around 20 mg l⁻¹ (in the absence of surfactant) to 60 mg l⁻¹ (in the presence of 4 mM Sapogenat T-300). For the PAH mixtures used in this work (containing up to seven-benzene ring PAHs), it was observed that, although the specific growth rate of strain VUN 10,010 increased by 30% from 0.010 h⁻¹ (in the absence of surfactant) to 0.013 h⁻¹ (in the presence of 5 g l⁻¹ Tergitol NP-10), the cell yield (around 83 mg l⁻¹) was largely independent of the presence or absence of Tergitol NP-10. These results suggest that low substrate solubility limited the PAH utilization rate but had little effect on the biomass yield of strain VUN 10,010 grown in PAH mixtures or that a nutrient was limiting in the culture medium.

A proposed mechanism to account for surfactant enhancement of PAH biodegradation (see Section 6.10.1) is that surfactants enhance the PAH mass transfer rate into the aqueous phase by increasing the solid phase dissolution rate *via* PAH incorporation into micelles; the dynamic exchange of PAH molecules between the micelle and the aqueous phase serves to replenish the supply of PAH compounds more quickly into the aqueous pseudophase as they are degraded (Liu *et al.*, 1995; Volkerling *et al.*, 1995). The more micelles in solution, the faster the replenishment rate of PAHs removed from the aqueous phase by biodegradation and, consequently, the faster the overall PAH biodegradation rate (Edwards *et al.*, 1992). The surfactant-enhanced PAH degradation and growth rates of VUN 10,010 observed in this work suggests that the PAH degradation rate of the surfactant-free cultures was restricted in part by the PAH dissolution rate, supporting the above hypothesis. However, two aspects of the results in this work cannot be explained in terms of PAH dissolution rate alone, these being:

- (i) the results show that surfactants reduced the PAH degradation lag period (Figures 6.10-6.12). An enhanced PAH dissolution rate cannot explain this observation since PAH utilization during the degradation lag period is very low, if at all.
- (ii) the degradation rates of phenanthrene and fluorene were substantially slower than pyrene in the mixed PAH control cultures which did not contain surfactant (Figure 6.12). Since both of the former PAHs have higher dissolution rates than pyrene, this result suggests that the rate-limiting step for biodegradation of phenanthrene and fluorene in the PAH mixture is not their dissolution rate into the aqueous pseudophase, rather, it is associated with the rate at which strain VUN 10,010 can catabolize these two compounds. However, a significant improvement in phenanthrene and fluorene degradation rates was observed when surfactant was added to these cultures which should not be possible if surfactants only enhance the PAH mass transfer rate into the aqueous pseudophase.

These observations suggest that there may be mechanisms involved in the surfactant enhancement of high molecular weight PAH degradation in addition to an increased PAH dissolution rate. This is supported by recent studies which found that PAH biodegradation in the presence of surfactants was greater than predicted by mathematical models which assumed that microorganisms did not have direct access to micellized PAHs (Guha and Jaffé, 1996a and 1996b; Liu *et al.*, 1995; Zhang *et al.*, 1997). Because of this outcome, the authors concluded that microorganisms have direct access to PAHs in surfactant micelles. A number of mechanisms, in addition to increased dissolution rate, have been proposed to explain the increased PAH degradation rate due to surfactant addition. Guha and Jaffé (1996b) proposed a model describing surfactant-enhanced biodegradation based on micellar-phase substrate being directly available to microorganism. Their hypothesis considered the following steps: (a) the PAH is transported by filled micelles from the bulk solution to within close proximity of the cells; (b) an exchange of the filled micelle with the hemi-micellar layer around the cell delivers

the PAH to the cell; (c) the PAH diffuses into the cell and is biodegraded. Other proposed mechanisms include an emulsifying action of the surfactant to increase the interfacial area and permit the effective contact between cells and substrate (Rittmann and Johnson, 1989), and membrane fusion of surfactant micelles (Miller and Bartha, 1989). In the latter case, the small micelles are thought to easily pass through the bacterial cell wall and their subsequent fusion with the cell membrane delivers the encapsulated hydrophobic substrate directly to the membrane-bound enzyme systems of the intact cells (Miller and Bartha, 1989). Surfactant-bacteria interactions also have a significant impact. Nonionic surfactants can form complexes with cell membrane proteins (Jahan *et al.*, 1997). Membrane proteins play a significant role in the transport of material across the cell wall. Surfactants may enhance this transport mechanism by formation of surfactant-protein complexes or alter lipid structures to improve diffusion rates. A mechanism which best describes the improved PAH degradation rate due to surfactant addition observed in this work cannot be deduced from the results in this thesis.

6.10.5 PAH Desorption From Soil Using Surfactants

The promising results demonstrated by surfactant addition to VUN 10,010 cultures, as well as those previously presented in Chapter 5 concerning the PAH degrading ability of co-cultures, prompted further investigation into the use of surfactants for improving high molecular weight PAH-soil bioremediation. Soil ecosystems are more complicated in their physicochemical and biological characteristics compared to liquid cultures and this affects the rate and extent of PAH biodegradation in soils.

In most cases, sorption is the rate-limiting step controlling both rate and extent of biodegradation in soil (Carriere and Mesania, 1995; Churchill *et al.*, 1995). The physical/chemical properties of PAHs and the size of the soil aggregate are the major variables which influence the rate of PAH adsorption/desorption. PAH bioavailability is decreased if the soil aggregates are larger and/or the number of aromatic rings in PAH molecules is greater than three (Pignatello and Xing, 1996;

Ramaswami and Luthy, 1997). PAH bioavailability is further reduced by aging of soil-waste matrices, providing more time for PAH diffusion into the aggregates (Hatzinger and Alexander, 1995). It has been noted that sorption of hydrophobic organic compounds into soils is highly correlated with the soil organic fraction and the rate of soil mineralization (Laor *et al.*, 1996; Pignatello and Xing, 1996; Carmichael *et al.*, 1997). The release of PAHs from soil particles therefore seems to be critical in meeting cleanup goals for the bioremediation of PAH-contaminated sites. Several investigations in the past few years have assessed the potential of surfactants in assisting in the treatment of PAH-contaminated soils. Most of this work has consisted of laboratory screening studies using soil-water suspensions or small column apparatus (Ganeshalingam *et al.*, 1994; Churchill *et al.*, 1995; Carriere and Mesania, 1995; Yeom *et al.*, 1996).

The major mechanism for the desorption of non-polar organic compounds in soils, sediments, and aquifers is hydrophobic binding (Weber Jr. *et al.*, 1983). It is well established that application of surfactants enhance the desorption of hydrophobic compounds, such as PAHs, from soils. Yeom *et al.* (1996) demonstrated that Brij 30 substantially increased the rate of PAH desorption from weathered MGP soil. The enhanced PAH mass transfer rate by Brij 30 was attributed to enhanced PAH diffusion into micelles rather than an increase in PAH aqueous concentration. This suggests that the mobilized PAHs from the soil were concentrated inside the micelle rather than in the aqueous phase. Micelle encapsulation of high molecular weight PAHs does, however, stimulate their degradation and mineralization rates as observed by liquid culture results in this chapter and results published by other workers (Liu *et al.*, 1995; Grimberg *et al.*, 1996; Guha and Jaffé, 1996a; Zhang *et al.*, 1997). Such information suggests that desorption of high molecular weight PAHs from soils would, as a consequence, increase their biodegradation rate. The results of this study show that for both PAH-contaminated and PAH-spiked soils, Tergitol NP-10 (2% and 5% w/w) increased the extent of pyrene and benzo[a]pyrene desorption from the soils and stimulated the biodegradation of these compounds as well as other high molecular weight PAHs in the soils.

Only a small percentage of a hydrophobic organic compound sorbed to soil is normally partitioned into the aqueous phase (Means *et al.*, 1980; Voice and Weber Jr., 1983; Laor *et al.*, 1996; Pignatello and Xing, 1996; Ramaswami and Luthy, 1997), and surfactants tested to increase the mobility of such chemicals usually have been used at levels much above their critical micelle concentration (Liu *et al.*, 1991). Ganeshalingam *et al.* (1994) demonstrated the effectiveness of surfactants to enhance the mobilization rate of methyl-naphthalene, anthracene, phenanthrene and pyrene from model soil systems. Ganeshalingam *et al.* (1994) reported that even though PAH solubility was enhanced with surfactant concentrations near CMC in liquid cultures, soil washing with near-CMC solutions failed to significantly enhance PAH removal from the soil. Surfactant concentrations well above the CMC were required for adequate PAH removal rates. The use of surfactants at high concentrations is not only expensive but may also inhibit microbial activity affecting their capacity to metabolize the polluting compounds. Hence, this study investigated the minimum surfactant concentration required to promote PAH desorption and biodegradation. The ability of surfactant to solubilize a PAH from soil is dependent upon various factors, as reported by Lui *et al.* (1991). These include (i) the interaction of PAH with surfactant monomers, (ii) the sorption of PAH on soil, (iii) the sorption of surfactant on soil and its effect on increasing the wettability of soil, and (iv) the partitioning of aqueous-phase PAH with the surfactant micelle.

Without surfactant addition, the initial amount of ^{14}C -pyrene and ^{14}C -benzo[a]pyrene added to the soils in this work was detected in the aqueous phase at concentrations less than 1% and 0.1%, respectively, when equilibrium was attained (after approximately 48-72 hours). The amount of PAH desorbed from soil was proportionally increased with an increase in surfactant concentration. For the same PAH and surfactant concentration, desorption of pyrene or benzo[a]pyrene was always higher in PAH-spiked soil compared to PAH-contaminated soil. This was probably due to interaction between soil composition and PAH noting that the PAH-spiked soil contained a high proportion of sand, which could reduce the number of hydrophobic binding sites in the soil and facilitate PAH mobilization. In PAH-contaminated soil, not only PAH compounds,

but other hydrocarbons as well as heavy metals were present; this may be a factor responsible for retarding mobilization of the tested PAHs. This result is also likely to stem from the observation that an increase in the amount of other hydrophobic organics in the soils decreases PAH mobilization (Voice and Weber Jr., 1983; Ganeshalingam *et al.*, 1994). Ganeshalingam and co-workers (1994) also demonstrated that in comparison to single PAH systems, PAH mobilization was significantly reduced when another hydrocarbon was present at high concentrations, most likely due to competition for a place within the hydrophobic core of the micelle. In the case of the soils used in this work, the PAH-contaminated soil contained a high proportion of total organic material compared to the PAH-spiked soil and the contaminated soil also contained weathered PAH contaminants which may have interacted with any spiked materials.

The most important surfactant parameter for mobilizing hydrophobic compounds in contaminated soil is the surfactant CMC. In general, only when micelles are present will significant desorption of organic compounds from the soil matrix take place. In environments such as soils and sediments, adsorption of surfactants to soil particulate surfaces results in much higher total surfactant concentrations being necessary to achieve micellization in pore water than would be necessary in clean water systems.

Tergitol concentrations used in this soil study varied from 0.5% to 5.0%. The overall effectiveness of Tergitol NP-10 in pyrene and benzo[a]pyrene mobilization from soils increased with increasing surfactant concentrations. Maximum PAH desorption occurred after approximately 48 and 72 hours for pyrene and benzo[a]pyrene, respectively. Concentrations of Tergitol NP-10 up to 5% not only increased the amount of both PAHs desorbed from soils, but also further stimulated the rate of biodegradation compared to controls that did not receive surfactant. However, there was no significant difference between the results when 2% and 5% of Tergitol NP-10 were applied even though at the higher surfactant concentration, *i.e.* 5%, a higher extent of PAH desorption was achieved. This suggests that the extent of PAH mobilization does not necessarily translate to an increased biodegradation rate. This outcome is most likely due to

limitations with the microbial PAH catabolism. The 5% Tergitol addition may not have further stimulated PAH degradation because the maximum microbial PAH catabolic rate had been achieved and PAH bioavailability was no longer a rate controlling step.

6.10.6 PAH Degradation in Surfactant-amended Soils

It has been clearly demonstrated by the results shown in this chapter that PAH biodegradation and mineralization rates in soils were substantially stimulated by the addition of the nonionic surfactant, Tergitol NP-10. The inoculated PAH-spiked soils amended with a surfactant concentration of 2% (w/w) showed an increase in the high molecular weight PAH removal rate which was much greater than those obtained from the control soil cultures which were not amended with the surfactant (Tables 6.10 and 6.11). For example, the degradation rate of benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene, in the presence of surfactant, by bacterial cultures of either consortium VUN 10,009 or *S. maltophilia* VUN 10,010 was enhanced by approximately 31-67%. The addition of 2% (w/w) Tergitol NP-10 to PAH-spiked soil also stimulated the degradation rate by of these PAHs by *P. janthinellum* VUO 10,201 (approximately 21-23%). The rate of benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene degradation by either co-culture A or co-culture B was improved by 21-45% in surfactant-amended PAH-spiked soil. Moreover, PAH mineralization rates in either PAH-spiked soil or PAH-contaminated soil was stimulated in the presence of Tergitol NP-10 (Table 6.12).

According to the literature on surfactant-amended PAH degradation in liquid cultures, there are conflicting views and evidence as to whether the presence of surfactants in soil enhances or inhibits the PAH degradation. Overall, the evidence favors the suggestion that solubilization of sorbed PAHs by surfactants can also enhance their biodegradation. In some cases, PAH degradation can be enhanced by surfactants even in the absence of a surfactant effect on PAH desorption. Aronstein *et al.* (1991) found that the extent of phenanthrene biodegradation was markedly increased by nonionic surfactants (Alfonic 810-60

and Novel II 1412-56) at a concentration of $10 \mu\text{g kg}^{-1}$ soil in both a mineral and organic soil, despite a lack of phenanthrene desorption enhancement in the organic soil. These surfactants also enhanced the mineralization of biphenyl in the organic soil at $100 \mu\text{g kg}^{-1}$ soil without having an effect on biphenyl desorption. In the work of Aronstein *et al.* (1991) it appears that even at very low concentrations, the nonionic surfactants were capable of promoting the mineralization of sorbed aromatic compounds in polluted soil, even in cases where surfactant-assisted desorption was not apparent. Further studies by Aronstein and Alexander (1992 and 1993) have confirmed this observation. Jahan *et al.* (1997) also observed enhanced mineralization of PAHs in soils in the presence of surfactants when desorption of the same PAHs by the surfactant was not significant. An explanation for this observation, which has not previously been proposed, may be that the surfactants enhanced the desorption of organic compounds other than PAHs in the soil which the microorganisms can use as a substrate. The enhanced released of organic substrate may stimulate microbial growth and, also, result in the stimulation of co-metabolic degradation or mineralization of the PAHs in the soils.

The degradation of PAHs in soil typically employs high cell population inocula (Trevors *et al.*, 1994). The reason is that high cell numbers may ensure survival of the introduced cells due to a lower possibility of the indigenous microorganisms outcompeting the introduced microbes for nutrients and other survival factors. The survival of *S. maltophilia* VUN 10,010 and *P. janthinellum* VUO 10,201 in non-sterile contaminated soil is difficult to measure, however, there was no evidence of the negative effects of competition between indigenous and introduced microorganisms on PAH degradation in this investigation.

Soil characteristics are known to play an important role in degradation and availability of compounds. Considerable differences in the biodegradation of phenanthrene have been observed between mineral and organic soils (Aronstein *et al.*, 1991). The experiments in this thesis showed a very slight difference between both soils in the rate of pyrene mineralization, however, substantial differences in rate and extent of benzo[a]pyrene mineralization was observed between the PAH-

spiked soil and PAH-contaminated soil. A higher rate and extent of benzo[*a*]pyrene mineralization was measured in the PAH-contaminated soil compared to the PAH-spiked soil, especially when Tergitol NP-10 was present. The higher benzo[*a*]pyrene mineralization rate in the PAH-contaminated soil was unexpected given the age of the soil and the presence of inhibitory compounds such as heavy metals. An explanation for this observation may be that, as described above, the higher organic content of PAH-contaminated soil could have provided the microflora with a greater supply of organic substrate, thereby increasing their metabolic activity and, consequently, PAH degradation rate.

9.10.7 Soil Detoxification by Means of Biodegradation

Biological treatment reduced soil mutagenicity in all the experiments in this chapter. Such results correspond to those previously reported in Chapter 5 (Section 5.9) regarding the reduction in mutagenic activity of liquid and soil cultures containing high molecular weight PAHs. However, the results obtained from experimental data presented in this chapter reveal that the reduction rate of soil mutagenicity is stimulated when surfactant (Tergitol NP-10) was present in the soils, either in the PAH-spiked soil or the PAH-contaminated soil. These results are consistent with previous reports that detoxification of PAH-contaminated soil (Weissenfel *et al.*, 1992; Belkin *et al.*, 1994; Tiehm *et al.*, 1997) can be achieved by surfactant enhanced biological treatment. Recently, Tiehm and co-workers (1997) reported on the enhancement of PAH mobilization and biodegradation by the addition of nonionic surfactant Sapogenat T-300, which corresponded to a substantial decrease in soil toxicity assayed by the Microtox™ test. In contrast to results in this thesis, studies by Belkin and co-workers (1994) showed that there was a decrease in PAH contaminants during the biodegradation of PAH-contaminated soils in column percolators, however, this was accompanied by an increase in genotoxic potential of the soil effluents as determined by Mutatox™ assay. However, the genotoxicity was abolished when a surfactant was added to the percolating fluids (the authors did not specify the type or name of the surfactant used in their studies). Conclusions drawn from the results in this chapter, as well as those presented elsewhere are that the combination of

surfactants and PAH-degrading microorganisms results in the stimulation of a reduction in soil mutagenicity, which is most likely due to a stimulation in the rate and extent of PAH degradation.

6.10.8 Concluding Remarks

The most significant result of this chapter is that the degradation of high molecular weight PAHs containing up to seven-benzene rings by *S. maltophilia* strain VUN 10,010, bacterial consortium VUN 10,009, *P. janthinellum* VUO 10,201 and fungal-bacterial co-cultures can be enhanced by the application of nonionic surfactants in liquids and soils. Furthermore, the PAH degradation rate is substantially improved when surfactants are present in single or mixed PAH cultures. The results stemming from the surfactant and co-culture work have direct implications for soil flushing technologies which have recently been shown to be highly successful when surfactant solutions are used to solubilize recalcitrant high molecular weight PAHs from weathered PAH contaminated sites (Churchill *et al.*, 1995; Joshi and Lee, 1996; Yeom *et al.*, 1996), but for which there have been no previous reports of microbial isolates capable of degrading the leachates from such operations. When bioreactors containing the co-culture are used in conjunction with soil-flushing techniques, it may be possible to effectively decontaminate PAH-polluted soils which would otherwise be highly recalcitrant to degradation using other bioremediation techniques.

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

The following conclusions regarding the microbial degradation of PAHs have been reached as a result of the work conducted in this thesis.

7.1.1 Isolation and Characterization of PAH-degrading Microorganisms

- Two PAH-degrading bacterial consortia, VUN 10,008 and VUN 10,009, which utilized pyrene as a sole carbon and energy source were enriched from PAH-contaminated soil A (coal gasification-polluted soil) and soil B (creosote-polluted soil), respectively.
- A combination of fungal and bacterial growth was observed in soil B-enriched cultures containing benzo[a]pyrene or dibenz[a]anthracene as a sole source of carbon and energy. However, no single organism could be isolated on these PAHs as a sole carbon and energy source.
- One fungal isolate was obtained from the isolation procedures; it was identified as *Penicillium janthinellum* and designated as strain VUO 10,201.
- Three pure isolates of PAH-degrading bacteria were isolated from bacterial consortium VUN 10,008 and one bacterial isolate was obtained from the consortium VUN 10,009. All of these isolates were originally identified as members of the genus *Pseudomonas* according to biochemical tests and were designated as strains VUN 10,010, VUN 10,012, VUN 10,011 and VUN 10,013, respectively.

- Identification of the four PAH-degrading bacterial strains was more precisely conducted using 16S rRNA gene sequence analysis. Based on this technique, the isolates were identified as *Stenotrophomonas maltophilia* VUN 10,010, *Pseudomonas fluorescens* VUN 10,011, *Stenotrophomonas maltophilia* VUN 10,012 and *Burkholderia* sp. VUN 10,013. The 16S rRNA gene sequence analyses, RFLP and DNA-DNA hybridization studies could not differentiate between *S. maltophilia* strains VUN 10,010 and VUN 10,012, the conclusion being that they are most likely the same strain.
- The four PAH-degrading bacterial isolates, as well as their parent mixed cultures, showed the ability to utilize pyrene as a sole source of carbon and energy. In addition to pyrene, these isolates also utilized fluorene and phenanthrene as a sole carbon and energy source. Dioxygenase activity was found to be present in these strains during PAH degradation. The above PAH-degrading bacteria demonstrated a versatility to utilize a wide range of substrates and to degrade various high molecular weight PAH compounds including chrysene, benz[a]anthracene, benzo[a]pyrene and dibenz[a,h]anthracene. However, they were unable to grow on these PAHs as a sole carbon and energy source. Among the four bacterial isolates, *S. maltophilia* VUN 10,010 was found to be the most productive strain in terms of substrate specificity, rapidness in of forming clear zones around colonies when sprayed with PAH solutions and bacterial growth on, and degradation of, various PAHs as sole source of carbon and energy.

7.1.2 PAH Degradation by Axenic and Fungal-bacterial Co-cultures

- Axenic cultures of either bacteria (VUN 10,009 or VUN 10,010) or the fungus (VUN 10,201) were able to degrade benzo[a]pyrene and dibenz[a,h]anthracene as a sole carbon and energy source. However, radiolabeled studies demonstrated that the benzo[a]pyrene was not

mineralized suggesting that these two compounds were only partially degraded under such conditions.

- Under co-metabolic conditions (*i.e.* the presence of a growth-supporting PAH such as pyrene), the degradation of high molecular weight PAHs by bacterial consortium VUN 10,009, *S. maltophilia* VUN 10,010 and *P. janthinellum* VUO 10,201 was substantially improved.
- In BSM containing pyrene or a PAH mixture (comprising three- to seven-benzene ring PAHs), bacterial consortium VUN 10,009 and *S. maltophilia* VUN 10,010 could co-metabolically mineralize benzo[a]pyrene to CO₂. In contrast, *P. janthinellum* VUO 10,201 could not mineralize benzo[a]pyrene under co-metabolic conditions.
- This thesis is the first published document to demonstrate microbial growth on PAHs containing five-benzene rings (benzo[a]pyrene and dibenz[a,h]anthracene) as a sole carbon and energy source. In this case, it was found that bacterial growth on these PAHs was always alongside the fungus, but the growth of these organisms did not occur on such PAHs when they were incubated separately. This observation suggests that the bacteria and fungus share a common catabolic pathway in utilizing high molecular weight PAHs as their growth substrate.
- This is the first report of the deliberate use of fungal-bacterial co-cultures to degrade PAHs. The results in this thesis have demonstrated that a *P. janthinellum* VUO 10,201 and bacterial consortium VUN 10,009 co-culture was able to mineralize and grow on benzo[a]pyrene as a sole carbon and energy source. Higher benzo[a]pyrene mineralization and degradation rates were achieved when *P. janthinellum* VUO 10,201 was co-cultured with *S. maltophilia* VUN 10,010. This result is significant since VUN 10,010 was isolated from a different site to VUO 10,201 indicating that fungal-bacterial cooperative mineralization of benzo[a]pyrene may not

restricted to species isolated from the same site, and hence selective pressures.

- Further investigations into the cooperative degradation of benzo[*a*]pyrene by fungal-bacterial co-cultures revealed more detail about the microbial relationship of the co-culture. These being:
 - (i) HPLC analysis of DCM extracts generated from benzo[*a*]pyrene degradation experiments identified the accumulation of a number of byproducts in axenic cultures of either *P. janthinellum* VUO 10,201 or the bacteria (*S. maltophilia* VUN 10,010 or bacterial consortium VUN 10,009). HPLC analysis of benzo[*a*]pyrene degradation experiments using the fungal-bacterial co-cultures identified not only the accumulation of the same byproducts as those observed in the axenic cultures, but it also demonstrated the subsequent disappearance of these byproducts and the appearance of byproducts which were not present in the axenic cultures. This data provides evidence of metabolic bottlenecks being curtailed by the shared PAH catabolisms of the fungus and bacteria and the functioning of catabolic pathways which were not operating under axenic conditions.
 - (ii) In fungal-bacterial co-cultures, *P. janthinellum* VUO 10,201 most likely plays the major role in the initial oxidation of the benzo[*a*]pyrene molecule with the bacteria utilizing the fungal byproducts as a growth substrate and subsequently mineralizing them to carbon dioxide and water.
 - (iii) The oxidation of benzo[*a*]pyrene by *S. maltophilia* VUN 10,010 results in byproducts which can be mineralized by *P. janthinellum* VUO 10,201, however, benzo[*a*]pyrene mineralization via this pathway appears to have a lesser role than that mentioned (ii) above during fungal-bacterial co-culture degradation of benzo[*a*]pyrene.

- The ability of the fungal-bacterial co-cultures to mineralize benzo[a]pyrene, and grow on and degrade other high molecular weight PAHs, was not restricted to microorganisms isolated from the same soils. *P. janthinellum* VUO 10,201 and *S. maltophilia* VUN 10,010 were isolated from different soils and yet their combination produced the best benzo[a]pyrene degradation and mineralization rates. This suggests that other fungal-bacterial combinations may be able to produce even better PAH degradation rates than those observed in this thesis and that the microorganisms used in the co-cultures probably do not need a common enrichment and isolation history.
- Co-metabolic mineralization of benzo[a]pyrene occurred in PAH-spiked soil and PAH-contaminated soil when inoculated with axenic cultures of either bacterial consortium VUN 10,009 or *S. maltophilia* VUN 10,010. Substantial amounts of $^{14}\text{CO}_2$ evolved from soil inoculated with axenic inocula of these bacteria. Co-metabolic benzo[a]pyrene mineralization may also have occurred in the soils inoculated with the co-culture, however, mineralization of this PAH also occurred as a result of co-culture activity in such soils since there was an absence of the mineralization lag period typical of soils inoculated only with the bacteria, and because substantially greater yields of $^{14}\text{CO}_2$ were achieved in the co-culture soils compared to the axenic bacteria-inoculated soils. This result demonstrated the stability of the co-culture relationship in an environment containing a number of inhibitory and stimulatory influences on the inocula.

7.1.3 Surfactant Enhanced PAH Biodegradation

- Nonionic surfactants; Brij 35, Igepal NP-10, Tergitol NP-10, Triton X-100 and Tyloxapol, which were less toxic to and not utilized by PAH-degrading microorganisms, were chosen for PAH degradation experiments due to their ability to significantly increase the apparent solubility of PAH

compounds (fluoranthene, pyrene and benzo[a]pyrene). The apparent solubility of pyrene and fluoranthene in BSM was increased by over 250-fold and the apparent solubility of benzo[a]pyrene was increased by a factor of 10^3 - 10^4 at a surfactant concentration of 10 g l^{-1} compared to their solubility at CMC.

- Lower MSR and higher $\log K_m$ values were observed for benzo[a]pyrene relative to those measured for fluoranthene and pyrene. This indicates that, on a molar basis, less benzo[a]pyrene was incorporated into the surfactant micelles from the nonpolar pseudophase, compared to fluoranthene and pyrene, and that a greater proportion of the total amount of benzo[a]pyrene in the micelle/aqueous pseudophases was embodied in the micelles. This result can be attributed to the much higher hydrophobicity of benzo[a]pyrene compared to that of fluoranthene and pyrene.
- Microbial growth and pyrene degradation by *S. maltophilia* VUN 10,010 was enhanced by the addition of Brij 35, Tergitol NP-10, Triton X-100 and Tyloxapol at concentrations up to 10 g l^{-1} . Igepal CA-630 at 5 g l^{-1} was inhibitory to pyrene degradation which correlates with its growth-inhibitory effect at this concentration on glucose-grown VUN 10,010. The specific growth rate on pyrene was 67% greater in the presence of Brij 35 or Tergitol NP-10 at 10 g l^{-1} than their absence. Enhanced PAH degradation was also observed when Brij 35 and Tergitol NP-10 were used in *S. maltophilia* VUN 10,010 cultures containing a single high molecular weight PAH or a PAH mixture. The maximum specific PAH degradation rate for most PAH compounds supplied as single or mixed PAH substrates was increased by surfactant addition.
- The surfactant-enhanced PAH degradation and growth rates of VUN 10,010 observed in this work suggests that the PAH degradation rate of the surfactant-free culture was, in part, restricted by the PAH dissolution

rate into aqueous phase, supporting the hypothesis of previous workers (Liu *et al.*, 1995; Volkerling *et al.*, 1996). However, there is evidence in this thesis to suggest that the above mechanism may not be the only factor behind surfactant-enhanced PAH degradation. This evidence being:

- (i) The lag period during high molecular weight PAH degradation was observed to be decreased in the presence of surfactant. An enhanced PAH dissolution rate cannot explain this observation since PAH utilization during the degradation lag period is very low, if at all.
- (ii) In the PAH mixtures without added surfactant, the degradation rates of phenanthrene and fluorene were substantially slower than pyrene. This suggests that their slower degradation rates were due to the VUN 10,010 catabolism since both of the former PAHs have a higher dissolution rate than pyrene *i.e.* dissolution rate was not responsible for their slower degradation rate otherwise they could be expected to be degraded at a rate at least equal to that of pyrene. However, in the presence of surfactant, the degradation rates of phenanthrene and fluorene were significantly increased which could not be the result of their enhanced dissolution rate since this did not appear to be controlling their degradation rate when the surfactant was absent.

The other mechanisms behind the enhanced PAH degradation rates due to surfactant addition could not be determined from the data in this thesis.

- A higher concentration of surfactant was required to solubilize PAHs in soil compared to those used in liquid media. The amount of PAH desorbed from soil proportionally increased with an increase in surfactant concentration. Furthermore, for the same PAH and surfactant concentration, desorption of pyrene or benzo[a]pyrene was always higher in PAH-spiked soil compared to the PAH-contaminated soil. This was probably due to interaction between the soil components and the PAHs noting that the PAH-spiked soil contained a high proportion of sand,

which could reduce the number of hydrophobic binding sites in the soil and facilitate PAH mobilization. In PAH-contaminated soil, not only PAH compounds, but other hydrocarbons as well as heavy metals were present; this may be a factor responsible for retarding mobilization of the tested PAHs.

- Tergitol concentrations used in the soil study varied from 0.5% to 5.0%. Concentrations of Tergitol NP-10 up to 5% not only increased the amount of pyrene and benzo[a]pyrene desorbed from soils, but also further stimulated their biodegradation rate compared to controls that did not receive surfactant. However, there was no significant difference between the results when 2% and 5% of Tergitol NP-10 were applied even though at the higher surfactant concentration a higher extent of PAH desorption was achieved. This suggests that the extent of PAH mobilization does not necessarily translate to an increased biodegradation rate. This outcome is most likely due to limitations with the microbial PAH catabolism.
- High molecular weight PAH biodegradation and mineralization rates by axenic cultures and fungal-bacterial co-cultures in soils were substantially stimulated by the addition of the nonionic surfactant, Tergitol NP-10.
- PAH degradation could be enhanced by surfactants even in the absence of a surfactant effect on PAH desorption from the soil. An explanation for this observation may be that the surfactants enhance the desorption of organic compounds other than PAHs in the soil which may stimulate microbial growth and, also, result in the stimulation of co-metabolic degradation or mineralization of the PAHs in the soils.
- A higher rate and extent of benzo[a]pyrene mineralization was measured in the PAH-contaminated soil compared to the PAH-spiked soil in the presence of Tergitol NP-10. The higher benzo[a]pyrene mineralization rate in the PAH-contaminated soil was unexpected given the age of the

soil and the presence of inhibitory compounds such as heavy metals. The higher organic content of PAH-contaminated soil may have provided the microflora with a greater supply of organic substrate, thereby increasing their metabolic activity and, consequently, PAH degradation rate.

- The results shown in this thesis have direct implications for soil flushing technologies which have recently been shown to be highly successful when surfactant solutions are used to solubilize recalcitrant high molecular weight PAHs from weathered PAH-contaminated sites (Churchill *et al.*, 1995; Joshi and Lee, 1996; Yeom *et al.*, 1996) but for which there have been no previous reports of microbial isolates capable of degrading the leachates from such operations. When bioreactors containing *S. maltophilia* VUN 10,010, bacterial consortium VUN 10,009, *P. janthinellum* VUO 10,201 or co-cultures are used in conjunction with soil-flushing techniques, it may be possible to effectively decontaminate PAH-polluted soils which would be highly recalcitrant to degradation using other bioremediation techniques.

7.1.4 PAH Detoxification

- Incubation of PAH-contaminated liquid soil cultures inoculated with VUN 10,009, VUN 10,010, VUO 10,201, co-culture A and co-culture B markedly reduced the mutagenicity of these polluted media. The greatest mutagenicity reduction rate in all PAH-contaminated media occurred when co-culture B was used for the decontamination process.
- The reduction rate of soil mutagenicity was stimulated when surfactant (Tergitol NP-10) was present in the soils, either in the PAH-spiked soil or the PAH-contaminated soil. The combination of surfactants and PAH-degrading microorganisms results in a substantial reduction in soil mutagenicity, this is most likely a consequence of a stimulation in the rate and extent of PAH degradation.

7.2 RECOMMENDATIONS FOR FURTHER WORK

7.2.1 Identification of PAH Catabolic Pathways

It is possible that bacteria may use different pathways to metabolize different PAHs. The results in Chapter 4 (Section 4.6.3) demonstrated that the PAH catabolism among the bacterial strains tested may be different for low molecular weight PAHs compared to high molecular weight compounds. Also, one PAH compound could be metabolized by different mechanisms according to the particular bacterial strain used. Further investigations into the identification of the PAH metabolic pathways in these bacteria for various PAHs are necessary which would provide a clearer picture on the evolutionary path of bacteria adapted to PAH degradation and identify catabolic bottlenecks in the bacterial isolates which may, in the future, be overcome by genetic or environmental manipulation.

The Use of Catabolic Intermediates as Inducers for PAH Catabolism

Following the identification of PAH catabolic pathways and intermediate production, the known intermediate compounds may be used as inducers to enhance the degradation rate of a particular PAH. As shown in Chapter 4 (Section 4.6.3), salicylic acid stimulated the dioxygenase activity and the degradation rate of some low molecular weight PAHs such as fluorene and phenanthrene, but not for the higher molecular weight PAH, pyrene. Although this thesis did not identify the catabolic pathways of these PAHs, it was assumed, based on the literature, that salicylic acid was likely metabolite produced during the breakdown of fluorene and phenanthrene. However this needs to be verified. The use of pathway intermediates as inducers for PAH degradation would be useful in practice scheme for PAH bioremediation, depending on the cost of such supplementation.

7.2.2 Investigation of High Molecular Weight PAH Mineralization Using Co-cultures

High Molecular Weight PAH Mineralization

It was postulated, based on microbial growth on high molecular weight PAHs, that, in addition to benzo[a]pyrene, the co-cultures may also be able to mineralize other high molecular weight PAHs such as benz[a]anthracene and dibenz[a,h]anthracene as a sole carbon and energy source. This may be investigated by incubation of the co-cultures with ^{14}C -labelled PAHs and the non-labeled compound as a sole carbon and energy source and monitoring the evolution of $^{14}\text{CO}_2$ and the radioactivity incorporated in the cell biomass. This would be required to ensure that the co-cultures are able to completely remove all high molecular weight PAHs from contaminated sites, rather than simply transform them to other compounds which may be more toxic

Co-culture Association

The discovery of a mechanism, *i.e.* fungal-bacterial co-incubation, by which microorganisms may grow on and mineralize benzo[a]pyrene provides an opportunity to identify a catabolic pathway by which this PAH, and possibly others, may be completely degraded. Such information would be important for identifying the current limitations of PAH-degraders which can, at the moment, only biotransform benzo[a]pyrene and are limited for practical use by their inability to grow on this compound. Such information could be used to either genetically or environmentally alter such PAH-degraders enabling their complete degradation of benzo[a]pyrene and possibly other recalcitrant PAHs. This could be performed by using a fraction collector coupled to the HPLC to obtain sufficient quantities of selected metabolic byproducts for NMR analyses. Alternatively, analysis of benzo[a]pyrene intermediates generated from axenic and co-culture incubations of *P. janthinellum* and *S. maltophilia*

could be performed using HPLC coupled to an electrospray MS or by using GC-MS.

Fungal-bacterial Co-culture Diversity

The fungal-bacterial combinations used in this thesis were not restricted to microorganisms isolated from the same site. *P. janthinellum* VUO 10,201 and *S. maltophilia* VUN 10,010 were isolated from different soils yet these organisms proved to be the best co-culture combination for the mineralization of benzo[a]pyrene. This suggests that the degradation of high molecular weight PAHs may be even further improved by the 'mixing and matching' of various PAH-degrading fungi and bacteria. Such experiments would provide information on the best 'groups' of fungi and bacteria which could be combined in co-cultures to ensure rapid bioremediation of PAH-contaminated sites.

Fungal-bacterial Stability

The fungal-bacterial co-cultures used in this thesis demonstrated an ability to rapid mineralize benzo[a]pyrene in PAH-spiked soil and PAH-contaminated soil. This indicates that the cooperative interaction of such co-cultures is relatively stable under the inhibitive and promotive pressures of the soil environment on microbial activity. However, before such co-cultures could be put to practical use, it would be appropriate to test the stability of the fungal-bacterial relationship in liquid cultures under condition which impose a considerable stress on microbial viability and/or activity. For example, benzo[a]pyrene mineralization by co-culture B in BSM could be tested by adding high concentrations of an easily metabolized substrate, *e.g.* glucose, and in a separate experiment, by adding inhibitory compounds that have a more adverse affect on one of the organisms, *e.g.* heavy metals to which bacteria may be more sensitive. In addition, PAH degradation by the co-cultures could be tested by using inocula containing large differences in the ratio of the biomass of each organism *e.g.* benzo[a]pyrene mineralization could be compared for cultures amended with inocula containing high fungal but low bacterial

populations, and inocula containing low fungal but high bacterial populations.

The Use of Fungal-bacterial Co-cultures to Degrade Other Xenobiotics

The success of the fungal-bacterial co-cultures in degrading high molecular weight PAHs may not be restricted to PAH degradation. Other xenobiotic compounds which are considered recalcitrant may be degraded by using fungal-bacterial co-cultures. This could easily be tested using the isolates obtained in this thesis but it would also be appropriate to use combinations of fungi and bacteria which are known to have some sort of degradative activity toward the xenobiotic in question. Such recalcitrant compounds could include nitro- and chloro-aromatic and composite pollutants such as crude oil.

7.2.3 Molecular Approaches to PAH Biodegradation

16S rRNA Gene Sequences

The 16S rRNA genes of the four PAH-degrading bacteria isolated in Chapter 4 were only partially sequenced. Sequences of approximately 50 bp each from the 5' and 3' termini are still unsequenced. These portions could be obtained by cloning the 16S rRNA gene into a plasmid vector and then using an appropriate pair of primers specific to the vector to sequence upstream and downstream. The remaining unsequenced portion of the 16S rRNA gene may contain a variable region that could identify a difference between *S. maltophilia* VUN 10,010 and VUN 10,012 which cannot be differentiated according to the data in this thesis. This is also useful for the construction of the phylogenetic tree of PAH-degrading bacteria.

PCR Detection of PAH-degrading Bacteria Based on 16S rRNA

Genes

Results from this thesis have shown that pure isolates of PAH-degrading bacteria, in particular *Stenotrophomonas maltophilia* VUN 10,010, can degrade high molecular weight PAHs and also co-metabolically mineralize benzo[a]pyrene when grown on pyrene in either liquid or soil media. Therefore, this bacterial strain is well placed to use for decontamination of PAH-contaminated sites. Studies to understand the movement, survival and growth of specific microbial populations in the environment require the use of methods which can detect and estimate the relative abundance of the specific strain when such strains are present in relatively low numbers in complex and diverse microbial communities. The 16S rRNA gene of VUN 10,010 was successfully amplified and analyzed in this work. For further application of this bacterial strain as an inoculum in bioremediation of PAH-contaminated sites, the development of PCR methods based on the 16S rRNA gene sequences is recommended for the quantitative detection of PAH-degrading strains (and also some other PAH-degrading strains which could be developed in a similar manner) in soils. This technique has potential application as described by many researchers (Picard *et al.*, 1992; Briglia *et al.*, 1996; Wang *et al.*, 1996).

Strain-specific DNA Probe for Detecting PAH-degrading Bacteria in PAH-contaminated Soils

In addition to PCR, the detection of the specific PAH-degrading bacteria *in situ* could be accomplished by the use of nucleic acid probes. The 16S rRNA gene-specific probe could also be designed to detect the specific PAH-degrading bacteria introduced into contaminated soil ecosystems. The specific probe could be accomplished through either the selection of variable region of the 16S rRNA gene (Briglia *et al.*, 1996) or by repetitive sequence-based (rep-PCR) that are subsequently cloned and used as a probe (Versalovic *et al.*, 1994).

Identification the Presence of Dioxygenase genes

As indicated in Chapter 4 (Section 4.6.3), the four PAH-degrading bacterial strains isolated in this thesis possess dioxygenase activity would be useful to identify the presence of this gene among these bacteria. This could be achieved by using PCR amplification techniques. PCR primers could be designed from the conserved regions of dioxygenase gene sequences from Gram negative strains available on database (GeneBank, NCBI). PCR products corresponding to the predicted fragment size could then be sequenced and analyzed. This gene or a specific part of the gene could be further used as a probe for the detection of PAH-degrading microorganisms in the environment and assist in the identification of the PAH-catabolic pathway by the isolates obtained in this thesis.

7.3.4 Identification of Mechanisms Involving Surfactant-enhanced PAH Biodegradation

The results in this thesis indicate that surfactants have a great impact on increasing solubility of PAHs and thus their biodegradation. However, more investigation is needed to identify the mechanism(s) involving the interaction between surfactants, PAHs and microorganisms. Frequently, PAH partitioning into surfactant micelles are reported as being unavailable and inhibit the accessibility of PAH substrates to microorganisms. An ultrafiltration technique could be used to determine the partitioning of a PAH between surfactant micelles and aqueous pseudophase, and its availability to microbial attack (Rouse *et al.*, 1994). This is useful for the prediction of the efficiency of PAH degradation by PAH-degrading microbes in either laboratory or field scale.

7.3.5 Bioremediation of PAH-contaminated Sites

There is significant potential for use of the PAH-degrading microorganisms, especially the co-cultures, isolated in this thesis in PAH bioremediation programs. However, further taking of their practical use

need to be taken into consideration before the isolates are used to bioremediate soils on a large scale.

Stability of PAH-degrading Potential in the Environment

Survival, activity and stability of PAH-degrading microbes, especially the co-cultures, in the PAH-contaminated soils are most important in remediation projects. It is considered that bioprocess optimization and engineering modifications could facilitate the effective use of powerful PAH-degrading strains if the inhibitory constituents of complex organic mixtures which are common by found in PAH-contaminated soils are identified. Therefore, inhibitory effects of various substrates such as the chemical components of creosote and heavy metals need to be assessed for their effects on microbial PAH degradation, mineralization and growth.

Development of Method Suitable for Surfactant-enhanced PAH

Biotreatment

The considerable improvement in high molecular weight PAH degradation resulting from surfactant addition needs to be trialed on a larger scale. Soil flushing techniques are currently proving effective for the removal of PAHs from soils, especially weathered soils, but a problem remains in the effective treatment of the PAH-laden leachates, which also contain surfactants, generated by the process. The co-cultures described in this thesis have been shown to rapidly degrade and mineralize PAHs in surfactant solutions and thus could be used in liquid bioreactors to degrade and mineralize the leathates resulting from soil flushing operations. Alternatively, the microorganisms could be suspended in surfactant solutions and injected directly into the soils.

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APPENDIX

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APPENDIX A

BACTERIAL IDENTIFICATION: MEDIA AND BIOCHEMICAL TESTS

The biochemical tests for bacterial identification were performed based on methods described by Collins *et al.* (1989) and Department of Microbiology, University of Melbourne (1995). Some of these tests are briefly described as follows:

A.1 Aesculin Hydrolysis

A test for hydrolysis of the glycoside aesculin to aesculetin and glucose. The aesculetin can react with ferric ions to form a dark brown/black complex.

Medium: Aesculin broth

Formula per litre of distilled water;

Nutrient broth No.2	25	g
Yeast extract (Oxoid)	3	g
Aesculin	1	g
Ferric citrate	0.5	g
Agar	12	g

Boil to dissolve ingredients, adjust pH and sterilize by autoclaving at 110°C for 25 min. Pour as plates.

Test: Aesculin broth was inoculated with a culture. Incubation was performed at 30°C and examined daily up to 7 days.

Result: Blackening in and around bacterial growth indicates hydrolysis of the aesculin.

A.2 Arginine Dihydrolysis

This biochemical reaction is known as the arginine dihydrolase reaction which involves the conversion of arginine to ornithine and ammonia accompanied by the production of ATP.

Medium: Arginine agar

Formula per litre distilled water;

Bacto Peptone	1.0	g
NaCl	5.0	g
K ₂ HPO ₄	0.3	g
Phenol red	0.01	g
L-arginine HCL	10.0	g
Agar	3.0	g

Dissolve by heating, dispense in Bijoux bottles, sterilize by autoclaving at 121°C for 15 min. The final color should be light pink.

Test: Bacterial cultures were stabbed in two tubes of arginine agar. The surface of one tube was covered with a 5 mm layer of sterile paraffin. Incubation was performed at 30°C from three days and up to two weeks.

Result: A positive reaction is shown by a color change to red in both tubes after incubation.

A.3 Carbohydrate Utilization Test

Medium: Ammonium salt sugar medium

Formula per litre;

(NH ₄) ₂ HPO ₄	1.0	g
KCl	0.2	g
MgSO ₄ ·7H ₂ O	0.2	g
Bromocresol purple (0.2% aq)	4.0	ml

Add solids to water, boil to dissolve, add indicator, sterilize by autoclaving at 121°C for 20 min. Cool to 50°C, add (filtered) sterile carbohydrate to a final concentration of 1.0 % (0.1 % aesculin; 0.2 % starch; these are sterilized by autoclaving. Dispense aliquots into sterile McCartney bottles. Allow to set with a deep butt and a short slope.

A.4 Fluorescein Production

Medium: Fluorescein medium

Formula per litre distilled water:

Proteose peptone	20	g
Glycerol	10	ml
Oxoid (No.1)	10	g
K ₂ HPO ₄	1.5	g
MgSO ₄ ·7H ₂ O	1.5	g
Agar	10	g

Dissolve ingredients by boiling and adjust to pH 7.2. Dispense as 15 ml agar slope in McCartney bottles

A.5 Gelatin Liquefaction

This is a test for the ability of an organism to produce gelatinase to hydrolyze gelatin.

Medium: Gelatin agar

Nutrient agar containing 5% bacteriological gelatin

Test: Bacterial cultures were inoculated into nutrient broth containing a denatured gelatin charcoal disc and incubated at room temperature for seven days to observe the digestion.

Result: A positive reaction for hydrolysis of gelatin is indicated by the release of charcoal granules, which fall to the bottom of the tube.

A.6 Indole formation

A test for the ability of an organism to split indole from the tryptophan molecule by the tryptophanase.

Medium: Peptone Broth

Formula per litre distilled water:

Bacteriological Peptone	10	g
NaCl	5	g

Dissolve ingredients in distilled water, adjust pH to 7.2 and autoclaved at 121°C for 15 min.

Test: Bacterial cultures were grown for two days in peptone broth. To the broth culture, a few drops of Kovac's reagent (5 g of *p*-dimethylamino-benzaldehyde in a mixture of 75 ml of amyl alcohol and 25 ml of concentrated sulphuric acid) to form a surface layer of 2-3 mm deep and shaken gently.

Result: A rose pink color in the top layer indicates indole.

A.7 MacCONKEY Agar

The selective medium acts as an indicator of lactose fermentation.

Formula per litre of distilled water:

Peptone	20	g
Lactose	10	g
Bile salts	5	g
Neutral red	0.075	g
Agar	12	g

Dissolve ingredients, adjust to pH 7.4 and sterilize by autoclaving at 121°C for 20 min. Cool and mix well before pouring plates.

A.8 Motility Test

Medium: Motility Medium

Formula:

Nutrient broth	1000	ml
Nutrient gelatin (Oxoid)	80	g
Agar	3	g

Dissolve agar in nutrient broth by boiling, adjust to pH 8.4 then add the gelatin. Sterilize by autoclaving at 115°C for 10 min. Pour as plates.

Test: Use straight wire to inoculate the centre of the plate, in which the wire should penetrate two-thirds of the depth of the medium.

Result: A non-motile organism grows only at the site of inoculation whereas a motile organism demonstrates a swarming growth extending through the medium of some distance from the original point of inoculation.

A.9 Nitrate Reduction Test

A test for the ability of bacteria to reduce nitrate substrate. Bacterial reduction of nitrate can occur via two major routes: (i) nitrate is reduced to ammonia, via nitrite, which then converted to amino acid and other organic cell components (assimilation) and (ii) dissimilatory nitrate reduction, in which nitrate or nitrite is used as a terminal electron acceptor under anaerobic conditions. This test is based on detecting nitrite after bacterial growth in nitrate-containing medium.

Medium: Nitrate broth

Formula per litre distilled water;

Nutrient broth	25.0	g
KNO ₃ (nitrite free)	1.0	g

Dispense 3.5 ml volumes in Bijou bottles, with Durham tube. Autoclave at 121°C for 15 min.

Test: Inoculate nitrate broth (Appendix A.2) medium and incubate overnight. Place a large drop of a culture on a clean slide and acidify the culture with a few drops of 1 N hydrochloric acid and add 0.5 ml each of a 0.2% solution of sulphanilamide and 0.1% N-naphthylethylenediamine hydrochloride.

Result: a pink color denotes the presence of nitrite. However, as some organisms further reduce nitrite, if no color is produced add a very small amount of zinc dust. Any nitrate present will be reduced to nitrite and

produce a pink color, i.e. the pink color in this part of the test indicates no nitrate reduction activity, while no color indicates that nitrate have been completely reduced.

A.10 Oxidase Test

This test is based on the presence of a cytochrome oxidase system in which the normal function is to transfer electrons from reduced cytochromes to oxygen forming water. The presence of cytochrome oxidase is indicated by the reduction of the colorless agent forming a purple compound.

Test: A colony from a fresh grown bacterial culture was picked up using a glass rod and placed onto a filter paper impregnated with a 1.0 % solution of tetramethyl-*p*-phenylenediamine dihydrochloride.

Result: A positive result is indicated by purple colouration of paper within 10 seconds.

A.11 Oxidation-Fermentation (O-F) Test

This test is also called Hugh and Leifson (HL) test. Some organisms metabolize glucose oxidatively, i.e. oxygen is the ultimate hydrogen acceptor and culture must therefore be aerobic. Others ferment glucose when the hydrogen acceptor is another substance; this is independent of oxygen and cultures may be aerobic or anaerobic.

Medium: Hugh and Leifson's medium

Formula per litre distilled water;

Bacteriological Peptone	2.0	g
K ₂ HPO ₄	0.3	g
NaCl	5.0	g
Glucose	10.0	g
Bromothymol blue (1.0 %)	3.0	ml
Bacteriological Agar	3.0	g

Dissolve ingredient by boiling, check the pH is 7.0-7.1, a deep green colour. Dispense a 5-ml volume in narrow test tubes and sterilize by autoclaving at 110°C for 25 min.

Test: Two tubes of Hugh and Leifson's medium (Appendix A.2) were heated in boiling water for 10 min to drive off any dissolved oxygen and quickly cooled on ice. Immediately after cooling, inocula were stabbed into two steamed tubes of medium with a straight wire. One of the inoculated tubes was overlaid with approximately 1 cm of sterile paraffin oil for anaerobic conditions. Incubation was performed at 30°C and examined daily for up to 14 days.

Result: An acidic (yellow) change at or near the surface of the unsealed tube indicates that the substrate is being oxidized by aerobic bacteria (oxidative metabolism). Acidity in the sealed tube indicates fermentative activity (fermentative metabolism).

A.12 Phenylalanine Deamination

This is a test for the oxidative deamination of phenylalanine to phenylpyruvic acid by enzymatic activity with resulting acidity. The aromatic amino acid phenylalanine undergoes oxidative deamination catalyzed by an amino acid oxidase enzyme (a flavoprotein) to yield the keto acid (phenylpyruvic acid).

Medium: Phenylalanine Agar

Formula per litre of distilled water

Yeast extract	3	g
L-phenylalanine	1	g
Na ₂ HPO ₄	1	g
NaCl	5	g
Agar	10	g

Dissolve all ingredients by boiling, dispense 3.5 ml volumes in Bijoux bottles and autoclaved at 121°C for 15 min. Solidify as a slant.

Test: Phenylalanine agar (Appendix A.2) was inoculated with a heavy bacterial inoculum and incubated overnight. Following the incubation, a few drops of 10% ferric chloride solution was poured over the bacterial growth.

Result: A positive result is the development of a green color in the syneresis fluid and the slant due to the interaction between the iron salts and the carboxyl group of the phenylpyruvic acid.

A.13 Starch Hydrolysis

This is a test for the ability of an organism to produce amylase and hydrolyze starch.

Medium: Starch Agar

Prepare 10% solution of soluble starch in water and steam for 1 hour. Add 20 ml of this solution to 100 ml of melted nutrient agar and pour plate.

Test: Bacterial culture was spot inoculated on starch agar (Appendix A.2) and incubated for 3-5 days.

Result: Hydrolysis of starch is indicated by cloudy zones around the bacterial growth. When plates were flooded with an iodine solution (1% w/v I_2 in 2% w/v KI) the zones become clear. Unchanged starch gives a blue color.

A.14 Urease Test

A test for the ability of an organism to produce the enzyme urease which split urea, forming two molecules of ammonia.

Test: Urea broth was heavily inoculated with bacterial culture and incubated for 3-12 hours. If the result is negative, further incubation is required up to 7-10 days.

Results: Hydrolysis of urea is indicated by a change of medium color from yellow to pink. The rise in pH and hence the color change in the phenol red indicator are due to the formation of NH_3 from the hydrolyzed urea.

APPENDIX B

MEDIA, BUFFERS AND REAGENTS FOR MUTAGENICITY ASSAY (THE AMES TEST)

All media, buffer and reagents described in this section are taken from Maron and Ames (1983).

B.1 Vogel-Bonner Medium E (50X)

Formula per litre;

Warm distilled H ₂ O (45°C)	670	ml
Magnesium sulfate (MgSO ₄ ·7H ₂ O)	10	g
Citric acid monohydrate	100	g
Potassium phosphate, dibasic (anhydrous) (K ₂ HPO ₄)	500	g
Sodium ammonium phosphate (NaH ₂ NH ₄ (PO ₄ ·4H ₂ O))	175	g

Add salts in the order indicated to warm water in a 2-litre beaker or flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to 1 litre. Distribute into two 1-litre glass bottles. Autoclave, loosely capped, for 20 min at 121°C. When the solutions have cooled, tighten the caps.

B.2 0.5 mM Histidine/Biotin Solution

Ingredients per 250 ml;

D-Biotin	30.9	mg
L-Histidine, HCl	24.0	mg
Distilled H ₂ O	250	ml

Dissolve the biotin by heating the water to the boiling point. This can be done in microwave oven. Sterilize by filtration through a 0.22- μ m membrane filter or autoclave for 20 min at 121°C. Store in a glass bottle at 4°C.

B.3 Top Agar

Ingredients per litre;

Agar	6.0	g
Sodium chloride (NaCl)	5.0	g
Distilled H ₂ O	1,000	ml

Dissolve the agar in a steam bath or microwave oven. Mix thoroughly and transfer 100-ml aliquots to 250-ml glass bottles with screw caps. Autoclave for 20 min with loosened caps. Slow exhaust. Cool the agar and tighten caps.

B.4 Salt Solution (1.65 M KCl + 0.4 M MgCl₂)

Ingredients per 500 ml;

Potassium chloride (KCl)	61.5	g
Magnesium chloride (MgCl ₂ .6H ₂ O)	40.7	g
Distilled H ₂ O to final volume of 500 ml		

Dissolve ingredients in water. Autoclave for 20 min at 121°C. Store in glass bottles at 4°C or room temperature.

B.5 0.2 M Sodium Phosphate Buffer, pH 7.4*Ingredients per 500 ml;*

0.2 M sodium dihydrogen phosphate (Na_2HPO_4)	60 ml
(13.8 g/500 ml)	
0.2 M disodium hydrogen phosphate (Na_2HPO_4)	440 ml
(14.2 g/500 ml)	

Mix two solutions with given volumes. Test the pH, if it is too low, add more 0.2 M disodium hydrogen phosphate to pH 7.4. Sterilize by autoclaving for 20 min at 121°C.

B.6 0.1 M NADP (Nicotine Adenine Dinucleotide Phosphate) Solution*Ingredients per 5.0 ml;*

NADP	383 mg
Sterile distilled H_2O	5 ml

Add NADP to pre-weighed sterile glass tubes with screw caps. Wrap the tubes with metal foil to protect against light and place in a jar with a tight fitting lid containing silica gel or other desiccant in the bottom. Store at -20°C. When need for making S9 mix, remove one tube from the jar, add the specified amount of water and mix by vortexing until the NADP has dissolved. Place the tube in an ice bath. This solution can be filter-sterilized, however, it is not necessary. Solutions of NADP stored in the freezer are stable for at least 6 months.

B.7 1.0 M Glucose-6-Phosphate*Ingredients per 10 ml;*

Glucose-6-phosphate (G-6-P)	2.82 g
Sterile distilled H_2O	10.0 ml

Pre-weighed aliquots of G-6-P are prepared as described for NADP and stored in desiccated jar in the freezer. Solutions of G-6-P can also be stored in the freezer and are stable for at least 6 months. If necessary, solutions may be filter-sterilized using 0.22- μ m membrane filter.

B.8 S9 Mix (Rat Liver Microsomal Enzymes Plus Co-Factors)

<i>Ingredients per 50 ml;</i>	<i>Standard S9 mix</i>		<i>High S9 mix</i>	
Rat liver S9 (Aroclor-1254-induced)	2.0	ml (4%)	5.0	ml (10%)
MgCl ₂ -KCl salts	1.0	ml	1.0	ml
1.0 M glucose-6-phosphate	0.25	ml	0.25	ml
0.1 M NADP	2.0	ml	2.0	m
0.2 M phosphate buffer, pH 7.4	25.0	ml	25.0	ml
Sterile distilled H ₂ O	19.75	ml	16.75	ml

Add the ingredients in the reverse order indicated above so that the liver will be added to a buffered solution. The solution must be prepared fresh and keep on ice. All ingredients should be chilled. Any left over S9 or S9 mix should be discarded. *Never refreeze S9.*

B.9 Ampicillin Solution (8 mg/ml)

Ingredients per 100 ml;

Ampicillin trihydrate	0.8 g
Sodium hydroxide (0.02 N)	100 ml

Sterilize ampicillin solution by filtering through a 0.22- μ m membrane filter. Dispense into aliquots and store at -20°C.

B.10 Crystal Violet Solution (0.1%)*Ingredients per 100 ml;*

Crystal violet	0.1 g
Distilled H ₂ O	100 ml

Store at 4°C in a glass bottle with a screw cap. Wrap the bottle with metal foil to protect against light.

APPENDIX C

BUFFERS AND REAGENTS FOR PROTEIN ASSAY

Reagent A

Dissolve 20.0 g of Na_2CO_3 in 1 litre of 0.1 N NaOH.

Reagent B

Dissolve 0.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 ml of 1.0 % (w/v) aqueous solution of K-Na-Tartrate (pH9.0).

Reagent C

Mix 50.0 ml of reagent A and 1.0 ml of reagent B. Prepare just before use.

Folin Ciocalteu Reagent (Diluted)

Mix 2.0 ml of concentrated Folin Ciocalteu reagent with 3.0 ml of deionized water.

Bovine Serum Albumin (BSA)

Prepare $100 \mu\text{g ml}^{-1}$ of BSA from 5.0 % stock solution.

0.45 M NaH_2PO_4 Buffer

Dissolve 54.0 g of NaH_2PO_4 in 1 litre of distilled water. Sterilize by autoclaving at 121°C for 15 minutes.

4.6 N NaOH

Dissolve 184.0 g of NaOH in 1 litre of distilled water.

0.1 N NaOH

Dissolve 4.0 g of NaOH in 1 litre of distilled water. Sterilize by autoclaving at 121°C for 15 min.

APPENDIX D

BUFFERS, REAGENTS AND ENZYMES FOR MOLECULAR BIOLOGY WORK

All media, buffers, reagents and enzyme preparations described in this section are taken from Sambrook and Maniatis, (1989).

5.0 M Ammonium Acetate

Dissolve 385.4 g ammonium acetate in 1 litre of distilled water

1.0 M Tris

Dissolve 121.14 g of Tris base in 800 ml of H₂O. Adjust the pH to the desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume of the solution to 1 litre with H₂O. Dispense into aliquots and sterilize by autoclaving.

20X SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H₂O. Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH. Adjust the volume to 1 litre with H₂O. Dispense into aliquots. Sterilize by autoclaving.

10 % Sodium Dodecyl Sulfate (SDS)

Dissolve 100 g of electrophoresis grade SDS in 900 ml of H₂O. Heat to 60°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 litre with H₂O. Dispense into aliquots.

0.5 M EDTA (pH 8.0)

Add 186.1 g of disodium ethylenediaminetetra-acetate. $2\text{H}_2\text{O}$ to 800 ml of H_2O .

Stir on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approximately 20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.

0.4 N NaOH

Dissolve 16.0 g of NaOH in distilled H_2O . Dispense into aliquots and sterilize by autoclaving.

10 M NaOH

Dissolve 400 g of NaOH in 1 litre of distilled water. Sterilize by autoclaving.

1.0 M Potassium Acetate (pH 7.5)

Dissolve 9.82 g of potassium acetate in 90 ml of pure H_2O (Milli-Q or equivalent). Adjust the pH to 7.5 with 2.0 M acetic acid. Add pure H_2O to 100 ml. Divide the solution into aliquots and store at -20°C .

3.0 M Sodium Acetate (pH 5.2)

Dissolve 408.1 g of sodium acetate. $3\text{H}_2\text{O}$ in 800 ml of H_2O . Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 litre with H_2O . Dispense into aliquots and sterilize by autoclaving.

5.0 M NaCl

Dissolve 292.2 g of NaCl in 800 ml of H_2O . Adjust the volume to 1 litre with H_2O . Dispense into aliquots and sterilize by autoclaving.

Denhardt's Reagent (50X)

Dissolve the following ingredients in succession in 500 ml of H_2O : 5 g of Ficoll, 5 g of polyvinylpyrrolidone (PVP), and 5 g of bovine serum albumin. Filter sterilize and store at -20°C .

Denaturing Solution

1.5 M NaCl, 0.5 M NaOH

Ethidium Bromide (10 mg/ml)

Add 1 g of ethidium bromide to 100 ml of distilled H₂O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution to a dark bottle and store at room temperature.

Gel-Loading Buffer

0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water

Hybridization Wash (Low Strengency)

0.2X SSC, 0.1 % SDS

Hybridization Wash (High Strengency)

2X SSC, 0.1 % SDS

Luria Broth (LB)

LB was used to culture bacteria for molecular biology-based studies. Medium contained per litre: 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride. pH was adjusted to 7.0 with 5 N NaOH, and sterilized by autoclaving for 20 min at 121°C, 15 lb sq⁻¹.

Lysozyme

Prepare a stock solution of lysozyme at a concentration of 50 mg/ml in water. Dispense into aliquots and store at -20°C. Discard each aliquot after use.

Neutralizing Solution

1.0 M Ammonium acetate, 0.02 M NaOH

Prehybridization/Hybridization Solution

5X Denhardt's reagent, 6X SSC, 0.5 % SDS, and 1.0 mg/ml denatured salmon sperm DNA.

Probe Washing Solution

Per 100 ml of probe wash solution: 0.5 ml of 20X SSC, 1.0 ml of 10 % SDS, 10.0 ml of 2.0 M Tris.Cl (pH 7.5). Adjust the volume to 100 ml by adding distilled water.

Proteinase K (stock solution 20 mg/ml in H₂O)

Dissolve 100 mg of Proteinase K in 0.5 M EDTA, pH 9.0. Autodigestion at 37°C for 1 hour before storing at -20°C.

RNAase (DNase free)

Dissolve 100 mg of pancreatic RNAase (RNAase A) in 10 ml of sterile TE buffer, pH 8.0. Heat in boiling water bath for 5 minutes, then switch off the water bath and leave the solution for another 10-15 minutes. Allow to cool slowly to room temperature. Dispense into aliquots and store at -20°C.

STE Buffer

0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1.0 mM EDTA (pH 8.0).

TE Buffer, pH 8.0

10mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0)

Tris-Acetate (TAE) Buffer; 50X

242 g Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0)

APPENDIX E

TABLE AND CALCULATION FOR MPN ESTIMATES

Table E.1 Most probable numbers for use with ten-fold dilutions and five tubes per dilution (from Alexander, 1965).

p_1	p_2	Most probable number for indicated values of p_3					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.10	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16	-

CALCULATIONS

To calculate the most probable number of organisms in the original sample. Select as p_1 the number of positive tubes in the least concentrated dilution in which all tubes are positive or in which the greatest number of tubes is positive. p_2 and p_3 represent the numbers of positive tubes in the next two higher dilutions. Subsequently, find the row of numbers in Table E.1 in which p_1 and p_2 correspond to the values observed experimentally. Follow that row of numbers across the table to the column headed by the observed value of p_3 . The figure at the point of intersection is the most probable number of organisms in the quantity of the original sample represented in the inoculum added in the second dilution. Multiply this figure by the appropriate dilution factor to obtain the MPN for the original sample.

As an example, consider the instance in which a ten-fold dilution with five tubes at each dilution yielded the following numbers of positive tubes after incubation:

10^{-5}	5
10^{-6}	5
10^{-7}	3
10^{-8}	1
10^{-9}	0

In this series, $p_1 = 5$, $p_2 = 3$ and $p_3 = 1$

For this combination of p_1 , p_2 and p_3 , Table B.1 gives 1.1 as the MPN of organisms in the quantity of inoculum applied in the 10^{-7} dilution. Multiplying this number by the dilution factor 10^7 give 11 million as the MPN for the original sample.