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Chapter 1

Introduction

Introduction

Global industry depends on fossil fuels as a primary energy source. South Africa is no exception in this regard as it is an industrialised country with a well-developed mining and fuel commerce. At the rate of consumption in the early 1970's, it was estimated that there coul be 20-40 years of crude oil resources remaining (Gold 1985). However, with advances in the technology of detection methods, drilling and mining, the availability of this natural resource has been extended. At present there are 53-55 billion tonnes of economically accessible coal reserves left in South Africa. Calculations following a Gaussian curve show that, with current technologies, and if coal mining in South Africa increases by 1.8% annually, peaks, and then drops by 1.8% annually, then the peak occurs in the year 2050 (Bredell Report 1987; Surridge[a], personal communication; Singh[b], personal communication). Due to fossil fuel imports and synfuel manufacturing within South Africa, there is a high risk of environmental pollution and consequently severe ecological disruption as a result of fuel by-products and spills in areas where storage, transport, refining, distribution, consumption and fossil fuel industry takes place.

Hydrocarbons have traditionally been considered to be of a biological origin, since methane and other longer chain hydrocarbons appear to be exclusively the result of biological processes. However, it is now known that the largest supply of carbon in the planetary system is in the form of hydrocarbons. Petroleum and coal contain a class of molecules known as hopanoids commonly found in bacterial cell walls (Gold 1985), thus it can be concluded that at some point all of these fuels originated, at least in part, from microbes. Based on this, the assumption can be made that biodegradation of these fuels has always been occurring to some

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extent. To extrapolate from this knowledge… the "biological evidence" within these hydrocarbons could be the reason that the adaptation of microbes to degrade them so readily occured upon technological industrialisation of the Earth and why phytoremediation is such an applicable method for polluted soil reclamation. The microorganisms, natural or genetically engineered, can mineralise toxic polycyclic aromatic hydrocarbons (PAHs) into carbon dioxide and water (Fig. 1).

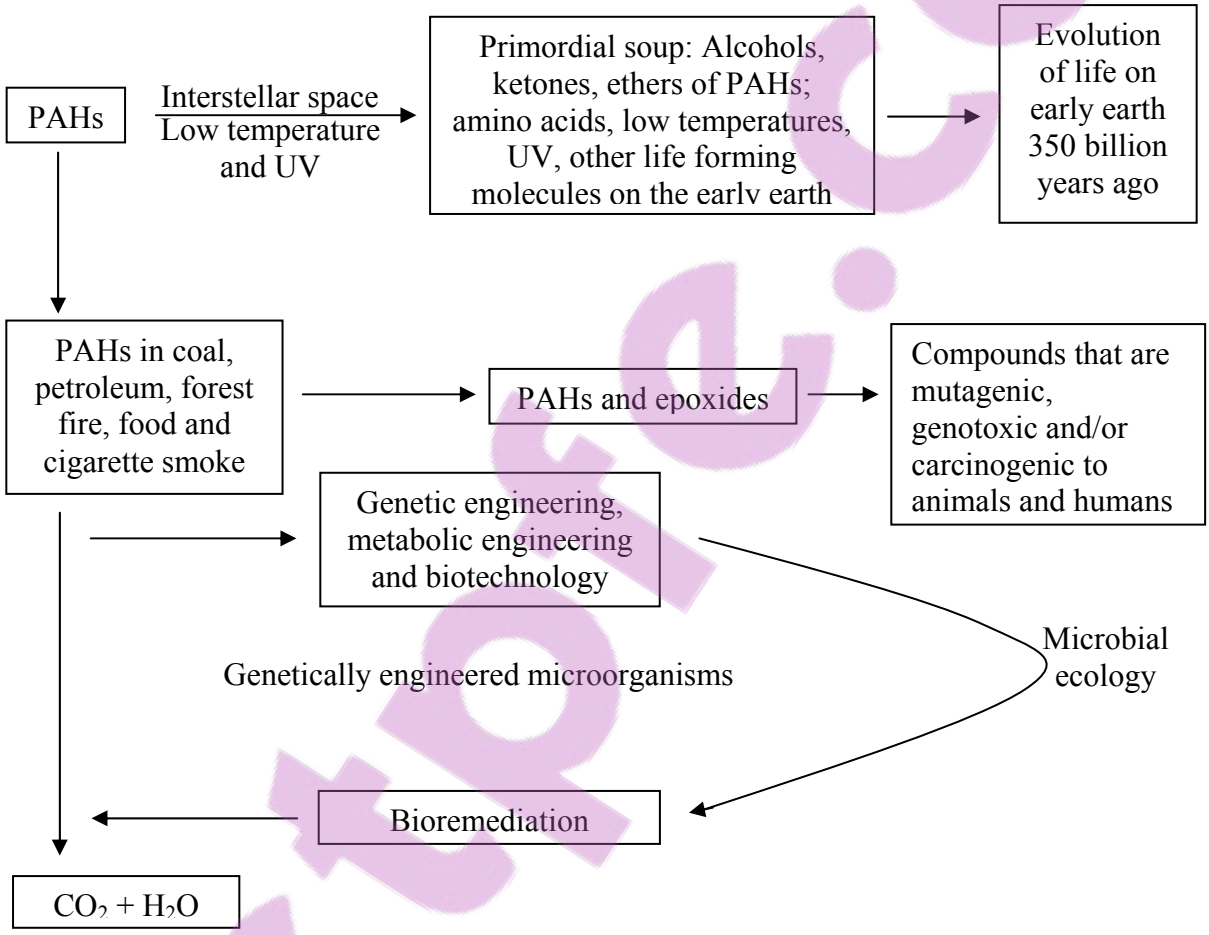


Figure 1: Fate, toxicity and remediation of PAHs in the environment (Samanta *et al*. 2002).

In petrol-polluted soil, benzene, toluene, ethylbenzene and xylene (BTEX) isomers are present in the water-soluble fraction causing pollution (Prenafeta-Boldú *et al.* 2002). However, the most notorious class of hazardous compounds found in petrol, diesel, oil, as well as coal-tar and its derivatives, are the polycyclic aromatic hydrocarbons (PAHs). PAHs are hydrophobic, chemical compounds consisting of fused aromatic rings, not containing

heteroatoms (any atom other than carbon or hydrogen) or carrying substituents, e.g. naphthalene, anthracene, phenanthrene, benzo(a)pyrene, coronene, pyrene, triphenylene, chrysene and benzo(ghi)pyrene (Wikipedia 2005a). Known to be carcinogenic, PAHs are formed by incomplete combustion of carbon-based fuels such as wood, coal, diesel, fat and tobacco. PAHs with up to four fused benzene rings are known as light PAHs, the simplest of these being benzocyclobutene (C_8H_6). Those containing more benzene rings are known as heavy PAHs and are more stable and more toxic. Two of the most commonly found aromatic hydrocarbons in pollutants are naphthalene and toluene.

Hydrocarbon sources of pollution affect the environment and specifically the soil. Soil disruption caused by pollution with these compounds decreases biodiversity and selects for microbial species better adapted to survive in the changed environment (Lindstrom *et al*. 1999; Kozdrój and Van Elsas 2001). Environmental changes due to this pollution affect the soil structure and fertility, and therefore the fauna and flora. Affected soils become relatively sterile to all but resistant microbial life forms. Certain indigenous microorganisms, including bacteria and fungi, are able to degrade PAHs in soil leading to *in situ* rehabilitation of polluted soils. The utilisation of such microorganisms for detoxifying and rehabilitating PAH-polluted soils provides an effective, economical, versatile and eco-compatible means of reclaiming polluted land (Guerin 1999; Bogan *et al.* 2001; Margesin and Schinner 2001; Mishra *et al.* 2001; Tesar *et al*. 2002).

The bioremoval capacity of a soil can be improved by inoculation with specific strains and/or consortia of microorganisms (Halden *et al.* 1999; Dejonghe *et al.* 2001), particularly those from the rhizosphere of plants, since they are less readily destabilised due to the buffering in the presence of their host plant, but nevertheless amenable, composition of the biotic and abiotic environment they inhabit (Bahme *et al*. 1988). BTEX isomers are the most amenable

to elimination from the environment by indigenous microorganisms. However, degradation can be impeded by the micronutrient balance within the natural system (Koizumi *et al.* 2002).

Remediation is usually limited by the amount of free carbon, phosphorus or nitrogen available (Bogan *et al.* 2001; Margesin and Schinner 2001; Röling *et al.* 2002). Nitrogen is the most important of these elements required under limited nutrient conditions, as it is used in the synthesis of proteins, nucleic acids and other cellular components. Elemental nitrogen present as an atmospheric gas is almost inert due to the stability of the triple bond between the two nitrogen atoms. Thus, elemental nitrogen must be "fixed" by bacteria in soil for plants, termites and protozoan organism growth (Deacon 2004). However, there are some exceptions to this synergistic nitrogen fixation relationship that exists between bacteria, plants, termites and protozoans. Struthers *et al.* (1998) reported that the herbicide atrazine is degraded by *Agrobacterium radiobacter* in soil without addition of extra carbon or nitrogen sources. Despite this, microbial community numbers can be increased by injecting soluble nutrients, like nitrogen sources, a few centimetres under the surface of the soil. Gaseous nitrous oxide has been used to supply nitrogen to polluted soils in the process of bioremediation (Bogan *et al.* 2001). Addition of nutrients to soil such as nitrogen fertilisers has been proven to enhance biodegradation of PAHs (Kasai *et al.* 2002).

The first culture-independent estimate of prokaryotic organisms in soil indicated the presence of 4600 distinct genomes in one gram of soil (Kent and Triplett 2002). Extracted DNA or RNA can, via molecular genetic techniques, facilitate microbial community analysis to be coupled with phylogeny (Blackwood *et al.* 2003). The uncultured diversity will reflect species closely related to known cultured organisms and also species from virtually uncultured lineages (Blackwood et al. 2003). Characterisation of genes of microbes involved in the degradation of organic pollutants has led to the application of molecular techniques in microbial ecology of polluted areas (Milcic-Terzic *et al.* 2001).

Molecular methods usually involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene. These methods include denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), single strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP). Most of these methods do not reveal diversity unless the community is very simple. This is due to only a very low amount of species indicated in rehybridisation or sequence analysis being visualised on a gel (Linderman 1988, Blackwood *et al.* 2003). However, DGGE in particular is applicable to the present study since diversity in PAH-polluted soils is expected to be low due to the high environmental selection pressure on the microbial species present. Catabolic gene probes can, furthermore be used in nucleic acid hybridisation analysis to characterise sequences (Nakatsu *et al.* 2000). DGGE also allows for the elucidation of major differences between communities and for testing of hypotheses on the basis of sample comparison (Blackwood *et al.* 2003).

Fairly recently developed, DGGE is an ideal molecular technique for monitoring microbial ecology. It relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks and Alleman 2002; Koizumi *et al.* 2002). DGGE allows a high number of samples to be screened simultaneously, thus facilitating more broad-spectrum analyses. PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The band pattern on the gel forms a genetic fingerprint of the entire community being examined (Gillan 2004). Resulting gel images can be digitally captured and used for species identification when samples are run against known standards (Temmerman *et al.* 2003). 16S rRNA genes are most commonly used to give an overall indication of the bacterial species

composition of a sample. Partial sequence of this gene has been analysed from as complex environments as soil (Throbäck *et al.* 2004).

DGGE allows for determining total community as well as specific community or gene diversity without further analysis and without elucidating particular individuals. It has been used in the identification of sequence variations in multiple genes among several organisms simultaneously (Muyzer *et al.* 1993). However, functional genes, having more sequence variation, can be used to discriminate between closely related but ecologically different communities. Rosado *et al*. (1998) used *Paenibacillus azotofixans nifH* species-specific primers in DGGE analyses of soil samples. They found that *nifH* is probably a multicopy gene in *P. azotofixans* and also identified intraspecific genetic diversity within this important functional gene. Following this, Milcic-Terzic *et al.* (2000) isolated diesel, toluene and naphthalene-degrading microbial consortia from diesel-polluted soils. Using PCR with gene-specific primers, they screened for the presence of the catabolic genes, *xylE* and *ndoB*, responsible for toluene/xylene and naphthalene biodegradation, respectively, from petroleum and diesel-polluted soils. These genes were targeted in order to assess the bioremediation potential of microbial consortia in petrol and diesel-polluted soils (Greer *et al*. 1993).

Some microorganisms, e.g. nitrogen-fixing microbes, are difficult to culture due to their specialised growth requirements and physiology limiting simultaneous cultivation of several species (Widmer *et al*. 1999). Molecular methods for identifying nitrogen-fixing *Bacteria* and *Archaea* are now available through the design of broad-spectrum highly degenerate primers. Widmer *et al*. (1999) designed a set of nested degenerate primers based on the amino acid sequence of the *nifH* gene. This is the general marker gene in nitrogen-fixing bacteria and encodes the enzyme nitrogen reductase. Similarly, Zehr and McReynolds (1989), Simonet *et al*. (1991) and Yeager *et al.* (2005), successfully designed three more sets of degenerate primers for universal targeting of the *nifH* gene in microorganisms. The *nif*

gene operon structure and regulation have been relatively conserved during evolution, making it a good candidate for focus in diversity studies (Gussin *et al.* 1986).

South Africa is an oil- and petrol-producing country with a large mining industry. However, what makes the potential threat of PAH pollution in the country unique, is that it produces synthetic fuel, which comprises approximately 40% of the final petroleum product. These production processes can lead to severe pollution of manufacture and mine sites. Soils at these sites are often rendered sterile for plant growth due to extensive pollution making rehabilitation essential. No biomolecular studies of polluted soils have yet been conducted in South Africa. Thus, five interdependent, molecular and/or DGGE-based studies were undertaken to gain a better understanding of species diversity, culturable and unculturable, and PAH degradation potential from PAH-polluted soils in South Africa.

The purpose of this thesis was to provide a basis for studies of microbial community diversity in PAH/PCB polluted soils in South Africa through the use of DGGE as a species diversity and richness evaluation technique and included the following objectives:

- To compare bacterial microbial community diversity in polluted and unpolluted soils at various sites in South Africa employing the techniques of DGGE, phylogenetic and distance studies.

- Comparison of community diversity between pro- and eukaryotes found within polluted and non-polluted soil at a site located in Mpumalanga Province, South Africa.

- To assess the possibility of PAH/PCB metabolism by the organisms within the samples being studied by determining the presence of *xylE* and *ndoB* genes, responsible for aerobic toluene/xylene and naphthalene metabolism, respectively.

- To identify, by sequence analysis of a portion of the 16S bacterial gene, eight isolates representing the most dominant culturable bacterial taxa found in polluted soils and to establish the hydrocarbon degrading capacity of the isolates using catabolic gene probes for *xylE* and *ndoB* genes.

- It was hypothesised that bioremediation could be enhanced by nitrogen addition to polluted environments. Thus the soils' capacity for nitrogen fixation was estimated by screening for the presence or absence of the *nifH* gene, the general marker gene of nitrogen-fixing bacteria.

Chapter 2

Literature review

Literature review

2.1 Introduction

2.1.1 The fossil fuel industry in South Africa

Energy to drive the South African economy is derived from various fossil fuel related sources, all of which can have a significant environmental impact. These fossil and other non-renewable fuel sources comprise oil, natural gas, coal, hydropower, nuclear power and biomass (Fig. 2). However, South Africa is unique in that it manufactures synthetic liquid fuel from coal and gas, known as synfuel (Fig. 3). Approximately 40% of South African liquid fuel requirements are met by synfuels, courtesy of Sasol (*ca*. 35%) and PetroSA (*ca*. 5%) (Surridge[1], personal communication). The synfuel industry was initially constructed to address supply security issues and this technology is now being exported, e.g. a new Sasol plant in Qatar (Surridge[1], personal communication).

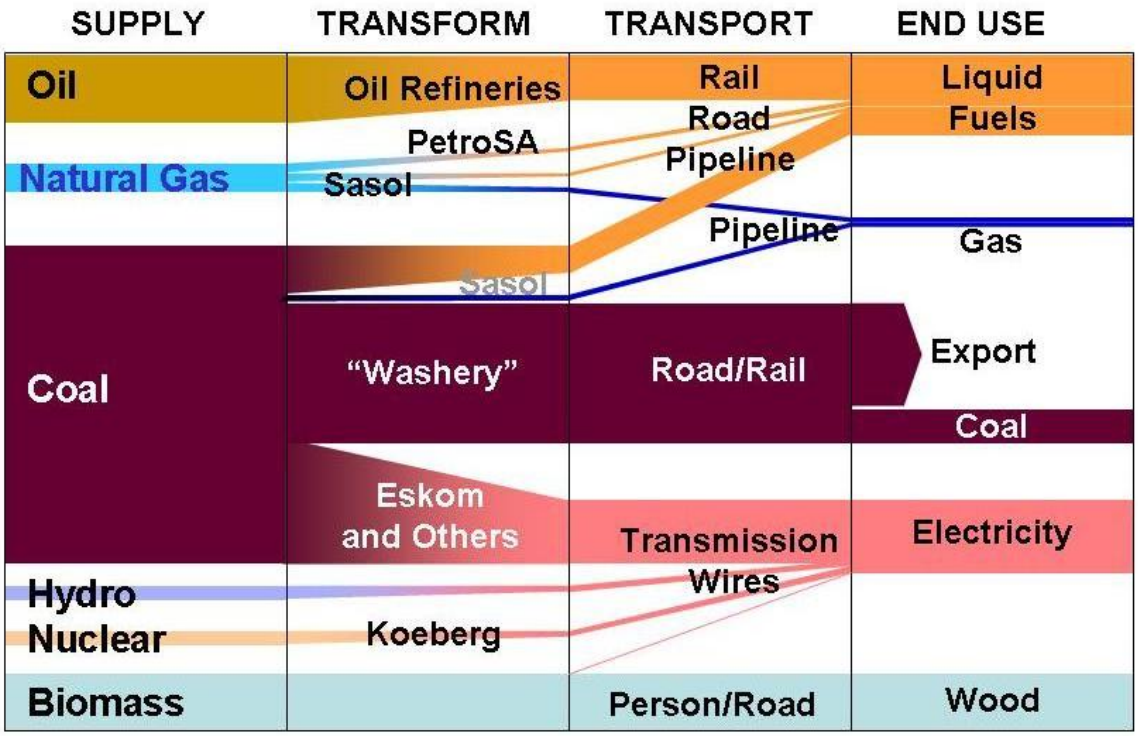


Figure 2: Energy flow from primary energy supply to final use – roughly to scale (Department of Minerals and Energy 2003).

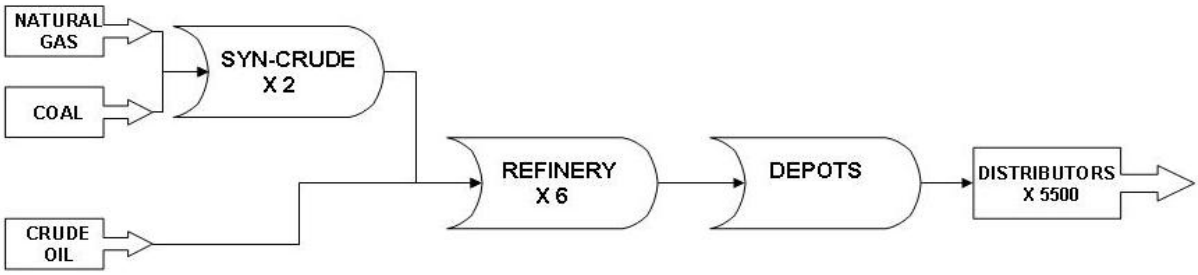


Figure 3: The petroleum product supply chain for South Africa (Surridge[1], personal communication).

The potential threat of crude oil leaks or spills from storage tanks is massive when considering that the approximate coastal crude oil storage capacity is at least 10.4 million barrels at the main storage unit in Saldanha Bay, plus operational stocks at the six refineries countrywide. Coastal and inland refined product storage must be maintained at 1.15 billion litres, a 21-day supply, since 20 billion litres of all fuel types are used annually in South

Africa (Surridge[1], personal communication). Possible leaks from high-pressure (maximum 100 bar petroleum products and 59 bar gas) underground petroleum and gas pipelines transporting fuel inland should also be considered, as 3000 km of pipelines ranging in diameter from 150-508 mm pose a potential threat to surrounding soil (Fig. 4). Total product throughput within the pipelines is 16 billion litres per annum liquid fuel and 450 million cubic metres of gas (Petronet SA 2005). Currently pipelines extend across five provinces of South Africa and construction is underway of a new multi-products pipeline between Durban and Gauteng (Petronet SA 2005). Approximately 5500 garages nationwide store refined fuel in underground storage facilities, hence posing a further risk of soil pollution should these tanks leak (Surridge[1], personal communication).

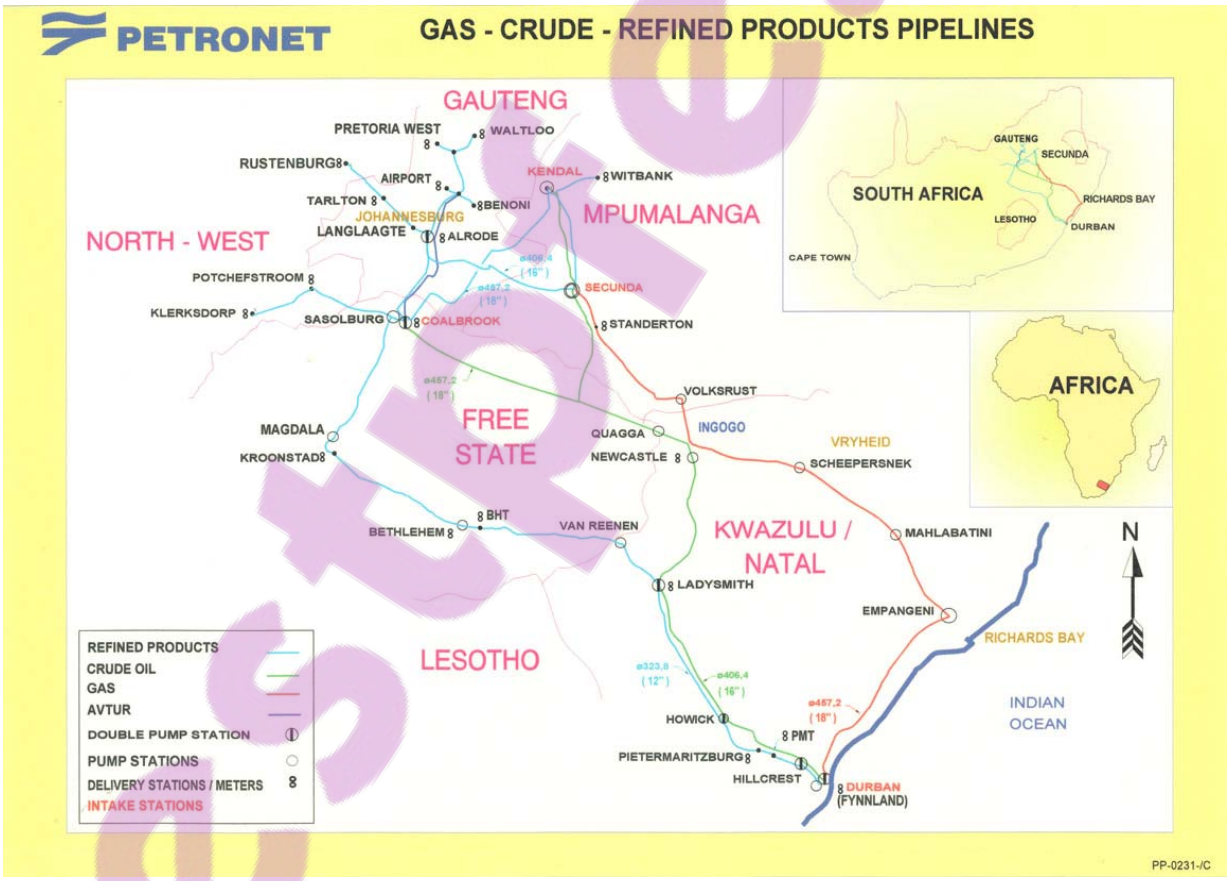


Figure 4: Areas in South Africa where refined oil products, crude oil, gas and avtur (non-jet engine aviation fuel) are delivered by pipeline, stored, transported and distributed (Petronet SA 2005).

Finally, the end-user, in the form of vehicles, is also a major potential source of pollution due to movement, during accidents and also as a result of oil and petrol leaks from engines. During 2005, South Africa was the best performing automobile market internationally, with domestic sales and production rising to all time highs. New vehicle sales amounted to 565 018 units, a 25.7% increase from 2004. During 2004 sales improved by 22.0%, reaching 449 603 vehicles compared with 368 470 units sold during 2003 (Fig. 5) (NAAMSA 2005). As a result of so many new vehicles coming onto the roads annually in South Africa, as well as the vehicles still on the roads at the end of each year, the potential for random point pollution caused by commercial and passenger vehicles can currently be assumed to increase by approximately 26% annually.

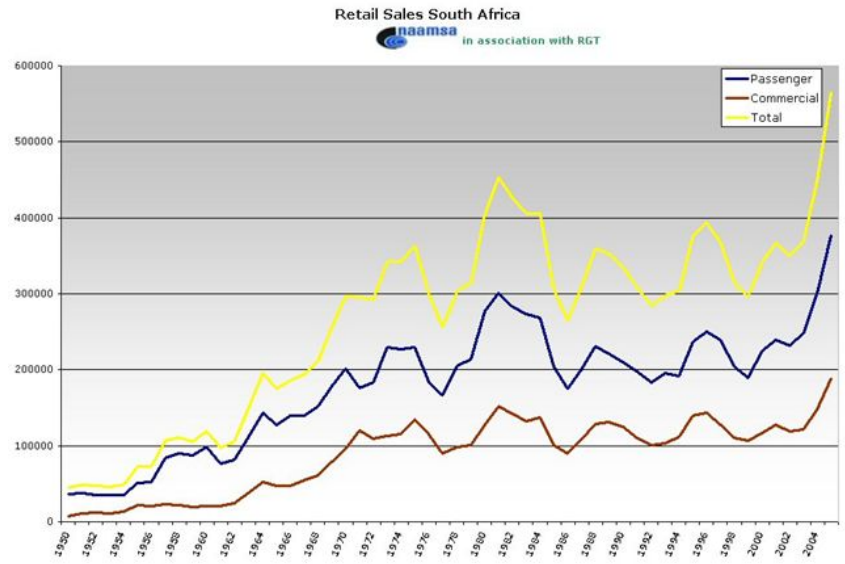
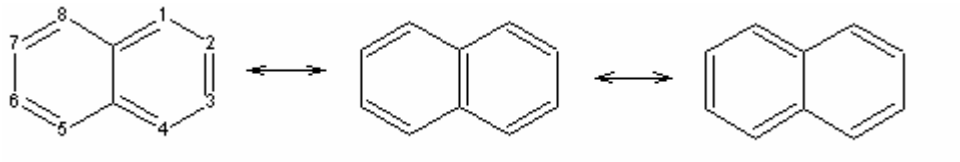


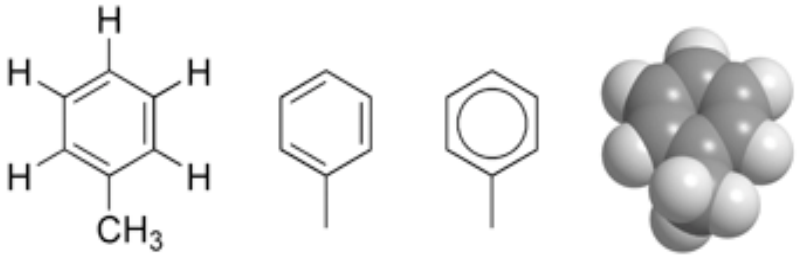
Figure 5: Passenger, commercial and total vehicle sales in South Africa from 1950 to 2005 (NAAMSA 2005).

Naphthalene and toluene, found in petroleum and diesel products, are two of the most common PAHs that are subject to biodegradation. Naphthalene is a crystalline, aromatic, white, solid hydrocarbon, it is volatile and forms a flammable vapour. The name is derived from the Latin *naphtha*, meaning liquid bitumen, and is of Semitic origin. It consists of two fused benzene rings, is classified as a benzenoid PAH, and is manufactured from coal-tar.

When converted to the phthalic anhydride, it is used in the manufacture of plastics, dyes and solvents, and as antiseptic and insecticide (Wikipedia 2005b):



Toluene, also referred to as methylbenzene or phenylmethane, is a clear, water-insoluble liquid. The name is derived from *toluol*, referring to tolu balsam, an aromatic extract from the tree *Myroxylon balsamum* (L.) Harms from which it was first obtained (Wikipedia 2005c). It is an aromatic hydrocarbon with a methyl side-chain, widely used as an industrial feedstock, octane booster in fuel, solvent in paints, rubber, printing, adhesives, lacquers, in leather tanning, disinfectants, and in the production of phenol, polyurethane foams and TNT (Wikipedia 2005c):



2.1.2 Soil health

Soil health can be defined as "the capacity of soil to function as a vital living system to sustain biological productivity, promote environmental quality and maintain plant and animal health" (Doran and Zeiss 2000). Productivity of conventional agricultural systems largely depends on the functional process of soil microbial communities (Girvan *et al.* 2003). These communities' structure and diversity are influenced by the soil structure and spatial distribution as well as the relationship between abiotic and biotic factors of microbial communities (Torsvik and Øvreås 2002). With the advent of various types of industries over the past 200 years, the ecology of earth's ecosystems has been severely disrupted. The commercialisation, extraction, refining, transportation, distribution and storage of petroleum

products have led to oil, petrol and diesel pollution of soils. In petrol-polluted water that may seep into soil, benzene, toluene, ethylbenzene and xylene (BTEX) isomers are present in the water-soluble fraction (Prenafeta-Boldú *et al.* 2002). This disruption has decreased biodiversity and selected for cosmopolitan microbial species better adapted to survive in the changed environment (Kozdrój and Van Elsas 2001). Not least impacted by these changes are the microbiota inhabiting the soil.

2.1.3 Pollution

Hydrocarbons are currently the main source of the world's energy resources due to the energy they produce when combusted. This also makes them the world's main source of pollution in the case of spills and waste products. There are essentially three types of hydrocarbons, viz. (i) aromatic hydrocarbons that have at least one aromatic ring, (ii) saturated hydrocarbons, including n-alkanes, branched alkanes and cycloalkanes that do not have double-, triple- or aromatic-bonds, and (iii) unsaturated hydrocarbons with one or more double- or triple-bonds between carbon atoms, referred to as alkenes and alkynes, respectively (Atlas 1981; Wikipedia 2006a). The most notorious class of hazardous compounds found in petrol, diesel, oil, as well as in coal-tar and its derivatives, are the PAHs. Polyphenols and PAHs are common industrial pollutants and are found as co-contaminants in the environment. They are hydrophobic organic compounds consisting of two or more benzene rings fused into a single aromatic structure. They may form naturally from burning of organic matter or from production and partial combustion of fossil fuels (Joner *et al.* 2002). Hopanes, complex alicyclic compounds, are of the most environmentally persistent components of petroleum spillage (Atlas 1981). Mammalian liver enzymes (cytochrome P-450 and epoxide hydrolase) oxidise certain PAHs to fjord- and bay-region diol-epoxides which, in turn, form covalent adducts with DNA (Bogan *et al.* 2001). Due to this, many PAHs promote effects similar to other carcinogens, once taken up by the body (Guerin 1999; Bogan *et al.* 2001). Sixteen

PAHs have been included in the United States Environment Protection Agency's priority pollutant list (Bogan *et al.* 2001).

2.1.4 Plants and phytoremediation

The presence of plant rhizospheres in hydrocarbon-polluted soils facilitates an increase in microbial numbers and metabolic activity within the soil. Studies have shown that root length, surface area, volume and diameter play a role in the rehabilitative effect of plants in crude oil-polluted soil (Merkl *et al.* 2005). Roots can also improve the physical and chemical properties of pollutant-stressed soil, besides increasing contact between microbes associated with plant roots and pollutants in the soil (Aprill *et al*. 1990). This effect was first described by Hiltner (1904), who defined the rhizosphere as the zone of soil in which microbes are influenced by plant root systems and where soil organisms have an impact on plants. Microbes isolated from the rhizosphere may have root growth-promoting or growth-inhibiting properties (Kuiper *et al*. 2004). Studies of plant species involved in phytoremediation have indicated that various grass species and leguminous plants are suitable for biodegradation. It is known that gram-negative rods such as *Pseudomonas* species dominate the rhizosphere (Kuiper *et al*. 2004). Some success in rehabilitation of hydrocarbon-polluted soils has been achieved by phytoremediation. It is defined as the use of plants to remove, destroy or sequester hazardous substances from the environment (Glick 2003). It has been documented that remediation of hydrocarbon-polluted sites is enhanced by cultivation of plants (Merkl *et al.* 2005).

Plants can reduce hydrocarbon levels in the soil, although the mechanism by which this happens is not yet entirely understood. Phytoremediation depends greatly on the stimulation of rhizosphere microorganisms by plant roots (Tesar *et al.* 2002). However, hydrocarbon uptake is limited by the lipophilicity of the hydrocarbons in question, which affects their

passage through the cell membrane. This uptake is thought to be attributed to increased microbial activity in polluted soils, as supported by community levels of degrading bacteria increasing during phytoremediation (Wünsche *et al.* 1995; Siciliano *et al.* 2003). BTEX isomers are the most amenable to elimination from the environment by indigenous microorganisms though degradation can be impeded by the natural ecological system (Koizumi *et al.* 2002). Most polluted environments are anoxic, and since aerobic degradation of hydrocarbons is faster than anaerobic processes, their removal can be less efficient in a polluted environment (Koizumi *et al.* 2002).

A variety of grass species, legumes and fast-growing trees such as poplar, alder and willow, with high transpiration rates, have been used in phytoremediation (Jordahl *et al.* 1997). Such plants have extensive root systems that provide large root surface areas available for soil contact. Merkl *et al.* (2005) proved that larger root surface areas are proportionately related to petroleum hydrocarbon degradation levels in the plant genera *Brachiaria*, *Cyperus* and *Eleusine*. Plant roots provide attachment sites to microbes and a source of nutrients, consisting mainly of organic acids, including amino acids, as well as sugars and complex carbohydrates, in the form of exudates (Mehmannavaz *et al*. 2002; Tesar *et al*. 2002). By way of example, Jordahl *et al*. (1997) reported that the number of microbes degrading benzene, toluene and xylene are five times higher in the rhizosphere of poplar trees than in surrounding soil. Successful rhizoremediation by plants depends on factors such as primary and secondary metabolites, colonisation, survival and ecological interactions with other organisms. In addition, the mucigel secreted by root cells, lost root cap cells, starvation of root cells and the decay of complete roots also provides nutrients (Reilley *et al*. 1996). Thus, plant roots have been suggested as a substitute for tilling of soil to incorporate additives and to improve aeration as a method of remediation (Kuiper et al. 2004). A broad phylogenetic range of bacteria, including the genera *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacteium*, *Norcardia*,

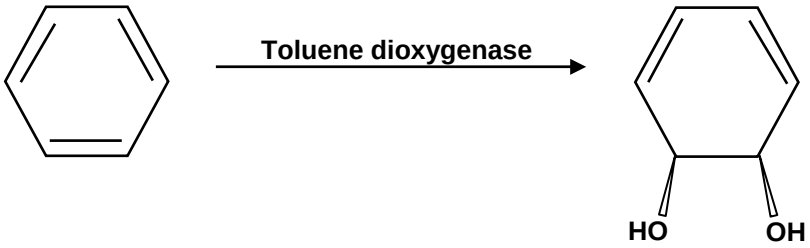
Pseudomonas, *Rhodococcus*, *Sphingomonas* and *Xanthomonas* are involved in the breakdown of hydrocarbons (Tesar *et al*. 2002).

Soil microbial communities are influenced by plant roots in various ways, e.g. excretion of organic compounds and competition for nutrients and attachment surfaces. Kuiper *et al*. (2004) reported that *Cyperus esculentus* L., *Eleusine coracana* (L.) Geartn. and *Brantha serratia* L. rhizospheres accommodate a large variety of bacteria. This probably is due to their ability to harbour large numbers of microorganisms on their highly-branched root systems. Plants with extensive root systems provide larger root-soil surface areas for attachment of microbes (Tesar *et al.* 2002). Plants influence soil pH, moisture and oxygen content by secretion of substances into the surrounding rhizosphere (Schroth and Hilderbrand 1964). Root exudates are common to all higher plants and are known to influence the abiotic and biotic environment of the rhizosphere (Schroth and Hilderbrand 1964). Studies characterising the culturable rhizosphere bacteria showed that plants have specific effects on communities. However, these bacteria represent only a very small component of those actually present in soil (Duineveld *et al.* 2001).

2.1.5 Biodegradation

Indigenous microorganisms, including bacteria and fungi, are able to degrade PAHs in soil, leading to *in situ* rehabilitation of the soils. Bioremediation of hydrocarbon-polluted soils using microbes for detoxification and rehabilitation is an efficient, economic and versatile environmental treatment. PAH-degrading microbes are pervasive in ecosystems where pollutants may serve as carbon sources, and seem to establish themselves soon after pollution occurs (Margesin *et al*. 2000). The reclamation of polluted land reduces the possibility that groundwater will become polluted, and enhances the rate of biodegradation (Gibson and Parales 2000; Mishra *et al.* 2001). It has been shown that hydrocarbon-degrading bacteria are

ubiquitously distributed in natural pristine environments. Wünsche *et al.* (1995), for instance, reported a 3.6% baseline community of hydrocarbon utilising bacteria that increased on addition of hydrocarbon pollutants. Thus, natural degradation of pollutants in low-risk oil-polluted sites is a cost-effective rehabilitation alternative to more traditional clean-up procedures (Gibson and Parales 2000; Margesin and Schinner 2001). Microbes have also been shown to use BTEX compounds as electron-donors in their metabolism, thereby facilitating pollution remediation in affected sites (Stephen *et al*. 1999). Supporting this, Wünsche *et al.* (1995) reported that substrate utilisation patterns in the Biolog system changed upon addition of hydrocarbons. Previously pristine soil bacterial communities shifted to a predominantly *Pseudomonas* population with hydrocarbon degradation capability, thus demonstrating a natural bioremediation adaptation potential. Similarly, Maila *et al*. (2005b), using a combination of Biolog™ and molecular methods, found that pollution removal by indigenous microbial communities at different soil levels was 48% in topsoil, 31% at 1m deep and 11% at 1.5m deep. Thus, PAHs and phenols have been shown to be biodegradable under appropriate conditions (Guerin 1999). However, the most readily degraded hydrocarbons are the n-alkanes with a relative molecular mass of up to n-C_{44} (Atlas 1981). Biodegradation of these n-alkanes commences via a mono-terminal attack, forming a primary alcohol, an aldehyde and a monocarboxylic acid. Further degradation is via β-oxidation forming a two-carbon unit, shorter fatty acids, acetyl co-enzyme A and CO_2 (Atlas 1981). Various bacteria are known to catabolise two- to four-ring PAHs as sole source of carbon, thus rendering them good candidates for site remediation applications (Bogan *et al.* 2001). This catabolism takes place using aromatic hydrocarbon dioxygenases within multicomponent enzyme systems (Samanta *et al*. 2002). Dioxygen is added to the aromatic nucleus of the PAH in question, forming an arene cis-diol as follows:



(Gibson and Parales 2000)

It has been hypothesised that metabolic engineering may improve microbial capacity for degradation of toxic compounds. However, the efficiency of naturally occurring organisms capable of this metabolism could be enhanced by optimising bioavailability, adsorption and mass transfer (Samanta *et al*. 2002). Widada *et al*. (2002) isolated 19 PAH-degrading bacterial species belonging to the genera *Ralstonia*, *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Comamonas*, *Flavobacterium* and *Bacillus* from environmental samples in Kuwait, Indonesia, Thailand and Japan. Enrichment cultures from these samples were supplemented with either naphthalene or phenanthrene as sole carbon source and multiple phenotypes, in terms of utilisation and degradation metabolism, were observed. Tesar *et al.* (2002) listed a broad range of bacterial genera capable of hydrocarbon breakdown, including *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Xanthomonas*. In addition to this, Riis *et al.* (2003) found certain bacteria capable of bioremediation of diesel-polluted soils under high salinities. Bacteria from the genera *Cellulomonas*, *Bacillus*, *Dietzia* and *Halomonas* rehabilitated soils with a salinity of up to 15% (Riis *et al*. 2003). Recently, Kleinsteuber *et al*. (2006) determined that salinity affects the dominant species in diesel-polluted soils differently, low salinity favouring *Sphingomonas* spp., higher salinities *Ralstonia* spp. and very high salinities the halophilic genera *Halomonas*, *Dietzia* and *Alcanivorax*. Some bacteria have been described to degrade specific PAHs in culture. Willison (2004), for instance, found a species designated *Sphingomonas* sp. CHY-1

capable of degrading chrysene as sole carbon source in culture after enrichment. More specifically, members of the *Providencia* genus are known to completely break down hexahydro-1,3,5-trinitro-1,3,5-tiazine (RDX) and nitroso-RDX, and have been used for this purpose in bioremediation (Kitts *et al.* 1994).

Ecto- and endomycorrhizal fungi are cosmopolitan and form symbiotic associations with the roots of plants (Linderman 1988). These endophytic fungi, particularly the ectomycorrhizae, aid plants in the absorption of nutrients from soil, especially immobile elements such as zinc, copper, sulphur, calcium, potassium, iron, magnesium, manganese, chlorine, boron and nitrogen. Absorption of phosphorus is enhanced by both ecto- and endomycorrhizae (Linderman 1988). Mycorrhizal fungi have been reported to reduce plant responses to other stresses such as high salt levels and noxious compounds associated with mine pollution, landfills, heavy metals and micro-element toxicity (Linderman 1988).

Bioremediation, by virtue of biodegradation, depends primarily on overcoming any nutrient limitations in the soil to be rehabilitated. Remediation of hydrocarbon-polluted soils is usually limited by the amount of free carbon, phosphorus and/or nitrogen present (Bogan *et al.* 2001; Margesin and Schinner 2001; Röling *et al.* 2002). However, Struthers *et al.* (1998) found that the herbicide atrazine could be degraded by *Agrobacterium radiobacter* in soil without addition of extra carbon or nitrogen sources, although inoculated cell numbers did not increase, indicating a state of survival rather than growth. Microbial community numbers can be increased by the injection of soluble nutrients just below the surface of the soil. This can, however, lead to excessive localised microbial growth in nutrient-injected areas, resulting in "biofouling" (Bogan *et al.* 2001). The use of gaseous formulations has been demonstrated to better distribute nutrients throughout a system for bioremediation purposes (Bogan *et al.* 2001). Rather than injecting nutrients, nutrient supplementing, particularly with nitrogen and phosphorus fertilisers, is known to enhance biodegradation of oil released into a marine

environment (Kasai *et al.* 2002). However, amendments to rectify nutrient deficiencies must be optimal as too high amounts may lead to eutrophication and too little may result in suboptimal biodegradation (Röling *et al.* 2002). Triethylphosphate (TEP) and tributylphosphate (TBP) are the safest phosphorus compounds that can readily be gasified and forced through deficient soil, whereas gaseous nitrous oxide has been used to supply nitrogen (Bogan *et al.* 2001). While not enhancing remediation of PAH-polluted soil, delivery of gaseous nutrients has been shown to expedite *in situ* remediation of soils polluted with chlorinated solvents, volatile organic compounds, C_4-C_{10} alkanes and monoaromatic hydrocarbons (Bogan *et al.* 2001). Lee *et al.* (2003) found that adding pyruvate at optimal levels to PAH-polluted soils as an additional carbon source, aided in the breakdown of PAHs (naphthalene used in model). They were able to determine the optimal concentrations of carbon sources for complete degradation of naphthalene by *Pseudomonas putida* G7.

Microorganisms intended for inoculation into polluted soils can be carried on various materials. Agricultural by-products are most commonly used to transfer microbes without affecting their degradative capacity (Mishra *et al.* 2001). In this respect, the rate and intensity of pollutant degradation in influenced by environmental factors such as the original indigenous microbial community, nutrient availability, oxygen levels, pH, temperature, moisture content, quality, quantity and bioavailability of pollutants, and soil properties (Margesin *et al.* 2000). Although bioremediation is the primary mechanism involved in removal of soil pollutants, other processes such as dispersion, dilution, sorption, volatilisation and abiotic transformation are also instrumental in the rehabilitation process (Margesin and Schinner 2001).

2.2 Rhizosphere

The rhizosphere is a niche that maintains indigenous soil microbial communities involved in the plant-soil nutrient cycle. It also plays a vital part in the survival of plants under adverse chemical soil conditions (Izaguirre-Mayoral *et al.* 2002). Phytoremediation uses rhizosphere technology in biodegradation enhancement. Plant health can be influenced by the promotion of production of phytohormones, furnishing of nutrients, nitrogen fixation, and the suppression of microbes detrimental to plants through antagonism (Da Silva *et al.* 2003). Siciliano *et al.* (2003) demonstrated that effective TPH phytoremediation systems promote the increase in numbers of bacteria with hydrocarbon catabolic genes. PAHs may be removed by volatilisation, photo-oxidation, sorption and leaching. This is enhanced by the presence of plants (Joner *et al.* 2002).

2.2.1 Exudates

Rhizosphere soil is modified with respect to pH, O_2, CO_2 and nutrient availability. Plants exude readily degradable substances into the soil that augment microbial activity in the rhizosphere (Schroth and Hildebrand 1964; Joner *et al.* 2002). These substances are released via volatilisation, leaching, exudation or decomposition and can influence the growth of other organisms in the soil, including that of nearby plants (Meissner *et al.* 1986).

The exact composition of root exudates in soil is unknown, mainly as a result of sloughing and autolysis of epidermal cells constantly affecting the environment (Schroth and Snyder 1961). However, three aspects of modified soil characteristics in the rhizosphere contribute to phytoremediation of organic pollutants, viz. higher microbial activity, higher oxidation potential, and modified microbial community (Joner *et al.* 2002). Plant secondary compounds (exudates) found in rhizosphere soil can include polyphenols and flavanoids. Some of these

compounds are suppressive to microbial growth while others enhance it (D'Arcy Lameta and Jay 1987). Thus, microbial communities within the rhizosphere are definitely affected by the type of root exudates produced by plants. In combination with bacterial PAH-degradative ability, plant roots contain soluble and wall-bound oxidative enzymes that are directly implicated in PAH-degradation (Joner *et al.* 2002). Phytoremediation systems, including the plant and its microbial rhizosphere community, can therefore be implemented as a means of increasing the hydrocarbon degradation potential of soil, but fertilisation is required for maximum results (Siciliano *et al.* 2003).

2.2.2 Microbial communities

The "population concept" is central to the fields of ecology, evolutionary biology and conservation biology. Krebs (1994) defined a population as "a group of organisms of the same species occupying a particular space at a particular time". Waples and Gaggiotti (2006) recently reviewed the definition of a population when considered in the context of ecological and evolutionary paradigms, and suggested several criteria for determining when groups of individuals are different enough to be considered separate communities. A natural population is bounded by ecological or genetic barriers only, for example within a local population individuals interact ecologically and reproductively. Based on this interaction, Waples and Gaggiotti (2006) concluded that a cluster of individuals without using locality sampling information detects true communities only under moderate to low gene flow. Therefore, for the purposes of this thesis, studying a large number of different species interacting within an environment will be referred to as studying a community. Thus, due to gene flow between communities within a community, it follows that the fairly recent advent of DNA markers has led to a great interest in studying natural communities genetically.

Soil microbial communities are relatively evenly distributed in unpolluted environments. However, Smalla *et al.* (2001) proved that there is a reduced evenness in the rhizosphere compared to unplanted soil. Zhou *et al.* (2002) examined microbial communities in 29 different soil types. They found that in low-carbon soils the diversity pattern of the surface soil was evenly distributed, while subsurface samples exhibited a distinct pattern. High-carbon soils, by contrast, displayed uniform diversity throughout the soil layers examined, indicating that spatial isolation differences in community structure could be overcome when the carbon content of a soil is high.

The general assumption stands that higher microbial diversity is proportional to an increased catabolic potential (Dejonghe *et al.* 2001). This can be extrapolated to imply that high species diversity leads to more effective removal of metabolites and pollutants from a substrate. Improving the bioremoval capacity of the soil by inoculating specific strains or consortia of microorganisms is referred to as bioaugmentation (Halden *et al.* 1999; Dejonghe *et al.* 2001). Two components constitute diversity in an environment, viz. total number of species present (species richness/abundance) and species distribution (species equitability) (Dejonghe *et al.* 2001). To promote and increase the degradative potential of a microbial community, competence for certain reactions under the conditions is required, implying that genes within the system need to be activated to participate in the energy flux of the environment (Dejonghe *et al.* 2001).

2.2.3 Assessment of species richness and diversity

Several methods are available to determine the richness of diversity in an environment, including different plating methods, light and fluorescence microscopy, and DNA and RNA analysis (Dejonghe *et al.* 2001; Duineveld *et al.* 2001; Torsvik and Øvereås 2002). There are some general limitations to be taken into account when studying microbial diversity. Spatial

heterogeneity is noteworthy since most environmental replicates consist of 1-5g of sample material, which does not give a true reflection of the spatial distribution of microorganisms (Kirk *et al*. 2004). Culturing colony-forming units (cfu) on different media was the most popular method for investigating microbial diversity. However, most bacteria targeted for isolation from environmental samples are difficult to culture due to constraints imposed by artificial media on which they are to be grown (Sekiguchi *et al.* 2002). Culture-based methods are tedious and certain organisms, e.g. mycobacteria, can take a long time before starting to grow. Only 1-10% of global bacterial species are culturable due to the selectivity of growth media and conditions (McCaig *et* al. 1999; Von Wintzingerode *et al.* 2002; Kirk *et al.* 2004). Less than 1% of microbes from soils in polluted environments are culturable (Stephen *et al*. 1999). Respiration analysis of individual cells within soil samples indicated higher numbers of metabolically active bacteria than the number of culturable bacteria (McCaig *et* al. 1999). Thus, both microscopy and plating lack the capacity to discriminate between multiple bacterial communities and to assess their diversity (Duineveld *et al.* 2001). Furthermore, should an organism be cultured on an artificial medium, substances produced by the organism in culture can either inhibit or stimulate growth of other microbes. These substances may have a markedly reduced effect once introduced into soil as an ameliorant due to pH, adsorption by clay and microbial utilisation, all of which can influence the rhizosphere (Schroth and Hilderbrand 1964).

Molecular methods have provided a more accurate view of species richness within diversity. Initially, random fragments of environmental genomic DNA were cloned and those containing rRNA genes were selected for sequencing (Dejonghe *et al.* 2001). The next advance in molecular analysis came when PCR was used to selectively amplify these rRNA genes from total microbial community DNA, using different sets of primers to amplify the genes from all types of organisms (Archaea, Bacteria, Eukarya) (Dejonghe *et al.* 2001; Torsvik and Øvereås 2002). Ahn *et al*. (1999) probed DNA from PAH-polluted soil for

naphthalene and other PAH metabolism. They found that most PAH-degrading bacteria had a NAH7-like genotype using the *nah*A probe, and only 15% were not detected using this probe. New gene probes were thus suggested for enumeration of PAH-degrades. The next logical step from this technology was that mixed PCR fragments could be cloned and sequenced or be separated and visualised by various fingerprinting techniques, e.g. DGGE (Dejonghe *et al.* 2001; Duineveld *et al.* 2001). However, these techniques are only as efficient as their methodologies, i.e. efficient cell lysis, maximum unsheared DNA extraction, unbiased PCR amplification and effective downstream analysis (Kirk *et al*. 2004).

2.2.4 Remediation

Several methods are available for determining the level of remediation in polluted soils. Screening for the disappearance of pollutants can be achieved by monitoring toxicity in a test organism for product or change. Classically, species used for toxicity response have been *Ceriodaphnia* (crustacean of the family Daphniidae) and *Pimephales promelas* Rafinesque (a fish, commonly known as "Fathead minnow", of the family Cyprinidae) in water, and several invertebrates in soils (White *et al.* 1998). However, analysis of microbial communities have since proved to be a far more comprehensive indicator of residual pollutants. Monitoring the return of a baseline community is used to indicate that the biological community of a soil is returning to normal (White *et al.* 1998). Li *et al*. (2006) found that species of tolerant bacteria in heavy metal-polluted soils increase in numbers with time and further pollution and can consequently be indicative of the level of heavy metal pollution and thus of soil quality.

Rhizosphere microflora are not easily destabilised due to the buffering effect of the biotic and abiotic surroundings they inhabit (Bahme *et al.* 1988). Research has shown, however, that the rhizosphere microflora can be altered by inoculation of plant roots with specific rhizobacteria. The capacity of the shift in microflora depends on several factors, e.g. the nature of the

introduced strain, the effectiveness of its colonisation and its ability to persist on root systems for a prolonged period (Bahme *et al.* 1988). The inoculum size and mode of delivery affects the community dynamics within the soil, i.e. community density declines proportionately to the distance from the point/source of inoculation (Bahme *et al.* 1988). Two delivery systems for applying rhizobacteria to underground plant organs have been described by Bahme *et al.* (1988), namely bacteria-impregnated granules that are mechanically incorporated into soil before planting, and low-pressure drip-irrigation systems containing the desired bacterial strain.

Burkholderia species are regularly isolated from plant rhizospheres, thus making them good potential agents for rhizoremediation. O'Sullivan and Mahenthiralingam (2005) found *Burkholderia* to be the predominant genus isolated from PAH-polluted soils. Of the various *Burkholderia* strains, six (CSV90, EML1549, K712, RASC, TFD2 and TFD6) also capable of 2,4-dichlorophenoxyacetate degradation. *B. xenovorans* strain LB400 is an aerobic degrader of polychlorinated biphenyls (PCBs) using the enzyme biphenyl-2,3-dioxygenase. This species can break down up to hexachlorinated biphenyls when supplemented with maltotriose esters to increase water solubility and hence bioavailability (O'Sullivan and Mahenthiralingam 2005). *B. vietnamiensis* strain G4 is able to co-metabolise trichloroethylene (TCE), which is an organic pollutant in groundwater aquifers, and toluene or phenol, using the enzyme toluene *o*-monooxygenase. Strain G4 has been extensively studied and is subject to two US patents, 4925802 and 5543317 (O'Sullivan and Mahenthiralingam 2005). Strain G4 preferentially degrades toluene in culture and therefore toluene levels have to be maintained to achieve maximum (100%) TCE biodegradation. Since toluene and phenol cannot be used during *in situ* environmental rehabilitation, a mutant of the G4 strain, PR1, which does not require additional nutrients, has been engineered to remove most TCE within a few weeks. Despite this, the G4 strain still proved to be a more efficient bioremediator. A mutant toluene *o*-monooxygenase gene was therefore spliced from G4 into *Escherichia coli* to

yield an organism with a higher rate of TCE degradation and with an enhanced PAH as well as naphthalene degradation capacity (O'Sullivan and Mahenthiralingam 2005).

Petrol and diesel, as well as crude oil spills in soils at fuel stations, have been found to be bioaugmented to a certain extent by members of the genera *Micrococcus, Corynebacterium, Flavobacterium, Bacillus* and *Pseudomonas* (Rahman *et al*. 2002). More specifically, pentachlorophenol was remediated with *Flavobacterium* and *Arthrobacter*, whereas augmentation of 2,4,5-trichlorophenooxyacetic acid with *Rhodococcus chlorophenolicus* and *Pseudomonas cepacia* accelerated its removal (Halden *et al.* 1999). Petroleum PAHs in a marine environment are known to be biodegraded by bacteria belonging to the genus *Cycloclasticus* (Kasai *et al.* 2002). Less species-specifically, Da Silva *et al.* (2003a) found a number of *Paenibacillus* species to have agricultural importance due to their ability to degrade several PAHs.

There has been much focus on the use of bacteria for bioremediation purposes in recent research. However, fungi may also play an important role in the rehabilitation process. In general, fungi are capable of tolerating harsher environmental conditions than bacteria and could well be involved in the degradation of petroleum hydrocarbons in soil (Prenafeta-Boldú *et al.* 2002). Da Silva *et al*. (2003b) isolated filamentous fungi from estuarine sediments in Brazil and monitored their ability to degrade PAHs, particularly pyrene, in culture. They found a *Cyclothyrium* sp. to be the most efficient, simultaneously degrading 74, 70, 59 and 38% of pyrene, phenanthrene, anthracene and benzo[a]pyrene, respectively. Additionally, toluene, ethylbenzene and xylene have been shown to be degraded by a *Cladophialophora* sp. (Prenafeta-Boldú *et al.* 2002).

2.3 Techniques for culture-independent assessment of microbial communities

Culturable proportions of bacterial communities from the environment are negligible compared with the number of species that are present. Thus, culture techniques for environmental bacterial community diversity analysis are becoming obsolete. Øvereås and Torsvik (1998) compared culturable bacterial diversity of agricultural soil communities with diversity obtained by molecular means. They found that molecular methods revealed a much higher bacterial diversity than classical isolation techniques, and concluded that bacterial diversity studies should embrace entire communities, not only the culturable portion.

Several molecular techniques have been developed to identify and determine species diversity of microorganisms without isolation (Kawai *et al.* 2002). PCR-based techniques are becoming increasingly popular for research ranging from diagnostic work to genome fingerprinting and probing (Torsvik and Øvereås 2002). PCR is regularly applied to assay environmental samples due to the ability of the technique to detect relatively small numbers of target organisms without requiring cell culture (Volossiouk *et al.* 1995). Thus, PCR can be used to target certain types of genes expected within specific communities and performing specialised functions. Sei *et al.* (2003) developed a set of primers for detecting and monitoring alkane-degrading bacteria. The primers were designed to target the homologous regions of alkane hydroxylase genes (alk genes) and thus assess the alkane-degrading potential of a particular environment. These primers were tested on communities capable of degrading n-alkanes, the major component of crude oil. According to Sei *et al*. (2003) it was found that shorter n-alkane chains were degraded first by Group I alkane-degrading bacteria, whereas Group III alkane-degrading bacteria degraded longer chains later. However, as with most techniques there are some drawbacks to using PCR, e.g. preferential amplification of certain types of sequences, chimeric sequence generation and false results due to pollution (Osborne *et al.* 2005). Despite this, PCR remains reliable and forms the base-technique for

most molecular work.

Ribosomal RNA (rRNA) molecules are used as molecular chronometers due to their high degree of structural and functional conservation. Consequently, domains within rRNA molecules harbour independent rates of sequence change (Kent and Triplett 2002). Phylogenetic relationships can be determined by examining these changes over time (Kent and Triplett 2002).

Initial assessment of soils, using culture-independent methodologies, revealed the presence of three main bacterial divisions, viz. *Proteobacteria*, *Fibrobacter* and low GC gram-positive bacteria (Kent and Triplett 2002). Specific genes coding for enzymes that are known to be involved in hydrocarbon catabolism have been identified. Widmer *et al.* (1998), realising the potential of environmental microorganisms, specifically *Pseudomonas* species, developed a PCR protocol for selective detection of *Pseudomonas* (*sensu stricto*) in the environment. They designed a highly-selective primer pair for the 16 rRNA genes of *Pseudomonas* species that was used with 91.7% efficacy for bacterial identification from the environment based on sequence phylogeny. Following this, Milcic-Terzic *et al.* (2001) and Whyte *et al.* (2001) combined culture-dependent methods and molecular analysis using hydrocarbon catabolic gene probes *alkB* (C_6-C_{32} n-paraffin degradation), *xylE* (toluene and xylene degradation) and *ndoB* (naphthalene degradation) to demonstrate the presence of hydrocarbon-degrading microbes in polluted soils.

Nitrogen-fixing microorganisms can be instrumental in hydrocarbon pollution bioremediation (see 2.1.4). However, they are difficult to culture due to their different growth requirements and physiology limiting simultaneous cultivation of separate species (Widmer *et al*. 1999). Molecular methods for identifying the presence of nitrogen-fixing *Bacteria* and *Archaea* are now available through the design of broad-spectrum highly degenerate primers. *nifH* is the

general marker gene in nitrogen-fixing bacteria and encodes the enzyme nitrogen reductase. It has an extensive database of sequences available for comparative purposes. Rosado *et al.* (1998) studied the diversity of *nifH* gene sequences in *Paenibacillus azotofixans* and found sequence divergence at DNA level, but more conserved sequence at protein level, hence the design of degenerate primers. Widmer *et al*. (1999) followed suit and designed two universal sets of degenerate primers for nested PCR, based on the amino acid sequence of the conserved *nifH* gene.

2.3.1 Microbial community analysis

Microbial community analysis, independent of culturing the organisms, involves the extraction of signature biochemicals from the environmental samples (Blackwood *et al.* 2003). The first culture-independent estimate of prokaryotic organisms in soil indicated 4600 distinct genomes in one gram of soil (Torsvik *et al*. 1990a). Extracted DNA or RNA can, via molecular genetic techniques, facilitate microbial community analysis to be coupled with phylogeny. The uncultured diversity will reflect species closely related to known cultured organisms and also species from virtually uncultured lineages (Blackwood *et al.* 2003).

Characterisation of genes of microbes involved in the degradation of organic pollutants has led to the application of molecular techniques in microbial ecology of polluted areas (Milcic-Terzic *et al*. 2001). Molecular methods usually involve the separation of PCR amplicons on the basis of DNA nucleotide sequence differences, most often the 16S rRNA gene. However, taxonomic resolution of 16S rDNA sequences can be insufficient for discriminating between closely-related organisms in e.g. cyanobacteria, where the rRNA 16S to 23S internal transcribed spacer (rRNA-ITS) provided better distinction between species (Janse *et al.* 2003). Molecular methods include DGGE, ribosomal intergenic spacer analysis (RISA), single strand conformation polymorphism (SSCP), amplified ribosomal DNA restriction analysis

(ARDRA) and terminal restriction fragment length polymorphism (T-RFLP). Several of these methods, such as SSCP, ARDRA and T-RFLP, do not reveal diversity unless the community is very simple, due to only a very small number of species indicated in rehybridisation or sequence analysis being visualised on a gel (Nakatsu *et al.* 2000; Blackwood *et al.* 2003). However, catabolic gene probes can be used in nucleic acid hybridisations to characterise sequences (Milcic-Terzic *et al*. 2001). Laurie and Lloyd-Jones (1999) probed a set of genes isolated from *Burkholderia* Sp. RP007 involved in PAH catabolism. They found that the *phn* locus, containing nine open-reading-frames, codes for enzymes degrading naphthalene and phenanthrene.

A rapid means of determining the relative abundance of common species present in a given sample, which do not need to be culturable, is provided by molecular techniques. Gelsomino *et al*. (1999) found after extensive molecular fingerprinting that similar soil types (clay, sand, loam, etc.) tend to contain similar dominating bacteria. Thus it is evident that soil type affects the microbial community present and not only the type of pollution to which they are exposed. Bundy *et al.* (2002) found that comparative bioremediation experiments on different soil types, all polluted with diesel, did not lead to the eventual development of a similar microbial community. They concluded that different soils have different inherent microbial potentials to degrade hydrocarbons. Molecular methods also allow for the elucidation of major differences between communities for testing of hypotheses on the basis of sample comparison (Blackwood *et al.* 2003). However, they do not always reveal the organisms primarily involved in the main energy flux of the system. Soil microbial ecologists suggest that only a few organisms are directly significant at a particular site (Dejonghe *et al.* 2001). If these organisms are targeted for non-culture analysis, more information could be revealed. For example, Leys *et al*. (2005) characterised fast-growing mycobacteria in PAH-polluted soils by means of PCR primers that targeted 16S regions of the *Mycobacterium* genome. PCR-DGGE was then used to distinguish between different species and ultimately in

elucidating the phylogeny (genetic relatedness) of the PAH-degrading species.

2.3.2 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a most appropriate molecular method for monitoring microbial community ecology. Wamberg *et al.* (2003) utilised DGGE to map the bacterial component in the pea (*Pisum sativum* L.) rhizosphere community, and observed that chemical changes in the rhizosphere during plant growth were mirrored by concomitant changes within the bacterial community present. MacNaughton *et al*. (1999) used DGGE to identify community members responsible for bioremediation of a crude oil spill and to monitor community changes and pollution level reduction over time. DGGE relies on variation in genetic sequence of a specific amplified region to differentiate between species within microbial communities (Banks and Alleman 2002; Koizumi *et al.* 2002). PCR product is electrophoresed through a polyacrylamide gel containing a linear DNA-denaturing gradient. The band pattern on the gel forms a genetic fingerprint of the entire community being examined (Gillan 2004). Most commonly, 16S rRNA genes are used to give an overall indication of the species composition of a sample. Partial sequence of this gene has been analysed from environments as complex as soil (Throbäck *et al.* 2004). Bodelier *et al*. (2005) screened the methane-oxidising bacteria from freshwater marshlands using combinations of existing 16S primers. They found that, when combined, direct PCR of universal and specific primers yielded community profiles identical to those obtained from nested amplification.

Although 16S gene analyses presently are the most informative for broad community analyses, other genes can also be examined for community diversity. Functional genes have more sequence variation and can be used to discriminate between closely-related but ecologically different communities. Throbäck *et al.* (2004) exploited the *nirS*, *nirK* and *nosZ* genes involved in denitrification as more discerning community biomarkers. DGGE has even

been extrapolated to applications in plant protection research, including analysis of gut flora of several insect pest species, phylloplane and rhizosphere communities associated with different plant varieties, and the impact of biopesticides on natural microflora (O'Callaghan *et al.* 2003).

2.3.3 Single-strand-conformation polymorphism

SSCP of DNA is used in mutation detection and analysis. It involves the separation of single-stranded PCR rDNA products with the same number of base-pairs but a different conformational structure, on a polyacrylamide gel (Dejonghe *et al.* 2001). This technique has been adapted for the analysis of, and differentiation between, cultivated pure-culture soil microorganisms and non-cultivated rhizosphere microbial communities (Schwieger and Tebbe 1998). Under non-denaturing conditions, single-stranded DNA folds into sequence-dependent secondary conformations. These structures render different electrophoretic motilities to the molecules that can then be separated on a non-denaturing polyacrylamide gel. SSCP can be used in conjunction with an automated DNA sequencer to differentiate between species using PCR products of 16S rRNA (Schwieger and Tebbe 1998). A limitation of using this technique for community DNA analysis is the high rate of reannealing after denaturation, especially at high DNA concentrations. Another constraint of SSCP is the appearance of two bands on electrophoretic gels as a result of only double-stranded PCR product being obtained. Characteristically, three bands are observed on gels, one of a double-stranded product and two of the single-stranded DNA molecules from PCR. In some instances, there may be four or more bands visible on the gel due to differing structural conformations, e.g. hairpin folding due to palindromic sequences. Likewise, physical conformation of products may be similar, causing them to overlap in the gel, resulting in fewer bands being visualised on a gel. Finally, heteroduplex DNA strands with a similar sequence adhere together, forming breathing heteroduplexes of two or more PCR products (Schwieger and Tebbe 1998).

2.3.4 Amplified ribosomal DNA restriction analysis

Another PCR-based DNA-fingerprinting technique, which makes use of restriction of amplified fragments, is amplified ribosomal DNA restriction analysis (ARDRA). This technique yields a high number of bands per species, and therefore cannot provide reliable genotypic characterisation at community level (Dejonghe *et al.* 2001). It is, however, particularly suitable for monitoring communities and assessing microbial diversity, and can focus on specific sub-groups within a community (Dejonghe *et al.* 2001). Lagacé *et al.* (2004) made use of 16S rDNA sequencing of ARDRA fragments for identifying bacterial communities in maple sap. The ARDRA profiles yielded a dendogram illustrating relationships between bacterial strains, and γ-proteobacteria were found to be dominant throughout the year.

2.3.5 Reverse transcription PCR

RT-PCR involves the extraction of RNA instead of DNA, and profiles the metabolically active microorganisms in a system (Dejonghe *et al.* 2001). It is a dual-step process. The first step entails the production of complementary DNA (cDNA) from a messenger RNA (mRNA) template using dNTPs and an RNA-dependent reverse transcriptase at 37°C. The second step involves the use of a thermostable transcriptase and a set of upstream and downstream DNA primers. Temperatures fluctuating between 38-95°C facilitate sequence-specific binding of the primers to the cDNA and allow transcriptase to produce double-stranded DNA. After approximately 30 cycles, the original RNA template is degraded by RNAse H, leaving pure cDNA in solution. It is now possible to simplify this process into a single step by using wax beads, containing the required enzymes, that melt at the higher temperatures releasing their contents.

Exponential amplification via RT-PCR provides a highly sensitive technique that can detect very low copy number RNAs. This technique is widely used in the diagnosis of genetic diseases and in the quantitative determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression, e.g. Northern blot.

2.3.6 Base-specific fragmentation and mass spectrometry

Base-specific fragmentation of PCR-amplified 16S rDNA followed by mass spectrometry of the fragment pattern is being used for rapid identification of bacteria (Von Wintzingerode *et al.* 2002). This method is inherently accurate and rapid, making it attractive as a tool for high-throughput microbe identification in pharmaceutical and industrial applications.

2.3.7 Signature lipid biomarker analysis/environmental nucleic acid probes

Signature lipid biomarkers can be used in biomas shift monitoring. Signature lipid biomarker analysis/environmental nucleic acid probes (SLB/ENAP) are relatively inexpensive molecular fingerprinting techniques used to ascertain a quantitative measurement of the microcosm. Chemical extraction of phospholipid fatty acids from the soil can be useful in determining the diversity within the soil and in estimation of the microbial biomass (Banks and Alleman 2002). It determines when community ecology becomes analogous to a known community that is considered to be safe (White *et al.* 1998). Total cellular phospholipid fatty acids (PLFAs) are not stored in cells and thus have a rapid turnover in communities. These make ideal markers for monitoring viable biomass within a community viz. an increase in cis/trans monoenic PLFAs in cells is indicative of toxic stress within bacterial communities and thus results in a change in their growth phase (Stephen *et al*. 1999). Specific PLFA biomarkers can be used to indicate broad microbial community diversity encompassing bacteria, fungi,

algae, gram-negative and -positive organisms, sphingomonads, actinomycetes and sulphate-reducing bacteria. Limitations of PFLA analysis include shortcomings in analysis of gram-negative communities. These profiles are dominated by monoenoic, saturated and cyclopropane fatty acids that are broadly distributed and thus fairly uninformative with regard to gram-negative population structure. This method has been combined with nucleic acid-based analysis such as DGGE to allow for better community elucidation (Stephen *et al*. 1999).

2.3.8 Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent method used to obtain a genetic fingerprint of a microbial community and has been shown to be effective in discriminating between microbial communities in various environments (Blackwood *et al.* 2003). Automation increases sample throughput and accelerates analysis of bacterial communities (Kent and Triplett 2002). PCR product of 16S rDNA is used for analysis (Dejonghe *et al.* 2001). One end of the PCR product is tagged with a primer carrying a fluorescent dye. It is then cut with a restriction enzyme to form terminal restriction fragments (T-RFs) that are separated by gel electrophoresis and visualised by excitation of the fluor (Dejonghe *et al.* 2001; Blackwood *et al.* 2003). A banding pattern is obtained, each band corresponding to one species or "ribotype" (Dejonghe *et al.* 2001). This provides quantitative data on each of the T-RFs in the form of size of base-pairs and intensity of fluorescence (peak height) (Blackwood *et al.* 2003). T-RF sizes can then be compared with a theoretical database obtained from sequence information (Blackwood *et al.* 2003), thus providing the species richness as well as community structure of the ecosystem (Dejonghe *et al.* 2001).

2.3.9 Other techniques

A method for detecting extracellular DNA in environmental samples has been developed by England *et al*. (2004). This method circumvents disruption of cell membranes by not employing the use of harsh chemicals or physical disruption of whole cells within samples. England *et al*. (2004) hypothesised that the persistence of extracellular DNA in the environment is partially due to the formation of soil-DNA complexes, whereby the naked DNA released upon cell death and lysis is protected from nuclease degradation by the soil particles to which it adheres. Extracellular DNA serves two purposes in the environment, that of a nutrient source and of a gene pool. This DNA was extracted by using a gentle relatively fast extraction method involving suspension and shaking of a 0.5g sample of leaf litter in 4ml of sodium pyrophosphate (pH 8) followed by several filtration and cleaning steps resulting in application-ready extracellular DNA.

Other techniques such as ribosomal intergenic spacer analysis (RISA), ITS-restriction fragment length polymorphism (ITS-RFLP) and random amplified polymorphic DNA (RAPD) provide complex community profiles that can be analysed for community composition studies (Kent and Triplett 2002). Detection and resolution of fragment analysis can be approached with a number of methods, including automated ribosomal intergenic spacer analysis (ARISA) and length heterogeneity PCR (LH-PCR) (Kent and Triplett 2002). Most probable number (MPN) is a specialised enrichment technique using relevant substrates to estimate the number of organisms in an environment capable of degrading specific pollutants (Banks and Alleman 2002).

A widely used approach to studying bacterial diversity is using clone libraries of 16S rRNA genes. The genes are collected from naturally occurring bacteria through PCR with universal 16S rRNA gene primers (Cottrell and Kirchman 2000). Cottrell and Kirchman (2000) studied

in situ marine microbial communities and found that data from a PCR-based clone library indicate that novel, uncultivated species are widespread in global oceans. However, clone libraries are effected by biases at each step of the method (including sample collection, cell lysis, nucleic acid extraction, PCR amplification, and cloning) and can deviate from the compositions of actual communities (Cottrell and Kirchman 2000). During PCR, using controlled mixtures of 16S ribosomal DNA, the relative abundance of targeted DNA molecules in the final PCR product can be affected by biases. Several precautions have been proposed for minimizing these biases during PCR; however, the amount of bias is not known for natural habitats.

2.3.10 Possible molecular pitfalls

Due to the low number of cultured microorganisms compared to the large numbers of unculturable microbes, microbial diversity cannot be implied by cultured diversity. Therefore PCR-based molecular techniques are favoured to give a better understanding of microbial communities in mixed samples. However, a review by Von Wintzingerode *et al*. (1997) indicated pitfalls of PCR-based genomic analyses. Briefly, they concluded that after initial sample collection several difficulties could be encountered during cell lysis, DNA/RNA extraction, PCR, separation of genes and sequence data analysis. These difficulties include the following:

- Insufficient cell lysis will skew an analysis if not all microbial DNA is released from cells in the sample.
- DNA/RNA can shear into fragments after release from cells during cleaning steps and may impact on post-extraction steps thereafter.
- PCR can be inhibited by co-extracted contaminants such as humic acids from soil that hamper the reaction of template and enzyme. Amplification efficiencies should be the same across molecules, thus assumptions must be made that:

- all molecules are equally accessible to primer hybridisation, the primer-template hybrids form with equal efficacy.
- the extension efficiency of the DNA polymerase is the same across templates.
- exhaustion of reaction components affects all templates equally.

Furthermore, the formation of PCR artefacts can occur due to the creation of chimeras between two homologous molecules, deletion mutants as a result of stable secondary structures, and point mutants because of misincorporation of bases by the DNA polymerase. In addition to this the possibility of contamination as a result of foreign DNA introduced into the reaction due to experimental error must be negated, this is monitored by the incorporation of negative control reactions containing no template DNA.

- Sequence analysis of 16S rDNA is usually done by comparison with previously identified sequences deposited on global databases. However, whether environmental sequences represent uncultured or novel organisms or remain unassigned to known taxa is yet to be determined. Many sequences on the database may be of low quality due to their length (only partial) or taxonomic ambiguity (Kirk *et al*. 2004).

In order to prevent these possible inaccuracies during molecular sample analysis, Von Wintzingerode *et al*. (1997) suggested that results of different extraction methods, PCR and cloning techniques be explored simultaneously to provide the most accurate results possible.

2.4 DGGE technique and application

Muyzer *et al.* (1993) introduced DGGE as a new genetic fingerprinting technique. This method is often preferred due to its capacity to provide rapid visual indications of community changes within a sample (Anderson *et al*. 2003). Bands can then be excised and sequenced. Sequence variation in rRNA has been used for elucidating phylogenetic relationships between

organisms and in designing probes for detecting microbial taxa (Muyzer *et al.* 1993). DGGE is used to determine the microbial genetic diversity and particularly the predominant communities in a sample (Muyzer 1999; Coclin *et al.* 2001; Stamper *et al.* 2003). Janse *et al.* (2003) concluded that it can also be used to determine the purity and uniqueness of isolated strains.

Denaturing gradient gels are used for the detection of non-RFLP polymorphisms (Helms 1990). Double-stranded fragments (200-700 basepairs), the products of PCR of rRNA genes (rDNA) with the same length but differing in base-pair sequences, are separated on an increasing denaturant gradient gel (Ferris *et al.* 1996; Nakatsu *et al.* 2000; Dejonghe *et al.* 2001; Kawai *et al.* 2002). A portion of DNA can be deemed suitable for DGGE analysis if it can be specifically amplified from the target organism, has adequate heterogeneity for good resolution and is part of a gene that has a large database of sequences already available (Janse *et al.* 2003). A factor that limits DGGE efficacy is the primer design. Sequences targeted should not yield a fragment much longer than 500 basepairs (bp) for successful analysis (Throbäck *et al.* 2004). At present 16S rDNA sequences form the ever-increasing, largest gene-specific data set available on internationally accessible databases (> 30 000), making tentative identification of unknown bacteria possible (Von Wintzingerode *et al.* 2002). Øvereås *et al.* (1997) were the first to analyse archaeal rDNA with DGGE. Using domain-specific sets of primers on samples from a meromictic lake in Norway, they found an increase in *Archaea* and a decrease in *Bacteria* the deeper they sampled.

Double-stranded DNA products that undergo electrophoresis through a DGGE gel are halted when they split into single strands due to a linearly increasing gradient of denaturants (Muyzer *et al.* 1993; Curtis and Craine 1998). The denaturants most commonly used are heat (constant 60°C), formamide (0-40%) and urea (0-7M) (Helms 1990). Initially, fragments move according to relative molecular mass. However, as the denaturation gradient increases

the fragments start separating as the hydrogen bonds between the double helix beging to break, this is known as melting (Helms 1990). This partial melting retards the progress of the DNA molecule through the gel, the resultant mobility shift differing for different sequences (Muyzer *et al.* 1993). The sequence of the PCR product separation on the gel determines the denaturant concentration at which this occurs (Ferris *et al.* 1996; Curtis and Craine 1998; Nakatsu *et al.* 2000). As denaturant concentrations increase, the DNA will dissociate completely into two separate strands (Helms 1990). Fragments do not partially melt in a zipper-like fashion, and specific portions of DNA fragment become single-stranded suddenly within a narrow denaturant range (Helms 1990; Muyzer *et al.* 1993). After double-stranded DNA dissociation the gel is stained with a DNA-intercalating dye that fluoresces under ultra-violet light. For the purposes of this review and work, SYBR gold nucleic acid gel stain was used. This stain is an asymmetrical cyanine dye with two fluorescence excitation maxima, *ca*. 300 and 495nm, when bound to DNA (Tuma *et al*. 1999). When used with 300nm transillumination and Polaroid black and white photography, SYBR gold is more sensitive during intercalation than ethidium bromide, forms dye-nucleic acid complexes *ca*. 70% higher than current counterpart dyes, produces up to a 1000-fold fluorescence enhancement, is as sensitive as silver staining but requires only one step, and does not influence subsequent molecular biology protocols (Tuma *et al*. 1999).

Narrowing the denaturant range can increase the sensitivity of DGGE, hence yielding fast, reliable and reproducible results (Fromin *et al*. 2002; Temmerman *et al.* 2003). Mobility rate in the polyacrylamide gel is determined by the physical shape of the fragment, which in turn depends on the denaturant gradient and fragment sequence, with partially melted fragments moving more slowly than those that are still double-stranded (Helms 1990). During analysis of a complex microbial community, a ladder of bands forms on the gel, each corresponding to an individual PCR-product of a specific sequence (Curtis and Craine 1998, Fromin *et al*. 2002). This allows for simultaneous detection of multiple 16S rRNA sequences (Ferris and

Ward 1997; Sekiguchi *et al.* 2002). The resulting gels can be probed with diagnostic oligonucleotides to identify specific sequences or bands, and may be excised, reamplified and sequenced (Ferris *et al.* 1996). The technique is sufficiently sensitive to detect as little as one base-pair difference in a sequence (Helms 1990). However, Gillan (2004) found that changes to the DGGE protocol can result in less robust results and thus should be standardised across particular sets of experiments. Alternatively, "markers" can be constructed from known species sequences and run alongside test samples to determine the identity of bands within the sample. Theunissen *et al*. (2005) demonstrated this when analysing probiotic microorganisms from yoghurt and lyophilised capsule and tablet preparations. Two markers with known lactobacilli and *Bifidobacterium* PCR-product were run adjacent to test samples and band patterns were then used for accurate and rapid species identification. Similarly, but more complex, Keyser *et al*. (2006) used a marker composed of five known methanogenic bacterial species to determine DGGE bands from an upflow anaerobic sludge blanket bioreactor that did not match the marker. These bands were then excised and sequenced, and a DGGE marker to monitor archeal members of the microbial consortium developed based on the sequence results.

Resolution of DGGE can be enhanced by incorporation of a GC-rich sequence into one of the primers to modify the melting behaviour of the fragment and allow for the majority of sequence variation to be detected in the denaturing gel (Ferris *et al.* 1996; Curtis and Craine 1998). A GC-clamp attached to the 5' end of a PCR product prevents complete melting during fragment separation in a denaturing gradient, and sensitises the technique enough to detect all single base changes in PCR fragments of 500bp (Heuer *et al*. 1997). Sheffield *et al*. (1989) found that attaching a GC-clamp of 40-45bp to primers allowed for the determination of single-base-mutations, previously only 40% distinguishable in DGGE analysis, to increase to 100%. Furthermore, Boon *et al.* (2002) included a GC-clamp to stabilise large fragments in all final reactions during nested PCR intended for DGGE analysis. However, despite the

advocation of the inclusion of a GC-clamp for melting stability during PCR-DGGE analysis under certain conditions the clamp can be disregarded. In this case, if no GC-clamp is added, it is recommended that the PCR product must have at least two melting domains (Chang bioscience 2004). Wu *et al.* (1997) found that GC-clamped products with a perfect melting curve yielded distorted smeared results when subjected to DGGE. They found that fragments containing a "high melting domain" provided better DGGE results when run without a GC-clamp, and concluded that if melting analysis of a PCR product predicts a high melting domain of <40bp, and differs by not more than 5°C melting temperature, then the fragment is suitable for DGGE analysis without a 5' GC-clamp.

Lanes of bands can be analysed utilising gel image software for more accurate results, using known pure culture isolates as standards for well-characterised environmental samples. Thus, gel images resulting from DGGE analysis can be digitally captured and used for species identification when samples are run against these known standards (Temmerman *et al.* 2003). These images can also be compared when samples are collected and analysed over a period of time, hence allowing monitoring of community structural changes with time (Van Hannen *et al.* 1999). Manual fine-tuning of the gel image completes the initial analysis and dendograms can be drawn to relate band pattern parallels (Fromin *et al*. 2002; Stamper *et al*. 2003). Software also calculates band densities necessary for determining the Shannon diversity index, where each band represents one species and the band intensity is proportional to the species abundance (Fromin *et al*. 2002; Stamper *et al.* 2003; Andreoni *et al*. 2004). Nübel *et al.* (1999) quantified diversity of oxygenic phototrophs within hypersaline microbial mats. The amount of bands per sample indicated species richness, whereas species abundance/"evenness" was determined by band intensity.

Limitations of DGGE include similar electrophoretic mobilities of phylogenetically related species sharing analogous sequences in the amplified area, and similar melting behaviour

between phylogenetically unrelated species (Smalla *et al.* 2001). Consequently, there may be more than one species represented by a single band on the DGGE gel. This has been demonstrated by Jackson *et al.* (2000) making use of site-directed mutagenesis to create *E. coli* 16S rDNA fragments differing by 1-4 base-pairs. Migration on DGGE gels consistently determined single base-pair changes, but multiple base differences proved to be more difficult to distinguish. Two of the sequences tested differing by two base-pairs only, showed identical migration patterns and could not be separated when run in a mixed sample. Furthermore, Vallaeys *et al*. (1997) reported that DGGE analysis of a 200bp fragment of 16S rDNA from rhizobia and methantrophs was difficult to elucidate due to low and high sequence polymorphism, respectively.

One also needs to take into account the method used for DNA extraction and purification when screening DGGE samples. Niemi *et al*. (2001) tested five different DNA extraction methods and three purification methods on rhizosphere soil samples destined for DGGE analysis. They found that the isolation and purification methods both had an effect on the final bacterial DGGE community structures of the samples. In addition to this, O'Callaghan *et al*. (2003) concluded that extracted DNA should be representative of the habitat, PCR bias must be taken into account as preferential amplification may occur due to inefficient primer annealing, and species determination should not be based on 16S rDNA sequences alone, although this is becoming increasingly more efficient as databases expand continually. There are, however, means of incorporating internal standards into the DNA extraction and PCR-DGGE process. Petersen and Dahllöf (2005) developed a protocol known as Internal Standards in Molecular Analysis of Diversity (ISMAD) that can monitor, and thus account for, experimental variability. A fluorescent 510bp PCR product is included in each sample prior to DNA extraction and recovered afterwards. PCR is monitored by adding non-competitive primers coding for a 140bp section of *Drosophila melanogaster* DNA to the same PCR as the sample. Together these internal controls reduced variation between replicate

samples during DGGE analysis. Despite these minor pitfalls, DGGE is still considered to be a reliable, reproducible, rapid and relatively inexpensive method for the simultaneous analysis of multiple samples and to map community changes over time (Muyzer 1999; Fromin *et al*. 2002).

2.4.1 Community diversity analysis

Most microbial diversity indices are based on plant and animal models, e.g. the Shannon and Simpson indices. As such, there is some difficulty in applying these indices to microbial models since they need a clear definition of species and unambiguous individual identification. This level of identification is difficult in bacteriology. An ideal bacterial index should encompass the following (Watve and Gangal 1996):

- Three important diversity dimensions, viz. species diversity, species richness/abundance and taxonomic distance between biotypes.
- Be based on a statistically justified parameter.
- Be insensitive to possible errors and variability of test results.
- Not be too sensitive for sample size.

According to this the use of Shannon algorithms to calculate microbial diversity according to DGGE gel fingerprints is acceptable. Dimensions such as diversity and richness/abundance can be determined from the number of bands and their intensity on the gel, respectively. Sequencing of each band on the gel can indicate taxonomic distance between biotypes. Diversity within the 16S rDNA is statistically well-documented and does account for possible errors and variability within the region that can be guarded by incorporating internal control standards. DGGE can be used for assessing anything from one sample individually to a large numbers of samples simultaneously.

DGGE allows for determining community as well as specific population diversity without

further analysis and without elucidating particular individuals (Muyzer 1999). It has also been used for the simultaneous identification of sequence variations in multiple genes among several organisms (Muyzer *et al.* 1993). Identity of community members can be further resolved by hybridisation of the gel with species/taxon-specific oligonucleotide probes to hypervariable regions of the sequence or by cloning and sequencing (Muyzer 1999). The gel can be used for direct analysis of genomic DNA by transferring separation patterns to hybridisation membranes, using capillary- or electro-blotting, and analysis with DNA-probes (Muyzer *et al.* 1993). PCR, with GC-clamp primers, can also be selectively employed to amplify sequences of interest, e.g. 16S, before DGGE is performed (Muyzer *et al.* 1993). Essentially, DGGE allows a high number of samples to be screened simultaneously, thus facilitating more broad-spectrum analysis. Kowalchuk *et al*. (1997) used DGGE to assess variation between different pathogenic fungal species within a taxon attacking the roots of *Ammophila arenaria* L. (marram grass). They amplified a 569bp region of the 18S rDNA gene by means of nested PCR with a GC-clamp on the final PCR. Upon assessing experimental and field/wild plants they were able to distinguish between species of fungi and detect a much higher level of diversity than in previous culture-based surveys.

2.4.2 Community dynamics studies

Due to multiple sample screening, DGGE allows for monitoring of the dynamics that microbial communities undergo during seasonal and environmental fluctuations in their habitat (Muyzer 1999). Ward *et al.* (1998) made use of 16S rDNA fragments in DGGE to study seasonal community changes of microbial communities within hot spring microbial mats. Subsequently, PCR-DGGE has been used to monitor seasonal changes in communities of bacterioplankton, the rhizosphere of chrysanthemum, post-viral bacterial lysis communities, diurnal behaviour of sulphate-reducing and phenol-degrading bacteria in activated sludge, as well as the impact of pesticide and herbicide applications on microbial

communities (Muyzer 1999). DGGE has even been applied in the mapping of communities of bacteria utilising organic-wastewater/sludge as fuel for a microbial electrochemical cell (Kim *et al*. 2004). Results indicated that microbial communities within the cell electrode differed from those in the sludge.

2.4.3 Molecular community mapping across varied environments

Culture techniques are important for the understanding of the physiology and function of microbes isolated from their natural environment. However, molecular tools can be used for monitoring enrichment cultures and facilitating the isolation of target communities from the environment (Muyzer 1999). Smalla *et al.* (1998) made use of DGGE and TGGE (temperature gradient gel electrophoresis) in the analysis of BIOLOG substrate utilisation patterns of two bacterial communities from potato rhizosphere and activated sludge. Both DGGE and TGGE showed enrichment of specific bacterial communities not evident from BIOLOG results. Prokaryotic communities are not the only type to be mapped. Foucher *et al.* (2004) determined nematode diversity in soil samples using 18S rDNA PCR-DGGE, and found a significant relationship between morphological and DGGE estimates of species richness. Marshall *et al*. (2003) tested PCR-DGGE primers for compost fungi, finding an α-elongation factor primer set targeting a portion of the 18S rDNA best for fungal community amplification. Similarly, Zuccaro *et al*. (2003) demonstrated the use of four sets of 18S primers in DGGE analysis for the identification of ascomycetes associated with algae in lichens on ferns.

2.4.4 Niche differentiation

Molecular microbial ecology is becoming more specialised, thus allowing analysis of specific functional communities within communities. Enzyme-coding genes are now being targeted

for ecological studies. They tend to display a higher level of sequence variation than the conserved 16S rDNA genes, which makes them more efficient molecular markers for phylogenetically similar but ecologically distinct communities (Muyzer 1999). In addition, targeting functional genes facilitates the study of specific activities within microbial communities. Milcic-Terzic *et al*. (2001) used genes of microbes involved in the degradation of organic pollutants for the application of molecular techniques in the microbial ecology of polluted areas. As more sequences of functional genes become available on databases worldwide, PCR-DGGE undoubtedly would deliver considerably more information regarding community structure and function.

2.4.5 Determining species diversity

Banding patterns on DGGE gels give an indication of species diversity when analysed using a visual gel analysis software package. For the purposes of the studies included in this thesis, DGGE gel image analysis was performed using the Gel2K program and fingerprints were analysed in a cluster investigation using CLUST (Norland 2004).

Bands excised from DGGE gels can be sequenced. The resulting sequences can then be used for comparative phylogenetic analysis to determine the evolutionary relationships between organisms in the community being analysed. Anderson *et al.* (2003) investigated a soil fungal community by DGGE of the ITS region (ITS1-F with a GC-clamp and ITS2 yielding a 300bp fragment), sequencing of bands, and BLAST result phylogeny of the resulting sequences. Phylogeny gives and indication of species diversity and not richness, since only one band is produced and picked from the gel per species (Van Hannen *et al.* 1999). By determining the closest relatives of unknown organisms the known characteristics can be inferred upon them (Ueda *et al.* 1995). The sequence data can also be used in the design of primers and probes for *in situ* identification of selected organisms.

2.5 Alternatives to PCR-based analyses

Microscopy and plate counts are traditional methods that are quick and inexpensive. Selective plating and direct viable counts can be used for providing information on the active heterotrophic portion of a community (Kirk *et al*. 2004). Methods are available that focus on physiological/metabolic characteristics of microbial communities, e.g. fatty acid methyl ester (FAME) profiles and phospholipid fatty acid analysis (Kent and Triplett 2002). Fluorescent *in situ* hybridisation (FISH) utilises fluorescent oligonucleotides to target rRNA sequences (Dejonghe *et al.* 2001). FISH can be used in conjunction with DAPI (4',6'-diamidino-2-phenylindole), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)-formazan, or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining for determining the contribution made by communities of interest to the total abundance or active cell count (Kent and Triplett 2002). However, FISH has a low throughput and this limits its application for comparison of high numbers of samples (Kent and Triplett 2002).

Various tests are also available for bacterial identification based on physiological reactions. Among these are the catalase reaction test, the oxidative-fermentative Hugh-Leifson test, Biolog and API, a standardised, miniaturised version of existing biochemical test techniques that is simple, rapid and reliable when used in conjunction with numerical identification with or without computer software programmes.

2.5.1 Morphology

Prudent morphological analysis of bacterial cells can yield important information about diversity, microbial abundance and two-dimensional spatial distribution of microbial community members. Computer-aided systems such as CMEIAS (Centre for Microbial Ecology Image Analysis System), is a semi-automated analytic tool that uses processing and

pattern recognition techniques (with microscopy) to gather information on size and shape of digital images of organisms and classify them into their morphotypes (Kent and Triplett 2002).

2.5.2 Catalase reaction

This is a test for production of the enzyme catalase by bacterial species. Hydrogen peroxide is a harmful by-product of metabolic processes, catalase catalyses its breakdown to water and oxygen. The enzyme has one of the highest turnover rates since one molecule of catalase can convert 83 000 molecules of hydrogen peroxide to water and oxygen per second (Wikipedia 2007). Although the catalase test alone cannot identify bacteria, combined with other tests it can aid in identification (Krieg *et al*. 1984). The test is performed by picking bacterial cells from pure cultures on agar plates, using sterile wooden toothpicks, and placed on clean microscope slides. One or two drops of 3% hydrogen peroxide are added to the bacteria and the formation of bubbles within 1min is regarded as a positive reaction.

2.5.3 Aerobic and anaerobic bacteria

The fermentative or oxidative nature of bacteria is determined using the Hugh-Leifson test (Hugh and Leifson 1953). Colonies from pure culture on agar plates are stab-inoculated in duplicate into sterile test tubes containing oxidative fermentative base medium (OFBM) with added glucose. The medium in one tube of each duplicate is covered with 1cm sterile liquid paraffin. Tubes are incubated at 37°C for 48h and a colour change from green to yellow is deemed a positive test result. Bacteria can be considered fermentative when the colour changes from green to yellow in both test tubes. Oxidative bacteria induce a colour change only in the test tube containing no liquid paraffin.

2.5.4 Identification using API and Biolog

API is a series of miniaturised metabolic tests deemed instrumental in bacterial species identification. Pure isolates from agar plates are subcultured on fresh agar medium for 48h. A sterile inoculation loop is then used to suspend cells in test tubes containing 0.85% NaCl. API strips are loaded with this suspension according to the manufacturer's instructions (OMNIMED (Pty.) Ltd.). Several different tests are available for use, e.g. API 50CH, API 20NE, API 20E, etc., based on different characteristics of bacterial species.

Garland and Mills (1991) developed a technique to assess the potential functional diversity of bacterial communities through sole carbon utilisation (SSCU) metabolic patterns. From this arose the gram-negative and gram-positive Biolog plate system that contained 95 different carbon sources and a control well for metabolic bacterial identification (Kirk *et al*. 2004). Biolog EcoPlate™ is specifically tailored for microbial community and ecological studies. Its development was initially prompted when Biolog GN microplates were inoculated with a mixture of microbes in culture and the community fingerprint characteristics were measured over time. Known as community-level-physiological-profiling this method proved to be effective in distinguishing spatial and temporal microbial community changes. The plates proved to be useful in assays of the normal community and to detect changes based on an introduced variable. These studies have been conducted with communities from soil, wastewater, activated sludge, compost and industrial waste. The Biolog EcoPlate contains the 31 most utilised carbon sources for soil community analysis, each of which is repeated in triplicate for data purposes. Communities of organisms yield a characteristic reaction pattern or "metabolic fingerprint". These patterns can be statistically analysed by computer software at defined intervals over 2-5 days, hence providing data about microbial community changes over time. This method has also been compared with other methods such as PLFA and proved to be more sensitive to important factors for instance temperature and water.

2.5.5 DNA reassociation

A non-PCR-based molecular technique has also been established on the basis of DNA melting and reassociation measurements. Comparative chemistry of genomes between species gives an indication of species diversity during DNA-DNA and DNA-RNA reassociation (Sanderson 1976). Purified DNA is split into fragments and thermally denatured so that the double-helix strands separate or "melt" and, by slowly cooling the DNA, reassociate or reanneal again. Following this, the rate at which the double-helix renatures is measured spectrophotometrically (Curtis and Sloan 2005). This rate is affected by the size and complexity of DNA, with large complex DNA reannealing the slowest. Originally, this method was used to estimate size and complexity of genomes from individual organisms. However, Torsvik *et al.* (1990a) reasoned that pooled genomic DNA from a microbial community might reanneal like the DNA from a large genome. They placed sheared total soil DNA in a French press to yield fragments with an average molecular mass of 420 000 daltons. It was then hypothesised that the heterogeneity of the DNA was a measure of genetic diversity of bacteria within the soil. Indeed, they showed that DNA extracted from soil reassociated so slowly that it resembled a genome 7000 times as large as the genome of a single bacterium (Curtis and Sloan 2005). It follows that there could have been at least 7000 different prokaryotic taxa in the sample of soil analysed.

Renaturation of the homologous single-stranded DNA follows second-order reaction kinetics (Torsvik *et al.* 1990a). The renatured DNA fraction is expressed as a product of the nucleotide concentration in moles per litre (C_ot), and time is measured in seconds. $C_ot_{1/2}$ under defined conditions is directly proportional to the complexity or genome size of the DNA, complexity being defined as the number of nucleotides in the genome of a haploid cell, excluding repetitive DNA. Based on this, $C_ot_{1/2}$ can be considered to be a diversity index measurement of bacterial communities, which would equate to indices based on phenotypic

analysis or species diversities.

DNA-reassociation has been used in combination with other molecular techniques such as DGGE to give a more complete idea of bacterial diversity within specific communities. Torsvik *et al*. (1998) investigated the community structure of natural, polluted and agriculturally perturbed environments. They compared DGGE diversity analysis of rRNA genes with total DNA reassociation to draw parallels between community diversity techniques. Their study indicated that total soil microbial diversity was 200 times higher than bacterial isolate diversity from the same samples and that farming and pollution played a significant role in reducing bacterial diversity.

2.6 Use of 16S rDNA sequences for parsimony and distance analysis.

Certain regions of rDNA sequences are highly conserved across all organisms whereas other regions may vary. The variability within these regions increases proportionately to the increase in the evolutionary distance between organisms, thus allowing for the determination of phylogenetic relationships between microorganisms (Nakatsu *et al.* 2000). Due to their usefulness as markers in phylogenetic studies, 16S rRNA genes have been the main target for prokaryotic ecological molecular surveys (Osborne *et al*. 2005).

Ribosomal RNA (rRNA) molecules are used as molecular chronometers because of their high degree of structural and functional conservation. As a result of this, domains within rRNA molecules harbour independent rates of sequence change. Phylogenetic relationships can be determined by examining these changes over time (Kent and Triplett 2002).

A large number of genes are available for phylogenetic studies on databases worldwide. Selected sequences should be appropriate, and can be affected by the following:

- Structural regions in the small and large subunit rRNA genes evolve at differing rates.
- Non-synonymous substitution rates at codon positions 1 and 2 are often slower than synonymous substitutions at position 3.
- Transitions occur more frequently than transversions.

Different substitution rates result in different levels of phylogenetic resolution in different areas of DNA. This should be taken into account when examining phylogenetic relationships at different taxonomic levels.

Patterns in sequence affect the suitability of data to be used in various phylogenetic tests:

- Phylogenetic signal: the level of conservation of sequence data.
- Saturation: multiple changes at the same site due to lineage splitting. Over time two sequences saturate due to multiple changes at certain sites. Increasing substitutions will have a diminishing effect on the sequences in question. A non-linear relationship develops between sequence divergence and time, leading to information loss to the phylogeny being examined.
- Base/codon composition.

At present 16S rDNA sequences form the ever-increasing, largest gene-specific data set available on internationally accessible databases (> 30 000), making tentative identification of unknown bacteria more possible (Von Wintzingerode *et al.* 2002). However, they are not always the most informative genes to select for study. Dauga (2002) investigated 16S and *gyrB* phylogenetic gene trees showing relatedness between *Enterobacteriaceae*. *gyrB* is a single-copy gene present in all bacteria. It has been proposed as a suitable genetic marker for identification of bacteria and encodes ATPase within the DNA-gyrase domain. Dauga (2002) found that *gyrB* trees proved to be more reliable determinants between closely-related species

than the 16S trees. 16S has nevertheless been used in the comparison and resolution of closely-related species. Anzai *et al*. (1997) found a 93.9% homology in 16S rRNA sequence homology between *Chryseomonas*, *Flavimonas* and *Pseudomonas* and on this basis proposed them synonymous. Similarly, Warwick *et al.* (1994) proposed that *Amycolata* and *Pseudonocardia* be classified in an emended *Pseudonocardia* genus on the basis of mixed clades emerging continuously from analysis of 16S data. *Pseudonocardia* has also, based on 16S sequence data, been observed to form a monophyletic unit with *Actinobispora* and it has been suggested that the latter genus be also incorporated into *Pseudonocardia* (Lee *et al*. 2000).

2.6.1 Characterisation of 16S region

The 16S gene of the bacterial genome holds the rDNA genetic code for the 16S subunit of the ribosome. Ribosomes are organelles in which translation of the genetic code (RNA to protein) takes place, and consist of two subunits of RNA and proteins (Tamarin 1996). Ribosome size is measured on the basis of its sedimentation rate during centrifugation in a sucrose density gradient. The unit of sedimentation is S, so designated after T. Svedberg, the developer of the method in the 1920s (Tamarin 1996). The 30S subunit of an *E. coli* ribosome comprises a 16S molecule of rRNA and 21 proteins (Tamarin 1996). This subunit of rRNA is encoded on the DNA of the bacterial cell and contains sequences that are highly conserved, thus allowing for sufficient resolution to distinguish between genera and species.

Advantages of using 16S rRNA gene sequences for analysis of microbial communities include the following:

- Essential component of ribosomes.
- Universal to all cell types.

- Universally conserved and variable taxon-specific sequences where the primary structure consists of conserved and variable sequences allowing for comparison of homologous positions of different species.
- Horizontal gene transfer not likely.
- Extensive databases (e.g. GenBank) of rRNA gene sequences exist.
- rRNA sequence-based "Tree of Life" provides a scaffold for comparison of unknown sequences from natural samples.
- Acts as a molecular chronometer.
- Allows for culture-independent analysis of unknown communities.

2.6.2 Characteristic base-pairs

There are two types of sequence data generated, viz. genomic DNA and expressed sequence tags (ESTs). Genomic DNA represents the genetic material of entire organisms in the form of genomes. The genomes are constructed from multiple experiments of high accuracy. However, ESTs are short pieces of DNA, usually 400-800bp, which are transcribed into mRNA and later translated into proteins. ESTs comprise 62% of the 38.9 million genetic sequences on GenBank, they are fairly easy to sequence and can be used to locate genes and their splice sites (Wu *et al*. 2005). Mapping of ESTs to known genomes has become more important in recent years for finding genes, EST clustering, alternative splice-sites and gene function. Wu *et al*. (2005) developed new computer software (EST mapper) which is 3-1000 times faster than current market software for aligning and clustering DNA sequences, and produces alignments of better quality.

Chapter 3

Materials and methods

Materials and methods

The objectives of this study were achieved by means of the following procedures:

3.1 Soil samples

Eight soil samples of approximately 50 g each were collected in 2003 in plastic sample bottles from a site in Free State Province (Samples 1-8 in Table 1). Soil samples 1 and 3-7 were collected from unpolluted and polluted top soil in the presence and absence of *Elusine coracana* and *Brantha serratia* plants (Table 1). Samples 2 and 8 were collected at different depths and were known to be polluted with diesel (Table 1). Samples taken from below the soil surface were collected using a soil auger. A further nine soil samples of approximately 50 g each from a pitch/oil/diesel/petrol/tar-polluted site in Mpumalanga Province were collected from approximately 5cm below soil surface in plastic sample bags in February 2004 (Samples 10-18 in Table 1). Soil samples at site 2 were polluted with different PAH-containing compounds and were rhizosphere and non-rhizosphere associated. Soil samples were taken either within the root zone or approximately 10 cm away from plant roots for rhizosphere and non-rhizosphere samples, respectively. The samples were transported to the laboratory and maintained at 4 ºC until total DNA could be extracted (max. 24 h). All soil samples were taken according to the simple random sampling protocol described by Tan (2005), and are considered to be representative of the environments from whence they came. However, broader spectrum sampling according to acknowledged systematic sampling protocols following standard operating procedures should be followed in soil sample collection in the future. A predetermined samlping area, having the same history, soil texture, colour and slope, should be targeted in a random zig-zag pattern and at least 20 samples collected (Zhang 2003).

Table 1: Soil collected for analysis from a site in Free State Province (site 1) and a site in Mpumalanga Province (site 2), South Africa. Soil samples taken at site 1 were collected one month after pollution by a leaking underground diesel pipeline. Soil samples at site 2 were collected from soil persistently polluted for approximately 10 years with different PAH/PCB-containing compounds.

Soil sample number	Area	Description
1	Site 1[c]	Diesel polluted topsoil with no plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
2	Site 1[c]	1m deep non-rhizosphere soil polluted with diesel Total petroleum hydrocarbon concentration 8 500 mg kg^{-1}
3	Site 1[c]	Diesel-polluted topsoil with *Elusine coracana* and *Brantha serratia* plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
4	Site 1[c]	Diesel-polluted topsoil with no plants growing nearby Total petroleum hydrocarbon concentration 25 000 mg kg^{-1}
5	Site 1[c]	Unpolluted topsoil with no plants growing nearby
6	Site 1[c]	Unpolluted topsoil with *Elusine coracana* and *Brantha serratia* plants growing nearby
7	Site 1[c]	Unpolluted topsoil with no plants growing nearby
8	Site 1[c]	1.5m deep non-rhizosphere soil polluted with diesel Total petroleum hydrocarbon concentration 28 000 mg kg^{-1}
9	Control[d]	Unpolluted reference loamy topsoil from University of Pretoria experiment farm
10	Site 2[d]	Unpolluted soil from *Bidens pilosa* rhizosphere pH 7.8, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}

[c] Soil samples taken to a depth of 10cm unless specified otherwise, the soil had a loamy texture.
[d] Soil samples taken to a depth of 5cm, the soil was a sandy loam (63.4% coarse, 21.1% silt, 13.9% clay).

Table 1 (continued)

Soil sample number	Area	Description
11	Site 2[d]	Unpolluted soil from *Brantha serratia* rhizosphere pH 7.9, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}
12	Site 2[d]	Unpolluted soil from *Cyperus esculentus* rhizosphere pH 7.8, mineral oil hydrocarbons 3 530 mg (kg dw) $^{-1}$, PAHs 190 mg (kg dw) $^{-1}$, volatile hydrocarbons 2.4 mg kg^{-1}, phenol index (pH 7.0) 96 ul l^{-1}
13	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *C. esculentus* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
14	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar) from *C. esculentus* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
15	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *B. serratia* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
16	Site 2[d]	Polluted soil (pitch/oil/diesel/petrol/tar) from *B. serratia* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}
17	Site 2[d]	Polluted soil (workshop oil) mulched with wood chips 10cm from *B. serratia* plant pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw) $^{-1}$, PAHs 1 200 mg (kg dw) $^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}

Table 1 (continued)

Soil sample number	Area	Description
18	Site 2[d]	Polluted soil (workshop oil) mulched with wood chips from *B. serratia* rhizosphere pH 7.7, mineral oil hydrocarbons 62 200 mg (kg dw)$^{-1}$, PAHs 1 200 mg (kg dw)$^{-1}$, volatile hydrocarbons 350 mg kg^{-1}, phenol index (pH 7.0) 1 300 ul l^{-1}

3.2 Bacterial isolates

Eight bacterial isolates, representing the dominant culturable taxa from the rhizosphere of weeds and from non-rhizosphere soil at site 2 in Mpumalanga Province, South Africa, with a *ca*. 10-year history of total coal-derived petroleum hydrocarbon pollution, were obtained in pure culture (Molobela 2005). The isolates from polluted soils were randomly designated SA1, SA2 and SA3 from *Bidens pilosa* L. rhizosphere, SA4 and SA8 from *Eleusine coracana* (L.) Geartn. rhizosphere, SA6 and SA7 from *Cyperus esculentus* L. rhizosphere, and SA5 from non-rhizosphere soil.

3.3 DNA extraction

Total soil DNA was extracted directly from soils samples using the BIO101 Fast DNA Spin kit (Soil) (Qbiogene Molecular Biology Products). DNA was maintained at –20 ºC at the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

3.4 Polymerase chain reaction

3.4.1 16S PCR

A portion of 16S bacterial gene of the rDNA was amplified by means of PCR from the total extracted soil DNA, using the primers:

K: PRUN518r: 5'ATT-ACC-GCG-GCT-GCT-GG3' (Siciliano *et al.* 2003)

M: pA8f-GC: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' (Fjellbirkeland *et al.* 2001)

These primers were found to be valuable in molecular ecological and systematics studies focussing on the 16S rRNA gene (Øvreås and Tosrvik 1998). Authentic *Escherichia coli* DNA (courtesy Dr A.K. Drønen[e]) and a reaction with no template DNA were included as positive and negative controls, respectively. Each PCR tube contained a total volume of 50µl: 40.75µl sterile distilled MilliQ water, 5µl PCR buffer with $MgCl_2$ (10x), 2µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 1µl template DNA (27ng μl^{-1}), 0.25µl hot start Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 51 °C and 1 min. at 72 °C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3)) agarose gel.

[e] A.K. Drønen, University of Bergen, Department of Biology, Bergen, Norway.

3.4.2 16S rDNA colony PCR

The 16S bacterial gene of each isolate was amplified by means of colony PCR, using the following primers:

K: PRUN518r: 5'ATT-ACC-GCG-GCT-GCT-GG3' (Siciliano *et al.* 2003)

M: pA8f-GC: 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' (Fjellbirkeland *et al.* 2001)

The M primer was designed specifically for DGGE analysis, hence the GC-clamp for stability. However, these primers were also used in PCR amplification and sequencing of the pure cultures.

A reaction with no template DNA was included as negative control. Each PCR tube contained a total volume of 25µl: 18.7µl sterile water, 2.5µl PCR buffer with $MgCl_2$ (10x), 2µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 0.5µl 10^{-1} bacterial suspension, 0.3µl Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 54 °C and 2 min. at 72 °C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % 1x TAE agarose gel.

3.4.3 Internal transcribed spacer sequence PCR

A portion of the internal transcribed spacer (ITS) gene sequence of the DNA from each samples was subjected to PCR using the primer set:

ITS1 : 5'CAT CGA GAA GTT CGA GAA GG3'

ITS4 : 5'TAC TTG AAG GAA CCC TTA CC3'

(White *et al*. 1990)

A reaction with no template DNA was included as negative control. Each PCR tube contained a total volume of 25µl: 18.7µl sterile SABAX water, 2.5µl PCR buffer with $MgCl_2$ (10x), 2.0µl dNTPs (2.5µM), 0.5µl primer K (50µM), 0.5µl primer M (50µM), 0.5µl template DNA (27ng μl^{-1}), 0.3µl hot start Taq (5U μl^{-1}). DNA amplification using the K and M primers was performed in a PCR thermal cycler using the following programme: 10 min. at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 2 min. at 72 °C, followed by 7 min. at 72°C, and then held at 4 °C. DNA amplification using the ITS primers was performed in a PCR thermal cycler using the following programme: 1min. at 92 °C, 30 cycles of 1min. at 92°C, 1min. at 50 °C and 1min. at 72 °C, followed by 5min. at 72 °C, and then held at 4 °C. PCR products were analysed on a 1 % TAE agarose gel.

3.4.4 *xylE* and *ndoB* gene fragment PCR

A *ca.* 400bp fragment from the *xylE* gene encoding catechol 2,3-dioxygenase, responsible for aerobic aromatic metabolism, from the *Pseudomonas putida* (ATTC 23973) TOL plasmid was amplified by means of PCR from soil DNA extracted above, using the primers:

Tol1 : 5'GTG-TCT-ATC-TGA-AGG-CTT-GG3'

Tol2 : 5'ATA-GAA-ACC-GAG-CAC-CTT-GG3'

(Milcic-Terzic *et al.* 2001)

A *ca.* 650bp fragment from the *ndoB* gene encoding naphthalene dioxygenase from *P. putida* (ATTC 17484) was amplified by means of PCR from soil DNA, using the primers:

Nah1 : 5'CAC-TCA-TGA-TAG-CCT-GAT-TCC-TGC-CCC-CGG-CG3'

Nah2 : 5'CCG-TCC-CAC-AAC-ACA-CCC-ATG-CCG-CTG-CCG3'

(Milcic-Terzic *et al.* 2001)

A reaction with no template DNA was included as a negative control. Each PCR tube contained a total volume of 25µl: 16.7µl sterile water, 2.5µl PCR buffer with KCl (10x), 2µl $MgCl_2$ (25mM), 2µl dNTPs (2.5µM), 0.5µl primer Tol/Nah 1 (50µM), 0.5µl primer Tol/Nah 2 (50µM), 0.5µl bacterial suspension (10^4cells ml^{-1}), 0.3µl Taq (5U μl^{-1}). DNA amplification was performed in a PCR thermal cycler using the following programme: 3 min at 95 °C, 40 cycles of 45 s at 94 °C, 45 s at 52 °C and 2 min. at 72 °C, followed by 5 min. at 72 °C, and then held at 4 °C. PCR product was cleaned by transferring the entire volume to a 0.5ml Eppendorf tube, adding 2µl of 3M sodium acetate and 50µl 95 % ethanol, and allowing it to stand on ice for 10 min. The suspension was centrifuged at 10 000 rpm for 30 min, the ethanol solution removed and the pellet rinsed in 150µl 70 % ethanol. After further centrifugation at 10 000rpm for 5 min, the ethanol was aspirated and the pellet dried under vacuum for approximately 10min. Following this, the pellet was resuspended in 20µl sterile deionised water. PCR product was analysed on a 1.6 % 1x TBE (89mM Tris, 89mM boric acid, 2mM EDTA (pH 8.0)) agarose gel.

3.4.5 *xylE* and *ndoB* gene fragment colony PCR

A 404bp fragment from the *xylE* gene encoding catechol 2,3-dioxygenase from the *P. putida* (ATTC 23973) TOL plasmid, and a 641bp from the *ndoB* gene encoding naphthalene dioxygenase from *P. putida* (ATTC 17484), were amplified by means of colony PCR from isolated species, according to the method and primers described in 3.4.4. A volume of 0.5µl bacterial suspension (10^4 cells ml^{-1}) was used as a template for PCR, after which the product was analysed as above.

3.4.6 *nifH* PCR

A portion of the *nifH* gene involved in nitrogen fixation was selectively amplified by means of nested-PCR from the total extracted soil DNA and from bacterial colonies, using the degenerate primers:

nifH (Forward A) : 5' GCIWTITAYGGNAARGGNGG 3'

nifH (Forward B) : 5' GGITGTGAYCCNAAVGCNGA 3'

nifH (Reverse) : 5' GCRTAIABNGCCATCATYTC 3'

(Widmer *et al.* 1999)

DNA sequence degeneracies are depicted using the International Union of Pure and Applied Chemistry Conventions (Liébecq 1992):

R : A/G

Y : C/T

W : A/T

V : A/C/G

B : C/G/T

N : A/C/G/T

I : Inosine used to reduce degeneracy in fourfold degenerate positions.

DGGE with product from nested PCR has been proven to be accurate by Bodelier *et al*. (2005), who determined species diversity within methanotrophic microbial communities. Two PCR reactions were performed on each sample, the first using primers *nifH* (Forward A) and *nifH* (Reverse) and the second using *nifH* (Forward B) and *nifH* (Reverse). Soil samples 9-18 (Table 1) and bacterial isolates from Molobela (2005) (See 3.2) were numbered according to their PCR results (Table 2). The PCR reaction component volumes were the same as in 3.4.4 and 3.4.5 using 0.5µl of a 10^{-1} bacterial suspension as template for the first reaction and 0.5 µl of this PCR product (*ca.* 27ng μl^{-1}) as template for the second reaction. DNA amplification was performed in a PCR thermal cycler using the following programme: 11 s at 94 °C, 40 cycles of 15 s at 92 °C, 8 s at 48 °C, 30 s at 50 °C, 10 s at 74 °C, 10 s at 72°C, followed by 10 min. at 72 °C, and then held at 4 °C. The PCR product was viewed on a 1 % TAE agarose gel. All reactions were performed in triplicate to negate possibilities of human or reagent error in PCR protocol.

Table 2: *nifH* PCR productnumbers of soil and bacterial samples collected from an unpolluted control site at the University of Pretoria experiment farm and from polluted and unpolluted areas at site 2 in Mpumalanga Province, South Africa (Table 1), with a history of crude-oil, pitch, diesel, petrol and tar pollution.

***nif* PCR product number**	**Corresponding soil sample or bacterial sequence (SA) number**	**Area state**[f]	**Description/Identification**
1	9	U	Unpolluted control soil from University of Pretoria experiment farm
2	10	U	Unpolluted soil from *Bidens pilosa* rhizosphere
3	11	U	Unpolluted soil from *Brantha serratia* rhizosphere
4	12	U	Unpolluted soil from *Cyperus esculentus* rhizosphere
5	13	P	Polluted soil (pitch/oil/diesel/petrol/tar) 10cm from *C. esculentus* plant
6	14	P	Polluted soil (pitch/oil/diesel/petrol/tar) from *C. esculentus* rhizosphere
7	15	P	Polluted soil (pitch/oil/diesel/petrol/tar), 10cm from *B. serratia* plant
8	16	P	Polluted soil (pitch/oil/diesel/petrol/tar), from *B. serratia* rhizosphere
9	17	P	Polluted soil (workshop oil) mulched with wood chips, 10cm from *B. serratia* plant
10	18	P	Polluted soil (workshop oil) mulched with wood chips from *B. serratia* rhizosphere
11	SA1	P, U	Bacterial isolate from *B. pilosa* rhizosphere in unpolluted soil and from *E. coracana* rhizosphere in polluted soil, groups with *Pseudomonas* genus
12	SA2	P	Bacterial isolate from *B. pilosa* rhizosphere, groups with *Providencia* genus

[f] U = unpolluted and P = polluted

Table 2 (continued)

***nif* PCR product number**	**Corresponding soil sample or bacterial sequence (SA) number**	**Area state[f]**	**Description/Identification**
13	SA3	P	Bacterial isolate from *B. pilosa* rhizosphere, groups with *Providencia* genus
14	SA4	P, U	Bacterial isolate from *E. coracana* and *C. esculentus* rhizospheres and from unpolluted soil void of plants, groups with *Staphylococcus* and *Bacillus* genera
15	SA5	P	Bacterial isolate from polluted soil with no plants growing, groups with *Pseudomonas* genus
16	SA6	P	Bacterial isolate from *C. esculentus* rhizosphere, groups with *Pseudomonas* genus
17	SA7	P	Bacterial isolate from *C. esculentus* rhizosphere, groups with *Pseudomonas* genus
18	SA8	P	Bacterial isolate from *E. coracana* rhizosphere, groups with *Pseudomonas* genus

3.5 DGGE

PCR product was subjected to DGGE according to the method described by Muyzer *et al.* (1993). Ten microlitres containing *ca.* 250ng of the various 16S and ITS PCR products was loaded per lane onto two 25-55 % denaturing gradient gels (Table 3). Similarly, 10µl (*ca.* 250ng) of *xylE* and *ndoB* products were loaded per lane onto a 30-60 % denaturing gradient gel. Finally, *nifH* nested-PCR products of the samples were loaded onto a 30-65 % denaturing gradient gel. Gels were run at 70 V for 17 h at a constant temperature of 60 °C. Image analysis was performed using the Gel2K (Norland 2004) programme and fingerprints were analysed in a cluster investigation using CLUST (Norland 2004).

Table 3: Denaturing gradient table showing volumes in millilitres of DSSA (denaturing stock solution A: 8 % acrylamide in 0.5x TAE (40mM Tris, 20mM acetic acid, 1nM EDTA (pH 8.3) buffer) and DSSB (denaturing stock solution B: 8 % acrylamide, 7M urea, 40 % formamide in 0.5x TAE buffer) mixed to form a gradient within the gel.

Denaturing percentage	DSSA (ml)	DSSB (ml)
25	10.9	3.6
30	10.2	4.4
35	9.4	5.1
40	8.7	5.8
45	8.0	6.5
50	7.3	7.3
55	6.5	8.0
60	5.8	8.7
65	5.1	9.4

Selected bands were picked under blue light from DGGE gels using a sterile micropipette tip. Each band was assigned a number for sequence analysis. The gel fragment was placed into 25µl filter-sterilised deionised water and allowed to stand overnight to dissolve. DNA from bands were then subjected to PCR, with respective primers, for sequencing purposes. Representative final sequences obtained were deposited into GenBank.

3.6 Sequencing

Sequencing the PCR product from the 16S colony PCR using the K and M primers above provided tentative species identification. Each isolate was sequenced in an Eppendorf tube containing 1µl clean PCR product, 2µl "Big Dye" (Roche) sequence mix, 0.32µl primer and 1.68µl filter-sterilised deionised water. The sequence PCR product was cleaned by adding 15µl sterile water, transferring the entire volume to a 0.5ml Eppendorf sequencing tube,

adding 2µl of 3M sodium acetate and 50µl 95 % ethanol, and allowing it to stand on ice for 10min. The tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150µl 70 % ethanol, and the tubes again centrifuged for 5 min at 10 000 rpm. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. Tubes were transferred on ice to the sequencer. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK). Partial sequences of the 16S eubacterial gene of the rDNA were obtained using the K primer above. Nucleotide sequence order was confirmed by comparison with the sequence obtained from the M primer of the corresponding sample.

Each sequence was subjected to a BLAST analysis on the GenBank database and matching hits, with e-values closest to 0.0 indicating a statistically plausible match, were selected for alignment. For samples 1-8, five matching hits with e-values closest to 0.0, were selected for alignment, whereas three matching hits closest to e 0.0 were selected for alignment from pure cultures SA1-SA8. In both cases, sequences of several species known to catabolise petrol, diesel, oil and other PAH and polyphenol-containing substances were included in the alignments. Sequences were aligned with Clustal X (Thompson *et al.* 1994) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping, MULPAR-effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was assessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention indices (RI) were determined for all data sets.

Phylogenetic trees of sequences from samples 1-8 were rooted with *Bacillus subtilis*, and with *B. subtilis*, *Thermotoga maritima* and *E. coli* as outgroups to the remaining taxa for the non-BLASTed and BLASTed results, respectively. Phylogenetic trees of sequences from pure cultures SA1-SA8 were rooted with *T. maritima* as outgroup to the remaining taxa. Bootstrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated. In sequences from soil samples 1-8, this was followed by a distance analysis using *B. subtilis* and *T. maritima* as outgroups to the analysed taxa. Two models of evolutionary base substitutions within PAUP were used to estimate evolutionary distances (Kimura 1981). This model also gives an approximation of evolutionary rates and divergence times, using formulae to determine base-substitution rates at each base of a codon.

Chapter 4

Results

Results

4.1 Phylogeny of microbial communities from crude oil-polluted soil according to DGGE profiles

DGGE with the 16S rDNA PCR product from soil samples 1-8 (Table 1) resulted in a gel displaying a denser (closer spaced and higher number) banded fingerprint pattern with a higher colour intensity in unpolluted soil (lanes 5-7) than in the diesel-polluted soils (lanes1-4 and 8) (Fig. 6).

Each band on the gel is assumed to be representative of only one distinct species, which was proved upon sequencing of the band. The gel also revealed a decrease in microbial diversity, i.e. number of bands, in subsoil layers (lanes 2 and 8) in comparison with topsoil. This was to be expected since these two samples should have very low species diversity as few organisms are present 1m and 1.5m deep in soil (Zhou *et al.* 2002). Of the polluted topsoil samples (lanes 1, 3 and 4), lane 3 DNA, extracted from the rhizosphere of plants growing in the soil, showed the greatest array of bands. This could have been due to plant root exudates enriching the soil around the roots, thus providing a nutrient boost within the polluted soil for microbes growing in the immediate vicinity of the roots. However, lane 4, which was from barren soil, displayed only slightly fewer bands than lane 3.

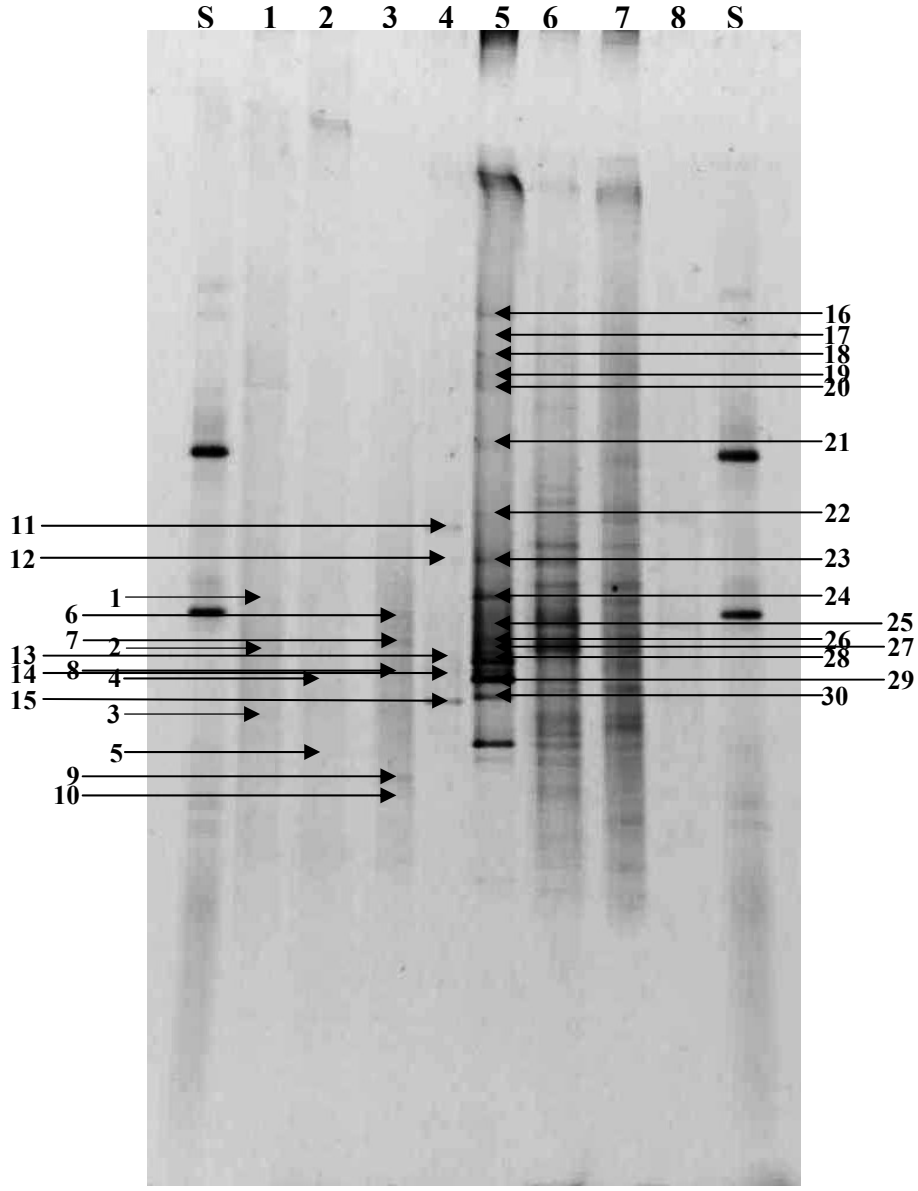
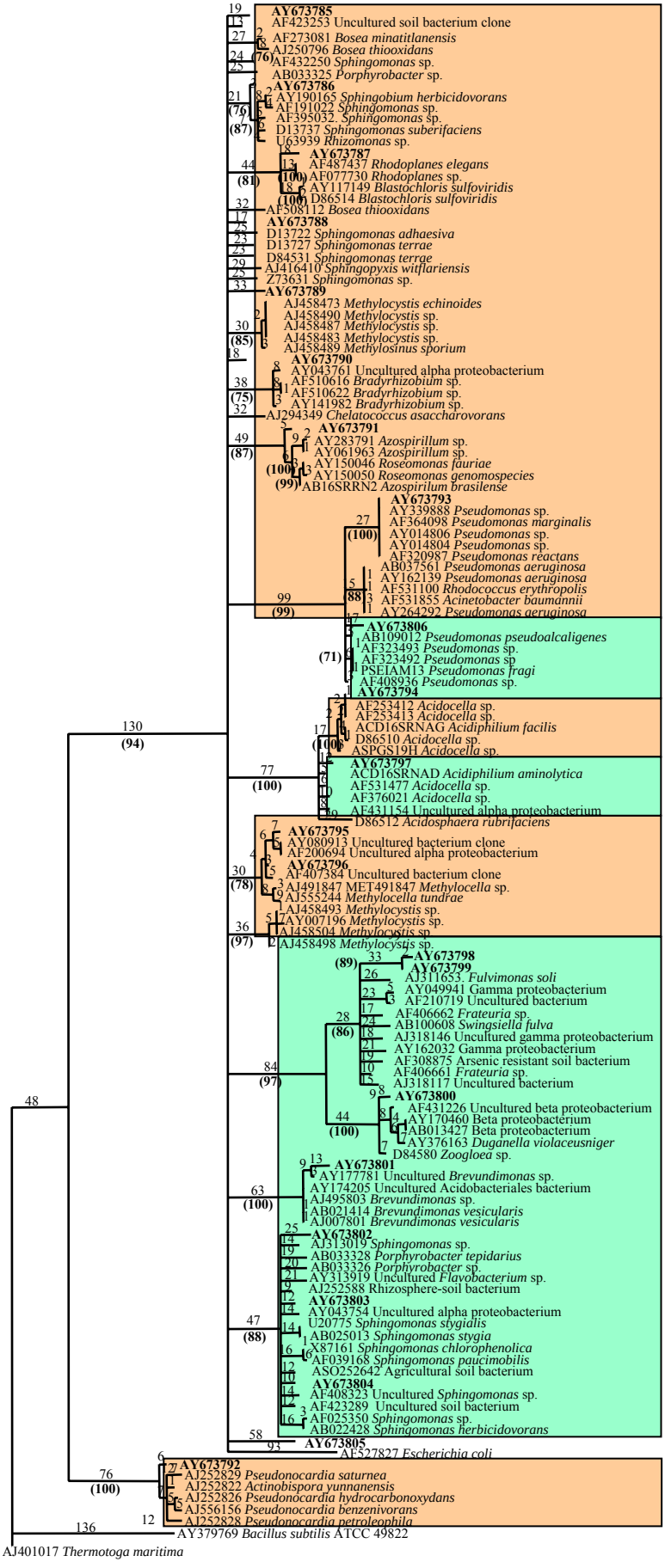


Figure 6: Denaturing gradient gel fingerprints resulting from assessing bacterial diversity between communities isolated from site 1 in Table 1, using a gradient of 15-55%. 16S rDNA PCR product separated according to base-pair sequence differences as a result of the denaturing gradient. Lanes 1-8 correspond with samples 1-8 in Table 1. Sequences 1-3 were taken from lane 1, 4-5 from lane 2, 6-10 from lane 3, 11-15 from lane 4 and 16-30 from lane 5. S represents the *Escherichia coli* standard used.

Distance (data not shown) and parsimony trees generated from sequence data displayed almost identical topography, within all of which distinct clade differentiation between polluted and unpolluted soil sequences was evident (Fig. 7). This is supported by the data

matrix comparing banding with lanes on the DGGE gel (Table 4), although, the matrix indicated that there were no sequences exclusively obtained from polluted soils. However several species were found solely in unpolluted soils viz. 16, 17, 19, 21, 22, 24, 26-30 (Table 4). Fig. 7 contains the sequences obtained from the gel with their corresponding BLAST results. Characters for this analysis were re-weighted to the consistency index, and only informative characters were included, missing and ambiguous characters as well as constant characters were excluded. The number of taxa included in this analysis was 134 thus making the required number of informative characters 264. The 271 informative characters presented in this phylogenetic analysis as well as the relatively high RI value obtained indicates significant signal within this data set. Sequences with e-values closest to 0.0 and displaying best alignments (data not shown) were compared with their most plausible matching organisms for tentative identification (Table 5). According to GenBank identification, many of these organisms are known to occur in hydrocarbon-polluted soils, e.g. *Sphingomonas adhaesiva*, *Sphingomonas terrae*, *Sphingopyxis witflariensis*, *Sphingomonas* sp., *Methylocystis* sp., *Pseudomonas* sp., *Pseudomonas marginalis*, *Acidocella* sp. and *Acidiphilium facilis*. The topography of the tree supports the tentative molecular identification of the unknown species isolated from polluted and unpolluted soils. The tentative identification of these species constitutes the first report of these microbes from polluted soils in South Africa.

Phylogenetic analysis of the sequences obtained from polluted soils (Fig. 8) showed a lower species diversity than those from unpolluted soils (Fig. 9). Species analysed from polluted soils appeared to be more closely related to each other as they grouped into one main clade, with AY673792 basal to that clade. Those from unpolluted soils showed a higher species diversity, i.e. genetically more distinct, as they grouped into three main clades, with AY673801 and AY673797 falling basal to these.



Parsimony informative characters 271
CI 0.231
RI 0.745
Number of trees 1
Tree length 2502
*g*1 -0.179919

Figure 7: Phylogeny of bacterial 16S rRNA gene amplicons recovered from the DGGE gel in Fig. 1 from soils collected at site 1 (Table 1). Sequences from polluted and unpolluted soils are indicated in orange and green areas, respectively (Distance values are indicated above branches and bootstrap values are indicated in brackets below).

Table 4: Data matrix of the banding pattern displayed on the DGGE gel (Fig. 6), showing the bands present in each lane and the identity of the most likely bacterial species that the band represents.

Sequence number	Lane number[g]								Most probable species
	1	2	3	4	5	6	7	8	
1	x		x	x	x	x	x	x	Uncultured soil bacterium clone
2	x	x	x	x	x	x	x		*Sphingomonas* sp.
3	x		x		x	x	x		*Sphingomonas* sp.
4		x		x	x	x	x	x	*Blastochloris sulfoviridis*
5		x		x	x	x	x		*Methylocystis* sp.
6	x		x		x	x	x	x	No identification available
7	x		x		x	x	x	x	*Bradyrhizobium* sp.
8	x		x		x	x	x		*Azospirillum* sp.
9			x			x	x		*Pseudonocardia saturnea*
10			x			x	x		*Pseudomonas* sp.
11				x	x	x	x	x	No identification available
12				x	x	x	x		*Acidocella* sp.
13			x	x	x	x	x	x	Uncultured alpha Proteobacterium
14	x			x	x	x	x	x	No identification available
15				x	x	x	x		*Methylocella* sp.
16					x	x	x		No identification available
17					x	x	x		*Acidocella* sp.
18	x				x	x	x		*Fulvimonas soli*
19					x	x	x		*Frateuria* sp.
20	x				x	x	x		*Duganella violaceusniger*
21					x	x	x		No identification available
22					x	x	x		*Brevundimonas* sp.
23	x				x	x	x		*Sphingomonas* sp.
24					x	x	x		*Sphingomonas* sp.
25			x		x	x	x	x	*Sphingomonas* sp.
26					x	x	x		No identification available
27					x	x	x		No identification available
28					x	x	x		*Pseudomonas aeruginosa*
29					x	x	x		*Pseudomonas pseudoalcaligenes*
30					x	x	x		No identification available

[g] Lanes from polluted and unpolluted soils are indicated in orange and green respectively.

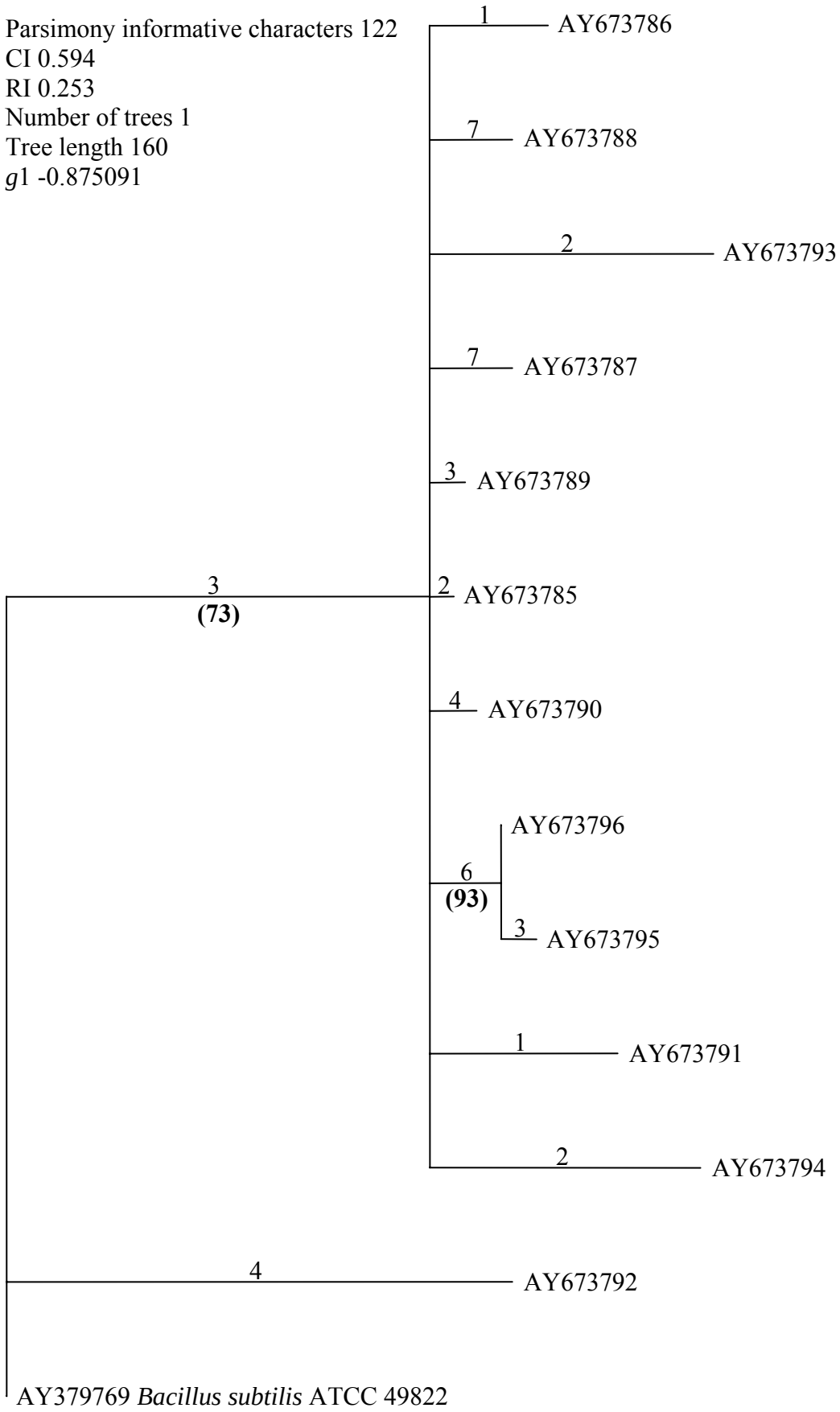


Figure 8: Phylogeny of the 16S eubacterial gene of rDNA from bands picked from the DGGE fingerprint in Fig. 1 of polluted soils sampled at site 1 (Table 1) (Distance values are indicated above branches and bootstrap values are indicated in brackets below).

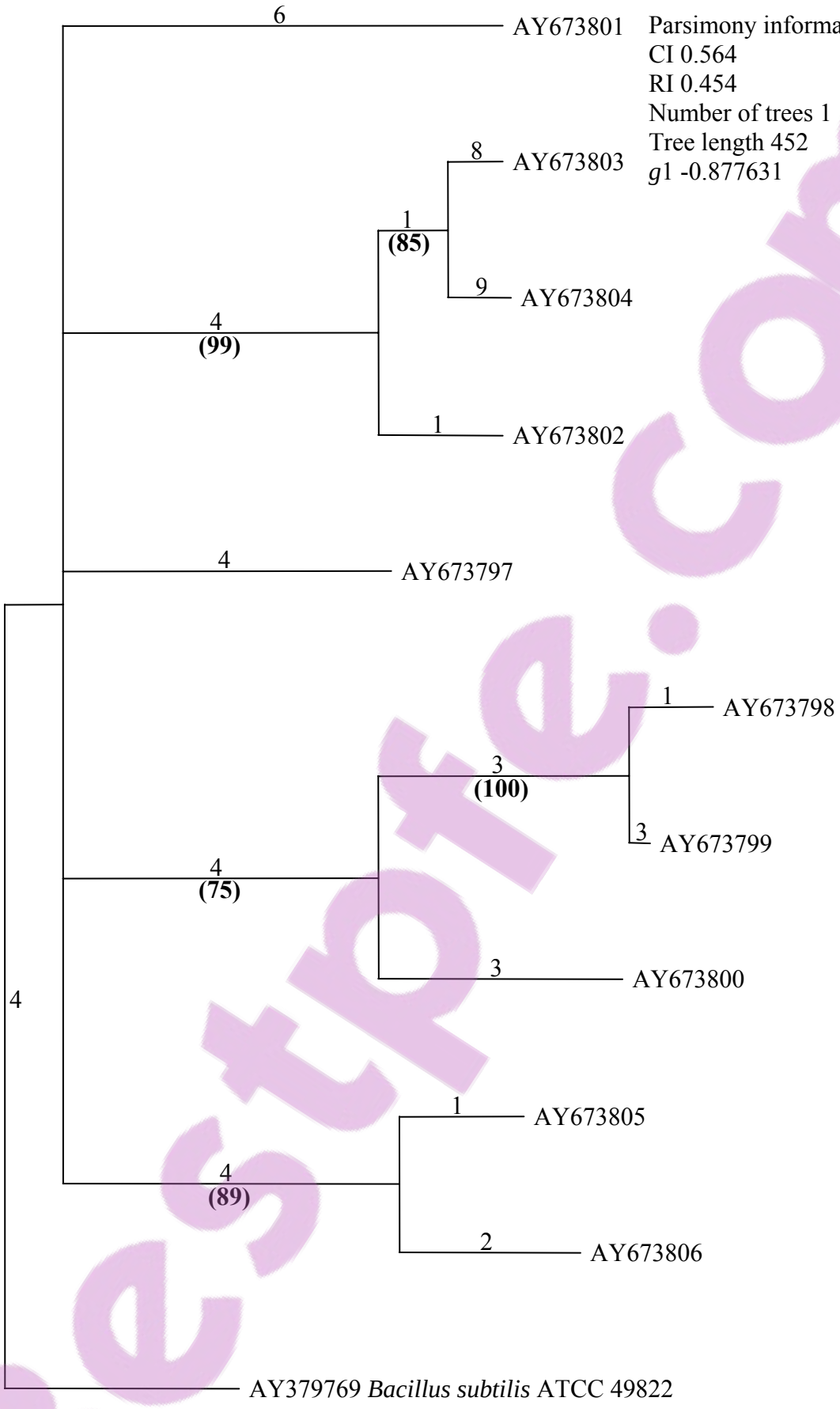


Figure 9: Phylogeny of the 16S eubacterial gene of rDNA from bands picked from the DGGE fingerprint in Fig. 1 of unpolluted soils sampled at site 1 (Table 1) (Distance values are indicated above branches and bootstrap values are indicated in brackets below).

Table 5: 16S eubacterial rDNA sequences from bands picked from the DGGE fingerprint in Fig. 1 of soils sampled at site 1 (Table 1). Bacterial species having the closest possible e-value for identification purposes and their descriptions are presented.

Deposited GenBank sequence number	DGGE gel band sequence number	Matching GenBank accession numbers	Closest species Identification	e-value	Description
AY673785	1	AF423253	Uncultured soil bacterium clone	-163	Bacteria; environmental samples.
AY673786	2	AF395032	*Sphingomonas* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; from Lake Vostok accretion ice.
		AY190165	*Sphingobium herbicidovorans*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingobium*; from environmental samples.
		D13737	*Sphingomonas suberifaciens*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; from corky root of lettuce.
		U63939	*Rhizomonas* sp.	0.0	Bacteria; Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; from marine bacterioplankton.
AY673787	4	D86514	*Blastochloris sulfoviridis*	-124	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Hyphomicrobiaceae *Blastochloris*.
AY673788	3	D13722	*Sphingomonas adhaesiva*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; polyethylene glycol-utilising bacteria.
		D13727	*Sphingomonas terrae*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingopyxis*; polyethylene glycol-utilising bacteria.

Table 5 (continued)

Deposited GenBank sequence number	DGGE gel band sequence number	Matching GenBank accession numbers	Closest species Identification	e-value	Description
		D84531	*Sphingomonas terrae*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingopyxis*; from ears of plants in Gramineae.
		AJ416410	*Sphingopyxis witflariensis*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingopyxis*; from activated sludge.
		Z73631	*Sphingomonas* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; marine ultramicrobacterium.
AY673789	5	AJ458490	*Methylocystis* sp.	-153	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Methylocystaceae *Methylocystis*; type II methane-oxidising bacteria isolated from various environments.
AY673790	7	AY141982	*Bradyrhizobium* sp.	-122	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae *Bradyrhizobium*; rhizobia.
AY673791	8	AY283791	*Azospirillum* sp.	-99	Bacteria: Proteobacteria Alphaproteobacteria Rhodospirillales Rhodospirillaceae *Azospirillum*; from wheat rhizosphere.
AY673792	9	AJ252829	*Pseudonocardia saturnea*	-171	Bacteria: Actinobacteria Actinobacteridae Actinomycetales Pseudonocardineae Pseudonocardiaceae *Pseudonocardia*.
AY673793	10	AY339888	*Pseudomonas* sp.	0.0	Bacteria: Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*; nitrobenzene biodegradation.
		AF364098	*Pseudomonas marginalis*	0.0	Bacteria: Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*; cold tolerant; from carrot.
		AF320987	*Pseudomonas reactans*	0.0	Bacteria: Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*; brown blotch disease of *Agaricus bisporus*.
AY673794	12	AF253412	*Acidocella* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhodospirillales Acetobacteraceae *Acidocella*; aromatic degradation.
		D30774	*Acidiphilium facilis*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhodospirillales Acetobacteraceae *Acidocella*.

Table 5 (continued)

Deposited GenBank sequence number	DGGE gel band sequence number	Matching GenBank accession numbers	Closest species Identification	e-value	Description
AY673795	13	AF200694	Uncultured alpha Proteobacterium	0.0	Bacteria: Proteobacteria Alphaproteobacteria; from environmental samples.
		AY080913	Uncultured bacterium clone	0.0	Bacteria; environmental samples; methylotroph communities in an acidic forest soil.
AY673796	15	AJ491847	*Methylocella* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Beijerinckiaceae *Methylocella*; novel methanotroph isolated from an acidic forest cambisol.
		AJ555244	*Methylocella tundrae*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Beijerinckiaceae *Methylocella*; novel methanotrophic bacterium from acidic tundra peatlands.
		AJ458498	*Methylocystis* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhizobiales Methylocystaceae *Methylocystis*; type II methane-oxidising bacteria isolated from various environments.
AY673797	17	D30771	*Acidiphilium aminolytica*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhodospirillales Acetobacteraceae *Acidocella*.
		AF376021	*Acidocella* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Rhodospirillales Acetobacteraceae *Acidocella*; microorganisms indigenous to acidic drainage waters at abandoned Norwegian copper mine.
AY673798	18	AJ311653	*Fulvimonas soli*	0.0	Bacteria: Proteobacteria Gammaproteobacteria Xanthomonadales Xanthomonadaceae *Fulvimonas*; from soil after enrichment on acetylated starch plastic.
		AF406662	*Frateuria* sp.	0.0	Bacteria: Proteobacteria Gammaproteobacteria Xanthomonadales Xanthomonadaceae *Frateuria*; from potato.
AY673799	19	AY162032	Gamma Proteobacterium	0.0	Bacteria: Proteobacteria Gammaproteobacteria.
		AF406661	*Frateuria* sp.	-175	Bacteria: Proteobacteria Gammaproteobacteria Xanthomonadales Xanthomonadaceae *Frateuria*; from potato.

Table 5 (continued)

Deposited GenBank sequence number	DGGE gel band sequence number	Matching GenBank accession numbers	Closest species Identification	e-value	Description
AY673800	20	AF431226	Uncultured beta Proteobacterium	-110	Bacteria: Proteobacteria Betaproteobacteria; environmental samples; lodgepole pine (*Pinus contorta*) rhizosphere soils from British Columbia forest soils.
		AY376163	*Duganella violaceusniger*	-106	Bacteria: Proteobacteria Betaproteobacteria Burkholderiales Oxalobacteraceae *Duganella*.
AY673801	22	AY177781	Uncultured *Brevundimonas* sp.	-129	Bacteria: Proteobacteria Alphaproteobacteria Caulobacterales Caulobacteraceae *Brevundimonas*; environmental samples; soil bacterial communities in California grassland.
AY673802	23	AJ313019	*Sphingomonas* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; from hexane degrading biofilters.
		AB033328	*Porphyrobacter tepidarius*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Porphyrobacter*.
		AB033326	*Porphyrobacter* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Porphyrobacter*.
		AY313919	Uncultured *Flavobacterium* sp.	0.0	Bacteria: Bacteroidetes Flavobacteria Flavobacteriales Flavobacteriaceae *Flavobacterium* environmental samples; from Tibetan plateau deep glacier ice.
		AJ252588	Rhizosphere soil bacterium	0.0	Bacteria
AY673803	24	U20775	*Sphingomonas stygialis*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae Novosphingobium; aromatic-degrading *Sphingomonas* isolates from deep soil subsurface.
		AB025013	*Sphingomonas stygia*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Novosphingobium*; from deep-sea sediments.
		X87161	*Sphingomonas chlorophenolica*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingobium*; pentachlorophenol-degrading.

Table 5 (continued)

Deposited GenBank sequence number	DGGE gel band sequence number	Matching GenBank accession numbers	Closest species Identification	e-value	Description
		ASO252642	Agricultural soil bacterium	0.0	Bacteria; environmental samples.
		AY043754	Uncultured alpha Proteobacterium	0.0	Bacteria: Proteobacteria Alphaproteobacteria; from British Columbia forest soils subjected to disturbance.
AY673804	25	AF039168	*Sphingomonas paucimobilis*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; lindane-degrading bacterium.
		AF025350	*Sphingomonas* sp.	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingomonas*; dicamba-degrading bacterium.
		AB022428	*Sphingomonas herbicidovorans*	0.0	Bacteria: Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae *Sphingobium*.
AY673805	28	AB037561	*Pseudomonas aeruginosa*	-107	Bacteria: Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*.
AY673806	29	AB109012	*Pseudomonas pseudoalcaligenes*	-138	Bacteria: Proteobacteria Gammaproteobacteria Pseudomonadales Pseudomonadaceae *Pseudomonas*; naphthalene-degrading bacteria from tar plant.

4.2 Comparative DGGE fingerprint analysis of pro- and eukaryotes in PAH-polluted soil

DNA was successfully extracted from samples 9-18 (Table 1). No protein contamination in the wells of the gel, or RNA contamination smears below the DNA bands, was evident (Fig. 10). PCR of 16S prokaryotic rDNA and ITS eukaryotic DNA yielded a *ca.* 550bp fragment of PCR product on the 1.5% TAE agarose gel (Fig. 11).

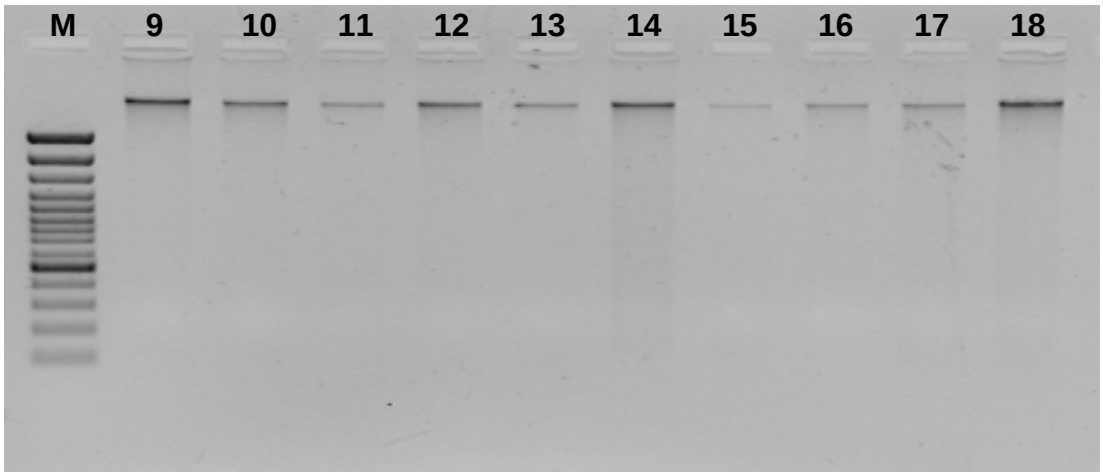


Figure 10: 1.5% TAE agarose gel showing high-quality, clean genomic DNA extracted from soil samples 9-18 at site 2 (Table 1) by means of the BIO101 Fast DNA Spin Kit for soil (M = 100bp marker, 9-18 = soil DNA from samples).

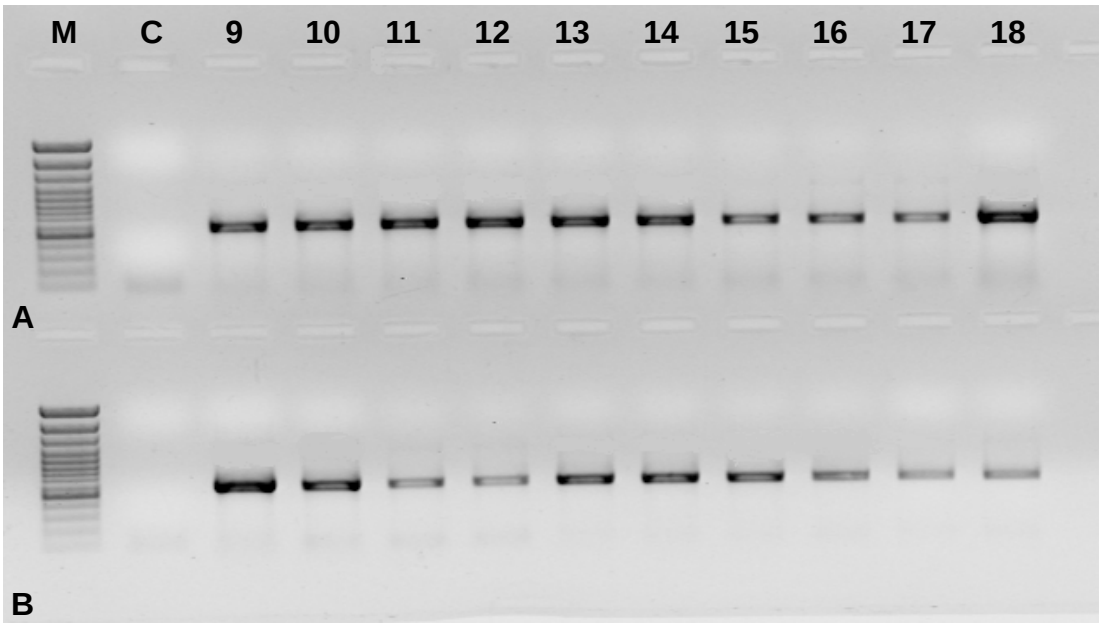


Figure 11: 1.5% TAE agarose gel showing (**A)** 16S rDNA PCR product of prokaryotic genomic DNA, and (**B)** partial internal transcribed spacer gene PCR product from eukaryotic genomic DNA, both *ca*. 550bp, from soil samples 9-18 at site 2 (Table 1) (M = 100bp marker, C = negative control, 9-18 = PCR product from samples).

DGGE yielded a gel showing clear multiple banding, forming a fingerprint in each lane (Fig. 12A and B). Due to the basis of DGGE, PCR fragments of the same size are separated into bands according to their sequence, the resulting fingerprint pattern being indicative of species diversity. From the gel a graphic cluster representation of the band pattern was drawn using Gel2K (Norland 2004). The programme does this by estimating band peak intensity along a lane. Peaks can be manipulated to ensure that, should more than one peak be registered per band, they can be grouped together. Dominant species per lane are indicated as dark prominent bands across the lane.

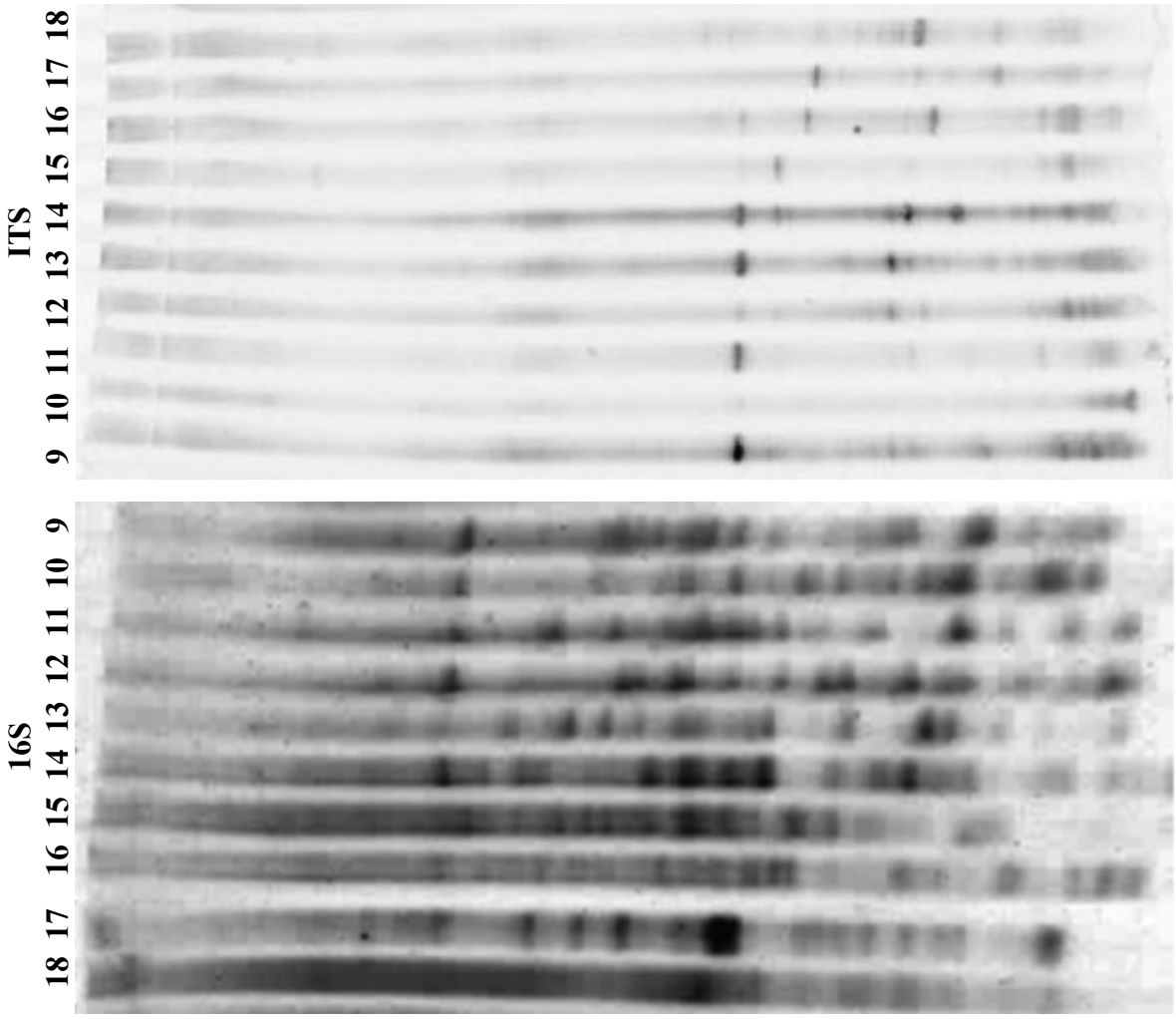


Figure 12A: DGGE gels, using a denaturant gradient of 15-55%. 16S rDNA (16S) and partial internal transcribed spacer sequence (ITS) PCR products separated according to base-pair sequence differences indicating community richness and diversity of pro- and eukaryotes in soils 9-18 (Table 1).

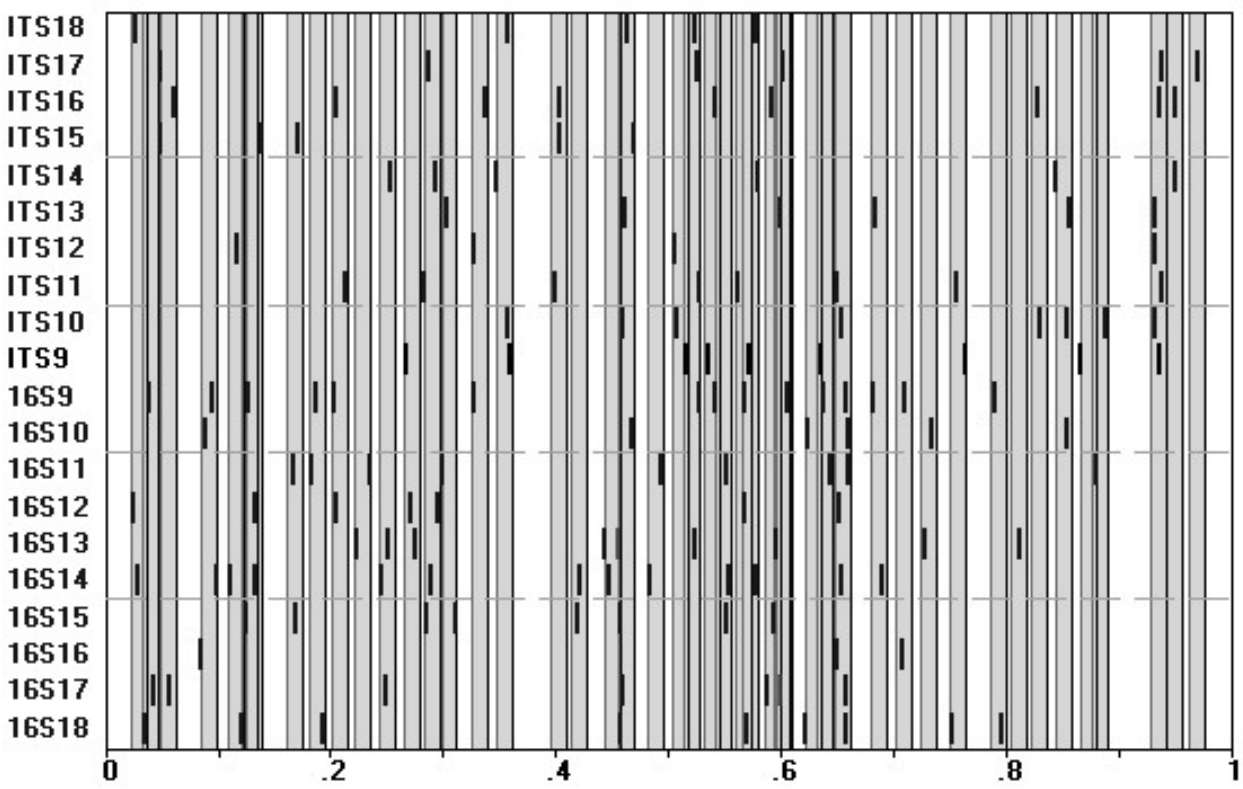


Figure 12B: Graphic representation of the DGGE gel, using a denaturant gradient of 15-55%. 16S rDNA (16S) and partial internal transcribed spacer sequence (ITS) PCR products separated according to base-pair sequence differences indicating community richness and diversity of pro- and eukaryotes in soils 9-18 (Table 1).

On average, more dominant bands were found for prokaryotes (mean 8.6 per sample) than for eukaryotes (mean 6.6 per sample) (Fig. 13), indicating that prokaryotic diversity was higher than that of eukaryotes. Species richness was also more evident within the prokaryotes, i.e. more dark/dominant species bands were observed within the prokaryotic gel lanes than in eukaryotic lanes.

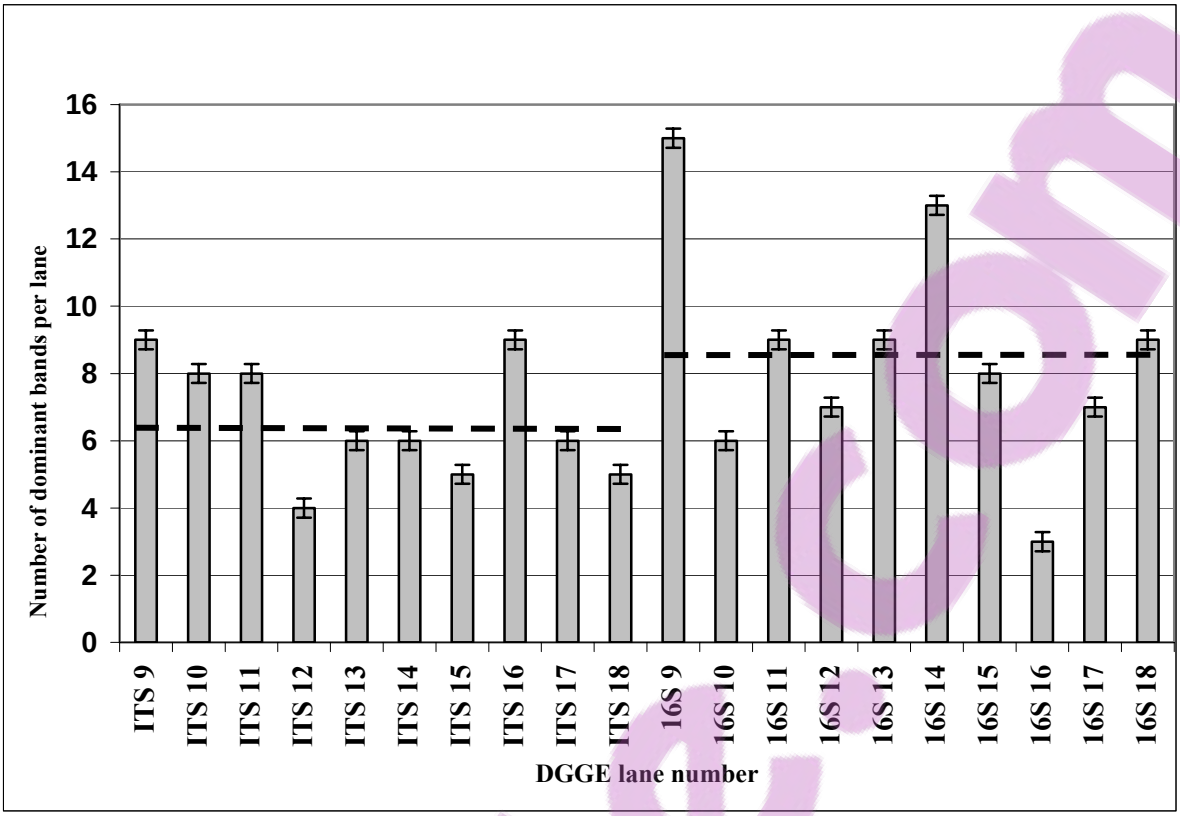


Figure 13: Number of dominant species per soil sample as indicated by DGGE of 16S rDNA (16S) and partial internal transcribed spacer sequence (ITS) PCR products. Note that the mean number of dominant species is 6.6 for eukaryotes and 8.6 for prokaryotes (dotted lines).

Following this, species diversity, and to certain extent species richness, were derived from the gel by compiling a dendogram (Fig. 14). The programme CLUST (Norland 2004) is based on Shannon index algorithms and groups the profiles of the species in each sample according to how similar in community composition the samples are. Thus, samples from similar environments would be expected to display analogous communities and group together in the CLUST dendogram.

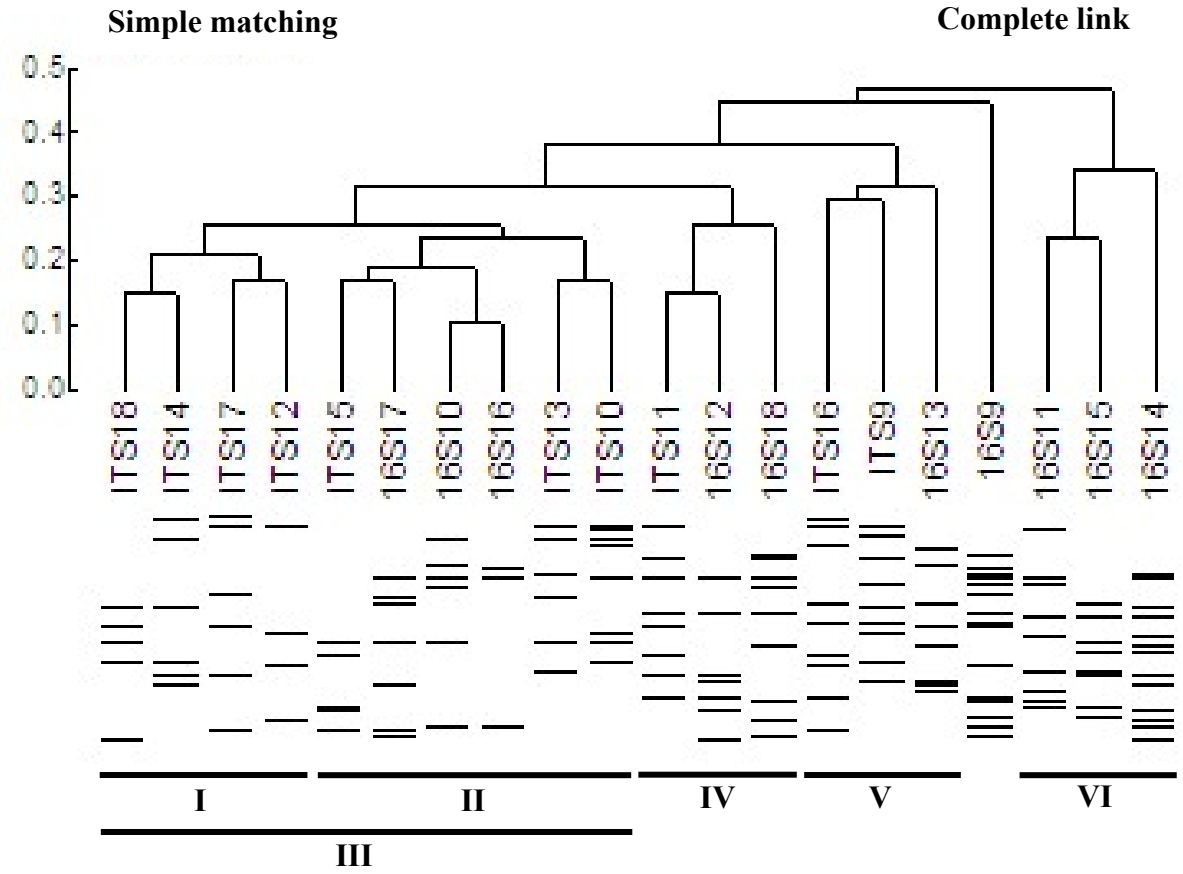


Figure 14: Cluster analysis of the banding pattern in Fig. 12, using a simple matching, complete link setting to separate communities according to species sequence differences (Thicker bands represent a brighter band on the gel, and therefore greater species richness).

4.3 DGGE analysis of toluene and naphthalene degraders in polluted soil

PCR of DNA extracted from samples 9-18 (Table 1, Fig. 11) with *ndoB* and *xylE* gene primers yielded a PCR fragment of *ca.* 650bp and 400bp, respectively on a 1.6% TBE agarose gel (data not shown). The DGGE gel showed clear multiple banding within each fingerprint in each lane (Fig. 15A and B). However, the gel had a great number of bands present and only dominant bands were therefore considered for diversity analysis. Control soil sample 9 harboured the most diverse *xylE* gene community, whereas the most diverse *ndoB* gene community was present in soils 17 and 18 polluted with machinery oil. On

average the gel showed a higher number of bands for the *xylE* gene (T) than for *ndoB* gene (N). The mean number of dominant species per soil sample was 13.6 among naphthalene degraders and 19.4 among toluene degraders. Dominant band numbers were compared across naphthalene and toluene degraders and, except for soil samples 17 and 18, all *xylE* gene products showed a considerably higher number of dominant species than *ndoB* gene products, possibly indicating a higher toluene than naphthalene degrading capacity across samples 9-16 (Fig. 16).

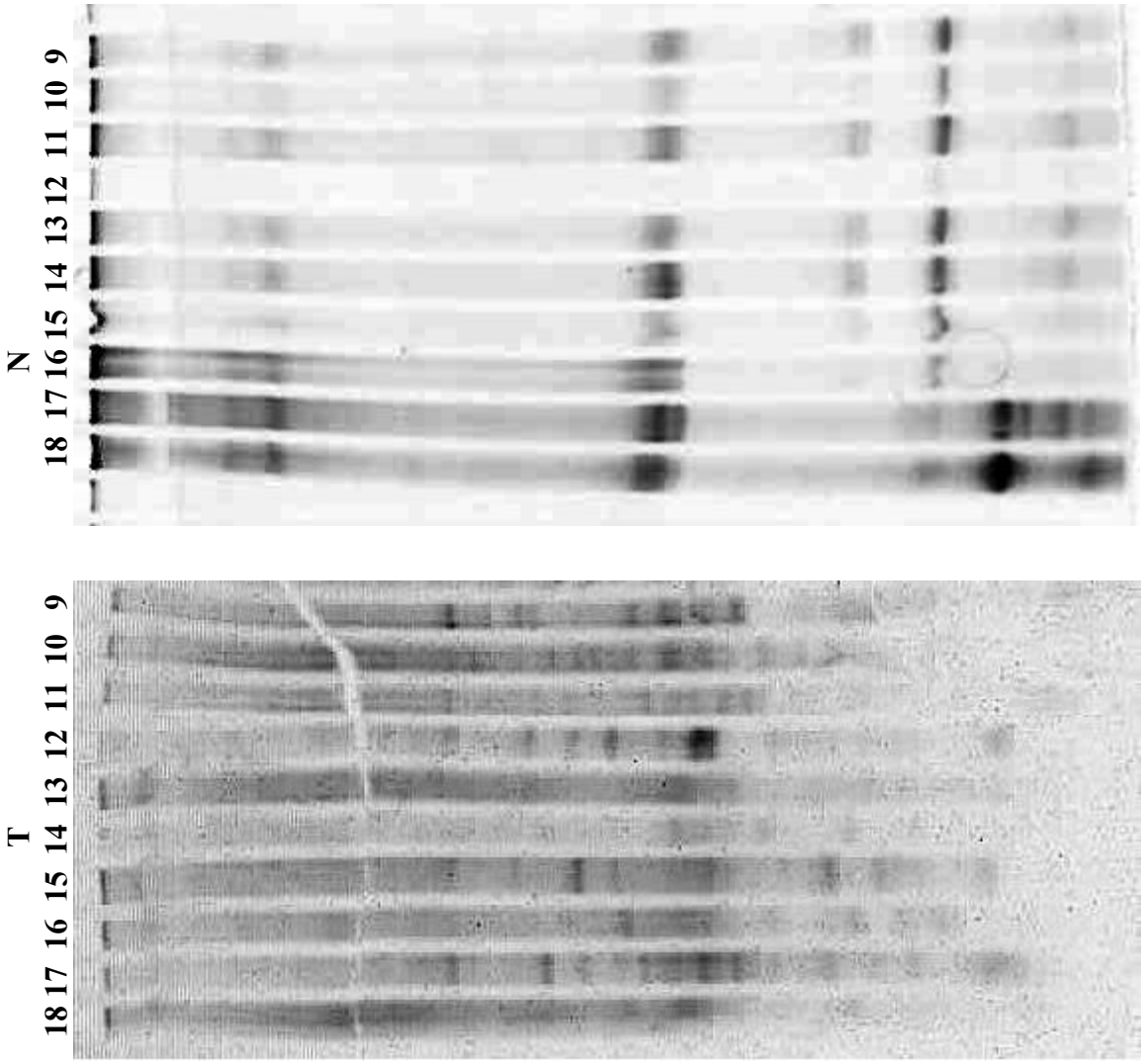


Figure 15A: DGGE gel, using a gradient of 30-65%, with *ndoB* gene (N) and *xylE* gene (T) PCR product amplified from DNA extracted from samples 9-18 (Table 1).

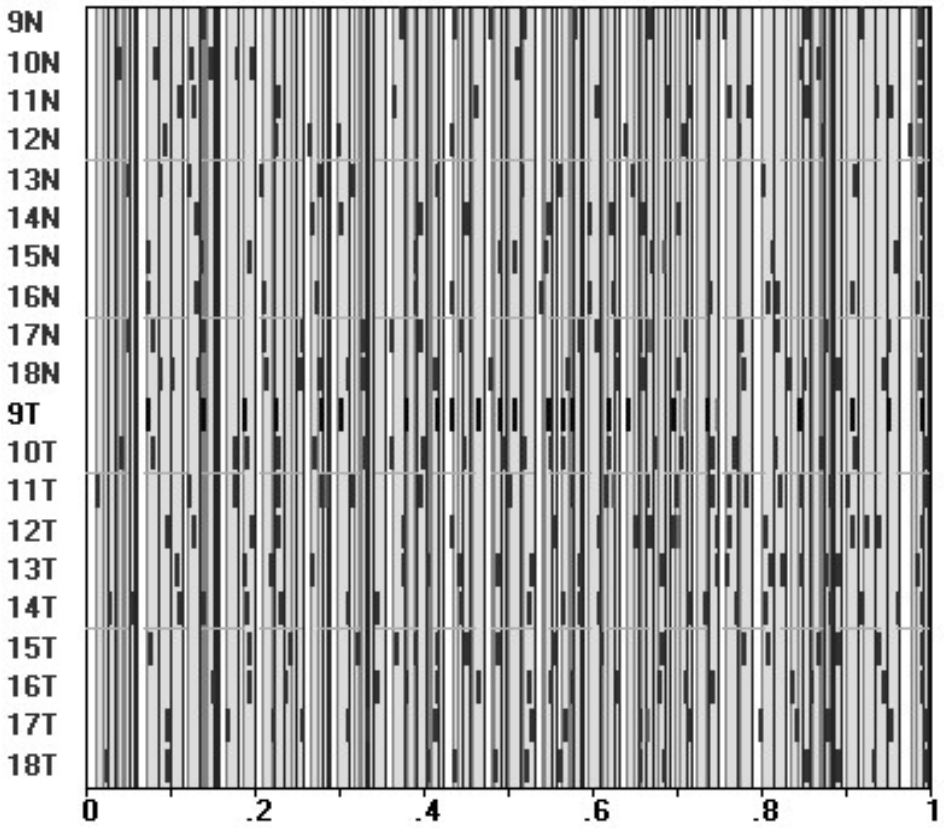


Figure 15B: Graphic representation of the DGGE gel, using a gradient of 30-65%, with *ndoB* gene (N) and *xylE* gene (T) PCR product amplified from DNA extracted from samples 9-18 (Table 1).

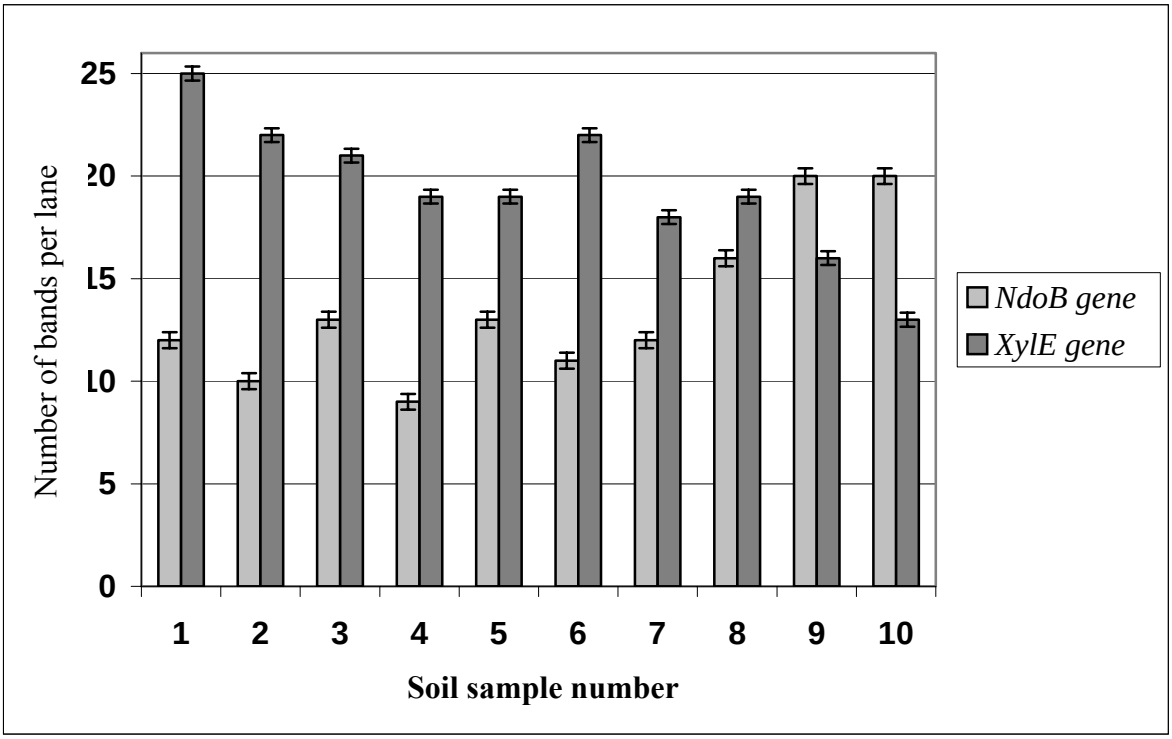


Figure 16: Number of dominant species per sample as indicated on the DGGE gel with *ndoB* gene and *xylE* gene PCR products from DNA extracted from samples 9-18 (Table 1) (Standard error for *ndoB* gene bands is 0.386 and 0.337 for *xylE* gene bands).

A combined dendogram of all communities across both *xylE* gene and *ndoB* gene diversity showed five main clades (Fig. 17). Clades I and V consisted mainly of *xylE* gene communities, while clade IV included mainly *ndoB* gene communities. Within clade IV there were two subclades (II and III), subclade II containing only *ndoB* gene communities and grouping three of the unpolluted soils and polluted soil 15.

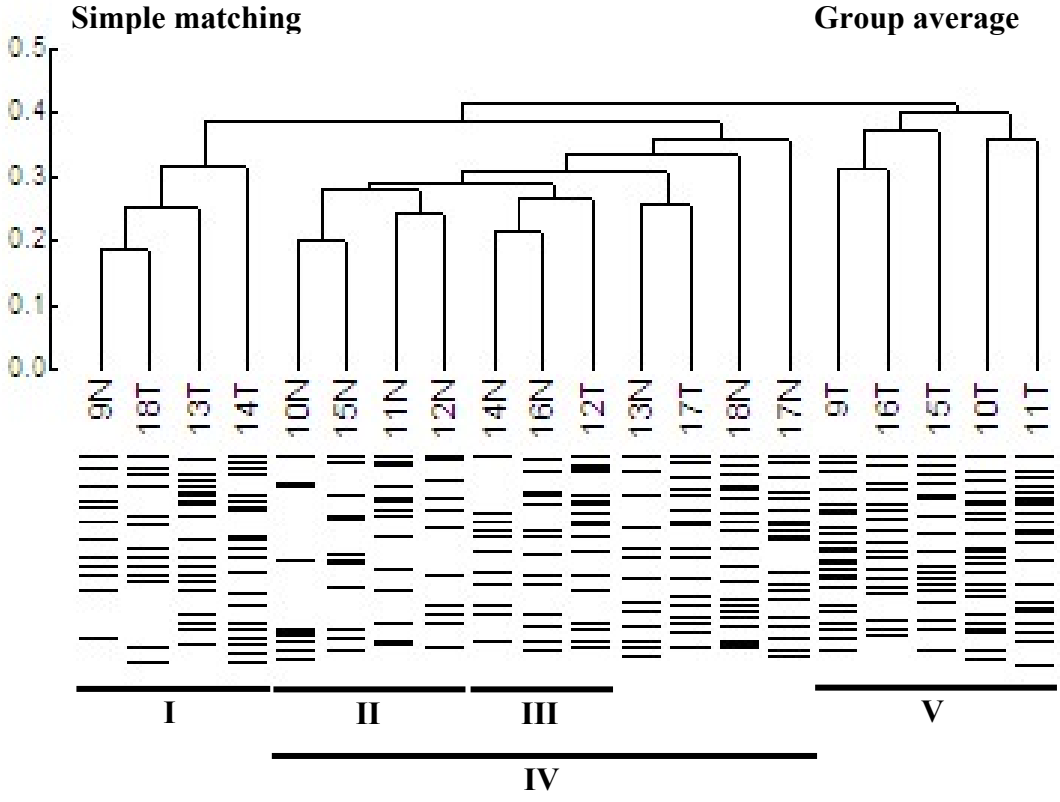


Figure 17: Cluster analysis of the banding pattern in Fig. 15 of *ndoB* and *xylE* gene PCR products, using a simple matching, group average setting to separate communities on the basis of species sequence differences from a multi-gene community (Thicker bands represent a brighter band on the gel, and therefore tentatively indicate a higher species richness).

The topography in Fig. 17 is also evident in clade I of the *ndoB* gene dendogram (Fig. 18). Within subclade III soils 14 and 16 grouped together as could be expected since they were both from rhizosphere soil (Table 1). The predominance of naphthalene degraders in clade IV indicates a similar genetic community profile across unpolluted and polluted soils. However, the majority of toluene degraders were split into two separate clades (I and V), those in clade I grouping with the naphthalene community from control soil 9.

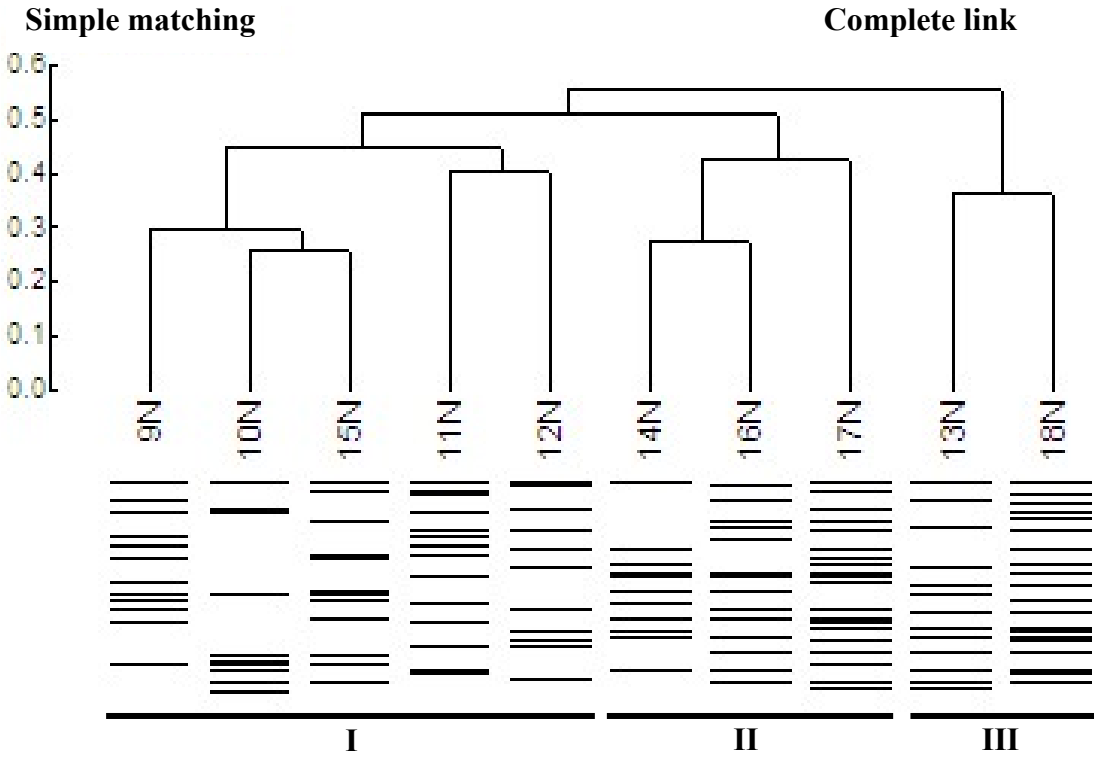


Figure 18: Cluster analysis of the banding pattern in Fig. 15 includes *ndoB* gene PCR products, using a simple matching, complete link setting to separate communities on the basis of species sequence differences from a single gene community (Thicker bands represent a brighter band on the gel, and therefore tentatively indicate a higher species richness).

The dendogram depicting *ndoB* gene diversity displayed three informative clades (Fig. 18). Clade I included mainly unpolluted soils grouping separately from polluted soils. Soils 11 and 12 clustered together as in clade II in Fig. 17, thus indicating that these soils contained very similar communities within the scope of the *ndoB* gene. Clades II and III contained polluted soils, with soils 14 and 16 grouping closer to each other than to soil 17, and soils 13 and 18 clustering together.

The final dendogram focusing on *xylE* gene diversity accommodated three informative

clades (Fig. 19). Clade I had soils 9, 13, 14, 17 and 18 assembling together. Except for soil 17 this is the same grouping found in clade I of Fig. 17, indicating a similar community profile. Clade II clustered soils 15 and 16 together, as was also evident in Fig. 17, clade V. Falling basal to the above clades was clade III containing the unpolluted soils 10, 11 and 12, hence corroborating the similarity of their community structures for *xylE* gene diversity.

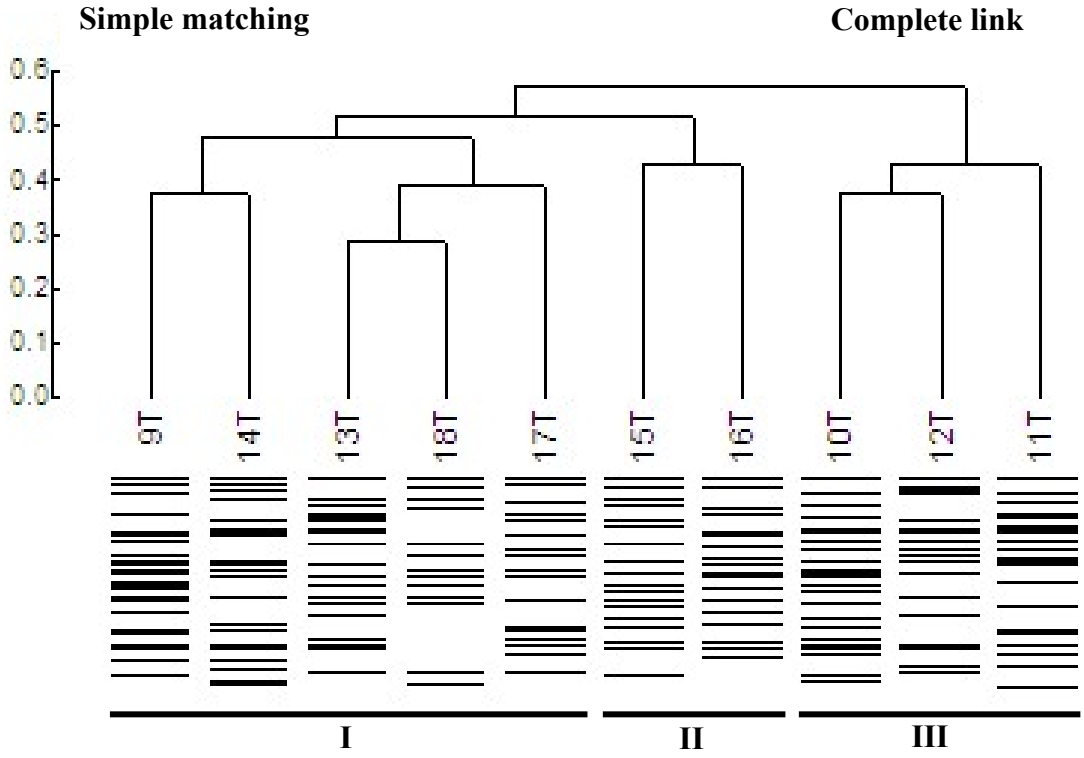


Figure 19: Cluster analysis of the banding pattern of the *xylE* gene PCR product in Fig. 15 using a complete link, simple matching setting to separate communities on the basis of species sequence differences from a single gene community (Thicker bands represent a brighter band on the gel, and therefore higher species richness).

4.4 Molecular identification of toluene and naphthalene degrading capacity of bacteria from PAH-polluted soil

All eight isolates yielded bands of *ca.* 400 (Fig. 20) and *ca.* 600bp when screened for the production of catechol 2,3-dioxygenase and naphthalene dioxygenase, respectively. The phylogenetic tree, containing these isolates, revealed two large clades rooted to *T. maritima* (Fig. 21). The larger of the two clades, containing various soil inhabitants and PAH degraders, housed six distinct sub-clades, viz. I (*Burkholderia*), II (*Sphingomonas*), III (*Pseudomonas*), IV (*Staphylococcus* and *Bacillus*), V (*Providencia*) and VI (*Burkholderia, Methylobacterium, Klebsiella, Rhodococcus* and *Pseudomonas*). Within this clade the genera *Burkholderia*, *Sphingomonas*, *Pseudomonas*, *Bacillus*, *Methylobacterium*, *Klebsiella* and *Rhodococcus* as well as *Vibrio* sp. are known to be involved in the metabolism of PAHs, particularly naphthalene and phenanthrene (Samanta *et al*. 2002; Kang *et al*. 2003).

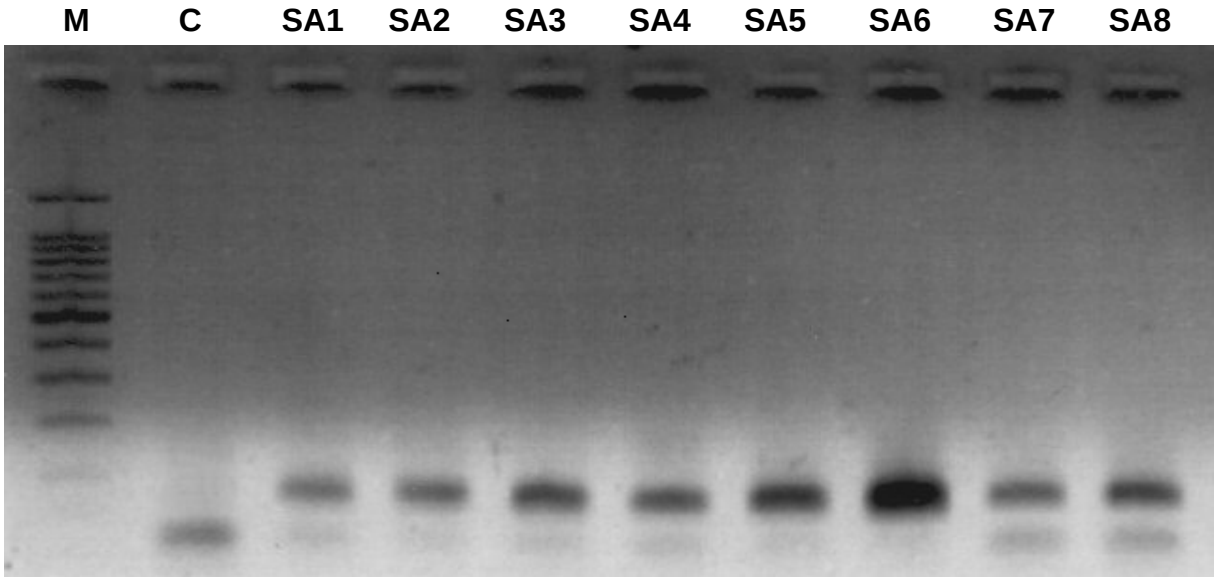


Figure 20: Bands of *ca.* 400bp on a 1.6% TBE gel showing production of catechol 2,3-dioxygenase (*xylE* gene) by bacterial isolates from a PAH-polluted soil in South Africa (M = λ marker, C = negative control, SA1-SA8 = isolates).

Five of the sequenced isolates (SA1, SA5, SA6, SA7 and SA8) clustered into the *Pseudomonas* sub-clade (III), one (SA4) into *Staphylococcus* (IV) and two (SA2 and SA3) into *Providencia* (V). None of the isolates clustered into the smaller of the two clades comprising mainly *Mycobacterium* species (Fig. 21). Most mycobacteria are capable of degrading PAHs but are not readily isolated and grow very slowly on artificial media (Prescott *et al*. 1999). There are, however, exceptions such as *M. tusciae*, *M. moriokaense*, *M. septicum* and *M. isoniacini*, which can grow rapidly in culture (Leys *et al.* 2005). The absence of *Mycobacterium* isolates in the present study was probably not due to the fact that they could not be isolated, but rather to the genus being very rare or not present in polluted soils in South Africa, as evident from a previous preliminary survey (Surridge *et al.* 2004).

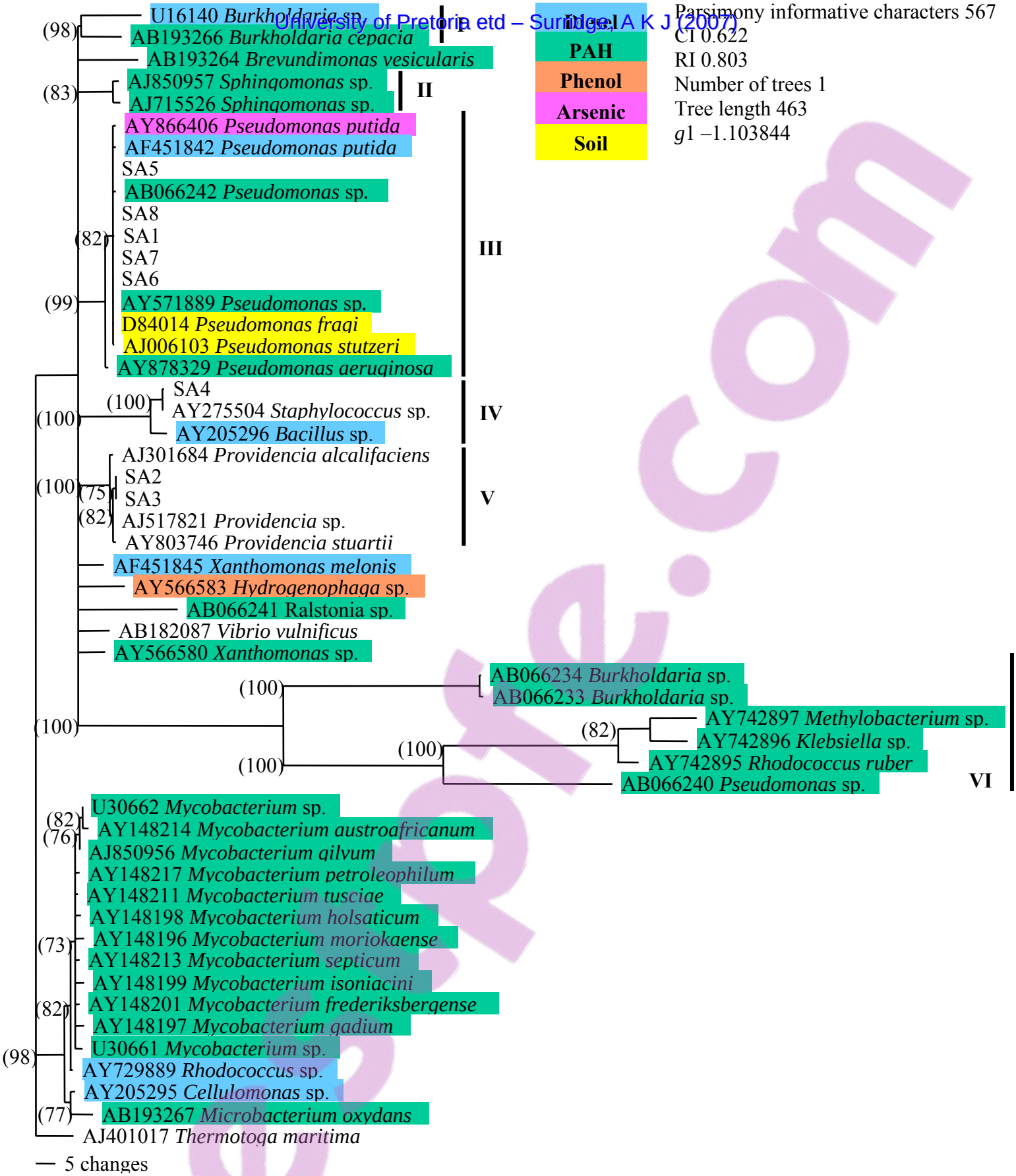


Figure 21: Phylogenetic tree (partial 16S eubacterial gene) of bacterial isolates (SA1-SA8) from a PAH-polluted soil in South Africa, their corresponding BLAST results and known PAH degraders.

4.5 DGGE community analysis of nitrogen-fixing bacteria in polluted soil

PCR was employed in a diagnostic capacity to determine the presence of nitrogen-fixing genes in the soil and pure culture DNA (Table 2). The presence of a PCR product was deemed a positive result. Positive results indicated that the *nifH* gene was present in the sample being tested. *nifH* nested-PCR performed on 10 soil and 8 pure culture (Fig. 22) DNA samples, yielded a *ca.* 370bp fragment on a 1.5% TAE agarose gel. All soil samples tested positive for the presence of *nifH* genes and thus nitrogen fixing capacity. Pure culture samples also tested positive for *nifH* genes, except for *nif*12 and *nif*18 (Fig. 22). *nif*12 was tentatively identified based on sequencing a portion of the 16S gene (4.4 above, sample SA2) as a member of the *Providencia* genus.

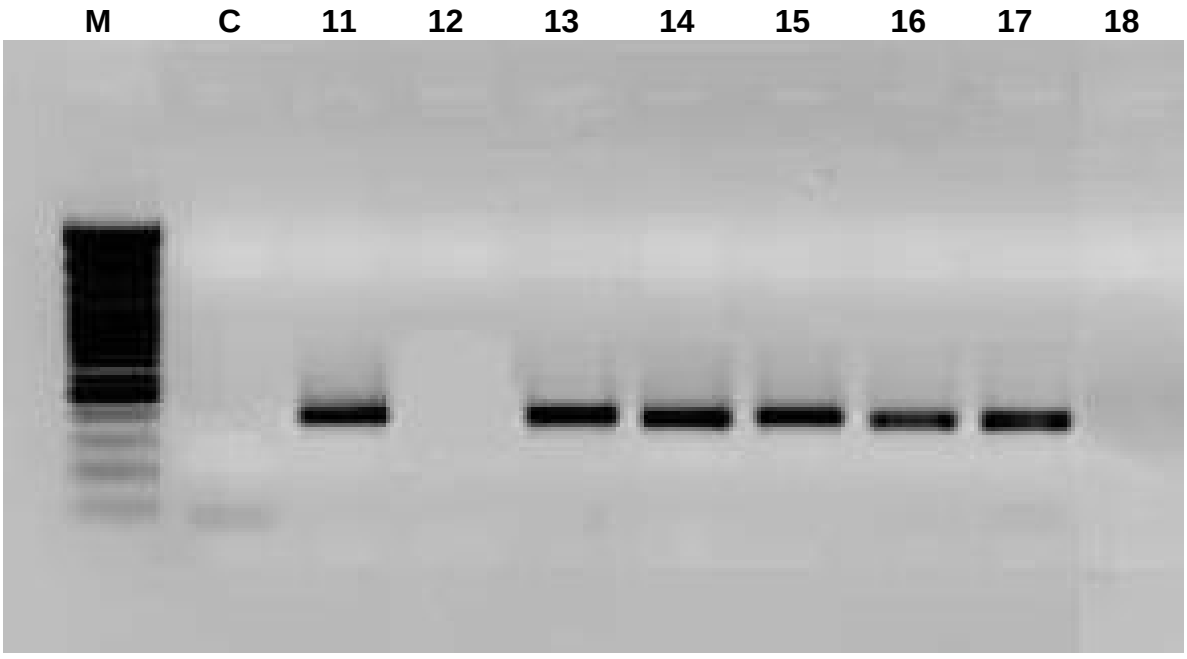


Figure 22: PCR product of *ca.* 370bp from nested-PCR using *nifH* degenerate primers (M = 100bp marker, C = negative control, 11-18 = *nif* PCR product of pure culture isolates (Table 2)).

DGGE fingerprint analysis revealed noteworthy species diversity, evident from a different dominant banding pattern within the *nifH* gene from the various soil samples screened (Fig. 23A and B). The mean number of dominant bands per lane for all soil samples tested was 7.2. Control soils sampled from unpolluted areas, as well as rhizosphere soils sampled from polluted areas, displayed on average a lower number of bands (mean 6.5 and 6.3 respectively) than the mean number per soil. All non-rhizosphere polluted soils displayed 8 or more bands (mean 9.0), indicating an above-average presence of nitrogen-fixing bacterial species.

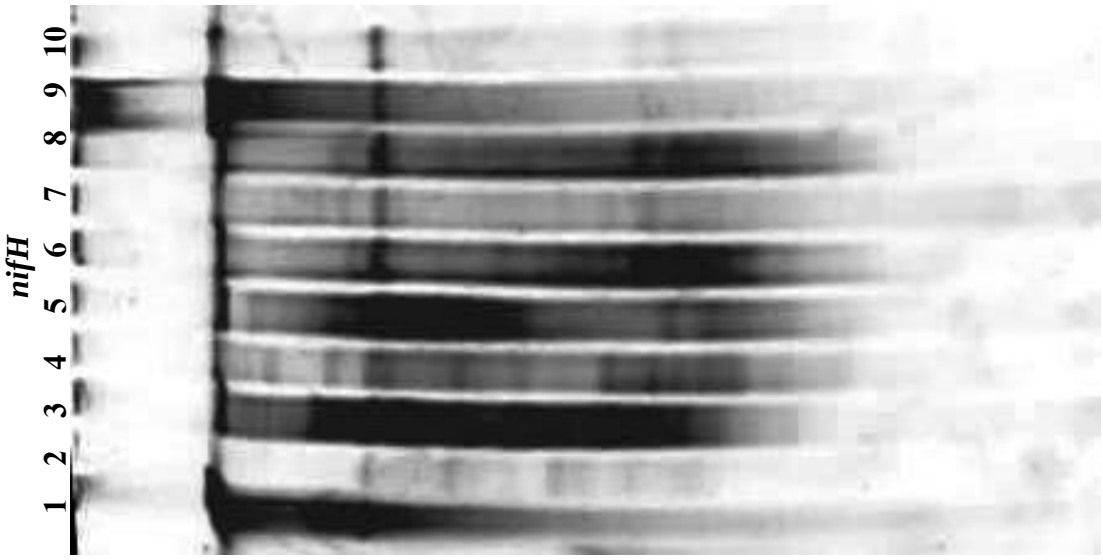


Figure 23A: DGGE gel of *nifH* PCR product from soil (*nifH* PCR product 1-10 Table 2) at 30-65% denaturant concentration.

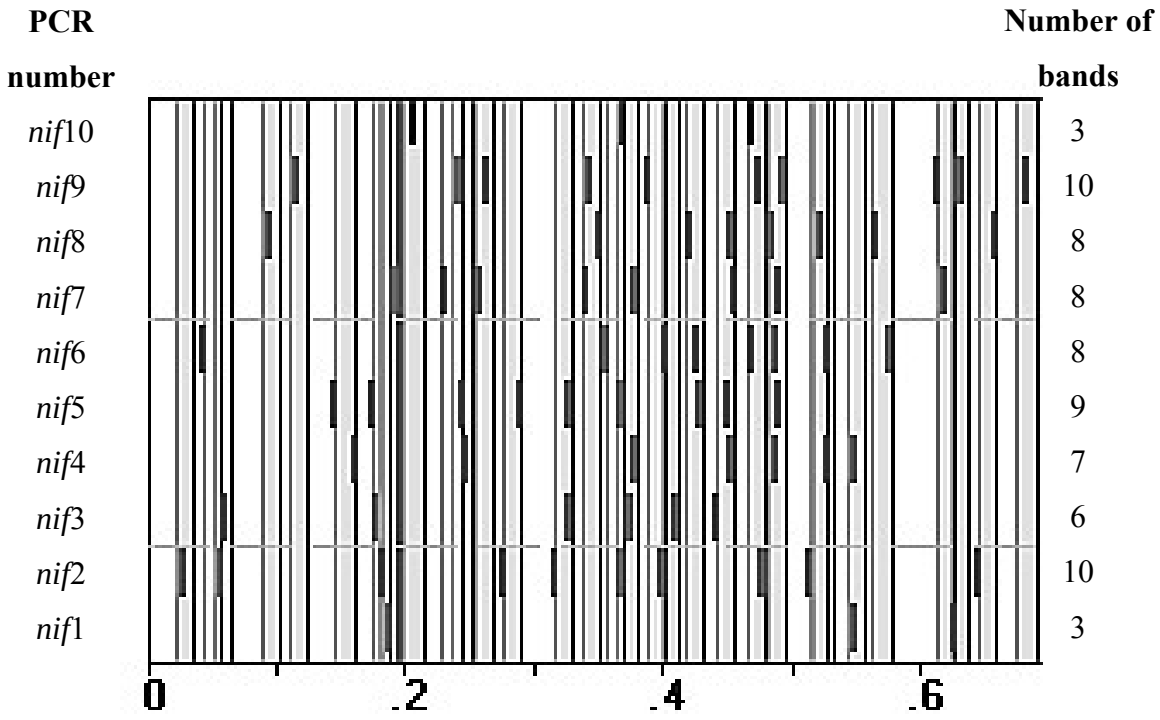


Figure 23B: Graphic representation of DGGE gel of *nifH* PCR product from soil at 30-65% denaturant concentration (Black bars represent dominant bands present in each sample).

Cluster analysis of the DGGE banding pattern revealed two main community sub-clades within the main cluster (I and II, Fig. 24), with *nif*2 and *nif*9 grouping outside. *nif*2 and *nif*9 displayed among the highest diversities of all the samples tested. *nif*9 also displayed the most dominant *nifH* gene presence as it had more intense banding. *nif*1, 3, 7 and 10 grouped in sub-clade I and *nif*4, 5, 6 and 8 in sub-clade II. Within sub-clade I, the community of *nif*3 and 10 from unpolluted and polluted *B. serratia* rhizosphere, respectively (Table 1), were the most similar. Also grouping in sub-clade I, but somewhat separated from *nif*3 and 10 were the *nif*1 (control) and *nif*7 (near *B. serratia* polluted rhizosphere). Sub-clade II included three samples (*nif*4, 5 and 6) from unpolluted and polluted soil under *C. esculentus*.

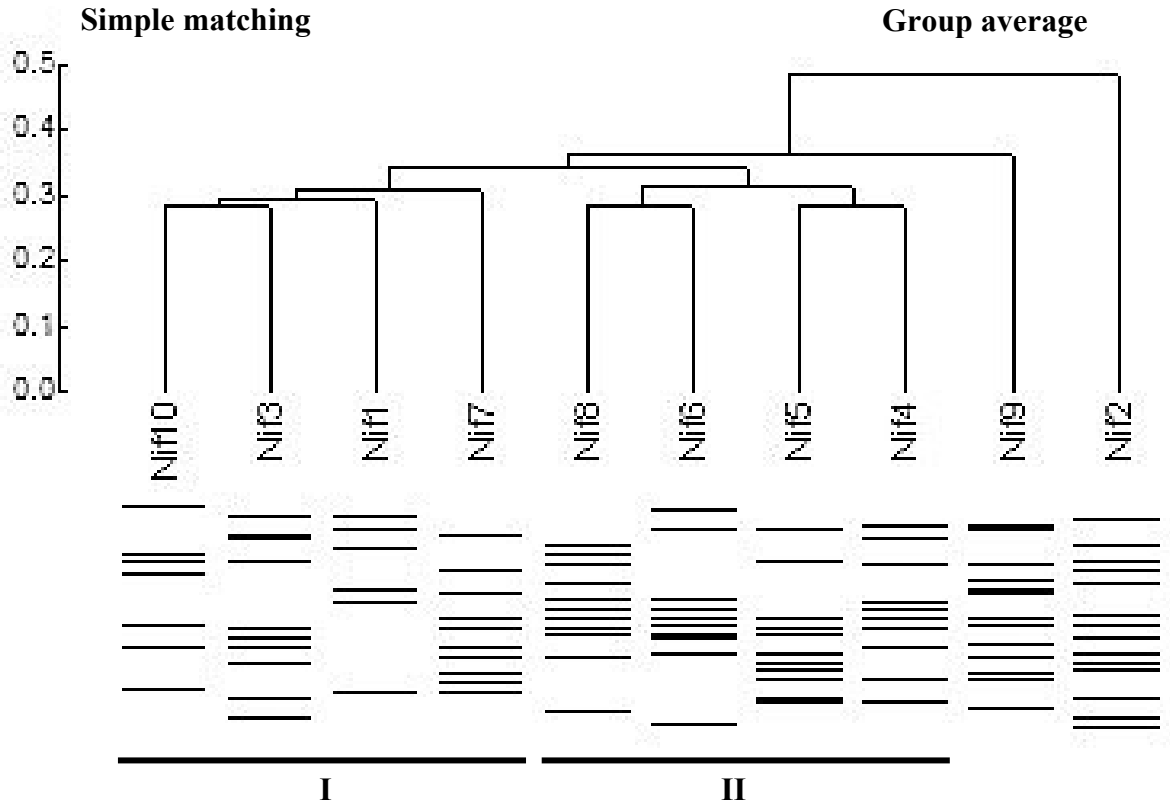


Figure 24: Cluster analysis of the banding pattern in Fig. 23, using a simple matching, group average setting to separate communities according to single gene species sequence differences (Thicker bands represent a brighter band on the gel, and therefore tentatively indicates a greater species richness).

Chapter 5

Discussion

Discussion

5.1 Phylogeny of microbial communities from crude oil-polluted soil according to DGGE profiles

It is evident from the density of the banding patterns observed on the gel (Fig. 6) that there was a higher microbial diversity in unpolluted than in polluted soils, as well as a decrease in microbial diversity in subsoil layers in comparison with topsoil. This was expected, since microorganisms present in polluted soil must be able to survive under PAH/PCB-induced stress conditions, with their growth furthermore limited by the availability of essential elements such as nitrogen (Röling *et al.* 2002). It is also known that few microbes are found deeper than 300mm into soil, especially when no plants are present (Zhou *et al.* 2002). It can be concluded that these microbes were either tolerating the presence of excess PAH/PCB or were in fact utilising them as a source of carbon. Utilisation of PAH/PCB requires specific metabolic pathways for obtaining carbon in an exploitable form. Competent microbial communities surviving and growing in oil-polluted soils can be considered metabolic generalists, differences in these community structures can be noted decades after an initial pollution event (Lindstrom *et al*. 1999). Bacteria capable of such growth have multi-component enzyme systems comprising of aromatic hydrocarbon dioxygenases (Gibson and Parales 2000). For example, toluene dioxygenase adds a dioxygen to the aromatic nucleus of benzene forming an arene-*cis*-diol-*cis*-1,2-dihydroxycyclohexa-3,5-diene (benzene-*cis*-diol) (Gibson and Parales 2000). These

specific enzymes enable growth, as opposed to survival, under aromatic hydrocarbon stressed conditions.

Based on phylogenetic relationships as well as distance analysis it was clear that the predominant representatives from the two soil types (polluted vs. unpolluted) grouped separately (Fig. 7). Supporting this were the two phylogenetic trees (Figs 8 and 9), indicating a closer relatedness and thus lower species diversity within the species isolated from polluted than from unpolluted soils. Topography of the beta and gamma bacteria shows them grouping in separate clades, this supports their classification as different evolutionary lineages. However, proteobacteria as whole seem to have a rather diverse phylogenetic grouping especially among the alpha and gamma groups (Purkhold *et al*. 2000; Sjöling and Cowan 2003; Davidov 2006). In these studies the phylogenetic groupings of the proteobacteria targeted show marked diversity from each other within clades and between clades. Purkhold *et al*. (2000) and Sjöling and Cowan (2003) both found that gamma clades could be very diverse forming bootstrap supported branches within clades. Purkhold *et al*. (2000) and Davidov *et al*. (2006) also show these definite branches forming almost completely separate clades within the alpha clade. The alpha bacteria are spread across clades in Fig. 7, possibly as a result of the lack of resolution for this specific taxonomic group, this could be due to limited number of specimen sequences or related species included in the analysis. However, different taxonomic contexts such as proteobacteria require different degrees of phylogenetic resolution (Woese 2000), thus perhaps the phylogeny within this tree is too broad for proteobacteria species diversity and more base pairs could be included to elucidate this. Nevertheless, Fig. 7 is showing that bacteria found in either polluted or unpolluted soils seem to group

together on the whole leading to "polluted" and "unpolluted" clades of organisms within the phylogram.

Preliminary tentative identification of the species representatives demonstrated that microbial equilibrium in polluted soils shifts to a different predominant group of closely-related organisms. This shift towards phylogenetic groupings displaying very similar consortia within polluted and unpolluted soils is in accordance with previous reports (Leys *et al.* 2004). Sequences obtained from DNA in lanes 1-3 (Fig. 6, samples 1-3 in Table 1) grouped closely together, except for sequence AY673793, a nitrobenzene-degrading *Pseudomonas* sp., that grouped in a clade with AY673806, a naphthalene-degrading *Pseudonomas* sp. Grouping together of nitrobenzene- and naphthalene-degrading bacteria by DGGE band sequencing has not been reported before.

Two main clades formed in both phylogenetic and distance trees and all sequences fell within one main clade, except for AY673792, which formed a clade on its own in both cases (Fig. 7; data not shown). AY673792 also fell basal to the main clade within the phylogenetic tree depicting species from polluted soils (Fig. 8). Its sequence grouped with *Pseudonocardia hydrocarbonoxydans*, *P. benzenivorans*, *P. petroleophila*, *P. saturnea* and *Actinobispora yuannanensis*. Many *Pseudonocardia* species are capable of oxidising complex hydrocarbons (Warwick *et al.* 1994; Lee *et al.* 2000). *P. hydrocarbonoxydans* degrades general hydrocarbon compounds, *P. benzenivorans* benzene, and *P. petroleophila* petroleum. *P. saturnea* and *A. yunnanensis* are both known to degrade aromatic hydrocarbons. From this information it can be concluded that this sequence (AY673792), in all likelihood,

represents an actinomycete that is capable of degrading polyaromatic hydrocarbons of the BTEX isomer group.

Other sequences from polluted soil samples that are also likely to be those of BTEX hydrocarbon degraders are AY673788, AY673789, AY673793, AY673794, AY673795 and AY673796. The hits that where obtained on GenBank BLAST searches indicate that, in accordance with Guerin (1999) and Tesar *et al*. (2002), there were many organisms utilising PAHs and PCBs in the polluted samples. In addition, alpha-proteobacteria were found to cluster with the above sequences. This is consistent with findings by MacNaughton *et al*. (1999) who detected alpha-proteobacteria only in crude oil-polluted soils. It is then plausible to assume that the microorganisms inherently present in these soils are capable of soil rehabilitation, and that pollution levels within the soils should be decreasing as PAHs/PCBs are being utilised. This remediation could occur until a certain point is reached when, perhaps, limiting factors such as seasonal climate change or exhaustion of an essential micronutrient will slow down the process (Ferris and Ward 1997; Ward *et al.* 1998; Muyzer 1999; Smalla *et* al. 2001; Koizumi *et al.* 2002). As the soil begins to return to a more natural, unpolluted state due to pollutant utilisation, new microbes will probably colonise the soil leading to an exponentially faster remediation process (White *et al.* 1998). Analysis of microbial communities has proved to be a far more comprehensive indicator of residual pollutants. For example, O'Sullivan and Mahenthiralingam (2005) found *Burkholderia* to be the predominant genus isolated from PAH-polluted soils capable of toluene degradation, but the population diminished as the pollutant level was reduced. Monitoring the return of a baseline community known to colonise unpolluted soil, e.g. several *Pseudomonas* spp. and

Sphingomonas spp., is used to indicate that the biological community of a soil is returning to normal (White *et al.* 1998).

In conclusion, it is evident that DGGE has indicated a decrease in microbial diversity in PAH/PCB polluted soils, as has been observed in other studies (Kozdrój and Van Elsas 2001). Andreoni *et al*. (2004) found that microbial soil biodiversity decreased with longer-term exposure to PAHs. However, soil pollution also selects for microorganisms with the ability to activate metabolic pathways, thereby allowing them to utilise the pollutants as an alternative source of carbon. Soils that become polluted with complex industrial hydrocarbons undergo a microbial community change that allows for a natural bioremediation process to be initiated.

5.2 Comparative DGGE fingerprint analysis of pro- and eukaryotes in PAH-polluted soil

It is estimated that there are more than 10^{16} prokaryotic cells in a tonne of soil. This is according to recent research which indicated that prokaryotic diversity is higher than previously thought by almost three orders of magnitude (Curtis and Sloan 2005). Measuring reservoirs of prokaryotic diversity, such as in soil, is challenging and it is widely accepted that disregarding the organisms themselves and focusing on their DNA is the most plausible option. Currently, diversity is estimated by targeting particular genes that occur in all the organisms being screened. Diversity is inferred by the number of different variants of these particular genes that can be cloned from a sample of environmental DNA (Curtis and Sloan 2005). However, unfortunately

the number of clones analysed provides only a small indication of the number of individual microbes present that could possibly be investigated.

Despite the possible major role of eukaryotes in bioaugmentation of soil, very little is known about which species are present (Meyer and Schmidt 2003). Literature contains studies of cultured soil eukaryotes, but these are of limited value because of flaws in methodology, inadequacy of surveys, technical problems with isolation and culture, and controversy of identification (Meyer and Schmidt 2003). DGGE is thus a more appropriate method to employ in diversity studies as it facilitates fingerprinting of communities at species level, hence allowing specific species to be targeted for diversity estimation. In view of this, prokaryotic and eukaryotic species diversity was compared and analysed concurrently in this study.

Prokaryotic species diversity and richness was notably higher than that of eukaryotes in the samples examined (Fig. 12). On average a 30% greater diversity was evident for prokaryotes than for eukaryotes (Fig. 13). Thus, at diversity level, a larger portion of prokaryotes appeared to be better able to adapt quickly to abrupt changes in the environment and/or in carbon source. The exception to the prokaryotic diversity dominance was sample 16, which had the lowest prokaryotic but nevertheless a high eukaryotic diversity overall, this may have been due to the soil pH being 3.9, by far the lowest of all the soil samples. The reason for the low pH is unclear, but most likely was not due to pollution since petrol, oil and diesel have a

pH of 5.7-6.0 (Jacobs[h], personal communication). Physico-chemical soil characteristics peculiar to the soil seems to be a more feasible explanation.

Although eukaryotes were found to be less diverse in this environment, their richness (indicated to an extent by band intensity) appeared to be very similar to that of prokaryotes. It is possible that low numbers of tolerant eukaryotic species were present in high numbers, creating a lower diversity but similar richness. This is supported by the work done by Lara (2005) who found, through 18S rRNA gene cloning, DGGE screening and artificial cultivation, that eukaryotes (protists) were present in higher numbers than prokaryotes in PAH-polluted soils. He attributed this to the fact that trophic webs are less complex in PAH-polluted soils due to a decrease in species diversity. However, it is also interesting to note that in the present study the prokaryote:eukaryote ratio in polluted and unpolluted soil was approximately the same. Many eukaryotes, such as various fungi, are capable of tolerating harsh environmental conditions than bacteria and are involved in the degradation of PAHs/PCBs in soil (Prenafeta-Boldú *et al.* 2002). Da Silva *et al*. (2003b) isolated filamentous fungi from estuarine sediments in Brazil and monitored their ability to degrade PAHs. They found a *Cyclothyrium* sp. to be the most efficient, simultaneously degrading 74, 70, 59 and 38% of pyrene, phenanthrene, anthracene and benzo[a]pyrene, respectively. Additionally, toluene, ethylbenzene and xylene have been shown to be degraded by a *Cladophialophora* sp. (Prenafeta-Boldú *et al.* 2002).

[h] A. Jacobs, ARC-Plant Protection Research Institute, Private Bag x134, Queenswood, 0121 Pretoria.

Diversity profiles for pro- and eukaryotes demonstrated a distinct cluster pattern evident in the dendogram (Fig. 14). Generally, prokaryotic profiles grouped together, probably due to their higher diversity and possibly closer relations (as shown in section 5.1). 16S9 was the most diverse community according to banding in the dendogram and this was expected since it is the unpolluted control soil. It was also the only community not clustering in a clade. Clade I contained only ITS communities with a low number of bands, indicating low diversity. Clade II contained three ITS and three 16S sequences, indicating very similar community diversity across these communities. Clade III displayed a sparse banding throughout the communities and had the majority of ITS communities represented. This clade had the lowest diversity of all the clades, due to most of the eukaryotes grouping within it. There was an increase in community diversity as clades branched off from Clade III. Clades IV, V and VI contained mainly 16S and this grouping was supported by an evident increase in diversity. Diversities within clade VI were the most similar to each other as these were basal to all other communities, although diversity was not as high as in 16S9. 16S9 is similar in community to clade V and VI, but grouped out due to a much higher species diversity.

The PAH breakdown potential of prokaryotic vs. eukaryotic communities was not determined. However, extrapolation of the DGGE data indicates that, due to the significantly higher diversity within prokaryotic communities, and their better adaptation potential within pollution stressed environments, the prokaryotic component of these samples would have the greatest PAH metabolism potential. This is in accordance with studies conducted by Atlas (1981) and Ahn *et al*. (1999) who showed that PAH/PCB degradation is not restricted to only a few bacterial

genera, but that many are in fact capable of survival and growth in a petroleum-hydrocarbon polluted environment. However, there have been cases where more fungal and protist genera than bacterial genera were found to degrade PAHs/PCBs in soil samples (Atlas 1981; Lara 2005). In many cases such as these, DGGE has proved to be the most valuable molecular tool in screening many diverse samples for different microorganisms.

5.3 DGGE analysis of toluene and naphthalene degraders in polluted soil

Different soils contain different microbial communities that have adapted to survive and develop within the specific environment in which they exist. However, Gelsomino *et al*. (1999) found after extensive molecular fingerprinting that similar soil types tend to contain comparable dominant bacteria. This was also the case in the present study, as unpolluted soils tended to group together, as did soil from the rhizosphere of the same species of plant.

The mean number of bands on the DGGE gel between the *xylE* and *ndoB* genes was higher for *xylE*, thus indicating a higher toluene degrading capacity, except in soils 17 and 18 (Fig. 16). Samples 17 and 18 were from soil mulched with wood chips and polluted with workshop oil, which is derived from tar and thus contains naphthalene, hence accounting for the higher naphthalene degrading capacity. Milcic-Terzic *et al.* (2001) found that consortia isolated from diesel-polluted soils possessed both catabolic genes. They determined that toluene-degrading consortia contained only the *xylE* gene, whereas the naphthalene-degrading consortia

possessed only the *ndoB* gene. Results of the current study demonstrated that both catabolic genes were present in all soil samples. However, a higher level of diversity among *xylE*$^+$ organisms indicated that there could have been a greater amount of toluene in samples 9-16. Similarly in samples 17 and 18 higher diversity among *ndoB*$^+$ organisms indicated the possibility of a predominantly naphthalene polluted environment. It was interesting to note a high toluene and naphthalene degrading capacity in soils 9-12 that contained no PAH/PCB pollution. This indicates that many soil dwellers contain the capacity for toluene and naphthalene degradation but that these genes are not always active. Siciliano *et al*. (2003) similarly found that there were often more *ndoB*$^+$ and *xylE*$^+$ organisms present in their unpolluted control soils than in the polluted soils being studied. Furthermore, Schneegurt and Kulpa (1998) reported that exposure to aromatic substrates caused an increase in inherent *ndoB* and *xylE* carrying plasmid levels in indigenous soil bacteria, leading to enzymatically catabolised degradation of naphthalene and toluene, respectively.

The grouping together of soil communities in clades according to the gene amplified was anticipated, due to the similar community structure expected when one gene is targeted for fingerprinting. However, soil community fingerprints showed some mixing of polluted and unpolluted profiles within single clades. This could have been due to different gene loci exhibiting similar gel profiles as a result of genetic similarity with respect to, for instance, G-C content. Similar electrophoretic mobilities of phylogenetically related species sharing analogous sequences could cause co-migration across samples within the amplified area, whereas a similar melting behaviour between phylogenetically unrelated species could cause bands pattern to show some level of homology (Smalla *et al.* 2001).

The *ndoB* gene dendogram (Fig. 18) showed a clustering of control soils 9, 10, 11 and 12 within clade I, and a cluster of polluted soils 14, 16 and 17 in clade II, in both cases possibly due to similar community structures within each clade. The assembly of 14 and 16 closer to each other probably can be ascribed to the fact that they were both from rhizosphere soil. Samples 13 and 18 grouped together in the dendograms of both *xylE* and *ndoB* genes. This grouping was also evident in clades I and IV of the dual-gene dendogram (Fig. 17). In this respect the community similarity seems to be stronger within the *xylE* gene (Fig. 19) as this grouping was reflected closest within its dendogram and the dual-gene dendogram. Samples 13 and 18 did not have common a rhizosphere or plant derivation, but shared a common pollutant, namely oil. It can thus be concluded that the *xylE* and *ndoB* genes are both needed for oil degradation, confirming the studies by Siciliano *et al*. (2003) and Luz *et al*. (2004). The common grouping of samples 15 and 16 in the *xylE* gene dendogram was also reflected in the dual-gene dendogram, which could be accounted for by the fact that these samples were from non-rhizosphere and rhizosphere soil under *B. serratia* in the same area, respectively. This clustering of samples (15 and 16) as a result of the *xylE* gene is in accordance with Siciliano *et al*. (2003), who found that change in the functional genetic composition of a community was not detectable by 16S rDNA, but was linked to specific functional genotypes (*ndoB*, *alkB* and *xylE*) with relevance to PAH degradation. In the context of the present study this implies that functional gene differences elucidated community dynamics, and response to PAH/PCB pollution, when targeting the *ndoB* and *xylE* genes. It is thus in agreement with Siciliano *et al*. (2003) who reported that phytoremediation systems increase the

catabolic potential of rhizosphere soils by influentially altering the functional composition of the microbial community.

On basis of the above results, it can be concluded that the polluted soil communities in South Africa have similar microbial communities, depending on their physical location, plant species proximity and soil conditions, viz. type of pollutants to which they are exposed. Examining metabolic gene diversity *in situ* has indicated that soils with one or more of the above factors in common tend to have a comparable community diversity within their functional genes (Milcic-Terzic *et al.* 2001; Siciliano *et al*. 2003). It was also evident that, even when not existing under PAH-polluted conditions, local soil microbial communities have the capacity to break down PAHs should pollution occur. This supports the finding by Wünsche *et al.* (1995) that substrate utilisation patterns in the Biolog system changed upon addition of hydrocarbons. According to them, previously pristine soil bacterial communities shifted to a predominantly *Pseudomonas* population with hydrocarbon degradation adaptation capacity.

5.4 Molecular identification of toluene and naphthalene degrading capacity of bacteria from PAH-polluted soil

The genera *Staphylococcus* and *Providencia* have not previously been reported to degrade PAHs and are also not common rhizosphere colonisers. Members of the *Providencia* genus can, however, completely break down hexahydro-1,3,5-trinitro-1,3,5-tiazine (RDX) and nitroso-RDX, and have been used to achieve such in

bioremediation (Kitts *et al.* 1994). In the present study it was initially assumed that *Staphylococcus* and *Providencia* were most likely present and surviving in polluted soil rather than performing PAH-degradation, but both have since been found to degrade PAH in culture (Molobela 2005, unpublished data). Members of these genera clustering with known PAH-degraders such as *Bacillus* in the present study, supports this. Furthermore, the presence of the *xylE* and *ndoB* genes within *Stapylococcus* and *Providencia* isolates has been reported (Kitts *et al.* 1994), which indicates that they may be opportunistic PAH degraders in environments providing none or very little of an alternative carbon source.

Pseudomonas, *Providencia* and *Staphylococcus* constituted a very narrow spectrum of taxa compared with the previous investigation of crude oil-polluted soil (5.1), albeit from a different site in South Africa, which indicated the presence of the following genera (in order of prevalence): *Pseudomonas, Sphingomonas, Methylocystis, Pseudonocardia, Acidocella, Azospirillum, Bradyrhizobium, Bosea, Roseomonas, Rhodoplanes, Blastochloris, Porphyrobacter, Sphingobium, Rhizomonas, Sphingopyxis, Acinetobacter, Rhodococcus, Acidiphilium, Methylocella, Actinobispora* and *Acidospheara* (Fig. 7). Except for *Pseudomonas*, none of these genera was isolated from any of the samples in the present study, either because they were not culturable or not present in sufficient numbers to allow isolation. *Pseudomonas* therefore appears to be the genus with the greatest potential as bioaugmentation agent, since it grows rapidly in culture and is a robust soil, rhizosphere and PAH-polluted site coloniser (Tesar *et al*. 2002; Kuiper *et al.* 2004). *Pseudomonas* dominated the bacterial population in the rhizosphere of *C. esculentus* and also occupied non-rhizosphere soil. *Cyperus* and *Eleusine* are associated with

phytoremediation of oil-polluted soils (Merkl *et al*. 2005), and species such as *C. esculentus* and *E. corocana*, as well as *B. pilosa*, are common invaders of PAH-polluted sites in South Africa (Maila *et al*. 2005a; Molobela *et al*. 2005). Of these three weed species, only *E. corocana* has thus far been found to be actively involved in phytoremediation of PAH-polluted soils in the country (Maila *et al*. 2005a). Although probably purely coincidental, it is interesting to note that *E. corocana* was also the only species from which *Staphylococcus* could be isolated.

Indigenous microbial communities inhabiting polluted sites are known to include species capable of bioaugmentation of the site, after being targeted, isolated and identified. The organisms within these communities are affected by biotic and abiotic factors that influence the ability of microbes to mineralise PAH and aliphatic compounds, but have proven to be effective in bioremediation at field scale (Samanta *et al*. 2002). Samanta *et al*. (2002) reported isolating a large number of naphthalene degrading microorganisms, including *Alcaligenes denitrificans*, *Mycobacterium* sp., *Pseudomonas putida*, *P. fluorescens*, *P. paucimobilis*, *P. vesicularis*, *P. cepacia*, *P. testosteroni*, *Rhodococcus* sp., *Corynebacterium venale*, *Bacillus cereus*, *Moraxella* sp., *Streptomyces* sp., *Vibrio* sp. and *Cyclotrophicus* sp. The identification of so many *Pseudomonas* species being capable of naphthalene degradation is consistent with earlier literature. Davies and Evans (1964) penned the first report of the catabolic pathway for naphthalene degradation by *Pseudomonas* spp. Consistent with the studies of Davies and Evans (1964) and Samanta *et al*. (2002), the present study found *Pseudomonas* to be the most common PAH-degrading genus, in this case identified and isolated from the rhizosphere of *C. esculentus*. However, it was established that, although less common, *Providencia* and *Staphylococcus* possess the

ability to mobilise PAH-degrading enzymes. Considering the well-established rhizosphere competence and PAH-degrading capacity of *Pseudomonas*, this genus nevertheless seems to be the best suited for bioaugmentation purposes in South Africa.

5.5 DGGE community analysis of nitrogen-fixing bacteria in polluted soil

PCR revealed all soil samples to include *nifH* genes, regardless of whether they were from rhizosphere soil or polluted sites or not. This was expected, as nitrogen-fixing organisms are not only common in unpolluted soils but also able to survive and grow in pollutant-stressed soils (K.A. Reynolds[i], personal communication). Diesel and other fuel spills result in a very high carbon to nitrogen ratio within affected soils (Eckford *et al*. 2002). Initially it was believed that such a situation could limit microbial degradation of PAHs. Eckford *et al*. (2002) found several consortia capable of PAH-degradation and nitrogen fixation under nitrogen-limited conditions in polluted Antarctic soils, whereas Rahman *et al*. (2002) observed a high level of inorganic nitrogen utilisers in samples taken from soils at petrol and diesel stations. Thus, from a pollution perspective, these studies support the presence of *nifH* genes in samples screened in the present study. However, isolates *nif*12 and *nif*18 did not respond to the *nifH* PCR (Fig. 22), hence supporting the apparent absence of this nitrogen fixation gene in the *Providencia* genus. *Providencia* belongs to the family *Enterobacteriaceae* and when subjected to metabolic tests is positive for the reaction nitrate to nitrite, this is the same reaction that is catalysed by denitrifying bacteria

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(Manos and Belas 2006). Thus *Providencia* is capable of performing the same functions as those of denitrifying bacteria. It is interesting to note that, although the remaining isolates were colony PCR'd from pure culture on agar plates, they still maintained the ability for nitrogen fixation.

According to DGGE profiles (Fig. 23), diversity differed between the various soil samples, evidently because of the different soil types and plant rhizospheres being sampled. The control soil (*nif*1) showed low nitrogen-fixing bacterial diversity, as could be predicted since most *nifH*-containing microorganisms are associated with plant roots, which were absent in this soil. Polluted, non-rhizosphere soils, by contrast, showed a fairly high diversity of nitrogen-fixing organisms. This is attributed to the survival of these microbes under stress, where they have no plants with which to associate and from which to gain habitat and nutrients. However, Muratova *et al*. (2003) found that the growth of microbes associated with the roots of lucerne (*Medicago sativa* L.) in PAH-polluted soil was greatly improved by addition of nitrogen. Fewer organisms are present in non-rhizosphere than in rhizosphere soils (Blagodatskaya *et al.* 2004), thus it follows that there will be less competition in non-rhizosphere soil despite more stressed living conditions. This cumulatively leads to an increased diversity, but not necessarily to increased species richness, when compared to rhizosphere soils. Also, microbes existing in a less nutrient-stressed environment, such as the plant rhizosphere, may have less need for nitrogen fixation due to indigenous root-associated microbes fixing nitrogen on their behalf. Conversely, microbes living some distance from plants will require an active *nifH* gene for nitrogen-fixation, as plant root nodules and associated bacteria are inaccessible.

Cluster analysis (Fig. 24) showed *nif*2 and *nif*9 falling basal to the other samples tested. This seems feasible as these two samples displayed the highest species diversity. However, it is interesting to note that these samples are exact opposites, *nif*2 was sampled from *B. pilosa* rhizosphere in unpolluted soil and *nif*9 from non-rhizosphere polluted soil. *B. pilosa* is known to have a rhizosphere rich in nitrogen-fixers and it therefore is plausible that species diversity may be high (Wolfe and Klironomos 2005). However, *nif*9 showed a much higher species richness than *nif*2, as indicated by the darker banding. This implies that the diversity of *nif*2 and *nif*9 was similar but that richness was higher in the non-rhizosphere polluted soil, possibly due to the reason stated above that more free-living microbes are forced to fix nitrogen under stressed living conditions.

Clade I of the cluster analysis showed *nif*3 and *nif*10 grouping together. They were both from *B. serratia* rhizosphere, which could explain the similarity in their rhizosphere communities. *nif*3 and *nif*10 were low in diversity, possibly due to nitrogen-fixing microbes being present mainly inside the plant roots. In the same clade were *nif*1 and *nif*7, both sampled from non-rhizosphere soils. *nif*7 showed a higher diversity, possibly because of its pollutant-stressed habitat, whereas *nif*1 was a control soil that is expected to have a high nitrogen-fixing capacity. Clade II grouped together *nif*6 and *nif*8, possibly because they were both rhizosphere soils and have similar diversity, their community fingerprints were sufficiently similar to conclude that the species within their respective communities are alike. The grouping together of *nif*4, *nif*5 and *nif*6 was expected as they were all associated with *C. esculentus*.

Nitrogen-fixing organisms were present in all the soils sampled. This was anticipated since free, available nitrogen is a limited resource in the soil environment as it easily returns to the atmosphere (Kaye and Hart 1997). Nitrogen fixation was also found to be present in 80% of the pure cultures isolated from polluted and unpolluted soils and rhizospheres. Although different rhizospheres and pollutants were examined, it was found that the highest *nifH* gene diversity of polluted soils existed within machinery oil-polluted, wood-chip-mulched, non-rhizosphere soil. This was also the most visibly and olfactorily highest level of polluted soil that according to site knowledge had been deliberately polluted severely for 10 years. It therefore appears that the more polluted the soil, the higher the free microbial nitrogen fixation diversity due to environmental stress, which is in accordance with Zepp *et al*. (2003) who found that PAH pollution of the environment alters the nitrogen cycling therein indirectly effecting the microbial communities.

Chapter 6

Conclusion

Conclusion

The purpose of this study was to evaluate the DGGE technique for determining microbial diversity in PAH/PCB-polluted soils. The technique proved to be appropriate and gave a good indication of different diversities, i.e. pro- and eukaryotes, across different gene groups viz. 16S, *ndoB*, *xylE* and *nifH*. A great deal of work has been done on determining bacterial soil diversity based on 16S DGGE analysis because 16S rRNA genes are most commonly used to give an overall indication of the bacterial species composition of a sample. A partial sequence of this gene from soil was analysed by means of DGGE by Throbäck *et al.* (2004). However more complex functional genes have also been targeted for determining microbial diversity among specific communities. Milcic-Terzic *et al.* (2000) successfully isolated diesel, toluene and naphthalene-degrading microbial consortia from diesel-polluted soils by screening for the presence of *xylE* and *ndoB* from petroleum and diesel-polluted soils using PCR with gene-specific primers. Similarly, the general marker gene in nitrogen-fixing bacteria has been targeted for community analysis by Rosado *et al*. (1998). They successfully used *Paenibacillus azotofixans nifH* species-specific primers in DGGE analyses of soil samples. In addition to this, Zehr and McReynolds (1989), Simonet *et al*. (1991), Widmer *et al*. (1999) and Yeager *et al.* (2005) successfully designed four new sets of degenerate primers for universal targeting of the *nifH* gene in microorganisms. All of these PCR-DGGE applications proved to yield valuable microbial diversity information.

This study revealed a great deal of information concerning microbial diversity, with respect to species and functional genes, in different soil environments. Representatives of South African soils were found to have a higher general microbial rhizosphere diversity than found in non-rhizosphere soils. This finding supports previous studies in the tropics, where it was established that plant roots play an important role in rhizoremediation of PAH/PCB-polluted soils by creating a haven for microorganisms involved in the process (Merkl *et al.* 2005). It has also been globally shown that the mere presence and diversity of these microbes directly improve the bioaugmentation of PAHs/PCBs in polluted soil (Glick 2003). DGGE was ideal for the analyses conducted in this study, as it is capable of screening multiple samples and genes, which can then be analysed simultaneously yielding many different types of information about the environments studied. Smalla *et al.* (1998), Marshall *et al*. (2003), Zuccaro *et al*. (2003) and Foucher *et al.* (2004) made use of several probes designed for PCR-DGGE in the analysis of different microbial rhizosphere communities, and found that it revealed far more community data than more conventional methods such as for example BIOLOG. In accordance with international literature (Milcic-Terzic *et al.* 2000), the *xylE* and *ndoB* genes identified diesel, toluene and naphthalene-degrading microbial consortia from PAH/PCB polluted soils in South Africa. These genes were targeted in order to assess the bioremediation potential of microbial consortia in petrol, diesel and crude oil-polluted soils (Greer *et al*. 1993). Finally, nitrogen-fixing capacity and species diversity in South African PAH/PCB polluted soil was successfully determined by targeting the *nifH* gene in DGGE analysis as supported by Rosado *et al*. (1998).

This thesis constitutes the first study of its kind in South Africa and has provided the basis for further more in-depth environmental microbial diversity studies. Now that the effectiveness of DGGE as a technique for assessing microbial diversity in the stressed environment of PAH/PCB polluted soils has been established, it can be applied to many more environments. Further such studies should include a higher number of samples across a wider range of environments, pollutants and other factors. South Africa has many unique environments, some of which are extreme, in which microbial diversity has not yet been studied, e.g. man-made vs. natural, urban vs. rural, desert vs. sub-tropical, industrial, mines, various types of underground sites, hot water springs, warm and cold ocean currents. The understanding of microbial community diversity, interaction and response to different environments is paramount to the application of microbes for the good of mankind. Until recently, the wealth of microbial diversity in the environment seemed immeasurable but with the application of techniques such as DGGE, considerably more data can be acquired to better understand microbes and their habitats.

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Resumē

Resumē

Fossil fuels are currently the primary industrial energy source on Earth. They are principally composed of complex hydrocarbons in either long-chain or cyclic conformation. Industrial use of petroleum, diesel, oil, tar and other coal-derived products inevitably leads to pollution of the environment. The most serious pollution is caused by polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) that are not easily removed from soil after a spill. Long-chain and cyclic conformation makes fossil fuel hydrocarbons difficult to break down. However, certain free-living soil microorganisms have adapted to utilising these PAHs/PCBs as a source of energy. In many cases, their efficacy is greatly enhanced by the presence of plants. By inhabiting the rhizosphere, microbes form a mutualistic relationship with the plant, receiving nutrients from it and in return providing a less polluted environment in which the plant can grow. The purpose of this study was to elucidate some of the microbial population diversity in PAH/PCB-polluted soils in South Africa through the use of denaturing gradient gel electrophoresis (DGGE).

In an initial study, DGGE was employed to separate soil communities in polluted and unpolluted soils into a genetic fingerprint, the main bands of which were sequenced and subjected to a BLAST analysis through a database for possible identification of species present. Phylogenetic and distance studies indicated that unpolluted soils have a far greater species diversity. It thus was evident that PAH/PCB pollution of

soil leads to a decrease in microbial diversity by selecting for microorganisms with the ability to activate metabolic pathways allowing them to utilise the pollutants as an alternative source of carbon.

Population diversity of pro- and eukaryotes found within polluted and non-polluted soils was compared. DGGE was employed to determine the genetic fingerprint of each population. Following this, dendogram analyses based on Shannon indices were done to determine PAH breakdown potential of prokaryotic vs. eukaryotic communities. A higher diversity and better adaptation potential were evident within prokaryotic than eukaryotic communities in pollution-stressed environments, indicating that the prokaryotic component of these samples had the greatest PAH-metabolism potential.

To determine the capacity for PAH/PCB metabolism by the organisms within the soil samples being studied, the presence of *xylE* and *ndoB* genes, responsible for toluene/xylene and naphthalene biodegradation, respectively, was determined. DGGE was performed to analyse genetic diversity between these two genes, based on community fingerprints. Polluted soil communities tended to have comparable community diversity within their functional genes, depending on their physical situation, plant species proximity and soil conditions. In general, soil contained indigenous microbes with a high natural potential for biodegradation of PAHs/PCBs.

A portion of the 16S gene of eight bacterial isolates representing the most dominant culturable taxa in the polluted soils was sequenced and analysed for identification purposes. These identifications were conducted in conjunction with the use of the

catabolic gene probes *xylE* and *ndoB* to establish the hydrocarbon degrading capacity of the isolates. *Pseudomonas*, from the rhizosphere of *Cyperus esculentus*, was the most common PAH-degrading genus found in this study. Considering the well-established rhizosphere competence and PAH-degrading capacity of *Pseudomonas*, this genus seems to be the best suited for bioaugmentation purposes in South Africa.

The presence of the *nifH* gene, the general marker gene of nitrogen-fixing bacteria in communities from unpolluted and polluted soils, was determined. It was hypothesised that bioremediation could be enhanced by nitrogen addition to polluted environments. Nested-PCR of the *nifH* gene was conducted on a diagnostic basis and was followed by DGGE of the product to determine the functional gene diversity within pollution-dwelling, nitrogen-fixing bacterial communities. Nitrogen-fixing microorganisms were present in all the soils sampled but, in only 80% of the pure cultures isolated from polluted and unpolluted soils and rhizospheres. Although different rhizospheres and pollutants were examined, it was found that of the polluted soils studied, most *nifH* gene diversity of polluted soils existed within machinery oil polluted, wood chip mulched, non-rhizosphere soil. Thus, it would appear that the more polluted the soil the higher the free microbe nitrogen fixation diversity possibly due to environmental stress.

Appendix 1

Presentations and publications arising from this research

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M.P. Maila, P. Randima, A.K. Drønen, **A.K.J. Surridge**, TE Cloete. 2004. Evaluation of microbial diversity of different soil layers at a contaminated diesel site. *International Journal of Biodegradation and Biodeterioration* **55**: 39-44.

A.K.J. Surridge, A.K. Drønen and T.E. Cloete. 2004. Phylogenetic and distance analyses of microbial communities from polluted soils in South Africa based on Denaturing Gradient Gel Electrophoresis (DGGE) profiles. South African Microbiology Society (SASM). Stellenbosch. 4-7 April 2004. Conference proceeding.

M.P. Maila, P. Randima, A.K. Drønen, **A.K.J. Surridge** and T.E. Cloete. 2004. Effect of total petroleum hydrocarbons on rhizosphere microbial communities estimated by CLPP and 16S rDNA fingerprints. South African Microbiology Society. Stellenbosch. 4-7 April 2004. Conference proceeding.

A.K.J. Surridge, I.P. Molbela, F.C. Wehner and T.E. Cloete. 2005. Molecular identification and confirmation of toluene and naphthalene degrading capacity of bacteria isolated from the rhizosphere of weeds in a polyaromatic hydrocarbon polluted soil in South Africa. Diffuse pollution specialist conference (WISA). 9-12 August 2005. Sandton Convention Centre, Johannesburg, South Africa. Conference proceeding and full paper submission.

A.K.J. Surridge, F.C. Wehner and T.E. Cloete. 2006. Denaturing Gradient Gel Electrophoresis (DGGE) community analyses of nitrogen-fixing bacteria found in petroleum, diesel, tar and oil polluted soil in South Africa. South African Microbiology Society. Council for Scientific and Industrial Research (CSIR), Pretoria. 10-12 April 2006. Conference proceeding.

W.F. Truter, N.F.G. Rethman, A. Smith, **A.K.J. Surridge** and K.A. Reynolds. 2006. Monitoring of rehabilitated mine land ameliorated with organic and industrial by-products. Diffuse pollution specialist conference 18 – 22 September 2006. Istanbul, Turkey. Conference proceeding and full paper submission.

A.K.J. Surridge, K.A. Reynolds, T.E. Cloete and D.A. Cowan. 2006. Phylogenetic analyses of microbial communities from South African fly ash based on Denaturing Gradient Gel Electrophoresis (DGGE) profiles. Coal Ash – A Valuable Resource. International Coal Ash Conference. Council for Scientific and Industrial Research (CSIR), Pretoria. 2-4 October 2006. Conference proceeding.

A.K.J. Surridge, F.C. Wehner and T.E. Cloete. 2006. Denaturing Gradient Gel Electrophoresis (DGGE) community analyses of nitrogen-fixing bacteria found in petroleum, diesel, tar and oil polluted soil in South Africa. *Microbial Ecology* Submitted for consideration.

A.K.J. Surridge, K.A. Reynolds, T.E. Cloete and D.A. Cowan. 2007. Phylogenetic and diversity analyses of microbial communities from South African fly ash based on

denaturing gradient gel electrophoresis (DGGE) profiles. World of Coal Ash – Science Applications and Sustainability. American Coal Ash Association. Covingtons Northern Kentucky Convention Center, Cincinnati, Ohio, USA.