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**RHIZOSPHERE MICROBIAL DIVERSITY IN PAH'S  
CONTAMINATED AND UNCONTAMINATED SOIL**

**BY**

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## DECLARATION

I declare that the thesis, which I hereby submit for the degree Msc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for the degree at this or any other tertiary institution.

.....



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## **SUMMARY**

### **Rhizosphere Microbial Diversity in PAH's Contaminated and Uncontaminated Soil**

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The intrusive and expensive nature of soil cleanup technologies like excavation and incineration created a need to search for alternative remediation technologies. Rhizoremediation and its associated microorganisms has the potential to cleanup contaminated soil in a 'non invasive' and cost-effective manner. The literature cites many benefits of the technology if implemented correctly. However, there is still a lack of knowledge concerning the interaction of the plants and microorganisms that are responsible for degrading the organic pollutants.

In this study, the potential for degrading Poly aromatic hydrocarbons (PAH'S) by rhizosphere bacteria was investigated. In addition, the hydrocarbon removal efficiencies of different plant rhizospheres were investigated. The metabolic and genetic profiles of soil bacteria in vegetated and non- vegetated soils were determined.

The results of the removal efficiencies of different plant rhizospheres showed that the removal of hydrocarbons was more effective in soil vegetated by different plant species. By using co-occurring (different) plant species, hydrocarbons were removed faster than when monoplanted were used. The number of hydrocarbon degrading bacteria in the rhizosphere increased during rhizoremediation of PAH's contaminated soil.

Analysis of the functional and genetic diversity in PAH's contaminated and non-contaminated rhizosphere and non-rhizosphere soil, using Biolog (physiological community level) and genetic diversity (polymerase chain reaction- denaturant gradient gel electrophoresis) was determined. The biolog did not revealed clear difference on substrate utilization profiles of the microbial communities in the rhizosphere and bulk soil. However, unlike the Biolog DGGE revealed slightly differences in both the metabolic and genetic profiles of the different soil samples.

The study on the feasibility of seeding bacteria capable of colonizing and surviving on the rhizosphere showed that *Pseudomonas putida* successfully colonized the rhizosphere of *Eleusine corocana*. The number of *P putida* increased during rhizoremediation of PAH's. These results suggest that bacteria with the ability to adhere and survive in the root zone can be engineered and seeded for rhizoremediation purposes. However, other factors such as the influence of soil type and organic matter content must be investigated to improve rhizoremediation technology.



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## CHAPTER 1

### INTRODUCTION

Industrialization has generated large amounts of hazardous waste. Indiscriminate dumping of this waste has resulted in a large number of environmentally damaged sites. Cost effective and efficient remediation of contaminated sites, notably water, soil, and sediments, requires a concerted multidisciplinary effort. The technology of bioremediation is proving to be one of the tools available for the treatment of contamination of the environment. Environmental awareness and efforts to cleanup these sites represent both a commitment to responsible stewardship of our limited natural resources and good business (Kuiper *et al.*, 2003).

Bioremediation involves the controlled use of microorganisms to mineralize hazardous organic chemicals (Bokvajova *et al.*, 1994). An important contribution to the degradation of pollutants is ascribed to rhizosphere microorganisms of plants used during phytoremediation or of plants that emerge as natural vegetation on contaminated sites. A plant can be considered to be a solar-driven biological pump, attracting water with its root system, accumulating water-soluble pollutants in the rhizosphere resulting in the degradation or translocation of the pollutant (Erickson, 1997). The contribution of the rhizomicrobial population to bioremediation is referred to as rhizoremediation (Anderson *et al.*, 1993; Schwab and Banks, 1994).

Rhizoremediation involves the symbiosis between plants and the rhizosphere microbial community responsible for degrading contaminants in soil and ground water. Microbial communities can affect chemical pollutants and the presence of chemical pollutants can also affect the microbial community structure. The chemicals can change the microbial community structure through selection of pollutant degraders or by toxicity to the microorganisms (Amellal *et al.*, 2001). Readily biodegradable pollutants can increase microbial numbers by stimulating growth by providing carbon and energy to the microorganisms (Long *et al.*, 1995).

There are several advantages that rhizoremediation has to offer. Plant cover reduces wind and water erosion and that may in itself prevent spreading of the pollution. Plants improve soil structure and exude oxidative enzymes that

may contribute to PAH's degradation. Plants can be instrumental in influencing the community structure and the diversity of microorganisms in the rhizosphere soil through the release of root exudates. Plant roots supply and constitute easily degradable carbon and energy that generally increases microbial activity in soil. This may lead to enhance degradation of organic pollutants through direct metabolism (Joner *et al.*, 2001). Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms. Microorganisms in turn influence the composition and the quantity of various root exudate components through their effects on root cell leakage, cell metabolism and plant nutrition. Based on differences in the root exudation and rhizodeposition in different root zones rhizosphere microbial communities can vary in structure and species composition in different root locations or in relation to soil type, plant species, nutritional status, age stress, diseases and other environmental factors. Soils harbouring a greater diversity of microorganisms are more likely to be resilient to stresses such as hydrocarbons or heavy metals or long term water–logging (Joner *et al.*, 2001).

Although PAH's may undergo photolysis, chemical oxidation and volatilisation, the major decomposition process is microbial degradation (Weissenfels *et al.*, 1990). Microorganisms are a fundamentally important component of the soil habitat where they play a key role in ecosystem functioning through controlling nutrient cycling reactions essential for maintaining soil fertility and also contributing to the maintenance of the soil structure.

Traditionally the diversity of bacteria was determined by culturing in the laboratory followed by a number of phenotypic tests; hence the true diversity of microorganisms in the soil remained unknown; because it depends on the ability of the microbial cells to grow on a particular media. This approach always results in an underestimation of the microorganisms since less than 10% of microorganisms in nature are culturable. Molecular techniques avoid the problems associated with culturing, because they utilize the total microbial community DNA extracted directly from the rhizosphere environment (Muyzer *et al.*, 1993). Although the importance of the rhizosphere microbial community

for degradation of pollutants has been recognized, very little is known about the exact composition of the degrading microorganisms (Kuiper *et al.*, 2003).

The overall objective of the study was to investigate microbial ecological aspects of rhizoremediation of PAH's contaminated soil. The overall objective has the following research aims:

- To determine the effect of co-occurring multi species plants on the remediation of PAH's in contaminated soil.
- Determination of the microbial community structure and its functional diversity of contaminated and uncontaminated rhizosphere and bulk soil using Biolog GN plates and Denaturing Gradient Gel Electrophoresis (DGGE).
- To determine the ability of *P. putida* to colonize and survive on the rhizosphere of *Eleusine corocana* in different conditions, and also to determine the effect of aromatic compounds on *P. putida* colonization.

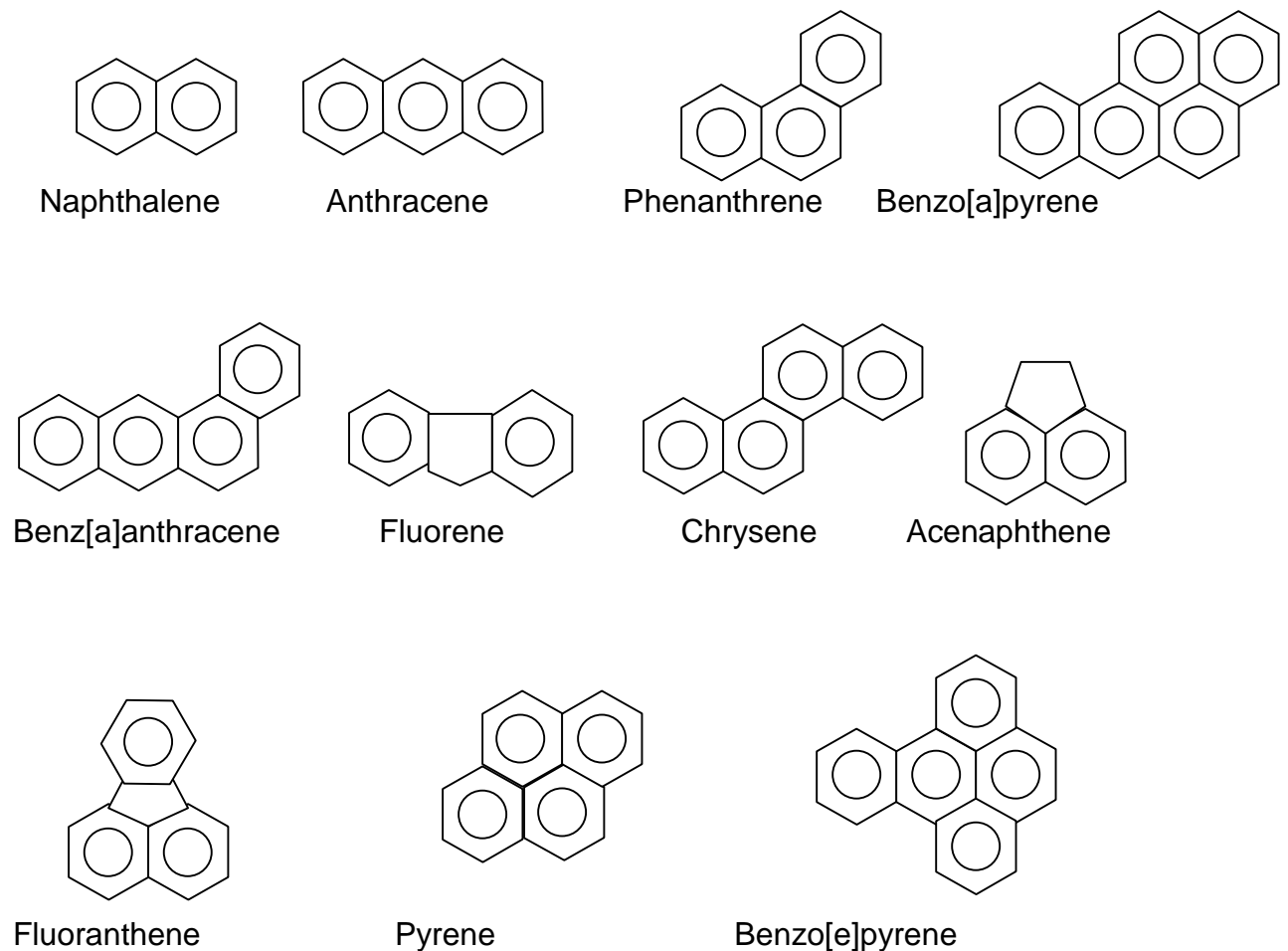
### LITERATURE REVIEW

#### 2.1 Polycyclic Aromatic Hydrocarbons (PAH's)

PAH's are chemical compounds that persist in the environment and cause environmental pollution. PAH's are non-polar hydrophobic neutral molecules with two or more fused benzene rings in linear step or cluster arrangements (Reilley *et al.*, 1996). PAH's are pollutants that are widely distributed in the environment by the energy generating and petroleum producing industries. The increased use of petroleum leads to contamination of the environment. The inadequate use of petroleum products, lack of proper storage and disposal of spilled oils and explosion of transformers cause contamination of the environment. PAH's are widely distributed in oil contaminated soil, ground water and sediments as a result of high aqueous solubility compared to other components of petroleum (Nakagawa *et al.*, 2000). The thermal and chemical stability of PAH's led to their wide spread industrial use which creates major problems of this lipid soluble toxic xenobiotic compound by persisting in the biosphere. PAH's are also known to be toxic, mutagenetic and carcinogenic to humans if they are exposed to it, which can lead to variety of chronic or acute diseases and birth effects on newborns (Reilly *et al.*, 1996).

##### 2.1.1 Definition

Polycyclic aromatic hydrocarbons (PAH's) are ubiquitous environmental contaminants that are formed by the incomplete combustion of organic materials, such as wood or fossil fuels. PAH's molecules are made up of three adjacent carbon atoms. In addition to PAH's that are composed of carbon and hydrogen atoms only, some PAH's contain heteroatoms such as nitrogen and sulphur. They form a large and heterogenous group and the most toxic PAH's are those molecules that have four to seven rings (Supid *et al.*, 2002).



**Figure 1.1:** Chemical structure of PAH's (Sander, 1997).

### **2.1.2 Sources and Physio-chemical Properties of PAH's**

The physical and chemical properties of PAH's are governed by the size (number of carbon) and shape (ring linkage pattern) of the individual molecule (Figure 1.1). PAH's are flat with rings of six carbon atoms and these molecules are colourless, white or pale yellow-green solids, and they often attach to particulate matter (e.g. Soot) (Chiapusio *et al.*, 2007). Benzo[e]pyrene, naphthalene, phenanthrene and other related PAH's are ubiquitous environmental contaminants (Figure 1.1).

PAH's are soluble in lipids and are insoluble in aqueous systems. PAH's are generally insoluble in water but can be readily dissolved in organic acids. This means that in aqueous environments PAH's are generally found adsorbed on particulates and humic matter, or dissolved in any oil matter, which may contaminate water, sediments and soil.

Vapour pressure for PAH's is low and decreases with an increase in molecular weight. The solubility of PAH's in water is inversely proportional to the number of rings it contains. As a result, high molecular weight PAH's (4 rings compounds) are almost exclusively bound to particulate matter, while lower molecular weight PAH's (3 ring compounds) can also be traced in water (Chiapusio *et al.*, 2007).

In the atmosphere and in the presence of sunlight, PAH's undergo photo oxidation, and it occurs much faster for free particles than bound compounds. PAH's in air can also be oxidized by ozone. PAH's are formed by the incomplete combustion of coal, oil, petrol, wood, tobacco, charboiled meats, garbage or other organic materials (Figure 1.1). Most of them have no use; a few of them are used in medicine, and for making dyes, plastic and pesticides. Naphthalene is used in the making of explosives, plastic, lubricants and moth repellent. Anthracene is used in dyes, insecticides and wood preservatives.

The primary sources of emissions of PAH's are petroleum refineries; fossil fuel power plants, coal-tar production plants, coking plants, bitumen and asphalt production plants, paper mills, wood product manufactures, aluminium production plants and industrial machinery manufactures. These emissions normally find the way into the atmosphere (Reilley *et al.*, 1996). PAH's can be formed from any naturally occurring fire, such as bushfires or forest fires. They occur in crude oil, shale oil and coal tars. They are also emitted from active volcanoes. The concentration of PAH's in the environment varies widely depending on the level of industrial development and contamination with petroleum products. PAH's contamination ranges from a low of 5 ng/g of soil in an undeveloped area to  $1.79 \times 10^6$  ng/g at an oil refinery (Cerniglia, 1992).

## **2.2 Rhizoremediation**

Rhizoremediation is a technique used to remediate environments contaminated with hazardous pollutants using plants. An important contribution to the degradation of pollutants is ascribed to microbes present in the rhizosphere of plants used during rhizoremediation or plants which are emerging as natural vegetation on the contaminated site. This contribution of the rhizomicrobial population is referred to as rhizoremediation (Schwab and



Banks, 1994). Although the importance of the rhizosphere community for degradation of microbial community has been recognized very little is known about the exact composition of the degrading population. The use of plants in combination with microbes has the advantage of causing an increase in microbial population numbers and metabolic activity in the rhizosphere. It can also establish an improvement of the physical and chemical properties of contaminated soil and an increase in contact between the microbes associated with the roots and the contaminants in soil (Anderson *et al.*, 1993; Kingsley *et al.*, 1994; Kuiper *et al.*, 2003).

### **2.3 Interaction between microorganisms and plants**

The interaction between plants and microbial communities in the rhizosphere is complex and has evolved to the mutual benefit of both organisms. Plants sustain large microbial populations in the rhizosphere by secreting substances such as carbohydrates and amino acids through root cells and by sloughing root epidermal cells. Also, root cells secrete mucigel, a gelatinous substance that is a lubricant for root penetration through the soil during growth. Using this supply of nutrients, soil microorganisms proliferate to form the plant rhizosphere (Anderson *et al.*, 1993).

The type of root exudates can influence the type of interaction between plants and soil microorganisms (Westover *et al.*, 1997). Specific interaction occurs when the plant exudes a specific compound(s) in response to the presence of the contaminant. Non-specific interaction occurs when typical plant exudates are chemically similar to the organic contaminant, resulting in an increased microbial activity and an increased degradation of contaminants. For example, the roots of red mulberry typically exude rhizosphere phenolics that help create a suitable environment for certain microbes, such as the PCB degrading bacteria *Alcaligenes eutrophus* H850, *Corynebacterium* sp. MB1 and *Pseudomonas putida* (Donnelly *et al.*, 1994; Hegde and Fletcher, 1996).

### 2.3.1 The Rhizosphere: Exudates, Nutrients and Root Colonization

The exudation of nutrients by plant roots creates a nutrient-rich environment in which microbial activity is stimulated. Plant root exudates contain sugars, organic acids and amino acids as main components (Vancura and Hovadik, 1965). In addition, the mucigel secreted by root cells, lost root cap cells, the starvation of root cells or the decay of complete roots provides nutrients (Rovira, 1956; Lynch and Whipps, 1990; Lugtenberg and de Weger, 1992). This stimulatory effect has been recognized for many years and was described for the first time by Hiltner in 1904, who defined the rhizosphere as the zone of soil in which microbes are influenced by the root system (Kuiper *et al.*, 2003). It is known that the rhizosphere is dominated by the gram-negative rods such as *Pseudomonas* species (Kuiper *et al.*, 2003). The ability to efficiently colonize plant roots depends on the different strains, indicating rhizosphere competence abilities.

**Table 2.1 Compounds Detected in Root Exudates (Schnoor, 2002)**

Compounds	Example of compounds
Carbohydrates	Glucose, fructose, sucrose, maltose, galactose, xylose, oligosaccharides
Amino acids	PAH's dissipation during phytoremediation, glucose, fructose, sucrose, maltose, galactose, xylose, oligosaccharides
Aromatics	Phenols, l-carvone, p-cymene, limonene
Organic compounds	Acetic acid, propionic acid, citric acid, butyric acid, valeric acid, malic acid
Volatile compounds	Ethanol, methanol, formaldehyde, acetone, acetaldehyde, propionaldehyde, methyl sulfide, propyl sulfide, allyl sulfide
Vitamins	Thiamine, biotin, niacin, riboflavin, pyridoxine, pantothenic acid
Enzymes	Phosphatase, dehydrogenase, peroxidase, dehalogenase,

### **2.3.2 Effects of the Rhizosphere Microbial Population on Plants**

Just as plant roots have a direct effect on the surrounding microbial populations; microorganisms in the rhizosphere have a marked influence on the growth of plants. In the absence of appropriate microbial populations in the rhizosphere, plant growth may be impaired (Atlas and Bartha, 1998). Microbial populations in the rhizosphere may benefit the plant by increasing recycling and dissolving of mineral nutrients, synthesis of vitamins, amino acids, auxins and gibberellins that stimulate plant growth (Atlas and Bartha, 1998). Microorganisms release antagonistic substances in the rhizosphere that allows the plant to enter an amensal relationship with other plants. Another role played by microorganisms involves their ability to reduce the phytotoxicity of contaminants to the point where plants can grow in adverse soil conditions, thereby stimulating the degradation of the pollutants (Walton *et al.*, 1994).

Plant growth-promoting rhizobacteria (PGPR) are free soil bacteria found on or near the roots of plants and can exert beneficial effects on the plants; either directly or indirectly (Chanway *et al.*, 1999). Directly, PGPR may provide plants with compounds synthesized by bacteria such as fixed nitrogen or phytohormone; they may facilitate the uptake of nutrients such as phosphorus and iron, or they may synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that lowers plant ethylene levels. Indirectly, PGPR facilitate plant growth by preventing or decreasing the deleterious effects of pathogenic microorganisms in the rhizosphere (Smalla *et al.*, 2001).

Microorganisms in the rhizosphere influence the availability of nutrients to the plants by solubilizing materials that are unavailable to plants like phosphorus. Some microorganisms produce organic chelating agents that increase the solubility of manganese and iron compounds making them more available to the plants. Microorganisms in the rhizosphere produce high concentrations of carbon dioxide that increases the solubility of calcium, which significantly increases the uptake of calcium by roots (Atlas and Bartha, 1998).

Microbial activators are microorganisms that activate the biological processes, by producing various biotic substances such as vitamins, auxins, amino acids, and other biocatalysts. Microbial activators increase the percentage of

germinating seeds, enhance the growth of the young plants, and often change the nature of the biochemical processes (Yang *et al.*, 2000).

### **2.3.3 The effect of Plant Root on the Microbial Population in the Rhizosphere**

Microbial growth in soil is limited by the availability of carbon. The rhizodeposition of easily available carbon makes the rhizosphere an area of high microbial activity. The composition and number of microorganisms present in the rhizosphere of different plants may differ due to variations in the quantity and quality of compounds exuded by different plants (Lynch, 1990; Soderberg *et al.*, 2002). Plant exudates also vary with physiological condition such as age, nutrient status and abiotic conditions such as temperature, soil structure, aeration and water content.

Plants and microorganisms are involved in the degradation of PAH's into alcohols, acids, carbon dioxide and water that are generally less toxic and less persistent than the parent compound (Eweis *et al.*, 1998). Though plants and microorganisms can degrade petroleum independently of one another, it is the interaction between the plants and microorganisms (the rhizosphere effect) that is the primary mechanism responsible for petrochemical degradation during rhizoremediation (Kuiper *et al.*, 2003).

Due to root exudates microbial population and activity are five to hundred times greater in the rhizosphere than in the bulk soil (Anderson *et al.*, 1993; Gunther *et al.*, 1996). This plant-induced enhancement of the microbial population is referred to as the rhizosphere effect and is believed to result in enhanced degradation of organic contaminants in the rhizosphere. Jordahl *et al.*, (1997) reported that the population of microorganisms capable of degrading benzene; toluene and xylene were five times more abundant in the rhizosphere of poplar trees than in the bulk soil.

## **2.4 Influence of Environmental Factors on Rhizoremediation**

Environmental factors affect or alter the mechanisms of rhizoremediation. Soil type and organic matter content can limit the bioavailability of petroleum contaminants (Walton *et al.*, 1994). Water content in soil and wetlands affect plant/microbial growth and the availability of oxygen required for aerobic

respiration. Temperature affects the rate at which various processes take place. Nutrient availability can influence the rate and extent of degradation in oil-contaminated soil. Finally, sunlight can transform parent compounds into other compounds, which may have different toxicities and bioavailability than the original compounds. These environmental factors causes weathering of certain fractions of the contaminant mixture with the end result being that only the more resistant compounds remain in the soil.

#### **2.4.1 The Effect of Soil Structure, Texture and Organic Matter Content**

Soil type is defined according to various characteristics including structure, texture and organic matter content. In terms of the influence of soil structure, Alexander *et al.*, (1997) identified that phenanthrene may be trapped within and adsorbed to the surfaces of nanopores (soil pores with diameters <100 nm) that are inaccessible to organisms.

Soil texture can also affect rhizoremediation efforts by influencing the bioavailability of the contaminant. For example, clay is capable of binding molecules more readily than silt or sand (Brady and Weil, 1996). Consequently, the bioavailability of contaminants may be lower in soils with high clay content. In support of this concept, Carmichael and Pfaender, (1997) found that soils with larger particles (e.g. sand) typically had greater mineralization of PAH's than soils with smaller particles (e.g. silt and clay), possibly due to the greater bioavailability of the contaminants in the sandy soils. The authors stated that they had anticipated this result since PAH's are known to adsorb to soil constituents and become unavailable to the microorganisms.

Soil organic matter binds lipophilic compounds, thereby reducing their bioavailability (Cunningham *et al.*, 1996). A high organic carbon content (>5%) in soil usually leads to strong adsorption and, therefore, low availability, while a moderate organic carbon content (1 to 5%) may lead to limited availability (Otten *et al.*, 1997).

Soil type may influence the quality or quantity of root exudates, which may influence rhizoremediation efforts. More specifically, research by Bachmann

and Kinzel, (1992) indicated an interrelationship between soil type and levels of amino acids, sugars and certain enzyme activities in the rhizosphere.

#### **2.4.2 The effect of Water and Oxygen Availability to Plants and Microbes**

Water and oxygen are important to the general health of plants and microbes (Eweis *et al.*, 1998). Water is not only a major component of living organisms; it also serves as a transport medium to carry nutrients to biota and to carry wastes away. If the moisture content of the soil is low, there will be a loss of microbial activity and dehydration of plants. Too much moisture results in limited gas exchange and the creation of anoxic zones where degradation is dominated by anaerobic microorganisms.

Interestingly, oxygen may be provided to the rhizosphere as plant exudates. The extent of oxygen-transfer from the root depends on the type of plant (Vance, 1996). Herbaceous wetland plants typically have a high capacity to transport oxygen from their leaves to their roots and then into the rhizosphere. It also allows wetland plants to support an enhanced bacterial population in the rhizosphere, which may help degrade organic contaminants. Conversely, non-wetland and woody plants have a poor capacity for the downward transport of oxygen.

#### **2.4.3 Effect of Temperature in Rhizoremediation**

Temperature affects the rate at which the various mechanisms of rhizoremediation take place. In general, the rate of microbial degradation or transformation doubles for every 10°C increase in temperature (Wright *et al.*, 1997; Eweis *et al.*, 1998). In an experiment involving oil bioremediation in salt marsh mesocosms, degradation of applied hydrocarbons averaged 72% during summer compared to 56% during winter, even though the winter exposure was 42 days longer (Wright *et al.*, 1997). The seasonal difference was thought to be the result of a 10 °C difference in temperature between the warm summer and cool winter periods. Simonich and Hites, (1994) reported that concentrations of PAH's in plants were higher during spring and autumn when ambient temperatures were relatively low compared to summer. Conversely, during the summer, when ambient temperatures were higher,

lower concentrations of PAH's were found in the plants (Simonich *et al.*, 1994).

#### **2.4.4 Effect of Nutrient Availability for Plant and Microbe Functionality**

Adequate soil nutrients are required to support the growth of plants and their associated microorganisms. This may be especially true during rhizoremediation efforts, when the plant/microbe community is already under stress from the contaminant. Eweis *et al.*, (1998) indicated that PAH's could reduce the availability of plant nutrients in soil. Low nutrient availability results from the fact that PAH's had high carbon content, but are poor suppliers of nitrogen and phosphorus. As soil microorganisms degrade the hydrocarbons, they use up or immobilize available nutrients (i.e. nitrogen and phosphorus) creating nutrient deficiencies in contaminated soil.

Biederbeck *et al.*, (1993) found that, following initial applications of an oily waste sludge to sandy soil, the soil had very low nitrate levels due to immobilization of nitrogen by rapidly growing populations of oil degrading bacteria as well as suppression of nitrogen-fixing bacteria. Two years following oil application, however, sludge treated plots contained more nitrate than untreated controls, presumably due to the gradual re-mineralization of the previously immobilized nitrogen. The trend was similar for phosphorus, which was initially low following incorporation due to immobilization by an expanding microbial biomass, but became more available one year later.

Petroleum hydrocarbons may also limit the accessibility of nutrients to plants and microorganisms by reducing the availability of water in which the nutrients are dissolved. Nutrient deficiencies in soil caused by PAH's may be offset by the application of fertilizer or green manure to the soil.

#### **2.4.5 Effect of Solar Radiation on Rhizoremediation**

Photomodification of PAH's by ultraviolet light can occur in contaminated water or on the surface of soil, increasing the polarity, water solubility and toxicity of the compounds prior to uptake by the plant (Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1997). PAH's that can be modified in this manner include anthracene, phenanthrene, benzo[a]pyrene, fluoranthene, pyrene and naphthalene. Enhanced toxic effects (such as reduced growth)

can also result from penetration of ultraviolet radiation into plant tissue, followed by photomodifications and photosensitizations of PAH's accumulated within these tissues (Duxbury *et al.*, 1997).

#### **2.4.6 Effect of Weathering on Rhizoremediation**

Weathering processes include volatilization, evapotranspiration, photomodification, hydrolysis, leaching and biotransformation of the contaminant. These processes selectively reduce the concentration of easily degradable contaminants, with the more recalcitrant compounds remaining in the soil. The contaminants left behind are typically non-volatile or semi-volatile compounds that preferentially partition to soil organic matter or clay particles, which limits their bioavailability and the degree to which they can be degraded (Bollag, 1992; Cunningham *et al.*, 1996).

### **2.5 Challenges Associated with Rhizoremediation**

Challenges associated with the rhizoremediation of PAH's include the establishment of appropriate plant and microbial communities on the contaminated site; effects of various hydrocarbon concentrations on plants and microbes; biotransformation and bioaccumulation of the contaminants together with disposal of contaminated biomass; implications regarding mixtures of inorganic and organic compounds at contaminated sites; and techniques to enhance rhizoremediation (Kuiper *et al.*, 2003). The following section provides information on these various challenges.

#### **2.5.1 Establishment of Appropriate Plants and Microorganisms for rhizoremediation**

Successful rhizoremediation of PAH' will require the establishment of appropriate plants and microorganisms at the contaminated site. Factors to consider include (Cunningham *et al.*, 1996).

- (i) The influence of contaminants on germination of plants or survival of transplanted vegetation;
  - (ii) The effectiveness of inoculating contaminated soils with microorganisms;
- and



(iii) The use of native versus non-native plants and microorganisms to remediate the site.

### *Seed Germination and Transplanting*

An important factor in establishing plants in an oil-contaminated site involves getting seeds to germinate. Seed germination is enhanced when soil is moist (but not too wet), the temperature is appropriate, and the soil is not toxic to the seeds (Cunningham *et al.*, 1996). A good way of knowing whether the plant being considered for rhizoremediation will germinate successfully is to carry out germination tests in the contaminated soil prior to planting (Cunningham *et al.*, 1996). For example, Epuri and Sorensen, (1997) tested the germination of four grasses exposed to approximately 220 mg of PAH's and 26 mg of PCBs per kilogram of soil (0.022% w/w PAH's and 0.0026% w/w PCBs). Of the four species, *Tall fescue* had the highest germination rate.

According to the results of germination of wheat and soybean in kerosene-contaminated soil (0.34% w/w) was delayed compared to germination in uncontaminated soil (Dibble and Bartha, 1979). However, the overall percentage of germination after 10 days was similar in both the contaminated and uncontaminated soil. The authors suggested that the slower rate of germination in the soil contaminated with kerosene may have been a result of decreased oxygen availability and, consequently, increased competition for oxygen between the germinating seeds and microorganisms.

### *Inoculation of Microorganisms in Soil*

There are conflicting reports on the effectiveness of inoculating contaminated sites with microorganisms. Cunningham *et al.*, (1996), states that it is a common experience for soil and plant inoculants to be out-competed by native microflora. They go on to say that this is even true in some symbiotic relationships (e.g. soybean and their *Bradyrhizobia symbiont*).

Other studies have found that inoculation enhances degradation of organic contaminants. Madsen and Kristensen, (1997) determined that soil inoculation with phenanthrene-degrading bacteria (a possible *Pseudomonas fluorescens*, an *Arthrobacter* species, and an unidentified species of gram-positive, rod-shaped bacterium) enhanced the degradation of [<sup>14</sup>C] phenanthrene

particularly in soils that had no known history of hydrocarbon contamination. It should be noted that a fairly large quantity of inoculum, at least  $10^6$  cells per gram of soil is required to establish the introduced bacteria. Grosser *et al.*, (1995) determined that the isolation, propagation and reintroduction of  $10^7$  colony-forming units of *Mycobacterium* species per gram of soil resulted in enhanced mineralization of phenanthrene, anthracene and pyrene above that found with just indigenous microbes. Siciliano and Germida, (1997) found that inoculating two *Pseudomonas* species onto meadow brome (*Bromus bierbersteinii*) increased degradation of 2-chlorobenzoic acids in the soil.

#### *Using Native versus Non-Native Plants and Microorganisms for Rhizoremediation*

Situations may arise where plants and microorganisms most appropriate for rhizoremediation may not be native to the contaminated site. A decision must then be made as to whether non-native plants or microorganisms should be introduced. The introduction of non-native biota into any ecosystem should not be taken lightly. Indeed, research indicates that 4 to 19% of non-native organisms introduced into natural and agricultural ecosystems in the United States have had severe adverse effects on both the environment and economy (Newman *et al.*, 1998).

Genetically modified (engineered) organisms (GMOs) are considered a type of non-native organism Kochetkov *et al.*, (1997). Preliminary studies are already being conducted concerning the engineering of organisms that could be used in rhizoremediation. Kochetkov *et al.*, (1997) have genetically modified two rhizosphere bacteria (*Pseudomonas putida* and *P. aureofaciens*) by supplying them with naphthalene-degradation plasmids. Unfortunately, it is difficult to identify beforehand the exact ecological consequences of introducing non-native and genetically engineered organisms into the environment.

The ecological risks associated with the use of non-native and genetically modified species in rhizoremediation can be avoided by using native species whenever possible. Whatever the plant chosen for rhizoremediation, the species must be well adapted to the soil and climate of the region making soil characteristics, length of growing season, average temperature, and annual

rainfall important considerations in rhizoremediation planning (Cunningham *et al.*, 1996; Newman *et al.*, 1998). Another advantage of using native plants is the fact that they are pre-adapted to the climatic and soil conditions at the site.

### ***2.5.2 Effect of PAH's on Plants and Rhizosphere Microorganisms***

Within certain concentration ranges, plants and microbes can tolerate PAH's, thus laying the foundation for the rhizoremediation of contaminated sites (Gerald, 2002). Rhizoremediation of PAH's may however be ineffective if concentrations of the contaminants are either too high (causing toxicity) or too low (resulting in poor bio availability).

#### *Effects of Low and High Concentrations of PAH's*

Initial low concentrations of contaminants may limit the extent to which rhizoremediation can further reduce contaminant levels. That is to say, if microbial uptake and metabolism of organic compounds ceases when the contaminant concentration reaches a certain level, the microorganisms will not be physiologically capable of reducing contaminant concentrations any further. Low concentrations may also cause microbes capable of degrading the contaminant to switch to alternative substrates or even result in the death of the microbes due to the lack of sustenance (Hrudey and Pollard, 1993). Similarly, readily biodegradable contaminants in groundwater may remain not degraded, or degrade only very slowly, if their bioavailability is limited to low concentrations.

Concentrations of contaminants that are too high, on the other hand, can cause toxic effects and may even kill exposed microorganisms and plants, again limiting the effectiveness of rhizoremediation. High concentrations of contaminants may prevent or slow the metabolic activity of microorganisms; this in turn would prevent the growth of new microbial biomass needed to increase or maintain degradation (Hrudey and Pollard, 1993). Various plants (red clover, alfalfa, birdsfoot trefoil, white clover, alpine bluegrass, tilesy sage, bering hairgrass, reed anarygrass and quackgrass) did not germinate or were killed shortly after germination given exposure to concentrations of an organic chemical mixture of 2000 mg per kg soil or more (Rogers *et al.*, 1996).

### *Concentrations of PAH's Tolerated by Plants*

Different plants can tolerate different levels of PAH's. Alfalfa can grow actively for up to one year in the presence of water saturated with toluene (e.g. 500 ppm) (Davis *et al.*, 1994). Rogers *et al.*, (1996) found that alfalfa, red clover, white clover, birds foot, trefoil, alpine bluegrass, Bering hairgrass, reed canary grass and quackgrass grew well in soil contaminated with less than or equal to 2000 mg per kg (0.2% w/w) of a mixture of organic chemicals. Whether the effect of the contaminant is beneficial or adverse depends, to a certain degree, on the concentration of the contaminant (Davis *et al.*, 1994).

### **2.5.3 Biotransformation, Bioaccumulation and Disposal**

Although biotransformation may ultimately lead to the mineralization of toxic contaminants, intermediate metabolites formed along the way may be more toxic than the original compound. The degradation of PAH's by fungi, for example, incorporates only one atom of oxygen, which can result in the production of carcinogenic epoxides (Sutherland, 1992). Therefore, under soil conditions favouring fungal activity, early PAH's metabolic products may actually increase the toxicity of the contamination. As degradation proceeds, however, the majority of fungal transformation would detoxify the PAH's compounds (Reilley *et al.*, 1996).

Toxic intermediate metabolites may build up in the soil as a result of either slow reactions by key bacteria or the production of "dead-end" products Davis *et al.*, (1994). Vinyl chloride, a cancer-causing agent, may build up during trichloroethylene biodegradation because bacteria can convert trichloroethylene to vinyl chloride relatively quickly, but the subsequent degradation of vinyl chloride occurs slowly. Dead-end products may form during cometabolism; that is the incidental metabolism of a contaminant may create a product that cannot be further transformed by bacterial enzymes.

In the cometabolism of chlorinated phenols, for example, dead-end products such as the toxic chlorocatechols sometimes accumulate in the soil (Carmichael *et al.*, 1997).

Bioaccumulation, the uptake and accumulation of a chemical by biota, may cause direct toxicity to the organism that accumulates the chemical or to a

consumer of that organism (Mackay, 1991). The disposal of contaminated plant matter is a common practice with plants that accumulate metals, since harvesting the plants effectively removes the contaminants from the site. However, no information concerning the disposal of plant tissues containing accumulated PAH's was found in the literature. This may reflect the fact that the issue is relatively new and, therefore, little to no research has been carried out to date. As well, there may be little interest in the topic since, as stated above, the few studies that do exist on accumulation of PAH's indicate that there is only minor uptake and accumulation of hydrocarbons in the stems and leaves of plants.

#### **2.5.4 The effect of Mixture of Contaminants on Rhizoremediation**

Soils from oil-contaminated sites may also be contaminated with metals, salts, and/or pesticides, thus complicating bioremediation efforts. Cunningham *et al.*, (1996) found that sodium salts as well as a variety of heavy metals (e.g. chromium, lead, mercury, zinc, nickel, copper and cadmium) are commonly encountered on sites contaminated with organics. However, relatively little information exists on the effect of mixtures of these contaminants on rhizoremediation efforts. In general, metal concentrations may inhibit microbial metabolism (Hrudey and Pollard, 1993), while high salinity levels can disrupt protein structures, denature enzymes and dehydrate cells (Atlas and Bartha, 1998).

Mixtures of organic contaminants may also cause difficulties in rhizoremediation. For an example, certain PAH's can inhibit the growth of bacteria that degrade other petroleum contaminants. More specifically, naphthalene competitively inhibits the growth of the phenanthrene-degrading bacteria *Acidovorax delafieldi* strain TNA921 and *Sphingomonas paucimobilis* strain TNE12 (Shuttleworth and Cerniglia, 1996). Mixtures of organic compounds can, however, promote microbial degradation of petroleum hydrocarbons, particularly if one or more components of the mixture are a cometabolite of others. Biota primarily degrading one type of organic compound may also degrade a second compound present at concentrations too low to independently support bacterial growth.

## 2.6 Methods Used to Study Microbial Diversity

Soil bacteria play a role in organic decomposition and are central to soil ecosystem processes maintaining plant primary productivity. In light of recent concerns regarding the impact of agriculture and climate change on biodiversity and ecosystem functioning, it is imperative to gain a more detailed understanding of the bacterial community ecology on remaining native grasslands (Griffiths *et al.*, 2002).

Soil microbial communities vary depending on soil physical and chemical properties, type and amount of plant cover and climate. It is well known that plants influence the biodiversity of bacteria in soils, through the release of compounds such as amino acids, sugars and growth factors in plant root exudates, thereby stimulating microbial activity and growth. Because bacteria respond differently to these compounds, differences in the composition of root exudates can influence the types of rhizosphere community (Dunfield *et al.*, 2000).

Most of soil microorganisms are still unknown and very little is known about the exact composition and diversity of degrading population in the soil. The molecular methods based on recovery of community DNA from soil offer a great potential for investigating the non- culturable part of complex microbial communities. These methods include Denaturing Gradient Gel Electrophoresis (DGGE) in which Muyzer *et al.*, (1993) separate amplified DNA fragments according to the melting properties. Another approach based on the Biolog system uses the ability of the soil to utilise a number of carbon substrates. This approach gives information about functional diversity of the soil and was first applied to complex communities by (Garland and Mills, 1991).

### 2.6.1 Denaturant Gradient Gel Electrophoresis (DGGE)

DGGE is an electrophoretic separation method based on differences in melting behaviour of double stranded DNA fragments. DGGE exploits the fact that identical DNA molecules, which differ by only one nucleotide within a low melting domain, will have different melting temperatures. When separated by electrophoresis through a gradient of increasing chemical denaturant (usually formamide and urea), the mobility of the molecule is retarded at the

concentration at which the DNA strands of low melt domain dissociate (Webster *et al.*, 2002). The branched structure of the single stranded moiety of the molecule becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC. DGGE uses 16S rRNA gene for identification because it is found in all bacteria. DGGE is also widely accepted as a marker for identifying because of the following reasons, 16S rRNA genes are in all prokaryotes and 16S rRNA genes differ between species (Niemi *et al.*, 2001).

### *Preliminary preparation*

A significant amount of preparative work must be undertaken before using the technique to screen for DNA in a particular gene. DGGE is usually performed on PCR products, primers must carefully be chosen so that the region to be screened for DNA has one or at the most two discrete melting domains (excluding the GC clamp). The GC clamp is usually positioned adjacent to the highest melting domain. Thus, full sequence data must be available so that a melt map of the molecule can be constructed, and primers can be designed to amplify a region of unit melting domain. The optimal gradient and gel running conditions must also be established (Muyzer *et al.*, 1993).

### *Computer Generation of Melt Maps and Primer Design*

The programs MELT87, MELT95 and MACMELT are used to generate a melt map from a known DNA sequence. The programs identify primer pairs that will amplify short segments of unit melting domain. Ideally the PCR products should be between 100 and 400bp in size. The program predicts the effect on the melting temperature of the PCR product when a GC clamp is positioned at one of the four ends of the molecule. The program SQHTX calculates the difference in melting temperature (TC) for the wild type molecule and any possible mutation and is used to determine the gel denaturing gradient concentration and run times for the PCR products. Alternatively, optimal gradient concentrations can be determined empirically by performing

perpendicular gel electrophoresis. In such an experiment the denaturing gradient is perpendicular to the direction of electrophoresis (Helms, 1990).

### *Optimisation of Gel Running Conditions*

The computer programs described above reduce the number of preliminary experiments required for optimisation of the gel running conditions. However, it is still necessary to run some preliminary gels to determine the optimal voltages and running times and to confirm that the optimal denaturing gradient has been chosen. These gels are sometimes referred to as travel schedule gels; the aim is to have well separated bands (normal and mutation positive control are simultaneously loaded on the gels), which are "focused" by the gradient. PCR products with two low melting domains require different gel conditions for the analysis of each domain. When optimised gel running conditions have been established the method can be used for DNA screening (Zwart and Bok, 2002).

**Table 2.2 Advantages and disadvantages of DGGE (Muyzer et al., 1993).**

<b>Advantages</b>	<b>Disadvantages</b>
High detection rate and sensitivity.	Additional primers may be required for sequencing
The methodology is simple and a non-radioactive detection method is used.	Primers are more expensive because of the 40 bases of GC clamp
PCR fragments may be isolated from the gel and used in sequencing reactions	Analysis of PCR fragments over 400bp is less successful.
	Genes that are exceptionally GC rich are not easily analysed by DGGE.

### **2.6.2 Community-level Physiological Profile (CLLP) Using Biolog**

The lack of knowledge about the diversity and function of microbial communities resulted in the need of effective methods to evaluate microbial community structure. Isolate based techniques (e.g. Plate count agar) have been previously used, but offering a limited biased view of microbial communities due to 1) the unculturability of many microorganisms



2) the multiple microbial interactions and difficulties of determining microbial function in situ 3) the selective nature of laboratory media (Gerland, 1997).

Many molecular (rRNA) and biochemical (signature lipid biomarker) techniques enable to identify and phylogenetic characterisation of microorganisms without cultivation. However, these techniques don't offer a clear interpretation or a view regarding the diversity of microbial communities and they are also time consuming and complex. Hence the Biolog system provides one to characterize microbial community without usual reliance on selective culturing and which will be less complex than molecular techniques (Ellis *et al.*, 2001).

Characterization of community-level substrate utilization via incubation of environmental samples in Biolog microplates has been increasingly applied in many areas of microbial ecology. Biolog microplates are based on the multivariate profile of colour production caused by utilization of carbon sources and concomitant reduction of tetrazolium dye (Garland and Mills, 1991).

Van Heerden *et al.*, (2002) used the Biolog technique to determine the microbial capacity of the natural and artificial created microbial community to utilise certain selected substrates to functional diversity. They interpreted the microbial community level carbon source utilisation patterns and related these to functional diversity and distribution of species abundance within the community. However, should the loss of substrate utilisation occur upon dilution, the Biolog technique reflects the extent of unevenness and decrease in functional diversity in the microbial community. Their results also indicate that there is a similarity of high substrate utilisation in lower dilutions ( $10^{-1}$  and  $10^{-2}$ ) that indicates high initial microbial diversity but not necessarily evenness of the species. It was concluded that the lower the similarity amongst dilutions, the lower the evenness and vice versa. Although the use of substrate utilisation profiles to characterise microbial communities has limitations, it can be used to determine functional diversity and evenness.

### **2.6.2.1 Factors Influencing the Biolog System**

Biolog plates have been useful as a metabolic fingerprint of bacteria communities. However, to interpret the Biolog fingerprint, it is of importance to know how different inoculum sizes and incubation conditions influence the tetrazolium reduction. Inoculation of bacteria in the Biolog plates is expected to result in growth and the rate of formazan development in individual wells has been assumed to represent growth of inoculated bacteria and to correlate positively with inoculum cell density (Gerland *et al.*, 1991).

#### *Effect of Incubation Time and Conditions on the Biolog System*

Bacterial biolog investigations have used fixed incubation temperatures between 15 and 28°C. The biolog system is sensitive to oxygen concentration; this can be indicated by undevelopment of formazan under anaerobic conditions, unlike where the development of formazan colour is attributed to the actively respiring dense cells not the total density of the cells, which indicate that the physiological state of cells can influence the rate of colour development (Proston-Mufham *et al.*, 2002). Furthermore, the period of microbial growth within the well may also lead to competition effects that again bias the substrate utilization profile. It can be of a good ideal if we can try to standardize inoculum levels by employing vital stains combined with epifluorescence microscopy as a means to actively cells can be introduced into each well (Hill *et al.*, 2000).

#### *Effect of Inoculum Cell Density*

The density of the initial inoculum is of most important to be standardized because it affects the rate at which colour develops in the wells and the time at which colour development should be measured. There will be no visible colour within the well until total number of cells able to utilize that substrate reaches approximately  $10^8$  cells/ml. If the number of cells directly inoculated into the wells may be below  $10^8$  cells/ml, there can be a substantial lag phase while the cells grow within the well. This may lead to false negatives if wells are read too early. Inaccurate physiological profiles may also result if samples are dominated by only few species capable of growing on a particular substrate (Hill *et al.*, 2000).

### *Ecological Relevance of Substrate*

The substrates found in the commercially available Biolog plates are not necessarily ecological relevant and most likely do not reflect the diversity of substrates found in the environment, which also become a problem. For an example, studies were made using plant root exudates as an additional carbon source substrate, but it was found that all of these exudates had allowed utilization rate suggesting there were utilized by microorganisms that were present in the soil in low number (Hill *et al.*, 2000).

### **2.7 Conclusion**

PAH's are chemical compounds that persist in the environment and thus cause environmental pollution. Their removal results in the improvement of air quality and the environment that is in return beneficial for the community. Other techniques such as incineration and land filling were expensive, so it is advisable to develop less expensive and more effective alternative techniques such as rhizoremediation for the removal of these harmful PAH's. The ecological relationship of plant and microbial community structure and the rhizosphere effect on the proliferation of degrading microbes, for effective removal of PAH's in the environment was investigated in this study.

The microbial diversity was expected to change from one environment to another, because the environment which contains abundant conducive substrates enhances the mutual relationship between plants and the rhizobacter where nitrogen will be fixed by rhizobacter for the plant growth and reproduction, and the plants excrete exudates for the growth of rhizobacter. Hence, this increases the productivity and colonization of rhizobacter depending on the ability of specific bacteria to adhere to the rhizosphere.

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### EFFECT OF MULTI SPECIES CO-OCCURRING PLANTS ON THE DEGRADATION OF HYDROCARBONS IN SOIL

#### 3.1 Introduction

Plant performance is affected by competitive interactions between adjacent plants. The interactions among plants may be important for the development of rhizosphere soil communities (Westover *et al.*, 1997). Studies on the importance of plants on stimulating degradation of hydrocarbons and the impact of hydrocarbons on the microbial communities have been studied using bulk and rhizosphere soil (April and Sims, 1990; Latour *et al.*, 1999). The use of plants in combination with microbes has the advantage of enhancing microbial population activities in the rhizosphere. It also improves the physical and chemical properties of contaminated soil and increases the contact between the microbes associated with the roots and contaminated soil (April and Sims, 1990; Kuiper *et al.*, 2003).

Plants can be instrumental in influencing the community structure and diversity of microorganisms in the rhizosphere soil through the release of root exudates. Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms (Joner *et al.*, 2001). Microorganisms in turn influence the composition and quantity of various root exudates components through their effects on root cell leakage, cell metabolism and plant nutrition. Based on differences in the root exudation and rhizodeposition in different root zones, rhizosphere microbial communities can vary in structure and species composition in different root locations or in relation to soil type (Soderberg *et al.*, 2002).

Soils harbouring a greater diversity of microorganisms are more likely to be resilient to stresses such as hydrocarbons or can vary in structure and species composition in different root locations or in relation to soil type, plant species (Joner *et al.*, 2001). The development of rhizosphere microbial

communities is influenced by different plant species and environmental factors affecting growth of the plants (Puentes *et al.*, 1999).

The use of plants to remediate PAH's in the environment has received considerable attention, because it is perceived to be environmentally friendly and inexpensive unlike traditional remediation that usually involves expensive excavation and removal of soil for treatment. The relationship of co-occurring plants and their impact on microbial structure and the efficiency in removing PAH's has been well studied. The objective of this study was therefore to determine the effect of co-occurring multi species plants on the remediation of PAH's in contaminated soil.

## **3.2. Materials and Method**

### **3.2.1. Plant Species used in this study**

Six plant species were evaluated for PAH's degradation. *Eleusine corocana*, *Bidens pilosa*, *Tagetes minuta*, *Branchiaria serrata*, *Panicum maximum* and *Smuts grass*.

### **3.2.2. Germination Test for determining Phytotoxicity**

For the germination test, seeds were purchased from AGRICOL (Pty) Ltd, SA. The soil used in this experiment was sandy loam soil taken from the CSIR site (Pretoria, SA). The soil was sieved through a 4mm sieve to remove small grasses, leaves and stones. The soil was contaminated with 300mg/kg mixture of Naphthalene and fluorene. For each plant treatment, 75ml of deionised water was added to 375g of soil to bring the moisture to 75% field capacity. The soil was mixed and divided into three polystyrene petri dishes (150mm x 25mm). For each petri dish, 50 seeds were sown and the plates were incubated at room temperature. Germination in different plates was recorded after 3 to six days of incubation as done by Maila and Cloete, (2002).

Germination was defined as an observable cracking of the seed coat with a measurable shoot production. The germination test was performed to evaluate the potential of a plant to germinate in contaminated soil. This information was used to select appropriate plants that could survive and grow in PAH's contaminated environment.

### 3.2.2 Contamination of Soil and Plant Growth

Soil was collected and contaminated as described above. Naphthalene and Fluorene was first dissolved in 100 ml of Chloroform and was then added to 1% of the soil by mass. The solvent was evaporated using a fume hood (air running at 0.6m/s). After evaporation, contaminated soil was mixed with 99% of uncontaminated soil.

The seeds of *Eleusine corocana* and *Branchiaria serrata* were sown in two different trays and incubated for five days to monitor germination of the seeds and seedling development. After germination seedlings were planted and the density of the plants in the pot was one plant per pot except where a mixture of the plants was used (that is two plants per pot *B. serrata* and *E. corocana*). The different treatments used in the study are shown in Table 3.1. For each treatment eight replicates were used. Plants were grown in the green house for eight weeks.

**Table 3.1 Treatments used in the study**

Treatments	Additions and Preparations			
	<i>E. corocana</i>	<i>B. serrata</i>	PAH's	Soil
TR1	X	–	X	X
TR2	–	X	X	X
TR3	X	X	X	X
TR4 control 1	–	–	X	X
TR5 control 2	X	X	–	X

X = added, - = exclusion/not added

Notes: Final PAH's concentration of each treatment was 300ppm.

### 3.2.3 Chemical Analysis

For each treatment plants were harvested after two weeks (Table 3.1). The rhizosphere soil was defined as the volume of the thin layer of soil immediately surrounded plant roots, and an active area for root activity and metabolism (Chin-hua and Palada, 2006). For hydrocarbon analysis, soil was first vigorously mixed using a spatula to evenly distribute the PAH's throughout the total mass of the soil. Total PAH's were extracted using the soil extraction method as described by Maila and Cloete, (2002).

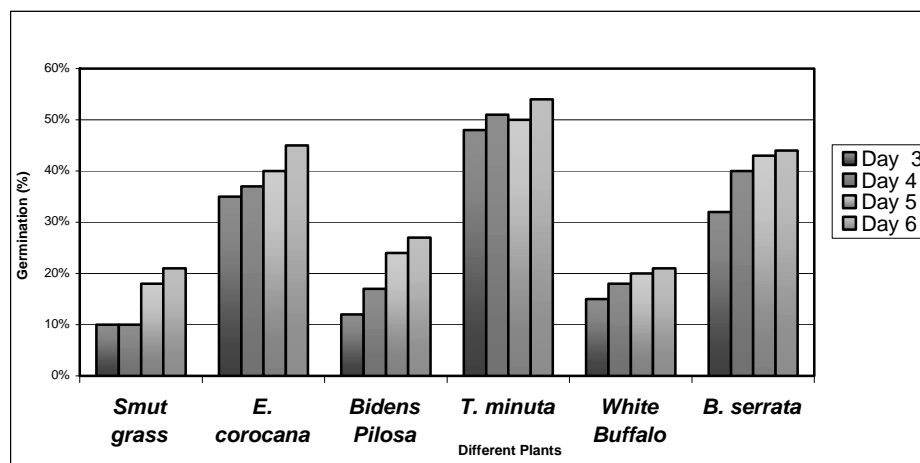
10g of soil was taken from the pot of each treatment and used in the analysis of PAH's. The methodology used for analysing of the PAH's was described in Maila and Cloete, (2002).

### 3.2.4 Microbiological Analysis

The bacterial suspension was prepared by mixing 10g of rhizosphere and bulk soil with 100ml of autoclaved 0.2% sodium pyrophosphate (pH 8.5). This suspension was agitated for 30 min at 140rpm and allowed to settle for 15 min. Dilutions were made from  $10^{-1}$  to  $10^{-5}$  using 0.85% sodium chloride (physiological saline solution). The total recoverable heterotrophs (TRH) and hydrocarbon degrading bacteria (HDB) were determined during the rhizoremediation experiment. Hydrocarbon degrading bacteria (HDB) were determined using bacteriological agar in which the hydrocarbon (PAH'S) acted as the only source of carbon energy. The plates were incubated for 72 h for TRHs and 7 d for biodegraders (EPA 164).

## 3.3 Results and Discussion

### 3.3.1. Germination Test and Plant Selection

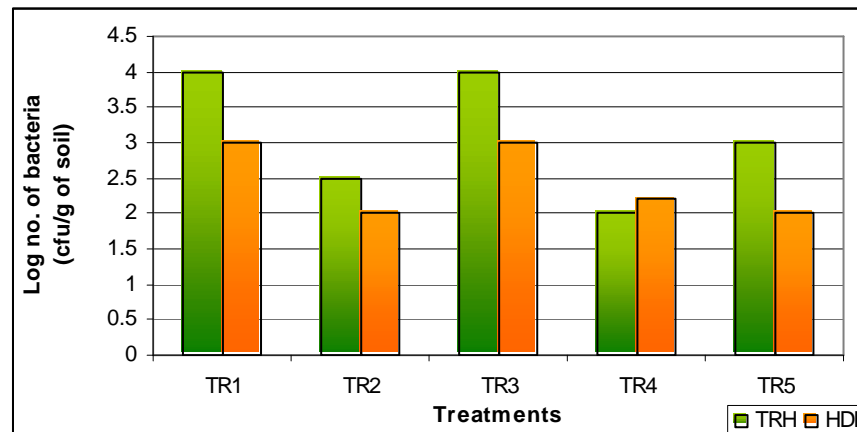


**Figure 3.1** The level of germination of different plants in PAH's (300ppm) contaminated soil.

The ability of the seeds to germinate in soil contaminated with 300ppm of Naphthalene and Fluorene was investigated. The germination level of *B. pilosa*, Smut grass and white buffalo grass was very low (10%) after three days of incubation, compared to that of *T. minuta*, *E. corocana* and *B. serrata*

which was 55%, 45% and 42% respectively (Figure 3.1). However, germination of all the seeds increased with an increase in time. The potential of *E. corocana* and *B. serrata* to germinate in the same environment indicated that these plants were tolerant to one another. *E. corocana* and *B. serrata* were therefore chosen as model plants for the phytoremediation experiment because of their dense roots and tolerance to PAH's.

### 3.3.2. Microbiological Analysis and PAH's Dissipation

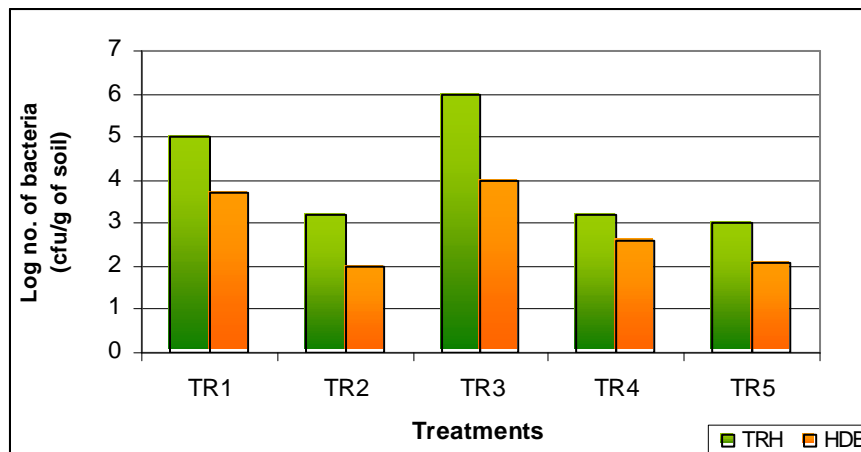


**Figure 3.2** The number of TRH's (Total Recoverable Heterotrophs) and HDB (Hydrocarbon Degrading Bacteria) after two weeks of phytoremediation.

**Key:** TR1 (PAH's soil, *E.corocana*), TR2 (PAH's soil, *B.serrata*), TR3 (PAH's soil, *E. corocana* and *B. serrata*), TR4 (uncontaminated soil), and TR5 (uncontaminated soil, *E. corocana* and *B. serrata*).

After two weeks of incubation the number of bacteria were lower than  $4,0 \times 10^1$  CFU/g of soil for all the treatments. HDB appears to be lower than TRH. This was probably because HDB in TR1, TR2, TR3 and TR4 were still adapting and acclimatising from the PAH's contaminated environment, whereas in TR5, HDB could be lower than TRH because there was no carbon source (PAH's) for them to grow.

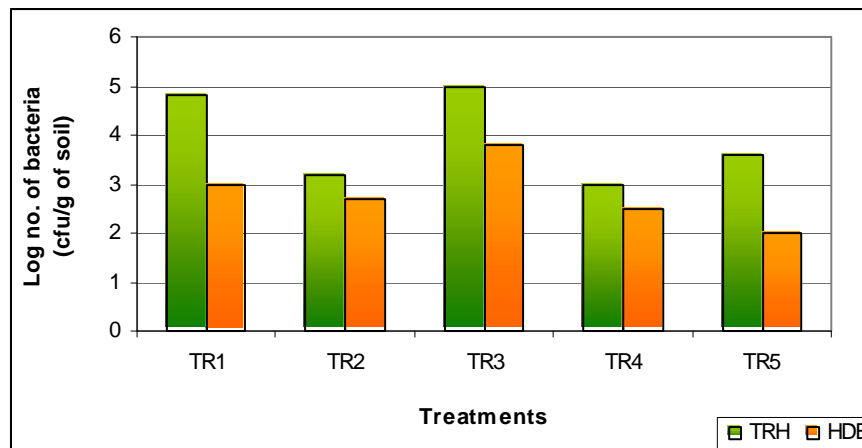




**Figure 3.3** The number of TRH's and HDB after four weeks of phytoremediation.

**Key:** TR1 (PAH's soil, *E.corocana*), TR2 (PAH's soil, *B.serrata*), TR3 (PAH's soil, *E.corocana* and *B. serrata*), TR4 (uncontaminated soil), and TR5 (uncontaminated soil, *E.corocana* and *B.serrata*).

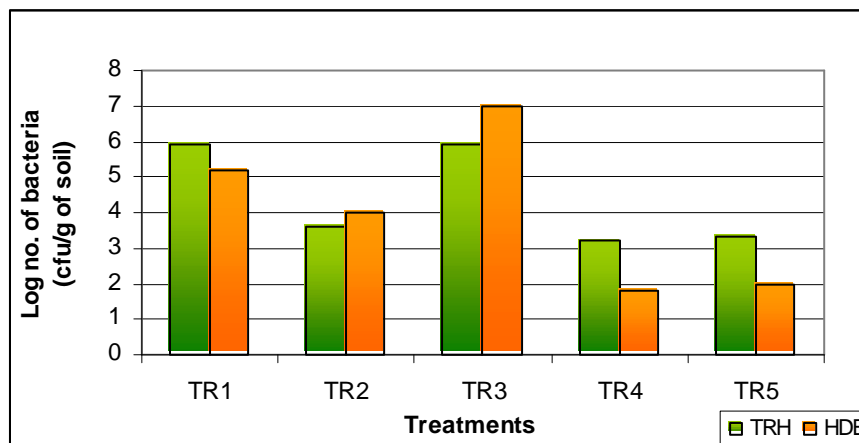
The number of bacteria increased from  $4,0 \times 10^4$  CFU/g of soil to a greater extent ( $6,0 \times 10^1$ ) in TR3 and TR1 ( $5,0 \times 10^1$ ) while there was a decrease in the number of bacteria in TR2, TR4 and TR5 ( $3,2 \times 10^1$ ,  $3,2 \times 10^1$ ,  $3,0 \times 10^1$  CFU/g of soil (Figure 3.2). This was attributed to the potential of multi-species co-cultured plants (in TR3) to interact commensally and produce abundant root exudates in addition to the carbon source (PAH's) that enhanced the proliferation of microorganisms (biodegrading bacteria). In TR1, there could be different exudates secreted by *E. corocana* and also the addition of carbon source (PAH's) that could enhance the growth of microorganisms.



**Figure 3.4** The number of TRH's and HDB after six weeks of phytoremediation.

**Key:** TR1 (PAH's soil, *E.corocana*), TR2 (PAH's soil, *B.serrata*), TR3 (PAH's soil, *E.corocana* and *B. serrata*), TR4 (uncontaminated soil), and TR5 (uncontaminated soil, *E.corocana* and *B.serrata*).

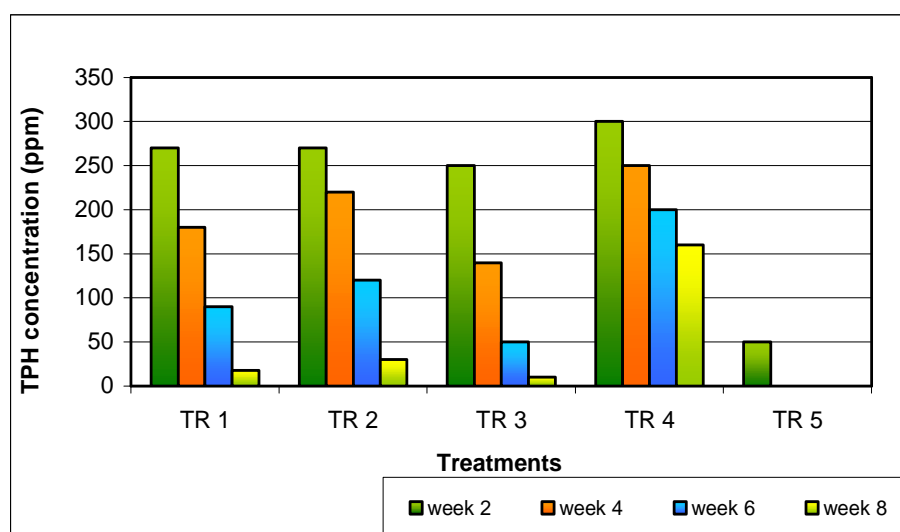
The number of bacteria increased from  $4,0 \times 10^4$  CFU/g of soil to a greater extent ( $5,0 \times 10^1$ ) in TR3 and TR1 ( $4.8,0 \times 10^1$ ) as compared to decreases in the number of bacteria in TR2,TR4 and TR5 (Figure 3.4). However, after six weeks of incubation there was a decline in number of bacteria in TR1 and TR3 as compared to after four weeks of incubation. This could attribute to some environmental factors during that specific period (e.g water availability).



**Figure 3.5** The number of TRH's and HDB after eight weeks of phytoremediation.

**Key:** TR1 (PAH's, soil, *E. corocana*), TR2 (PAH's, soil, *B. serrata*), TR3 (PAH'S soil, *E. corocana* and *B. serrata*), TR4 (uncontaminated soil), and TR5 (uncontaminated soil, *E. corocana* and *B. serrata*).

The study revealed that the number of microorganisms in TR3 were higher than in all other treatments followed, by TR1, TR2, TR5 and TR4 respectively (Figure 3.2 - 3.5). The study also indicated that plants had an effect on the growth of total recoverable heterotrophs (TRH) and hydrocarbon degrading bacteria (HDB). In TR3 initially, the number of TRH ( $4,0 \times 10^1$ ) was higher compared to HDB ( $3,0 \times 10^1$ ) (Figure 3.2). After eight weeks of incubation the number of HDB ( $7,0 \times 10^4$ ) was higher than the number of TRH ( $6,0 \times 10^1$ ) (Figure 3.5). This could be due to the fact that, TR3 had an additional substrate (PAH's) which the HDB could use as a carbon and energy source and could also be attributed to the toxic effect of the PAH's to other heterotrophs. However, with time, microbes became acclimatized and could use the pollutant as a substrate to survive and grow. The HDB count increased with time and the HDB count in TR2 was higher than the TRH's counts. This indicates that the number of biodegraders in the soil microcosms increased due to favourable environmental conditions. Bacterial counts were high in planted treatments TR1, TR3 ( $5,1 \times 10^1$ ,  $7,0 \times 10^1$  CFU /g of soil compared to unplanted treatments (TR4  $1,8 \times 10^4$  CFU /g of soil) (Figure 3.5). This was attributed to the fact that plants can release 10 to 20 percent of total carbon fixed in photosynthesis through their roots into the soil, which is rich with biodegradable macromolecules that stimulate microbial growth (Gerald, 2002). Thus heterotrophic microorganisms in the rhizosphere can be up to 100 times more than in the bulk soil (Atlas and Bartha, 1992; Gerald, 2002).



**Figure 3.6** PAH's dissipation during phytoremediation.

There was 90% PAH's removal in TR3 compared to TR1 and TR2 (Figure 3.6). The concentration of PAH's during week 2 in TR3 was 280ppm and this was reduced to 10ppm after 8 weeks of incubation. In TR1 and TR2, the concentration also decreased from 300ppm to 15ppm and 20ppm respectively. In TR4 more than 50% of the pollutant was still in the soil after 8 weeks of incubation. This reflected that vegetation had an impact on the removal of PAH's in the environment. This is in line with the results obtained by April and Sims (1990). The treatment with the large number of HDB also resulted in the highest PAH's removal.  $7 \times 10^1$  CFU/g of soil bacterial numbers were observed in 'multi-vegetated' treatments (TR3) compared to the mono-planted (TR1 and TR2) and the control treatment (TR4). These patterns of different microbial growth was attributed to the influence of co-occurring plants able to interact commensally with each other and release exudates that enhances bacterial proliferation. It has been hypothesized by Lynch and Whipps, (1990) that root exudates may improve both the bioavailability and biodegradation of hydrophobic organic compounds such as PAH's through the release of structural analogues for induction of enzymes in the microbial rhizosphere, these results support this hypothesis.

### **3.4 Conclusion**

Plants play an important role in the dissipation of PAH's in the environment and in this study this was observed in TR1, TR2 and TR3 in which the planted treatment removed the PAH's effectively compared to the non-planted soil treatments. In addition multi-planted treatment (TR3) performed better than the mono-planted treatments (TR1 and TR2). The feasibility of co-occurring plants to grow, survive and improve the interaction of microbes associated with the roots and contaminated soil indicated an efficient removal of PAH's in the environment. These findings support the concept of rhizosphere remediation, whereby the planting, inter-cropping and maintenance of selected plant species at a contaminated site will foster development of a microbial community that is conducive to contaminant degradation.

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## CHAPTER 4

### EVALUATION OF MICROBIAL DIVERSITY IN SOIL AT VEGETATED AND NON-VEGETATED HYDROCARBON CONTAMINATED SITES.

#### 4.1 Introduction

Soil bacteria play an important role in organic decomposition and are central to soil ecosystem processes which maintains plant primary productivity. In light of recent concerns regarding the impact of agriculture and climate change on biodiversity and ecosystem functioning, it is imperative to gain more detailed understanding of the bacterial community ecology (Griffiths *et al.*, 2002). Soil microbial communities vary depending on soil physical and chemical properties, type and amount of plant cover and climate.

It is well known that plants influence the biodiversity of bacteria in soils, through the release of compounds such as amino acids, sugars and growth factors in plant root exudates hence microbial activity and growth are stimulated (Muyzer, 1993). Because bacteria respond differently to these compounds differences in the composition of root exudates can influence the types of rhizosphere bacterial community. One of the major factors that can influence roots exudation is plant stress (defoliation event or insect attack, or more insidious reduction in plant nutrient availability), which can radically alter both the quantity and quality of root exudates with consequences for the soil microbes (Clegg and Murray, 2002).

In many studies, an important contribution to the degradation of PAH's is ascribed to microbes present in the rhizosphere of plants used during phytoremediation or of plants that are emerging as natural vegetation on contaminated site. Although the importance of the rhizosphere community for degradation of PAH's has been recognized, very little is known about the exact composition of the degrading population (Kuiper *et al.*, 2003). Rhizoremediation relies on the symbiosis between plants and rhizosphere microbial community to degrade contaminants in the soil and ground water.

Although it has been demonstrated that microbial communities can affect chemical PAH's, the presence of chemical pollutants can also affect the microbial community structure.

The chemicals can alter the community structure through selection of pollutants degraders or through acute toxicity to microorganisms (Amellal *et al.*, 2001). Readily biodegradable pollutants can increase population densities by promoting growth through providing carbon and energy to microorganisms in oligotrophic environments (Long *et al.*, 1995). Actively growing roots also furnish and constitute easily degradable carbon and energy that generally increase microbial activity in soil, which lead to enhance degradation of organic pollutants through direct metabolism (Joner *et al.*, 2001). Although polycyclic aromatic hydrocabons (PAH's) may undergo photolysis, chemical oxidation and volatilization, the major decomposition process is microbial degradation (Weissenfels *et al.*, 1990). The rhizosphere microbial communities are important for the ecosystem, both in relation to direct interaction with plants and with regard to nutrient and organic matter cycling (Soderberg *et al.*, 2002).

Traditionally the diversity of bacteria was determined firstly by culturing in the laboratory followed by a number of phenotypic tests; hence the true diversity of microorganisms in the soil remained unknown. This problem has now been circumvented through application of molecular biological approaches in the nucleotide Sequences analysis of ribosomal RNA genes (16S rRNA genes in bacteria). These approaches avoid the problems associated with culturing, because it utilises the total microbial community DNA extracted directly from the rhizosphere soil (Soderberg *et al.*, 2002).

Most of soil microorganisms are still unknown and very little is known about the exact composition and diversity of degrading population in the soil. The molecular methods based on recovery of community DNA from soil offers a great potential for investigating the part of complex microbial communities that cannot be cultured. These methods includes Denaturing Gradient Gel Electrophoresis (DGGE) in which amplified DNA fragments are separated according to the melting properties (Soderberg *et al.*, 2002); but it was first applied to complex microbial communities by Muyzer, (1993). Another approach based on the Biology system which uses the ability of the microorganism to utilise a number of sole carbon substrates as a community profile, and the oxidation of those substrates are periodically monitored by measuring the concomitant reduction of tetrazolium dye. The oxidation of the



substrate depends on both the composition and the density of the inoculum used, and hence the pattern of substrate used may only reflect the functional characteristics of organisms that are able to grow in the Biolog GN plates wells under the assay conditions used (Smalla *et al.*, 2001). This approach gives information about functional ability of the microbial community and was first applied to complex communities by Garland and Mills, (1991).

In this study we investigated the physiological characteristics of rhizosphere microorganisms associated with two different plants, and the findings obtained were compared to the results of the microbial structure of bulk soil to determine whether the presence of a particular plant influences the free-living rhizosphere microbial population structure. In this experiment we used Biolog GN plates and DGGE for the analysis of the microbial community structure of contaminated and uncontaminated rhizosphere and bulk soil.

## **4.2 Materials and Methods**

### **4.2.1. Rhizosphere and Bulk Soil Sampling**

Rhizosphere and bulk soil samples were collected in sterile bags from historically contaminated and uncontaminated sites in Secunda (South Africa). The sites were contaminated with hydrocarbons originated from the diesel and oil residues from their refinery plants. The degree of contamination on the sites was not analysed because the aim of the study was to investigate the microbial diversity on vegetated and non-vegetated contaminated sites. The Rhizosphere soil samples were collected from soil vegetated by *Eleusine corocana* and *Branchiaria serrata*. Samples were transported and stored at 4°C to avoid possibilities of microbial multiplication until used.

### **4.2.2. Measurement of CLPP using Biolog GN2 Microtitre Plates**

Community-level physiological profile was assessed using the method originally described by Garland and Mills, (1991). The bacterial suspension was prepared by mixing 10g of rhizosphere and bulk soil with 100ml of autoclaved 0.2% sodium pyrophosphate (pH 8.5) in a 250ml Erlenmeyer flask. This suspension was agitated for 30 minutes at 140rpm in a shaker and allowed to settle for 15 minutes. Dilutions were made from 10<sup>-1</sup> to 10<sup>-3</sup> using 0.85% physiological saline.

Each well was inoculated with 150  $\mu\text{l}$  (approximately  $10^5$  cells  $\text{ml}^{-1}$ ) bacterial suspension in Biolog GN2 plates. The plates were incubated up to 3 days at  $20^\circ\text{C}$  and the colour development was measured every 24 hours using a microtitre plate reader (Bio-Tek ELx800) at 600nm. Plates with an average well colour development (AWCD) were used in the statistical calculations (Statistica for windows release 5.0).

#### ***4.2.3. DNA Extraction and PCR Amplification of 16S rDNA Fragments for DGGE Analysis.***

Total DNA was isolated and purified from the rhizosphere and bulk soil samples using fast DNA kit (Bio 101, North America & Europe) as described in the protocols of the manufacturer. Contaminated and uncontaminated soil samples were obtained from the planted and unplanted soil sites, a total of four rhizosphere samples and two bulk soils were analysed in the experiment.

The 16s rDNA fragments were amplified by PCR with the primer pair of 0,2  $\mu\text{M}$  K and M for eubacteria. Each PCR was performed with a model 480 DNA thermal cycler. Regions within 16S rDNA were amplified by using 25 $\mu\text{l}$  reaction mixtures containing 2.5 $\mu\text{l}$  of the Taq polymerase fragment, deoxynucleotide triphosphate at a final concentration of 0,2 mM, 3.75 mM  $\text{MgCl}_2$ . PCR amplification was done by using an initial denaturation step ( $94^\circ\text{C}$  for 5 min), followed by 35 cycles consisting of 1 minute at  $94^\circ\text{C}$ , 1 minute of primer annealing at  $54^\circ\text{C}$  and 1 minute of primer extension at  $72^\circ\text{C}$ . The resulting PCR products were analysed on a 1% agarose gel.

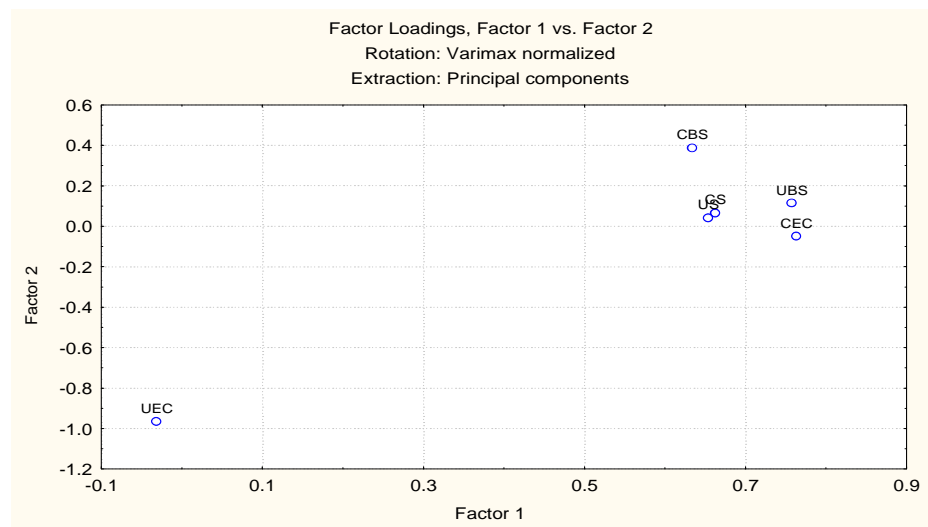
#### ***4.2.4. DGGE Analysis***

To assess PCR amplified products DGGE was used and done as described by Muyzer, (1993). DGGE was performed using Hoefer SE600 vertical dual cooler system (Hoefer Scientific, San Francisco, CA). PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 0.5x TAE (20 mM Tris, 10mM acetate, 0.5mM Na-EDTA, pH 7.4). The 8% (wt/vol) polyacrylamide gels (bisacrylamide gel stock solution, 37.55:1; BioRad Laboratories, Inc) were prepared with a 20 to 55% gradient of denaturant (urea and formamide) and allowed to polymerise overnight. The electrophoresis was run at  $60^\circ\text{C}$ , first for 10 min at 20 V, and subsequently for 17h at 200V. After electrophoresis, the

gels were stained for 15 min in SYBR Green I nucleic acid gel stain rinsed in distilled water for 1 min and photographed with a Polaroid MP4 Land camera. The gels were evaluated using a software program developed by Svein Norland (Department of Microbiology, University of Bergen), where presence/absence of bands was recorded. Clustering is based on the simple matching algorithm, while the dendrogram is drawn applying the group average method.

## 4.3 Results and Discussion

### 4.3.1 Microbial Community Physiological Profiles



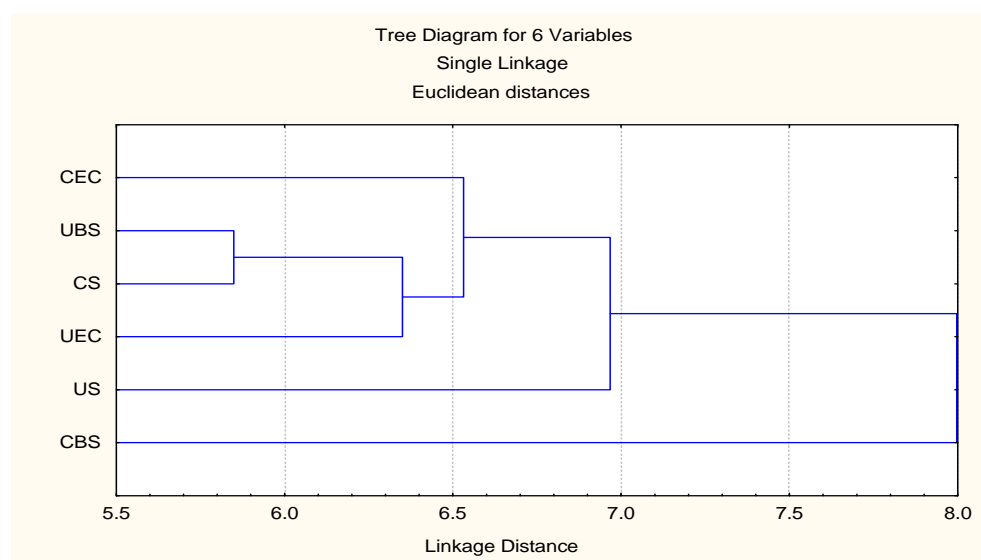
**Figure 4.1** Principle component analyses (PCA) of different rhizosphere and bulk soil samples

Notes: Planted contaminated soil with *Eleusine corocana* (CEC), planted uncontaminated soil with *Eleusine corocana* (UEC), planted contaminated soil with *Branchiaria serrata* (CBS), planted uncontaminated soil with *Branchiaria serrata* (UBS), unplanted contaminated soil (CS) and unplanted uncontaminated soil (US)

The PCA did not reveal clear difference in substrate utilization profiles of the microbial communities in the rhizosphere and bulk soil. However, UEC was the only treatment that appears to be different to the other samples, which indicate that the microbial functional diversity of UEC is different from the other samples. Cluster analysis shows CBS to be different to the other samples (Figure 4.2). This could be attributed to Biolog system that put emphasis on functional diversity that cannot be absolutely related to species diversity. These suggest that the studies on microbial community analysis

should be done using more than one technique due to the inherent limitation of the different methods.

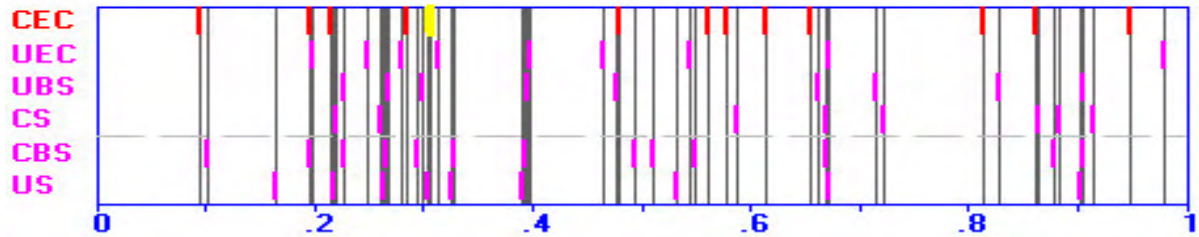
PCA was performed to characterize the functional diversity amongst the samples taking into account the response of 96 wells indicated by the absorbance values. Two principal factors were isolated from the individual CEC, UEC, CBS, UBS, CS and US pattern. Factor one was related to the absorbance values of the wells, while factor two was related to the incubation time. US were more closely related to CS than to UEC, CEC, UBS and CBS respectively (Figure 4.1). This was ascribed to the fact that plants can release different substrates that stimulate the metabolic activities of certain species that differs from the substrates of unplanted environment. The substrate utilization patterns of the rhizosphere soil of different plants were slightly different (UBS, CBS differs from UEC CEC). This was attributed to the fact that different plants excrete different types of exudates that could be selected by microbial community that resulted on having different species in different rhizosphere. This finding is also in line with the findings made by Muratova *et al.*, (2002) who reported that the degradative potential of two different plants species (Alfafa and reed) were different.



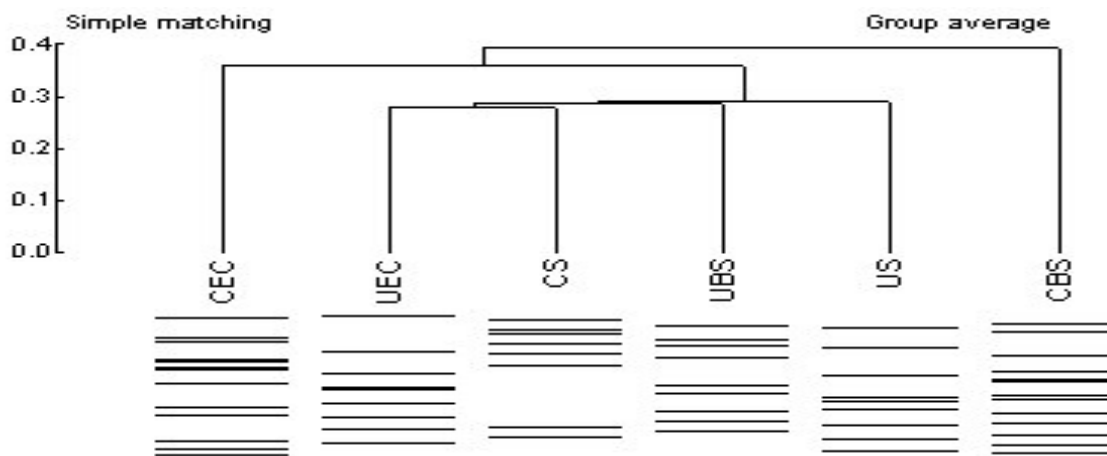
**Figure 4.2** Cluster Analysis of different rhizosphere and bulk soil samples.

The functional diversity of different samples was further analysed using hierarchical clustering (Figure 4.2). The dendrogram revealed clustering of all samples except CBS. However, CS is more closely related to UBS, whereas US, UEC and CEC were clustered distantly from each other.

### 4.3.2 DGGE Profiles



**Figure 4.3.a** Graphical representation of the DGGE gel



**Figure 4.3.b** Cluster analysis of different treatments (lines under each treatment represent a band)

The results show that CEC has more diversity than UEC; CEC has 10 bands whereas UEC has 8 bands, and CEC has 3 thicker bands than UEC having only 1 thick band. The same phenomena could also be seen in CBS (11) having more band than UBS (7). The thickness of the band indicates the high number of the species and the number of the bands represents functional diversity. Unlike DGGE the Biolog system revealed a close functional relationship of all samples except that of UEC and CBS as revealed by PCA and Cluster analysis respectively. Vegetated soil (UEC, CEC, UBS and CBS) also revealed to have more bands than non-vegetated soil (US and CS). Vegetated soil had 8, 10, 9 and 11 bands whereas non-vegetated has 8 and 7 bands respectively. This was ascribed to the roots of the plants that can act as a substitute for the tilling of soil to incorporate additives (nutrients) and to improve aeration to introduce oxygen into deeper soil layers through specialized root vessels, aerenchyma (Kuiper *et al.*, 2003).

This will enhance the availability of nutrients and oxygen to the microorganisms. In addition, plants also release a variety of photosynthesis derived organic compounds (Anderson *et al.*, 1993). These root exudates contain water soluble, insoluble, and volatile compounds including sugars, alcohols, amino acids, proteins, organic acids and certain enzymes that enhance the metabolism of microorganisms (Kuiper *et al.*, 2003).

Some organic compounds in root exudates may serve as carbon and nitrogen sources for the growth and long-term survival of microorganisms that are capable of degrading organic pollutants. The genetic profiles of the rhizosphere soil of the two different plants (*E. corocana* and *B. serrata*) were also different in both contaminated and uncontaminated soil; their bands are positioned in different levels (Figure 4.3.b) that indicate that they are comprised with different genetic profile resulting in different functional diversity. The results show that there was a slightly difference in the number of microbial communities in *E. corocana* and *B. serrata* planted soils; CEC has 10 bands and CBS has 11 bands. This was attributed to the different roots exudates produced by the two plants that may select for specific bacteria in the root zone.

#### **4.4 Conclusion**

Analysis of the microbial diversity using functional diversity (Biolog GN2) and genetic diversity (PCR-DGGE of 16S rDNA) did not complemented each other in revealing the differences in microbial communities in both the bulk and rhizosphere soils. There were differences in both metabolic and genetic profiles of the contaminated and non-contaminated soil (PCR-DGGE). In addition both techniques revealed slightly differences in microbial communities harboured in the rhizosphere of different plants. This study suggests the use of multiple techniques to analyse microbial community due to the inherent limitations of the different methods.

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## CHAPTER 5

### ROOT COLONIZATION OF *ELUESINE COROCANA* BY SELECTED BACTERIA IN CONTAMINATED AND UNCONTAMINATED SOIL

#### 5.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are free-living bacteria found on or near roots of plants and can exert beneficial effects on plants growth either directly or indirectly. Directly, PGPR provide plants with compounds synthesized by bacteria such as fixed nitrogen or phytohormones. They facilitate the uptake of nutrients such as phosphorus and iron and they may synthesize the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that lowers plant ethylene levels. Indirectly, PGPR facilitate plant growth by preventing the deleterious effects of pathogenic microorganisms in the rhizosphere (Shishido *et al.*, 1999; Chanway *et al.*, 2000; Ma *et al.*, 2001). The rhizosphere is the narrow zone of soil surrounding the root that is subject to influence by the root. Rhizobacteria are plant-associated bacteria that are able to colonize and persist on roots. Some strains of plant growth-promoting rhizobacteria can effectively colonize plant roots and protect plants from diseases caused by a variety of root pathogens and growth promotion of plants through direct stimulation of growth hormone (Bashan and Holguin, 1995; Kumari and Srivastava, 1999). Such beneficial or plant health-promoting strains are emerging as promising bio-control agents. They are suitable as soil inoculants either individually or in combination and may be compatible with current chemical pesticides. Inoculating seeds or plants with microorganisms has been successfully used for the control of pathogens (Bacilio-Jiménez *et al.*, 2001).

*Pseudomonas* are ubiquitous and have been isolated from different habitats, like different plant leaves and roots, soil types, fresh and marine waters, animal skin and tissues (Molina *et al.*, 2000). The ability of *Pseudomonas* to colonize a variety of habitats reside on their capacity to adhere to solid particle, their motility and their metabolic versatility which allows them to use many natural and xenobiotic compounds such as C-, N-, S- and P- sources (Dawson and Chang, 1992; Ramos *et al.*, 1994; Molina *et al.*, 2000).

Although employing indigenous microorganisms and plants to treat contaminated soils often requires the identification of suitable pre-existing natural systems the bacteria known to colonize specific plants may be engineered to gain more control over the process (Yee *et al.*, 1998). Root colonizing bacteria that is genetically engineered to degrade pollutants may also retain their competitive advantage in the rhizosphere compared with the indigenous non-root colonizing bacteria (Ma *et al.*, 2001).

The potential of certain *Pseudomonas putida* species to promote plant growth or suppress plant pathogens has been the subject of many studies (Thomashow and Weller, 1988; Glandorf *et al.*, 1992; Yee *et al.*, 1998). For these *Pseudomonas* species to be effective as biological control agents, their efficient colonization of the rhizosphere is a prerequisite, however, mechanisms of root colonization have yet to be elucidated. The objectives of this study are to determine the ability of *P. putida* to colonize and survive on the rhizosphere of *Eleusine corocana* in different conditions, and also to determine the effect of aromatic compounds on *P. putida* colonization.

## **5.2 Materials and Method**

### **5.2.1. Bacteria**

*Pseudomonas putida* was cultured from a contaminated rhizosphere soil collected in sterile bags from a historically contaminated Secunda site (in Mpumalanga Province, SA). The plate count method using tryptic soy agar was used, and the bacteria was identified using API 20NE as described by the manufacture (API systems, Biomerieux, France). It was confirmed as Polyaromatic hydrocarbons (PAH'S) degrader by growing it in bacteriological agar embedded with naphthalene, 1% cyclohexamine, to inhibit fungal growth and was incubated at 27<sup>0</sup>C for 7 d. The purity of *P. putida* was confirmed by culturing it on Pseudomonas base agar embedded with glycerol and SR103 (sigma) at 27<sup>0</sup>C for 24 h. Isolates were transferred to 2ml of liquid bacteriological medium containing SR103 and glycerol for 72 h at 27<sup>0</sup>C. After two days, 5ml of glycerol and one vial of SR103 were added to avoid the depletion of food for the bacteria. The optical density (OD) was measured until the concentration of approximately 3.0 X 10<sup>6</sup> cfu/ml and 2.5 OD was obtained for the inoculation of each treatment.

A single type of bacterium (a pure culture) was chosen in the study to avoid any complications from synergistic or antagonistic effects.

### **5.2.2. Seed Inoculation and plant growth Conditions**

*E. corocana* seeds were purchased from Agricol Ltd (Pretoria, SA). Seeds were surface sterilized by immersion in 95% ethanol for 5 min, washed 3 times in sterile deionised water, then soaked in 1.5% sterile sodium hypochlorite for 10 minutes, and then thoroughly rinsed with sterile distilled water. Since the seeds of the grass were frequently observed to be infected after germination (Glandorf *et al.*, 1992), sterilized seeds were germinated by incubating them on wet sterilized sponges for 48h at room temperature. The sponges were immersed in 0.3% tryptic soy broth (Difco) to detect contamination of the seeds (Glandorf *et al.*, 1992). The sponges were sterilized by soaking them in 3% sodium hypochlorite solution for 30 min, rinsed, and individually autoclaved in foil covered beaker. Non-infected germinated seeds were selected and aseptically immersed in 15ml of bacterial culture in a sterile petri dish under lamina flow hood for 4h and allowed to dry.

### **5.2.3. Soil and planting conditions**

Root colonization of *E. corocana* was studied in the sandy loam soil collected from the CSIR campus (Pretoria, SA). The soil was sieved through 4mm sieve and exposed to the sun for 4 d. 600g of this soil was added to 10cm x 5cm sterilized plastic pots. Sterilized germinated seeds were immersed in 5ml of pure culture under fume hood. Germinated seeds with pure culture were planted using sterile forceps and the pure culture liquid was poured directly in the sterilized pots containing 600g of soil. 475ml of sterilized tap water was added to the pots. The pots were placed in a green house and watered with 240 ml of sterile tap water every 24h. The treatments used in the experiment are shown in (Table 5.1).

**Table 5.1 Treatments used in the experiment**

Treatments	<i>P. putida</i>	Naphthalene	<i>E.corocana</i>	Nutrients
TR1	X	-	X	X
TR2	X	-	X	-
TR3	X	X	X	X
TR4	X	X	X	-
TR5	-	-	X	-

X=inclusion/addition; - = exclusion/not added

The treatments were prepared and analysed as done by Maila and Cloete, (2002) in triplicates. The soil was contaminated with 300ppm of naphthalene. Naphthalene was chosen because it is more cost effective compared to other PAH's and easily degradable by the strain isolated. Nutrients embedded were prepared as shown in (Table 5.1). Dilutions per litre in deionised water were mixed as follows: solution A- 10ml, B -3ml, C, D and E- 5ml. The bacteria were cultured as described above and the initial Colony Forming Units/g soils in each treatment were determined (Table 5.2).

**Table 5.2 Nutrients solutions used in the experiment**

Trace mineral solution	Compounds	Concentration (g/l)
A	Na <sub>2</sub> HPO <sub>4</sub>	10
	KH <sub>2</sub> PO <sub>4</sub>	10
B	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.5
C	MgSO <sub>4</sub>	0.4
D	CaCl <sub>2</sub> .2H <sub>2</sub> O	0.05
E	EDTA	0.0086
	FeSO <sub>4</sub> .7H <sub>2</sub> O	0.01
	ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.004
	MnSO <sub>4</sub> .H <sub>2</sub> O	0.01
	CuSO <sub>4</sub> .5H <sub>2</sub> O	0.0015
	Co(NO <sub>3</sub> ) <sub>2</sub> .6H <sub>2</sub> O	0.0008
	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> . 4H <sub>2</sub> O	0.0001

#### **5.2.4. Sampling and Bacterial Enumeration**

Samples were harvested by removing the roots with soil closely attached to it during day 7, 14 and 21, after planting. Individual plants in duplicate were sampled and after gently removing seedlings from the pots, roots were separated from the shoots. For each treatment one plant was for bacterial enumeration and the other was for Scanning Electronic Microscopy (SEM) analysis. To ensure contamination free cultures during sampling periods, un-inoculated control plants were sampled at each sampling time. Ten grams of rhizosphere soil (soil closely attached to the roots) with their roots were placed in 250ml Erlenmeyer flask and 100 ml of 0.2% of sodium pyrophosphate (pH 8.5) was added, the mixture was shaken for 30 minutes on a rotary shaker at 240 rpm. Rhizosphere soil suspensions were serially diluted (10-fold) in 0.85% sodium chloride. 0,1ml aliquots were spread on selective media with glycerol as a carbon source (Molina *et al.*, 2000). *P. putida* were plate counted and the bacterial population was considered as minimum population levels. The optical density (OD) of the bacteria was taken to compare with the bacterial count results. STATISTICA for Windows Release 5.1 was used for statistical analysis.

### **5.2.5. Bacterial Sampling for SEM**

Roots samples were obtained from five different treatments. The location chosen was about 1cm long from growing root tip described in Yang and Crowley, (2000). Samples were harvested once for each treatment in three weeks resulting in 15 total samples analysed. During the growth of new roots, exudates secreted in the zone of elongation behind the root tips support the growth of the primary root colonizers that utilize easily degradable sugars and organic acids (Yang and Crowley, 2000). Each root was collected with the adhering soil. Roots samples were placed in 1ml of liquid bacteriological medium containing SR103 and glycerol for 24hrs at 27<sup>0</sup>C in a petri dish.

### **5.2.6. Scanning Electron Microscopy (SEM)**

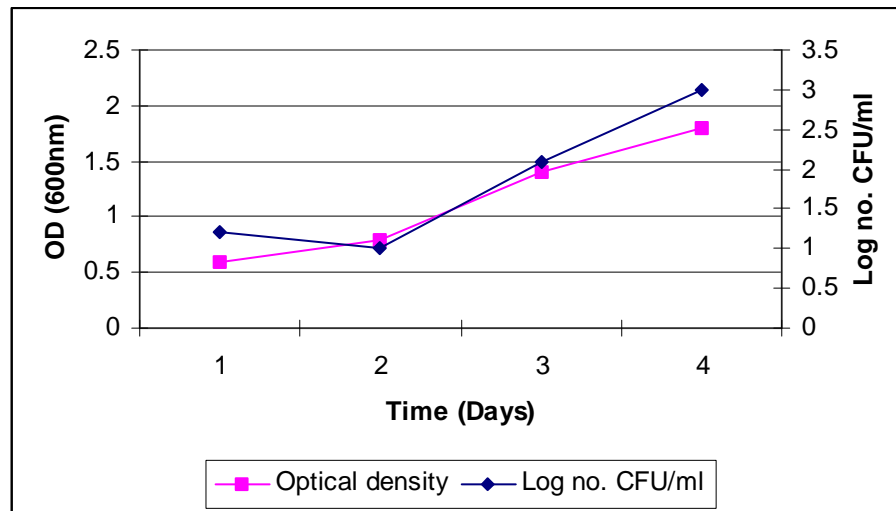
Root isolates were prepared for SEM observation as follows:

Two roots (1cm long) of fifteen samples were fixed in 2.5% glutaraldehyde (0.07M PO<sub>4</sub> buffer, pH 7.4) for 2h at room temperature. Samples were washed three times in the same buffer over 30 min and post fixed in 0.5% of aqueous osmium tetroxide for 2h. The fixed specimen were rinsed in distilled water and dehydrated through a graded ethanol series (30%, 50%, 70%, 90% and 100%) three times; critical point dried in liquid carbon dioxide. Dried specimens were mounted on aluminium specimen stubs with double sticky and sputter coated with gold. Samples were viewed under a JEOL-840 SEM at an acceleration voltage of 20-25kv.

## **5.3 Results and Discussion**

### **5.3.1. Colony Forming Units and OD Determination**

*Pseudomonas putida* was cultured from a historically hydrocarbon contaminated soil. Both the CFU and OD were determined before inoculating the soil microcosms (Figure 5.1). The number of *P.putida* and the OD increased with time of incubation (Figure 5.1). The OD was measured until the concentration of approximately 10<sup>6</sup> CFU/ml was obtained.



**Figure 5.1** Number of CFU/ml and OD value of *P. putida* before inoculation in the soil.

### 5.3.2. Pollutant Removal and Bacterial count

**Table 5.3** Naphthalene removal in soil (mg/kg soil; average  $\pm$ Std dev)

Treatment	Time (Weeks)			
	Week 0	Week 1	Week 2	Week 3
TR1	0	0	0	0
TR 2	0	0	0	0
TR 3	300 $\pm 0.77$	210 $\pm 1.0$	90 $\pm 0.33$	0
TR 4	300 $\pm 0.77$	250 $\pm 1.35$	120 $\pm 0.98$	20 $\pm 1.34$
TR 5	0	0	0	0

The removal of naphthalene in the TR3 treatment was higher than in the TR4 treatment (Table 5.3). The removal efficiency in the two treatments was 70% and 60% respectively after two weeks of incubation. This was attributed to the availability of the nutrients in the TR3 treatment compared to TR4. The biodegraders require a good balance of both the macro and micronutrients to remove the organics in soil. According to Genou *et al.* (1994) high hydrocarbon removal can be attained using the C:N:P ratio of 100:10:1. Naphthalene was

undetectable in the TR3 treatment while 7% of the pollutant was still in the soil in the TR4 treatment after three weeks of incubation (Table 5.3).





**Table 5.4 The bacterial count in different treatments**

Time (Weeks)	CFU/g soil
<b>Treatment 1 (TR1)</b>	
0	$2.54 \times 10^3$
1	$2.53 \times 10^2$
2	$5.90 \times 10^4$
3	$1.41 \times 10^7$
<b>Treatment 2 (TR2)</b>	
0	$2.80 \times 10^3$
1	$8.30 \times 10^3$
2	$2.01 \times 10^7$
3	$1.70 \times 10^8$
<b>Treatment 3 (TR3)</b>	
0	$2.80 \times 10^3$
1	$2.12 \times 10^1$
2	$8.71 \times 10^4$
3	$9.67 \times 10^6$
<b>Treatment 4 (TR4)</b>	
0	$2.73 \times 10^3$
1	$2.10 \times 10^3$
2	$1.84 \times 10^5$
3	$3.10 \times 10^7$
<b>Treatment 5 (TR5)</b>	
0	0
1	0
2	0
3	0

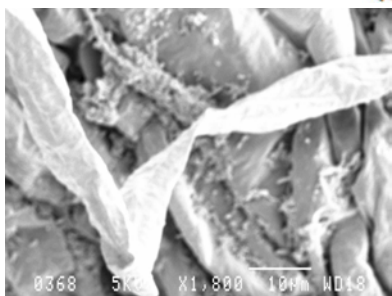
After planting the seedling in the pots, samples were taken to determine the number of *P. Putida* during day zero using plate counting technique. The results revealed that there was no significant difference between initial inoculation and after planting in the number of *P. Putida* in TR1, TR2, TR3, TR4 (Table 5.4).

The results of the bacterial count in different treatments revealed that it was feasible for *P. putida* to proliferate and survive in the roots of *E. corocana* in both contaminated and uncontaminated soil.

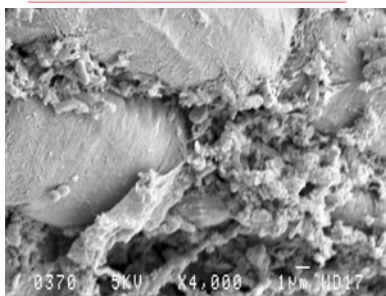
The counted viable cells/g soil after three weeks of inoculation in TR3, TR4, TR1 and TR2 indicated a growth from three to six, seven and eight Log no. CFU/g of soil respectively (Table 5.4). TR3 indicated the effect of naphthalene and nutrients on the proliferation of the bacteria with time. After the first week (Table 5.4) there was a die-off (from  $9.54 \times 10^3$  decreased to  $2.53 \times 10^2$ ) of some bacteria; but this changed as the number of bacteria started to improve in the second week (from  $2.53 \times 10^2$  increased to  $5.90 \times 10^4$ ) of incubation (Table 5.4). Unlike TR3, the number of *P. putida* in TR2 and TR4 increased from  $9.80 \times 10^3$  to  $1.70 \times 10^8$  and  $3.10 \times 10^7$  respectively. However, initially TR4 ( $9.80 \times 10^3$ ) had more number of bacteria than TR3 ( $9.54 \times 10^3$ ); both treatments received the same materials except that TR3 had additional nutrients. This shows that more bacteria in TR4 was not degrading PAH's as compared to TR3 in which 100% of PAH's had been removed as compared to 93% in TR4. The ability of microorganism to survive in the environment is defined as it's persistence to withstand the prevailing conditions; this was attributed to the ability of *P. putida* showing its ability to withstand the existing environmental conditions.

### **5.3.3 Scanning Electronic Microscopy**

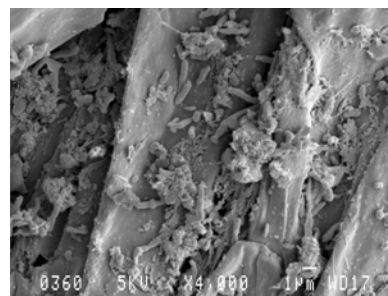
The changes in the number of *P.putida* in different treatments are shown in (Table 5.4).



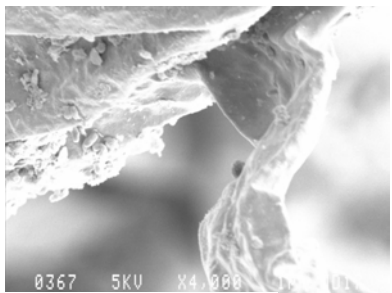
TR 1 (Week 1)



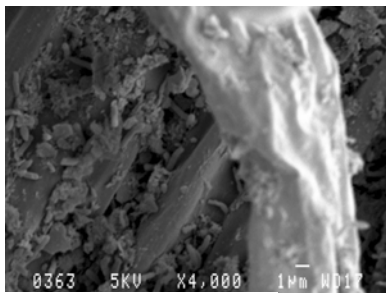
TR 1 (Week 2)



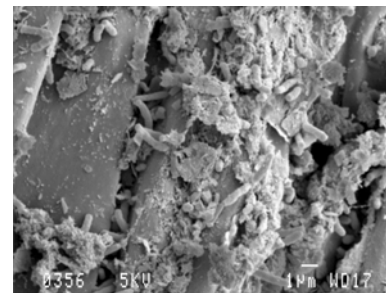
TR 1 (Week 3)



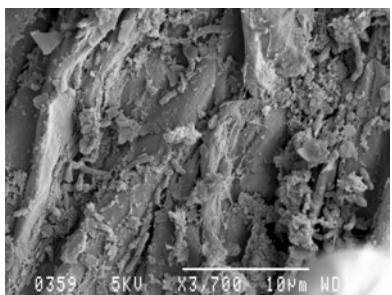
TR 2 (Week 1)



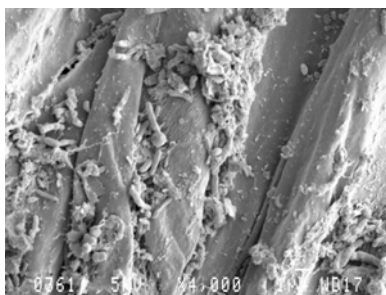
TR 2 (Week 2)



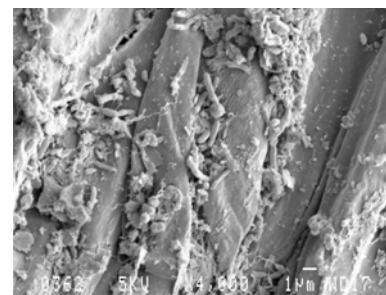
TR 2 (Week 3)



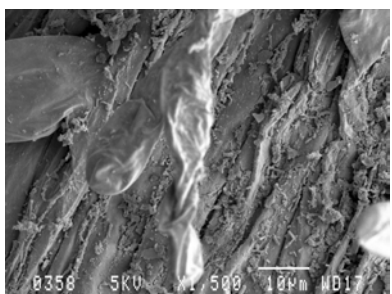
TR 3 (Week 1)



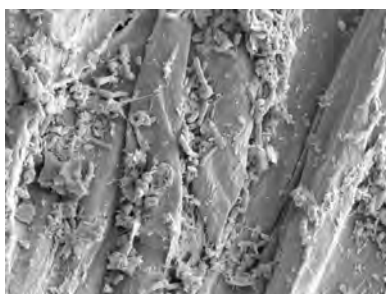
TR 3 (Week 2)



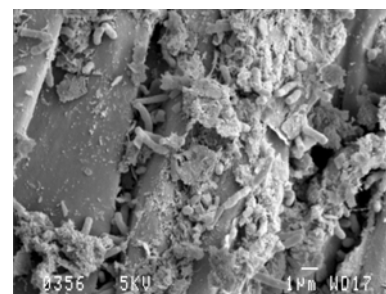
TR 3 (Week 3)



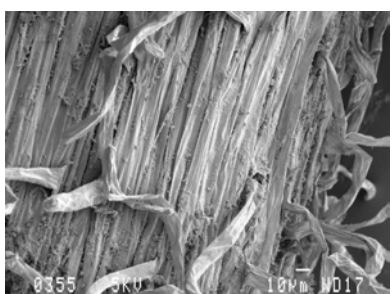
TR 4 (Week 1)



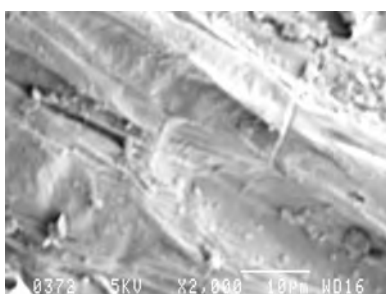
TR 4 (Week 2)



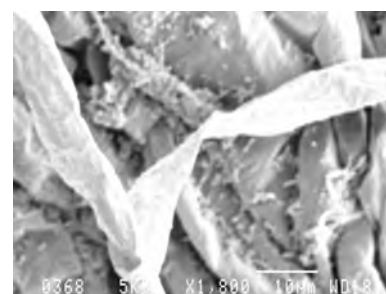
TR 4 (Week 3)



TR 5 (Week 1)



TR 5 (Week 2)



TR 5 (Week 3)

**Figure 5.4** Cell morphology of *P. putida* as revealed by the scanning electron microscope (SEM) in different treatments

In this study, *P. putida* was subjected to screening in order to ascertain its feasibility to colonize the roots of *E. corocana* plant in different conditions. SEM was useful in visualising the morphological structure of the bacteria that are able to colonize the roots of *E. corocana*. TR1 after one week revealed less attachment of bacteria to the root, but after two weeks more bacteria were attached to the rhizosphere. These results of SEM complimented that of colony count as the number of bacteria increased from  $2.53 \times 10^2$  to  $5.9 \times 10^4$  during the first two weeks. After week one, there was low root colonization of bacteria in TR2 compared to week two and three. In TR2, SEM did not complement the results of plate colony count (Table 5.4). There was a decrease in the number of bacteria from  $8.30 \times 10^3$  to  $2.01 \times 10^7$  and  $1.70 \times 10^8$  respectively (week 1 to 3). In TR3, there was a low colonization of bacteria as compared to week two and three, the root colonization increased with time. In this treatment, SEM complemented the findings of plate count method were the number of bacteria increased from  $2.12 \times 10^1$ , to  $9.67 \times 10^6$  during the first three weeks. TR4 had low root colonization after week one, but this has increased with time during the period of incubation.

Examination by SEM revealed that roots that were inoculated and embedded with nutrients and naphthalene had abundant colonized bacteria (TR3) than other treatments. These results corroborated those of rhizosphere enrichment cultures using plate count technique (Table 5.4). The extensive colonization of the rhizosphere was ascribed to the affinity of the bacteria to the exudates secreted by the plant and the prevailing conducive environment (addition of Naphthalene which was possibly used as a carbon source and nutrients) that is favourable to the growth of the bacteria. The result obtained in this study also demonstrated a distinct ability of *P. putida* to colonize and grow differently with time in different conditions (Figure 5.4).

#### **5.4 Conclusion**

The ability of *Pseudomonas* species to adhere to different surfaces is well known and documented. Therefore the results obtained using SEM and plate count methods scientifically justify the feasibility of *P. putida* to colonize rhizosphere in different conditions. This has also been observed with different strains of *Pseudomonas* species, (De Weger *et al.*, 1987; Simons *et al.*, 1996; Molina *et al.*,

1999). The inoculation of pollutant degrading bacteria on plant seed can be an important additive to improve the efficiency of rhizoremediation. The SEM results in (figure 5.4) supports the hypothesis that the root rhizosphere of some plant species provides an ecological niche that favours their survival and persistence in natural microbial community. Root colonizing bacteria have to be genetically engineered to degrade pollutants and should also retains their competitive advantage compared with the indigenous non-root colonizing bacteria. In summary this study demonstrates that inoculation of *P. putida* in the roots of *E. corocana* and able to colonize its rhizosphere is feasible. The bacteria survived and were capable of colonizing the rhizosphere of *E. corocana* and able to reduce naphthalene.

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## CHAPTER 6

### 6. Discussion and conclusion

The seeds *T. munita*, *E. corocana* and *B. serrata* were able to germinate in the presence of up to 300ppm of PAH's (Naphthalene and Fluorene) The germination level of *B. pilosa*, Smut grass and white buffalo grass was very low (10%) after three days of incubation, compared to that of *T. minuta*, *E. corocana* and *B. serrata* which was 55%, 45% and 42% respectively. However, germination of all the seeds increased with an increase in time. *E. corocana* and *B. serrata* germinated faster than *B. pilosa*, Smut grass and white buffalo grass and able to germinate as co-occurring plants when compared to other plants. *E. corocana* and *B. serrata* were chosen as model plants for the phytoremediation experiment because of their dense roots and tolerance to hydrocarbons.

More number of microorganisms was observed to be higher in TR3 than in all other treatments followed, by TR1, TR2, TR5 and TR4 respectively (Figure 3.2 - 3.5). It was also observed that plants had an effect on the growth of total recoverable heterotrophs (TRH) and hydrocarbon degrading bacteria (HDB). In TR3 initially, the number of TRH ( $4,0 \times 10^{10}$ ) was higher compared to HDB ( $3,0 \times 10^{10}$ ). After eight weeks of incubation the number of HDB ( $7,0 \times 10^4$ ) was higher than the number of TRH ( $6,0 \times 10^{10}$ ) in TR3. This could be due to the fact that, TR3 had an additional substrate (PAH's) that the HDB could use as a carbon and energy. Some bacteria could not tolerate the conditions in the beginning and most probably died. However with time microbes became acclimatized and could use the PAH's as a substrate to survive and grow.

More efficient removal of pollutants was observed on co-cultured as compared to mono-planted and unplanted treatments respectively. This was attributed the communal interaction of the plants with each other and the release exudates that enhanced bacterial growth, creating an environment for the biodegraders to remove the pollutants. The study is in line with the findings by April and Sims (1990) where there was an efficient removal of pollutants in co-cultured plants than in monoplanted. It has been hypothesized that root exudates may improve both the bioavailability and biodegradation of hydrophobic organic compounds such as



PAH's through the release of structural analogues for induction of enzymes in the microbial rhizosphere (Lynch and Whipps, 1990). The results of the current study revealed that plants had a positive effect on the growth of microorganisms. Planted treatments had more microorganisms present than the unplanted treatments due to the fact that plants release exudates that are rich in biodegradable macromolecules that stimulate microbial growth. Rhizoremediation is recognized as a suitable tool to restore contaminated sites (Kuiper *et al.*, 2003). The combination of using plants that have dense root system that can transport biodegraders deeply in contaminated soil and ability of communal of co-cultured plants can contribute in the restoration of polluted site.

The PCA did not revealed clear difference on substrate utilization profiles of the microbial communities in the rhizosphere and bulk soil. However, UEC was the only treatment that appears to be different to the other samples, which indicate that the microbial functional diversity of UEC is different from the other samples. Cluster analysis shows CBS to be different to the other samples (Figure 4.2). This could be attributed to biolog system put emphasis on functional diversity that cannot be absolutely related to species diversity. These suggest that the studies on community of analysis should be done using more than one technique due to the limitation of different method. However, there is relative similarity in clustering as revealed by biolog.

DGGE profiles of amplified 16S rDNA fragments from DNA extracted from rhizosphere and bulk soil bacterial fractions revealed differences in the DNA fingerprint of different samples (CEC, UEC, CBS, UBS, CS and US). The results had show that CEC has more diversity than UEC; CEC has 10 bands whereas UEC has 8 bands, and CEC has 3 thicker bands than UEC having only 1 thick band. The same phenomenon was observed in CBS (11) having more band than UBS (7). The thickness of the band indicates the high number of the species and the number of the bands represents functional diversity. However, unlike DGGE the biolog revealed a close functional diversity of all samples except that of UEC (PCA) and UBS (Cluster analysis). Vegetated soil (UEC, CEC, UBS and CBS) also revealed to have more bands than non-vegetated soil (US and CS). Vegetated soil had 8, 10, 9 and 11 bands whereas non-vegetated has 8 and 7 bands respectively. This was

ascribed to the roots of the plants that can act as a substitute for the tilling of soil to incorporate additives (nutrients) and to improve aeration to introduce oxygen into deeper soil layers through specialized root vessels, aerenchyma (Kuiper *et al.*, 2003).

Analysis of the microbial diversity using functional diversity (Biolog GN2) and genetic diversity (PCR-DGGE of 16S rDNA) did not complemented each other in revealing the differences in microbial communities in both the bulk and rhizosphere soils. This study suggests the use of multiple of techniques to analyse microbial community due to limitations on different methods.

*Pseudomonas putida*, a hydrocarbon biodegrader, was evaluated for its potential to colonise the roots of *Eleusine corocana* in both the hydrocarbon contaminated and uncontaminated soil. The influence of nutrient addition to the soil was also investigated. TR1 after one week reveals less attachment of bacteria to the root, but after two weeks more bacteria was attached to the rhizosphere. These results of SEM complimented that of colony count as it shows that after one week to two weeks of incubation grows from  $2.53 \times 10^2$  to  $5.9 \times 10^4$  respectively. TR2 after week one there was a low root colonization of bacteria as compared to week two and three. Unlike in TR1, here the SEM did not complement with the results of colony count (Table 5.4). In TR2 PCA shows a decrease in number of bacteria from  $8.30 \times 10^3$  to  $2.01 \times 10^7$  and  $1.70 \times 10^8$  respectively. In TR3 there was a low colonization of bacteria as compared to week two and three, the root colonization increase with time. In this treatment SEM had compliment the findings of PCA were number of bacteria increases from  $2.12 \times 10^1$ ,  $8.71 \times 10^4$  and  $9.67 \times 10^6$  respectively. TR4 has low root colonization after week one, which had increased with time after week two and three. PCA results also revealed the same pattern of bacterial counts increasing with time from week one, two and three ( $2.10 \times 10^3$ ,  $84 \times 10^5$  and  $3.10 \times 10^7$  respectively). The extensive colonization of the rhizosphere was ascribed to the affinity of the bacteria to the exudates secreted by the plant and the prevailing conducive environment (addition of Naphthalene which was possibly used as a carbon source and nutrients) that is favourable to the growth of the bacteria. The result obtained in this study also demonstrated a distinct ability of *P. putida* to colonize and grow differently with time in different conditions.

Scanning Electronic Microscopy (SEM) and plate count method was useful in visualising the morphological structure and number of bacteria that are able to colonize the roots of *E. corocana*.

While this study showed the potential of seeding a root colonizing PAH's degrader to contaminated soil, more studies are required using molecular approaches as well as the more persistent organic pollutant to complement the existing results. Also, long-term studies should be considered to understand the impact of seeding the root colonizing PAH's degrader on the existing microbial populations.

In conclusion, plants have a large effect on the dissipation of pollutants in the environment. The feasibility of co-occurring of different species to grow, survive and improve the interaction of microbes associated with the roots and contaminated soil indicated an efficient removal of PAH's in the environment. Analysis of microbial diversity using functional diversity (Biolog GN2) and genetic diversity polymerase chain reaction –denaturing gradient electrophoresis (PCR-DGGE) of 16 S rDNA revealed the non-cultivated functional and structural population diversity, showing a distinct efficiency of roots exudates of particular plant on the diversity of microbes.

The results obtained in this study (using SEM and culture based methods) suggest that it is feasible to add biodegraders that are root colonisers in the soil to improve rhizoremediation. The inoculation of pollutant degrading bacteria on plant seed can be an important additive to improve the efficiency of rhizoremediation. Root colonizing bacteria can be modified genetically to improve their pollutant removal capacity and then added to contaminated soil to aid phytoremediation. However, the modified organisms should also retain their competitive advantage compared with the indigenous non-root colonizing bacteria.

From the findings of the study it seems to be reasonable to conclude that, rhizoremediation is a potential technology that is environmentally friendly, less expensive to remediate polluted environment. It also needs further investigations on the plants that have the feasibility to grow and survive in different geographical areas and still perform effectively.