Chapter 1

Introduction

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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).

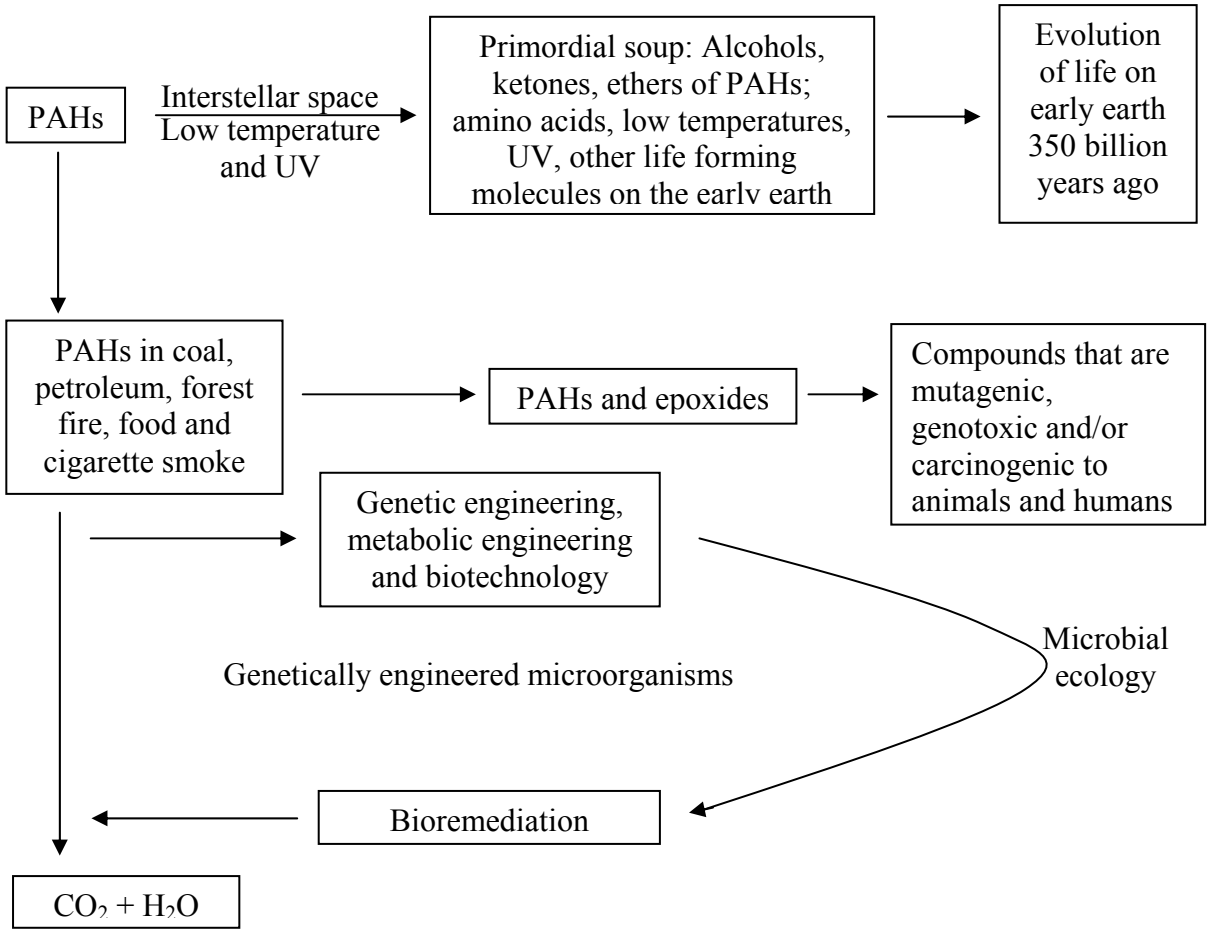

Figure 1: Fate, toxicity and remediation of PAHs in the environment (Samanta *et al*. 2002).

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.