UNIVERSITY OF CALGARY

CHARACTERISATION OF MICROBIAL COMMUNITIES DEGRADING C5+ HYDROCARBONS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

August, 2000

Abstract

The effect of C5+, a mixture of aromatic and alicyclic hydrocarbons, on soil microbial communities was studied with reverse sample genome probing (RSGP). The master filter used for RSGP was expanded to contain genomes of 55 bacterial standards, of which 82% could grow on C5+ hydrocarbons. The effects that incubation with single C5+ hydrocarbons, or a C5+ mixture, had on soil communities was determined. Both contaminated and uncontaminated soils developed communities where a few standards often initially became predominant. C5+ hydrocarbon incubated enrichments from contaminated and uncontaminated soils developed similar communities as a function of time. The rate constants of hydrocarbon removal by soil microcosms, enrichments and synthetic consortia were determined. Enrichments were the most efficient, but soil microcosms and synthetic consortia were also capable of removing hydrocarbons. The recalcitrant C5+ component DCPD was found to inhibit growth of naphthalene-degrading bacteria. *Pseudomonas* sp. Q5, a known DCPD-oxidiser, was least affected.

Acknowledgements

I would like to thank Dr. G. Voordouw for the last two years of guidance. It has truly been an enjoyable time. I would also like to thank Anita, Yin, Johanna, Anne, Casey, Brant and Mehdi, as well as everyone else who came and went in the lab, who taught me everything and made my time here all the more entertaining.

Of course, I could not have made it this far without the support of my parents through all these years of school. I'll never be able to repay you, but know that I appreciate it immensely and love you.

Dedicated to my grandfather, who helped teach me critical thinking and introduced me to science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

AQDS	9-10-Anthraquinone-2,6-disulphonic acid
BTEX	benzene, toluene, ethylbenzene and xylenes
bp	base pairs
BSA	bovine serum albumin
ca.	approximately
Ci	Curie
CoA	coenzyme A
CPD	cyclopentadiene
dATP	deoxyadenosine-5′-triphosphate
DCPD	dicyclopentadiene
dCTP	deoxycytidine-5′-triphosphate
ddNTP	di-deoxynucleoside-5′-triphosphate
dGTP	deoxyguanosine-5′-triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine-5′-triphosphate
EDTA	ethylenediamine tetra-acetic acid
FISH	fluorescent *in situ* hybridisation
PLFA	phospholipid fatty acid analysis
GC	gas chromatography
h	hours
HEPES	N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]
HMN	2,2,4,4,6,8,8-heptamethylnonane
kbp	kilobase pairs = 10^3 bp
λ	lambda
min	minute
MSM	minimal salts medium
Δp	proton motive force
ΔpH	chemical proton potential
RDP	ribosomal database project
PSL	phosphostimulable luminescence
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RSGP	reverse sample genome probing
SDS	sodium dodecyl sulphate
sp.	species (singular)
spp.	species (plural)
SSC	standard sodium citrate
SVE	soil vapour extraction
TE	tris-EDTA
TNT	2,4,6-trinitrotoluene
Tris	tris(hydroxymethyl)methylamine

TY	tryptone-yeast extract
V	volts
vs.	versus
v/v	volume per volume
W	watts
w/v	weight per volume
w/w	weight per weight
$\Delta\psi$	electrical potential

Chapter 1

BACKGROUND AND OBJECTIVES

1.1 Ethane Pyrolysis

One of the most economically important petrochemicals in the world is ethylene (Chen *et al*, 1997), which can be polymerised into polyethylene, a commonly used plastic. Ethylene is obtained by a process known as ethane pyrolysis, or ethane cracking. In this process, ethane is treated with temperatures up to 1140 °K, as well as high pressures (Chen *et al*, 1997). Many higher molecular weight unsaturated by-products are formed under these conditions. One of the major by-products is referred to as the C5+ stream. C5+ consists of benzene (45% w/w), dicyclopentadiene (DCPD) (13% w/w), cyclopentadiene (CPD) (7% w/w), toluene (6% w/w), styrene (3% w/w), naphthalene (1.3% w/w) (Figure 1-1) and other aromatic hydrocarbons in smaller amounts (Stehmeier, 1997). C5+ can be further purified into its individual components, which can be used for other industrial purposes. DCPD, for example, is used to make the polymer Metton, which is used in marine and electrical applications (Breslow, 1990 and Stehmeier *et al*, 1995).

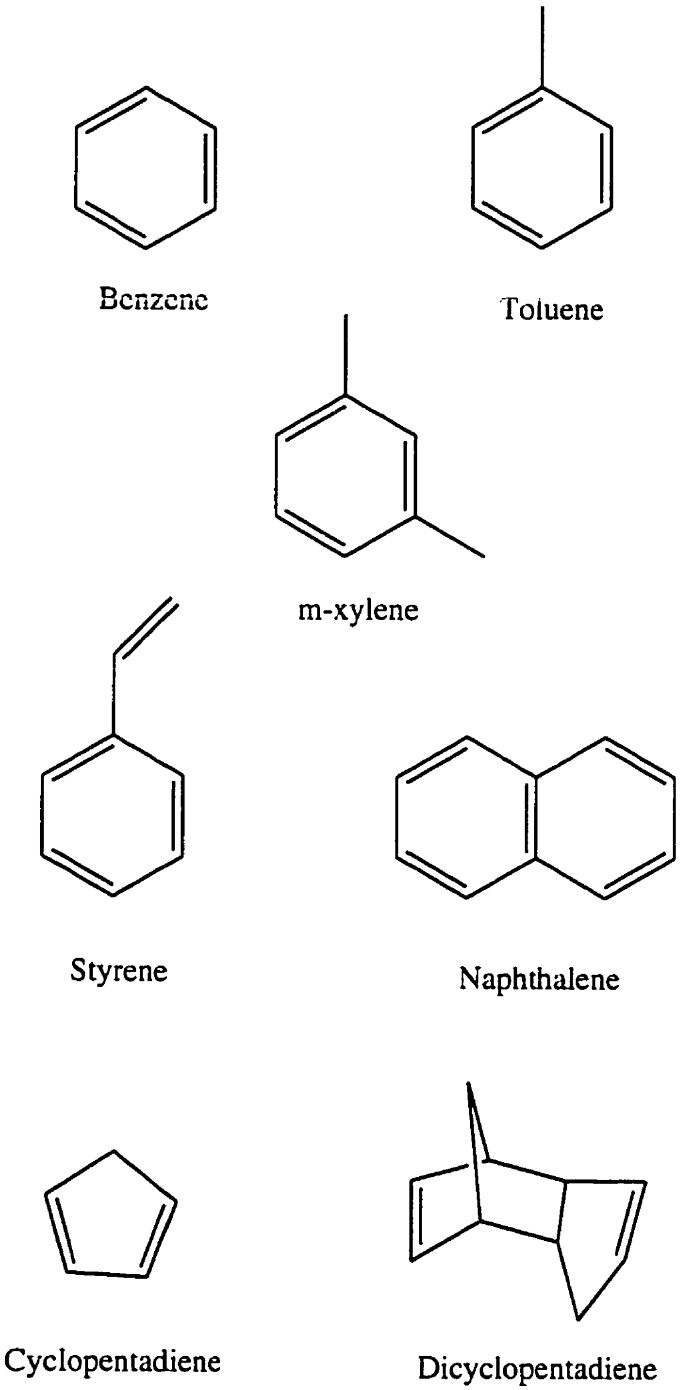


Figure 1-1. Major components of C5+.

1.2 Biodegradation

Biodegradation is a naturally occurring process, which leads to the removal of hydrocarbon pollutants by living organisms. It relies on the metabolic activities of diverse organisms, especially bacteria. Many bacteria are able to degrade hydrocarbons as part of their metabolism. In many cases, bacteria are able to derive energy and carbon for the production of biomass from the breakdown of the contaminant. The hydrocarbon can often be completely mineralised to water and carbon dioxide in the presence of oxygen, which is used for ring cleavage, and serves as the final electron acceptor (Gibson and Subramanian, 1984). It is well known, for example, that many bacteria are able to grow in the presence of hydrocarbons, such as benzene, when these are the sole carbon and energy source (Gibson, 1993). In other cases, bacteria are capable of degrading the contaminant to a derivative which is less damaging to the environment (Stehmeier, 1997). For example, Vorbeck *et al* (1998) found that under nitrogen limited conditions with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source, isolates were found which were able to remove one of the nitro-groups. In the process, the aromatic ring was saturated. Though the other nitro-groups remained and the species was not further broken down, the toxicity of the derivative was lower than the TNT because the aromatic ring had become saturated (Cerniglia, 1984).

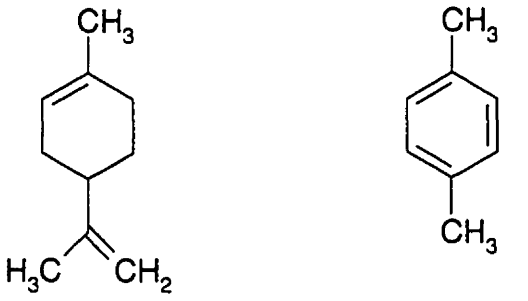
Virtually all organic compounds come from photosynthetic primary production making plant material a first link in the temporal sequence of organic hydrocarbons. In this temporal sequence, plant material is converted to humic acids, and then to fossil fuels (Holden and Firestone, 1997). For this reason many

structural analogues, which are structurally similar or identical to the polluting hydrocarbon, exist and are derived from plant material. Take for example limonene, which is a common plant terpenoid. Limonene is structurally similar to *p*-xylene (Figure 1-2). The extensive and diverse presence of genes that code for enzymes capable of degrading polluting hydrocarbons has most likely evolved from genes coding for the enzymes in microbial communities degrading these naturally occurring structural analogues (Holden and Firestone, 1997). It could be hypothesised that in an area where limonene is present regularly, the ability may already exist in the microbial community to degrade *p*-xylene.

1.2.1 Biodegradation of Aromatic Hydrocarbons

Two pathways by which BTEX (benzene, toluene, ethylbenzene and mixed xylenes) and naphthalene hydrocarbons are mineralised by microorganisms are well known. The *tod* pathway (Figure 1-3) is most commonly used by known aromatic hydrocarbon-degrading bacteria. This pathway uses a dioxygenase to add two oxygen atoms to adjacent carbons on the aromatic hydrocarbon ring to form catechol (Harayama and Rekik, 1989 and Tsao *et al*, 1998). The catechol ring can then undergo ortho- or meta-cleavage by a second dioxygenase. Ring cleavage leads to products which are able to enter the TCA cycle (Gibson, 1993). In this manner, hydrocarbons can be oxidised to carbon dioxide and water in order to provide energy to the bacterial cell, or alternatively can be used to provide precursors for biosynthetic pathways.

The second general pathway known is termed the *tol* pathway. This pathway is not as common, nor as well understood, as the *tod* pathway. In the *tol* pathway,



Limonene p-xylene

Figure 1-2. An example of a plant derived hydrocarbon analogue. Limonene is a common product of plant metabolism, and serves as a substrate analogue for the polluting hydrocarbon p-xylene. Adapted from Holden and Firestone (1997)

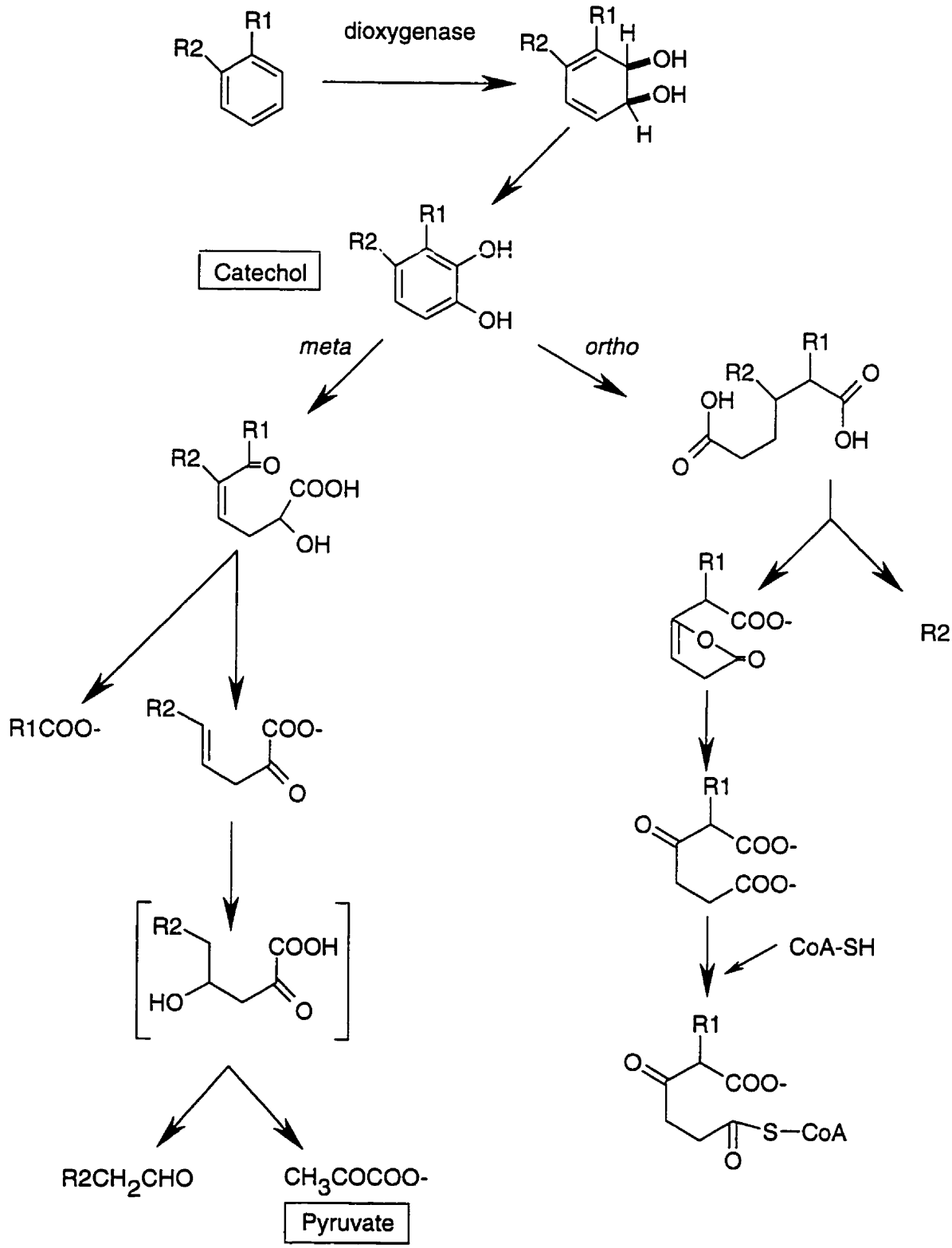


Figure 1-3. A general scheme for the *tod* degradation pathway. Benzene (R1+R2=H), toluene (R1=CH_3, R2=H), ethylbenzene (R1=CH_2CH_3, R2=H) and *o*-xylene (R1 +R2 = CH_3) can all be degraded by this pathway. Adapted from Gibson (1993) and Ellis *et al* (2000).

attack initially occurs on the methyl/ethyl substituent of the aromatic ring with a monooxygenase (Tsao *et al*, 1998). The resulting oxygenated structure from the *tol* pathway then joins with coenzyme A (CoA) via the oxygenated methyl/ethyl substituent. The resulting benzoyl-CoA undergoes reduction and hydration, causing the ring to cleave, forming a saturated acyl-CoA. β-oxidation follows, with the release of acetyl-CoA units (Harwood and Gibson, 1997). These acetyl-CoA units can then be used for energy production or biosynthesis.

Naphthalene biodegradation is also quite well understood. The best known pathway for the degradation of naphthalene is the pathway encoded by the NAH7 plasmid (Eaton, 1994; Figure 1-4). Naphthalene degradation follows the same general pathway as that of the single ring aromatics. This includes attack by dioxygenases, ring cleavage and production of products that can enter the TCA cycle. One of the products of naphthalene degradation is catechol, which can be degraded by gene products of the *tod* pathway (Figure 1-3).

Genes conferring the ability to degrade hydrocarbons can be present on plasmids or the chromosome (van der Meer *et al*, 1992). Additionally, bacteria that degrade hydrocarbons differ in the types of hydrocarbons that they are able to degrade. Some bacteria are very specific, while others can degrade a variety of hydrocarbons. It is often possible for a bacterium that is able to degrade a limited number of hydrocarbons to increase this number by the introduction of a single gene. Indeed, comparisons of major known pathways for the catabolism of aromatic compounds reveals that while initial conversion steps for aromatics are carried out by varied enzymes, all are converted to a limited number of intermediates which are then acted upon by a standard set of enzymes (van der

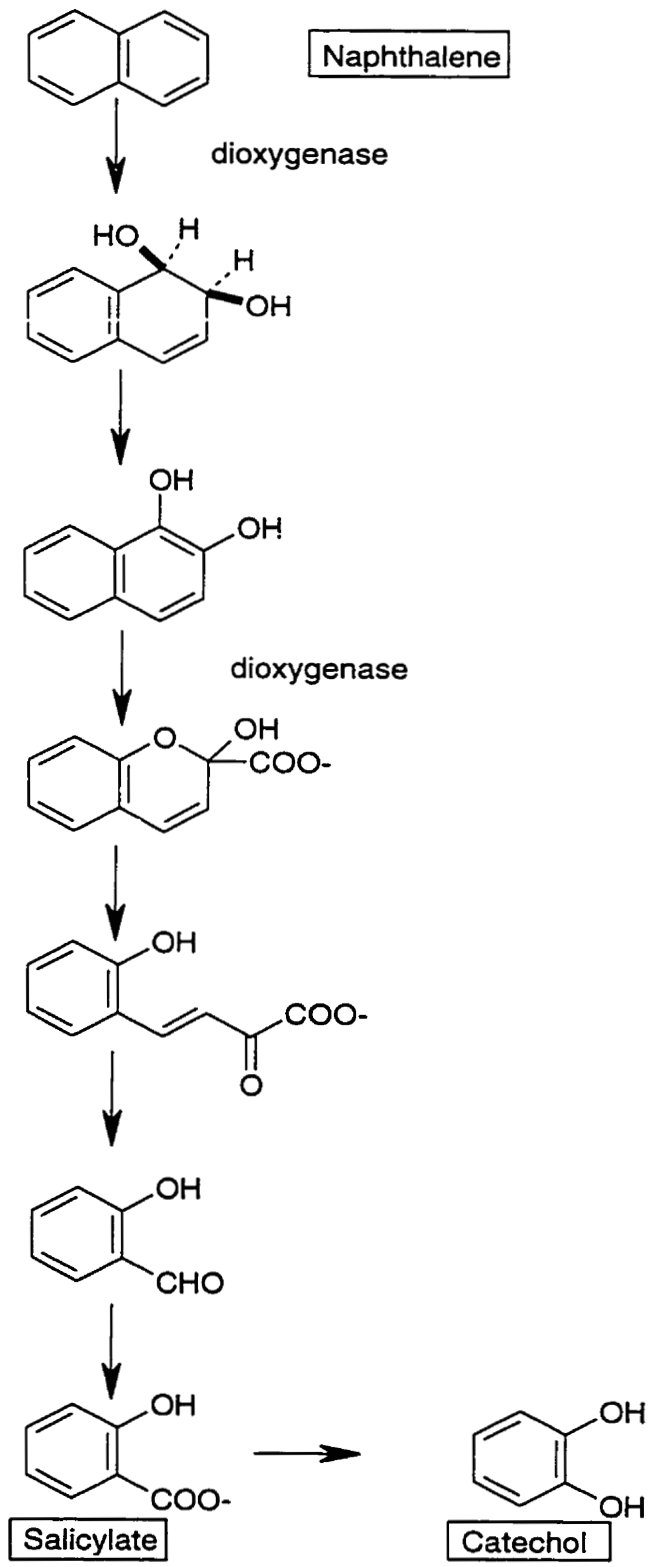


Figure 1-4. The degradation of naphthalene catalysed by gene products of the NAH7 plasmid. Adapted from Eaton (1994) and Ellis *et al* (2000).

Meer *et al*, 1992).

Oxygen is the most favourable final electron acceptor, as the greatest amount of free energy is attainable from substrates when it is used (Holden and Firestone, 1997). As explained above, oxygen also participates in the chemical reactions by which hydrocarbons are degraded. Because oxygen is used for both degradation of compounds as well as in the production of free energy from the oxidation of the derived metabolites (e.g. pyruvate), the available oxygen canbe rapidly depleted when biodegradation is occurring. This is especially true when biodegradation is occurring in groundwater, as the amount of oxygen in water (2.7×10^{-4} mol/L; 25°C) can be rapidly depleted as oxygen does not rapidly redissolve in groundwater (Tinoco *et al*, 1995 and Holden and Firestone, 1997). This is because groundwater is relatively stagnant, and is not mixed to increase the diffusion of oxygen. The availability of oxygen for aerobic growth is therefore often limiting for the removal of aromatic hydrocarbons from the environment. However, biodegradation of aromatic hydrocarbons has also been observed under anaerobic conditions, with nitrate, carbon dioxide, sulphate, manganese, AQDS (9,10-anthraquinone-2,6-disulphonic acid) and ferric iron serving as final electron acceptors (Shen, 1997). This indicates that while aerobic degradation is important, anaerobic metabolism can also play a role in aromatic hydrocarbon degradation, and if it is found that aerobic biodegradation is slow or limited because of oxygen availability, biodegradation may still occur by other processes involving anaerobic metabolism.

1.2.2 Biodegradation of Dicyclopentadiene

DCPD is formed by a Diels-Alder condensation of CPD (Figure 1-5). DCPD is classified as an alicyclic hydrocarbon, which is a cyclic non-aromatic hydrocarbon. Some bacteria are known to degrade a variety of alicyclic hydrocarbons, yet to date there have been no bacteria isolated that are capable of growth using CPD, or the more prevalent dimer DCPD, as the sole carbon and energy source. Experiments with incubation of microbial communities in the presence of ^{14}C-labelled DCPD have resulted in up to 2% $^{14}CO_2$ formation after 25 days incubation. However, most ^{14}C was found as oxygenated derivatives of DCPD (Stehmeier *et al*, 1996) (Figure 1-6). The formation of oxidised derivatives of DCPD may not be due solely to microbial activity, however, as some derivatives were shown to form in the absence of microorganisms (Stehmeier *et al*, 1996). Additional work by Stehmeier (1997) indicates that DCPD is more likely to be mineralised by microbial consortia than by single species. The process remains slow and other carbon and energy sources must be present, as DCPD can not solely be used to sustain growth.

Work on *in situ* C5+ bioremediation has shown that the majority of the components within C5+, with the exception of DCPD, are readily and efficiently biodegraded. Stehmeier *et al* (1999) performed studies at a C5+ spill site. Hydrocarbon samples were taken at the site after the initial spill (Figure 1-7; July), 116 days later (Figure 1-7; Nov) as well as at timepoints in between the two dates. Calculations performed taking into account losses due to volatilisation concluded that at the site, 87% of the hydrocarbon was bioremediated (Stehmeier *et al*, 1999). Approximately 60% of the DCPD that was at the site initially remained, however it

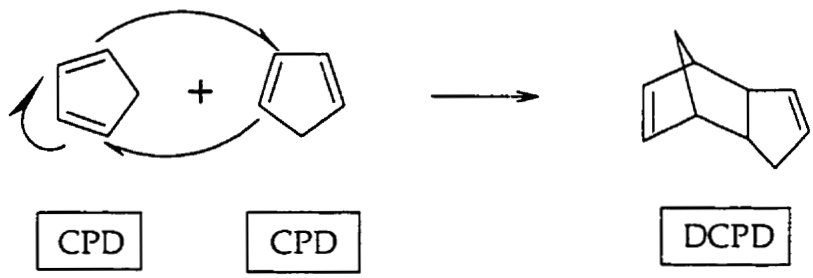


Figure 1-5. Diels-Alder condensation of two molecules of cyclopentadiene (CPD) to form DCPD.

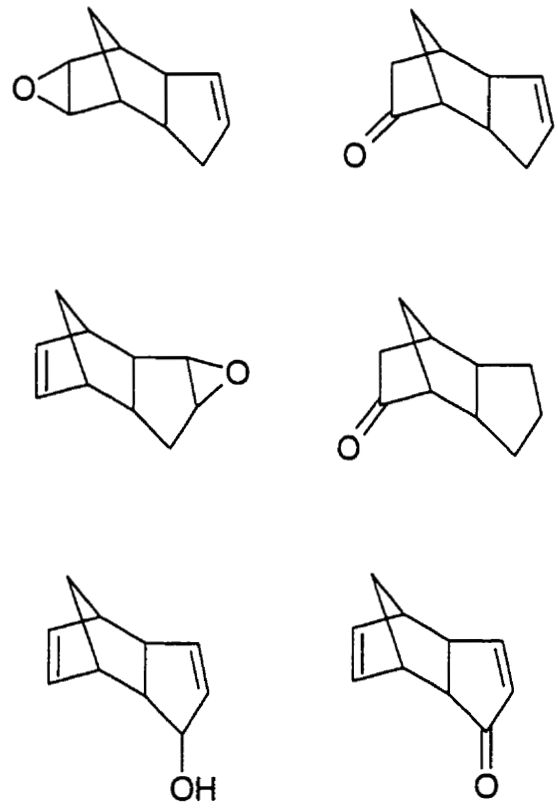


Figure 1-6. Oxygenated derivatives of DCPD. Adapted from Stehmeier *et al* (1996).

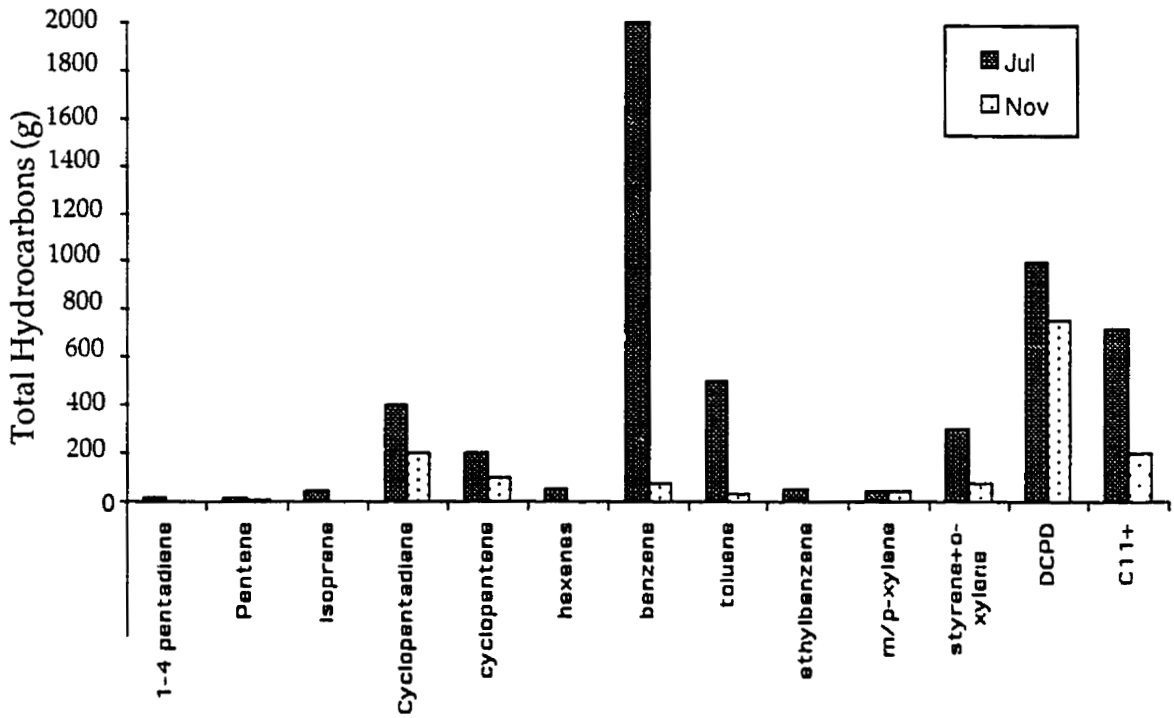


Figure 1-7. Total hydrocarbons from C5+ spill site. Estimated from measurements taken at the site of a C5+ spill after a spill occurred (July) and four months later (Nov). Taken from Stehmeier *et al* (1999).

was found that 29% of the original DCPD present was in fact biodegraded. Stehmeier *et al* (1999) also showed that the presence of other C5+ components increased the rate of DCPD bioremediation, indicating that DCPD co-metabolism was likely occurring.

There is some preliminary evidence that oxidation of DCPD may occur cometabolically with oxidation of naphthalene by *Pseudomonas* sp. Q5 (Table 3-1). Shen *et al* (1998) found that during continued soil culture incubations in the presence of DCPD, Q5 was the dominant species present according to RSGP. One possibility is that a dioxygenase involved in the oxidation of naphthalene (Figure 1-4) may also be able to oxidise DCPD. It has been shown that NDO has relaxed substrate specificity, which allows dihydroxylation reactions to be catalysed not only on naphthalene (Jeffrey *et al*, 1975), but also on other polycyclic aromatic hydrocarbons such as anthracene and phenanthranene (Jerina *et al*, 1976), benzocycloalkenes (Resnick and Gibson, 1996), toluene and ethylbenzene (Lee and Gibson, 1996).

While DCPD is not as toxic as benzene or toluene because its carbon atoms are more saturated (Section 1.2.3), the perception of contamination and toxicity is present in the public because of its acute smell, and low odour threshold of 5 ppb. Due to the odour problem, bioremediation of C5+ is not complete until the removal of all DCPD has been achieved, which, currently, is the rate limiting step. Oxygenated DCPD derivatives do not have as strong an odour as DCPD. However their toxicity has yet to be determined (Stehmeier, 1997).

1.2.3 Hydrocarbon Toxicity

Hydrophobic hydrocarbons, including aromatics and alicyclics, are toxic to cells. It is believed that the most significant mechanism of toxicity involves effects on the cell's cytoplasmic membrane: hydrocarbons are able to damage the membrane integrity. They do this by partitioning into the central hydrophobic portion of the membrane bilayer. Accumulations of these compounds within the bilayer can disturb the interactions between the acyl chains of the phospholipids, which will alter the fluidity of the bilayer, the integrity of the bilayer, and may eventually cause swelling of the bilayer. This can cause a number of deleterious effects. One effect of altering the integrity of the membrane is that it may lead to an increase in the passive flux of protons across the membrane. The proton motive force (Δp) is important for the formation of ATP, the main unit of usable energy for the cell. The Δp relies upon the electrical potential ($\Delta\psi$), as well as the chemical proton potential (ΔpH) across the cytoplasmic membrane (Gennis, 1989). If the membrane becomes leaky and allows a passive flux of protons, then the Δp will be destroyed. Additionally, the concentration gradients of other ions such as sodium, which are important for secondary transport processes such as uptake of certain nutrients and excretion of endproducts, will be destroyed (Sikkema *et al*, 1995). In addition to maintaining ion gradients, the cytoplasmic membrane also forms a matrix for various proteins. Altering the membrane can cause the proteins to denature, and thereby cause loss of functions (Sikkema *et al*, 1995), including inactivation of membrane-bound protein function, membrane leakiness and loss of proton motive force (Sikkema *et al*, 1995). The extent to which a particular

lipophilic hydrocarbon affects a cellular membrane varies with the physical parameters of the hydrocarbon as well as with the composition of the cellular membrane.

Toxicity of hydrocarbons varies with the vapour pressure and viscosity of the hydrocarbon. It has been shown that hydrocarbons with higher vapour pressure have a higher toxicity, while those with a higher viscosity are less toxic (Sikkema *et al*, 1995). The main determinant of toxicity, however, is its solubility, or bioavailability. The only way for hydrocarbons to interact with bacteria and be available for metabolism or cause toxic effects is for the hydrocarbon to pass through the aqueous phase (Sikkema *et al*, 1995). Therefore, those hydrocarbons with higher solubility will have higher toxic potential. Single ring aromatics have relatively high solubility when compared to other cyclic hydrocarbons with greater numbers of carbon atoms, or those with a higher degree of saturation (Sikkema *et al*, 1995). This being the case, the majority of the components within C5+ are quite toxic.

The composition of the bacterial cytoplasmic membrane varies for different species, as well as according to conditions of growth within a single species. Because of these differences, bacteria have varying tolerances for lipophilic hydrocarbons. Gram-negative bacteria are generally much more tolerant to hydrocarbons than gram-positive bacteria. This is because of the presence of the outer membrane in gram-negative bacteria in addition to the cytoplasmic membrane. The outer membrane is a barrier that allows small hydrophilic molecules to pass, and is surprisingly resistant to passage of hydrophobic molecules (Sikkema *et al*, 1995). This resistance is attributed to the hydrophilic

lipopolysaccharide (LPS) portion of the outer membrane. Because of the highly hydrophilic nature of the LPS, hydrophobic molecules are hindered from passing through to the cytoplasmic membrane.

Bacteria have the ability to alter their tolerance to lipophilic hydrocarbons. One method of doing this is by changing the fatty acid composition of their membranes. While the hydrocarbon itself influences the amount that enters the membrane (partitioning), it is the composition of the phospholipids that has a major effect on the partitioning (Sikkema *et al*, 1995). For example, it has been shown that by changing the composition of the fatty aids in a lipid bilayer from myristoyl to stearoyl lipids, the partition coefficient of lindane is reduced 50-fold (Sikkema *et al*, 1995). Therefore, by modifying the cytoplasmic membrane phospholipids, bacteria can tolerate different levels of lipophilic hydrocarbons. Additionally, the cell wall may alter its hydrophobicity. By causing the cell wall to decrease in hydrophobicity, it may be able to shield the cytoplasmic membrane from the lipophilic compounds (Sikkema *et al*, 1995).

The differences and strategies outlined above leads to microorganisms that are able to tolerate and grow under widely differing concentrations of hydrocarbons. This was demonstrated by Hubert *et al* (1999), who isolated seven strains that were able to grow using toluene at different concentrations as the sole carbon and energy source. Some of these were able to grow at vapour phase concentrations of toluene as low as 0.05 mg/L, and were inhibited by concentrations of 5 mg/L. Others were unable to grow at concentrations below 5 mg/L, and survived quite well up to concentrations of 45 mg/L. However, none of the isolates was able to survive in a saturated atmosphere of toluene (146

mg/L). This demonstrates that toluene is highly toxic at very high concentrations. These observations are in agreement with work published by Gibson *et al* (1970), Yarmoff *et al* (1988) and Sikkema *et al* (1994).

1.2.4 Advantages of Bioremediation

Natural attenuation, which uses naturally occurring organisms capable of bioremediation and no additional treatment, is often the preferred method of contamination cleanup. The main reason for this is cost. Other methods may also rely upon bioremediation, but they attempt to increase the rate of hydrocarbon removal by increasing availability of nutrients to the bioremediating organisms. Bioventing, for example, is the process of injecting air through drilled ports into the soil where a spill has occurred. This increases the amount of available oxygen within the spill site, increasing the rate of diffusion of oxygen to the organisms performing the bioremediation. This increase in oxygen availability will increase the rate of bioremediation (Section 1.2.1). The increased flow of air through the soil will also likely increase the rate of volatisation of the hydrocarbons. Examples of methods that do not rely upon bioremediation are bioslurping and soil vapour extraction (SVE). Both of these methods remove contaminating hydrocarbons by volatilisation. In bioslurping, the contaminated groundwater is removed and a vacuum is applied to volatise the contaminating hydrocarbons. In SVE, air from the soil is extracted, which contains volatilised hydrocarbons.

Both the initial set-up cost, as well as the annual operations and maintenance cost, are a factor of 2 to 10 times lower for natural attenuation than for other available methods (Downey *et al*, 1999). Bioremediation does take longer to

complete than other methods, however in the vast majority of cases natural biodegradation alone has been shown to be sufficient to remove BTEX before human or ecological receptors are impacted (Downey *et al*, 1999).

1.2.5 Relevance of Laboratory Experiments to Activities in the Field

In comparison to *in situ* field studies, laboratory experiments are quicker and easier to control. Laboratory experiments can be important for predicting the potential of bioremediation in the field, especially by detecting the presence of organisms capable of degrading the compounds of interest (Greene, 1999). Studies have been performed which compare the *in situ* degradation to laboratory experiments (Nielsen *et al*, 1995a; 1995b and Fu *et al*, 1996). Nielsen *et al* (1995b) performed experiments to determine if aromatic and chlorinated aliphatic compounds were able to be biodegraded effectively in both laboratory and *in situ* conditions. In aerobic experiments, they found that *in situ* degradation potentials were very similar to laboratory degradation potentials for the tested compounds. They also found that anaerobic *in situ* and laboratory degradation potentials were similar, though phenolics tended to be degraded or transformed more often *in situ*. They attributed this to the lack of Fe (III) as a terminal electron acceptor in the laboratory experiments (Nielson *et al*, 1995a).

Fu *et al* (1996) compared biodegradation rates of phenol in various types of laboratory experiments. Soil wafers were made by mixing soil and water to give a uniform biomass concentration. The water in the soil was then evaporated until the remaining wafer (0.2 cm thick) of soil had the desired moisture content. The remaining water is small in amount and stationary, which reduces mass transfer of

solutes within the liquid phase (Fu *et al*, 1996). This will cause the system to resemble *in situ* conditions more closely. It was found that this system most closely resembled bioventing (Section 1.2.4), where oxygen limitation in the soil is minimised in order to speed up biodegradation. This is likely because the small size of the wafer does not hinder oxygen diffusion, with a uniform concentration of oxygen present throughout the wafer (Fu *et al*, 1996). Compacted soil tube reactors were found to resemble *in situ* bioremediation the best, and had the slowest rates of the tested laboratory conditions. The compacted soil was held in porous glass tubes that allowed the free movement of oxygen through the tubes, and that were 1.6 cm inner diameter and 6 cm in length. Because of the increased width of the soil tubes (1.6 cm) over the width of a soil wafer (0.2 cm), oxygen diffusion within the soil became limiting (Fu *et al*, 1996). Fu *et al* (1996) found that as the complexity of the system increased, such as becoming dependent on diffusion of nutrients, the rates of biodegradation decreased. However, they also found that with more complex laboratory systems, rates that were determined could more reliably be applied to *in situ* situations.

1.3 Characterising Microbial Communities

Soil microbial communities are complex. Microorganisms rarely occur as pure cultures under natural conditions. Soils have some of the most diverse communities, with a single gram of soil having up to ca. 4000 microbial species present (Torsvik *et al*, 1990). As explained in Section 1.2, bacteria can tolerate and degrade different ranges of hydrocarbons. Because of this wide diversity of tolerances and abilities, biodegradation of spilled hydrocarbons is quite successful

(Downey, *et al*, 1999). A major obstacle that remains to be overcome is understanding how the bacteria within a soil community respond when subjected to a hydrocarbon spill, and how the community facilitates the hydrocarbon removal.

Understanding the growth rates, substrate range, toxicity, as well as studying the metabolic processes involved in degradation are among the analyses that can be performed when single culturable species are isolated. However, gaining an understanding of how a bacterial strain behaves in the environment, as part of a community, is not possible with pure culture studies. In order to understand community dynamics other methods are required.

1.3.1 Identifying Bacterial Isolates

The current standard molecular method for identifying and relating microbial species is by comparison of their 16S rRNA gene sequences. 16S rRNA genes have areas of high and low conservation; these differences are used to give information on phylogeny, or species relatedness. Species that are closely related have 16S rRNA genes that are very similar in both the highly and poorly conserved regions, while species that are distantly related will have unique sequences in the highly variable regions. To find the sequence of the 16S rRNA genes in bacteria, total DNA is extracted from the species of interest and the 16S rRNA genes are amplified using the polymerase chain reaction (PCR). There are highly conserved regions near either end of the 16S rRNA gene, so standardised primers for the amplification of the entire gene are available (Olsen *et al*, 1986). The 16S rRNA gene sequences are then determined. The total length of the 16S rRNA genes in

bacterial species is ca. 1 500 base pairs. To compare a new sequence to those that are known, the new sequence is read in 7-oligomer sections, moving along the sequence one base at a time. The 7-mers are next translated into an integer for comparison purposes. The 7-mer integers are compared to a library of integers from the pre-read 7-mers of all the 16S rRNA sequences with the database with which the unknown is being compared. A one-dimensional array is made by comparing the integers of the unknown sequence to the sequences in the library. Similarity scores (S_{ab}) for the compared sequences are calculated, and those sequences which are of highest similarity to the unknown sequence are presented (Maidak *et al*, 2000).

Sequences of 16S rRNA genes can also be compared using similar algorithms to that explained above. In this case, an evolutionary distance is calculated by a program that gives a certain score for each change in bases between the sequences. A phylogenetic tree can then be produced by comparing the evolutionary distances between 16S rRNA gene sequences and producing branches which best fit the data (Felsenstein, 1995). Identification of novel and/or as yet uncultured species is performed by submitting and comparing the 16S rRNA sequences to a database of 16S rRNA sequences of known species. The relative homologies obtained for the unknown species can be used to identify closely related known species (if these exist). It is often possible to infer properties of the unknown species based on the known properties of its close relatives (Schmidt *et al*, 1991).

1.3.2 Molecular Biological Techniques

There are a number of molecular biological techniques that attempt to characterise the dynamics of a microbial community. These techniques rely on the characterisation of indicator molecules such as DNA, RNA or fatty acids. These indicator molecules can suggest various aspects of the community dynamics that occur within the soil.

1.3.2.1 General Methods

Phospholipid fatty acid pattern analysis (PLFA) involves extraction of fatty acids from a sample. Methyl esters of the fatty acids are made and then characterised and quantified by gas chromatography/mass spectrophotometry (GC-MS). The fatty acid profiles of a community are then defined (Bååth *et al*, 1992). By changing the incubation conditions of a community, the fatty acid profile will also change. For example, when Hansen *et al* (1999) incubated a soil community with varying amounts of toluene (0 – 100 mg/L of soil solution), the amount of myristic acid (14:0) initially decreased from 0.05 nmol/g dry soil at 0 mg/L toluene to 0.045 nmol/g dry soil when 20 mg/L of toluene was present. When toluene was increased to 100 mg/L, however, myristic acid (14:0) increased in concentration to 0.07 nmol/g dry soil. This technique does not show how individual species react; only changes in the community as a whole are obtained. Hansen *et al* (1999) found that when toluene concentrations increased, fatty acids as a whole increased in concentration. This is likely due to the overall increase in growth of microorganisms with degrading ability due to the increase of an available carbon source for growth.

PLFA has also been linked with ^{13}C-tracing (Hanson *et al*, 1999). When a soil community was incubated with 30 mg/L of ^{13}C-labelled toluene, incorporation of ^{13}C was biased towards certain fatty acids. For example, after incubation, over 70% of pentadecanoic acid (15:1, double bond location unknown) recovered was ^{13}C-labelled. Palmitic acid (16:0), on the other hand, was only 10% ^{13}C-labelled, even though it made up 15% of the recovered fatty acids as compared to 6% for pentadecanoic acid. With this additional analytic capability, subgroups of the community can be identified, making the procedure more specific. However, it is still not possible to follow individual species with this method.

Gene probes have been used to look for specific activities within a community. This method uses a labelled (^{32}P or fluorescence) probe designed to target a specific gene known to be involved in the activity of interest (for example, toluene degradation). This probe is hybridised with total community DNA. If the gene being targeted is in the community of interest, it will hybridise with the probe. Gene probing has the advantage that catalytic functions of the community can be determined relatively quickly, and different catalytic functions can be measured with different probes (Wolcott, 1992). An important aspect of this method is the design of the probe. Many short probes are subject to non-specific hybridisation and therefore have limited specificity (Wolcott, 1992). A probe which has low specificity may hybridise with regions not specific to the gene, overestimating its presence in the community DNA (Shen, 1997). A complication of this method is that diverse species can have genes of very dissimilar sequence identity that perform very similar functions (Amann *et al*, 1995). Soil communities have a great assortment of species within them, and as such, the likelihood of such

genes being present is great. This will prevent the detection of diverse genes performing similar functions to the function of interest, other than those for which clones are available. For example, Sayler *et al* (1985) used probes based on the NAH plasmid to track a population that was capable of growing on naphthalene as the sole carbon and energy source. They found that only a few of the strains appearing on selective media for naphthalene degradation would react with the probe.

Efforts to characterise microbial communities based on the 16S rRNA composition have also been made. This can give an estimate of the overall diversity of the bacterial species present in a community. The genes for 16S rRNA in a DNA sample are amplified by PCR from a community of interest. In order to identify the species present, the PCR products are separated by cloning into plasmids and transferring into *Escherichia coli*. Each separate *E. coli* colony will contain one PCR product from a single species that was present in the original community (Amann *et al*, 1995). This cloned PCR product can then be purified and sequenced. In this manner, many of the 16S rRNA sequences that were in the original community DNA can be determined without the need for cultivation of individual species.

Another route to determine the 16S rRNA sequences of species present in a bacterial community uses reverse transcriptase to amplify 16S rRNA directly (rather than the 16S rDNA). The complementary DNA (cDNA) is then cloned and sequenced. Using the actual 16S rRNA as the initial template holds the advantage that there are often more copies of rRNA present than of the DNA (Ludwig and Schleifer, 1994).

There are certain cautions to be considered when performing community 16S rRNA gene sequencing. PCR of rRNA (or genes) mixed with other rRNA (or genes) is shown to be biased, with preferential amplification of certain rRNA sequences over others. As such, certain 16S sequences are likely not amplified (Amann *et al*, 1995). The cloning step is also biased. Attempts have been made to limit the restriction enzymes used to prevent cutting of the 16S fragments, but the possibility of bias cannot be ruled out (Amann *et at*, 1995). If the 16S fragment is cut during cloning, it will be lost. Additionally, it is likely that different 16S fragments will have different cloning efficiencies (Amann *et al*, 1995). Despite these cautions, sequencing of 16S rRNA genes of a total community is currently the best method for determining the total diversity of bacteria present in communities without culturing.

Attempts have been made to determine the relative amounts of each species present in communities using 16S rRNA sequence information. This involves examining sequence databases and finding a sequence unique enough to make a specific 16S rRNA probe targeted towards the species of interest. Additionally, a universal probe that will hybridise with total 16S rRNA is needed. A dot blot of total community rRNA (or DNA) with the probes is then performed. The relative amount of a specific species present is determined by comparing the hybridisation strengths of the universal and species specific probes. This method has a low detection limit, with 0.1%-1% (w/w) of total nucleic acid present needing to be the target, but it is only the 16S rRNA (or DNA) that is measured. 16S rRNA genes vary in copy number from 1 to 10, so absolute cell numbers cannot be determined when probing 16S rRNA genes. The amount of 16S rRNA in a cell varies with at

least one order of magnitude with cell growth rates, so while probing the actual 16S rRNA can give information about changes in growth rates, cell numbers cannot be determined (Amann *et al*, 1995).

One method that uses 16S rRNA sequences to quantify actual cell numbers is fluorescent *in situ* hybridisation (FISH). FISH uses fluorescently labelled 16S rRNA probes, which are hybridised against whole cells. The cells stain brightly because 16S rRNAs are generally present in many copies. Different probes can be attached to different dyes, and by combining FISH with flow cytometry, populations of different species can be determined by automated separating and counting of the different dyes that are present. While this system works well for aquatic samples, the method is difficult to perform for soil samples because removing non-cellular particles, while at the same time keeping cells intact, is quite problematic (Amann *et al*, 1995). Additionally, the number of individual strains that can be separated and quantified in a single experiment is limited by the number of different dyes available for attachment to the separate probes, which are also limited in number. The complete 16S rRNA sequence of the target species must be known before a probe can be designed.

One final concern about using 16S rRNA gene sequences to monitor microbial communities is that bacteria can have similar 16S rRNA genes, yet be unrelated. For example, *Bacillus globisporus* and *Bacillus psychrophilus* have less than 50% genomic DNA similarity, but their 16S rRNA gene sequences are identical (Fox *et al*, 1992). Genomic DNA-DNA hybridisation has the highest fidelity, and is therefore used to determine whether two bacteria are of the same species. In order

for two bacteria to be classified as belonging of the same species, their genomic DNA must have >70% homology (Wayne *et al*, 1995).

1.3.2.2 Reverse Sample Genome Probing (RSGP)

RSGP (Figure 1-8) is a method that characterises microbial communities by using entire genomes as probes. As each bacterium in a community usually contains one set of chromosomal DNA, it is possible to relate the extent of genome hybridisation to relative cell numbers.

For RSGP, DNA from pure bacterial strains must be obtained, usually by culturing organisms from the microbial community of interest (Voordouw, 1998). The cross-hybridisation between chromosomal DNAs from community isolates is then tested. To do this, the hybridisation intensity (ΔPSL) of radiolabelled DNA to its own DNA is set to 100%. The ΔPSL of the radiolabelled DNA to any of the other DNAs is compared to the fixed 100% value ΔPSL of the radiolabelled DNA to itself. The relative amount of this hybridisation to other DNA is termed cross-hybridisation and is expressed as a percentage (Telang *et al*, 1997). Isolates that have relatively little cross-hybridisation with each other are collected. These collected isolates are referred to as standards (Voordouw *et al*, 1993). Isolates that have genomes with high cross-hybridisation are considered to be one standard. A standard thus represents a set of bacterial genomes that cannot be distinguished by RSGP. RSGP cannot distinguish between genomes with greater than 80% cross-hybridisation (Voordouw, 1998). The genomes that make up a standard generally have cross-hybridisations that are greater than 70%. This makes all strains within a species belong to the same standard (Wayne *et al*, 1987). Known amounts of

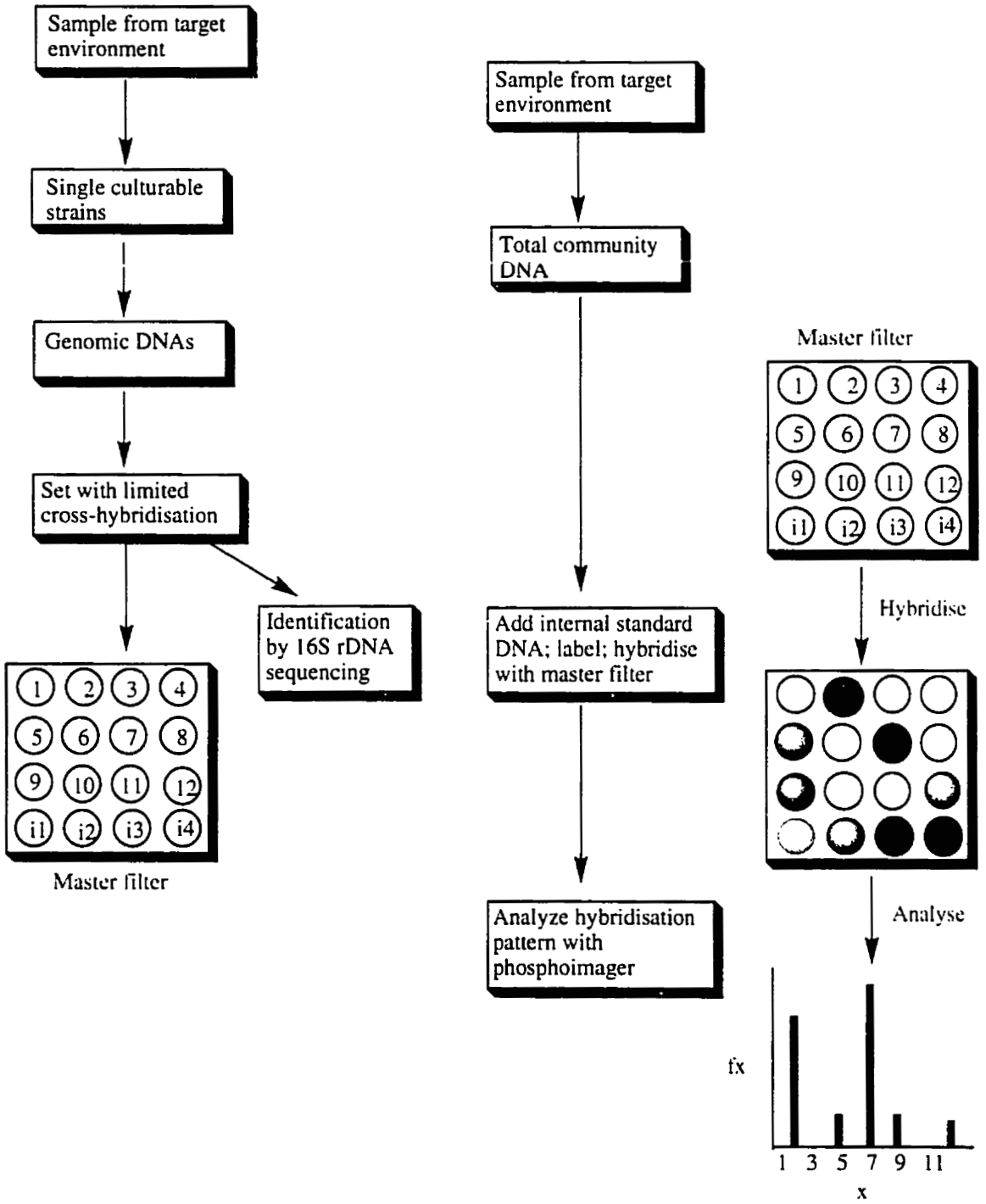


Figure 1-8. Overview of the RSGP procedure.

chromosomal DNA from each of these standards are then fixed to a new filter; the master filter (Figure 1-8, circles 1-12). Also placed on the master filter are known amounts of denatured phage-λ DNA (Figure 1-8, circles i1-i4), which is included as an internal standard (Voordouw, 1998).

For quantitation, the amount of genome hybridisation must be compared to that of the internal standard. RSGP uses the ratio of the hybridisation constant of λ DNA to itself (k_λ) to the hybridisation constant of each standard to itself (k_x). Because genomes for bacteria vary greatly in size, the ratio of hybridisation constants (k_λ/k_x) for the standards will vary. Standards that have higher genomic complexities will generally have higher k_λ/k_x ratios. This is because higher genomic complexity will result in a lower amount of the labelled probe hybridising to the standard on the filter. The amount of labelled λ DNA hybridising to the λ DNA on the filter acts as a constant internal standard and therefore the ratio k_λ/k_x will be higher. Once the hybridisation constant ratios of each of the standards on the master filter have been determined, it is possible to determine the fraction of each standard in an unknown mixed DNA sample (f_x). It should be noted that the calculated f_x value might contain contributions due to cross-hybridisation. Some of the cross-hybridisation may be from standards not represented on the filter (Voordouw *et al*, 1993).

The ability to determine fractions of a great number of standards (as many as are represented on the master filter) within a mixed sample by a single hybridisation experiment is the benefit that this method holds (Voordouw, 1998).

It has been shown that multiple RSGP tests of a single sample provide reproducible patterns (Shen *et al*, 1998).

The requirement of first culturing bacteria for DNA extraction and placement on the master filter is the main disadvantage of RSGP (Voordouw, 1998). However, as the species represented on the master filter are culturable, this provides the opportunity to use traditional microbial methods to study bacteria that become predominant under certain conditions, and try to understand what advantages they may possess in the environment being studied.

1.4 Objectives

Alberta has one of the world's largest ethylene pyrolysis plants. At this plant, 10^6 to 10^7 litres of C5+ are produced per year (Stehmeier *et al*, 1996). Given these large quantities, spills of C5+ occasionally occur during transport and handling, mainly on the plant site itself. Earlier research has shown that the majority of C5+, with the exception of DCPD, is readily and efficiently biodegraded (Section 1.2.1 and Section 1.2.2). Although DCPD biodegradation does not appear to be as efficient as biodegradation of the rest of C5+, it does appear to occur, but requires a microbial community and may be co-metabolic. In an attempt to further expand on these earlier results, the objective of this research was to use molecular microbial methods to characterise the soil microbial community involved in C5+ bioremediation. This general objective was met by completing the following steps:

i) Expand the master filter used for RSGP analysis of the soil microbial community and include bacterial standards grown in C5+ components as a sole

carbon and energy source. Standards that had been previously obtained for the master filter were obtained by growth on rich media.

ii) Characterise the newly isolated bacterial standards by 16S rRNA gene sequencing and determination of C5+ substrate specificity.

iii) Use RSGP with the newly expanded master filter to analyse the effect of soil incubations with single and mixed C5+ hydrocarbons.

iv) Analyse the effect of incubation of soils with a C5+ mixture followed by incubation with DCPD only with RSGP. This simulates the time course of a natural spill where the majority of C5+ component hydrocarbons are removed and DCPD remains (Figure 1-7). Standards that prevail under these conditions are candidate DCPD degraders.

v) Monitor enrichments of soils in C5+ with RSGP. Determine the rates of degradation of C5+ components by these enrichments.

vi) Analyse the possibility that DCPD is metabolised or cometabolised with naphthalene by organism Q5.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Reagents

Chemicals. Dicyclopentadiene (95% pure), naphthalene (>99% pure), HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]), polyethylene glycol, urea, ethidium bromide, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), 2,2,4,4,6,8,8-heptamethylnonane (HMN) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company. Toluene (99.5% pure), styrene (99% pure), benzene (99% pure), *m*-, *o*- and *p*-xylenes (99% pure), tris-(hydroxymethyl)-methylamine (Tris), ethylene diamine tetra acetic acid (EDTA) and sodium dodecyl sulphate (SDS) were obtained from BDH Chemicals. Salicylate was from Matheron, Coleman and Bell. Acrylamide was from Boehringer Mannheim. High gelling temperature (HGT) agarose, with gel strength greater than 1 200 g/cm^2, was from EM Science. Ficoll was from Pharmacia Biotech. Vacuum pump oil 19, a 100% parafinnic oil with a density of 0.85 g/cm^3 was from VWR Scientific. Compressed gases were from Praxair Products Inc. Agar and rich media components were from Difco. Most other chemicals were reagent grade from BDH, Fisher or Sigma.

Biochemical reagents. The Klenow fragment of DNA polymerase I, Taq polymerase, λ DNA (0.5 mg/mL) and lysozyme were purchased from Pharmacia. Hybond-N hybridisation transfer membrane was purchased from Amersham. RNAse, from bovine pancreas, and Sepharose 4B were obtained from Sigma. Proteinase K was from Roche Diagnostics. Salmon sperm DNA was from USB Amersham. Deoxyoligonucleotides, used as primers for PCR and sequencing reactions were synthesised by the regional DNA Synthesis Laboratory of the University of Calgary. All other biochemical reagents were obtained from Sigma, Boehringer Mannheim, Difco Laboratories, Fisher or BDH.

Radioisotopes. [α ^{35}-S]dATP (10 mCi/mL; 400 Ci/mmol) was from Amersham and [α^{32}-P]dCTP (10mCi/mL; 3 000 Ci/mmol) was from ICN.

2.1.2 Environmental Samples

Soil samples. Three sets of soil samples were used, all of which were taken from an ethylene pyrolysis plant near Joffre, Alberta. The first set was collected from a constructed pile of soil contaminated by C5+ in 1997. Samples collected from the North or South end of this pile were given the designations N and S. The second set of contaminated soil samples was collected by Les Stehmeier and Karim Jaber in the fall of 1999. Samples were collected in close proximity to a building where C5+ was spilled on various occasions. All samples (A0.5, A1, A2, A3, B1, C1, C2 and E1) were taken from a depth of 90-120 cm at the locations indicated in Figure 2-1.

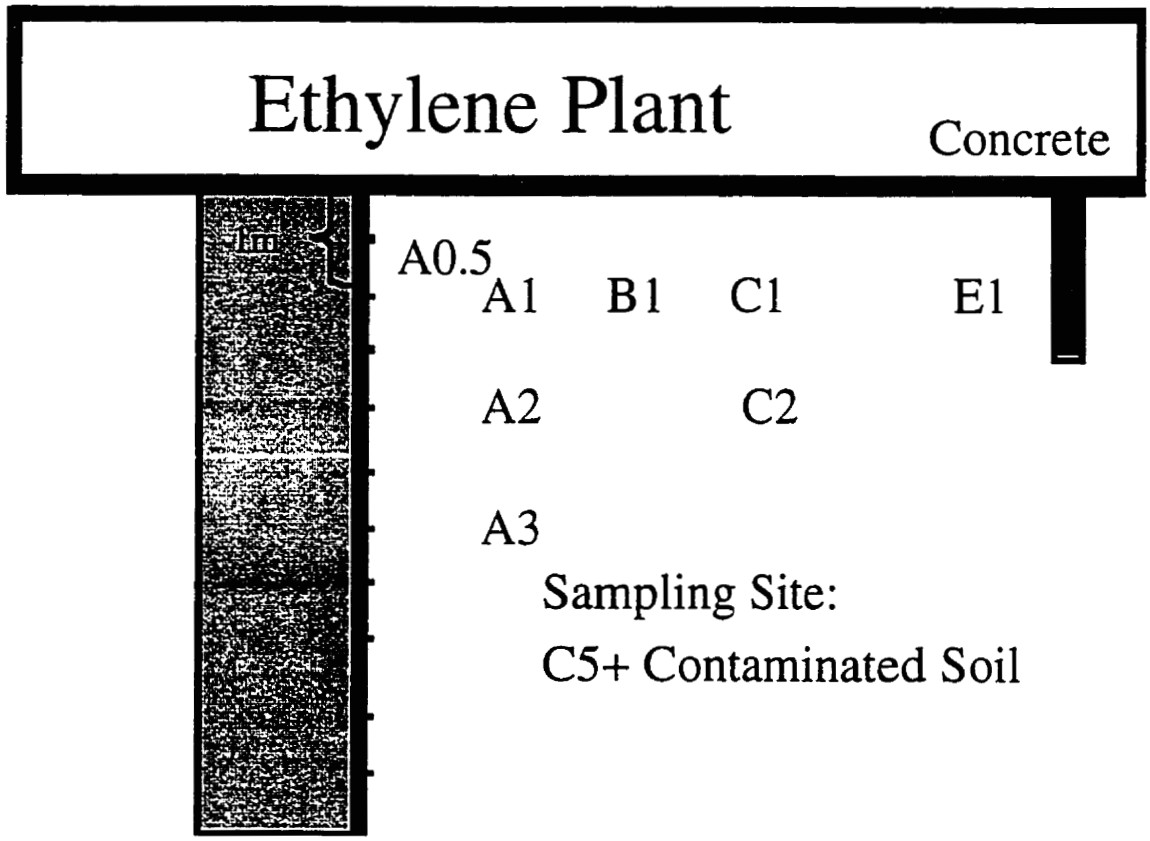


Figure 2-1. Diagram of a contaminated soil sampling site. Located near an ethylene pyrolysis plant. A core sampler was used, and all samples were taken at a depth of 90-120 cm.

The third set of soil samples was collected in the summer of 1999 by Anne Greene and Les Stehmeier from Nova Research & Technology Corp. in Calgary. They were taken from subsurface drillings in areas that had previously not been contaminated with C5+. These samples were given the designations 320, 330 and 350. Samples 320 and 330 were taken from depths of 12.5 m, from outer edge piezometer wells, where monitoring for subsurface contamination occurs. Sample 350 was a homogenisation of soils taken from depths of 5.5 m and 14 m at a site where a new polyethylene plant is planned.

2.1.3 Buffers and Growth Media

Buffers. TE buffer is 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0; 1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2; lysis buffer is 0.15 M NaCl, 0.1 M EDTA, pH 8.0. 10X buffer for PCR amplification of 16S rRNA genes is 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl; pH 9.0. 5X buffer for sequencing using the fmol sequencing kit is 250 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0.

Growth media. Tryptone yeast extract medium (TY, pH 7.4) contained 10 g bactotryptone, 5 g of yeast extract and 5 g NaCl per litre of H_2O. Minimal salts medium (MSM) (Shen, 1997) and B+NP medium (Fedorak and Grbic'-Galic', 1991) were used for soil incubations and enrichments. The compositions of these media are outlined in Table 2-1.

Table 2-1. Composition of MSM and B+NP medium. Per litre of water, all in grams unless indicated otherwise.

Ingredient	MSM	B+NP
KNO_3	-	2.0
$NaNO_3$	4.0	-
K_2HPO_4	-	0.5
Na_2HPO_4	0.5	-
KH_2PO_4	1.5	-
Na_2SO_4	-	2.0
$FeSO_4 \bullet 7H_2O$	0.0011	1 grain
$MgSO_4 \bullet 7H_2O$	0.2	0.2
NH_4Cl	-	1.0
$CaCl_2 \bullet 2H_2O$	0.01	-
Trace metals	-	1 mL

2.2 Analysis of Microbial Communities by RSGP

2.2.1 Enrichment and Isolation of Bacterial Strains

Bacterial strains were isolated by enrichment on a specific C5+ component hydrocarbon, followed by dilution plating. For growth on benzene, styrene or xylenes (containing *o*-, *m*- and *p*-xylene) 5 g aliquots of previously contaminated soil samples were placed in test tubes with 10 mL of MSM. The soil and MSM were mixed by vortexing and placed in a dessicator containing the hydrocarbon of interest for two weeks (Figure 2-2). All incubations were performed at room temperature (ca. 20°C). The hydrocarbon was diluted with vacuum pump oil (vpo) to concentrations of either 10% (w/w) (styrene and xylenes) or 1% (w/w) (benzene). The aromatic compound enters the vapour phase and then the aqueous phase, becoming available to the microorganisms. Vacuum pump oil dilutes the aromatic hydrocarbon and, due to Henry's law, lowers its vapour pressure. The low concentration of the aromatic compound in the vapour phase leads to a correspondingly lower concentration in the aqueous phase (Tinoco *et al*, 1995). Table 2-2 gives approximate concentrations of some hydrocarbons in each phase at equilibrium. Vpo, which is a mixture of high molecular weight aliphatic hydrocarbons, is used as a solvent because its vapour pressure is negligible, and so no vpo enters the medium aqueous phase (Hubert *et al*, 1999).

After two weeks of incubation, the test tubes were removed from the dessicator. The tubes were again vortexed, and the large particles were allowed to settle (1-2 min). The supernatant was taken and serially diluted (10^{-2}, 10^{-4}, 10^{-6}). The dilutions were plated on MSM plates, which were placed back in dessicators

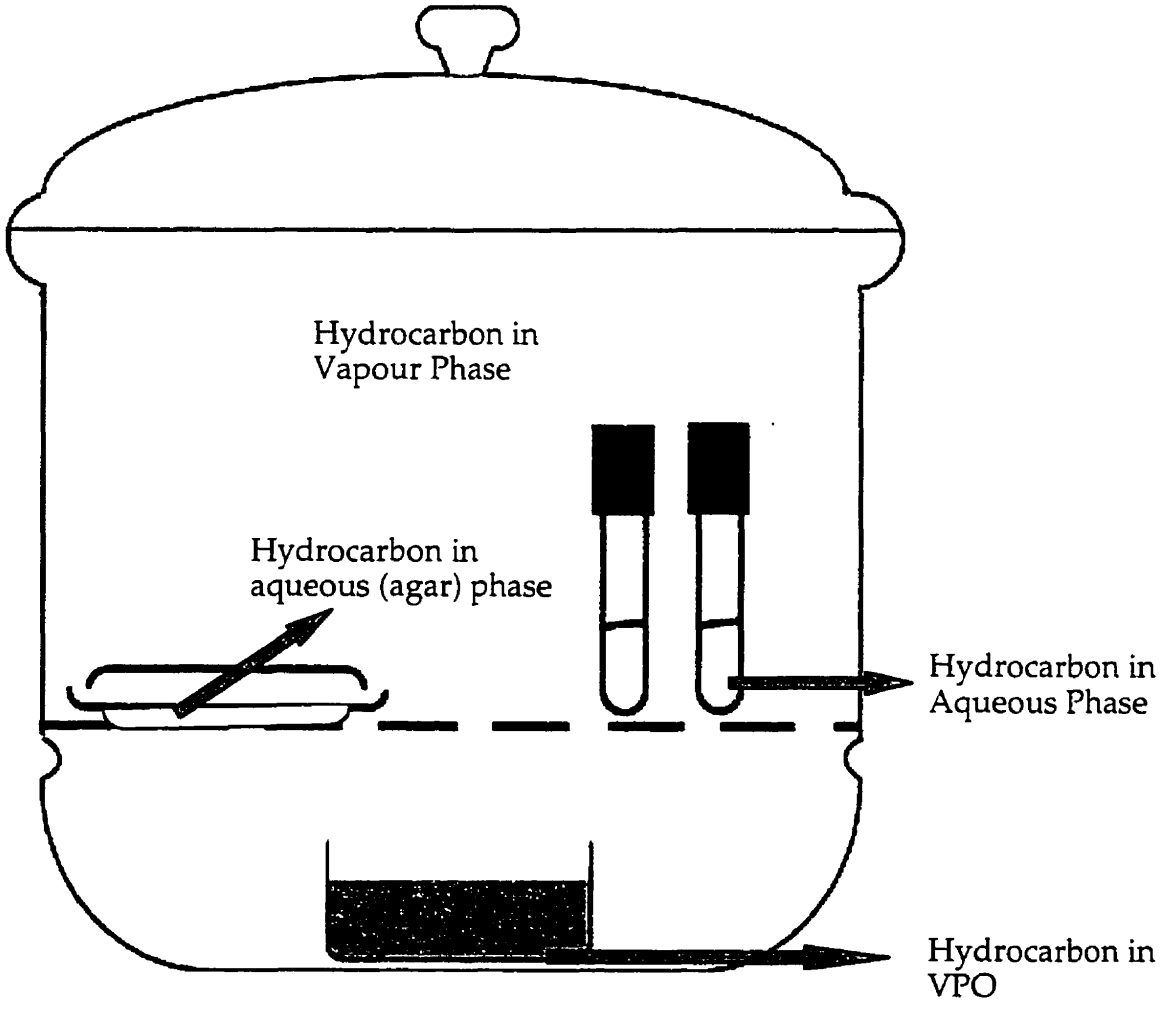


Figure 2-2. Dessicator set-up. Hydrocarbon is placed in vacuum pump oil (vpo) at the bottom of the dessicator. An equilibrium between the hydrocarbon in the vpo, vapour phase and aqueous phase of the media present is reached.

Table 2-2. Approximate concentrations (C) of C5+ hydrocarbons in vapour and aqueous phases equilibrated with different dilutions of hydrocarbon in vpo.

Hydrocarbon	% (w/w) in vpo	Cvapour (mg/L)	Cwater (mg/L)
Benzene	100	422	1850
	10	42	184
	1	4.2	18
Toluene	100	170	540
	10	45	143
	1	5	16
Xylenes	100	48	160
	10	4.8	16
	1	0.5	1.7
Styrene	100	28	228
	10	2.8	23
	1	0.3	2.4
DCPD	100	11.9	15
	10	1	1.3
	1	0.1	0.13
Naphthalene	100	0.6	34
	10	0.06	3.4
	1	0.006	0.34

with the appropriate atmosphere of hydrocarbon and incubated for a further two weeks. The plates were then removed and colonies were picked. These were grown in liquid TY medium and then streaked on TY plates to evaluate purity. The ability of colony purified strains to grow on the hydrocarbon used for isolation was then tested by streaking each strain on two MSM plates. One set of plates was incubated in dessicators with just vpo, whereas the other set was incubated in dessicators with the same hydrocarbon concentrations as used for the enrichments. Growth in the hydrocarbon atmospheres but not in the vpo only atmosphere verified the ability to use the hydrocarbon as the sole energy and carbon source.

Strains able to degrade naphthalene were obtained by first enriching 5 g of soil in 10 mL of MSM amended with salicylate (0.69 g/L), an intermediate in the degradation of naphthalene (Figure 1-4), for two weeks at room temperature. The supernatant was next serially diluted and plated on MSM plates containing salicylate (0.69 g/L) and allowed to grow for two weeks. The colonies that developed were picked and grown up as described earlier. To test for growth on naphthalene, purified isolates were streaked on MSM plates, which were placed in dessicators with 10% w/w naphthalene in vpo. These were compared to strains streaked on MSM plates and incubated in dessicators with vpo only.

2.2.2 Master Filter Preparation and Determination of k_λ/k_x

Purified strains were grown in 50 mL of TY medium, and genomic DNA was isolated by the method described by Marmur (1961), modified to include digestion with DNAse free RNAse and Proteinase K (Voordouw *et al*, 1990). The DNA pellets were dissolved in TE. The concentrations of the DNA, which was

determined by a fluorimetric method as described by Voordouw *et al* (1993), ranged from 50 to 500 ng/μL. Part of the DNA recovered from each strain was diluted to approximately 50 ng/μL in TE and 2 μL (100 ng DNA total) were spotted on a Hybond-N membrane. These were used for genomic cross-hybridisation testing as described in section 1.3.2.2, using the high-stringency dot blot procedure described by Voordouw *et al* (1991). For probe preparation, 100 ng of heat denatured chromosomal DNA from a single strain, 0.1 ng heat denatured λ DNA, 6 μL of primer extension (PE) mix containing random hexamers (Voordouw *et al*, 1992), 2 μL of Klenow polymerase (2U/μL) and 2 μL of [α^{32}-P]dCTP were combined in a total volume of 30 μL labelling mixture. PE mix was made by adding 44 μL of 0.9 M HEPES, 0.1 M $MgCl_2$ (pH 6.6), 25 μL of 1 M Tris-HCl (pH 7.4), 10 μL of 0.1 M dithiothreitol, 4 μL of 50 mM dGTP, 50 mM dATP and 50 mM dTTP and 10 μL of random hexanucleotides (10 μg/μL) in 100 μL. Once a labelled probe was obtained, it was boiled and hybridised to the filter under conditions described in section 2.2.4. The hybridisation patterns obtained were analysed quantitatively using MacBAS v2.2 software (Fuji Photo Film, Inc, 1995). The hybridisation values (I_x) obtained were normalised by dividing by the amount of DNA spotted for each individual standard (c_x). The value of this net hybridisation (I_x/c_x) was used for determination of cross-hybridisation values. Strains with genomic DNAs that cross-hybridised greater than 70% were considered to represent the same standard. The DNAs from these strains were pooled if necessary. Chromosomal DNA from new standards were next cross-hybridisation tested against the 35 standards isolated and defined in previous work (Table 3-1,

Standards 1-35) (Shen *et al*, 1998). Those with cross-hybridisation greater than ca. 70% with the existing set were considered to be already represented on the master filter. New standards without significant cross-hybridisation with the existing set were added to the master filter by spotting 2 μL of heat denatured genomic DNA on a Hybond-N membrane. The exact amounts of DNA spotted on the filters are listed in Table 3-2. The master filter also contained denatured bacteriophage λ DNA in amounts of 10, 20, 40, 60, 80, 100, 200 and 400 ng (Figure 3-1). The denatured DNAs were fixed to the Hybond-N membrane by heating at 80°C for 15 min. They were then covalently linked to the membrane by irradiation with UV light (8000 $\mu W/cm^2$, 312 nm) for 3 min (Voordouw *et al*, 1992).

The ratio of hybridisation constants (k_λ/k_x) of each standard added to the master filter was determined by probing the master filters with a labelled mixture of known amounts of standard DNA (100 ng) and λ DNA (2.5 ng). Following hybridisation and determination of hybridisation intensities I_x and I_λ from the net Phospo-stimulatable Luminescence (PSL) values, the ratio was calculated using the following formula (Telang *et al*, 1997):

$$(k_\lambda/k_x) = (f_x/f_\lambda) \times (I_\lambda/c_\lambda) \times (I_x/c_x)^{-1} \qquad \textbf{(Equation 2-1)}$$

where k_λ and k_x are the hybridisation constants of λ and standard x, respectively, f_λ and f_x are weight fractions of λ and standard DNA used in the probe (2.5/102.5 and 100/102.5, respectively) and c_λ and c_x are the amounts (ng) of denatured λ and standard DNA spotted on the master filter.

2.2.3 Identification of New Standards by 16S rRNA Gene Sequencing

New standards were characterised by partial 16S rRNA gene sequencing. The genes were amplified by the polymerase chain reaction (PCR) to a 1.4 kb product. Primers f8 and r1406 (Table 2-3) were used for the PCR amplification in a Perkin Elmer GeneAmp 2400 PCR system. A 25 µL reaction volume was used which contained 50 ng isolate genomic DNA, 2.5 µL of 10 x Buffer, 1.25 µL of 50 mM $MgCl_2$, 2 µL of dTTP, dATP, dCTP and dGTP (2.5 mM each), 0.5 µL of each of the primers f8 and r1406 (10 pmol/µL) and 0.13 µL of Taq polymerase (5 U/mL). Amplification was performed by 15 cycles of 30 sec at 94°C, 30 sec at 60°C (with temperature decreasing 0.5°C per cycle) and 60 sec at 72°C followed by 15 cycles of 30 sec at 94°C, 30 sec at 54°C and 60 sec at 72°C. After PCR amplification, 60 µL of 30% (w/v) of polyethylene glycol (PEG) and 50 µL of 2.5 M NaCl were added to the PCR reaction mixture. After incubation at 37°C for 10 min, the PCR product was collected by centrifugation (14 000 x g, 15 min), washed with 70% ethanol and air dried. The amplified DNA was dissolved in 12 µL TE, of which 2 µL was used to visualise it on a 1% HGT agarose gel stained with ethidium bromide (0.1 µg/mL).

The amplified product was sequenced directly with the Promega fmol sequencing system using [α^{35}-S]dATP and primers EUB or p76 (Table 2-3). In this method, four PCR reactions are carried out in seperated reaction tubes for each primer used to sequence. In each reaction tube (T, C, G, and A), 0.75 µL amplified 16S rRNA gene template from reactions above, 0.75 µL primer (1 pmol/µL), 1.25 µL 5X buffer, 0.25 µL Taq polymerase and 0.125 µL [α^{35}-S] dATP were added.

Table 2-3. Target sites and sequences of primers used for 16S rRNA PCR and sequencing.

Primer	Sequence (5′→3′)	Target Position[a]	Reference
f8	TGAGCCAGGATCAAACTCT	8-26	Hicks *et al*, 1992
r1406	ACGGGCGGTGTGT(A/G)	1406-1392	Olsen *et al*, 1986
EUB338	GCTGCCTCCCGTAGGAGT	338-355	Amann *et al*, 1992
P76	GCCAGC(A/C)GCCGCGGT	517-531	Shen, 1997

[a]Numbering is for the 16S rRNA gene of *Escherichia coli*.

Additionally, to each tube a mixture of deoxynucleotides (dNTP) and dideoxynucleodtides (ddNTP) were added. In each reaction tube, all four dNTPs were added (dTTP, dCTP, dGTP and dATP) at a concentration of 20 μM, however in each a different ddNTP was added. In tube T, there was 600 μM ddTTP, in tube C 200 μM ddCTP, in tube G 30 μM ddGTP and in tube A 350 μM ddATP. PCR was performed on the reaction tubes with 30 cycles of 30 sec at 95°C, 60 sec 72°C. The PCR product was next run on a polyacrylamide gel at 50 W (1400 to 1900 V) for 3.5 to 4.5 hours. The polyacrylamide gel was then dried and exposed to X-ray film for 3 to 7 days. During the PCR process, Taq polymerase would occasionally incorporate a ddNTP into the PCR product instead of the regular dNTP. When this occurs, Taq polymerase can no longer extend the PCR product, and the PCR product will be of a fixed length. This happens in each of the reaction tubes for each of the respective nucleotides (T, C, G and A). The polyacrylamide gel is able to separate DNAs with a single base pair difference in length. This, combined with the knowledge of which ddNTPs were in which reaction tube, allows the developed X-ray film of the polyacrylamide gel to be read as a DNA sequence, one base pair at a time. Approximately 250 nucleotides were able to be read for each primer. The sequences obtained with different primers often overlapped to give 350 to 450 bp of continuous sequence.

In two cases, for standards LQ27 and LQ33, the amplified 16S rRNA gene product was sequenced by the University of Calgary's DNA Sequencing Lab. The closest homologue to the standard for which the 16S rRNA sequence was determined was obtained by its comparison with 16S rRNA gene sequences

available in the RDP database using the program SIMILARITY_RANK (Maidak *et al*, 1994) or in the GenBank database with the program BLAST (Altschul *et al*, 1990).

2.2.4 Extraction of Total Community DNA From Soil

DNA was extracted from soil samples using a modification of a method described by Shen (1997), that was adapted from a technique described by Tsai & Olsen (1991). Soil samples were combined with 0.5 g of acid washed polyvinylpolypyrrolidone (PVPP) and 0.1% (w/v) sodium pyrophosphate was added to a final volume of 15 mL. This was mixed vigorously by vortexing. PVPP was acid washed as described by Holben *et al* (1988). Approximately 100 g of insoluble PVPP was mixed with 1 litre of 3 M HCl and incubated overnight. The HCl was removed by vacuum filtration through Whatman Number 1 qualitative filter paper. The PVPP was then washed with 20 mM potassium phosphate, stirred and filtered as before. The washing was repeated until the PVPP/potassium phosphate suspension was pH 7. The filtered PVPP was then dried overnight.

Soil particles and PVPP were removed by centrifugation at 1000 x g for 10 min at 4°C. The soil particles were washed a second time with 0.1% (w/v) sodium pyrophosphate. The supernatants from the washings were combined and centrifuged at 15 000 x g for 20 min at 4°C to collect bacterial cells. The cell pellet was resuspended in 2 mL of lysis buffer. Early soil extractions included lysozyme (15 mg/mL) and incubation for 60 min at 37°C. However, a paper by Miller *et al* (1999) comparing various methods of soil DNA extraction reported lower yields

from soil samples when lysozyme was used. This step was excluded from later soil extractions.

Sodium dodecyl sulphate (SDS) (1.5 mL of 25% (w/v)) was next added and the cells were subjected to three cycles of 20 min at -70°C and 20 minutes at 65°C in order to release the DNA. Following addition of 0.4 mL of 4 M sodium perchlorate and 2 mL of chloroform-isoamyl alcohol (24:1) the DNA was extracted by gentle end-over-end mixing on a rotating wheel for 60 minutes. Centrifugation at 6 000 x g for 10 min followed, after which the top aqueous phase was collected. The DNA was precipitated with 2 volumes of cold 95% ethanol at -70°C for one hour or -20°C overnight. A crude DNA pellet was obtained by centrifugation at 12 000 x g for 20 minutes at 4°C. The pellet was brownish in colour because of co-extraction of humic acids that were not removed by the PVPP. The DNA was dissolved in 200 µL of TE buffer and 5 µL were visualised on 1% HGT agarose gel stained with ethidium bromide (0.1 µg/mL).

Humic acids are organic molecules which are formed during the microbial decomposition of plant material, which are themselves resistant to further decomposition (Fredrickson, 1999). Humic acids interfere with labelling of the DNA by DNA polymerases, and DNA-DNA binding (Tebbe and Vahjen, 1993). DNA-DNA binding and DNA polymerases are important in the RSGP process. Therefore, the humic acids, which were co-extracted with the soil DNA, needed to be removed. The humic acids were removed using a column purification technique described by Jackson *et al* (1997). Approximately 0.1 cm^3 of acid washed glass wool was packed into the bottom of a 1 mL syringe. The remainder of the syringe was filled with Sepharose 4B. This was done by pipetting Sepharose 4B in

an ethanol suspension into the syringe followed by centrifugation (1 000 x g for 4.5 minutes) until a Sepharose 4B column of ca. 0.9 cm^3 was obtained. After washing of the columns by loading 100 µL of TE, followed by centrifugation and repeating this four times, crude DNA (200 µL) was loaded and centrifuged at 1 000 x g for 4.5 min. The effluent was collected and the column was eluted by loading 100 µL of TE and spinning, 5 to 6 times, collecting the effluent after each spin. Sepharose 4B is a chromatography reagent made of agarose beads with diameters ranging from 45 to 165 µm. These beads contain pores, which are too small to allow large DNA molecules in. Therefore the DNA goes around the beads of the column and rapidly appears in the early fractions. Humic acids appear later because they are smaller and are detained by the matrix (Berg, 1963).

From each of the collected fractions, 5 µL was run on an 1% HGT agarose gel, which was stained with ethidium bromide (0.1 ug/mL). DNA and humic acids were both visible on the ethidium stained agarose gel. Fractions containing DNA but no humic acids were pooled, treated with 15 µL of RNAse (1mg/mL) at 37°C for 30 min followed by 4 µL of Proteinase K (14 000 U/mL) for 30 min at 37°C and extracted with 1/3 volume of TE saturated phenol by gentle end-over-end inversion for 30 min. After centrifugation (5 min, 14 000 x g) the top aqueous layer was removed and precipitated with 2 volumes 95% ethanol at -70°C for 1 hour or -20°C overnight. The DNA was obtained by centrifugation (14 000 x g for 15 min at 4°C), air dried and redissolved in 20 µL TE.

2.2.5 Labelling of Purified Community DNA and Filter Hybridisation

Master filters were prehybridised for 2 hours at 68°C in hybridisation solution, made by combining 30 mL of 6 x SSC, 5 mL 10% (w/v) SDS, 10 mL of 50 x Denhardt's solution (5 g Ficoll 400, 5 g PVP and 5 g BSA in 100 mL water) and 0.5 mL of 10% (w/v) of denatured salmon sperm DNA in a total volume of 100 mL. DNA probes were made by combining 100 ng of purified genomic DNA and 2.5 ng λ DNA and labelling with the random hexamer extension method using [α^{32}-P] dCTP as described in more detail in section 2.2.2. After boiling of the probe, it was hybridised with a master filter for 16 hours at 68 °C (Voordouw *et al*, 1990; 1991). After hybridisation, the master filter was washed in 1 x SSC for 15 min at 20°C, followed by a second wash with 1 x SSC and 0.2% (w/v) SDS at 68°C for 1 hour. The master filter was then dried with 3MM paper and wrapped in Saran Wrap for quantitative analysis.

2.2.6 Quantitation of Hybridisation Data

Probed master filters were exposed to BAS-III Imaging plates for 2-4 hours, which were then scanned with a Fuji BAS 1000 Bio-Imaging analyser. MacBAS 2.2 software was used to determine the PSL of each spot of DNA on the filter. A background (PSL-B) reading was also taken. The net hybridisation intensity (I_x or I_λ) was determined as (PSL - PSL-B) for each spot on the filter. The weight percentage, f_x, of each standard DNA was found by rearranging Equation 1 to:

$$f_x = (k_\lambda / k_x) \times (I_x / c_x) \times (I_\lambda / c_\lambda)^{-1} \times f_\lambda. \qquad \textbf{(Equation 2-2)}$$

After calculating the f_x values they were normalised by setting $\Sigma f_x=1$. Each f_x value was represented as a percentage of the total. The normalised f_x values were plotted versus the standard number x in a bar diagram (RSGP profile; Figure 1-8) representing all the standards on the master filter (Voordouw, 1998). RSGP profiles, from different soil samples, were compared by dendrograms, generated with the cluster analysis program SYSTAT v7.0. With this program, each RSGP profile obtained is entered into the database as a case. The f_x values are the variables within the cases. The f_x variables between cases (RSGP profiles) are compared and single linkage trees are formed using normalised Euclidean distances. Cases that are most similar will be treed together, while those with lower similarity will tree at a greater distance (SPSS Inc., 1997).

2.3 Monitoring Biodegradation of C5+ Compounds

2.3.1 Soil Incubations and Enrichments

For soil incubations, 1 g of the soil of interest was combined with 5 mL of MSM in a sterile 20 mL glass test tube. These were placed in a dessicator with the hydrocarbon of choice, appropriately diluted with vpo (Figure 2-2). The hydrocarbon concentrations and incubation conditions are given below.

2.3.1.1 Soil Incubations in Individual Hydrocarbons

A number of soil incubations were performed in atmospheres of single C5+ components. These included benzene (0.2, 1 and 10% (w/w) in vpo), toluene (0.1, 1, 10 and 100% (w/w) in vpo), styrene (0.2, 1, 10 and 100% (w/w) in vpo), *m*-xylene (0.2, 1, 10 and 100% (w/w) in vpo and naphthalene (1, 10 and 100% (w/w)

in vpo). Incubations were performed at room temperature (ca. 20°C) for periods of 2 to 12 weeks.

2.3.1.2 Soil Incubations in C5+ Followed by Incubation in DCPD Only

The C5+ used was a synthetic mixture of 56% (w/w) benzene, 25% (w/w) DCPD, 7.5% (w/w) toluene and 4% (w/w) each of *m*-xylene, styrene and naphthalene. Incubations and enrichments were performed with 1% of the synthetic C5+ mixture in vpo, as explained in section 2.3.1.1 for 2 to 12 weeks. Following incubation in C5+ for 2 to 4 weeks, some soils were transferred to 1% (w/w) DCPD in vpo and incubated for a further 2 to 9 weeks.

2.3.1.3 Soil Enrichments

Soil enrichments were started with 2 g of soil and 10 mL of MSM. They were incubated in dessicators with 1% (w/w) C5+ in vpo. Every fortnight a 20% inoculum was transferred (2 mL culture slurry into 8 mL medium). After two transfers, the medium was changed to B+NP. This medium contains additional trace metals that may be needed since by this time the soil was absent. After four transfers (2 months), the transfer inoculum was changed to 10%.

2.3.2 Rate analysis

The rates of hydrocarbon removal from soil microcosms, enrichment cultures, synthetic consortia and some individual standards were studied in either 158 or 125 mL serum bottles. Soil microcosms were started by placing 25 g of soil in a serum bottle with 40 mL of MSM, sealing with composite Teflon (inside) and butyl rubber (outside) stoppers. Synthetic C5+ mixture was added as a source of hydrocarbons. 2 µL (approximately 14.2 µmol benzene, 2.4 µmol DCPD, 1.6 µmol

toluene, 1.4 µmol m-xylene, 0.7 µmol styrene and 0.4 naphthalene) gave a final concentration in the approximately 60-mL microcosms of 350 µM. Sterile control microcosms were made in the same manner as the viable microcosms; however, the sterile controls were autoclaved on three consecutive days, after which mercuric chloride was added to a final concentration of 2.5 mg/L. Synthetic C5+ mixture was added after the sterile control microcosms had been autoclaved.

The oxygen present in the serum bottle headspace was sufficient for complete mineralisation of the synthetic C5+ mixture added to the microcosms. Mineralisation of the added hydrocarbons was predicted to require 172 µmol O_2 (Table 2-4). At room temperature, air contains 1 mmol O_2 per 117 mL, therefore 20 mL air contains the required 172 µmol O_2. The headspace in the serum bottles used for the tests was 98 mL in the 158 mL bottles and 65 mL in the 125 mL bottles. This was more than sufficient.

Enrichment cultures (Section 2.3.1.3) were grown for an additional two weeks in a 1% C5+ atmosphere prior to rate analysis. The cells from 10 mL were harvested by centrifugation (2 500 x g, 10 min, 4°C) and resuspended in 1 mL of B+NP. The suspension was injected into 125 mL serum bottles containing 60 mL B+NP medium. The serum bottles were then sealed with Teflon (inside) and butyl rubber (outside) stoppers and 2 µL of C5+ was added.

Studies with synthetic consortia were performed similarly. Three synthetic consortia were made. SC-41 contained 41 organisms on the master filter that would readily grow in TY medium under aerobic conditions (all standards except 4, 21, 22, 23, 24, 28, 30, 31, 32, 33, 34, 35, 50 and 53). SC-9 contained nine organisms from the master filter (standards 11, 25, 26, 35, 37, 38, 39, 40, 45, 47 and 52) which

Table 2-4. Oxygen requirements for the mineralisation of compounds present in synthetic C5+ mixture.

Compound	Balanced equation for mineralisation
benzene	$C_6H_6 + 7.5O_2 \rightarrow 6CO_2 + 3H_2O$
toluene	$C_7H_8 + 9O_2 \rightarrow 7CO_2 + 4H_2O$
xylenes	$C_8H_{10} + 10.5O_2 \rightarrow 8CO_2 + 5H_2O$
styrene	$C_8H_8 + 10O_2 \rightarrow 8CO_2 + 4H_2O$
DCPD	$C_{10}H_{12} + 13O_2 \rightarrow 10CO_2 + 6H_2O$
naphthalene	$C_{10}H_8 + 12O_2 \rightarrow 10CO_2 + 4H_2O$

were present at $f_x \geq 5\%$ of the RSGP profile in at least two of the enrichments after transfers four to six. SC-5, which contained 5 organisms from the master filter (3, 11, 25, 38 and 52) which were present in ≥2% in all enrichment RSGP profiles. The synthetic consortia were made by taking glycerol stocks of the standards for each consortium, growing the standards separately in 5 mL TY for 5 days at room temperature. Volumes of 1 ml of selected cultures were then combined, centrifuged, washed twice with B+NP medium and finally resuspended in 3 ml of B+NP medium. For determination of C5+ degradation rates, volumes of 0.5 ml of resuspended synthetic consortia were injected into 158-ml serum bottles containing 60 ml B+NP medium and 2 µL C5+ hydrocarbon (final concentration approximately 300 µM). Sterile controls were prepared by autoclaving on 3 consecutive days, followed by injection of mercuric chloride (final concentration 2.5 mM).

For the analysis of naphthalene removal by master filter standard Q5, it was grown up in 75 mL of BN+P medium in a dessicator with a saturated naphthalene atmosphere. They were prepared as the enrichment microcosms above and 1 mL of resuspended culture was placed into 158-ml serum bottles containing 60 ml B+NP medium. Napthalene was introduced by injecting 2, 5 or 10 µL of naphthalene saturated 2,2,4,4,6,8,8-heptamethylnonane (HMN) into the microcosm. HMN is a non-polar solvent that is itself recalcitrant to degradation by microorganisms (Allen *et al*, 1999).

Kinetic constants were determined using a method described by Metcalf and Eddy (1979). If a plot of the compound concentration vs. time was linear, the data were consistent with zero order kinetics. If the plot of the natural logarithm of the

compound concentration vs. time was linear, the data were consistent with first order kinetics.

2.3.3 Analytical Methods

Hydrocarbon analysis was performed with a Hewlett-Packard HP5190 gas chromatograph (GC) equipped with a flame ionisation detector (FID). 10 µL of headspace from the samples was injected into the GC. The column used was an 0.5 µ RTX-5 (Restek, Brockville, ON) of 30 m x 0.2 mm ID. The flow rate of the carrier gas, He, was 1.23 mL/min. FID airflow was 400mL/min and H_2 flow 30 mL/min. The injector temperature was 210°C and the detector temperature was 250°C. Oven temperature was 60°C for 3 min, followed by an increase of 10°C per min to a final temperature of 200°C for 5 min. Retention times and peak areas were determined by a HP 3392A Integrator. Peaks were identified by injecting known standards and recording their retention times.

Chapter 3

RESULTS AND DISCUSSION

3.1 Additions to the RSGP Master Filter

3.1.1 Isolation of Bacteria and Identification of Standards for RSGP Master Filter

After incubating soil in MSM + salicylate for two weeks followed by serial dilution plating, 15 bacterial strains capable of growth on MSM agar plates amended with salicylate were obtained. Chromosomal DNA from the isolates was tested for cross-hybridisation and 5 genomically distinct bacterial standards were identified. Cross-hybridisation testing with existing standards (Section 3.1.2.1) reduced the number of new standards to 4 (Tables 3-1 and 3-2: standards 52-55), which were added to the master filter. Ten new standards which could grow on benzene, styrene or xylenes as the sole carbon and energy source (Greene *et al*, 2000) were similarly added to the master filter (Tables 3-1 and 3-2: standards 42-51) at the same time. Standards 1-35 had been isolated earlier mainly on rich media (Shen *et al*, 1998), and standards 36-41 were isolated on MSM with toluene as the sole carbon and energy source (Hubert *et al*, 1999). The layout of the master filter for the performance of RSGP for the evaluation of C5+ degrading soils following these additions is shown in Figure 3-1. Continued isolation of bacteria from the soil samples would have resulted in more standards being obtained. However, many of the recently isolated bacterial strains were already represented on the

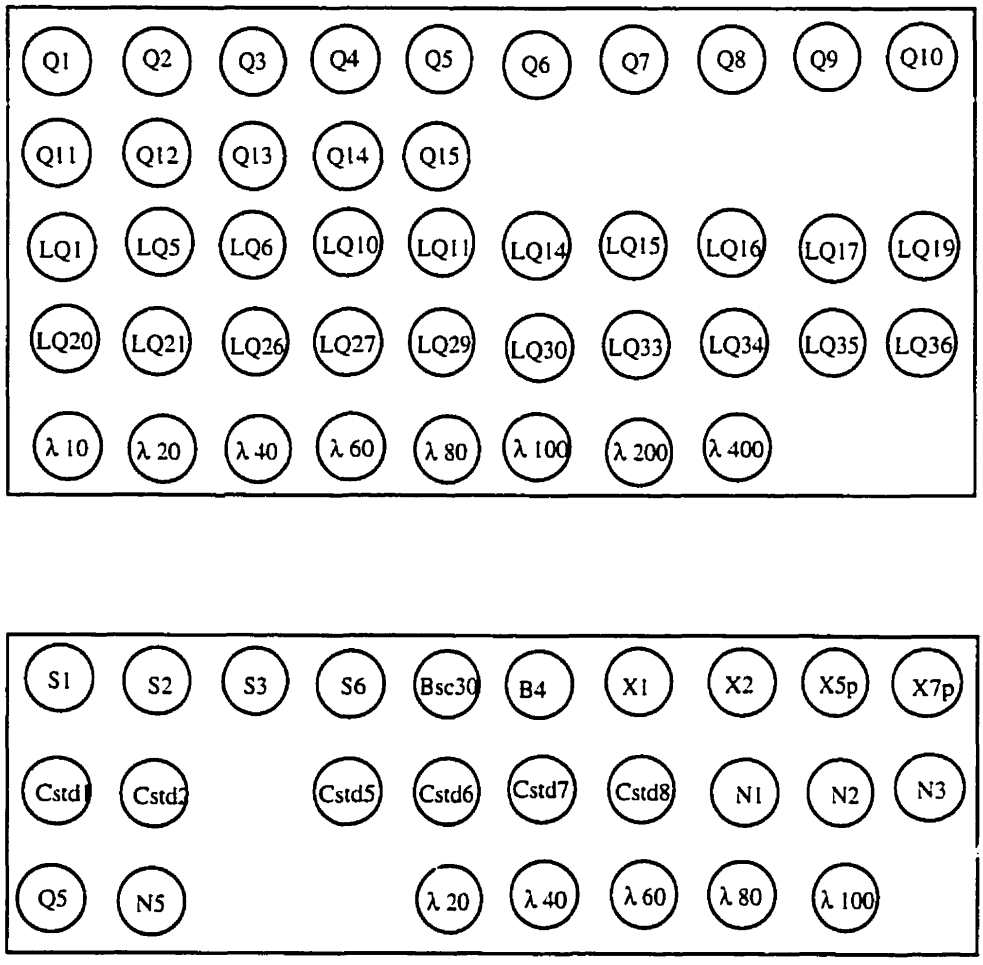


Figure 3-1. Layout of master filter used in the characterisation of C5+-degrading microbial communities. The genomic DNAs of 55 standards are spotted on Hybond-N nylon membranes known as 'filters' in the order shown, with the newly isolated standards on the second (bottom) filter. Tables 3-1 and 3-2 describe the standard organisms. The bottom rows of each filter contain denatured λ-DNA in the amounts shown (ng), which is used to allow quantification of genomic DNA hybridisation to the master filter (Section 2.2.6).

Table 3-1. Standards for which DNA was spotted on the master filter.

Pos.[a]	Name[b]	Medium[c]	S_{ab}[d]	Nearest homolog[e]
1	LQ1	PTYG	0.802	*Bordetella bronchiseptica*
2	LQ5	PTYG	0.375	*Pseudomonas syringae*
3	LQ6	PTYG	0.396	*Azospirillum* sp.
4	LQ10	PTYG	0.598	*Sphingomonas parapaucimobilis*
5	LQ11	PTYG	0.769	*Bacillus macroides*
6	LQ14	PTYG	0.615	*Xanthomonas campestris*
7	LQ15	PTYG	0.580	*Bacillus pseudomegaterium*
8	LQ16	PTYG	0.861	*Pseudomonas syringae*
9	LQ17	PTYG	0.931	*Agrobacterium rubi*
10	LQ19	PTYG	0.349	*Flavobacterium lutescens*
11	LQ20	PTYG	0.816	*Pseudomonas syringae*
12	LQ21	PTYG	0.791	*Bordetella parapertussis*
13	LQ26	PTYG	0.865	*Bordetella parapertussis*
14	LQ27	PTYG	0.783	*Bordetella bronchiseptica*
15	LQ29	PTYG	0.843	*Bordetella parapertussis*
16	LQ30	PTYG	0.935	*Sphingomonas yanoikuyae*
17	LQ33	PTYG	0.917	*Stenotrophomonas maltophilia*
18	LQ34	PTYG	0.969	*Pseudomonas flavescens*
19	LQ35	PTYG	0.972	*Pseudomonas flavescens*
20	LQ36	PTYG	0.965	*Pseudomonas flavescens*
21	Q1	HDM, benzene	0.851	*Rhodococcus* sp.
22	Q2	PTYG	0.879	*Bacillus cereus/thuringiensis*
23	Q3	PTYG	0.763	*Nocardioides luteus*
24	Q4	PTYG	0.683	*Flavobacterium ferrugineum*
25	Q5	HDM, naphthalene	0.779	*Pseudomonas syringae*
26	Q6	HDM, styrene	0.836	*Rhodococcus globerulus*
27	Q7	TY	0.745	*Pseudomonas syringae*
28	Q8	TY	0.879	*Bacillus benzeovorans*
29	Q9	TY	0.809	*Bacillus polymyxa*
30	Q10	TY, anoxic	0.504	*Bacteroides distasonis*
31	Q11	TY, anoxic	0.498	*Bacteroides heparinolyticus*
32	Q12	TY, anoxic	0.846	*Clostridium xylanolyticum*
33	Q13	TY, anoxic	0.337	*Clostridium* sp.
34	Q14	Medium C, anoxic	0.582	*Desulfovibrio longus*
35	Q15	Medium C, anoxic	0.571	*Desulfovibrio desulfuricans*
36	Cstd1	MSM, toluene	0.715	*Pseudomonas putida*
37	Cstd2	MSM, toluene	0.816	*Pseudomonas* sp. isolate LX1
38	Cstd5	MSM, toluene	0.761	*Pseudomonas stutzeri*
39	Cstd6	MSM, toluene	0.796	*Microbacterium lacticum*
40	Cstd7	MSM, toluene	0.823	*Pseudomonas fluorescens*
41	Cstd8	MSM, toluene	0.857	*Pseudomonas putida*
42	S1	MSM, styrene	0.889	*Pseudomonas* sp.
43	S2	MSM, styrene	0.872	*Pseudomonas corrugata*
44	S3	MSM, styrene	0.920	*Pseudomonas syringae*

45	S6	MSM, styrene	0.935	*Pseudomonas aureofaciens*
46	Bsc30	MSM, benzene	0.870	*Pseudomonas agarici*
47	B4	MSM, benzene	0.884	*Alcaligenes* sp.
48	X1	MSM, *o-*, *m-*, *p*-xylenes	0.946	*Alcaligenes* sp.
49	X2	MSM, *o-*, *m-*, *p*-xylenes	0.872	*Pseudomonas* sp.
50	X5p	MSM, *o-*, *m-*, *p*-xylenes	0.895	*Rhodococcus marinoascens*
51	X7p	MSM, *o-*, *m-*, *p*-xylenes	0.839	*Microbacterium laevaniformans*
52	N1	MSM, naphthalene	0.935	*Alcaligenes* sp.
53	N2	MSM, naphthalene	0.658	*Arthrobacter oxydans*
54	N3	MSM, naphthalene	0.825	*Pseudomonas pseudoalcaligenes*
55	N5	MSM, salicylate	1.000	*Pseudomonas* sp. strain BI*7

[a]Position of standard DNA on filter.
[b]Name assigned to standard at time of isolation.
[c]Medium used for isolation. Standards 30 – 35 were isolated under anaerobic conditions
[d]Similarity coefficient for query and matching sequences (Maidak *et al*, 1994).
[e]Closest homolog in the RDP database as determined with the program SIMILARITY_RANK (Maidak *et al*, 1994).

Table 3-2. Survey of standards. Concentration of standard DNAs on master filter, k_λ/k_x values and substrate specificity.

Pos.[a]	Name[b]	C[c]	k_λ/k_x[d]	σ[e]	Hydrocarbons[f]
1	LQ1	142	238	20	-
2	LQ5	158	180	10	ben
3	LQ6	152	283	4	ben, tol, sty, nap
4	LQ10	164	112	11	-
5	LQ11	158	46	2	ben, tol, xyl
6	LQ14	142	61	12	xyl
7	LQ15	148	68	24	tol, nap
8	LQ16	158	95	1	ben, tol, sty, xyl, nap
9	LQ17	158	112	5	ben, tol, nap
10	LQ19	158	157	14	-
11	LQ20	158	210	30	ben, tol, xyl
12	LQ21	138	294	0	ben
13	LQ26	158	166	25	-
14	LQ27	158	149	36	tol
15	LQ29	192	208	32	-
16	LQ30	162	200	5	ben, tol
17	LQ33	174	249	14	-
18	LQ34	152	170	13	ben, tol
19	LQ35	170	160	6	-
20	LQ36	158	109	6	ben, tol
21	Q1	174	574	98	ben, tol, sty, xyl
22	Q2	156	60	6	nd[g]
23	Q3	174	330	32	nd[g]
24	Q4	148	93	10	nd[g]
25	Q5	198	161	45	ben, nap
26	Q6	170	202	5	ben, tol, sty, xyl, nap
27	Q7	180	151	12	ben, tol, sty
28	Q8	111	51	7	nd[g]
29	Q9	166	73	7	nd[g]
30	Q10	42	72	10	nd[g]
31	Q11	66	80	8	nd[g]
32	Q12	156	65	6	nd[g]
33	Q13	170	61	17	nd[g]
34	Q14	120	127	6	nd[g]
35	Q15	166	94	8	nd[g]
36	Cstd1	66	56	2	ben, tol
37	Cstd2	76	255	27	ben, tol
38	Cstd5	90	226	54	ben, tol, xyl
39	Cstd6	80	127	43	ben, tol
40	Cstd7	90	92	19	ben, tol
41	Cstd8	80	120	22	ben, tol
42	S1	62	17	6	ben, sty, xyl, nap
43	S2	80	113	55	ben, sty
44	S3	86	71	12	sty

45	S6	106	81	7	sty
46	Bsc30	80	54	2	ben, tol, xyl
47	B4	62	91	12	ben, tol, sty, xyl
48	X1	70	74	37	ben, tol, xyl
49	X2	96	70	36	ben, tol, xyl
50	X5p	96	91	3	ben, tol, sty, xyl
51	X7p	88	47	21	ben, tol, sty, xyl
52	N1	80	218	70	ben, nap
53	N2	80	101	16	ben, tol, nap
54	N3	80	123	26	ben, nap
55	N5	100	189	28	-

[a]Position of denatured chromosomal DNA on master filter.
[b]Name assigned at time of isolation.
[c]Amount of denatured chromosomal DNA (ng) spotted on the filter
[d]Ratio of hybridization constants for bacteriophage λ and genomic DNA x from equation 1 (Section 1.3.1.2).
[e]Average deviation between duplicate measurements of k_λ/k_x.
[f]Hydrocarbons each standard was able to grow on. Ben is benzene; tol is toluene; sty is styrene; xyl is a mixture of *o*- *p*- and *m*- xylene; nap is naphthalene; – indicates no growth.
[g]Not determined.

master filter. Further isolation of standards would have resulted in a diminishing return, so the development of the master filter was not continued.

3.1.2 Characterisation of Master Filter Standard Organisms

3.1.2.1 Cross-hybridisation of Chromosomal DNAs

DNAs from the new standards (36 through 55) were cross-hybridisation tested against all standard DNAs that were now on the master filter (Figure 3-1). For these experiments genomic DNA of a new standard was mixed with λ DNA, labelled and hybridised with the master filter (Section 2.2.2). The cross-hybridisation was determined quantitatively as explained in Section 1.3.2.2 in duplicate to ensure reproducibility. Some of the results are shown in Figure 3-2. Standard 39 had less than 5% cross-hybridisation with any of the other standards on the master filter (Figure 3-2). Standard 39 is the only one that had *Microbacterium* sp. as the nearest homologue according to its 16SrRNA sequence. Other standards showed higher levels of cross-hybridisation because their DNA interacted with DNAs of standards that were of the same genus. Standard 46, for example, cross-hybridised between 10 and 20% with DNA from other *Pseudomonas* spp. (Figure 3-2). However, cross-hybridisation with DNA from standards that are not *Pseudomonas* spp. was below 5%. Higher cross-hybridisation between standards belonging to the same genus is to be expected. Assuming that bacterial strains must have >70% cross-hybridisation to be considered the same species (Wayne *et al*, 1987), it follows that DNAs from organisms of the same genus but belonging to different species can still cross-hybridise up to 69%. Standards included all strains which cross-hybridised ≥ 70%. Therefore, all strains constituting a standard represent the same species. This implies that not all strains

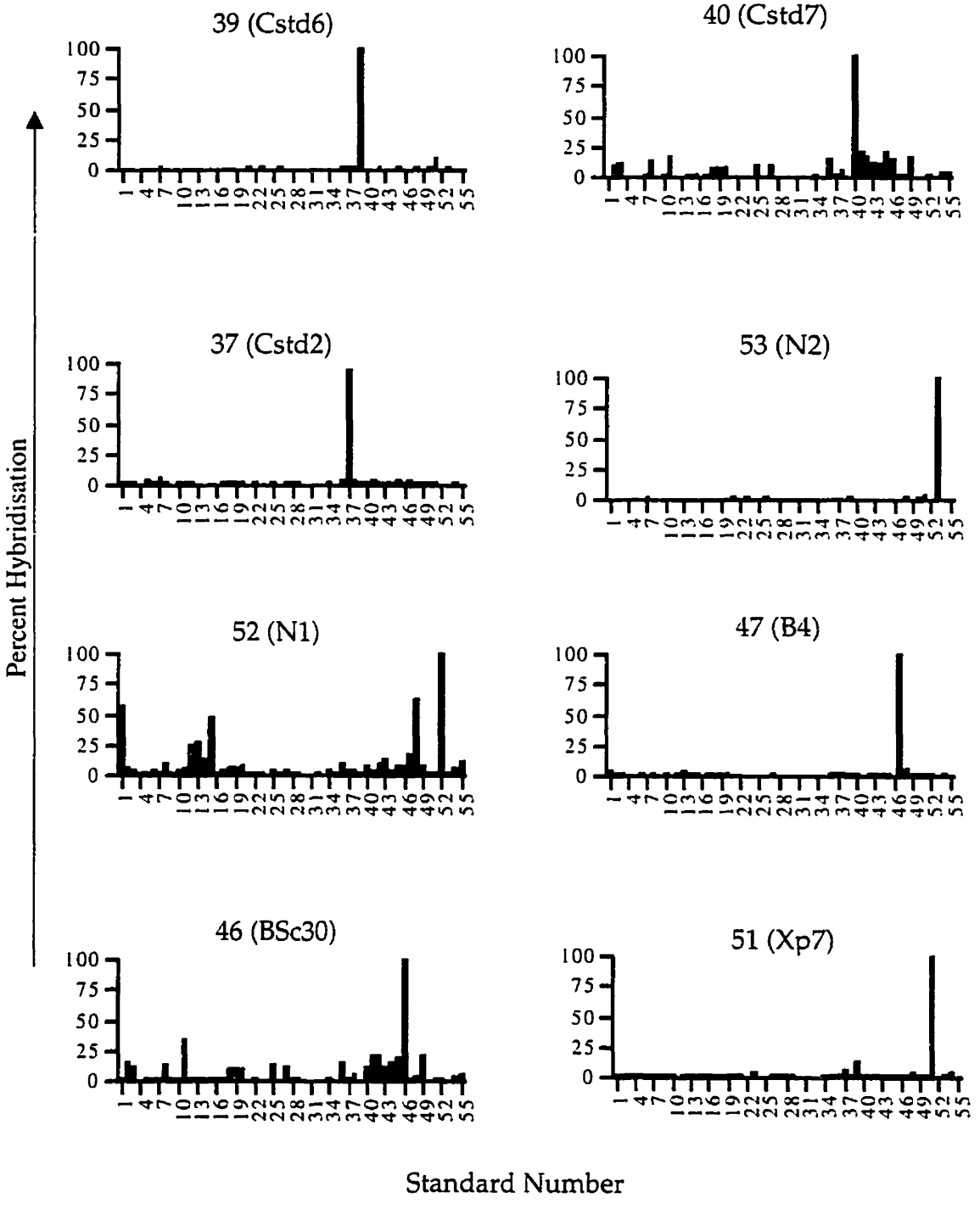


Figure 3-2. Cross-hybridisation between selected master filter standards with the other standards on the master filter. In each case, the relative hybridisation of the tested standard DNA to the other standard DNAs on the filter was calculated and was referred to as cross-hybridisation. It is expressed as a percentage of the hybridisation of the tested standard DNA to itself, which was set at 100%. The numbers are the position of the standard on the master filter (Tables 3-1 and 3-1).

belonging to a given species need to be represented on the master filter. If a particular strain is lacking, an increase in its f_x value can still be monitored by RSGP, provided that another strain with >70% genomic cross-hybridisation is present on the filter. Because strains within a species are highly genetically similar (Brock and Madigan, 1991 and Wayne *et al*, 1987), an increase in f_x would be due to an increase of an organism, or a collection of organisms, with a nearly identical genotype of the strain isolated and characterised.

When RSGP is performed on a synthetic consortium in which every bacterium is known, then the cross-hybridisation data can be used to correct the f_x values obtained (Shen, 1997). Unfortunately, in soil samples a great number of the bacterial species are not easily cultured by traditional methods (Torsvik *et al*, 1990). For this reason, the degree of cross-hybridisation between standard DNAs on the master filter were generally selected to be <20% so cross-hybridisation would interfere minimally with the interpretation of RSGP results. In one case, standard 52 (Table 3-1: *Alcaligenes* sp. N1), some cross-hybridisations were relatively high (Figure 3-2) especially with standard 48 (Table 3-1: *Alcaligenes* sp. X1), with which standard 52 cross-hybridised ~60%. Peculiarly, standard 52 also cross-hybridised strongly with standards 1 and 15 (Figure 3-2), both *Bordetella* spp. (Table 3-1). These high amounts of cross-hybridisation have to be kept in mind when interpreting RSGP results.

The k_λ/k_x values determined for the new standards are indicated in Table 3-3. The average k_λ/k_x values for all the standards on the master filter are shown in Table 3-2. These have a large range, from 17 to 574. As explained in Section 1.3.2.2, genome complexity is the major reason for these differences. As can be

Table 3-3. k_λ/k_x determinations and lengths (L) of assembled 16S rRNA gene sequences for standards 14, 17, and 36-55.

Standard	kλ/kx #1	kλ/kx #2	kλ/kx ave	σ[1]	L (bp)	Position[2]
14	nd[3]	nd[3]	149	36	1150	27-1177
17	nd[3]	nd[3]	249	14	1120	27-1137
36	54	58	56	2	nd[4]	n/a
37	229	282	255	27	397	356-752
38	280	171	226	54	nd[4]	n/a
39	170	84	127	43	nd[4]	n/a
40	111	74	92	19	nd[4]	n/a
41	98	142	120	22	nd[4]	n/a
42	25	10	17	6	406	356-762
43	70	156	113	55	393	356-749
44	92	50	71	12	394	356-750
45	89	73	81	7	268	356-624
46	56	52	54	2	180	356-536
47	114	69	91	12	427	356-783
48	115	33	74	37	280	356-636
49	111	29	70	36	390	356-746
50	94	89	91	3	341	356-697
51	68	26	47	21	345	356-701
52	148	288	218	70	380	356-736
53	117	85	101	16	288	356-644
54	97	148	123	26	365	356-721
55	217	162	189	28	156	356-512

[1]Average deviation between duplicate measurements of k_λ/k_x.
[2]Part of 16S rRNA sequence represented based on *E. coli* numbering
[3]Determined by Shen (1997)
[4]Determined by Shen *et al* (1998)

seen in Table 3-2, the k_λ/k_x values for standards which have 16S rRNA homologues of the same genus have similar k_λ/k_x values. For example, the k_λ/k_x values for the standards that have *Pseudomonas* spp. as the nearest RDP homologues ranged from 50-150, for *Bacillus* spp. homologues k_λ/k_x were all under 100 and for *Bordetella* spp. homologues k_λ/k_x ranged from 150 to 300. This suggests that the genomes of these groups have a similar complexity. This is expected as a genus is a collection of species with similar genomes (Brock and Madigan, 1991).

3.1.2.2 Identification of New Standards by 16S rRNA Sequence

The new standards 36-55, as well as standards 14 and 17 that were not previously identified, were subjected to partial 16S rRNA gene sequencing as explained in Section 2.2.3. The lengths of assembled sequences (L) obtained are listed in Table 3-3. These were used to search the RDP database. The closest homologues found in the RDP database, and the similarity scores (S_{ab}) calculated by the program SIMILARITY_RANK (Maidak *et al*, 1994), are given in Table 3-1. The range of S_{ab} values was between 0.337 and 1.00 (a perfect match). The lower values may not allow accurate classification at the genus level. The reason that some of the sequence matches are poor is likely that the number of determined sequences in the RDP database is still quite limited. Less than 1% of the species in soils have been isolated and not all isolated strains have had their 16S rRNA genes sequenced (Wayne *et al*, 1987 and Maidak *et al*, 2000). Some of the low S_{ab} values, in the range of .300, may have also been the result of poorly determined DNA sequences from the 16S rRNA genes.

Partial 16S rRNA sequences were used to search against the RDP database for 53 of the 55 standards. Standards 14 and 17 (LQ27 and LQ33) had 70% of their 16S

rRNA gene sequences determined, for the others it was a smaller fraction (Table 3-3). It is possible that there are dissimilarities in segments of the 16S rRNA gene that were not sequenced. A standard and its nearest RDP homologue are probably highly related when good similarity coefficients (S_{ab} > 0.8) were obtained. Increasing 16S rRNA sequence homology between two strains also generally correlates with increasing genomic DNA-DNA hybridisation (Ward *et al*, 1998), although there are exceptions (Section 1.3.2.1). The correlation between genomic DNA-DNA hybridisation and 16S rRNA sequence identity can be demonstrated with standard 14, which was classified initially as a *Bordetella* sp. based on its cross-hybridisation with DNA from other *Bordetella* spp. on the master filter (Shen *et al*, 1998). Upon comparing the 16S rRNA sequence of standard 14 to the RDP database, its classification as a *Bordetella* sp. was confirmed (Table 3-1).

3.1.2.3 Growth of Bacterial Standards on Individual C5+ Components

Standards were tested for their ability to grow on the C5+ components benzene, toluene, styrene, xylenes and naphthalene as sole carbon and energy source. Fourty-four of the 55 standards represented on the master filter were tested by streaking liquid cultures in TY on MSM plates and incubating the plates in dessicators with 1% or 10% of the hydrocarbon in vpo. The results are summarised in Table 3-2. Of the 44 standards, 36 grew on one or more C5+ components as a sole carbon and energy source. Benzene was the most commonly used compound (31 of the 36 standards). Toluene was used by 23, styrene by 12, xylenes by 14 and naphthalene by 10 of the 36 standards. Hydrocarbon utilisation appeared to be common in organisms isolated from soils that were previously contaminated with C5+. The list may not be entirely definitive, as the bacteria

were only allowed two weeks to grow before the plates were removed from the hydrocarbon atmosphere. Some standards may require a longer acclimation period. In addition, hydrocarbon degradation genes are often on plasmids (van der Meer *et al*, 1992). Most of the earlier isolated standards were isolated and maintained on rich medium. Under these conditions, it is possible that the plasmids carrying the genes to degrade hydrocarbons were lost (Stuart-Keil *et al*, 1998). Despite these uncertainties, this list gives a general idea of the degradative abilities of the standards represented on the master filter, and can help with interpreting RSGP profiles. All of the *Alcaligenes* spp. standards tested were able to grow on hydrocarbons (Table 3-1 and 3-2). The most common genus on the master filter is *Pseudomonas*, and nearly all of the tested *Pseudomonas* spp. were able to grow on hydrocarbons, with many able to grow on many hydrocarbons (Tables 3-1 and 3-2). The standards that are *Bordetella* spp. were generally unable to grow on hydrocarbons (Tables 3-1 and 3-2).

It is interesting to see that of the standards able to grow using a hydrocarbon, the majority (31/36) were able to grow on benzene. Since benzene is the most predominant hydrocarbon in C5+ (45%, Section 1.1), it appears that microorganisms in contaminated soils are adapted to use this most prevalent hydrocarbon. Of the other C5+ hydrocarbons usable for growth a similar trend can be seen. Toluene, styrene and naphthalene, present in decreasing concentrations in C5+ (Section 1.1), are used by master filter organisms in the same order of frequency. The exception was with xylenes, of which 14 standards could use for growth, yet xylenes make up less than 1% of C5+ (Stehmeier *et al*, 1999).

3.2 Analysis of the Effects of Hydrocarbons on Soil Microbial Communities

3.2.1 DNA Extraction

Soils were incubated in dessicators with mixtures of hydrocarbons in vpo. RSGP was used to monitor the effects of hydrocarbons on soil microbial communities. An example of crude DNA samples extracted from incubated soils and analysed by agarose gel electrophoresis is shown in Figure 3-3. The gel shows that RNA and humic acids were also present. As the humic acids interfere with processes required for RSGP (Section 2.2.4), they were removed. Figure 3-4 shows the results of DNA purification with a Sepharose 4B column. Chromosomal DNA passes through the column first. Humic acids elute after the nucleic acids. The first two or three fractions were typically collected to obtain a purified DNA preparation free of humic acid interference.

3.2.2 Determination of Baseline RSGP Profile

DNA extractions of soils without incubation did not give high enough yields for RSGP analysis (Figure 3-5: gel A). For this reason, two week incubations in MSM in dessicators containing only vpo were used to give baseline RSGP profiles. Reasonable amounts of DNA for RSGP analysis were obtained following incubation in MSM in most, but not all, cases (Figure 3-5: gel B). Under these conditions, the only carbon sources available as a substrate for growth are those already in the soil, since vpo is unavailable due to low vapour pressure (Section 2.2.1). The growth that occurred during these incubations was due to the increased availability of water and nutrients (nitrate, phosphate) during the incubations.

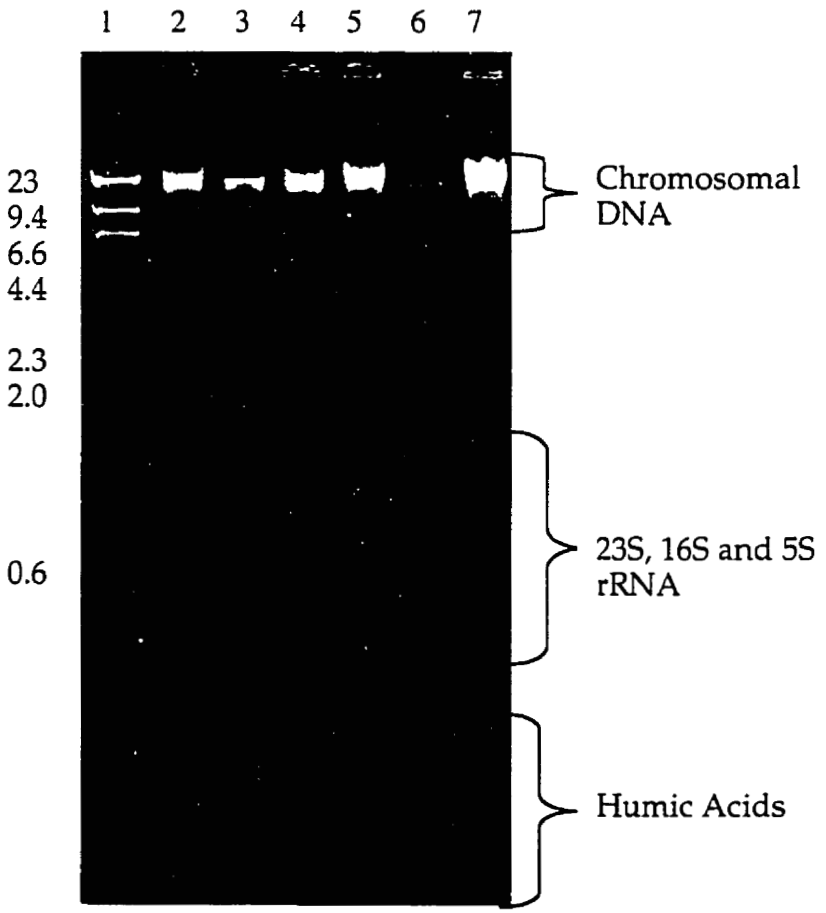


Figure 3-3. Agarose gel of DNA obtained from the soil DNA extraction protocol. Lane 1 contains 500 ng HindIII digested λ-DNA; sizes in kilobase pairs (kbp) are on left. Lanes 2-7 (soils A1, A3, C1, 320, 330 and 350, respectively) are crude DNAs extracted from soils incubated for 2 weeks in C5+ then 9 weeks in DCPD plus naphthalene. Note that rRNA and humic acids were co-extracted with DNA.

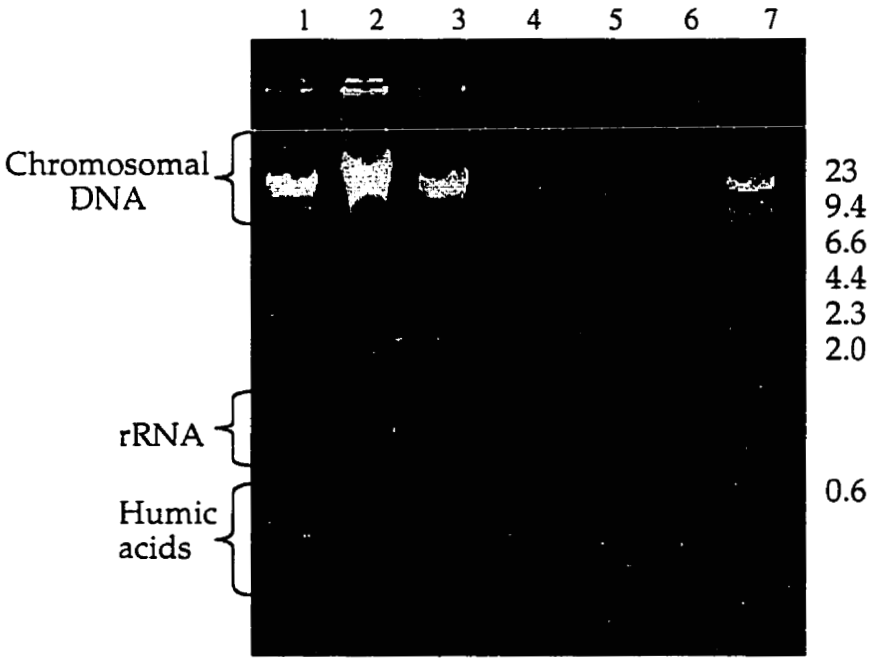


Figure 3-4. Agarose gel of Sepharose 4B column fractions. Lanes 1-6 contain fractions 1-6, respectively, from column purification of DNA extracted from soil. Lane 7 contains 500 ng HindIII digested λ-DNA; sizes in kbp are on the right. DNA was obtained from soil A3 after incubation for 4 weeks in C5+.

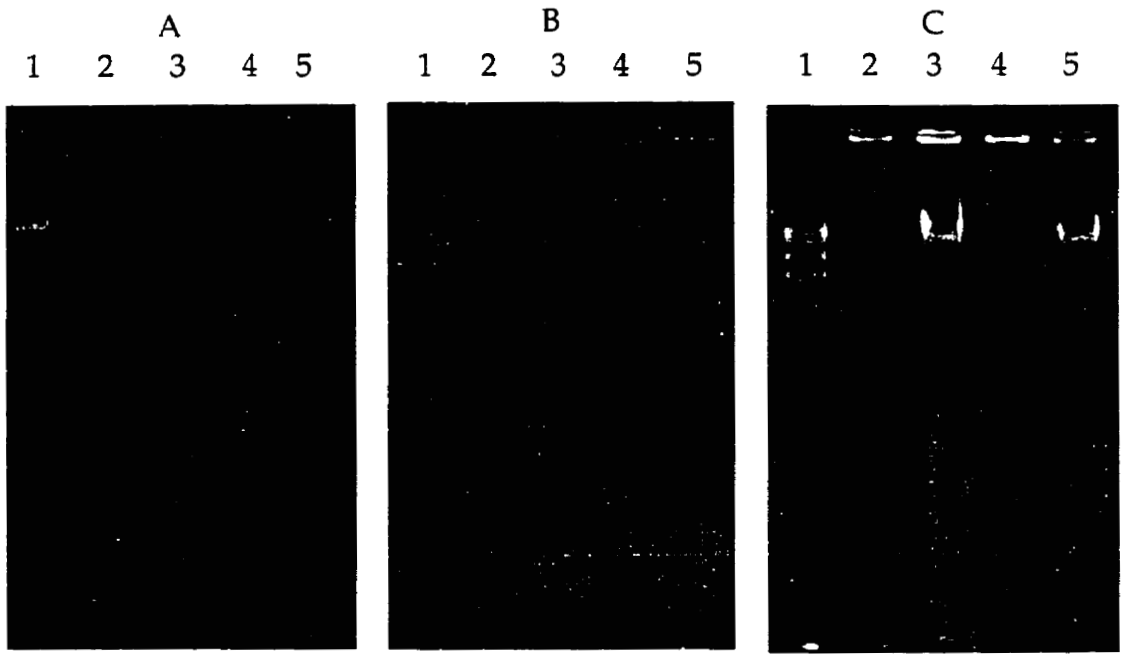


Figure 3-5. Agarose gels of products of DNA extractions from soils. Soils A1, A3, C3 and E3 (lanes 2, 3, 4 and 5, respectively) were not incubated (A), incubated in MSM for two weeks (B) and incubated in MSM with 1% C5+ in vpo for two weeks (C). The amount of DNA extracted from C is greater than B, which is greater than A. Lane 1 is λ-Hind III digest marker.

Some RSGP profiles from these incubations are shown in Figure 3-6. The community distribution was rather even for most soils. All standards were present at f_x values of 5% or lower. Higher f_x values were only seen when a C5+ component was also added as a carbon source. One exception is seen with the vpo RSGP profile of soil 320 (Figure 3-6). It shows that standard 27 (*Pseudomonas* sp. Q7) was present at an f_x=14%, suggesting that it made up a large fraction of the community in soil 320 before incubation with hydrocarbons. Standard 27 is able to use benzene, styrene and toluene as sole carbon and energy sources (Table 3-2). Standard 27 did appear during incubation with low concentrations of single hydrocarbons (Figure 3-7: panels 1 and 7), however it did not appear with incubations of hydrocarbons present at 1% or higher. This implies that it can compete effectively under conditions with little or no hydrocarbons, but not under conditions of moderate to high concentrations of hydrocarbons.

When soils were incubated with a hydrocarbon, the amount of DNA that was extracted increased above the amount extracted when the soil was incubated with vpo alone (Figure 3-5: B and C). This implies that the hydrocarbon could be used to support additional growth.

3.2.3 Effects of Incubating Soils with Single C5+ Hydrocarbons on the Microbial Community Composition

Contaminated soils were incubated with single C5+ components (Section 2.3.1.1) for two weeks. Incubations with different concentrations of single C5+ components were performed to determine which of the master filter standards became dominant under these conditions. A summary of results for these incubations is shown in Figure 3-7. In most cases, one or two standards were

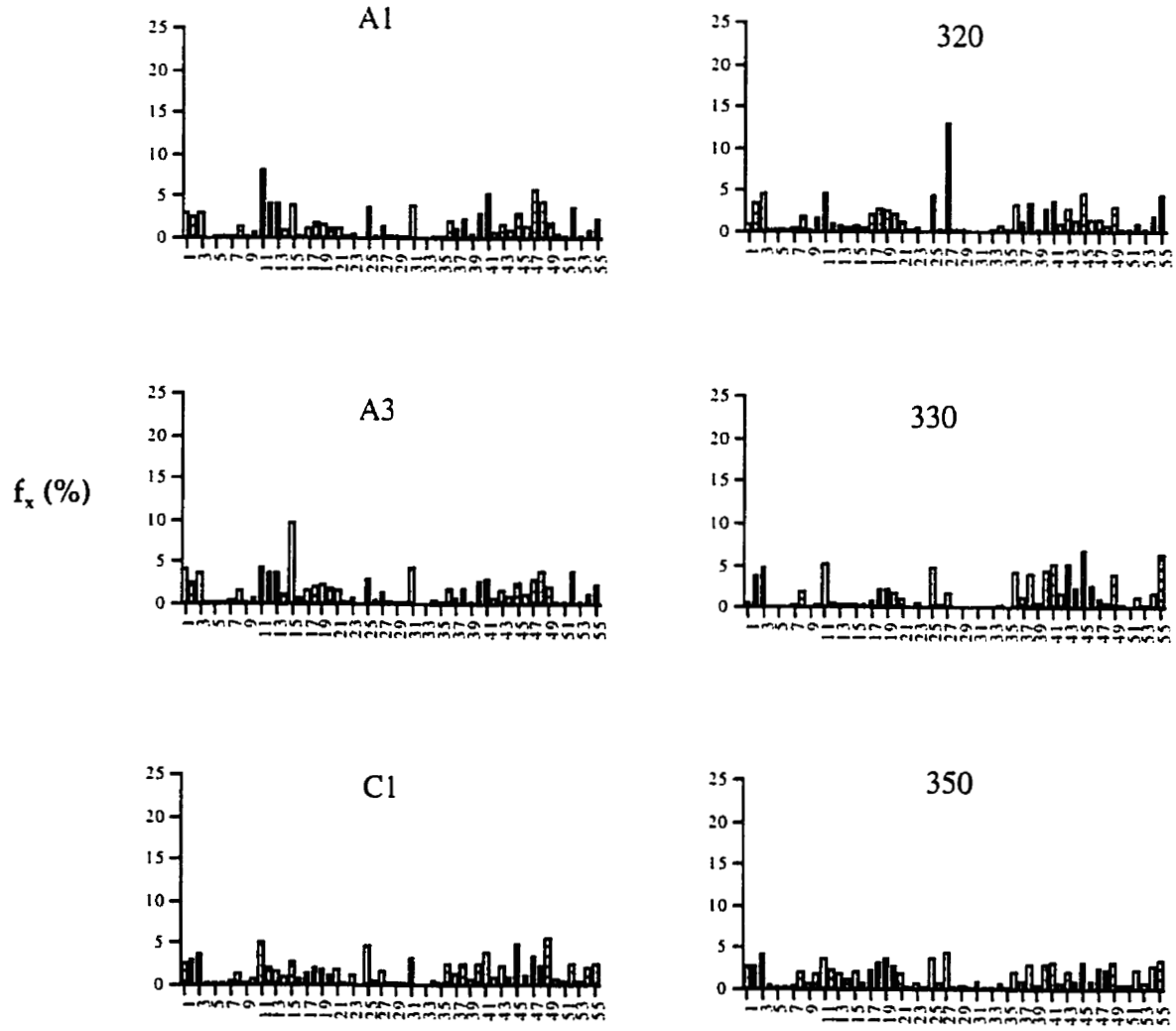


Figure 3-6. RSGP profiles of microbial community obtained from incubations in vpo. Previously uncontaminated (320-350) and previously contaminated (A1-C1) soils were used.

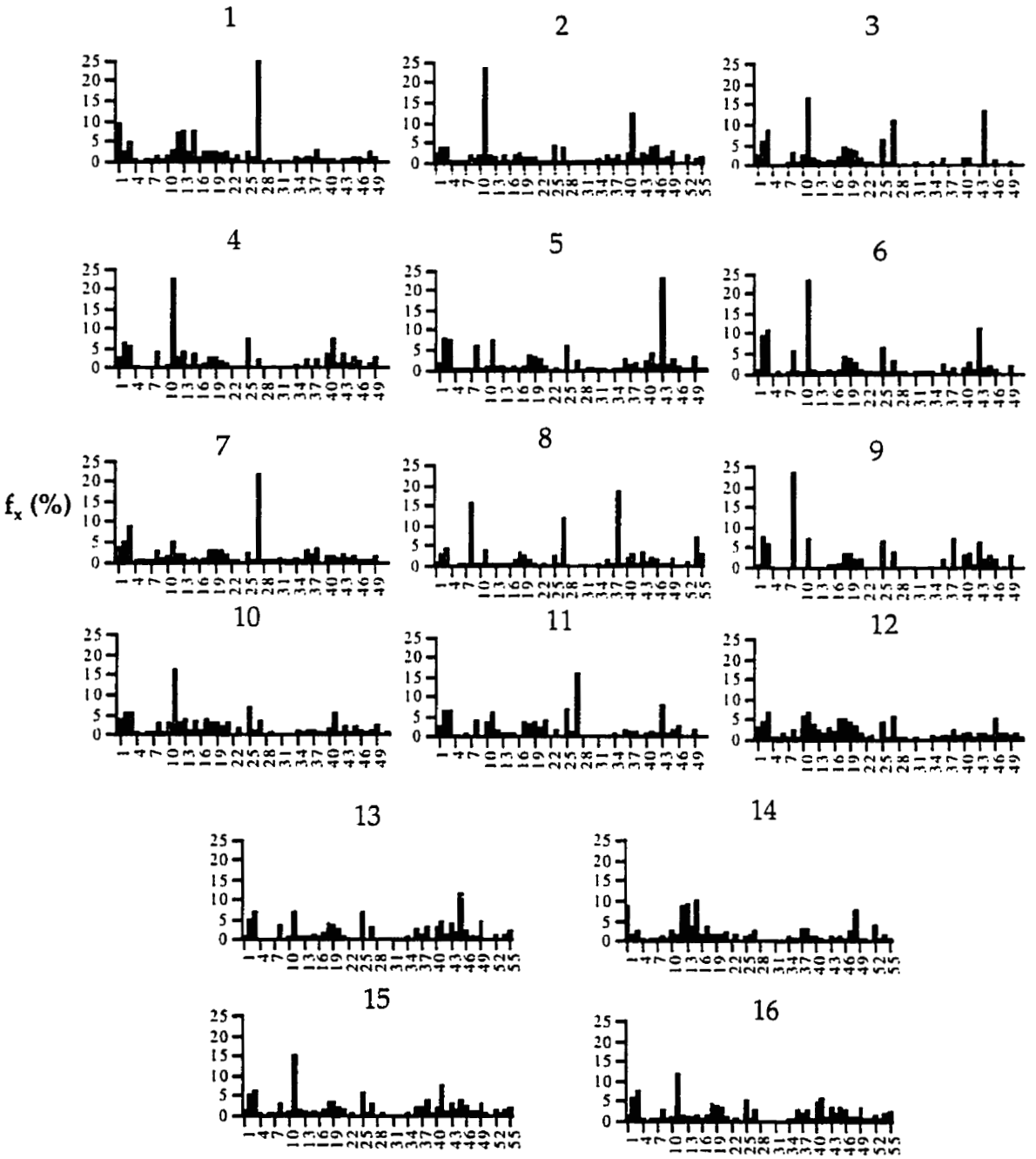


Figure 3-7. RSGP profiles of the microbial community in soils incubated in single C5+ hydrocarbons for two weeks. Profiles 1-3: soil N, 0.1, 1 and 10% benzene; 4-6:soil N, 0.2, 10 and 100% styrene; 7-9: soil S, 0.1, 10 and 100% toluene; 10-12: soil N, 0.2, 10 and 100% xylenes; 13-14: soil E1, 5 and 100% naphthalene; 15-16: soil C1, 5 and 100% naphthalene.

predominant with f_x values of up to 25% when different percentages of each hydrocarbon in vpo were present. The standard that predominates under each of the shown conditions are listed in Table 3-4. It is interesting to note that the same standard can predominate with different hydrocarbons. For example, standard 11 (*Pseudomonas* sp. LQ20) predominated in some of the incubations with benzene, toluene, xylenes and naphthalene, but not in incubations with styrene. Standard 11 (*Pseudomonas* sp. LQ20) is able to degrade benzene, toluene and xylenes (Table 3-2). Its predominance in some of the naphthalene incubations suggests that standard 11 (*Pseudomonas* sp. LQ20) can use some by-products of naphthalene degradation, even if it is not the primary degrader.

A second round of single hydrocarbon incubations was performed, with separate incubations of benzene, toluene, styrene, naphthalene, xylenes or DCPD (all 1% (w/w) in vpo). In this set of experiments, RSGP profiles were monitored over a longer period. Some of the resulting RSGP profiles are shown in Figure 3-8. The results showed two types of responses. The first type is shown by the benzene and naphthalene incubations in Figure 3-8. In this response, one or two standards were predominant with f_x values well above 8% for the entire period of incubation (naphthalene) or for two and four weeks with a more even distribution at 12 weeks (benzene). In the second type of response, illustrated by the DCPD incubation in Figure 3-8, different predominant standards were observed for each of the time points investigated. One possible reason for the differences in time dependence is that in the first case (naphthalene and benzene) the hydrocarbon is readily degradable, setting up a stable food web in which certain organisms predominate. In the case of DCPD the hydrocarbon is not readily degradable, giving shifts in

Table 3-4. Predominant standards after 2 weeks of incubation in various concentrations of individual C5+ hydrocarbons.

Hydrocarbon	Predominant standard
0.1% benzene	27 (*Pseudomonas* sp. Q7)
1% benzene	11 (*Pseudomonas* sp. LQ20)
10% benzene	11(*Pseudomonas* sp. LQ20)
	44 (*Pseudomonas* sp. S3)
0.2% styrene	11(*Pseudomonas* sp. LQ20)
10% styrene	43 (*Pseudomonas* sp. S2)
100% styrene	11(*Pseudomonas* sp. LQ20)
0.1% toluene	27 (*Pseudomonas* sp. Q7)
10% toluene	38 (*Pseudomonas* sp. Cstd5)
	8 (*Pseudomonas* sp. LQ16)
100% toluene	8 (*Pseudomonas* sp. LQ16)
0.2% xylenes	11(*Pseudomonas* sp. LQ20)
10% xylenes	27 (*Pseudomonas* sp. Q7)
100% xylenes	none
5% naphthalene	11(*Pseudomonas* sp. LQ20)
	45 (*Pseudomonas* sp. S6)
100% naphthalene	11(*Pseudomonas* sp. LQ20)
	15 (*Bordetella* sp. LQ29)

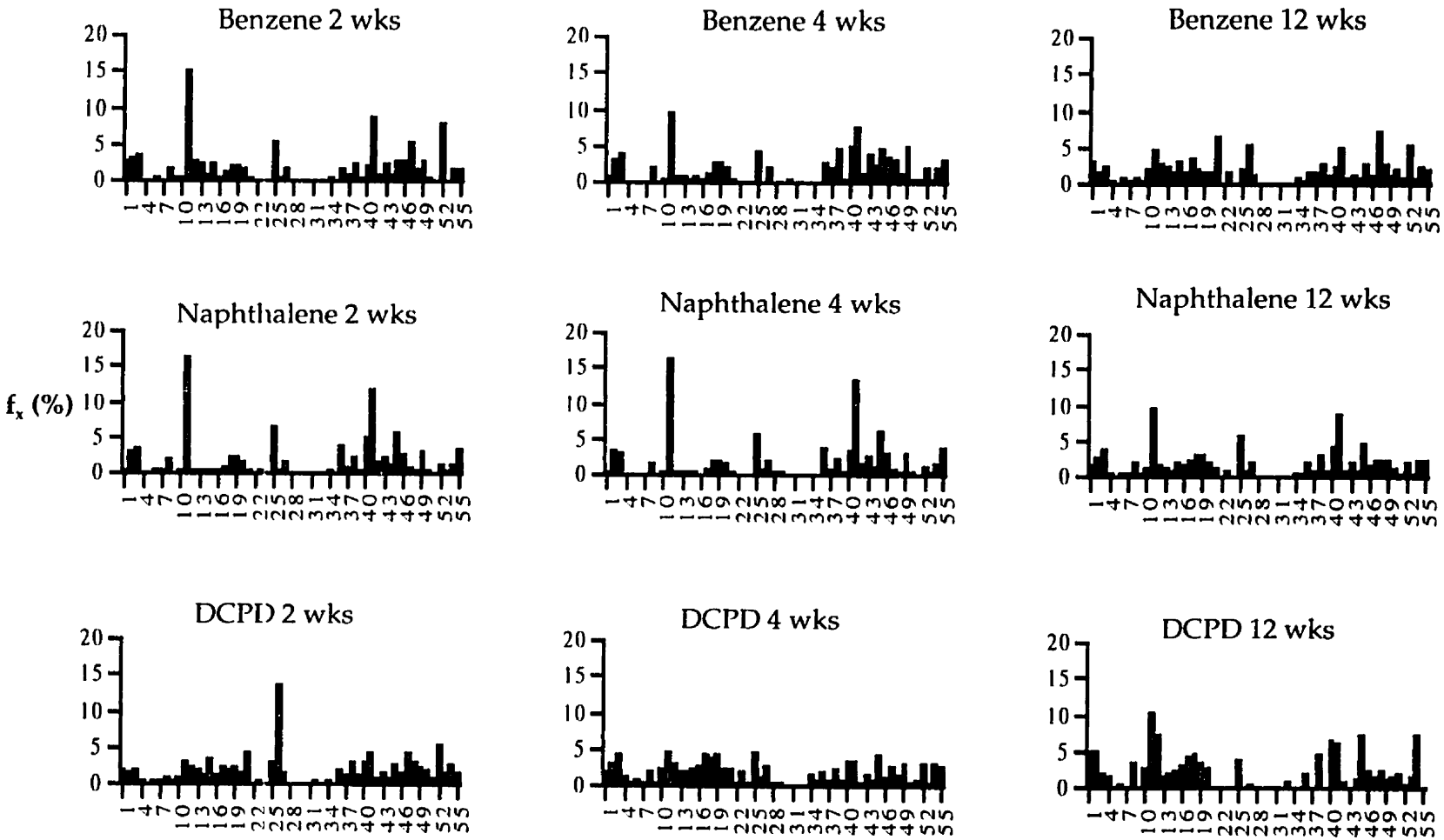


Figure 3-8. RSGP profiles of the microbial community obtained from incubation of soils in single C5+ components for 2, 4 and 12 weeks. Results shown are for soils A1 (benzene), 330 (naphthalene) and C3 (DCPD).

community composition as degradable carbon sources present in the soil become exhausted. It was seen that incubations for similar lengths of time under similar conditions (2 weeks) gave similar community profiles. For example, compare Figure 3-7, panel 2 and Figure 3-8 benzene, 2 weeks; Figure 3-7, panel 15 and Figure 3-8 naphthalene 2 weeks.

3.2.4 Effects of Incubation with C5+ on Microbial Community Composition

All soil incubations were performed in dessicators containing 1% of a synthetic C5+ mixture in vpo. Three uncontaminated soils (320, 330 and 350) and three contaminated soils (A1, A3 and C1) were incubated for different periods of time. Some of the resulting RSGP profiles obtained are shown in Figure 3-9. RSGP for incubations without C5+ for the same soils are shown in Figure 3-6 (Section 3.2.2).

The RSGP profiles for the C5+ incubations (Figure 3-9) show some trends. The profiles after 2 weeks of incubation show increases in one or two standards to above 8% for both uncontaminated and contaminated soils. Standards 11 (*Pseudomonas* sp. LQ20) in soils 350, A1, A3 and C1, standard 41 (*Pseudomonas* sp. Cstd8) in soils A1, A3 and C1, standard 25 (*Pseudomonas* sp. Q5) in soils 320 and C1, and standard 36 (*Pseudomonas* sp. Cstd1) in soil 320 all showed increases. Five out of the six profiles after 4 weeks of incubation showed significant shifts. The community from soil A1 was dominated by standard 47 (*Alcaligenes* sp. B4) at 4 weeks; standard 41 (*Pseudomonas* sp. Cstd8) also still had an f_x value greater than 8%. In soil 320, standard 25 (*Pseudomonas* sp. Q5) had an increase in f_x value from 9% to 13% at 4 weeks. In soil 330, enough DNA became available to perform RSGP; standards LQ20 and Q5 were predominant with f_x values of 10%. Three

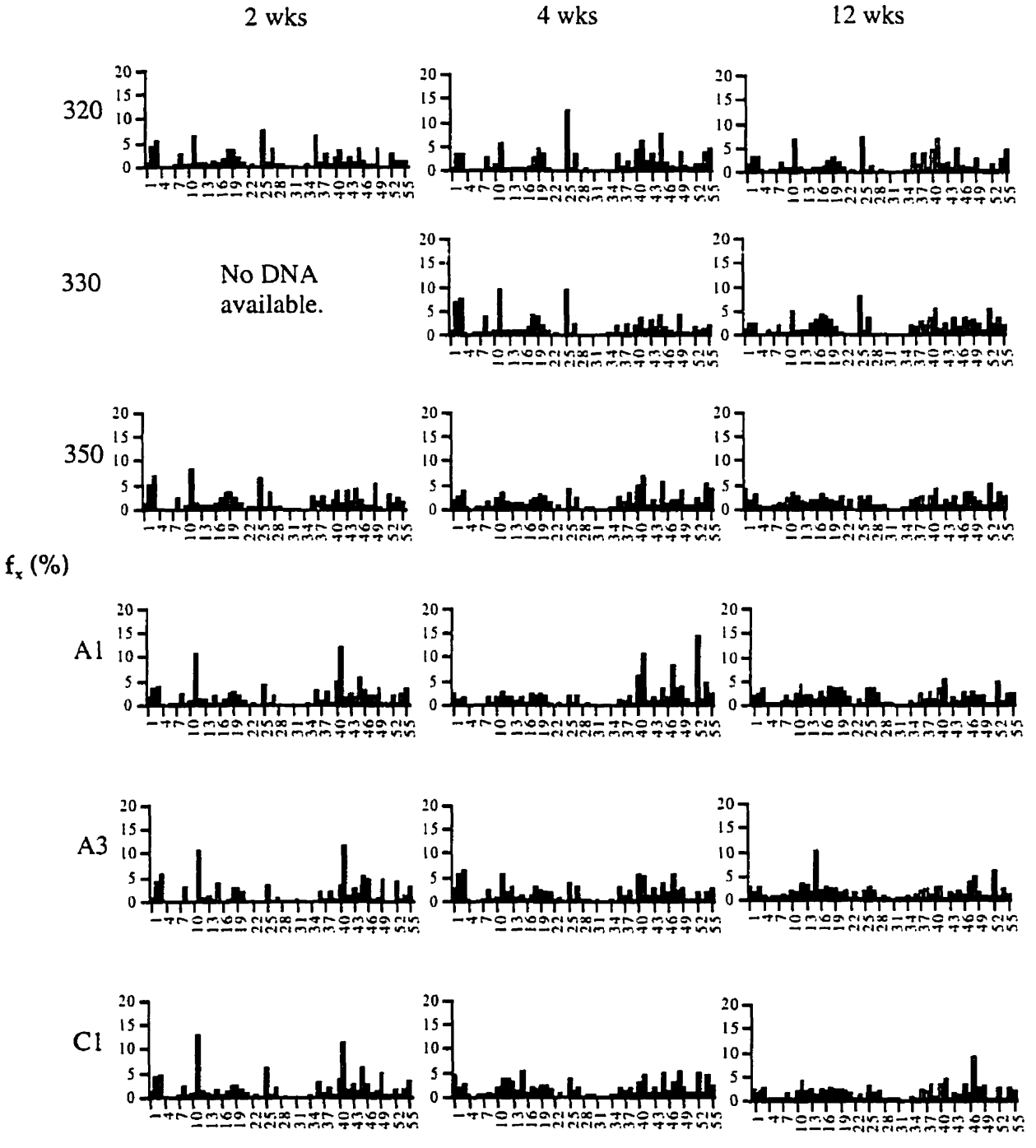


Figure 3-9. RSGP profiles of the microbial community obtained from soils (320, 330, 350, A1, A3 and C1) incubated in 1% C5+ for 2, 4 and 12 weeks.

other soils (350, A3 and C1) returned to a more even community composition at 4 weeks. After 12 weeks of incubation, most soils had a more evenly distributed community than at 2 weeks, especially 350 and A1.

3.2.5 Incubation of Soils with C5+ Followed by Incubation with DCPD Only

After two and four weeks of C5+ incubation, some soils were moved to dessicators with 1% DCPD only in order to emulate *in situ* conditions, where eventually only DCPD remains (Figure 1-7). The effect of switching to DCPD only on the soil community composition is seen in a dendrogram comparing the RSGP profiles of C5+ incubations with those for cultures switched to DCPD only (Figure 3-10). Four clusters are indicated in the dendrogram based upon the predominant standards in each RSGP profile (Figure 3-10). Cluster I contains RSGP profiles in which standard 25 (*Pseudomonas* sp. Q5) became dominant and was present with an f_x of greater than 10%. In the cluster II profiles standard 11 (*Pseudomonas* sp. LQ20), and sometimes standard 41 (*Pseudomonas* sp. Cstd8), were dominant with f_x values above 10%. In cluster III, standard 25 (*Pseudomonas* sp. Q5) and either standard 11 (*Pseudomonas* sp. LQ20), standard 41 (*Pseudomonas* sp. Cstd8), or both were major community components, but with f_x values below 10%. Cluster IV contains RSGP profiles in which the community was evenly distributed, with no clear standard dominating. The profiles in the dendrogram (Figure 3-10) which are not part of a cluster each had at least one dominant standard (f_x above 8%), which was neither standard 11 (*Pseudomonas* sp. LQ20) nor standard 25 (*Pseudomonas* sp. Q5).

In a few instances, certain standards became predominant during DCPD incubations: standard 47 (Alcaligenes sp. B4) in soil 330, standard 52 (Alcaligenes

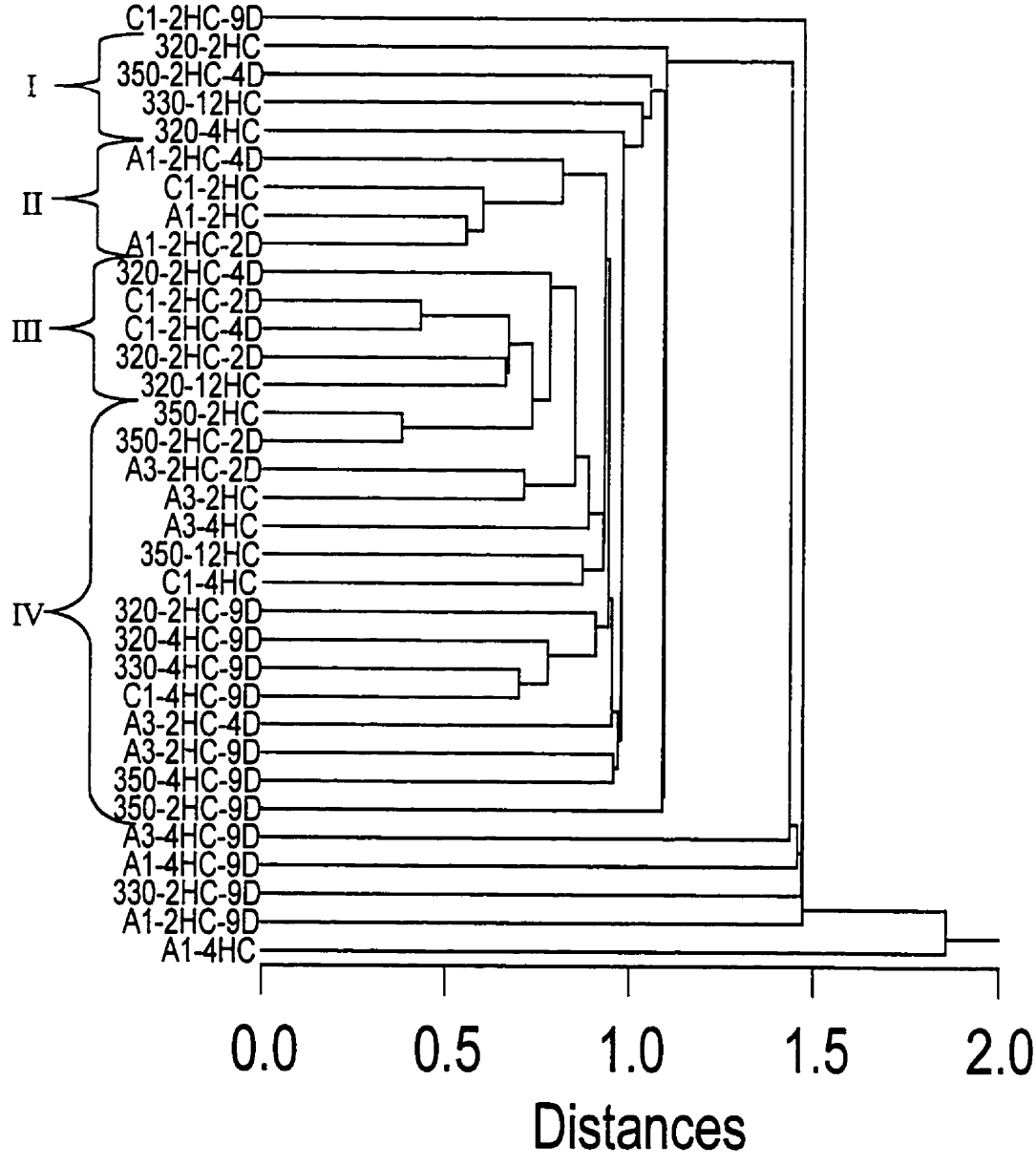


Figure 3-10. Dendrogram of RSGP profiles of microbial communities obtained from soils incubated in C5+ for 2, 4 or 12 weeks (2HC, 4HC or 12HC), and in C5+ for 2 or 4 weeks before transfer to DCPD for 2, 4 or 9 weeks (2D, 4D or 9D). I-IV indicate clusters of RSGP profiles that have similar characteristics.

sp. N1) in soil A1, standard 54 (Pseudomonas sp. N3) in soil C1 and standard 37 (*Pseudomonas* sp. Cstd2) in soils A1 and A3. There was no common denominator between the profiles that showed a predominant standard after 9 weeks of DCPD incubation. Standards 52 (*Alcaligenes* sp. N1) and 54 (*Pseudomonas* sp. N3) were able to grow on naphthalene (Table 3-2). Standard 25 (*Pseudomonas* sp. Q5), which also grows on naphthalene has been implicated in DCPD oxidation via co-metabolism (Shen *et al*, 1998). Standard 47 (*Alcaligenes* sp. B4) can degrade benzene, toluene, styrene and xylenes, but not naphthalene and standard 37 (*Pseudomonas* sp. Cstd2) can degrade benzene and toluene. Thus, there is some overall support for the notion that dominant standards may be able to degrade DCPD with naphthalene-degrading enzymes.

In order to further investigate the possibility of DCPD co-metabolism with naphthalene, soils were incubated with C5+ for 2 or 4 weeks and then transferred to dessicators with 1% naphthalene plus 1% DCPD. The same soil samples were used in this experiment as were used in the transfer to DCPD only experiment. The RSGP profiles are compared in a dendrogram (Figure 3-11). Cluster I has profiles with even community distributions. Cluster II has as the major community members. Clusters III and V have standard 25 (*Pseudomonas* sp. Q5) as the major community member. In cluster III it has an f_x value ≤10%, but in cluster V it has an f_x value >10%. Cluster IV has no predominant standard, but there are a number of *Pseudomonas* spp. standards present, with few other standards present. Microbial community compositions of soils, which were incubated for 2 or 4 weeks in C5+ and then for 9 weeks in DCPD plus naphthalene, did not cluster together. Only two out of twelve community profiles obtained after 9 weeks of incubation in

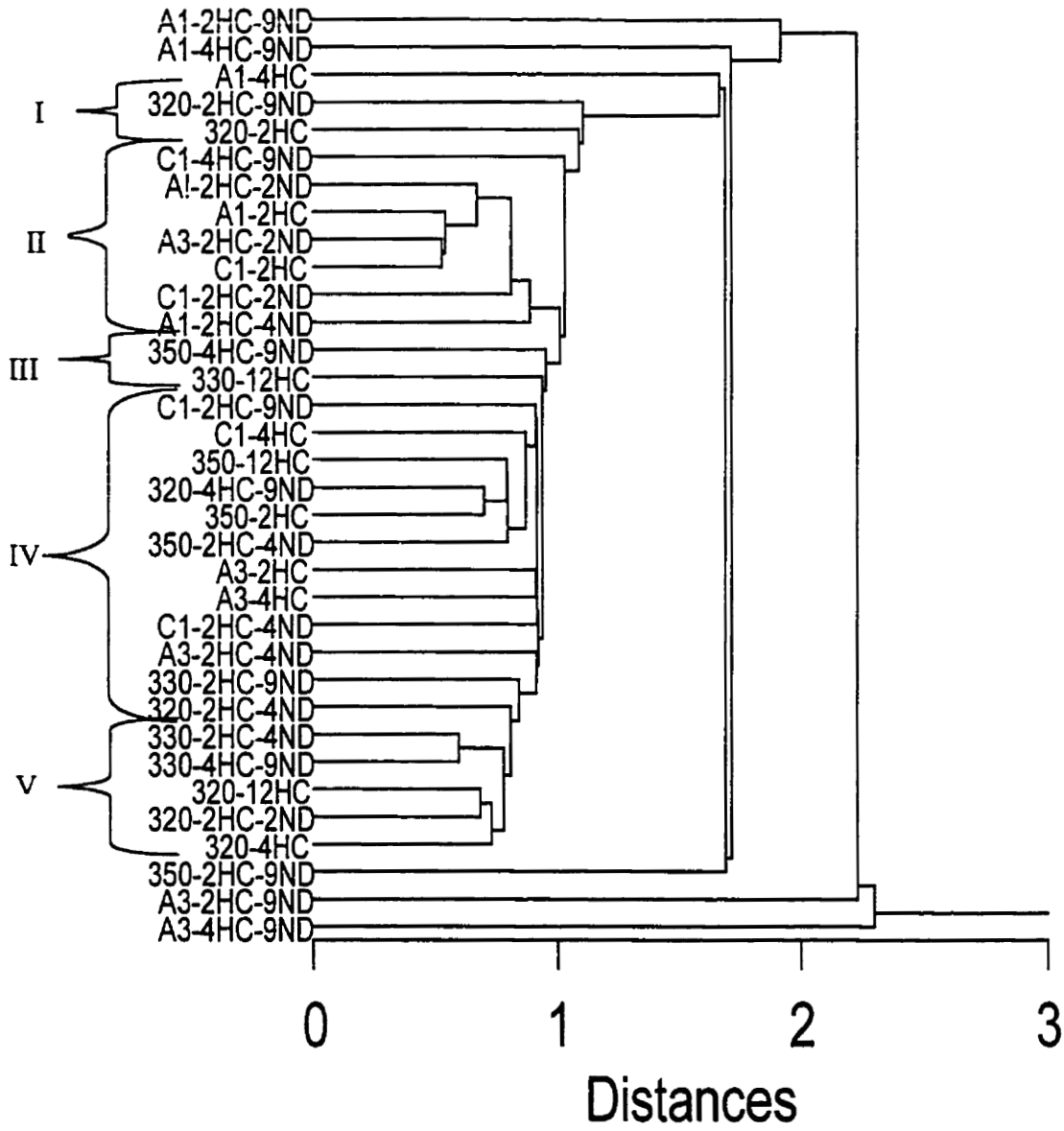


Figure 3-11. Dendrogram of soils incubated in C5+ for 2, 4 or 12 weeks (2HC, 4HC or 12HC), and in C5+ for 2 or 4 weeks before transfer to DCPD plus naphthalene for 2, 4 or 9 weeks (2ND, 4ND or 9ND). I-V indicate clusters of RSGP profiles which have similar characteristics.

DCPD plus naphthalene showed standard 11 (*Pseudomonas* sp. Q5) as a significant community component (Figure 3-12: 330-4HC-9ND and 350-4HC-9ND), which tree in Figure 3-11 in clusters III and IV, respectively. Four of the twelve soils incubated for 9 weeks had no dominant community member, although the community was made up mainly of *Pseudomonas* spp. (Figure 3-11; cluster IV). In the communities from five of the twelve soils incubated for 9 weeks in DCPD plus naphthalene, a single standard other than standard 25 (*Pseudomonas* sp. Q5) was dominant. They were A1-2HC-9ND, A1-4HC-9ND, 350-2HC-9ND, A3-2HC-9ND and A3-4HC-9ND (Figure 3-11 and Figure 3-12). All of the standards that became predominant in these profiles were from a different genus. Standards 47 (*Alcaligenes* sp. B4) and 52 (*Alcaligenes* sp. N1) were major community members in some of the incubations in DCPD only at 9 weeks as well (Section 3.2.4). This suggests that these standards may have been capable of utilising DCPD in some way. The majority of the RSGP profiles for 9 week incubations in DCPD plus naphthalene, which showed no predominant community members, were in cluster IV (Figure 3-11). In this cluster, even though there were no predominant standards, there was a tendency for only *Pseudomonas* spp. to appear, suggesting that *Pseudomonas* spp. in general were more likely to be able to flourish in the presence of DCPD and naphthalene.

3.2.6 Discussion

Although the number of standards present on the master filter (55) was small relative to the number of species thought to be present in the average soil sample (4 000 per g; Torsvik *et al*, 1990), significant responses of the soil microbial community to incubation with hydrocarbons could be registered. Interestingly,

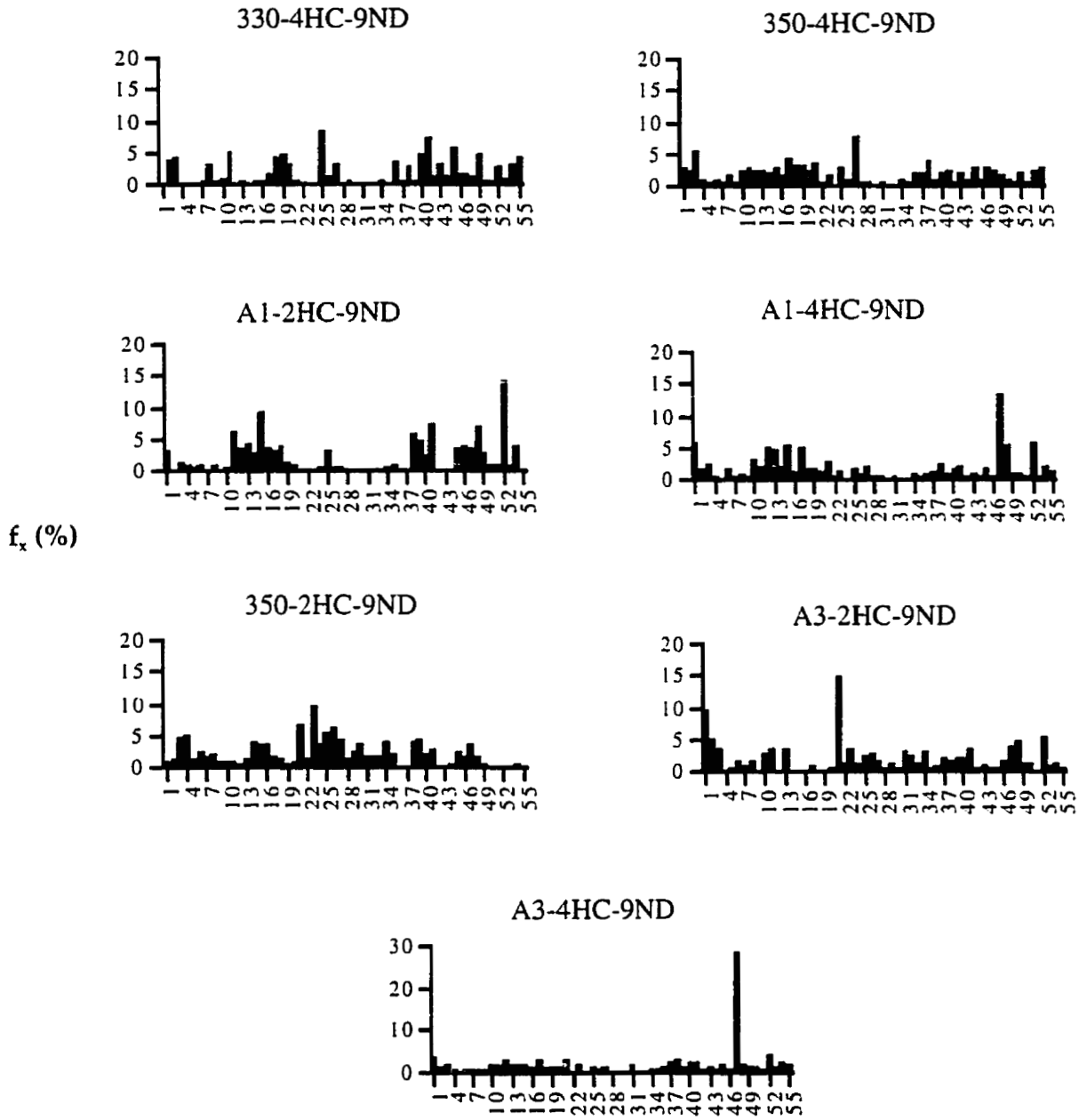


Figure 3-12. RSGP profiles of some soils incubated in C5+ for a period of 2 or 4 weeks (2HC or 4HC) and then switched to naphthalene plus DCPD for 9 weeks (9ND).

in nearly every soil that was tested, at least one standard became predominant during 2 weeks of incubation with a single C5+ component, with the exception of DCPD. The dominant community member often increased from $f_x < 5\%$ to $f_x = 10$-15%, and in some cases up to $f_x = 25\%$ (Figure 3-6). These increases in f_x value indicate high metabolic activity of the dominant standard.

Most of the standards that were predominant when soils were incubated with single hydrocarbons were able to grow on those hydrocarbons as a sole carbon and energy source (Table 3-2), although there were some exceptions. For example, standard 11 (*Pseudomonas* sp. LQ20) became predominant in soil C1 at naphthalene concentrations of 5% and 100% (Figure 3-7: panels 15 and 16). Standard 11 (*Pseudomonas* sp. LQ20) grew on benzene, toluene and xylenes, but did not grow on naphthalene during plate growth tests (Table 3-2). Assuming that the plate method (Section 3.1.2.3) for correctly determined the C5+ substrate specificity of standard 11 (*Pseudomonas* sp. LQ20) this must mean either that other organisms in the community must initiate degradation of naphthalene with excretion of degradation products that can be used by standard 11 (*Pseudomonas* sp. LQ20), or that the microorganism detected in the soil community was highly related to standard 11 (*Pseudomonas* sp. LQ20) but had catabolic genes imparting the ability to use naphthalene.

It is of interest to note that despite the fact that 36 genomically distinct hydrocarbon degraders have been isolated in this work, the response of the soil microbial community to incubation with either single hydrocarbons (Figure 3-7: benzene, panel 2 and 3; toluene, panel 4 and 6; xylenes, panel 10 and naphthalene) or 1% C5+ (Figure 3-9) is so similar with standard 11 (*Pseudomonas* sp. LQ20) being

dominant in 13 of 24 communities that emerged at 2 weeks. A possible explanation is that when C5+ hydrocarbons are introduced (single or mixed), they may inhibit growth of many of the members of the soil microbial community because of their toxicity (Sikkema *et al*, 1995). Then community members may still metabolise C5+ hydrocarbons to oxygenated intermediates that are excreted as part of their detoxification. For example, Langbehn and Steinhart (1995) have shown that when hydrocarbons are oxidised in a soil environment, a number of metabolites are formed. These include aliphatic organic acids, diacids and aromatic ketones. As well, the turnover of cell materials will occur because of cell lysis (Langbehn and Steinhart, 1995). Standard 11 (*Pseudomonas* sp. LQ20) may become predominant because it can withstand C5+ hydrocarbon toxicity better than other hydrocarbon degraders and is thereby able to utilise the catalytic potential (e.g. naphthalene degradation) of other community members. As explained in Section 1.2.1, the degradation of hydrocarbons is generally started by specific enzymes for that compound, but they are then acted upon by a central set of enzymes (van der Meer, *et al*, 1992). Those microorganisms with increased f_x values were not shown to grow on the specific C5+ hydrocarbon compound provided, but were shown to grow on other C5+ compounds, may have used degradation intermediates from organisms that had enzymes to perform initial breakdown of the supplied hydrocarbon.

However, when the incubation was continued past two weeks, other standards adapt (e.g. by changing the composition of their membrane lipids (Section 1.2.3)) and so the RSGP profiles changed to a more even community distribution. This could also involve increased expression of degradative enzymes

(Gibson and Subramanian, 1984), genetic changes allowing increased degradative ability (van der Meer *et al*, 1992), or a combination of all three processes. When a shift in the community occurred during continued incubation (e.g. Figures 3-8 and 3-9: 2, 4 and 12 weeks), the new predominant master filter standard was likely more competitive once it had become adapted to the prevailing conditions. Often multiple standards became more efficient, once adapted, creating a more even community distribution.

The results from both single and mixed C5+ hydrocarbon incubations show that communities undergo large changes when C5+ hydrocarbons are introduced. Other groups have found shifts in communities when hydrocarbons were introduced. For example, Hansen *et al* (1999) showed, with PLFA, that when soils were incubated with toluene for 5 days, the soil community underwent large changes in composition. Results presented here, however, show that during long term soil incubations with hydrocarbons the initial changes are not continuously propagated, but rather the community continues to be dynamic and undergo fluctuations in its composition. So, from these results, no single recognisable community capable of degrading C5+ hydrocarbons, when present singly or mixed, is evident. This is not unprecedented. Stoffels *et al* (1998) showed with FISH that different communities, dominated by either Proteobacteria of the alpha and beta subclasses or bacteria belonging to the gamma subclass of the class Proteobacteria, were capable of effective degradation of contaminating hydrocarbons in fermentors or trickle-bed reactors.

During long term incubations, the soil communities not only continued to be dynamic, but they also appeared to be increasing in complexity. As incubation

periods were extended, fewer master filter standards were predominant, and the community seemed to be moving towards a more complex community where many standards were present. A community becoming more complex during long term incubations with hydrocarbons has been shown in other work. Van Hamme *et al* (2000) used a batch culture derived from hydrocarbon contaminated soil to study microbial diversity during incubation with crude oil. They found that a greater diversity of species were able to be isolated from the culture with increased incubation periods of up to 7 days.

No DCPD-degrading microorganism was indicated by this research. This is in agreement with previous studies, which have suggested that there are no culturable species able to use this compound as a sole carbon and energy source (Spanggord *et al*, 1979, van Breemen *et al*, 1993 and Stehmeier, 1997). Soil communities, incubated with DCPD over nine weeks, generally developed an even population distribution (Figure 3-10). This result is in agreement with the work done by Stehmeier (1997), which suggested that DCPD bioremediation required cometabolism of other (hydro)carbon sources, was slow, and was performed by a microbial consortium.

The results from the DCPD and DCPD plus naphthalene incubations demonstrated that standard 25 (*Pseudomonas* sp. Q5), an organism previously implicated in DCPD oxidation (Shen *et al*, 1998), was not the most prevalent microorganism under all circumstances. However, standard 25 (*Pseudomonas* sp. Q5) did appear as a major component of the community in soils 320 and 350 during incubation in 1% DCPD and soils 320, 350 and C1 during incubation in DCPD plus naphthalene. Additionally, these experiments suggest that standards

47 (*Alcaligenes* sp. B4), 52 (*Alcaligenes* sp. N1) and 54 (*Pseudomonas* sp. N3), as well as other *Pseudomonas* spp. in general, may either play a role in DCPD degradation or are not inhibited by the presence of DCPD. Again, the finding that no single standard was predominantly present during incubation conditions agrees with earlier research, indicating that DCPD degradation requires a community of bacteria (Stehmeier *et al*, 1999).

3.3 Enrichment Cultures Obtained from Soils in a C5+ Atmosphere

Enrichment cultures were obtained by transferring soil microcosms in MSM and incubating in a 1% C5+ atmospheres fortnightly. At the time of each serial transfer (Section 2.3.1.3), DNA was extracted and RSGP was performed. The community compositions resulting from enrichment of 3 contaminated (A1, A3 and C1) and 3 uncontaminated (320, 330 and 350) soils are presented in a dendrogram (Figure 3-13). This dendrogram is again divided into clusters based upon the predominant standards in each RSGP profile. In cluster I, an *Alcaligenes* sp., either standard 47 or standard 52, was the major constituent of the community. In clusters II and IV, the RSGP profiles showed the communities were evenly distributed with no dominant master filter standards. Standard 25 (*Pseudomonas* sp. Q5) was dominant in all the RSGP profiles in cluster III. RSGP profiles in cluster V were dominated by an *Alcaligenes* sp. and a *Pseudomonas* sp. (standards 52 and 11, respectively). It is of interest to note that the community compositions derived from both contaminated and uncontaminated soils developed similarly with time. The RSGP profiles obtained at 2 and 4 weeks were in either cluster V, or in one of the clusters with an even distribution (II and IV). Those obtained after 4 or 6 weeks were generally in cluster I. After 6 to 8 weeks most enrichments displayed an even distribution, but at 8 to 11 weeks most profiles were in cluster III (standard 25 dominant). Eventually, at 15 to 17 weeks, all enrichment RSGP profiles were in cluster I, which was dominated by an *Alcaligenes* sp. (either standard 47 or 52).

It is interesting to note that the enrichment cultures went through cyclic phases of dominance in which the *Pseudomonas* spp. and *Alcaligenes* spp. were

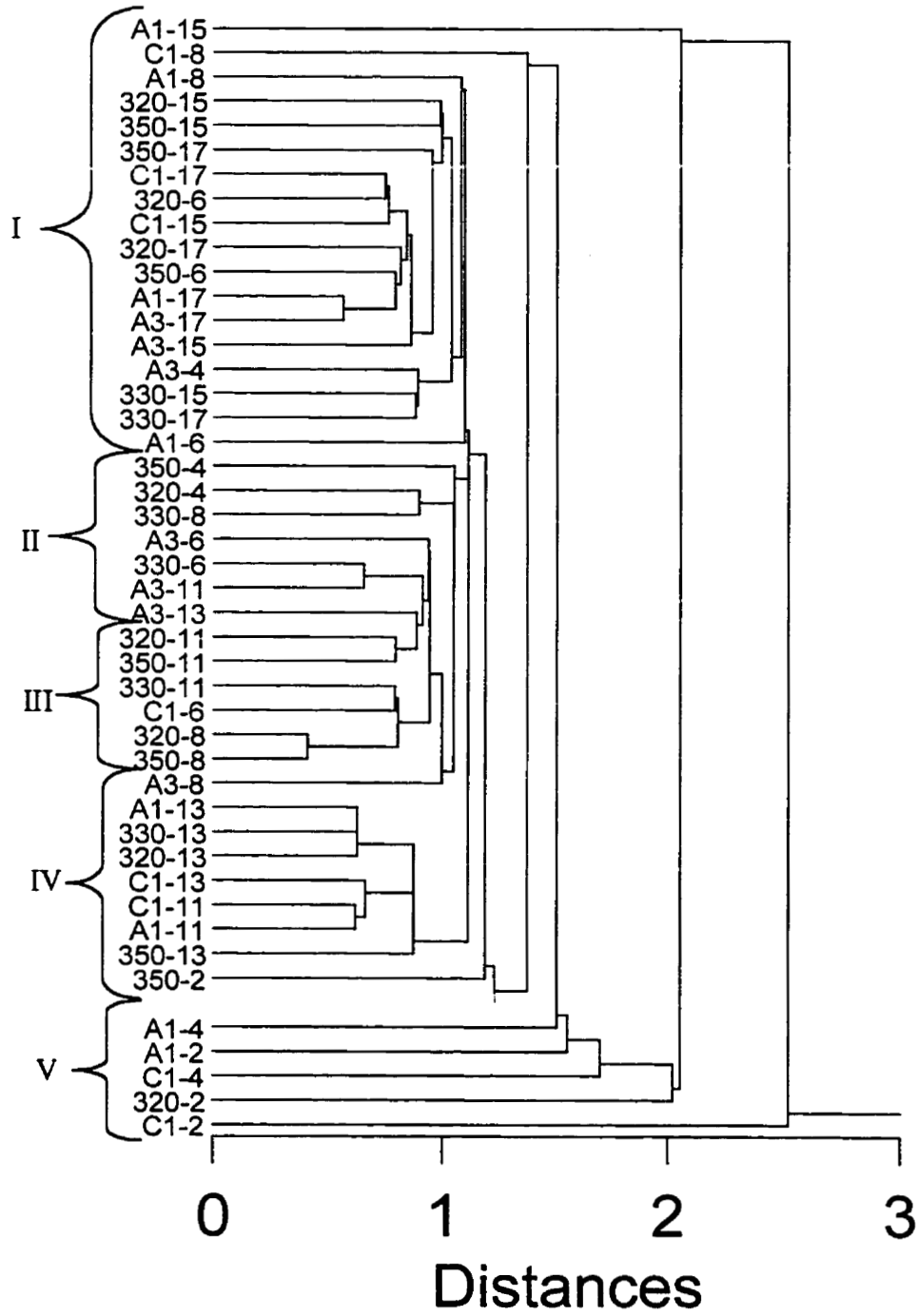


Figure 3-13. Dendrogram obtained by comparing RSGP profiles of soil enrichment cultures. Soil used for enrichment, followed by incubation times (weeks) are listed. I-V indicate clusters which have RSGP profiles with similar characteristics.

alternately the most dominant species. One possible reason for this cycling is a predator-prey relationship. Beveridge *et al* (1997) demonstrated that gram negative bacteria have a tendency to bleb off membrane vesicles, which often contain hydrolytic enzymes such as peptidoglycan hydrolases. Using a *Pseudomonas aeruginosa* model system, it was proposed that these vesicles could be predatory to other bacteria within a biofilm (Beveridge *et al*, 1997). In a batch culture, where the density of cells can become quite high, as well as in soils, sediments, water and animal models, similar processes can occur (Zusheng *et al*, 1998).

An alternative reason for the cycling as already discussed in Section 3.2.6 is the occurrence of selective hydrocarbon toxicity and adaptation, as well as the formation of end products, which may be inhibitory. The second set of standards may be able to use the end products of C5+ degradation of the first set. When the end product concentration decreases, the first set of standards can become active again. Alternatively, one set of standards may have a steady rate of growth, and only the second set is fluctuating in growth, becoming more and less predominant than the first in a cyclic nature.

A large inoculum (initially 20%, then 10%; Section 2.3.1.3) was used when transferring the enrichments. This large inoculum was used to maintain community complexity rather than artificially shrink it. After 2 transfers, 4% of the original culture would remain, after 4 transfers 0.16%. Bacteria that had no activity under the initial incubation conditions would likely be lost after a few transfers. If a smaller inoculum was used, the continued community cycling dynamics might not have been seen because those with little initial activity may have been lost.

It is interesting that the community composition in both contaminated and uncontaminated soils followed similar paths with time, and that by 15 weeks all enrichments had similar RSGP profiles. This shows that the ability to grow using C5+ hydrocarbons as a sole carbon and energy source is inherent within the soils sampled even if they had not been previously contaminated. This is not unprecedented, as uncontaminated soils have been shown to be capable of degrading *p*-xylene and naphthalene (Eriksson *et al*, 1999) as well as biphenyl and polychlorinated biphenyls (Mohn *et al*, 1997). Knowing that soils, which have not previously been contaminated with hydrocarbons, contain the potential ability to degrade hydrocarbons is important in terms of reducing the impact on human health and ecological factors, as well as lowering the cost of cleaning up contaminating hydrocarbons, which can be a large economic concern (Downey *et al*, 1999).

3.4 Kinetics of C5+ Removal

The rates of C5+ hydrocarbon removal by microbial communities obtained from contaminated and uncontaminated soils were measured. Unlike the experiments in dessicators for the determination of community composition where a continuous supply of hydrocarbon was available, the communities were in sealed serum bottles and a fixed amount of hydrocarbon was supplied for the kinetic studies.

3.4.1 Soils

Details of the experimental set-up are outlined in Section 2.3.2. Uncontaminated soils 320, 330 and 350, and a contaminated soil mixture were used. All contaminated soil samples were from a 15 m^2 sampling site (Figure 2-1) and were all within 90-120 cm of the surface. This sampling site was near a building at a polyethylene plant and was highly travelled, disturbed and mixed during plant operations. For this reason, the samples within this area were all quite homogenous, and it was believed a homogenisation of the contaminated samples would give good average rates of degradation for the small sample area. For each soil type, two viable cultures and one sterile control were incubated. The results of these experiments are shown in Figure 3-14. The average lag times were considered to be the amount of time that elapsed between the addition of hydrocarbon and the beginning of a major decrease in hydrocarbon concentration (Table 3-5). An example of the plots used in the determination of the rate constants (Section 2.3.2) is shown in Figure 3-15.

Lag times were shortest for microcosms from soils contaminated with C5+: 0.5 hours for xylene, styrene and naphthalene, and 12 hours for benzene

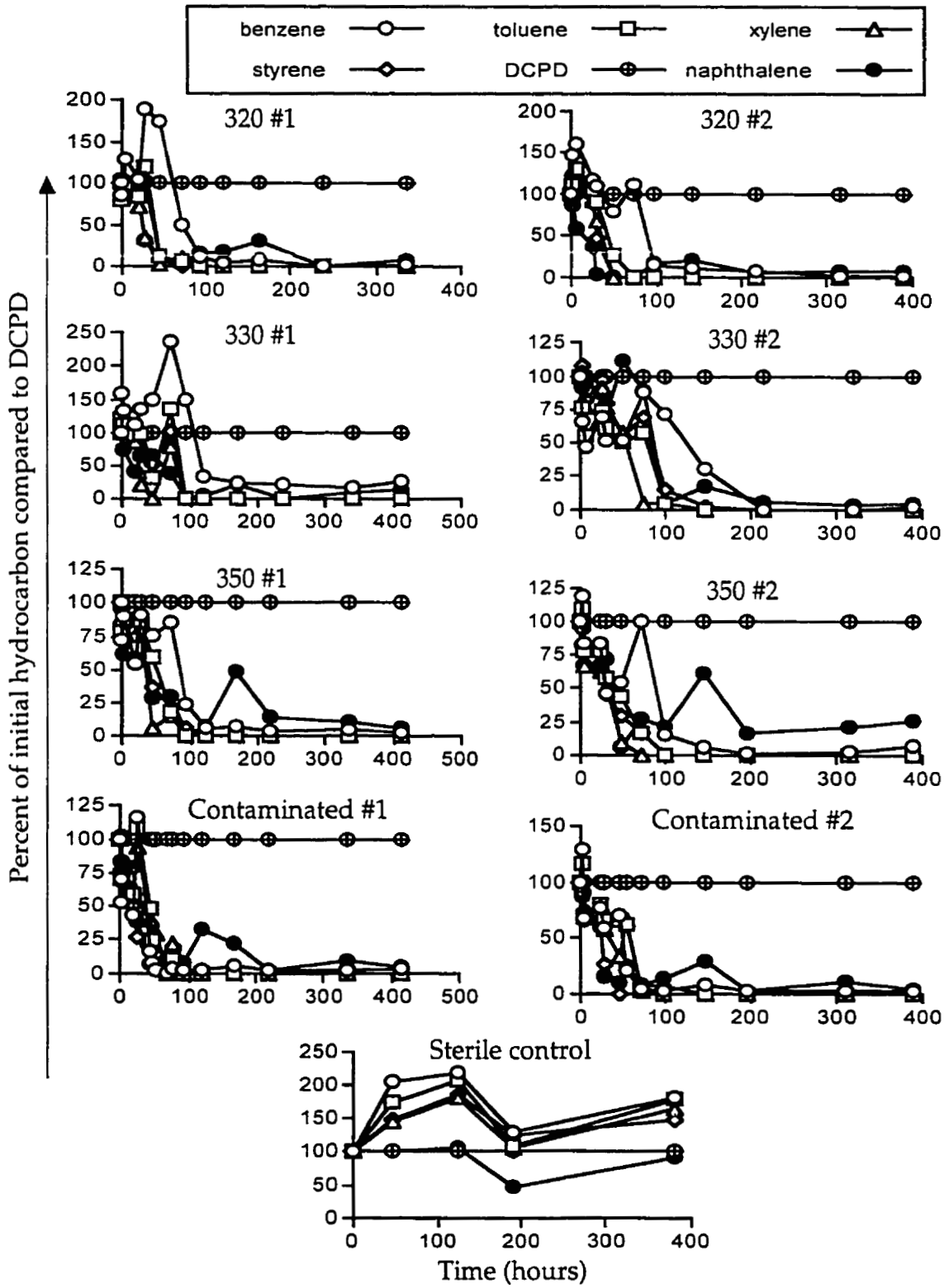


Figure 3-14. Removal of C5+ by soil microcosms as determined by GC. The contaminated soil was a mixture of soil samples as explained in the text. DCPD is used as an internal standard (100%).

Table 3-5. Average lag times (t) and first order rate constants (k) of removal of hydrocarbons from C5+ mixture by soil microcosms, enrichments and synthetic consortia.

Culture	t (h)					k (d^{-1})[b]				
Identity[a]	Ben[c]	Tol[c]	Xyl[c]	Sty[c]	Nap[c]	Ben[c]	Tol[c]	Xyl[c]	Sty[c]	Nap[c]
320	3.5	25.3	2.5	13.5	2.5	0.91	1.9	1.68	2.4	1.66
330	82.5	16.3	0.5	14	46.5	0.46	1.18	1.20	1.20	0.98
350	2.3	13.5	13.5	13.5	0.5	0.55	0.84	1.15	0.82	0.98
contaminated	12	12	0.5	0.5	0.5	2.28	2.04	0.74	1.10	1.32
E-320	0.5	0.5	1.0	1.0	0.5	1.92	1.03	0.38	0.84	0.30
E-330	0.0	0.0	24.5	24.5	15.0	1.09	0.79	2.30	0.16	0.83
E-350	0.0	0.0	13.0	0.0	10.3	2.71	1.16	2.40	0.55	0.40
E-A1	0.0	0.0	12.5	0.0	0.0	1.74	1.91	0.94	3.25	1.46
E-A3	0.5	0.0	2.8	0.0	0.5	4.88	3.55	2.30	2.38	1.49
E-C1	2.8	9.0	5.5	5.5	1.0	3.67	3.02	1.19	2.47	0.92
C-5	1.8	2.5	3.5	2.5	0.5	3.37	1.66	0.35	2.25	2.57
C-9	0.3	2.0	3.3	2.0	1.3	3.40	1.63	1.02	2.22	1.21
C-41	1.7	2.8	3.5	1.2	3.3	3.78	2.47	0.58	2.83	0.81

[a]E=enrichment culture from designated soil, C=synthetic consortia with 5, 9 or 41 master filter organisms; contaminated = mixture of contaminated soils as explained in the text; 320, 330, 350 and contaminated are soil microcosms.
[b]First order rates are shown as more degradation rates can be described by first order than by zero order.
[c]Ben=benzene, Tol=toluene, Xyl=*m*-xylene, Sty=styrene, Nap=naphthalene

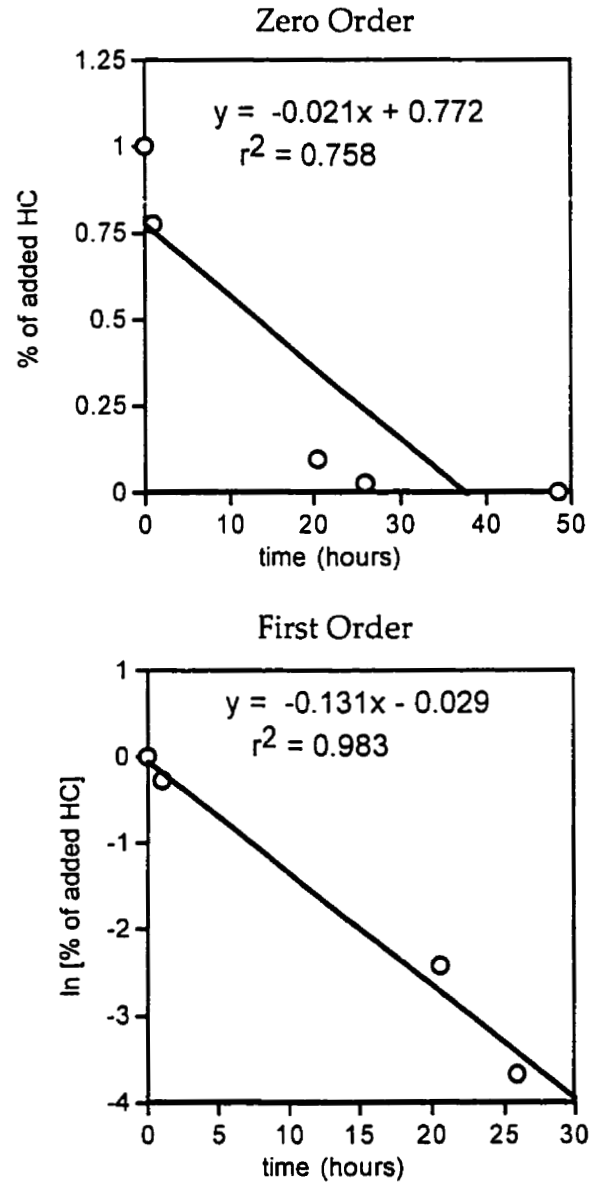


Figure 3-15. Example of plots used to determine kinetic rate constants. Benzene removal data from enrichment of soil 350. R^2 value for first order plot is better than for zero order plot, so first order kinetic rate constant is used to describe removal of benzene from enrichment (k=0.131).

and toluene. The longest lag times were with microcosms from uncontaminated soil 330: 0.5 hours for xylene, 14 and 16.3 hours for styrene and toluene, but 46.5 and 82.3 hours for naphthalene and benzene (Table 3-5). In all viable microcosms the C5+ components were mostly removed within 100 to 200 hours of incubation, with the exception of DCPD. The lack of DCPD removal was expected and consistent with earlier results (Stehmeier *et al*, 1998). The previously contaminated soil microcosms removed the hydrocarbons fastest with larger first order rate constants (k) for benzene and toluene (Table 3-5), and with hydrocarbon removal nearly complete within 75 hours (Figure 3-14). Toluene, styrene and xylene were removed to below detectable levels given sufficient incubation, but naphthalene and benzene were not (Figure 3-14); minimal levels of these compounds usually remained.

3.4.2 Enrichments and Synthetic Consortia

The rates and lag times for hydrocarbon removal of soil enrichment cultures obtained by serial transfer in dessicators containing 1% C5+ in vpo (Section 3.3) were also measured in sealed serum bottles (Figures 3-16 and 3-17). Additionally, synthetic consortia were made by combining master filter standards (Section 2.3.2). Consortium C-41 contained all the organisms from the master filter that were readily cultured in rich medium under aerobic conditions. Consortia C-5 and C-9 contained organisms that were frequently major community components during enrichment experiments (Section 3.3). Hydrocarbon removal by these synthetic consortia is shown in Figure 3-18.

Enrichments were able to remove all hydrocarbons, with the exception of DCPD, within 100 hours in most cases (Figures 3-16 and 3-17). The enrichments

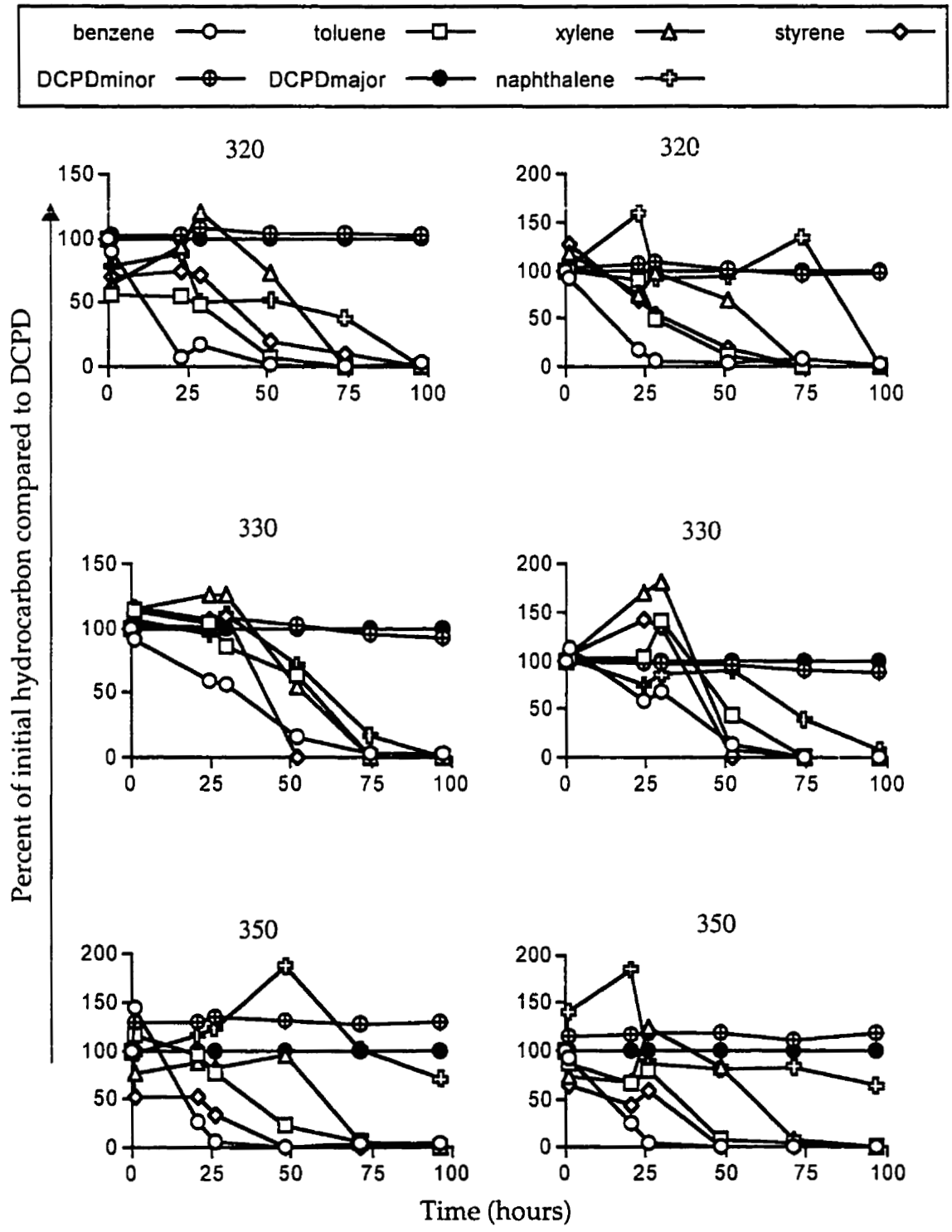


Figure 3-16. Removal of 2 μL of C5+ hydrocarbons by enrichments from uncontaminated soils in sealed serum bottles. DCPD appeared as two peaks; the major DCPD peak is used as an internal standard (100%).

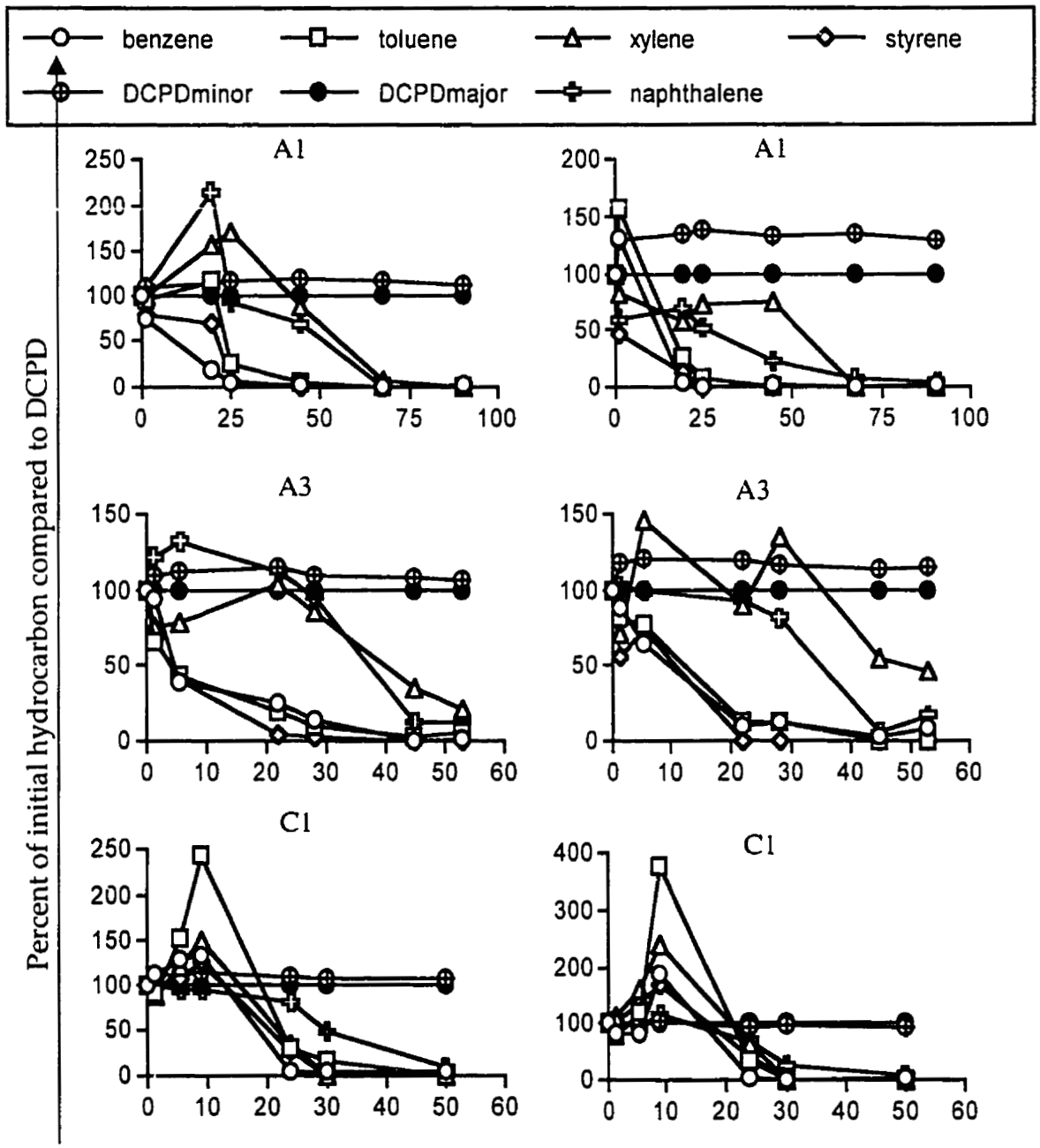


Figure 3-17. Removal of 2 µL of C5+ hydrocarbons by enrichments from contaminated soils in sealed serum bottles. DCPD appeared as two peaks; the major DCPD peak is used as an internal standard (100%).

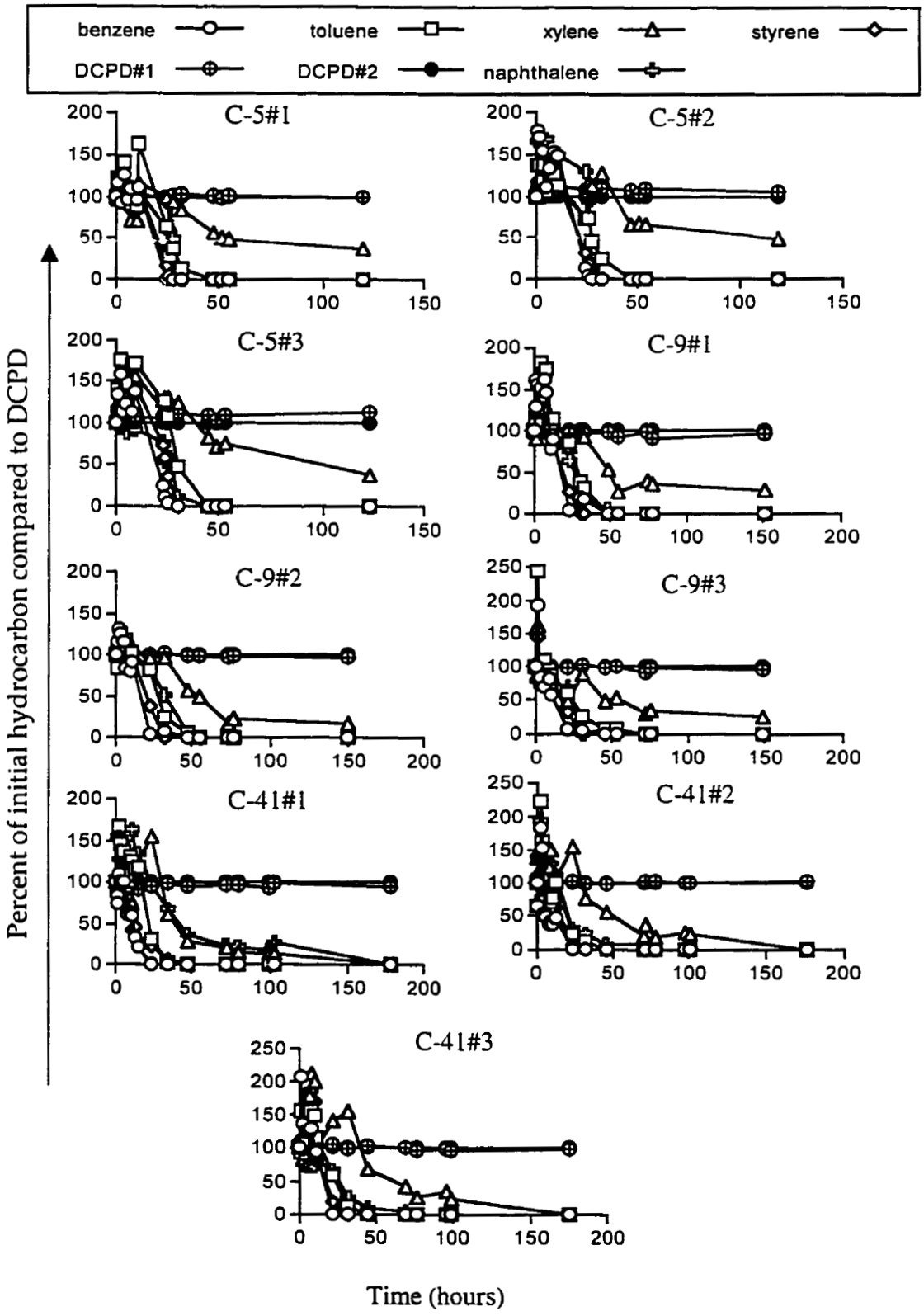


Figure 3-18. Removal of 2μL C5+ hydrocarbons from synthetic consortia in sealed serum bottles. DCPD is used as an internal standard (100%).

from uncontaminated soils removed hydrocarbons more slowly than enrichments from contaminated soils. The enrichment from uncontaminated soil 350, did not remove naphthalene after 100 hours of incubation (Figure 3-16). The enrichments from contaminated soils removed all of the hydrocarbons (except DCPD) within 100 hours (Figure 3-17) and were generally faster than the uncontaminated enrichments. Enrichment C1 was the fastest, with all the hydrocarbons removed within 50 hours of C5+ addition.

The synthetic consortia had difficulty removing *m*-xylene. In all three consortia, it was the slowest to be removed from the medium (Figure 3-18). The other hydrocarbons, with the exception of DCPD, were readily removed within 50 hours in all consortia. For consortium C-41, *m*-xylene was removed within 175 hours (Figure 3-18).

The average lag times and first order degradation rate constants for hydrocarbon removal by the enrichments and the synthetic consortia were calculated (Table 3-5). When the lag times are compared, it can be seen that they are generally much lower for the enrichment cultures than for either the synthetic consortia, or the soil microcosms (Table 3-5). The rate constants for removal of C5+ hydrocarbons were generally greatest for the synthetic consortia and the enrichments from contaminated soils. They were smaller for microcosms from contaminated soils and from enrichments from uncontaminated soils. The rate constant for removal of benzene, the main component of C5+, was lowest for microcosms from uncontaminated soils (Table 3-5). All three synthetic consortia had similar lag times and rate constants of hydrocarbon removal (Table 3-5). The order in which the hydrocarbons were removed was also determined and is given

in Table 3-6. The order of removal is a function of both the lag time and the first order rate constant.

3.4.3 Discussion

In these studies rates of C5+ hydrocarbon removal were studied with soil microcosms, soil enrichment cultures and synthetic consortia. Some of the results obtained can be understood by considering the prior exposure time to C5+ of these samples. In the case of soil microcosms and synthetic consortia this exposure was absent or minimal (the contaminated soil microcosms had obviously been exposed to C5+ at some point). However, in the case of soil enrichment cultures, adaptation to the presence of C5+ occurred over a lengthy period (20 weeks). We would thus expect that lag times for soil microcosms are longer than for enrichment cultures, especially for uncontaminated soils. The results in Table 3-5 indicate this to be the case. The lag times for microcosms from uncontaminated soils reflect the time needed for an active hydrocarbon-degrading microbial population to be induced. Once that has been accomplished the first order rate constants were similar (Table 3-5), with the exception of benzene (Table 3-5). The similar rates may be caused by the similar community profiles that developed in uncontaminated and contaminated soils incubated under similar conditions, with C5+ as a sole source of carbon and energy (Section 3.2). Other groups have shown that uncontaminated soils are capable of degrading hydrocarbons. Eriksson *et al* (1999) showed that natural uncontaminated potting soil was capable of degrading the hydrocarbons *p*-xylene and naphthalene at 20°C. Mohn *et al* (1997) have shown that uncontaminated Arctic soils were capable of degrading biphenyls and polychlorinated biphenyls.

Table 3-6. Order of removal of C5+ hydrocarbons from microcosms. DCPD, which was never observed to be removed, is not included.

Culture Identity[a]	Hydrocarbon Removal Order				
	Ben[b]	Tol[b]	Xyl[b]	Sty[b]	Nap[b]
320	4	2	3	1	5
330	5	2	1	3	4
350	4	2	1	3	5
contaminated	1	4	3	2	5
E-320	1	4	5	2	3
E-330	1	3	5	2	4
E-350	1	3	5	2	4
E-A1	1	2	4	3	5
E-A3	1	4	3	2	5
E-C1	1	3	4	2	5
C-5	1	3	5	2	4
C-9	2	2	4	1	3
C-41	1	2	3	2	4

[a]E=enrichment culture from designated soil, C=synthetic consortia with 5, 9 or 41 master filter organisms.

[b]Ben=benzene, Tol=toluene, Xyl=*m*-xylene, Sty=styrene, Nap=naphthalene

However, Mohn *et al* (1997) also found that previously contaminated soils were capable of degrading contaminating hydrocarbons faster and more extensively than uncontaminated soils. The results for the soil microcosms obtained here show that both previously contaminated and uncontaminated soil microcosms degraded the added hydrocarbons at similar rates once the initial lag times were overcome (Table 3-5; Figure 3-14). The uncontaminated soil microcosms, however, did have longer lag times than the contaminated soil microcosms (Table 3-5), which caused the overall time required to remove the introduced hydrocarbons to be longer for the uncontaminated soil microcosms (Figure 3-14). The contaminated soil microcosms likely had a shorter lag time because of a higher population of hydrocarbon degraders due to *in situ* selection for the degrading population, as explained at the start of this section. A second reason for the decreased lag time would be that the hydrocarbon degraders within the soil were more physiologically or genetically prepared to encounter C5+ hydrocarbons (Mohn *et al*, 1997). This could also explain why enrichment cultures had greater rates and much smaller lag times than observed for the soil microcosms (Table 3-5).

Despite the similarity in community composition following many transfers in MSM equilibrated with 1% C5+ (Figure 3-13), the rates for degradation of the enrichments from contaminated soils were higher than for enrichments from uncontaminated soils. The additional *in situ* selection the contaminated soils experienced before enrichment may have contributed to this (Mohn *et al*, 1997). The rates obtained for the enrichments (Table 3-5) compare to rates obtained for degradation of mixed hydrocarbons obtained by other workers. Yerushalmi and

Guiot (1998) found first order degradation rate constants of 2.4 d^{-1} and 3.1 d^{-1} for benzene and toluene when degraded as part of a mixture of hydrocarbons in gasoline.

The rates obtained with synthetic consortia were comparable to those found for enrichment cultures, with the exception of those for *m*-xylene, which were quite low. The synthetic consortia also had longer lag times than those observed for the enrichment cultures, but shorter than found for the soil microcosms. Although the synthetic consortia contained standards able to grow on mixed xylenes (Section 2.3.2 and Table 3-2), they removed *m*-xylene poorly. It is possible that the standards in the synthetic consortia were better able to use *o*- or *p*-xylene. It has also been reported that xylene is slowly removed by cometabolism in instances where microorganisms are unable to grow on a xylene as sole carbon and energy source (Chang *et al*, 1993). With *Pseudomonas* sp. strain PPO1, it was found that when benzene and *p*-xylene were present, metabolic intermediates of *p*-xylene would accumulate in the medium (Oh *et al*, 1994). Alvarez and Vogel (1991) showed that xylene removal was stimulated by the presence of toluene, but that the presence of xylene in turn inhibited the removal of toluene and benzene. It may be that in the results presented, slower cometabolic processes contributed to *m*-xylene removal in the case of the synthetic consortia because fewer microorganisms were present. The rates of *m*-xylene removal in general were slower than for the other hydrocarbons in all cases. Nielsen and Christensen (1994) have also shown that xylene degradation is generally slower than degradation of other BTEX compounds.

The rates of hydrocarbon removal by the three synthetic consortia were all quite comparable, with the exception of those for *m*-xylene (Table 3-5). This implies that rates of hydrocarbon removal were not dependent on the high numbers of microorganisms being present. The most prevalent standards according to RSGP analysis of enrichment cultures were used in the synthetic consortia C-9 and C-5. Removing the master filter organisms that were not prevalent according to RSGP analysis did not affect the rate of hydrocarbon removal by the microcosm (Table 3-5). This shows that the standards that were most prevalent according to RSGP during enrichment were likely the most active, and contributed most to determining the rates of hydrocarbon utilisation.

Mixed hydrocarbons have been shown to be degraded with lower rates than single hydrocarbons. Alvarez and Vogel (1991) found that toluene could inhibit the removal of benzene when a single microorganism was present, but not with a mixed community. They also showed that when benzene and toluene were present, benzene was used preferentially over toluene in studies with single microorganisms. Chang *et al* (1993) and Oh *et al* (1994) obtained similar results. As shown in Table 3-6, benzene was nearly always the first hydrocarbon to be removed by the contaminated soil microcosms, as well as by all enrichments and synthetic consortia tested. It is also the hydrocarbon present in the greatest amount in C5+ (Section 1.1: 45%). Styrene was second, toluene third, naphthalene was usually fourth and xylene was most often the last to be removed (Table 3-6). This generally follows the percentages of these hydrocarbons in C5+ (Section 1.1). These percentages also relate to the numbers of master filter standards that were able to grow using C5+ hydrocarbons as an energy and carbon source (Section

3.1.2.3). The uncontaminated soil microcosms, however, did not follow the same pattern and benzene was fourth or fifth to be removed by these microcosms, with xylene usually being removed first (Table 3-6). It appears that exposure to C5+ before testing for hydrocarbon removal not only reduced the lag time required to begin hydrocarbon degradation (Table 3-5), but also influenced the order in which hydrocarbons were removed from the microcosms.

3.5 Effects of DCPD on Standard 25 (*Pseudomonas* sp. Q5)

DCPD is recalcitrant to degradation. To date, no bacterial isolate capable of growth on DCPD as the sole carbon and energy source has been reported. Studies by Shen *et al* (1998) have indicated that oxidised derivatives of DCPD may be formed by standard 25 (*Pseudomonas* sp. Q5). Further investigations into the properties of standard 25 (*Pseudomonas* sp. Q5) were performed.

3.5.1 Growth of Standard 25 (*Pseudomonas* sp. Q5) in the Presence of DCPD

Standard 25 (*Pseudomonas* sp. Q5) was unable to grow using DCPD as the sole carbon and energy source, but was shown to grow in the presence of naphthalene (Table 3-2). Standard 25 (*Pseudomonas* sp. Q5) was tested for its ability to grow in the presence of naphthalene plus DCPD (Figure 3-19). All standards which were tested were able to grow in the presence of 1% naphthalene and 1% DCPD (Figure 3-19). When grown in 1% naphthalene plus 10% DCPD, little effect was seen in the growth of the standards tested (Figure 3-19). Standard 25 (*Pseudomonas* sp. Q5) was still able to grow in the presence of 50% (w/w) DCPD and 1% (w/w) naphthalene in vpo. The other master filter standards that were tested for growth on naphthalene in an atmosphere containing DCPD were inhibited by concentrations of DCPD of 50% (Figure 3-19). DCPD had a much lower toxicity effect on standard 25 (*Pseudomonas* sp. Q5) than any other standard that was tested. This is possibly caused by the ability of standard 35 (Pseudomonas sp. Q5) to detoxify DCPD by forming oxidised derivatives as demonstrated previously (Shen *et al*, 1998). Thus standard 25 (*Pseudomonas* sp. Q5) is viable under conditions where DCPD exists in high amounts, and a readily usable carbon (such as naphthalene) is present in lower amounts.

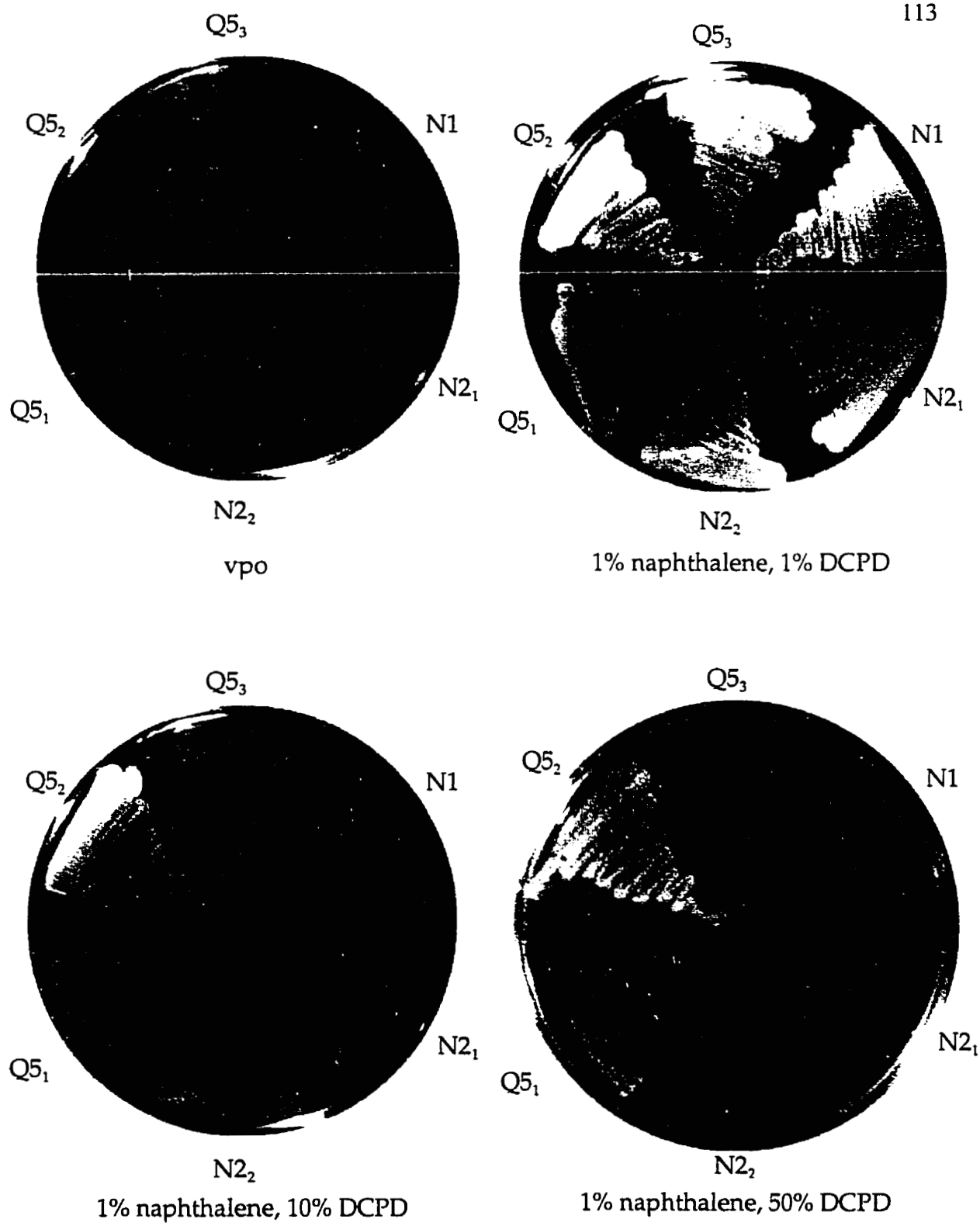


Figure 3-19. Growth of standard 25 (Pseudomonas sp. Q5) on MSM plates in the presence of naphthalene and DCPD. $Q5_1$, $Q5_2$ and $Q5_3$ are all strains making up standard 25 (*Pseudomonas* sp. Q5). $N2_1$ and $N2_2$ are strains making up standard 53 (*Arthrobacter* sp. N2). Plates were incubated at room temperature for 2 weeks.

3.5.2 Effect of DCPD on Naphthalene Degradation by Standard 25 (*Pseudomonas* sp. Q5)

Standard 25 (*Pseudomonas* sp. Q5) was incubated in sealed serum bottles to test the rates at which it removed naphthalene. Additionally, the effect of DCPD on the rate of naphthalene removal by Q5 was tested. As seen by the results in Figure 3-20 and Table 3-7, increasing naphthalene concentrations increased the lag time required by Q5 to start removal of naphthalene. The first order rates of naphthalene removal were also decreased as the amount of naphthalene introduced was increased (Table 3-7). This increase in acclimation required and reduced rates of removal could have been due to toxic effects of higher concentrations of naphthalene (Section 1.2.3). The additional toxicity of the aromatics on standard 25 (*Pseudomonas* sp. Q5) would have reduced its ability to remove the naphthalene from the medium. HMN, the solvent used to dissolve the naphthalene (Section 2.3.2), has been shown to increase the oxidation rate of naphthalene when present in small amounts (Allen *et al*, 1999). However, HMN is a nonpolar solvent and may have contributed to toxicity effects on standard 25 when the amounts present increased (Section 1.2.3).

The addition of DCPD to the sealed microcosms appeared to decrease the rate of naphthalene removal (Table 3-7). It is possible that this was due to toxic effects of DCPD. However, as the total amount of hydrocarbon present was 2 μL for both the naphthalene alone incubation, and the naphthalene plus DCPD incubation (Figure 3-20), these effects are likely minimal. A second possibility for the decreased rate of naphthalene removal is that DCPD was a competitive inhibitor of the enzymes responsible for the removal of naphthalene. Naphthalene

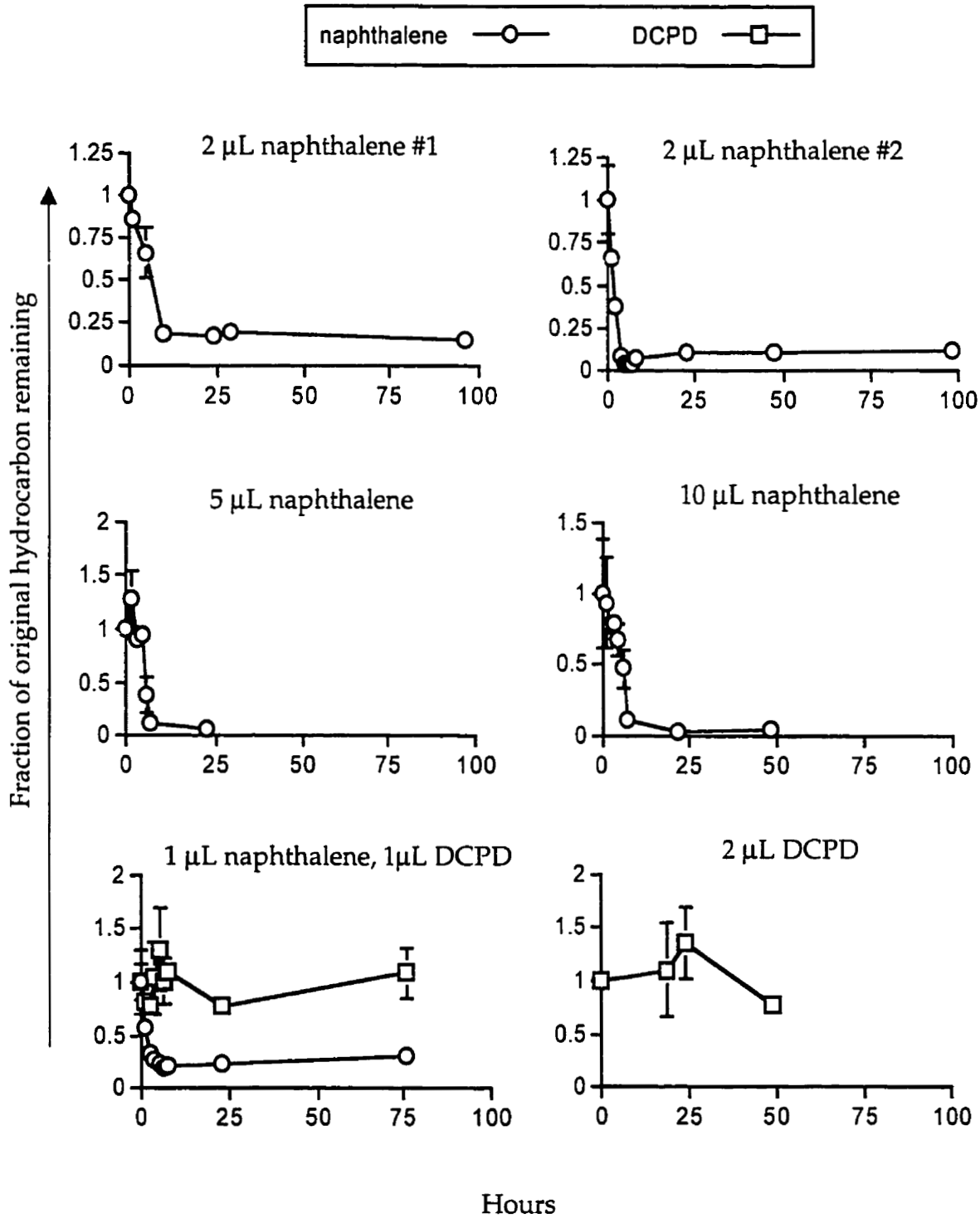


Figure 3-20. Removal of naphthalene and DCPD from standard 25 (*Pseudomonas* sp. Q5) cultures in sealed vials as determined by GC. Results are an average of 3 replicate cultures. The fraction of naphthalene present, compared with naphthalene concentration at time zero is shown.

Table 3-7. Lag times (t) and first order rate constants (k) for degradation of naphthalene by standard 25 (*Pseudomonas* sp. Q5).

Incubation conditions	t (h)	k (h^{-1})
2 µL naphthalene	0	.731 ± 0.100
5 µL naphthalene	1.5	.143 ± 0.008
10 µL naphthalene	3.5	.177 ± 0.014
1 µL naphthalene, 1 µL DCPD	0	.413 ± 0.052

dioxygenase (NDO) has been shown to posses relaxed substrate specificity (Section 1.2.2). If DCPD was indeed able to fit into the active site of NDO, it was not oxidised rapidly, as it remained at initial levels during the experiment (Figure 3-20). This shows that if oxygenated derivatives of DCPD are formed by NDO, or similar enzymes, this process occurs slowly. This agrees with earlier research (Shen *et al*, 1998; Stehmeier *et al*, 1999). To determine if oxygenated derivatives of DCPD are indeed formed, longer term incubations of standard 25 (*Pseudomonas* sp. Q5) with naphthalene and DCPD (2 to 4 weeks) need to be performed (Shen *et al*, 1998).

3.6 Conclusions

Microorganisms capable of degrading the hydrocarbon components of C5+, with the exception of DCPD, are readily obtained from soils that have had previous exposure to C5+. Using RSGP, it was shown that during incubations without an additional hydrocarbon, an even community with no single standard being predominant, was generally obtained after 2 weeks of incubation. After incubating soils with single or multiple components of C5+ hydrocarbons for 2 weeks, changes in the microbial community were observed, with one or a few standards becoming predominant. After longer incubations, the soil microbial communities generally became more evenly distributed. During kinetics experiments, it was found that uncontaminated soils had longer lag periods before initiation of hydrocarbon removal than soils that had previously been contaminated with C5+. However, once the lag periods had elapsed, the rates of C5+ removal were similar for both types of soils.

Soils were enriched in the presence of C5+ as the sole hydrocarbon source, and the development of the enrichment community was followed by RSGP. Soils which had previously been contaminated with C5+ and uncontaminated soils both developed similar community profiles after 15 weeks of enrichment. The profiles showed that an *Alcaligenes* sp., either standard 47 (*Alcaligenes* sp. B4) or 52 (*Alcaligenes* sp. N1), was predominant in the community. The kinetics of C5+ removal by the enrichments was determined. The enrichments had greatly reduced lag times when compared to the initial soil cultures, and the rates of hydrocarbon removal were similar to those of the soils.

Artificial consortia, which consisted of those standards that were predominant during serial enrichment in C5+, demonstrated rates of removal which were similar to those of both soils and enrichments. This suggests that there is a reliance on a specific sub-community in the soil for the majority of the hydrocarbon removal performance.

Further studies with DCPD and standard 25 (*Pseudomonas* sp. Q5) showed that it tolerates high concentrations of DCPD. As well, kinetics experiments performed with standard 25 (*Pseudomonas* sp. Q5), suggest that DCPD may cause competitive inhibition naphthalene degradation, possibly through interaction with an enzyme involved in naphthalene degradation, such as naphthalene dioxygenase (NDO).

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