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IMPACT OF TROPICAL PLANTS ON MICROBIAL ACTIVITY AND DIVERSITY IN SOIL CONTAMINATED WITH PETROLEUM HYDROCARBONS

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ABSTRACT

The effect of plants (milo, oleander and buffelgrass) and a hexadecane and phenanthrene mixture (1 g and 200 mg/kg soil, respectively) on the diversity and activity of hydrocarbon-degrading bacteria in a sandy coastal soil was investigated. Two/third of hexadecane was degraded after 56 days. Hydrocarbon depletion was not plant-enhanced but was retarded slightly by milo and buffelgrass. Lipase activity, an alkane-metabolism indicator, increased during rapid hexadecane depletion (days 0-56). The diversity of the dominant hexadecane-degrading bacteria was based on partial sequencing of 16S rDNA. On day 0, mainly *Alphaproteobacteria* were found. By day 56, *Gammaproteobacteria* dominated the contaminated samples whereas similar numbers of *Alphaproteobacteria* and *Gammaproteobacteria* genotypes dominated the uncontaminated samples. *Alcanivorax* was found in all contaminated samples except for buffelgrass rhizospheres, which harbored only *Pseudomonas* sp. IMT40. With little hexadecane left by day 114, similar abundances of *Alphaproteobacteria* and *Gammaproteobacteria* genotypes occurred in all samples. *Alcanivorax* had virtually disappeared.

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CHAPTER 1

INTRODUCTION

1.1 Environmental pollution problem

A consequence of industrialization is the contamination of soil and watersheds with various pollutants. The US has one of the most widespread contamination problems in the world, but it is also the leader in defining and implementing standards for remediation processes.

In a recent report by the US Environmental Protection Agency (EPA), it was estimated that 294,000 sites will be in need of remediation within thirty years at an average cost per site of $209 billion (EPA, 2004a). Currently there are 57,000 sites in federal facilities with potential contamination problems (EPA, 2006). Military bases often have multiple contamination problems that are difficult to remedy (NRC, 2003).

Under environmental law, the EPA can collect taxes from industries to fund cleanup of some contaminated sites. In the 2004 fiscal year, the EPA collected $4.8 billion to use in environmental cleanup and projected 1 billion pounds of contaminants to be reduced,

treated or properly managed (EPA, 2004b). There are currently 1,238 sites nationwide that require long-term remedial actions and financial help from the government.

Some of the most common chemicals found in contaminated sites are petroleum hydrocarbons from ruptured underground storage tanks (Schwab and Banks, 1994; ATSDR, 1999). Petroleum hydrocarbon contamination, even though not considered of high-risk to human-health or difficult to remediate, continues to be of great concern given the number of sites affected. Currently 200,000 sites among the 500,000 land areas that cannot be redeveloped are impacted by petroleum leakage (EPA, 2004a).

1.2 Composition of Petroleum Hydrocarbons

Petroleum and petroleum products are complex mixtures of hydrocarbons (compounds consisting of carbon and hydrogen atoms), other organic compounds, and relatively small concentrations of metallic constituents (Van Hamme et al., 2003). Refined petroleum products contain a combination of these compounds in various amounts. Hydrocarbons (HC) comprise the majority of the components in most petroleum products and can be grouped into saturated hydrocarbons, unsaturated hydrocarbons, and aromatics (ASTDR, 1999; Weisman, 2003).

Petroleum and most petroleum products are primarily composed of saturated hydrocarbons. These are comprised of single C-C bonds arranged as straight chains,

(alkanes), branched chains (isoalkanes) and cyclic compounds (cycloalkanes). Diesel fuel is a commonly used refined petroleum product that contains 30% straight and branched alkanes (Weisman, 2003). A model straight-chain alkane present in crude oil and major component in diesel #2 fuel is hexadecane ($C_{16}H_{34}$) (ATSDR, 1999).

Aromatic compounds are also major components of diesel fuel. These are based on a ring structure of six carbons (benzene). Two or more rings fused together result in polycyclic aromatic hydrocarbons (PAHs). PAHs are present in crude oil and most petroleum products. They can remain in the environment for long periods of time and can be a major risk to human health (Weisman, 2003; NRC, 2003). Phenanthrene, a PAH comprising three rings is a common example in petroleum hydrocarbon studies (Adachi et al., 1999).

1.3 Bioremediation

Many sites are cleaned each year by using a variety of physical (e.g. incineration, thermal desorption), chemical (e.g., reduction/oxidation processes) and/or biological methods. The methods vary in cost and effectiveness, and they can be either *in-situ* or *ex-situ* technologies. *In-situ* applications involve the treatment of the affected area in place without removal, while *ex-situ* techniques require prior excavation. *In-situ* techniques are usually more economical than *ex-situ* techniques, but they have the disadvantage of requiring more time and they are affected by the heterogeneity of the location (e.g.

hydraulic conductivity and contaminant distribution) (Blackburn and Hafter, 1993). There is also less certainty of the complete removal of the contaminant, given the difficulty of verifying the effectiveness of the treatment. *Ex-situ* techniques have other disadvantages besides high costs. The excavation and removal process presents the risk of further human exposure to the contaminant (NRC, 2003). Physicochemical treatments also destroy the soil structure thus leaving it biologically inactive (McGrath et. al., 2001). It is common to employ a combination of both *in-situ* and *ex-situ* techniques to treat an affected site. Ideally, remediation technologies should be of relatively low cost, address large surface areas, and deal with a wide range of contaminants (Blackburn and Hafter, 1993; Cunningham et al., 1996).

Bioremediation is one of the primary technologies for both *in situ* and *ex situ* soil treatment used by the EPA (Weisman, 2003). This technique involves the use of microorganisms, either naturally or commercially available, to breakdown the contaminants (Nichols et al., 1997). Bioremediation is especially useful for petroleum hydrocarbon contamination, because it provides a complex environment that favors the growth of a consortium of microorganisms (van Hamme et al., 2003), many of which are able to degrade one or more of the multiple components in petroleum products. Bioremediation allows large contaminated areas to be treated at a relatively low cost and low maintenance, and has become a more accepted technique in recent decades.

Among the different types of bioremediation, three of the most commonly used are: natural attenuation, biostimulation, and bioaugmentation. In natural attenuation, treatment

areas are only monitored to determine whether the contaminant is being degraded. Biostimulation involves the optimization of the area (e.g. by adding water and nutrients) in order to encourage the naturally occurring microbial population to degrade the contaminants. Maintaining appropriate oxygen levels and nutrients concentration speeds up the remediation process (Kaplan and Kitts, 2004; Chaîneau et al., 2005). There are some instances where even with biostimulation the degradations levels remain low due to low numbers of microbial degraders or to the complexity of the contaminant. In these cases, bioaugmentation can be a useful technique. This type of bioremediation introduces a specific consortium of microorganisms to improve the capacity of contaminant removal (Gentry et al., 2004; El Fantroussi and Agathos, 2005). However, bioaugmentation is not recommended for soils contaminated with petroleum hydrocarbons since there are many microorganisms that naturally utilize petroleum hydrocarbons as a source of carbon. Furthermore, competition with indigenous microbial populations in soil for oxygen and nutrients (Crowley et al., 1997) makes it difficult for the introduced microorganisms to thrive (Gentry et al., 2004). A combination of the techniques may be a possibility in some cases (El Fantroussi and Agathos, 2005). To increase the survival of the introduced microorganisms, Gentry et al. (2004) discuss the development of methods including the introduction of remediation genes through horizontal gene transfer, of enzymes purified or encapsulated in dead microbial cells, and the application of genetically engineered microbes. Nevertheless, these are possibilities that must be studied further before their application to the environment (Top et al., 2002).

The techniques of natural attenuation, biostimulation and bioaugmentation, were compared in a study by Bento *et al.* (2005), which highlights the difficulty of developing efficient bioremediation strategies. Total petroleum hydrocarbon (TPH) degradation was monitored in soils from Long Beach, California (USA) and Hong Kong (China). They determined that for each soil, a different technique was effective. The Hong Kong soil (pH 7.7 and organic carbon concentration of 38.1 g/kg) had better degradation rates with natural attenuation, while the Long Beach soil (pH 6.3 and organic carbon concentration of 15.1 g/kg) did better when a microbial consortium was introduced.

The complexity of the soil environment doesn't allow for an easy determination of the proper technique to use in the remedial process. Detailed studies of the qualities of the particular soil matrix, of the contaminant and of the microbial populations in that soil, need to be conducted for each case in order to design a proper remediation plan (Atlas and Cerniglia, 1995).

1.4 Phytoremediation

In some cases, the bioremediation process has been further enhanced by the use of green plants to help remove, contain or render harmless the environmental pollutants, a technique called phytoremediation (Anderson et al., 1993; Shaw and Banks, 1994; Cunningham et al., 1996). An important benefit of using plants in bioremediation is that plant cover reduces wind and water erosion, reducing the spreading of the contaminated

soil (Joner et al., 2001). Microorganisms degrading petroleum hydrocarbon compounds require oxygen to most efficiently conduct the breakdown. Diatomic oxygen concentrations tend to decrease with increasing depth in soil, and ultimately its low availability may limit heterotrophic activity (Crowley et al., 1997). Plants remove excess water from soil through continuous transpiration, allowing access to air in the roots area and therefore reducing limitations in biodegradation.

In the zone of soil around plants roots, or rhizosphere, microorganisms are presented with a relatively high concentration of nutrients by rhizodeposition, a combination of compounds from the lysis of dead root cells and exudates released by the roots (Anderson et al., 1993; Schwab and Banks, 1994; Shann and Boyle, 1994; Cunningham et al., 1996; Singer et al., 2003). The rhizosphere has been shown to be a zone of increased microbial activity, and the number of microorganisms in rhizosphere soil versus the number of microorganisms in bulk soil can be expressed quantitatively as the R/S ratio. This ratio can be several orders of magnitude higher in the rhizosphere; this is the rhizosphere effect (Anderson et al., 1993; Cunningham et al., 1996).

Many studies have been conducted using different plants in various environments to gain a better understanding of rhizosphere-enhanced biodegradation processes. A recent study on enhanced rhizosphere degradation with perennial ryegrass and alfalfa found an increased number of rhizosphere bacteria in the hydrocarbon-contaminated soil and of bacteria capable of petroleum degradation (Kirk et al., 2005).

The catabolic enzymes of hydrocarbon degrading microorganisms are possibly induced by the presence of other carbon sources (Egli, 2002) in the rhizodeposits and by analogs (e.g. phenolics, waxes, etc.) secreted by the plant (Singer *et al.*, 2003; Shaw and Burns, 2005). The plant may also increase the availability of the pollutants by producing biosurfactants, which further enhance the hydrocarbon biodegradation process (Anderson *et al.*, 1993).

There are, however, many limitations to phytoremediation. In some cases, rhizodeposition may potentially impair biodegradation of organic contaminants if degrading microorganisms preferably use the rhizodeposits over the contaminant (Crowley et al., 1997). Moreover, the production of exudates is known to be accompanied by mineral nutrient depletion in the rhizosphere, increasing competition for nutrients between degrading and non-degrading bacteria (Corgié et al., 2003). The positive effect of the rhizosphere in biodegradation has also been found to be dependent on the plant species and the age of the plant (Shann and Boyle, 1994; Huang et al., 2004; Sun et al., 2004; Shaw and Burns, 2005). Limitations also include age of the contamination and depth at which the pollutant is located, among other factors (Anderson et al., 1993; Cunningham et al., 1996; Hutchinson et al., 2001; Tang et al., 2004). Continuing studies in phytoremediation will help understand the complexity of the process for its more efficient application to contaminated environments.

Even though many microorganisms are able to metabolize some petroleum hydrocarbons, not all have the initial enzymes necessary to breakdown the compounds

(Corgié *et al.*, 2003). Some microorganisms growing on carbon sources from rhizodeposits may transform hydrocarbon contaminants by co-metabolic processes, i. e., the microbe does not gain energy from the changed compound (Horvarth, 1972; Anderson *et al.*, 1993; Cunningham *et al.*, 1996). Microbial consortia are more efficient in the remediation of recalcitrant organic pollutants (Cunningham et al., 1996). The following are genera known to degrade hydrocarbons (Atlas and Cerniglia, 1995):

Achromobacter
Acinetobacter
Aeromonas
Alcaligenes
Arthrobacter and other coryneforms
Bacillus
Corynebacterium
Erwinia
Flavobacterium
Klebsiella
Lactobacillus
Leucothrix
Micrococcus
Moraxella
Mycobacterium
Nocardia
Peptococcus
Pseudomonas
Sarcina
Sphaerotilus
Spirillum
Streptomyces
Vibrio
Xanthomonas

The selection of plants to use in phytoremediation should not only take into account their resistance to potentially toxic levels of the contaminant and environmental stress (Huang et al., 2004), but also the possible associations that the plants have with microorganisms. Microorganisms may develop specific root-colonizing relationships with the plant that can help protect it (Anderson et al., 1993; Basil et al., 2004) from toxic compounds and even to increase its root biomass in the presence of the pollutant (Huang et al., 2004). Though the number of microorganisms may be the same in the rhizosphere of different plants, a higher degradation potential may accompany some associations (Jones et al., 2004). Our knowledge of the types of bacteria associated with a particular plant species in a specific environment will help us not only to select the plants for tolerance to the contaminant and environmental stresses, but also to select plants that harbor a more efficient degrader population.

1.5 Soil lipase activity as an indicator of hydrocarbon degradation and overall activity of hydrocarbon-degrading microorganisms

1.5.1 Use of soil enzymes to determine microbial activity

In a previous experiment in this soil from the Hickam Air Force site (Jones et al., 2004), reported that high numbers of hydrocarbon-degrading microorganisms in planted and unplanted soil were not always indicative of the microbes' ability to degrade

petroleum hydrocarbons. Thus, measurement of a suitable enzyme activity might be a better indicator of microbial degradative performance.

Dehydrogenase activity, which is associated with live cells and provides an assessment of microbial respiration, has been widely used to determine microbial activity in soil (Frankenberger and Johanson, 1982; Alef and Nannipierri, 1995; Tate, 1995; Margesin et al., 2000b; Maila and Cloete, 2005). However, the sensitivity of the dehydrogenase-activity assay may not be sufficient to detect differences between contaminated and uncontaminated treatments when the level of contaminant is low (Robert, 1996).

A more interesting enzyme in the context of petroleum-hydrocarbon bioremediation evaluation may be lipase, which was proposed by Margesin et al. (1999, 2000a) as a means of monitoring hydrocarbon degradation in the field. Lipase production by hydrocarbon-degraders was negatively correlated with the disappearance of hydrocarbons from soil and positively correlated with the evolution of carbon dioxide from the contaminated soil (Margesin and Schinner, 2001). Lipase activity in soil was associated with the presence of petroleum hydrocarbons, the activity being very low in uncontaminated soil (Margesin and Schinner, 2001). Petroleum hydrocarbons that contain alkanes seem to elicit lipase activity. Diesel fuel, which consists of a high proportion of alkanes, produced lipase activity when soil was contaminated with 5,000 or 10,000 mg kg^{-1}. No lipase activity was detected when the soil was contaminated with a

mixture of PAH (500 mg of phenanthrene and 500 mg of naphthalene/kg of soil) (Margesin et al. 1999; 2000a).

1.5.2 Role of lipase in microorganisms

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) hydrolyze of triacylglycerols to glycerol and free fatty acids, or a mixture of the latter and diacylglycerols and monoacylglycerols (Jaeger et al., 1994; Snellman et al. 2002; Gupta et al., 2004; Ko et al., 2005). In nature, microorganisms produce extracellular lipases to hydrolyze fats and oils in organic matter to fatty acids and glycerol prior to absorption by the cell. Most of the studies of microbial lipases indicate that a high proportion of the microbial lipases are extracellular (Breuil et al., 1978; Jaeger et al., 1994; Saxena et al., 1999; Kanwar et al., 2002) or cell-bound (Breuil et al., 1978). Some lipases are produced intracellularly. Breuil et al. (1978) found small amounts in *Acinetobacter lwoffi* whereas Chen et al. (1994) reported various amounts of intra- vs. extracellular lipase in a yeast, depending on the types of alkanes used for growth. The ratio of extracellular lipase over total lipase for the yeast was 0.56, 0.33, and 0.32 for kerosene, gas oil, and liquid paraffin, respectively.

Unlike other enzymes, lipases are hydrophobic (Gupta et al., 2004) and act at the oil-water interphase (Harwood, 1989; Shabtai and Daya-Mishne, 1992). They are stable in organic solvents and can function at high temperatures (70°C) in industrial processes (Harwood, 1989; Gupta et al., 2004).

1.5.3 Uptake and metabolism of alkanes

It is generally accepted that hydrocarbon-degrading microbes either attach to insoluble hydrocarbon droplets, as seen in *Acinetobacter calcoaceticus* RAG-1---which produces fimbriae for attachment (Rosenberg et al., 1982), or produce emulsifying compounds to promote hydrocarbon solubilization and uptake (Leahi et al., 2003).

Britton (1984) reported the various phases involved in the metabolism of alkanes by *Pseudomonas putida* (formerly *Ps. oleovorans*) occurred as follows: the alkane reaches the lipid bilayer of the cytoplasmic membrane where it is transformed into an alcohol by a hydroxylase. After lateral diffusion through the membrane, the alcohol reacts with an alcohol dehydrogenase and is oxidized to an aldehyde. The aldehyde is subsequently oxidized to a fatty acid (FA) either in the membrane or the cytoplasm. The β-oxidation of the FA occurs in the cytoplasm.

Alvarez (2003) showed that *Actinobacteria* such as *Rhodococcus*, *Nocardia*, *Gordonia*, and *Dietzia*, grown on alkane hydrocarbons under N-limiting conditions, produce triacylglycerols (TAG) for storage. In this cases, alkanes were oxidized to fatty acids (FA), which were broken down by β-oxidation to acetyl CoA in order to synthesize the FA portion of the TAG *de novo*, or the alkanes of adequate length were oxidized to FA (e.g. palmitic acid, 16 C) and directly incorporated into TAG.

Conversely, Leahi et al. (2003) reported that under high nutrient conditions (i.e. of high N, P, S) two *Acinetobacter* grown on hexadecane (16 C) produced membrane-bound vesicles, originating from the outer membrane. They suggested hexadecane-filled vesicles were taken inside the cell to form inclusions of storage material. They called this process a "luxury consumption "of hexadecane.

1.5.4 Link between growth on alkane-type of hydrocarbons and increased lipase activity in microorganisms

Formation of lipase by microorganisms growing on hydrocarbons as sole source of carbon has been reported in several studies, although hydrocarbons are not a substrate for lipase. Takahashi et al. (1963) showed that all the filamentous fungi and yeasts they tested produced extracellular lipase when growing on kerosene. Chen et al. (1994) noted the formation of various amounts of intra- and extracellular lipase in the yeast *Trichosporon fermentans* WU-C12 growing on 3 petroleum hydrocarbons. Addition of surfactants, which also contain a long chain of alkane, enhanced the production of lipase in most cases. Breuil et al. (1978) convincingly demonstrated that growth on alkanes had to occur for lipase production to take place in the bacterium *Acinetobacter lwoffi* grown on defined medium. They showed that during growth on hexadecane, cell-bound and exocellular lipases in the supernatant were induced whereas very little intracellular lipase was formed. When *A. lwoffi* and *Pseudomonas aeruginosa* were grown on a mixture of

ethanol and hexadecane, diauxic growth (i.e., when one substrate interferes with the utilization of the second substrate) resulted. All the ethanol was consumed before growth on hexadecane began. Lipase induction started with growth on hexadecane. These authors also tested 56 strains of various genera for growth on hexadecane and production of lipase and concluded that all bacteria capable of growing on hexadecane produced lipase. None of the bacteria that did not produce lipase could grow on hexadecane. Presumably a test for lipase production (Ko et al., 2005) could be used to screen microbes for potential alkane degraders. However, not all lipolytic microbes are alkane degraders (Breuil et al., 1978).

It is not clearly understood why lipase is produced when microbes are growing on alkanes and other compounds such as fatty acids or when non-hydrolysable detergents are present in the medium (Breuil et al., 1978; Shabtai and Daya-Mishne, 1992; Hooker et al., 1997; Kanwar et al. 2002; Gupta et al., 2004). Fatty acids are the product of lipase activity on fats and oils and would be expected to act as catabolic repressors. Indeed, they have been shown to repress lipase formation in some bacteria such as *Ps. aeruginosa* and *Acinetobacter calcoaceticus* (Gupta et al., 2004). However most studies indicate that alkanes, triacylglycerols, hydrolysable esters, fatty acids, tween detergents, bile salts and glycerol act as lipase inducers (Breuil et al., 1978; Shabtai and Daya-Misne, 1992; Hooker et al., 1997; Gupta et al., 2004).

Since alkanes are metabolized via the formation of fatty acids in the cell and fatty acids are well known to be inducers of lipase, it is possible that the fatty acids derived

from alkanes are the actual inducers of lipase (Dr. Tung Hoang, personnel communication). We hypothesize that because lipase is the extracellular enzyme that normally enables the cell to be provided with the hydrolysis products of fats and oils in the environment, fatty acids will be viewed by the cell as derived from extracellular lipase activity, and thus will act as inducers of lipase formation. Thus, it seems reasonable to expect that lipase will continue to accumulate in the environment until most of the alkane inducer, which supports alkane-degraders' growth, is degraded. Other authors (Hooker et al., 1997) have proposed that lipase induction be due to adsorption of lipase to emulsified drops of oily material and continuous removal of the lipase from the cell wall. No matter the process, lipase would be produced until low concentrations of alkanes are reached.

1.5.5 Soil lipase activity as an indicator of the progress of bioremediation of petroleum hydrocarbon-contaminated soils

Margesin et al. (1999; 2000b) were the first to evaluate the potential of lipase activity measurements to monitor petroleum-hydrocarbon degradation in the processes of natural attenuation and engineered bioremediation where nutrients (N-P-K) were not added or added, respectively. Lipase activity increased markedly during the first 30 days of the experiment, while the concentration of diesel contaminant decreased rapidly in the fertilized treatment. In the non-fertilized treatment, the lipase activity increased slowly while the concentration of diesel decreased slowly over a period of 120 days. The degradation of hydrocarbons shows a quasi-first order reaction where the rate of disappearance of the contaminant is dependent on its concentration. Thus the increase in lipase activity was a good indicator of the decrease in diesel concentration that took place during the bioremediation process. Ideally, when the degradation process comes to an end, enzyme production by microorganisms should slow down, thus indicating the end of the reaction. In this case, high lipase activity was maintained from day 30 to the end of the experiment (120 days) in the fertilized treatment. This probably reflects the tendency of enzymes released in soil to become adsorbed to soil particles, such as clays, and to soil organic matter, such as humus (Tabatabai, 1994). Once adsorbed, the enzymes become more recalcitrant to degradation by microorganisms and their activity may persist beyond the time the hydrocarbons have been degraded. This is a drawback of all soil enzymes whose activities do not depend on a live cell. Different soils will have different

proportions of sand, silt and clay and different amounts of organic matter. Therefore the behavior of the lipases will depend on the soil used in the experiment. This is shown in a soil from an Alpine area where lipase activity peaked at the beginning of the experiment and neared zero after approximately one year (Margesin and Schinner, 2001).

The advantage of the lipase activity measurement over another enzyme activity such as dehydrogenase and fluorescein diacetate hydrolysis (Margesin et al., 2000a) is that the lipase is induced by growth on alkane-type hydrocarbons. Thus, increases in lipase activity reflect the activity of microorganisms directly involved in the degradation of the contaminant (see section 1.5.4). On the other hand, dehydrogenase activity and fluorescein diacetate hydrolysis reflect the general activity of soil microbes.

1.6 Assessing microbial diversity using DNA-based techniques

1.6.1 PCR amplification of the small subunit of rRNA genes

Since first proposed, the use of the small subunits of the ribosomal RNA molecules to study phylogenetic relationships among microorganisms has become common practice in microbiology (Lane et al., 1985; Olsen and Woese, 1993; Olsen et al., 1994; Pace, 1996). This approach has enabled the determination of microbial community composition in a

variety of environments (e. g. Nüsslein and Tiedje, 1999; Nakatsu et al., 2000; Kuske et al., 2002; Schmalenberger and Tebbe, 2002; Koizumi et al., 2003).

The molecular tools available continue to improve and new techniques are frequently being developed. However, it must be conceded that these tools have weaknesses and biases that can shape our current assumptions (Forney et al., 2004). From the extraction of DNA to the sequence analysis (Ashelford et al., 2005), one must remember to keep in mind the limitations of each system during the interpretation of the results (Schneegurt and Kulpa, 1998; Kirk et al. 2004; Forney et al., 2004). Universal eubacterial primers have been designed from available sequences and they do not target all bacteria (Forney et al., 2004). Nevertheless, molecular techniques using 16S rDNA sequences give us an overview of the microbial diversity.

Ercolini et al. (2001) compared culture-independent and culture-dependent methods to study the microbial populations occurring in natural whey cultures generally used as starters in the production of Mozzarella cheese in Southern Italy. The direct extraction of total DNA and subsequent amplification of the 16S rDNA V3 region by PCR coupled with denaturing gradient gel electrophoresis, revealed only four *Lactobacillus* spp. However, with the use of scrapings from plates two other *Lactobacillus* spp. were discovered. Their study highlights one of the disadvantages of direct DNA extraction with culture-independent methods, the selective amplification by PCR techniques (Reysenbach et al., 1992; Suzuki and Giovannoni, 1996). A similar study in soil by Ellis et al. (2003) confirms these findings. This is partially due to the fact that culturing gives

the opportunity to less dominant bacteria to grow and allows their detection by PCR techniques.

Both approaches have their limitations and might be a source of bias (Kirk et al. 2004), but when used in conjunction they can complement each other in the analysis of the results (Watanabe et al., 1998; Ercolini et al., 2001; Ellis et al. 2003; Joseph et al., 2003).

1.6.2 Denaturing gradient gel electrophoresis (DGGE) of the variable 16S rDNA regions

DGGE is an effective tool used to accurately separate PCR-amplified products according to their G+C content allowing the detection of even single-base substitutions (Muyzer et al., 1993; Schneegurt and Kulpa, 1998). Since its introduction into microbial ecology in 1993 (Muyzer et al., 1993), DGGE analysis has been applied to thousands of studies for different purposes in a variety of settings (Muyzer and Smalla, 1998) and it has proven useful in determining the bacterial diversity in complex environments (e.g., Webster et al. 2003; Wilms et al., 2006) from which isolation of all the associated microorganisms is difficult or impractical (Fromin et al., 2002). The use of the DGGE technique has also proven effective in monitoring changes in the community composition in an environmental setting (Randazzo et al. 2002; Crump et al., 2003; Throbäck et al., 2004).

In soil, Griffiths et al. (2000) employed DGGE for the analysis of 16S rRNA as an indicator of microbial activity. They described a direct method for co-extracting DNA and RNA from soil to compare the diversity using reverse transcription-PCR (RT-PCR) of 16S rRNA and PCR of 16S rDNA. The profiles indicated differences between the active bacterial community and the total diversity present in soil. Cocolin et al. (2001) also compared DNA and RNA profiles in a recent application of the DGGE technique to food microbiology, and distinguished microorganisms involved in the natural fermentation of Italian sausages, as well as the dominant *Lactobacillus* spp. responsible in the process.

Rhizospheres of various plants have also been studied. Normander and Prosser (2000) studied the community composition of a developing barley phytosphere and found differences in the bacterial community depending on the different habitats of the root zone. More recently, Stout and Nüsslein (2005) studied the diversity of the bacterial community associated with the roots of the aquatic angiosperm *Lemna minor* in cadmium-contaminated waters. A wide variety of bacteria were observed from the DGGE profiles with a dominant *Betaproteobacteria* group known for its high tolerance to heavy metals.

In bioremediation-related studies, Hamamura et al. (2005) analyzed the diversity of the bacterial community present in acidic soils in Yellowstone National Park. Soils studied were associated with natural hydrocarbon seeps, thereby underlining the

importance of the bacteria present in this environment. DGGE profiles revealed distinct bacterial populations established during the degradation of hexadecane with dominant bands affiliating with the genera *Acidiphilium*, *Acidocella*, and *Acidisphaera*. Another study used DGGE to identify an anaerobic bacterium capable of the ortho-dechlorination of a PCB (Cutter et al., 2001).

In a phytoremediation study with ryegrass and alfalfa, Kirk et al. (2005) determined that both perennial ryegrass and alfalfa increased the total number of bacteria in the rhizosphere. Perennial ryegrass seemed to support the increased abundance of hexadecane-degraders. In contrast, alfalfa selected diesel-degraders. Similar results of plant-specific microbial populations in the rhizosphere have been reported (Jones et al. 2004).

The studies mentioned above demonstrate that DGGE is a reliable, reproducible and rapid tool to analyze a large number of samples simultaneously (Nakatsu et al. 2000, Kirk et al., 2004). The DGGE technique is, however, dependent on DNA extraction protocols and PCR biases, and might represent only the most abundant populations in the DGGE profile (Forney et al. 2004; Kirk et al., 2004). Limitations in the DGGE technique also include the presence of multiple copies of the 16S rDNA in most bacteria (Wang and Wang, 1997) and co-migration of bands from microorganisms with 16S rDNA of different sequences but same G+C content (Muyzer and Smalla, 1998; Kirk et al., 2004; Throbäck et al., 2004). One must also keep in mind that the DGGE technique detects differences in the community structure that are not quantitative but qualitative (Seghers et

al., 2003; Basil et al., 2004). Despite its limitations, this fingerprinting technique makes it possible to detect major differences between communities (Muyzer and Smalla, 1998) and to obtain sequences from predominant populations without the need of cloning libraries (Throbäck et al., 2004).

1.7 Use of DGGE to analyze the distribution of specific functional genes in microbial populations

Very few studies have used DGGE to study functional genes. Targeting these genes in PCR further enhances our knowledge of the structure of the microbial community and the microbe's role within the community (Schneegurt and Kulpa, 1998; Nakatsu et al., 2000; Fjellbirkeland et al., 2001; Torsvik and Øvreås, 2002). Catabolic gene primers would also be useful in reducing limitations of the DGGE technique by targeting important bacterial groups that are not the most abundant groups in the community (Seghers et al. 2003).

The use of functional genes to investigate the methanotroph community in agricultural soils has been attempted (Fjellbirkeland et al., 2001). Genes encoding for the methane monooxygenase enzyme (pMMO) and the methanol dehydrogenase enzyme (MDH) as well as the V_1 to V_3 region of the 16S rDNA were targeted in PCR-DGGE. However, Fjellbirkeland et al. (2001) were unable to separate bands for sequencing after

obtaining weak amplifications from PCR, highlighting the difficulty of using degenerate primers in complex environments with the DGGE technique.

Wawer and Muyzer (1995) applied DGGE to detect sulfate-reducing bacteria in environmental samples by targeting the gene encoding [NiFe] hydrogenase. The technique allowed them to successfully determine the diversity of *Desulfovibrio* species present in bioreactors using an important enzyme in the hydrogen metabolism and dissimilatory metal reduction by sulfate-reducing bacteria.

Specific primers have been used to amplify the gene encoding the largest subunit of the multicomponent phenol hydroxylase to study phenol-degrading bacteria in activated sludge (Watanabe et al., 1998). The PCR products obtained were separated by TGGE, a gradient gel electrophoresis technique that uses temperature instead of increasing denaturant concentration to separate the amplified products by their G+C content.

Throbäck et al. (2004) re-evaluated previously designed primers targeting the functional genes *nirS*, *nirK*, and *nosZ*, which encode for enzymes involved in the denitrification pathway, for use in DGGE analysis. In contrast to the study by Fjellbirkeland et al. (2001), the introduction of the GC-clamp at the end of one primer for each set of primers did not affect the amplification efficiency for either isolates or environmental samples.

Other research on PCR-DGGE applied to functional genes includes work by Kowalchuk et al. (1997) on the ammonium monooxygenase gene (*amoA*) in ammonia-oxidizing bacteria (AOB) in coastal sand dunes. More recently, Briones et al. (2002) studied the composition and activity of AOB in rice roots through the *amoA* gene.

Little has been done in terms of applying DGGE to catabolic genes in petroleum bioremediation technologies. This may be due to the fact that many bacteria are known to degrade hydrocarbons, and there is likely high sequence divergence between genes encoding the enzyme systems used to breakdown contaminants (Rojo, 2005).

As mentioned above (section 1.2), hexadecane (straight-chain alkane) and phenanthrene (three-ring PAH) are proxy prototypes for studying the microbial degradation of petroleum hydrocarbons. Targeting the genes involved in alkane and PAH metabolism, together with the DGGE technique, could help us detect the important participants of hydrocarbon degradation in the complex environment like the rhizosphere; such findings would enhance future phytoremediation studies.

1.7.1 Alkane hydroxylase system

The enzyme complex involved in the degradation of alkanes in *Pseudomonas putida* GPo1 (previously *Ps. oleovorans*) was found to be encoded in the OCT plasmid over

thirty years ago (Chakrabarty et al., 1973) and it continues to be a model for other alkane hydroxylase systems (Van Hamme et al., 2003).

In brief, the complex consists of a membrane-bound monooxygenase (AlkB) and two soluble proteins, rubredoxin and rubredoxin reductase. Electrons are transferred from NADH by the rubredoxin reductase to the rubredoxin and then to the hydroxylase (van Beilen et al. 2003; Rojo, 2005). The hydroxylase uses an atom of oxygen to convert the alkane into an alcohol (see Fig. 1.1), while the other atom is reduced to H_2O (Shanklin and Whittle, 2003; van Beilen et al. 2003; Rojo, 2005).

Some homology exists between the alkane hydroxylase systems of other bacteria and that in *P. putida* GPo1, mainly among pseudomonads (van Beilen et al., 2002). However, many species have been found to have a very different complex of enzymes involved in the degradation of alkanes. For example, the *alkM* gene of *Acinetobacter* sp. ADP1 encoded for a quite distinct alkane hydroxylase system with low homology to any other alkane hydroxylases known (Ratajczak et al., 1998). Sequences of *alkB* genes in Gram-positive strains were also quite different (51%) from the alkane hydroxylase gene sequence of *P. putida* GPO1 (van Beilen et al., 2002). Moreover, the genes for some alkane hydroxylase homologues are arranged in a different manner than those in *P. putida* GPO1 and have other ways of regulating the expression of these genes (Chen et al., 1996; Ratajczak et al., 1998; Marin et al., 2001; van Beilen et al., 2003; van Hamme et al. 2003).

The diversity among alkane hydroxylase genes extends not only across bacterial species, but also across strains. Many strains have been found to have multiple alkane hydroxylases (Smits et al., 2002; van Beilen et al., 2003; Rojo, 2005). *Rhodococcus erythropolis* and *R. fascians* were found to have at least three different *alkB* homologues (van Beilen et al., 2002). *Acinetobacter* sp. ADP1 has two alkane hydroxylase complexes (*alkM*a and *alkM*b) controlled by *n*-alkane chain length (van Hamme et al. 2003). In *P. aeruginosa* PAO1, however, the two *alkB* genes have very similar substrate ranges and instead are induced at different stages of growth (Rojo, 2005). Lateral gene transfer has been suggested as the most likely explanation for the high diversity of the genes and the presence of several *alkB* homologues (Rojo, 2005).

It is important to note that not all alkane-degrading bacteria can assimilate long-chain alkanes; since *P. putida* GPo1 in pure culture can only degrade C_5-C_{12} *n*-alkanes, the metabolism of alkanes in this range is relatively well understood. Nevertheless, due to their persistence in petroleum-contaminated sites, identifying long-chain alkane-degraders and understanding how their enzymes operate is important in the field of bioremediation. Recently, van Beilen et al. (2005) proposed a model for alkane metabolism and uptake. According to this model, degraders of *n*-alkanes shorter than C_{12} have a bulky aminoacid residue in a key position of the enzyme structure that prevented longer alkanes from binding to the hydrophobic pocket of the enzyme (Rojo, 2005; van Beilen et al., 2005).

Many studies have used catabolic gene probes to detect alkane-degraders (Vomberg and Klinner, 2000; Siciliano et al., 2003), some with limited success (Whyte et al., 1996; Kohno et al. 2002). Some primers have also been designed (Chénier et al., 2003; Kohno et al., 2002) including some with high degeneracy (Smits et al., 1999), to screen for different alkane hydroxylase systems. Many questions remain on the diversity and distribution of the alkane hydroxylases, and more work needs to be done in designing primers and probes with high specificity to obtain more reproducible and reliable results.

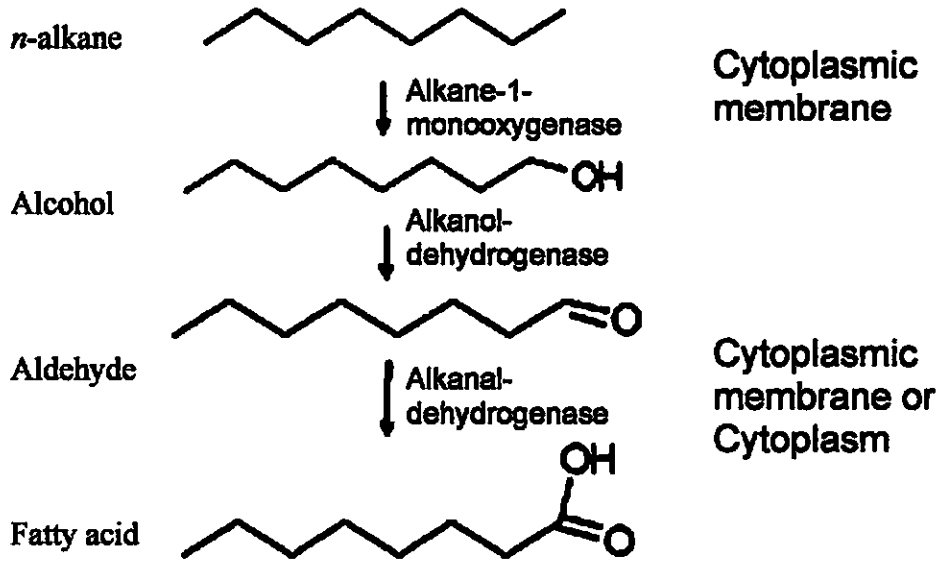


Figure 1.1. Simplified pathway for the degradation of alkanes before they enter the ß-oxidation pathway.

1.7.2 Enzymes involved in the degradation of phenanthrene

The biodegradation of PAHs is exemplified once more by the genetic information obtained from *Pseudomonas putida*. The plasmid NAH7 of *P. putida* strain G7 contains the genes encoding for the pathway for naphthalene conversion to salicylate (Herrick et al., 1993; Ferrero et al., 2002). The main enzyme involved in this system is naphthalene dioxygenase, which introduces molecular oxygen into the aromatic nucleus to form *cis*-naphthalene dihydrodiol (Hamman et al., 1999; van Hamme et al., 2003). This broad specificity enzyme is also involved in the catalysis of the phenanthrene degradation, as well as other PAHs (Takizawa et al., 1994; Iwabuchi and Harayama, 1997).

Some Gram-positive strains, and other bacteria, have been found to have a PAH degradation pathway that differs from that in *P. putida* G7 (Churchill et al., 1999; Khan et al., 2001; Krivobok et al., 2003; Van Hamme et al., 2003). This involves a second set of enzymes that convert phenanthrene from the intermediate 1-hydroxy-2-naphthoate to *o*-phtalate (Fig. 1.2) (Takizawa et al., 1994; Iwabuchi and Harayama, 1997). Iwabuchi and Harayama (1997, 1998) described the enzymes used in the *o*-phtalate route by *Nocardioides* sp. strain KP7 (Fig. 1.2). These enzymes are not involved in the degradation of naphthalene. Aromatic ring cleavage of 1-hydroxy-2-naphthoate is catalyzed by 1-hydroxy-2-naphthoate dioxygenase encoded in the *phdI* gene (Iwabuchi and Harayama, 1997, 1998; Adachi et al., 1999).

Genes involved in the metabolism of phenanthrene and other PAHs can be found both on the chromosome and on plasmids, and a high degree of sequence divergence occurs between degrader strains (Lloyd-Jones et al., 1999; Van Hamme et al. 2003). As with the alkane hydroxylase enzyme complex, lateral gene transfer and genetic recombination has probably contributed to the crucial progression of these exceptional enzyme systems (Laurie and Lloyd-Jones, 1998; Van Hamme et al., 2003). Attempts to determine the sequence diversity of PAH-degrading genes are limited by our knowledge and the few sequences available (Lloyd-Jones et al., 1999; Meyer et al., 1999).

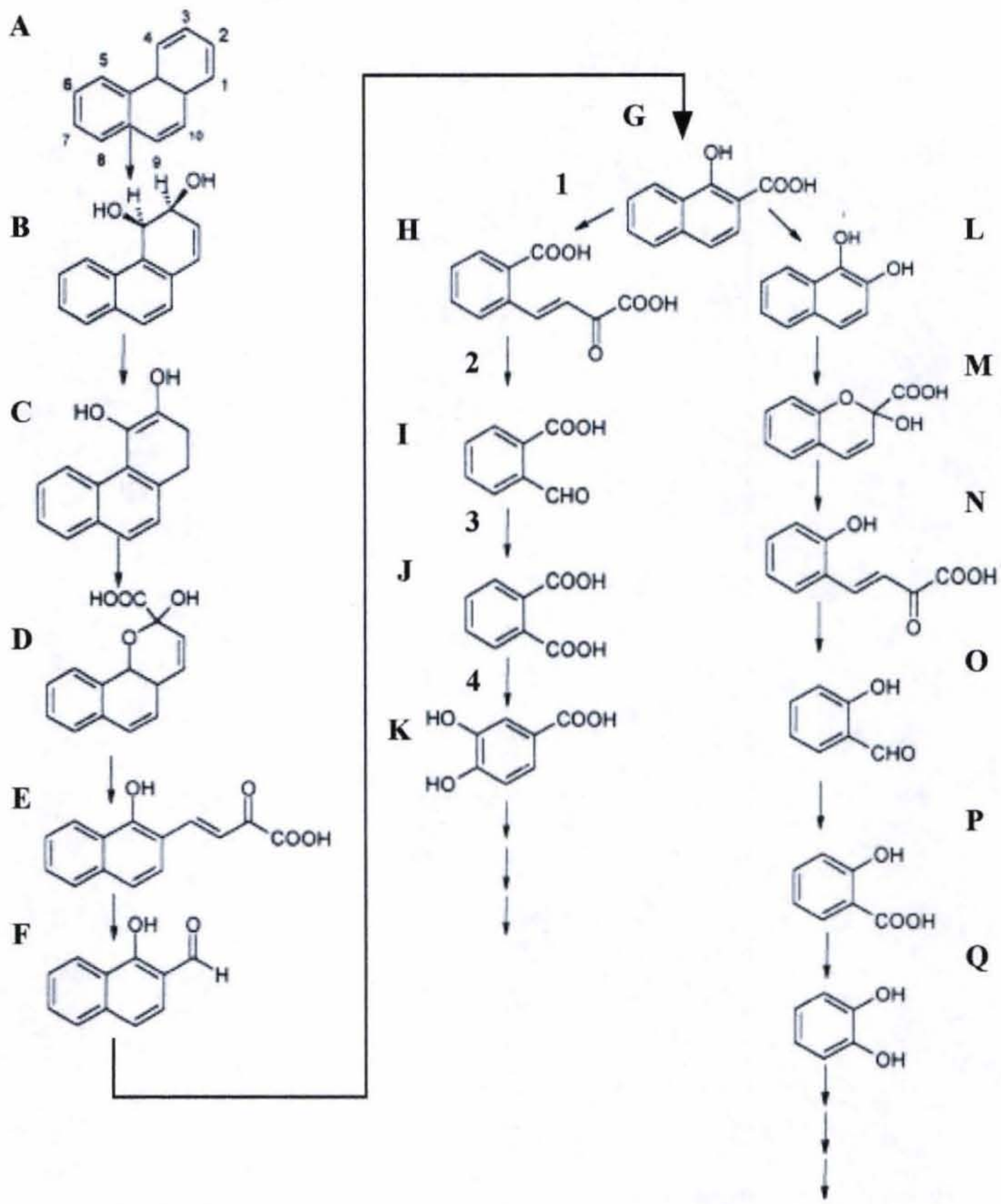


Figure 1.2. Pathways for degradation of phenanthrene. Phenanthrene is degradaded by *Nocardioides* sp. KP7 and *Mycobacterium* sp. PYR-1 via the *o*-phtalate route with a unique set of enzymes not shared with the naphthalene degradation pathway (Iwabuchi and Harayama, 1997; Pinyakong et al., 2000; Krivobok et al., 2003). The enzymes thought to be involved in the catalysis of each step in the *o*-phtalate route are as follows: (1) 1-hydroxy-2-naphthoate dioxygenase; (2) 2'-carboxylbenzalpyruvate hydratase-aldolase; (3) 2-carboxybenzaldehyde dehydrogenase; (4) *o*-phthalate dioxygenase. Chemical designations: A, phenanthrene; B, *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene; C, 3,4-dihydroxyphenanthrene; D, 2-hydroxy-2*H*-benzo[*h*]chromene-2-carboxilic acid; E, *trans*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid; F, 1-hydroxy-2-naphthaldehyde; G, 1-hydroxy-2-naphtoic acid; H, 2'-carboxylbenzalpyruvic acid; I, 2-carboxybenzaldehyde; J, *o*-phthalic acid; K, protochatechuic acid; L, 1,2-dihydroxynaphtalene; M, 2-hydroxychromene-2carboxylate; N, *trans-o*-hydroxybenzylidenepyruvate; O, salicylaldehyde; P, salicylic acid; Q, cathecol.

1.8 Scope of the project

This study was undertaken to provide information regarding the microbial lipase activity (indicator of alkane degradation) and the diversity of the bacteria involved in the degradation of petroleum hydrocarbons in planted and unplanted coastal soil from the John Rogers Tank Farm site at Hickam Air Force Base, Honolulu, Hawaii.

1.8.1 Relevance of findings from a previous phytoremediation investigation

A previous study of phytoremediation of the same soil artificially contaminated with 10,000 mg of No.2 diesel/kg of soil and 1% NaCl in a greenhouse experiment (Jones et al., 2004; Sun et al., 2004) indicated that:

1) The hydrocarbon-degraders in the rhizospheres of 3 tree species (milo, kiawe, and kou), out of 7 species of trees and shrubs tested, significantly reduced the concentration of diesel in soil (40% on average) when compared to unplanted soil.
2) The hydrocarbon-degraders in the rhizospheres of one tree species (iron wood) and 3 shrub species (oleander, beach naupaka, and false sandalwood) were ineffective at reducing diesel concentration.

3) Microbial populations responded differently to the contaminant and the plant rhizospheres. Total aerobic bacteria preferred plant root exudates and sloughed-off cells to the diesel contaminant. Phenanthrene and pristane-degraders preferred the hydrocarbons to the plants products. Diesel-degraders had no preference and responded to the same extent to the rhizodeposition and to the diesel contaminant.
4) In the contaminated treatments the numbers of hydrocarbon degraders were similar in the rhizospheres of the effective trees and in the rhizospheres of the ineffective shrubs. Thus it seemed that qualitative differences might exist among the root exudates and root debris of the different plants, fostering the growth of effective or ineffective hydrocarbon degraders. According to the proposed conceptual model, the effective microbes would prefer hydrocarbons to root exudates whereas the ineffective ones may be more inclined to use the root exudates rather than the hydrocarbons.

1.8.2 Goals and objectives of this study

The long-term goal of this research is to identify the major groups of hydrocarbon-degrading bacteria using model hydrocarbons in the alkane and PAH categories to facilitate the design of future experiments aimed at monitoring plant impact on the dynamics and catabolic activities of these populations in field conditions.

The specific objectives of this bench-top research were:

1) To evaluate the usefulness of measurements of lipase activity instead of the size of hydrocarbon-degrading microbial populations to monitor petroleum hydrocarbon degradation in soil;

2) To determine the diversity of the dominant hydrocarbon-degrading bacteria under planted and unplanted conditions based on their 16S rRNA gene sequences; and

3) To verify the hydrocarbon-degrading capabilities of those bacteria identified above by determining the presence in their genome of genes reported to be involved in hydrocarbon catabolism.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental design

In this study the impact of two factors, the presence of plant roots and of hydrocarbon contaminants, was examined with respect to microbial numbers, lipase activity, and bacterial diversity. The plants were chosen on the basis of their behavior in previous experiments performed in the greenhouse by our group (Sun et al., 2004). From those studies, milo (*Thespesia populnea)* was determined to be an efficient tree, while oleander (*Nerium oleander)* was an inefficient shrub in phytoremediation of coastal soil contaminated with diesel fuel. Buffelgrass (*Cenchrus ciliaris*) was the predominant grass at the field site; it was found to grow vigorously when fertilizer and irrigation were applied to the field. In preliminary experiments (Sun et al., 2004) buffelgrass was shown to have some potential for phytoremediation provided salinity and diesel fuel concentration were low. Due to the fibrous nature of their roots, grasses have been good candidates for phytoremediation (Banks et al., 2000). Buffelgrass might be useful in the phytoremediation of deep-seated hydrocarbons if their root exudates can be flushed down the soil profile to the contaminated zone (Tang et al., 2004).

A defined mixture of diesel fuel constituents was used to permit the study of specific hydrocarbon-degrading microbial populations. It consisted of 1,000 mg of hexadecane and 200 mg of phenanthrene/kg of site topsoil. The characteristics of this sandy loam, which is a 1:1 mixture of topsoil and subsurface soil, have been described previously (Sun et al., 2004). According to the soil survey map produced by USDA and the University of Hawai'I Agricultural Experimental Station, the soil at the Hickam Air Force Base site is classified as fill land (mixed). It may consist of mixed dredged marine material and material from other areas and would be used for "urban development" and not for agriculture. Therefore it has no "capability classification".

To simulate the conditions in the field, where the roots grow in topsoil of low salinity then reach a zone of higher salinity, the pots consisted of two 15.24 cm-long sections of PVC pipe (internal diameter, 5.1 cm) held together by 2 pieces of duct tape. The top section of all pots contained untreated soil. The bottom section contained soil moistened with 1% NaCl which was either contaminated with hexadecane and phenanthrene or uncontaminated. A piece of mosquito net, secured with duct tape, was positioned at the bottom of the lower-pot section to hold the soil in place (Fig. 2.1).

The pots were placed in a growth-room equipped with Sunbrella lighting systems (Environmental Growth Chambers, Chagrin Falls, OH). Each Sunbrella unit houses one 400W high-pressure sodium-vapor lamp and one 400W metal-halide lamp which provide light in the 550-650 nm region and the UV and blue region, respectively (Krizek et al., 1998). The average photosynthetic photon flux at 0.6 m from the lamp banks was 215

μmol of photons s^{-1} m^{-2}. The photoperiod was 16 hours, based in a 24-hour cycle, and the temperature 26°C.

Four replicate pots were prepared per treatment for each of the time points when the 4 replicates were sacrificed. The three time points were days 0, 56, and 114 based on previous results (Tang et al., 2004). The pots were organized on a grid in a completely randomized design.

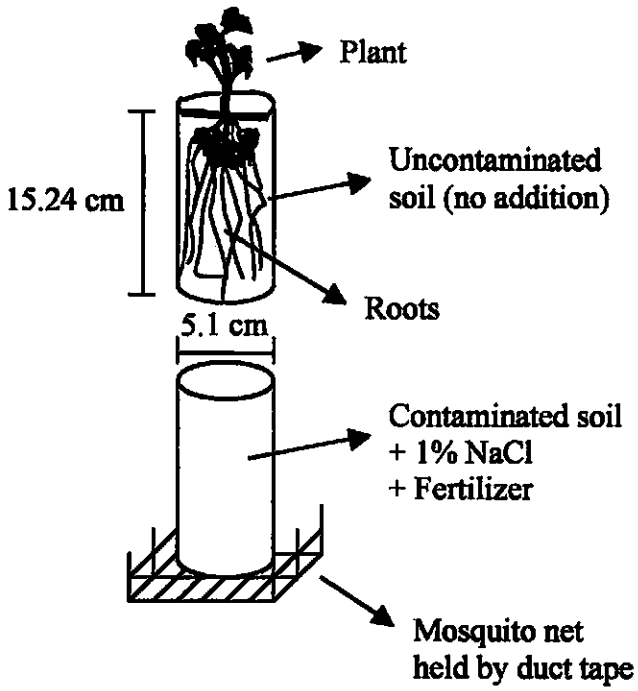


Figure 2.1. Schematic diagram of pot setup on day 0.

Different watering regimes for planted and unplanted treatments were used to maintain the soil moisture content at *ca.* 50% water holding capacity in all pots to provide

similar aeration in all treatments and to make sure there was no effluent. The volume of fluid (fertilizer or water) delivered per pot was: 20 mL for milo, 10 mL for oleander and buffelgrass, and 5 mL for unplanted soil. Fertilizer was applied every other day. Peters professional fertilizer (30-10-10) (United Industries Corporation, St. Louis, MO), which contained 126 mg N, 1.5 mg P, and 35 mg K/L, alternated with a mix of 1 mM $Ca(NO_3)_2$ and 0.1 mM $FeCl_3$ (28 mg N and 0.1mg Fe/L). The plants received water on alternate days.

2.2 Soil preparation for bottom pots

Each bottom pot contained 385g of air-dry soil equivalent. Batches of 7 kg of air-dry soil were prepared at a time. Uncontaminated batches of soil received 1138 mL of Peters fertilizer solution (420 mg fertilizer/L) in which 11.5 g NaCl were dissolved (1% NaCl) to bring the soil to 50% water-holding capacity.

The contaminated soil was prepared as follows. A glass container was used to mix 500 g of air-dry soil with a filter-sterilized solution of 1.43 g phenanthrene dissolved in 20 mL of acetone in a chemical flow hood. The acetone was left to evaporate, with frequent mixing, for 2 hours. This soil was then added to 6.5 kg of air dry-soil, and the two soils were mixed. Filter-sterilized hexadecane (9.4 mL) was added and carefully mixed with the soil. The bottom pots were then filled with contaminated or uncontaminated soil. A portion of the soil was left in the refrigerator at 4°C for 24 hours

for the day 0 microbial counts. Another portion was frozen at -70°C for the day 0 lipase determination.

2.3 Plant and soil preparation for top pots

Milo and buffelgrass were started as seeds 3 months prior to the experiment. Milo seeds required treatment with sand paper to germinate. They were germinated in the dark on moist silica sand. Seedlings were planted in Hickam soil in individual pots. Buffelgrass seeds were sown in Hickam soil at several per pot.

Oleander plants were prepared from cuttings treated with hormone powder and fungicide. They were grown in potting soil under two layers of shading cloth until the roots were vigorous (*ca.* one month). They were grown in full light for another month. All plants were routinely fertilized as described above.

All plants were transplanted to the top PVC pots on day 0. Milo was transplanted as one plant per pot with the Hickam soil in which it was growing, leaving *ca.* 2 cm of soil devoid of roots at the bottom of the top pot. Buffelgrass was also transplanted in the same manner after thinning to 5 seedlings per pot. Oleander roots were washed free of potting soil and were transplanted to the PVC top pots filled with Hickam soil. Unplanted soil, which had been treated like the milo and buffelgrass soil during plant growth, was transferred to PVC top pots.

A thin strip of mosquito net was placed on the bottom of the top PVC pots to indicate the separation between the untreated top pot soil and the treated (salt or salt + contaminant) bottom pot to facilitate harvest of the correct part of the root and soil. The top PVC pots were then attached to the bottom sections with 2 pieces of duct tape after removal of the mosquito net from the bottom of the top pots.

2.4 Determination of plant biomass at harvest

At harvest the plant green parts were cut at soil level. Plant height was measured to the apical meristem and dry weight was determined after 3 days at 65°C. For buffelgrass the height was deemed the height of the longest stem in the pot. The duct tape holding the top and bottom sections of the pots was removed and the roots were cut with scissors at the intersection of the two parts. The roots in the top sections of the pots were washed free of soil and dried at 65°C. The bottom section of the pot was emptied onto a piece of Kraft paper and the roots with attached rhizosphere soil were carefully separated from the rest of the soil for microbial enumerations (see section 2.7). The roots were gently shaken to leave a thin layer of rhizosphere soil (<1 mm) on the root. After collecting samples for microbial enumerations, the roots from the bottom sections of the pots were washed and dried as described above. The remainder of the soil was thoroughly mixed and a portion stored at 4°C until used for hydrocarbon concentration determination (see section 2.5). A portion was frozen at -70°C for soil lipase analysis (see section 2.6).

2.5 Hydrocarbon extraction from soil and analysis

The procedure of Banks et al. (2000), with slight modifications, was used to extract the hydrocarbons from soil and for analysis of hydrocarbons by gas chromatography.

2.5.1 Hydrocarbon extraction from soil

Unplanted soil and rhizosphere soil were air-dried for 12 hours after removal of roots from the lower pots (see section 2.4). One gram of air-dried soil was placed in a Teflon centrifuge tube together with 15 mL of methylene chloride and 12 μL of a 10 mg/mL solution of *o*-terphenyl (Restek Corporation, Bellefonte, PA) as surrogate standard. The tubes were shaken for 1 hour on a Burrell wrist-action shaker at the highest setting. The supernatant was transferred to a 40-mL Qorpak glass vial which had a Teflon-lined septum inserted in the screwed cap. A second extraction was carried out by adding 15 mL of methylene chloride to the centrifuge tube and shaking for 1 hour. The centrifuge tube was spun at 4,000 x *g* for 10 minutes and the supernatant was pooled with the first one in the Qorpak vial. Solid Na_2SO_4 was treated at 450°C for 7 h to combust organic matter and dry the salt. Five grams of Na_2SO_4 was added to the liquid to remove any moisture and the vial was shaken several times. The extract was withdrawn with a glass pipette and filtered through a 0.5-μm pore-size nylon Acrodisc filter using a

glass syringe. The samples were concentrated to 1 mL by solvent evaporation in a Brinkmann/Büchi rotary evaporator. Controls consisting of methylene chloride and *o*-terphenyl were run simultaneously to determine the recovery of terphenyl from the soil. Terphenyl being a surrogate for the hydrocarbons, the recovery of hexadecane and phenanthrene was determined from the surrogate recovery.

2.5.2 Hydrocarbon analysis

For gas chromatography (GC) 1 mL of hydrocarbon extract from soil was mixed with 10μL of a 2-mg/mL solution of 5-α-androstane as internal standard (Restek Corporation, Bellefonte, PA). One μL of extract was automatically injected into a Perkin-Elmer Autosystem gas chromatograph equipped with an Rtx-5 capillary column (30 m x 0.53 mm i.d., Restek Corporation, Bellefonte, PA) and a flame-ionization detector. The oven temperature was programmed to stay at 50°C for 2 minutes, to ramp to 300°C 15 degrees per minute, and to maintain the final temperature for 5 minutes. The injector temperature was 250°C and the detector temperature was 300°C (Banks et al., 2000, with slight modifications). GC analyses were conducted by Marisa Toma in Dr. Traci Sylva's lab (MBBE).

2.6 Soil lipase activity determination

Soil lipase activity was determined using the colorimetric method of Margesin et al. (2002) based on the hydrolysis of the colorless *p*-nitrophenylbutyrate (pNPB) to yellow *p*-nitrophenol (pNP) and butyrate by soil lipase. Both reagents were purchased from Sigma, St. Louis, MO.

2.6.1 Standard curve preparation

Due to adsorption of *p*-nitrophenol to soil, the standard curve was prepared with sterile soil (autoclaved on 3 consecutive days for 90 minutes) at 50% of water holding capacity added to the reagents. The moisture content of the sterile soil was the average moisture content of the soil samples.

The stock pNP solution for the standard curve was 100 μg/mL of 100 mM sodium phosphate buffer at pH 7.23. The pNP was first dissolved in an amount of 2-propanol, representing 1% of the total volume of phosphate buffer. The standard curve was prepared in triplicate, each with a dilution series containing 0 (blank), 1, 25, 50, 75, 100, and 125 μg of pNP per tube (Table 2.1). Each tube received an appropriate volume of stock solution and the total volume was brought to 5 mL with phosphate buffer (see Table 2.1).

The 5-mL aliquots of pNP diluted in phosphate buffer were placed in capped polypropylene centrifuge tubes which were then pre-warmed in a water bath at 30°C for 10 minutes. Sterile soil (0.1 g) was added and the tube contents mixed. Tubes were incubated for exactly 10 minutes then cooled on ice for 10 minutes. All tubes were centrifuged at 5,000 x *g* at 4°C for 5 minutes and the supernatant was placed in a capped test tube kept on ice. The liquid was poured into a cuvet and the absorbance was immediately read at 400 nm. The blank (0 µg pNP/tube) was used to zero the spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Fig 2.2 shows the standard curve.

Table 2.1. *p*-Nitrophenol standard curve set up.

Tube no.	1	2	3	4	5	6
µg of pNP/ tube	0	25	50	75	100	125
µL of stock pNP sol./tube [a]	0	250	500	750	1000	1250
mL of buffer/tube	5	4.75	4.5	4.25	4	3.75
Sterile soil (g)	0.1	0.1	0.1	0.1	0.1	0.1

[a] Stock solution of pNP: 100µg pNP/mL of 100mM sodium phosphate buffer at pH 7.23

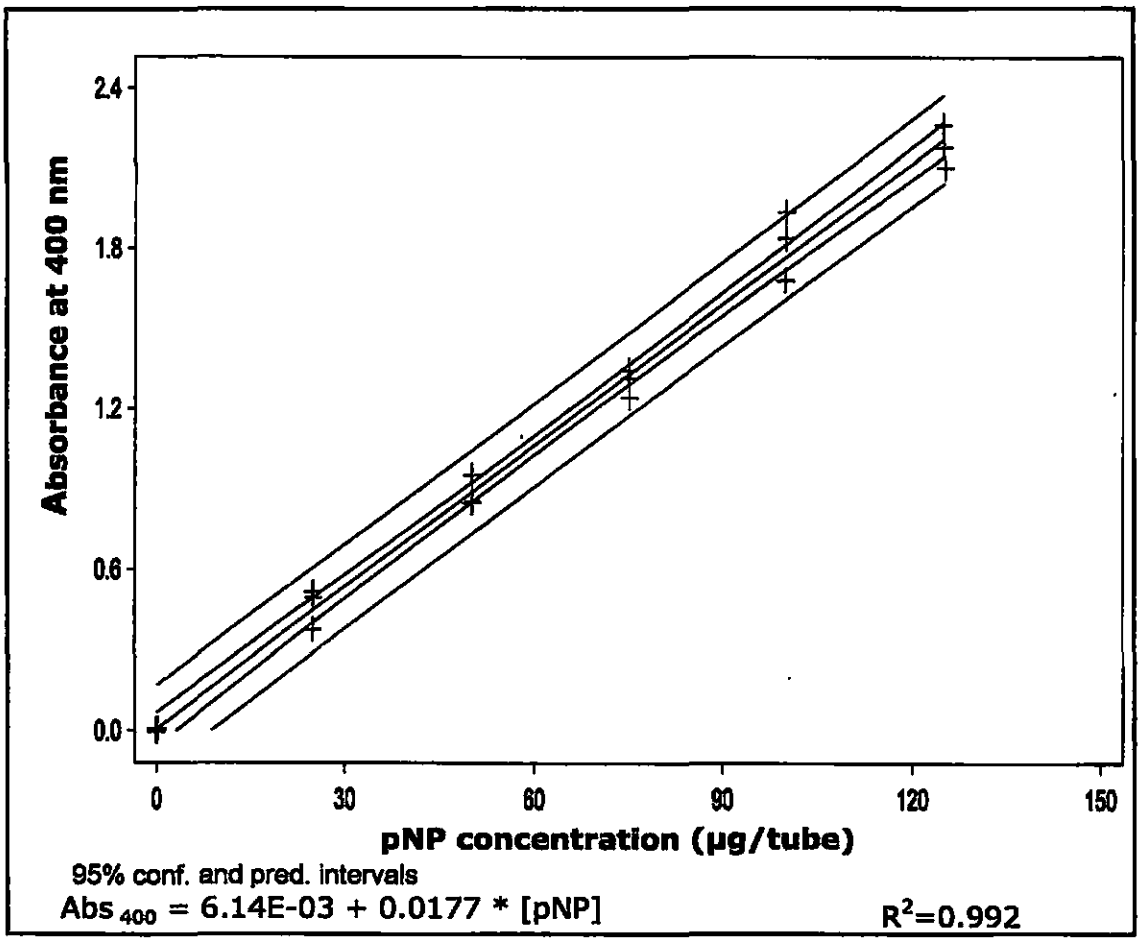


Figure 2.2. *p*-Nitrophenol standard curve

2.6.2 Determination of lipase activity in soil samples

A portion of a frozen soil sample was brought to room temperature. Duplicate polypropylene tubes were prepared with 0.1g of soil sample and 5 mL of phosphate buffer. One blank (without pNPB), to zero the spectrophotometer, was prepared with

sterile soil. A control sterile soil with pNPB was added to each series of samples to determine the auto hydrolysis of pNPB to pNP.

All tubes containing soil and phosphate buffer were pre-warmed in a water bath at 30°C for 10 minutes. Then 50 µL of a 100 mM solution of pNPB in 2-propanol at -20°C was added to all tubes except the blank. After incubation in the water bath for 10 minutes the tubes were cooled on ice for 10 minutes and centrifuged at 5,000 x *g* for 5 minutes. The supernatant fluid was transferred to a test tube kept on ice, and then to a cuvet for reading the absorbance at 400 nm.

The dry weight of an aliquot of the soil was determined at 105°C. The activity of the lipase [µg pNP/(g dry soil x 10 min)] was determined from the standard curve. The average amount of pNP obtained with 7 controls was subtracted from each sample value.

2.7 Enumeration of bacteria in soil and rhizospheres

Ten-fold dilutions of unplanted and rhizosphere soil were made in a solution of 0.5 g of K_2HPO_4 and 5.1 g of NaCl per liter. The first dilution of soil (10 g of soil/95mL of solution) was shaken for 1 hour on a Burrell wrist-action shaker (highest setting) to disperse the microbial cells. For the rhizosphere soil the root(s) with adjacent soil (see section 2.4) were placed in a milk-dilution bottle containing 95 mL of solution and shaken for 1 h., then serially diluted. The actual dilution in the first bottle was determined

by removing the root, pouring all of the soil suspension in a tared aluminum dish, and weighing the oven-dry soil (110°C until dry) in the known volume of fluid (Timonin, 1940). All counts were normalized to the number of cells per gram of oven-dry soil.

All media were supplemented with 1% NaCl; the pH was adjusted to *ca.* 7.5. All plates were incubated at 30°C.

2.7.1 Heterotrophic bacteria

Total aerobic heterotrophic bacteria were enumerated (spread plates) after 3 days of incubation on R2A agar medium (Greenberg et al., 1985), supplemented with 100 μg of cycloheximide mL^{-1} to inhibit growth of fungi.

2.7.2 Hexadecane-degrading bacteria

Hexadecane-degrading bacteria were enumerated in liquid mineral Bushnell-Haas medium (Bushnell and Haas, 1941) by the most-probable-number (MPN) technique (Mills et al., 1978). Twenty-four well tissue-culture plates (Brown and Braddock, 1990) received 2 mL of Bushnell Haas medium supplemented with micronutrients (Pochon and Tardieux, 1962) and vitamins (Bogardt and Hemmingsen, 1992). A drop of filter-sterilized hexadecane (5 μL) (Aldrich, Milwaukee, WI) was added to all wells except

those in the control plates to detect possible growth on organic material in the sample. Growth on control plates free of hexadecane was always negligible. Decimal dilutions of samples (0.5 mL) were added to 5 wells per dilution. The 6th well in the sample plates received 0.5 mL of diluent and 1 drop of hexadecane to verify the sterility of hexadecane. MPN plates were incubated for 28 days and scored for presence or absence of microbial growth. The tables by Alexander (1982) were used to compute the MPN of hexadecane degraders.

2.7.3 Phenanthrene-degrading bacteria

Phenanthrene-degrading bacteria were enumerated on overlay plates modified from Bogardt and Hemmengsen (1992). Mineral medium prepared with 1.5% Noble agar supplemented with vitamins and 100 μg cycloheximide mL^{-1} was used in the bottom layer. Six mL of the same medium, devoid of vitamin and cycloheximide, mixed with 0.2 mL of a solution of phenanthrene in acetone (11.3 mg/mL acetone) constituted the overlay. After evaporation of the acetone at 30°C for 24 hours, 0.1 mL of a sample dilution was spread on the surface of the agar. The plates were examined for clear halos around the colonies and positive colonies were enumerated everyday for 15 days.

2.8 Characterization of isolates of alkane and phenanthrene-degrading bacteria

Some isolates capable of degrading hydrocarbons were isolated from the Hickam site during previous experiments (Jones et al., 2004) and some phenanthrene degraders were isolated from the phenanthrene-enumeration plates during this project. None of these isolates except PM600 (phenanthrene-degrading bacterium) have been characterized previously. Isolates grown on half-strength tryptic-soy-agar (TSA) medium were examined for Gram reaction, colony and cell morphology, and pigment production (Smibert and Krieg, 1981).

Capability to grow on No.2 diesel fuel (Union 76 service station), mineral oil (White oil #9, which contains a mixture of alkanes, mostly 20-28 C in length), hexadecane, and pristane (branched alkane) as sole C source was studied in tubes containing Bushnell-Haas medium and 5 μL of sterile hydrocarbon. Cultures in tryptic soy broth were washed in water and cells were introduced into the tubes at a level of 10^6 cells/mL. Control with cells but without hydrocarbons and vice versa were prepared to determine potential growth on dead cells and the sterility of hydrocarbon, respectively. The tubes were incubated at 30°C and inspected for growth for 28 days.

Potential phenanthrene-degraders, which produced a clear halo on phenanthrene plates, were further tested by a modification of the method of Wrenn and Venosa (1996),

which allows the observation of yellow-colored metabolic products after aromatic ring fission. Plates of half-strength TSA were covered with a suspension of cells from a broth culture and incubated for 2 days at 30^0C. Dry cotton swabs were rolled lightly over the culture and were agitated in tubes containing 10 mL of a solution of 5 g K_2HPO_4 and 1 g NaCl/L until the turbidity was near 1 on the McFarland $BaSO_4$ scale (*ca.* 2 x 10^7 cells/mL). Twenty-four well tissue-culture plates were used for the assay. The wells contained 1 mL of 2X Bushnell-Haas medium (see section 2.7.2). One mL of the diluted cell suspension was added to each well to make the medium 1X. Sterile phenanthrene crystals were obtained by filter-sterilizing a phenanthrene solution in acetone followed by evaporation of the acetone (loose cap) in a fume hood. To each well, 1.1 mg of phenanthrene crystals was added with a sterile spatula. Triplicate wells were prepared on 2 separate occasions for each strain tested. Negative controls were prepared with strains known to be free of phenanthrene-degrading enzymes. The plates were incubated on a shaking platform for 3 weeks at 26°C. All wells developing a yellow color were recorded as positive for aromatic ring fission.

2.9 Genomic DNA extraction from isolates and cells from MPN wells

Sub-samples and colonies from pure cultures were collected and transferred to a sterile 1.5 ml Eppendorf tube containing 750 µl of sterile water. For the MPN plates, the contents of positive wells from the last dilution were pooled and transferred to a sterile

1.5 ml Eppendorf tube. Each tube was centrifuged at 15,000 rpm for 5 minutes and all supernatant water discarded. Pellets were kept at -20°C until they were processed.

DNA was extracted using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA) according to manufacturer's protocol, with minor adjustments. A Vortex Genie 2 model G-560 (Scientific Industries, Inc., Bohemia, NY) was used instead of a Bead Beater to avoid shearing DNA. Extracts were visualized by agarose gel electrophoresis to confirm successful extractions using lambda DNA as molecular weight marker.

2.10 PCR amplification

Primers used in this study are listed in Table 2.2. Amplifications of the V_6 to V_8 region of the small subunit of the ribosomal RNA gene were performed using the univeral primers set 968f and 1346r (379 bp amplification products) (Table 2.2). To improve the detection of sequence variations of amplified DNA and to avoid complete denaturing of the products by subsequent DGGE (Muyzer et al., 1993), a 40-bp GC-rich clamp was attached to the forward primer (Nübel et al., 1996). The amplification product with the clamp was ~419 bp long.

Genes encoding for alkane hydroxylase were targeted by two sets of primers, one for the *alkM* gene and a second one for the *alkB* gene (Kohno et al., 2002), while another set of primers (Chénier et al., 2003) contained inosine in their sequence for degeneracy.

Two different sets of primers for the *alkB* gene were re-designed from primers of Chénier et al. (2003) following the results of 16S rDNA sequence analysis conducted for this study. Sequences in the database for known degraders of long-chain alkanes were aligned using the ClustalX program (Version 0.1) (Ramu & Co., 2003). The first set of primers was designed within the target region of Chénier et al. (2003) from conserved regions of *Alcanivorax borkumensis* AP1, *Pseudomonas fluorescens* CHA0, *Pseudomonas aeruginosa* PAO1 and *Pseudomonas* sp. isolate PFSW607.

Table 2.2. PCR primers used in this study

Primer [a]	Sequence (5' to 3')	Target gene	Amplification Product (bp)	Reference organism	Reference
968F [b] 1346R	AACGCGAAGAACCTTAC TAGCGATTCCGACTTCA	16S rDNA (V6-V8)	379 (419 bp with clamp)	Universal	Nübel et al. (1996)
ALK-2 F ALK-2 R	GAGACAAATCGTCTAAAACGTAA TTGTTATTATCCCAACTATGCTC	*alkM*	271	*Acinetobacter* sp. ADP1	Kohno et al. (2002)
ALK-1 F ALK-1 R	CATAATAAAGGGCATCACCGT GATTTCATTCTCGAAACTCCCAAAC	*alkB*	185	*Pseudomonas putida* P1; *P. putida* GPo1; *P. aureofaciens* RWTH529	Kohno et al. (2002)
CF CR	CIGIICACGAIITIGGICACAAGAAGG IGCITGITGATCIIIGTGICGCTGIAG	*alkB*	549	*Rhodococcus* sp. Q15	Chénier et al. (2003)
Ab-F Ab-R	TTTCAAGGTGGAGCATGTGCG ACGACGCTTGGCGTAAGCATG	*alkB*	491	*Alcanivorax borkumensis* AP1; *Pseudomonas fluorescens* CHA0; *P. aeruginosa* PAO1; *Pseudomonas* sp. PFSW607	This study
Nd-F Nd-R	CGGCCCATGAGCTGGGTCACAAGAAGG GGCGTGATGGTCGGAGTGTCGCTGCAG	*alkB*	549	*Nocardioides* sp.; *Mycobacterium* sp.	This study

[a] F=forward;R=reverse

[b] 40-bp GC-rich clamp was attached (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG) for DGGE (Nübel et al., 1996)

The second set of primers was modified from the primers as Chénier et al. (2003) to target *Nocardioides* and *Mycobacterium* found in our samples by 16S rDNA analysis. This was accomplished by replacing the inositol by bases specific for *Nocardioides* and *Mycobacterium*.

Attempts were made to design primers for the *phdI* gene encoding for 1-hydroxy-2-naphtoate 1,2-dioxygenase by aligning available sequences of *Nocardioides* sp. KP7 (Iwabuchi and Harayama, 1997, 1998) and *Mycobacterium* sp. PYR-1 (Stingley et al., 2004).

PCR reaction mixtures of 50 μL contained approximately 10 ng of DNA template, 1 μl of each primer (0.2 pmol μL^{-1}), 1 μL of 40mM solution of dNTP's (Promega, Madison, WI), 5μl of 10X cloned-*Pfu* Buffer and 1.25 U of *Pfu* polymerase (Stratagene, La Jolla, CA). *Pfu* polymerase was used for 16S rDNA amplifications and *Taq* polymerase (Promega, Madison, WI) was used for the other gene targets. To minimize amplification inhibition (Kreader, 1996) and non-specific annealing of the primers (Reysenbach et al., 1992), 5 μL of bovine serum albumin (Sigma chemical Co., St. Louis, MO) (final concentration of 200 ng μl^{-1}) and 5 μL of acetamide (Sigma chemical Co., St. Louis, MO) (0.5% wt vol^{-1}) were added to the mixture. PCR amplifications were performed in an Eppendorf 5332 Mastercycler® Personal as follows: 3 min of initial denaturation at 94°C; 30 cycles of 1 min of denaturation at 94°C, 1 min annealing at 55°C for 16S rDNA, 53.5°C for *Alcanivorax alkB*, and 58°C for *Nocardioides alkB*, and 1 min of extension at 72°C; and a final extension at 72°C of 7 min. The PCR fragments were

analyzed by agarose gel electrophoresis (1% agarose) using PCR markers (Promega, Madison, WI) and visualized by staining with ethidium bromide (Bio-Rad Laboratories, Inc., Hercules, CA) incorporated into the agarose (1 μL of 10 mg mL^{-1} ethidium bromide per 100 mL agarose). All amplifications were done at least twice. PCR products were cleaned using the UltraClean™ PCR Cleanup Kit (MO BIO Laboratories, Inc., Solana Beach, CA) following manufacturer's instructions.

2.11 DGGE analysis

Amplified DNA products of pooled MPN-well samples and isolates were resolved on DGGE gels using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). PCR products from *Lactococcus lactis* ATCC 11454, and isolates *Sphingomonas* sp. PM601, *Rhizobium* sp. 806, and *Mycobacterium* sp. 808 were used as markers for DGGE analysis.

One-millimeter thick, 16 x 16 centimeters 8% polyacrylamide gel containing a 30 to 55% urea-formamide denaturing gradient (100% denaturant = 7M Urea and 40% formamide) were poured using a gradient delivery system model 475 (Bio-Rad Laboratories, Inc., Hercules, CA).

Approximately 350 ng of the amplified PCR product were electrophoresed in 1X Tris-Acetic Acid-EDTA (TAE), pH 8, at 150V and 60°C for 6 hours. Migration patterns

were visualized by staining with 1:2000 SYBR® Green I (Invitrogen, Carlsbad, CA) in fresh 1X TAE buffer, pH 8, for 10 minutes followed by UV transillumination. Images were recorded with a Polaroid camera and 667 Polaroid film.

2.12 Sequencing and computer analyses of partial 16S rRNA and *alkB* genes

Bands from DGGE gels were excised with a sterile scalpel blade and placed individually into sterile 0.5 mL PCR tubes containing 20 μL sterile ultrapurified water. DNA was eluted at 4°C for 24 hours. PCR amplifications from eluted DNA amplifications were performed as described above with the same primers (Table 2.2) without the GC-clamp. PCR products were purified with UltraClean™ PCR Cleanup Kit (MO BIO Laboratories, Inc., Solana Beach, CA) according to the manufacturer's instructions. Approximately 100 ng of the PCR products were prepared for sequencing with 3.2 pmol of forward primer (without clamp) in a total of 12 μL. Sequencing was performed at the Greenwood Molecular Biology Facility and the Center for Genomic, Proteomic, and Bioinformatic Research Institute at the University of Hawai'i at Mānoa. Sequences were viewed using the chromatograph-viewing program FinchTV (Version 1.3.1) (Geospiza Inc., 2004-2005) and subsequently compared to sequences in the GenBank database through the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Derived nucleotide sequences for each of the target genes were aligned using

the ClustalX program (Version 0.1) (Ramu & Co., 2003). Phylograms were prepared in the PHYLIP program (Version 3.6.5) (J. Felstein, University of Washington, Seattle, 2004). Maximum likelihood analysis was conducted, taking into account the evolutionary changes over time, and tree topology was evaluated by bootstrap analysis using 500 replicates.

2.13 Statistical analyses

The Statistix program (version 8) (Analytical Software, Tallahassee, FL) was used for all statistical analyses. Hydrocarbon concentrations were analyzed by one-way ANOVA on each sampling day followed by specific t-tests between days. Lipase activity data were not normally distributed and were analyzed by the non-parametric Wilcoxon Rank Sum Test. Microbial counts were compared by a two-way ANOVA to determine the effect of plants and hydrocarbons on the numbers of microorganisms of interest after transformation to the base-10 logarithmic scale. For each group of microorganisms a two-way ANOVA was conducted on pooled counts from the 3 plants. One-way ANOVA compared the counts in the 3 rhizospheres followed by least-significant-difference (LSD) comparison of means or two-tailed t-tests on pairs of treatments of interest. A significance level of 0.05 was used in all comparisons.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Degradation of petroleum hydrocarbon contaminants over time

3.1.1 Hexadecane degradation

Concentration of hexadecane in soil decreased in all treatments (planted and unplanted) (Fig. 3.1A). In contrast to results obtained in previous experiments (Sun et al., 2004), milo did not enhance hydrocarbon depletion. A one-way ANOVA, carried out on all contaminated treatments on days 56 and 114, indicated that the hexadecane concentration was significantly lower in unplanted soil and soil planted to oleander than in soil planted to milo and buffelgrass. No significant difference was found between unplanted soil and soil planted to oleander. On day 56, an average of 178 mg of hexadecane/kg of soil (20% of the concentration at the start of the experiment) was left in the unplanted soil and in soil planted to oleander. Soils planted to milo and buffelgrass contained the same concentration of contaminant, 323 mg of hexadecane/kg of soil (37% of the concentration at the start of the experiment). On day 114, an average of 59 mg of hexadecane/kg of soil (7% of the concentration at the start of the experiment) was detected in unplanted soil and in soil planted to oleander. Soils planted to milo and

buffelgrass had virtually identical concentrations of hexadecane, 126 mg/kg of soil on average (14% of the concentration at start). Thus, milo and buffelgrass retarded the degradation of hexadecane as the concentration of hexadecane on days 56 and 114 was approximately twice the concentration in the unplanted soil and soil planted to oleander. A significant decrease in hexadecane concentration occurred between day 56 and 114 as indicated by a t-test on the contaminant concentration in unplanted soil.

The results obtained in this experiment, where an attempt was made to maintain similar levels of soil moisture in all of the treatments, indicate that microbes in unplanted soil are capable of degrading hexadecane on their own (Fig. 3.1A). The results obtained with oleander, which did not increase the level of hexadecane degradation, are similar to the results obtained with diesel fuel in a previous experiment (Sun et al., 2004). However, the results obtained for milo contrast with those of previous experiments in the greenhouse (Sun et al., 2004) where the unplanted soil, contaminated with diesel, was irrigated in the same manner as the planted soil to simulate natural conditions. Under natural conditions, the amount of rain received by all treatments would be the same. This would result in wetter conditions in unplanted soil, which would impair degradation of hydrocarbons. Buffelgrass, which like milo, decreased microbial degradation of hexadecane in this experiment, has not been tested previously with diesel fuel in salt – containing soil and it is not known how it behaves under natural conditions.

Beside differences in soil moisture between this experiment and the previous one (Jones et al., 2004), other reasons for milo to have performed differently could be the

nature of the hydrocarbons (diesel fuel vs. hexadecane) and the concentrations of the hydrocarbons (10,000 mg diesel/kg of soil vs 1,000 mg hexadecane/kg of soil).

These conflicting results suggest that the role of the plant is complex and may have to be tested under various conditions.

3.1.2 Phenanthrene degradation

Fig. 3.1B indicates that phenanthrene was rapidly degraded under planted and unplanted conditions; no phenanthrene was detected in any treatment on day 56. Thus the role of the plants in the degradation of phenanthrene cannot be determined from this experiment.

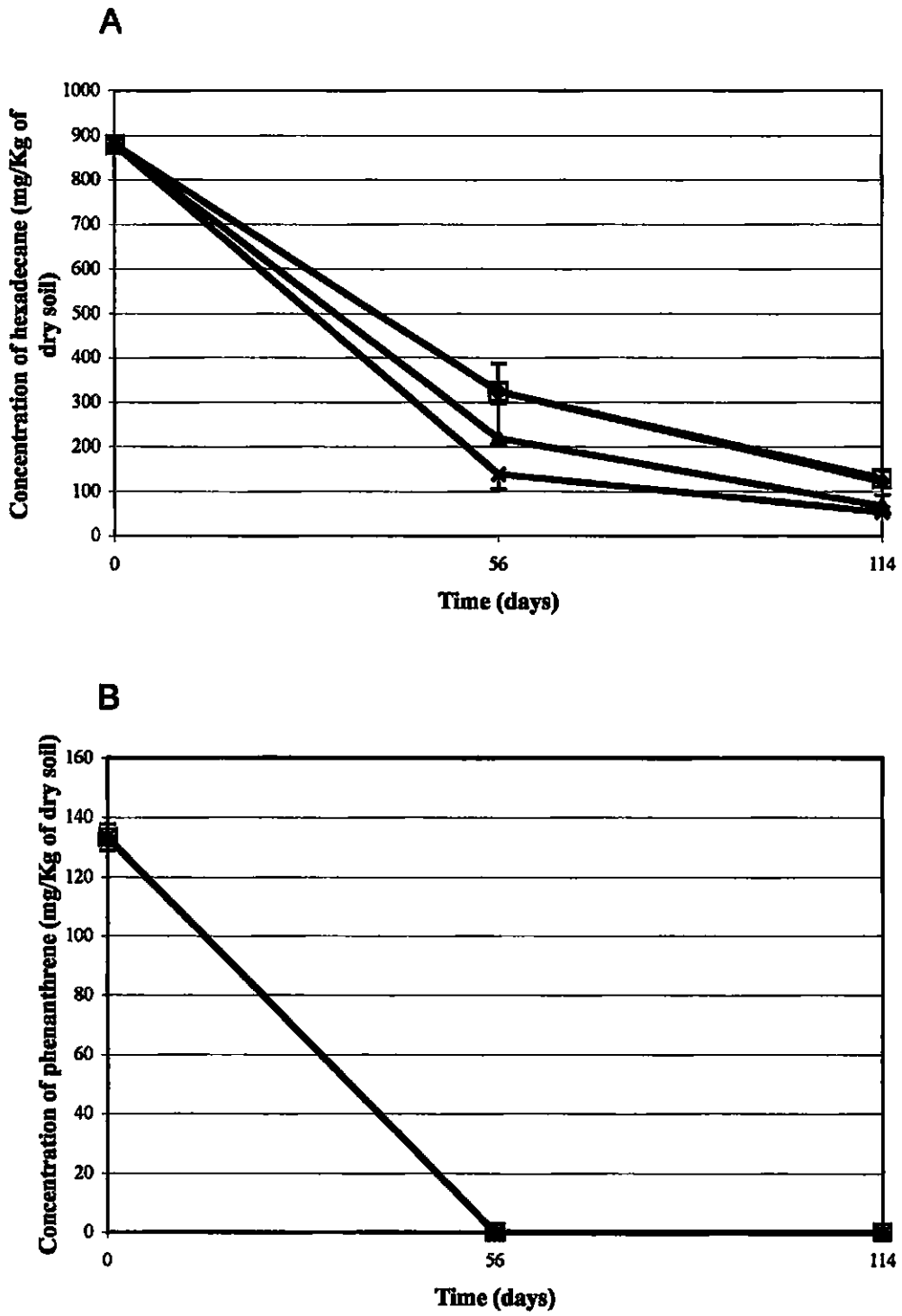


Figure 3.1. Depletion of hexadecane (A) and phenanthrene (B) in planted and unplanted treatments. ×, unplanted soil; □, Milo; ▲, Oleander; ◆, Buffel grass.

3.2 Response of plants to hydrocarbon contaminant

The impact of the hydrocarbon contaminants on the plants was determined at 56 and 114 days (Table 3.1). In general the growth of the contaminated plants was similar to the growth of the uncontaminated plants on both days. Milo plant height and dry weight of the root in the lower pot, where the root was in direct contact with the contaminant, were negatively affected by the contaminant on day 56. Between day 56 and 114 the concentration of contaminant was lower and milo was not affected on day 114. In a previous experiment (Sun et al., 2004) milo was not affected by 10 g of diesel fuel/kg of the same soil after 98 days. Milo had a mild leaf fungal infection in this experiment, for which it was treated. It is possible that this stress made the plants more sensitive to the contaminant. The impact of contaminant on buffelgrass is more difficult to explain. Shoot dry weight was significantly lower in contaminated than in uncontaminated soil on day 56. However no other parameter, such as root dry weight in the lower pot where the root would be more impacted by the contaminant, was affected. On day 114 the whole root dry weight was significantly decreased by the contaminant whereas the dry weight of the root in the lower pot was unaffected. The opposite would be expected.

The results obtained for plant height and root dry weight of milo on day 56 (Table 3.1) indicate that the two-section pot design allows for the firm establishment of roots in upper uncontaminated soil. Although the roots in the contaminated lower section of the pot are negatively affected by the contaminant, the root system as a whole is not affected.

This seems to suggest that plants that can be strongly rooted in the upper section of the soil profile in the field could survive if a portion of their roots is growing in a subsurface contaminated zone.

Upon transplantation to the upper pot on day 0 the roots of all plants reached approximately to 2 cm from the bottom of the upper pot. Root penetration through the lower pot at harvest times for both the contaminated and uncontaminated lower pots is shown in Table 3.2. By day 56 most roots were approximately 2 cm from the bottom of the lower pot and some reached to the bottom. By day 114 all roots reached the bottom except for 2 plants of contaminated oleander, which were 2 cm away from the bottom. When the roots did not reach the bottom, the bottom soil devoid of roots was discarded prior to mixing the soil from the whole pot for hydrocarbon analysis. A picture of the roots at day 56 is shown in Fig. 3.2.

Table 3.1. Impact of contaminant (1000 mg hexadecane + 200 mg phenanthrene /kg of soil) on plant growth (n=4)

A. Plant height (cm)

Day & Status	Milo		Oleander		Buffelgrass	
	Mean	SD	Mean	SD	Mean	SD
Day 56						
Uncont.	32	1.95	10.8	1.1	33.8	6.0
Contam.	28	1.83	13.2	1.5	32.7	8.5
	* [a]		NS		NS	
Day 114						
Uncont.	39.8	3.52	15.75	2.47	39.25	10.5
Contam.	39.5	1.00	14.83	2.75	42.25	5.8
	NS		NS		NS	

[a] t-test significance: * $p<0.05$; NS, not significant

Table 3.1. (Contd.) Impact of contaminant (1000 mg hexadecane + 200 mg phenanthrene /kg of soil) on plant growth (n=4)

B. Shoot dry weight (g)

Day & Status	Milo		Oleander		Buffelgrass	
	Mean	SD	Mean	SD	Mean	SD
Day 56						
Uncont.	5.54	0.16	2.31	0.14	2.54	0.16
Contam.	4.97	0.68	3.43	0.38	2.05	0.18
	NS		* [a]		*	
Day 114						
Uncont.	6.52	1.03	5.14	1.43	3.32	0.48
Contam.	6.75	0.35	5.85	0.91	3.02	0.23
	NS		NS		NS	

Table 3.1. (Contd.) Impact of contaminant (1000 mg hexadecane + 200 mg phenanthrene /kg of soil) on plant growth (n=4)

C. Root dry weight (g) in bottom pot

Day & Status	Milo		Oleander		Buffelgrass	
	Mean	SD	Mean	SD	Mean	SD
Day 56						
Uncont.	0.42	0.13	0.14	0.02	0.92	0.19
Contam.	0.21	0.11	0.06	0.05	0.64	0.17
	*		NS		NS	
Day 114						
Uncont.	0.68	0.17	0.29	0.12	2.07	0.31
Contam.	0.60	0.06	0.23	0.13	1.64	0.26
	NS		NS		NS	

Table 3.1. (Contd.) Impact of contaminant (1000 mg hexadecane + 200 mg phenanthrene /kg of soil) on plant growth (n=4)

D. Whole root dry weight (g)

Day & Status	Milo		Oleander		Buffelgrass	
	Mean	SD	Mean	SD	Mean	SD
Day 56						
Uncont.	3.02	0.48	0.98	0.09	3.06	0.60
Contam.	3.03	0.32	1.36	0.35	2.54	0.13
	NS		NS		NS	
Day 114						
Uncont.	4.87	0.50	2.79	1.74	6.36	0.72
Contam.	4.74	0.33	3.22	0.85	5.31	0.33
	NS		NS		*	

Table 3.2. Depth of root penetration in the lower pot at harvest times

Plant	Depth of penetration (cm from the bottom) of roots in the lower pot:		Root characteristics
	Day 56	Day 114	
Milo	0-2	0	Roots throughout the pot. Brittle lateral roots.
Oleander	0-2.5	0-2	Fine roots distributed throughout the pot.
Buffelgrass	0	0	Main root in a palisade configuration along the pot walls. Network of lateral roots in the middle of pot.

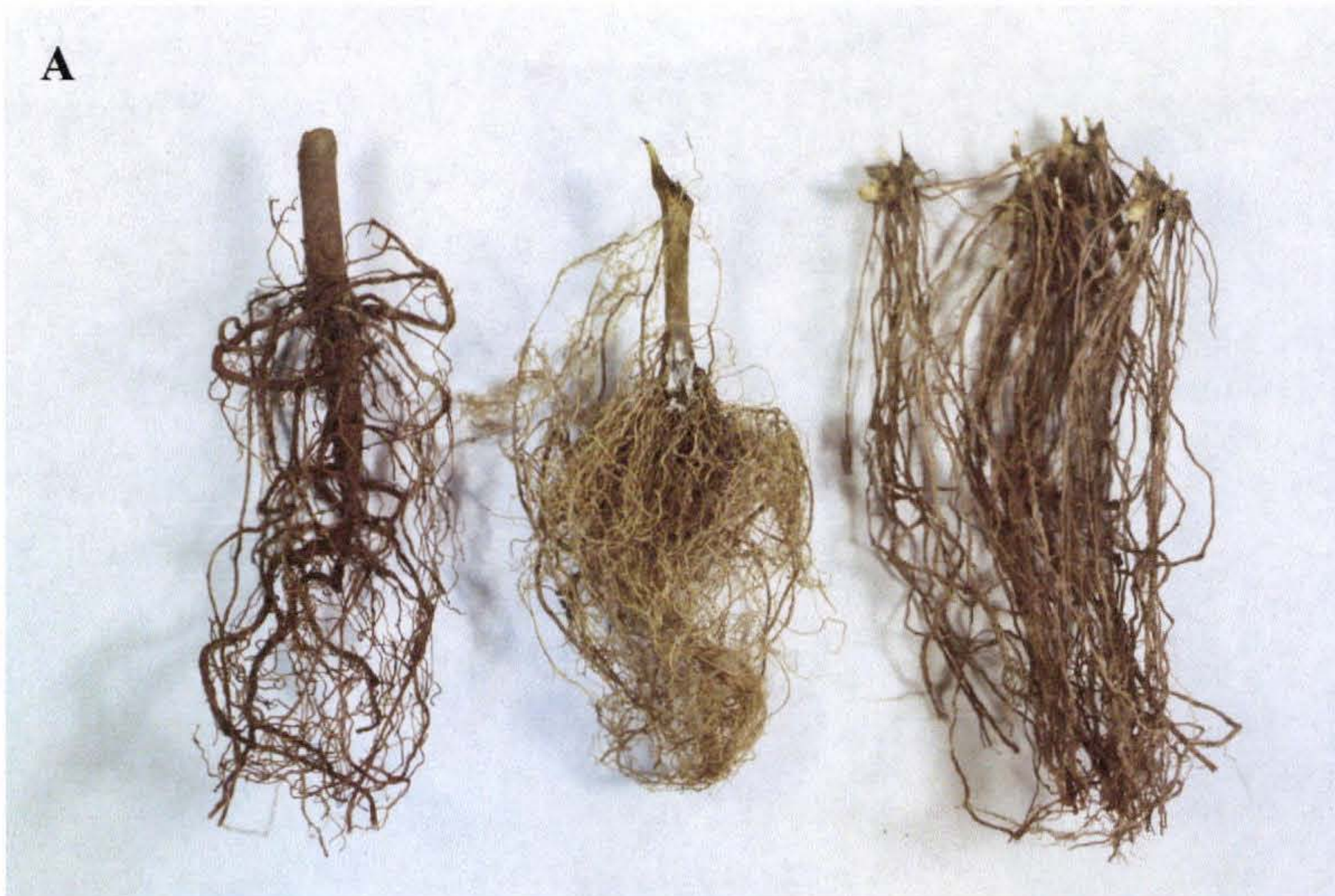


Figure 3.2. Roots of contaminated treatments on day 56. A. Top roots grown in uncontaminated soil. B. Bottom roots grown in contaminated soil supplemented with 1% NaCl, after removal of rhizosphere soil for microbial enumerations. From left to right: milo, oleander and buffelgrass.

3.3 Response of bacterial populations to petroleum hydrocarbons and plant roots

A two-way ANOVA was carried out on the counts of individual groups of bacteria at day 56 and day 114 to determine the effects of hydrocarbon contamination and planting on the counts of heterotrophic bacteria, hexadecane-degrading bacteria, and phenanthrene-degrading bacteria (Table 3.3). Data from the 3 plants were pooled for the two-way ANOVA test. The data will be discussed individually in the following sections.

Table 3.3. Significance of the effect of plants and petroleum hydrocarbons (1000 mg hexadecane + 200 mg phenanthrene/kg of soil) indicated by a two-way analysis of variance of the log of the microbial counts/kg of soil 56 and 114 days after the start of the experiment.

Factors Tested	Value and significance of F tests on the log of counts/kg soil for:					
	Heterotrophic Bacteria		Hexadecane-degrading Bacteria		Phenanthrene-degrading Bacteria	
	Day 56	Day 114	Day 56	Day 114	Day 56	Day 114
Plant[a]	45.6 **[b]	54.8 **	39.2 **	25.4 **	36.7**	NA[d]
Hydrocarbon	4.4 *	1.6 NS[c]	39.9 **	31.7 **	50.9**	NA
Interaction	6.3 *	1.4 NS	7.5 *	12.6**	2.3 NS	NA

[a] Log values of the microbial counts in the rhizosphere of the 3 plants were pooled for the analysis
[b] Levels of significance: *, $p<0.05$; **, $p<0.01$
[c] NS, not significant
[d] NA, not applicable; due to inequality of variances ANOVA could not be performed

3.3.1 Heterotrophic bacteria counts

On day 0 the counts of heterotrophic bacteria in contaminated soil were significantly lower (37%) than the counts in uncontaminated soil (Fig. 3.3A). This suggests that the addition of hydrocarbons to the soil may be harmful to some or all heterotrophic-bacterial populations. Also, the levels of the heterotrophic populations in uncontaminated unplanted soil remained constant from day 0 to day 114. This suggests that the heterotrophic bacteria did not grow on soil organic matter. A low level of organic carbon (0.9%) was found in this soil (Sun et al., 2004). In contaminated unplanted soil there was a significant increase in the levels of heterotrophic bacteria on day 56 as compared to day 0.

The two-way ANOVA using pooled plant rhizosphere counts on day 56 (Table 3.3) indicates a higher plant effect than hydrocarbon effect and a small interaction. The statistical results may be explained using Fig. 3.3A. The sole plant effect on day 56 is the difference between the levels of heterotrophic bacteria in unplanted uncontaminated soil (white bars) and the levels of those bacteria in the rhizospheres of the uncontaminated respective plants (white bars). The sole hydrocarbon effect is the difference between the levels of heterotrophic bacteria in uncontaminated (white bars) and contaminated soil (black bars) on day 56. The plant effect is significantly higher than the contaminant effect.

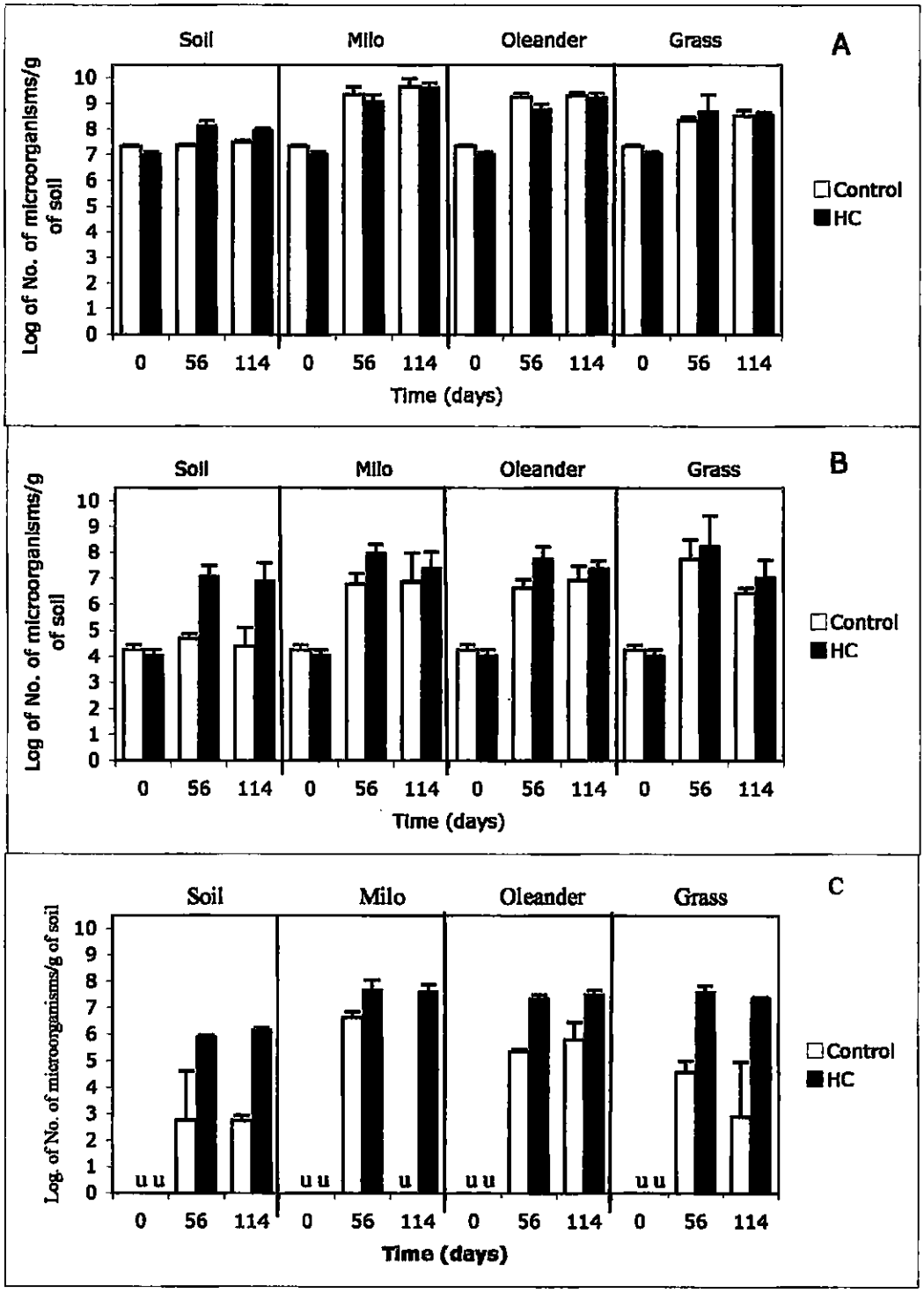


Figure 3.3. Levels of heterotrophic bacteria (A), hexadecane-degraders (B), and phenanthrene degrading bacteria (C) in soil and rhizospheres. u, undetectable. Error bars are standard deviations (n=4).

In contrast, there is no hydrocarbon effect in the planted treatments (white vs. black bars for each plant). The difference in contaminant effect in unplanted and planted soil is the basis of the interaction. The preference of the heterotrophic bacteria for rhizodeposition is in agreement with the results of a previous study using diesel fuel (Jones et al., 2004).

The two-way ANOVA using pooled plant rhizosphere counts on day 114 (Table 3.3) indicates that there was only a plant effect. In both the uncontaminated and contaminated planted treatments (Fig. 3.3A) the heterotrophic bacteria counts were significantly higher than in unplanted soil. The two uncontaminated woody plant rhizospheres were significantly more favorable to heterotrophic bacteria than the grass rhizosphere on days 56 and 114. So were the counts of heterotrophic bacteria in contaminated rhizospheres on day 114. In contaminated rhizospheres on day 56, all plants behaved similarly (one-way ANOVA and LSD tests on subsets of data, $p<0.05$, n=4).

As expected, counts in uncontaminated and contaminated unplanted soil were not significantly different on day 114 since there was virtually no hexadecane left in the soil (Fig. 3.1A). No elevated numbers of starvation-surviving "generalists" were observed in contaminated bare soil on day 114 (Heterotrophic bacteria counts were not significantly higher in contaminated than uncontaminated bare soil according to the two-way ANOVA) (Fig. 3.3A).

3.3.2 Hexadecane-degrading bacteria counts

Unlike the heterotrophic bacteria, the hexadecane degraders were not adversely affected by the hydrocarbons on day 0 (Fig. 3.3B). Also, the level of hexadecane degraders in uncontaminated soil remained virtually the same throughout the experiment. This indicates that the hexadecane degraders did not use the *in situ* organic carbon pool, at least to a significant degree. In contaminated unplanted soil the level of hexadecane degraders increased significantly between day 0 and 56.

The two-way ANOVA using the 3 pooled plant-rhizosphere counts on day 56 (Table 3.3) indicates a similar effect of plant and hydrocarbon and a small interaction between plant and hydrocarbon effects. The magnitude of the sole plant effect, the difference between the levels of hydrocarbon degraders in unplanted uncontaminated soil (white bars) and the levels of those bacteria in the rhizospheres of uncontaminated plants (white bars), was similar to the magnitude of the sole hydrocarbon effect (difference between contaminated and uncontaminated unplanted soil counts on day 56 (Fig. 3.3B). This indicates that the hexadecane degraders have no preference for either the rhizodeposition or the hexadecane. Thus they behave like diesel-degraders reported in a previous experiment (Jones et al., 2004). The contaminant effect was higher in the unplanted than in the planted treatments (Fig. 3.3B), which accounts for the interaction between plant and contaminant effects.

The counts of hexadecane degraders were significantly higher in the plant rhizospheres (three plant average) than in unplanted soil under uncontaminated conditions on day 56. A one-way ANOVA followed by an LSD test among the log counts of hexadecane degraders in uncontaminated rhizospheres indicated that buffelgrass supported higher counts of hexadecane degraders than the woody plants (Fig 3.3B).

T-tests comparing the levels of hexadecane degraders in contaminated and uncontaminated rhizospheres of each plant indicated a hexadecane effect for both milo and oleander but not for buffelgrass at day 56. Thus, it seems that in the rhizosphere of contaminated buffelgrass the hexadecane degraders may preferably use the rhizodeposits whereas in the rhizosphere of milo and oleander they may use both the rhizodeposits and the hexadecane. Supportive evidence for phylogenetic differences between hexadecane degraders from the rhizosphere of woody plants and those from the rhizosphere of buffelgrass, as seen by 16S rDNA partial sequences, is presented in section 3.5.2.4.2.

The pattern of hydrocarbon depletion (Fig. 3.1A) and the pattern of the changes in size of the populations of hexadecane degraders (Fig. 3.3B), indicate a significant increase in degraders in planted and unplanted treatments concomitant with a marked hydrocarbon depletion on day 56. This negative correlation is typical of the hydrocarbon biodegradation process (Wang and Bartha, 1990; Atlas, 1991).

A two-way ANOVA, using pooled plant rhizosphere counts, carried out on the hexadecane degraders on day 114 showed, as on day 56, both an effect of plant and hydrocarbon and some interaction (Table 3.3). Under uncontaminated conditions there was a higher number of hexadecane degraders in the plant rhizospheres than in unplanted soil but no difference in numbers was found among the three plants (Fig 3.3B).

In unplanted soil, there was a positive effect of contamination on the counts of hexadecane degraders (Fig. 3.3B) although there was virtually no hexadecane left in the soil on day 114 (Fig. 3.1A). Conversely, hexadecane degraders did not respond to the contaminant in the presence of plants on day 114 (the counts for each plant were similar in contaminated and uncontaminated rhizospheres) (Fig 3.3B).

By day 114, the concentration of hexadecane had dropped to very low levels (*ca.* 100mg/kg of soil), as the degradation curve is usually a first-order reaction type (as suggested by Fig. 3.1A). Thus the very small concentration of hexadecane had virtually no effect on the hexadecane degrader counts in the three planted treatments (Fig. 3.3B). However, in the unplanted treatment, despite the virtual absence of hexadecane and the inability of the hexadecane populations to grow on soil organic matter, the levels of hexadecane degraders remained high (Fig. 3.3B) compared to their levels in uncontaminated soil (*ca.* 2.5 log units higher). These results suggest that in unplanted soil, growth on hexadecane had conferred the ability on hydrocarbon-degrading bacteria to remain in the environment in a starvation survival stage (van Elsas and Oberbeek, 1993). Studies are needed to determine which sources of carbon extend the likelihood of

survival of microbes in the absence of nutritional resources. Our data may indicate that bacteria, capable of degrading hydrocarbons are specialists with some physiological characteristics lacking from the heterotrophic "generalists". Alvarez (2003) and Alvarez et al. (2001) have shown that non-sporulating *Actinobacteria* were capable of producing triacylglyceride storage material while growing on various alkanes under conditions where nitrogen was limiting. Alvarez et al. (2000) have determined that stored triacylglycerides could be used during periods of starvation by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Stored triacylglycerides have also been proposed as a carbon source for antibiotic production by *Streptomyces* (Olukoshi and Packter, 1994). In light of Alvarez' group results, it seems plausible that some of our hydrocarbon-degraders may be able to store high-energy compounds such as triacylglycerides while growing on alkanes. These storage compounds could be used during starvation periods and could confer an advantage to the microbes capable of synthesizing them. Tests need to be performed in the future to determine the nature of the storage products in our hexadecane degraders.

In conclusion, in this project, no correlation was found between the numbers of hexadecane degraders in a particular treatment (planted and unplanted) and the degree of hydrocarbon decrease (Fig 3.3A and 3.1A). During the active degradation of hexadecane (day 56) the counts of hexadecane degraders were significantly higher (average of 1 log unit) in the rhizospheres than in unplanted soil but no increase in degradation of hexadecane occurred in planted treatments. On day 114 (Fig 3.3), counts in planted and unplanted treatments were not significantly different (planted treatment counts were two-

fold higher on average than the unplanted treatment counts). However, the concentration of hexadecane in the pots planted to milo and buffelgrass, although low, were significantly higher than those in the unplanted soil and soil planted to oleander (Fig. 3.1A).

These results confirm what was seen previously in this soil for diesel fuel biodegradation; the large size of the hydrocarbon-degrading populations in contaminated rhizospheres was not always indicative of active biodegradation activity (Jones et al., 2004). Thus it seems that in planted treatments the levels of hydrocarbon degraders, such as alkane degraders, are not a reliable indicator of hydrocarbon depletion because the alkane degraders have no preference for hydrocarbon or rhizodeposition and may increase in numbers in response to plants.

In view of these results, alternative indicators of hydrocarbon depletion in the presence of plants need to be examined. In the following sections soil lipase activity of hexadecane degraders will be investigated as well as the qualitative differences among the hexadecane degraders in soil and rhizospheres of the 3 plants.

3.3.3 Phenanthrene-degrading bacteria counts

The phenanthrene-degrader counts on day 0 were below detection level (*ca.*1000 cells/g of soil) for valid statistical analysis and were recorded as "undetectable" (Fig.

3.3C). In uncontaminated unplanted soil, the levels of phenanthrene degraders were barely at detection limit levels on days 56 and 114. Thus they were also incapable of growing on soil organic matter.

A two-way ANOVA using the phenanthrene-degrader counts from pooled-plant data on day 56 indicates both a plant and a hydrocarbon effect without interaction between the two effects (Table 3.3). This means that the counts of phenanthrene degraders were significantly higher 1) under planted than unplanted conditions across contaminated and uncontaminated conditions and 2) under contaminated than uncontaminated conditions across planted and unplanted conditions. The hydrocarbon effect was higher than the plant effect indicating the preference of phenanthrene degrading bacteria for the hydrocarbon as seen in a previous experiment with diesel contaminant in the same soil (Jones et al., 2004).

A one-way ANOVA carried out on the log counts of phenanthrene-degrading bacteria in the rhizosphere of the 3 uncontaminated plants on day 56 showed differences between the plants (Fig. 3.3C). An LSD test indicated the milo rhizosphere harbored significantly higher counts than the oleander rhizosphere and the oleander rhizosphere harbored higher counts than the buffelgrass rhizosphere. There were no differences among the counts in the rhizospheres of the 3 plants under contaminated conditions. Thus one can deduce from these two relationships that the phenanthrene effect in the 3 rhizospheres is higher for buffelgrass than for oleander and higher for oleander than for milo. Presumably the more useful plant to promote an increase in counts of phenanthrene

degraders under uncontaminated conditions and a switch to growth on phenanthrene when the root penetrates into a contaminated area (model proposed by Jones et al., 2004) would be buffelgrass.

On day 114, the high variability in the data precluded the use of a two- way ANOVA and the results were analyzed by t-tests between data of interest (Fig. 3.3C). Under uncontaminated conditions only oleander maintained significantly higher numbers of phenanthrene degraders than the numbers in unplanted soil. A hydrocarbon effect was found in the contaminated rhizospheres of oleander and buffelgrass by carrying out t-tests between the counts in the rhizospheres of each uncontaminated and contaminated plant. Since there was no plant effect on the phenanthrene degraders in milo rhizospheres on day 114, the counts in contaminated rhizospheres were considered as hydrocarbon effect.

Thus, although all of the phenanthrene had already disappeared by day 56 (Fig. 3.1A), there was a strong hydrocarbon (phenanthrene) effect on the phenanthrene-degraders in contaminated treatments on both day 56 and day 114 under both planted and unplanted conditions (Fig. 3.3C). Since the plant rhizospheres did not sustain high levels of phenanthrene degraders under uncontaminated conditions except for oleander on day 114, it is unlikely that the phenanthrene degraders were growing on rhizodeposition in the rhizospheres of milo and buffelgrass. In unplanted soil the phenanthrene degraders would not be able to grow since they did not grow on soil organic matter in uncontaminated bare soil throughout the experiment (Fig 3.3C). Thus it seems they were capable of remaining for several weeks in starvation survival stage. We have observed

this situation previously in a soil from a site in Pearl Harbor after complete bioremediation when virtually no organic carbon was left, and no dehydrogenase activity was detected. However, 10^8 live phenanthrene-degrading bacteria/g of soil were detected (Robert, 1996). These results suggest that in unplanted soil and in the rhizosphere of milo and buffelgrass, growth on phenanthrene had conferred the ability to the phenanthrene-degrading bacteria to remain in the environment in a starvation survival stage, as seen for hexadecane. The metabolic basis for the persistence of high numbers of phenanthrene-degrading bacteria in the environment after the phenanthrene is gone remains to be elucidated. The heterotrophic bacteria (total bacteria or generalists), which showed a preference for plant-derived compounds (Table 3.3), did not display an increase in numbers in contaminated unplanted soil as compared to uncontaminated soil on day 114 (Fig. 3.3A). It is tempting to speculate they failed to produce populations with special storage material to be used during starvation periods because they preferred the rhizodeposition.

3.4 Evaluation of lipase activity as an indicator of the activity of alkane-degrading microorganisms and biodegradation of alkane-type hydrocarbons

It was shown in the previous study (Jones et al., 2004) and in this study (section 3.3.2) that alkane degraders in this soil have no preference for alkanes or rhizodeposition.

Thus some alkane degraders may grow on plant compounds instead of degrading alkanes and counts of alkane degraders may not mirror alkane degradation.

Soil lipase activity, which was shown in the introduction (section 1.5) to reflect the activity of the alkane degraders, because lipase production is induced by growth on alkanes, may provide a more accurate indication of the progress of bioremediation. Under our experimental conditions where pH and temperature are optimal and substrate concentration saturating (Margesin et al., 2002), the reaction is zero order with respect to substrate. Thus enzyme activity is proportional to the amount of enzyme (Tabatabai, 1994; Alef and Nanipierri, 1995; Bell and Bell, 1988). In this experiment the increase in lipase activity, which occurs under contaminated conditions, is due to an increase in lipase production during growth of the hexadecane degraders, and thus reflects the activity of this population of microorganisms.

The changes in lipase activity occurring over time in planted and unplanted treatments are shown in the presence or absence of hexadecane contamination (Fig. 3.4). Most of the data were not normally distributed; therefore they were analyzed by the non-parametric Wilcoxon rank sum test where pairs of samples are compared. Lipase activity in hexadecane-contaminated samples was significantly higher than in uncontaminated samples at all sampling times. These results agree with those of Margesin et al. (2000a) for a soil contaminated with 10,000 mg of diesel/kg of soil. They found a high lipase activity in contaminated soil but no activity in uncontaminated soil or soil contaminated with 1,000 mg PAH/kg soil. It seems that the hexadecane degraders became active very

rapidly. Lipase activity on day 0 in contaminated soil was already significantly higher than in uncontaminated soil. This was just 24 hours after the soil was treated with contaminant, water, and fertilizer and left at room temperature. Lipase activity has not been used so far as an indicator of hydrocarbon degradation in the presence of plant roots. Our results show that microbial lipase production in planted soil in the absence of contaminant is minimal and will not interfere with lipase activity measurements in contaminated soil.

The period of lipase activity increase in contaminated treatments between day 0 and day 56 corresponded to the period when hexadecane concentration dropped from a 100% to an average 30% of the original concentration (Fig. 3.1A). In contaminated soil on day 56, the hexadecane concentration was significantly lower in unplanted soil and oleander rhizosphere than in the rhizospheres of milo and buffelgrass. Since extracellular lipase production is induced by alkane uptake and metabolism (as explained in section 1.5) it is plausible that lipase production be proportional to the concentration of hydrocarbon available at the time of lipase activity measurement. According to this rationale one would expect the levels of lipase activity to be lower for unplanted soil and soil planted to oleander than for soil planted to milo and buffelgrass. Values of lipase activity for milo and buffelgrass were virtually equal while the values for oleander and unplanted soil are grouped together at a lower value (Fig 3.4). However, the 4 treatments were found not to be significantly different from each other by the Wilcoxon rank sum test. Therefore the lipase activity assay was not capable of separating the two groups of data.

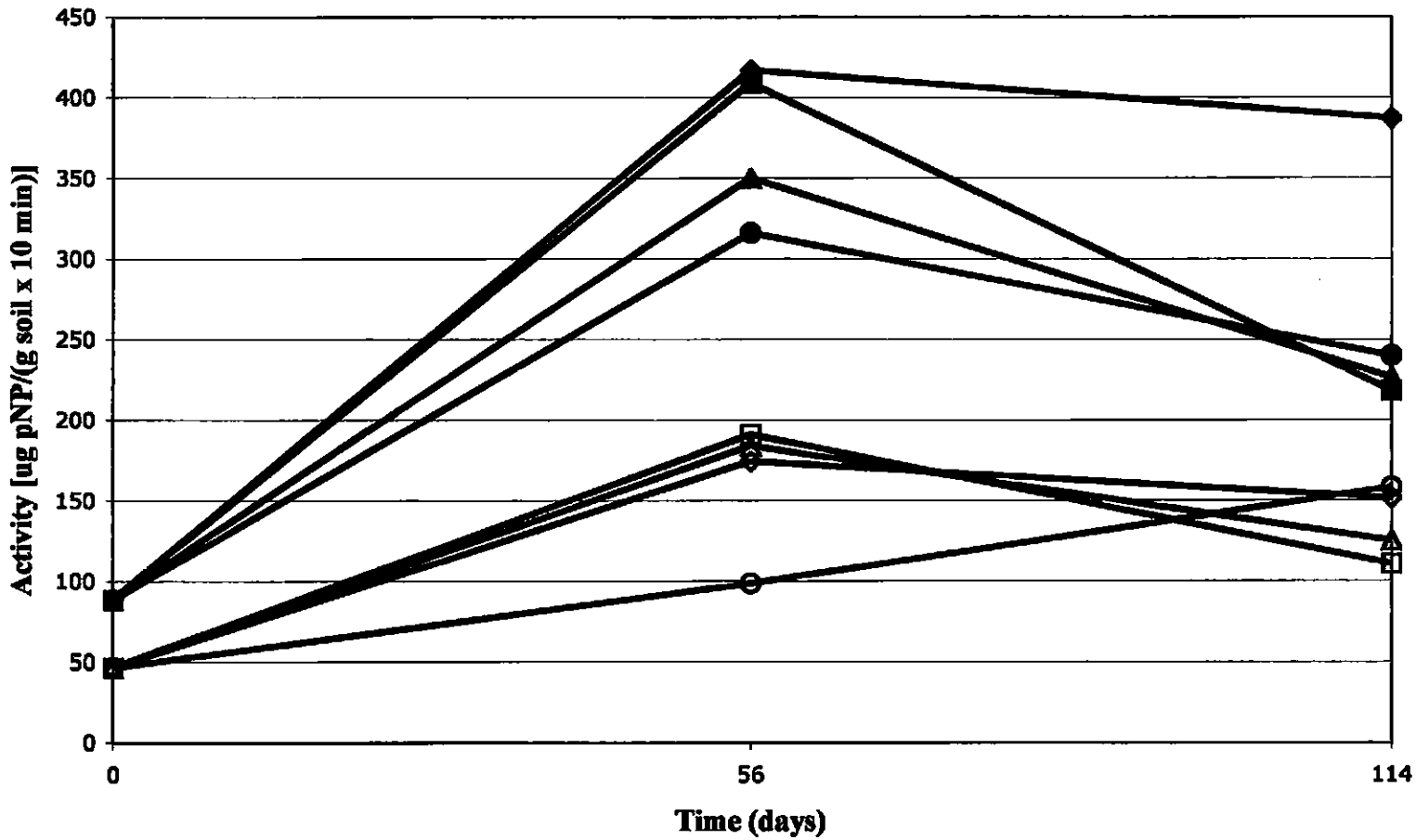


Figure 3.4. Lipase activity in contaminated (closed symbols) and uncontaminated (open symbols) planted and unplanted treatments. Symbols: (○,●) unplanted soil; (□,■) Milo; (△,▲)Oleander; (◇,◆) Buffelgrass. Average CV=20.9% (n=4).

The high variability in the data (coefficient of variation was 20.9%) may have been a major factor. To decrease the variability one could use a higher amount of soil (0.5 g instead of 0.1 g) in the assay and more subsamples (here 3 were used) to reduce the effect of soil heterogeneity. A second source of variability is the production of condensation water on the cuvet in the spectrophotometer due to the low temperature of the sample. A spectrophotometer that could hold the sample near 0°C would improve the technique. However, this factor was probably not responsible for much of the variability because the standard curve (Table 2.1, Fig. 2.2) showed very little variability in the absorbance readings.

In contaminated soil on day 114, the lipase activity remained as high as on day 56 for buffelgrass. Conversely, lipase activity decreased significantly from the values on day 56 for the soil planted to the two woody plants and the bare soil. In all treatments on day 114, the concentration of hexadecane had decreased to 10% on average of the original concentration and the rate of hydrocarbon degradation had slowed down between day 56 and day 114 (Fig 3.1A). However the difference in hexadecane concentrations in soil between the two days was still significant. It would be expected that less lipase would be formed during this period and that some of the lipase produced previously would start being degraded in soil. A possible explanation, which needs more study, is that buffelgrass-derived soil organic matter, such as root exudates, may be different in nature from that of the other plants and may have adsorbed lipase to a higher extent. Thus, soil microorganisms would have degraded less lipase by day 114. In support of the release of different organic compounds by different roots are data regarding phenolic compounds.

Liste and Alexander (1999) have shown high levels of phenolic compounds in the roots of corn, sunflower, pea, and soybean and low levels in the roots of wheat and radish. Whitehead et al. (1982) reported that grasses produced more phenolics than dicotyledonous species. In our lab phenolics from tropical wooden plant roots were extracted and milo was found to contain more phenolics than kou and false sandalwood (Tang et al., 2004). However, buffelgrass roots from our Hickam experimental site contained significantly higher amounts of phenolics than the milo roots (F. Robert, unpublished data). Thus it is possible that buffelgrass growth causes the production of soil organic matter that is different from that of the woody plants and might bind lipase more tightly thus resulting in a lesser degree of lipase degradation with time.

In uncontaminated soil on day 56, lipase activity in the planted treatments was significantly higher than in unplanted soil. This is plausible since it is well known that plant leaves and stems are covered with waxy cuticles, which are mixtures of monoesters of fatty alcohols and long chain fatty acids, long chain alkanes (C25-C35 or longer), and other related compounds (Dinel et al., 1990).

In uncontaminated soil on day 114, there was no significant difference in lipase activity among the planted and unplanted treatments. Between day 56 and 114 the lipase activity of the planted treatments significantly decreased whereas the lipase activity of the unplanted soil did not change significantly as per the Wilcoxon rank sum test, probably due to the variability in the data.

In conclusion, although lipase activity measurements in this experiment did not permit separation of the plant treatments according to their effect on the depletion of hexadecane in the soil, it nonetheless indicated the progression of bioremediation, i.e. lipase activity increased during the major decrease in hexadecane and once low levels of hexadecane were reached, lipase activity either decreased or remained stable. Further experiments with more sampling points and more subsamples would provide a more accurate view of lipase activity as an indicator of hydrocarbon depletion. Lipase activity should also be determined in planted soil under circumstances where plant roots have a positive effect on hydrocarbon depletion (Sun et al., 2004) to test further the ability of lipase activity to differentiate between plants that are efficient or inefficient for phytoremediation.

3.5 Diversity of hexadecane-degrading bacteria determined by PCR-DGGE analysis of partial sequences of 16S rDNA.

The variable V6-V8 region of the 16S rRNA gene was chosen because it produces longer amplification products than the V1 or V3 regions (which are more variable), while reportedly producing good DGGE profiles (Yu and Morrison, 2004). Schmalenberger et al. (2001) showed higher discrimination among their PCR-single-strand-conformation polymorphism (SSCP) profiles of bacteria in the corn rhizosphere with the V6-V8 region than with other variable regions.

3.5.1 Use of partial 16S rDNA sequences to identify hexadecane-degrading bacteria isolates

Pure cultures of hydrocarbon-degrading bacteria were prepared from MPN and phenanthrene plates inoculated with soil from pots used in a previous experiment (Jones et al., 2004), and were examined in this study for their hydrocarbon-degrading capabilities and other features (Table 3.4). These bacteria were identified by sequencing the V6-V8 region of the 16S rDNA and were used as reference strains in section 3.6.

Table 3.4. Characteristics of putative hydrocarbon-degrading bacteria cultivated from Hickam Air Force Base soil (Jones et al., 2004) and determined in this study.

Isolate Number	Isolate Name	Partial 16S rDNA sequence analysis [a]		Gram staining & morphology (1/2 TSA)	Rate of growth (30°C)	Colony			Degradation potential [b]
		Possible ID	Identity (%)			Color	Shape	Texture (gum production)	
418	HBPRI4	*Mycobacterium* sp. SRB1151-113 (AB010910)	99.1	G+, rods	slow	orange	round, convex	dull (-)	Die, MO, Hex, Pri
428	MDPRI5	*Mycobacterium confluentis* (AJ634379)	99.7	G+, rods	very slow	yellow	round	dull (+/-)	Die, MO, Hex, Pri
429	MOPRI6			G+, rods	very slow	pink-orange	round, flat	dull, dry (-)	Die, MO, Hex, Pri
443	PKC32D1	*Cellulosimicrobium* sp. TUT1242 (AB188222)	100	G+, cocci	normal	light yellow	round, convex	dull-waxy (+/-)	Die, MO, Hex, Pri
	PBN606	*Pseudomonas indica* strain IMT40 (AF302796)	100	G-, rods	fast	beige center w/clear edges	round, flat	glossy (+)	Die, Hex
	PFSW607	*Pseudomonas* sp. (AM114531)	99.7	G-, rods	fast	white	irregular, flat	glossy (+)	Die, Hex
	PNOD613	*Sinorhizobium* sp. WK6 (AY196140)	99.7	G-, rods	normal	beige	oval, flat	glossy (+)	Phe, Die [c], Hex [c]

[a] GenBank Accession Number in parentheses

[b] Die, Diesel Fuel No.2; MO, Mineral Oil; Hex, Hexadecane; PRI, Pristane; PHE, Phenanthrene (halos on phenanthrene plates)

[c] Inconsistent results

3.5.2 Effect of plants and hydrocarbons on the diversity of dominant hexadecane-degraders as a function of time.

3.5.2.1 DNA extraction from pooled MPN wells

DNA was extracted from pooled positive MPN wells at the highest dilution (e.g. Fig. 3.5, 2 wells at the10^{-7} dilution). The rationale was that, in theory, the last positive well of an extinction-dilution series contains only one genotype of interest. Each positive well at this dilution represents a dominant genotype. Different positive wells at this dilution may contain either the same genotype or different genotypes. Thus for the example (Fig. 3.5), the two-pooled wells may contain one single genotype or two genotypes. If we had 5 positive wells the maximum number of expected different genotypes would be 5. Pooling the wells considerably reduced the workload. In general, the actual number of genotypes detected by sequencing of PCR-DGGE bands was equal to or smaller than the maximum predicted number (Table 3.5). Only in soil on day 0 were the actual numbers of genotypes higher than the predicted numbers. Day 0 soil had been moist for only 24 hours and the dry surface soil at the site was hydrophobic. Some clay particles carrying more than one microbe might have been entrained into a higher dilution. Also, there is more soil in the dilution bottles of unplanted soil than in rhizosphere soil, which increases the chances of having more soil particles in the last positive MPN well.

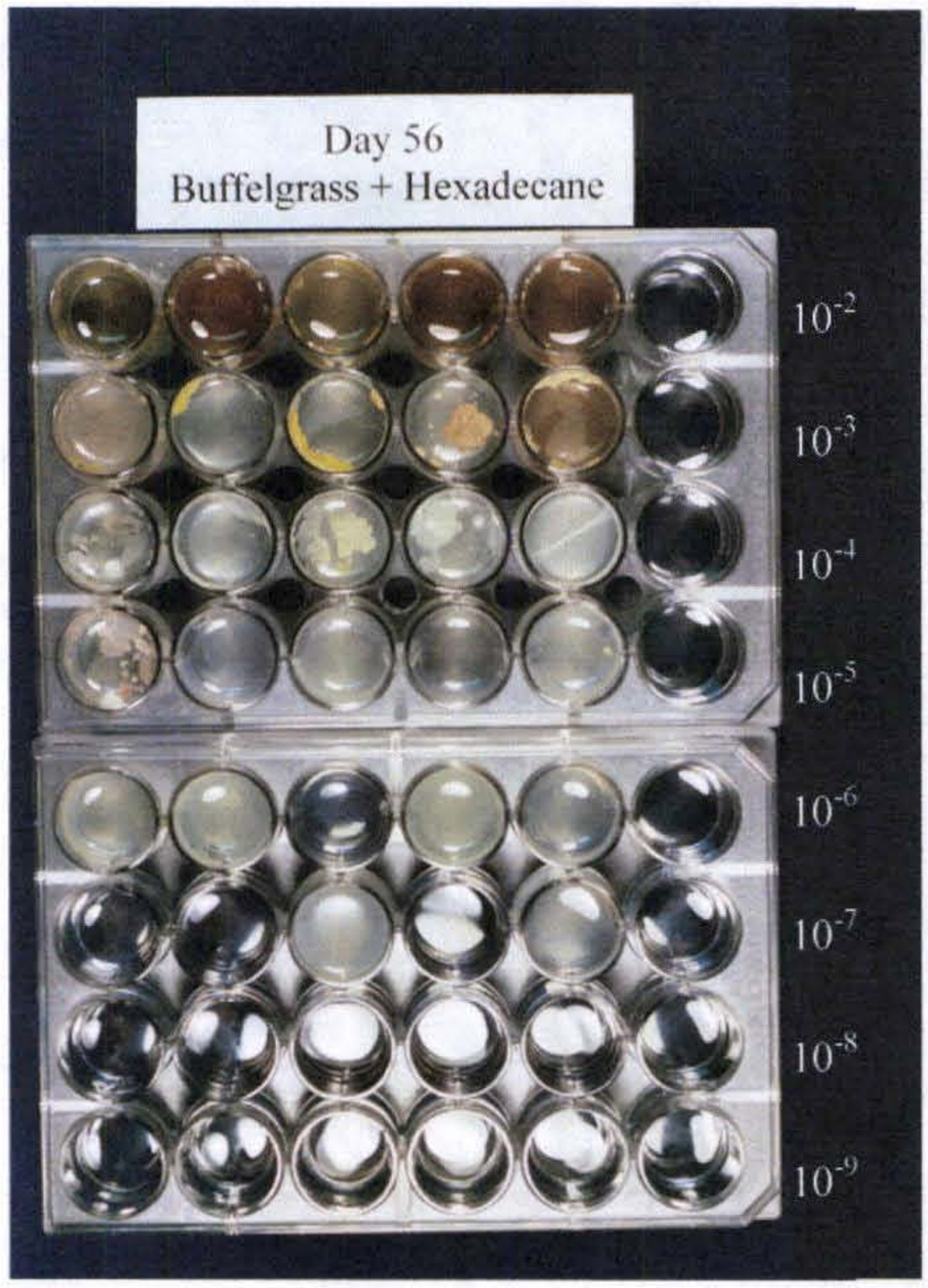


Figure 3.5. A typical MPN plate showing two positive wells at the greatest dilution (10^{-7}) i.e., positive for hexadecane-degraders.

Table 3.5. Representative values for predicted and actual numbers of genotypes in DNA from pooled MPN wells at the greatest dilution positive for hexadecane degraders (examples from first trial).

Day	Treatment (sample)	No. of pooled positive wells	Predicted No. of genotypes	Actual No. of genotypes
0	Soil -HC (SC2,10^{-5})	2	≤ 2	3
0	Soil +HC (SD2,10^{-5})	1	1	3
56	Soil -HC (SC2, 10^{-4})	4	≤ 4	2
56	Milo -HC (MC2, 10^{-6})	1	1	1
56	Oleander -HC (NOC1,10^{-5})	2	≤ 2	1
56	Buffelgrass -HC (BGC4, 10^{-8})	1	1	1
56	Soil +HC (SD1, 10^{-7})	4	≤ 4	1
56	Milo +HC (MD1, 10^{-6})	5	≤ 5	1
56	Oleander +HC (NOD1, 10^{-6})	5	≤ 5	2
56	Buffelgrass +HC (BGD2, 10^{-9})	1	1	1

3.5.2.2 Quality of DNA extracted from MPN plate wells by the Mo Bio technique

A representative 1% agarose gel with DNA samples extracted with the Mo Bio soil DNA extraction kit is shown in Fig. 3.6. DNA was of high quality; fragments size was *ca.* 23,000 bp.

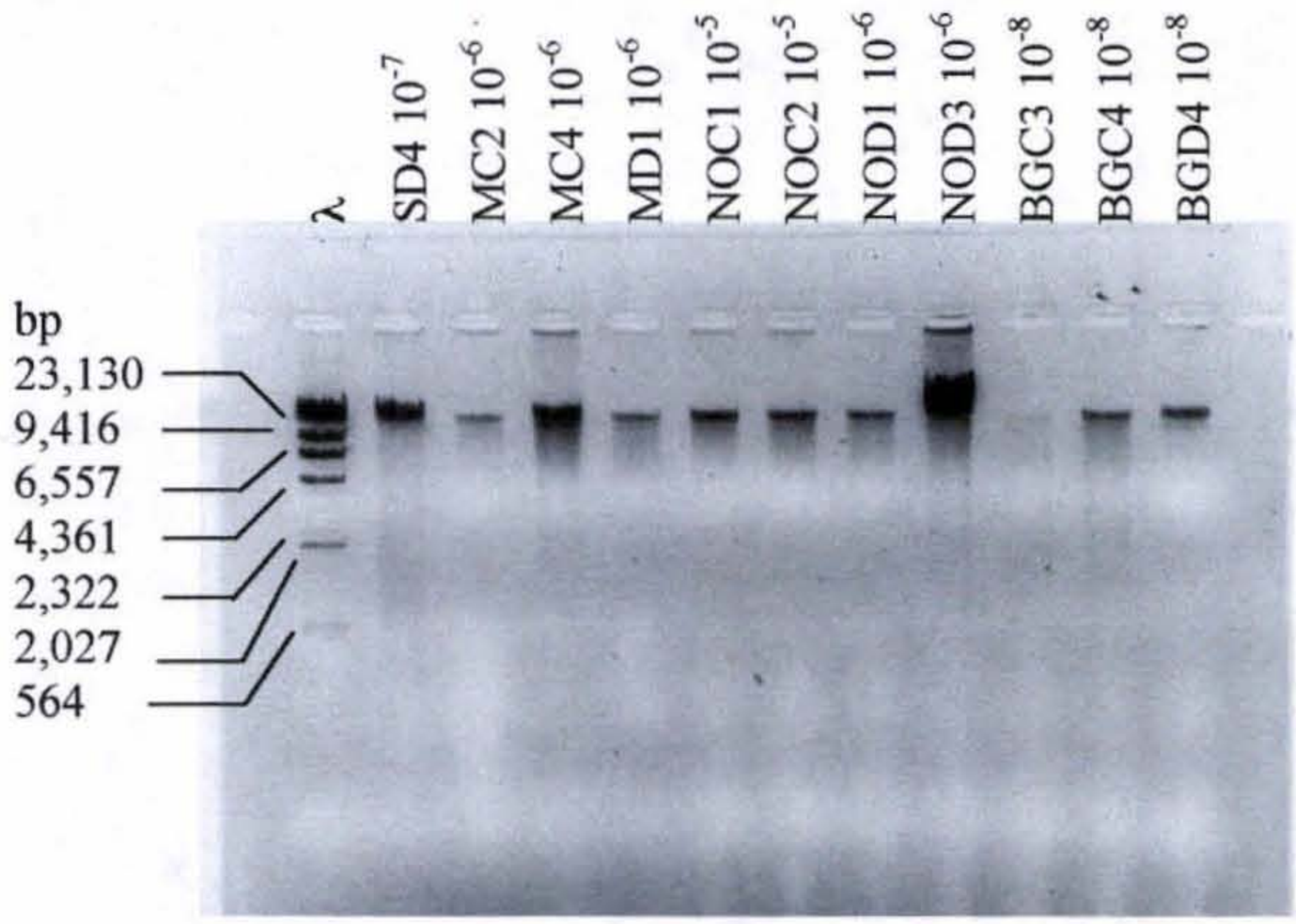


Figure 3.6. DNA extractions from day 56 MPN samples. Extractions were performed using the MoBio Soil DNA Extraction Kit (see section 2.10).

3.5.2.3 PCR products obtained with universal primers for the V6-8 region of 16S rDNA

A representative 1% agarose gel with PCR products obtained from various MPN samples of hexadecane degraders is shown on Fig. 3.7. The expected product was 419 bp long.

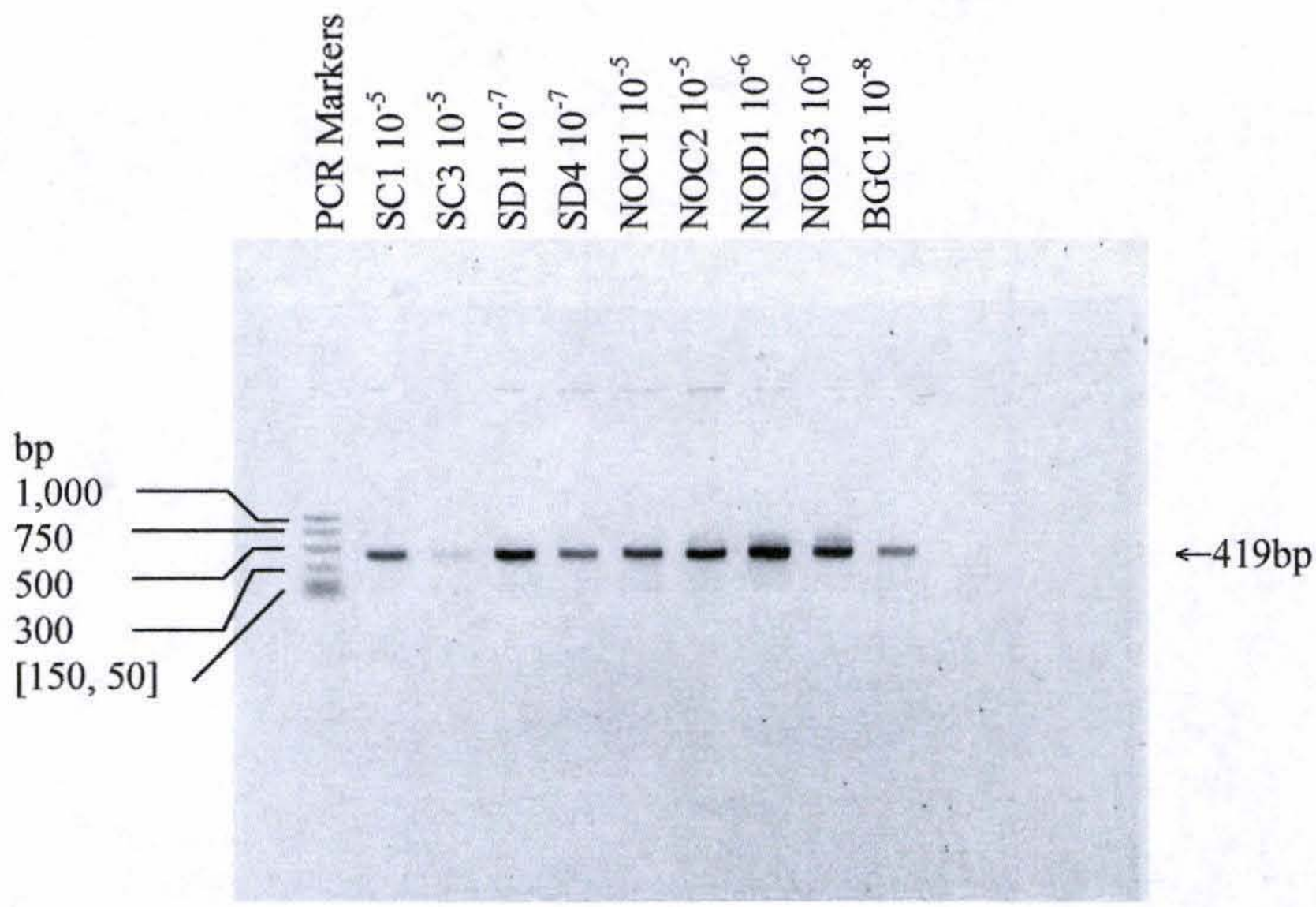


Figure 3.7. PCR amplified products for the V6-V8 region of the 16S rRNA gene from day 56 MPN samples.

3.5.2.4 Diversity of dominant hexadecane degraders as determined by sequencing of DGGE V_6-V_8 16S rDNA bands

Two PCR amplifications were conducted on DNA sample followed by DGGE and sequencing of bands on the DGGE gel. The closest phylogenetic neighbors were identified through the BLAST algorithm at the NCBI website. Data are presented as representative DGGE gel profiles and tables describing each genotype identified in the whole experiment.

3.5.2.4.1 Day 0

A representative day-0 DGGE gel is presented (Fig. 3.8) as are the hexadecane-degrading bacteria genotypes found on day 0 (Table 3.6) The hydrocarbons had only been in contact with the soil for 24 h at room temperature on day 0 therefore it is unlikely that the contaminant could have had a major impact on the soil microbes yet. The genotypes detected are probably those of hexadecane degraders present in the soil prior to the introduction of the contaminants (Table 3.6). It should be noted that the majority of the hexadecane-degraders from day 0 belonged in the *Alphaproteobacteria* group. Our results contrast with those of McNaughton et al. (1999) who reported the absence of *Alphaproteobacteria* from control soil devoid of hydrocarbons at a coastal soil site in Delaware. *Sphingomonas* is a soil bacterium adapted to oligotrophic environments (Johnsen et al., 2005) and is a known hydrocarbon-degrader. Members of this genus have been shown to degrade mostly aromatic compounds, but *Sphingomonas paucimobilis* was reported by Vomberg and Klinner (2000) to degrade decane and hexadecane in liquid cultures. *S. paucimobilis* DNA hybridized to their *alkB* probe. *Sphingomonas* is listed by van Beilen et al. (2003) as a genus whose members degrade aliphatic hydrocarbons. *Sphingomonas* sp. strain HXN200 degrades hexane and hydroxylates 4-to-6 ring alicyclic compounds (van Beilen et al., 2003, 2006). *Erythrobacter* and *Alterierythrobacter* are Sphingomonadales whose activities toward hydrocarbons have not been reported. *Caulobacter* is a prosthecate bacterium usually found in water.

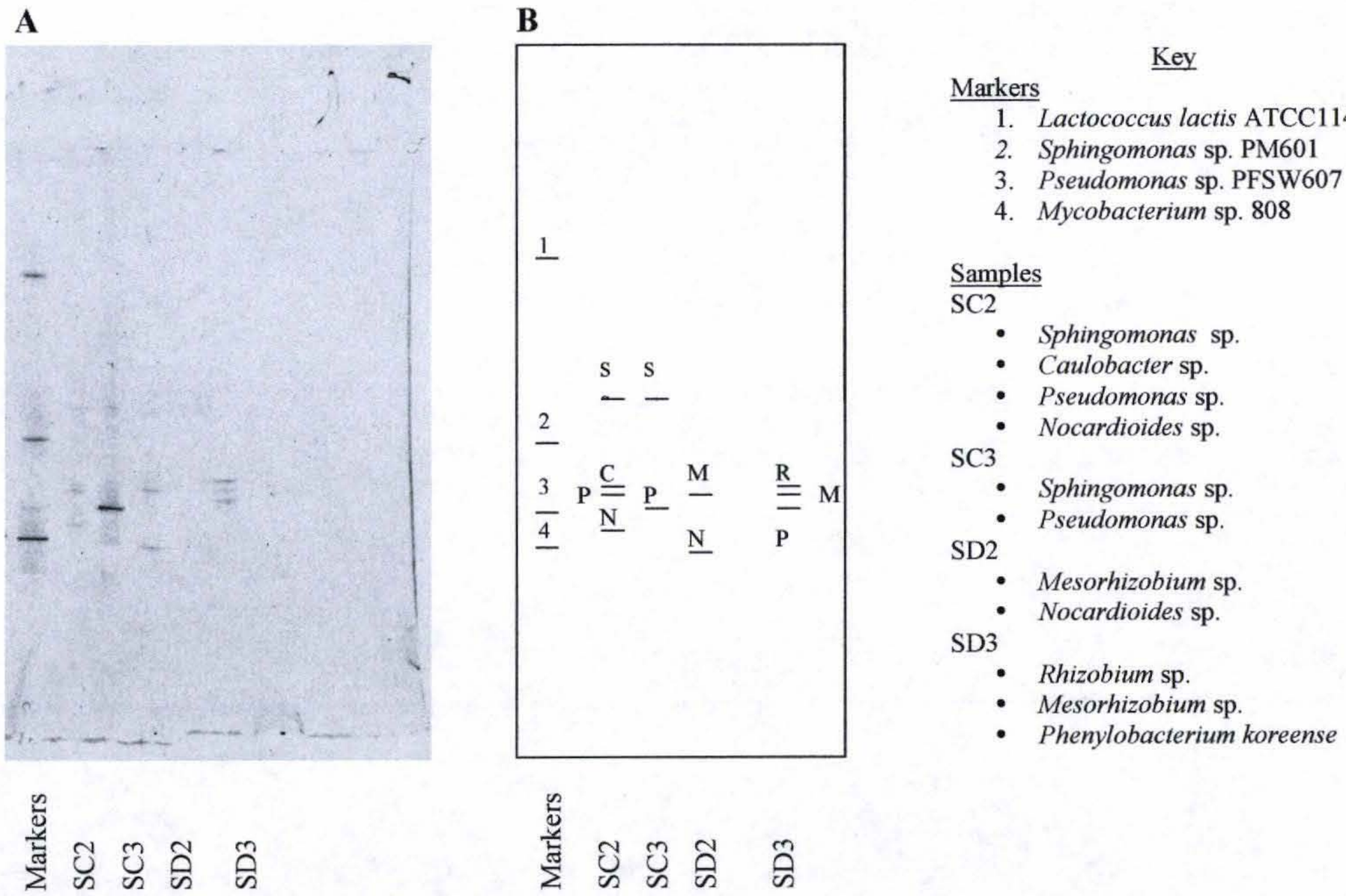


Figure 3.8. DGGE fingerprints (A) and schematic of the band position for 16S rDNA fragments from day 0 MPN samples. Letters above the bands represent the first initial of the names listed in the key. For sample codes see Appendix A.

Since the soil used in this study is a coastal soil and the tide is felt 4 feet below, *Caulobacter* and other aquatic bacteria may be expected in this soil. *Phenylobacterium koreense* is a newly described species in the *Caulobacteraceae* (Aslam et al., 2005). *Nocardioides* is a Gram-positive bacterium with a high G+C content, a normal inhabitant of soils and is known to degrade alkanes (Vomberg and Klinner, 2000). Several "types" of rhizobia (e.g., *Rhizobium* sp. and *Sinorhizobium* sp.) were found in this soil. Their sequences in the V6 to V8 region of the 16S rDNA gene appear very similar and it is possible that the number of different genera is not as high as reported. Rhizobia hydrocarbon-degradative abilities have been little studied. *Rhizobium* sp. was reported as a hydrocarbon degrader in an Argentinian soil heavily contaminated with mineral oil (Wünsche et al., 1997). The authors did not indicate which hydrocarbons were degraded. *Rhizobium galegae* pure cultures obtained from enrichment of soil slurries with phenanthrene were among the best degraders of this PAH (Andreoni et al., 2004). Bodour et al. (2003) reported *Rhizobium* and *Sinorhizobium* growing on phenanthrene plates but did not investigate further. A strain of *Sinorhizobium* sp. (PNOD613) isolated from the rhizosphere of an oleander plant contaminated with diesel fuel (Jones et al., 2004), degraded phenanthrene but grew inconsistently on diesel and hexadecane (Table 3.4). More studies are needed in this area. *Ramlibacter* is a cyst-producing bacterium recently isolated from a sandy soil in the Tunisian desert (Heulin et al., 2003). It may have a potential for alkane degradation because it shares the characteristic of some hydrocarbon-degraders (e.g. *Alcanivorax*) of growing on very few C sources such as 3-4 C organic acids and Tween 40 and 80. The *Pseudomonas* genus includes several species capable of degrading various hydrocarbons.

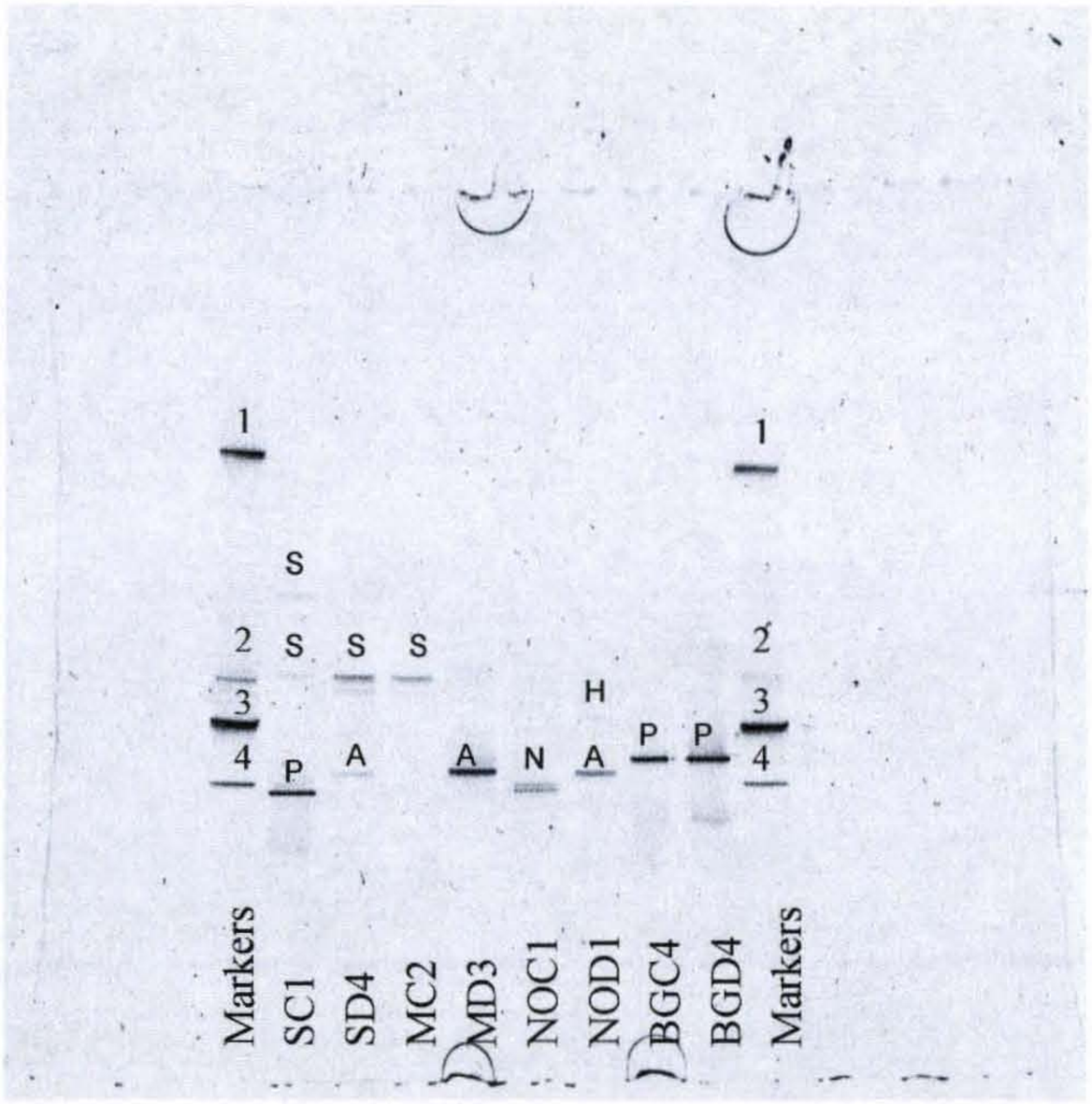
Table 3.6. BLASTn analysis of the 16S rDNA V6-8 sequence from DGGE bands of dominant hexadecane-degrading bacteria

Origin	Phylogenetic assignment	Nearest neighbor and GenBank Accession No.	Identity (%)
Day 0			
Soil -HC	*α-Proteobacteria*	*Sphingomonas sp.* 44/40 **AY571829**	95
	α-Proteobacteria	*Erythrobacter* sp. MBIC411 **AB035544**	96
	α-Proteobacteria	*Alterierythrobacter epoxidivorans* strain JCS350 **DQ304436**	98.7
	α-Proteobacteria	*Caulobacter fusiformis* **CFJ227759**	97.2
	γ-Proteobacteria	*Pseudomonas* sp. **AY683884**	97.4-100
	γ-Proteobacteria	*Pseudomonas* sp. clone DS109 **DQ234192**	100
	Actinobacteria	*Nocardioides* sp. MFC-A **AB183421**	95
Soil +HC	*α-Proteobacteria*	*Sphingomonas* sp. 44/40 **AY571829**	95
	α-Proteobacteria	*Rhizobium* sp. DUS752 **DQ316231**	99
	α-Proteobacteria	*Rhizobium* sp. R-24658 **AM084043**	99.4
	α-Proteobacteria	*Mesorhizobium* sp. **AY230775**	100
	α-Proteobacteria	*Mesorhizobium* sp. casi-1 **AY490133**	99.4
	α-Proteobacteria	*Phenylobacterium koreense* **AB166881**	99.7
	β-Proteobacteria	*Ramlibacter* sp. HTCC332 **AY429716**	99.4
	Actinobacteria	*Nocardioides* sp. EN47 **AY148082**	99

3.5.2.4.2 Day 56

A representative day-56 DGGE gel is presented (Fig. 3.9) as are the hexadecane-degrading bacteria genotypes found on day 56 (Table 3.7). Under uncontaminated conditions across the treatments there was a mixture of *Alphaproteobacteria* and *Gammaproteobacteria* genotypes (6 and 7 genotypes, respectively). *Sphingomonas* (*Alphaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*), and *Nocardioides* (*Actinobacteria*) are genera that were already detected on day 0.

Pseudomonas indica IMT40 was found consistently in the rhizosphere of buffelgrass, under both contaminated and uncontaminated conditions. *Ps. indica* was isolated from an Indian oil field and described for the first time by Pandey et al. (2002) as capable of using butane and other short-chain hydrocarbons up to C10. No longer-chain alkanes were offered as C sources by these authors. However, one of our isolates (PBN606) was provisionally identified as *P. indica* (Table 3.4) and could grow on hexadecane and diesel fuel No. 2. *Pseudomonas azotifigens* is a newly described species, capable of fixing nitrogen, and phylogenetically close to *P. indica* (Hatayama et al., 2005). *Hydrocarboniphaga effusa,* a recently described genus of alkane and aromatic hydrocarbon degraders (Palleroni et al., 2004) was found in unplanted soil and the milo rhizosphere. This species is cosmopolitan, and strains have been isolated from a petroleum-hydrocarbon contaminated soil in New Jersey, from activated sludge in Japan, and from a biofilter in Canada (Palleroni et al., 2004).



Key

Markers

1. *Lactococcus lactis* ATCC11454
2. *Sphingomonas* sp. PM601
3. *Rhizobium* sp. 806
4. *Mycobacterium* sp. 428

SC1 10^{-5} (1)

- *Sphingomonas* sp.
- *Sphingomonas* sp.
- *Pseudomonas* sp.

SD4 10^{-7} (2)

- *Sphingomonas* sp.
- *Alcanivorax* sp.

MC2 10^{-6} (1)

- *Sphingomonas* sp.

MD3 10^{-7} (2)

- *Alcanivorax* sp.

NOC1 10^{-5} (2)

- *Nocardioides sp.*

NOD1 10^{-6} (5)

- *Hydrocarboniphaga* effusa
- *Alcanivorax* sp.

BGC4 10^{-8} (1)

- *Pseudomonas* sp.

BGD4 10^{-9} (1)

- *Pseudomonas* sp.

Figure 3.9. DGGE fingerprints for 16S rDNA fragments from day 56 MPN samples. Letters above the bands represent the first initial of the names listed in the key. For sample codes see Appendix A.

Mycobacterium sp. was found in unplanted soil. This genus is well known to degrade hydrocarbons. In the rhizosphere of buffelgrass bacteria usually associated with plants were detected, e.g., *Agrobacterium tumefaciens* and *Xanthomonas campestris*. *Xanthomonas* can degrade hydrocarbons (Atlas and Cerniglia, 1995; Wünsche et al., 1997). *Devosia* (Nakagawa et al., 1996) is classified in the *Hyphomicrobiaceae* within which some members are common in saltwater. No information regarding the ability of *Devosia* and *Ahrensia* (*Rhodobacteraceae)* to degrade hydrocarbons is available.

In contaminated samples on day 56, a distinct shift toward the *Gammaproteobacteria* occurred and the diversity of the hexadecane degraders decreased. The dominant genus present in all contaminated samples except for the buffelgrass rhizosphere was *Alcanivorax*. *Alcanivorax* is a marine bacterium described for the first time by Yakimov et al. (1998). *A. borkumensis*, the type strain, was isolated from the North Sea; its genome has been sequenced (Golyshin et al., 2003). Other species have been described from around the world. The genus seems restricted in the number of C sources it can use, but straight-chain and branched alkanes are the major ones, while organic acids such as pyruvic acid can also be used. *A. borkumensis* produces "special" glycolipids as surfactants (Yakimov et al., 1998). *Alcanivorax* spp. appear to be dominant members of the microbial community during degradation of oil spills at sea when N and P are added (Kasai et al., 2002; Röling et al., 2002; Hara et al., 2003; Harayama et al., 2004; Head et al., 2006). It was also a significant player in the bioremediation of oil in the tidal area below the surface of mudflat beaches in the United Kingdom (Röling et al., 2004). This a cosmopolitan alkane degrader that seems to behave like an r-strategist,

being hardly detectable in non-polluted water and rapidly increasing in numbers during the early phase of bioremediation. *Alcanivorax* numbers rapidly decrease after biodegradation of the alkanes (Head et al., 2006; Kasai et al., 2002). Therefore *Alcanivorax* is probably not among the hexadecane degraders that persisted in high numbers in contaminated unplanted soil, in which virtually all the hexadecane had been degraded by D114 (Fig. 3.1A and 3.3B). *Achromatium* was found in the rhizosphere of milo and oleander. *Achromatium oxaliferum* is a very large coccoid colorless sulfur bacterium, which can reach 100 μm in diameter, contains sulfur globules and calcium carbonate inclusions, and has not been cultivated for over one 100 years (Holt et al., 1994). It lives in fresh and saltwater sediments at the interphase between oxic and anoxic layers (Glöckner et al., 1999). A smaller species, *A. minus*, was recently described by Glöckner et al. (1999) in acidic Lake Fuchskuhle in Germany on the basis of detection by 16S rDNA. Nothing is known regarding *Achromatium* ability to degrade hydrocarbons. Since *Achromatium* was detected in our MPN wells, it must have been able to use either hexadecane or dead cells. Buffelgrass stood apart from all the other treatments in that it harbored only *P. indica* IMT40 as the dominant hexadecane degrader in its rhizosphere. The R2A plates inoculated with rhizosphere samples from buffelgrass also showed colony types that were distinctly different from the colony types of the other treatments. We are attempting to isolate the major genera of hexadecane degraders from contaminated soil and rhizospheres on day 56 or after cessation of the most active hexadecane degradation period.

Table 3.7. BLASTn analysis of the 16S rDNA V6-8 sequence from DGGE bands of dominant hexadecane-degrading bacteria

Origin	Phylogenetic assignment	Nearest neighbor and GenBank Accession No.	Identity (%)
Day 56			
Soil -HC	*α-Proteobacteria*	*Sphingomonas* sp. 44/40 **AY571829**	95
	α-Proteobacteria	*Sphingomonas* sp. HI-K4 **DQ205308**	100
	α-Proteobacteria	*Ahrensia* sp. DFL-11 **AJ582083**	98
	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	97.9
	γ-Proteobacteria	*Hydrocarboniphaga effusa* **AY363245**	99.1
	Actinobacteria	*Mycobacterium* sp. **AF408960**	100
Milo -HC	*α-Proteobacteria*	*Sphingomonas* sp. 44/40 **AY571829**	95
	γ-Proteobacteria	*Pseudomonas* sp. **AM114531**	100
	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	98.2
	γ-Proteobacteria	Marine bacterium YTBM8-1A **AY626832**	99.7
	γ-Proteobacteria	*Hydrocarboniphaga effusa* AP103 **AY363245**	99.4
Oleander -HC	*γ-Proteobacteria*	*Pseudomonas azotifigens* **AB189452**	98.6
	γ-Proteobacteria	*Pseudomonas* sp. **DQ237950**	98.5
	Actinobacteria	*Nocardioides* sp. MFC-A **AB183421**	100
Buffelgrass -HC	*α-Proteobacteria*	*Agrobacterium tumefaciens* B8S **AY850392**	100
	α-Proteobacteria	*Devosia* sp. PIC-C22 **DQ227786**	96
	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	96.9-99.7
	γ-Proteobacteria	*Xanthomonas campestris* **X95917**	99.7

Table 3.7 (Contd.). BLASTn analysis of the 16S rDNA V6-8 sequence from DGGE bands of dominant hexadecane-degrading bacteria

Origin	Phylogenetic assignment	Nearest neighbor and GenBank Accession No.	Identity (%)
Day 56			
Soil +HC	α-Proteobacteria	*Sphingomonas* sp. 44/40 **AY571829**	95
	γ-Proteobacteria	*Alcanivorax* sp. NT N136 **AB167044**	94
Milo +HC	γ-Proteobacteria	*Achromatium* sp. JD13 **AF129551**	97.5
	γ-Proteobacteria	*Alcanivorax* sp. CBF L53 **AB166953**	93.8
Oleander +HC	γ-Proteobacteria	*Achromatium* sp. JD13 **AF129551**	94.2
	γ-Proteobacteria	*Alcanivorax* sp. CBF L53 **AB166953**	94.1
	γ-Proteobacteria	*Hydrocarboniphaga effusa* **AY363245**	98.4
Buffelgrass +HC	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	99.4-100

The percentage of hydrocarbon degrading-bacteria among the total heterotrophic bacteria grown in R2A (Table 3.8) will be interpreted using bacteria identified on day 56 (Table 3.7), to tentatively explain the differences in hexadecane-degrading ability of soil and rhizospheres [hexadecane was more efficiently degradaded in the unplanted soil and oleander rhizospheres than in the rhizospheres of milo and buffelgrass (Fig. 3.1A)]. If we focus on the percentage of hexadecane degraders in contaminated soil on day 56 we can see that the unplanted and oleander treatments have similar percentages (9.3 and 9.5, respectively) of degraders. In contrast, the percentage of hexadecane degraders in contaminated milo treatments is 4.7. Assuming that *Alcanivorax* is the major player in the degradation of hexadecane in the soil, milo, and oleander treatments (Table 3.8), *Alcanivorax* would be similarly represented in the soil and oleander treatments. This would be expected to lead to similar decreases in concentrations of hexadecane on day 56 (Fig. 3.1A). *Alcanivorax* would also dominate the milo treatment, but its representation is only half (4.7%) of the representation of *Alcanivorax* in soil and oleander treatments. Thus the level of degradation of hexadecane in the milo treatment lead by *Alcanivorax* would be half of the degradation in the soil and oleander treatments, which is what Fig. 3.1A indicates. Contaminated buffelgrass treatment contained the highest proportion of hexadecane degraders (35%) (Table 3.8) but the dominant degrader detected in this treatment was *Pseudomonas indica* IMT40. In section 3.3.2 the results indicated that the hexadecane degraders in the contaminated buffelgrass treatment preferred the rhizodeposition to hexadecane. Pandey et al. (2002) report that *P. indica* is capable of using many sugars and other sources of carbon as well as alkanes. This suggests that *P. indica* may be a less effective hydrocarbon degrader than *Alcanivorax*, which degrades

very few other C sources than alkanes (Yakimov et al., 1998) and therefore would preferentially use hexadecane.

Table 3.8. Prevalence of hydrocarbon-degraders among viable heterotrophic bacteria in soil and rhizospheres.

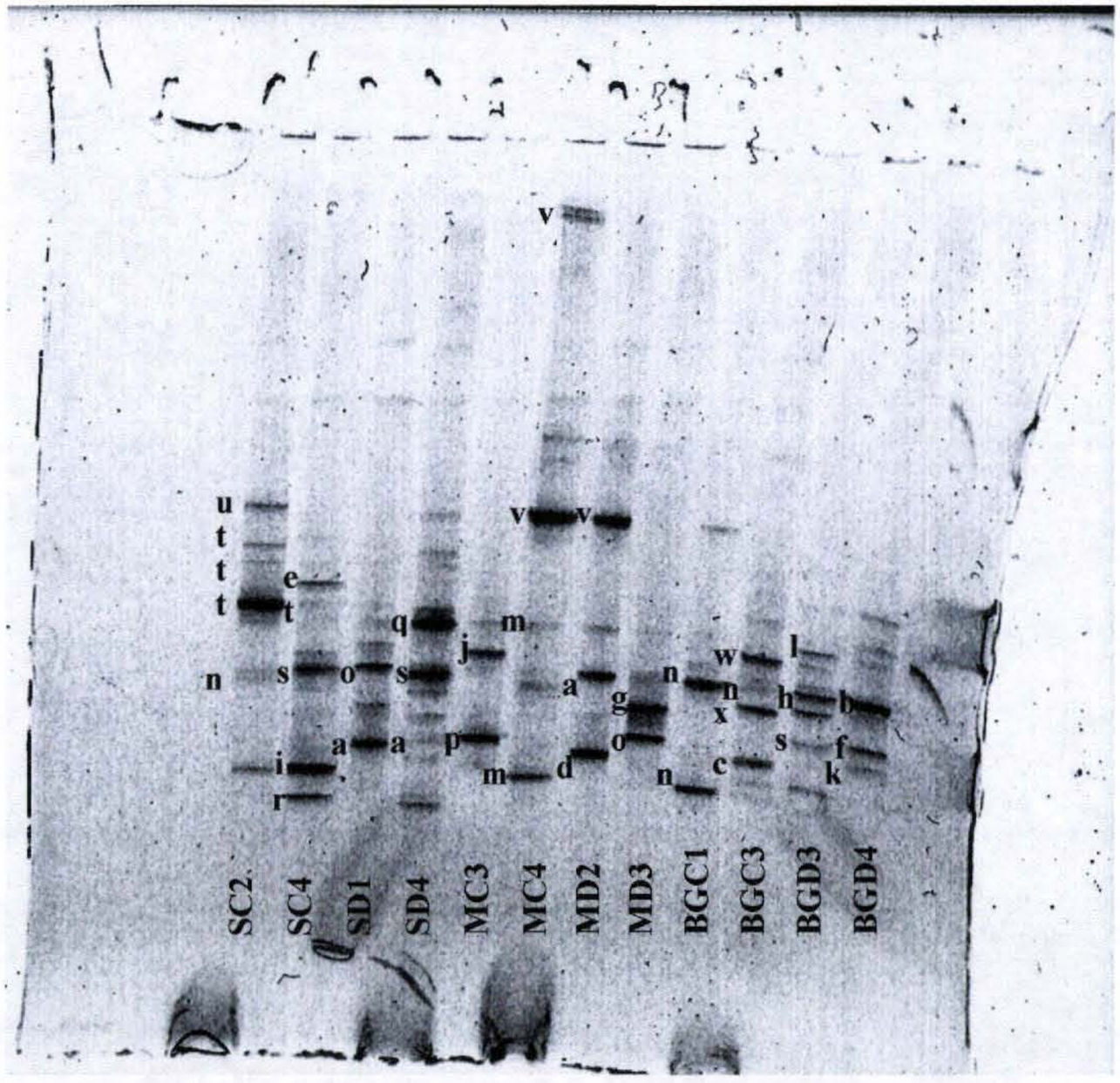
Treatment	Percent hydrocarbon degraders:					
	Hexadecane-degrading bacteria			Phenanthrene-degrading bacteria		
	D0	D56	D114	D0	D56	D114
Soil (-HC)[a]	0.1	0.2	0.1	*ca.* 0	0.002	0.001
Soil (+HC)	0.1	9.3	9.0	*ca.* 0	0.6	1.7
Milo (-HC)		2.7	0.2		0.2	*ca.* 0
Milo (+HC)		4.7	0.6		4.0	9.6
Oleander (-HC)		2.4	0.4		0.01	0.03
Oleander (+HC)		9.5	1.4		3.8	1.8
Buffelgrass (-HC)		25.0	0.8		0.02	*ca.* 0
Buffelgrass (+HC)		35.0	3.0		8.2	6.6

[a] –HC, without hydrocarbons; +HC, with hydrocarbons

3.5.2.4.3 Day 114

A representative day-114 DGGE gel is presented (Fig. 3.10) as are the hexadecane-degrading bacteria genotypes found on day 114 (Table 3.9). Under uncontaminated

conditions and among all treatments 8 *Alphaproteobacteria* and 7 *Gammaproteobacteria* genotypes were detected. Most were similar to those already detected on days 0 and 56, bacteria that are common in soil and rhizospheres. Among the distinct genotypes detected on day 114 were *Rhodococcus*, *Bacillus*, *Sphingopyxis*, *Ochrobactrum*, *Stappia*, and *Alcaligenes*. *Rhodococcus* and *Bacillus* are known to degrade some petroleum hydrocarbons (Peressutti et al., 2003). *Sphingopyxis* is a recently described genus, which was previously part of the *Sphingomonas* genus (Takeuchi et al., 2001). *Ochrobactrum* is a Gram-negative genus closely related to *Rhizobium*; its habitat is water and soil. *O. lupini* is capable of nodulating lupine and of nitrogen fixation (Trujillo et al., 2005). *Ochrobactrum* may be capable of degrading alkanes because it can hydrolyze Tween 20 and 80 (Holt et al., 1994). Peressutti et al. (2003) report *Ochrobactrum* as a hydrocarbon degrader, but they detected it in uncontaminated soil only. However, Wünsche et al. (1997) considered *O. anthropi* as capable of using hydrocarbons and reported it in a soil that was undergoing *in-situ* bioremediation. *Stappia* is a recently created genus to include all marine forms of *Agrobacterium* (Uchino et al., 1998); members of this genus are known to oxidize carbon monoxide. No information is available regarding its use of hydrocarbons. *Alcaligenes* is a common soil and rhizosphere bacterium, which is known to degrade hydrocarbons (Atlas and Cerniglia, 1995).



Key

a. *Achromatium* sp.
b. *Agrobacterium tumefaciens*
c. *Alcaligenes* sp.
d. *Alcanivorax* sp.
e. *Bacillus* sp.
f. *Devosia* sp.
g. *Hydrocarboniphaga effusa*
h. *Mesorhizobium* sp.
i. *Mycobacterium* sp.
j. *Ochrobactrum* sp.
k. *Prosthecomicrobium* sp.
l. *Ps. aeruginosa*
m. *Ps. azotifigens*
n. *Ps. indica*
o. *Ps. stutzeri*
p. *Pseudomonas* sp.
q. *Rhizobium* sp.
r. *Rhodococcus* sp.
s. *Sinorhizobium* sp.
t. *Sphingomonas* sp. 44/40
u. *Sphingomonas* sp. HI-K4
v. *Sphingopyxis* sp.
w. *Stappia* sp.
x. *Xanthomonas* sp.

Figure 3.10. DGGE fingerprints for 16S rDNA fragments of day 114 MPN samples. Letters besides the bands correspond to the names listed in the key. See Appendix A for sample codes.

In contaminated treatments on day 114, the genotypes detected were very different from the ones on day 56. An almost equal number of *Alpha-* and *Gammaproteobacteria* genotypes were found (9 *Alphaproteobacteria* vs. 8 *Gammaproteobacteria*) and among the *Gammaproteobacteria*, *Alcanivorax* was found only in the milo rhizosphere. This parallels the concentration of hexadecane left in the milo treatment, which was higher than the remaining concentration in soil and oleander treatments (Fig. 3.1A). Thus at the end of the experiment, when the hexadecane concentrations were low in all treatments, there was a change in genotype composition, characterized by a return to a higher proportion of *Alphaproteobacteria* genotypes as in uncontaminated treatments on days 56 and 114. This parallels the literature reports (Kasai et al., 2002; Head et al., 2006); here *Alcanivorax* disappeared from treatments such as soil and oleander when hexadecane became scarce on day 114. Bacteria such as the rhizobia (e.g. *Rhizobium* sp.) and various *Pseudomonas* spp. made a general come back. These bacteria are probably capable of degrading hexadecane on the MPN plates but survive in the pots by growing on rhizodeposits (planted treatments) or by living off accumulated fatty products (unplanted soil). One species was detected first on day 114, *Prosthecomicrobium pneumaticum,* a prosthecate bacterium found in soil and fresh and marine waters (Holt et al., 1994). Its hydrocarbon degradative abilities are unknown. By day 114, the specialized hydrocarbon degrader *Alcanivorax,* which was dominant on day 56 had virtually disappeared and a variety of non-specialized hydrocarbon degraders prevailed. On day 114, the contaminated rhizosphere of buffelgrass had also lost its distinctive dominant hexadecane degrader, *Pseudomonas indica* IMT40, which was replaced by species from several other genera belonging mostly in the *Alphaproteobacteria*.

Table 3.9. BLASTn analysis of the 16S rDNA V6-8 sequence from DGGE bands of dominant hexadecane-degrading bacteria

Origin	Phylogenetic assignment	Nearest neighbor and GenBank Accession No.	Identity (%)
Day 114			
Soil -HC	*α-Proteobacteria*	*Sphingomonas* sp. 44/40 **AY571829**	95
	α-Proteobacteria	*Sphingomonas* sp. S37 **AF367204**	99.4
	α-Proteobacteria	*Sinorhizobium* sp. S005 **AF285963**	99.7
	γ-Proteobacteria	*Pseudomonas azotifigens* **AB189452**	98.8
	Firmicutes	*Bacillus* sp. LCSAOTU11 **AF506060**	99.7
	Actinobacteria	*Mycobacterium* sp. JS624 **AY162029**	97.7
	Actinobacteria	*Rhodococcus* sp. OUCZ16 **AY785730**	98.5
Milo -HC	*α-Proteobacteria*	*Sphingopyxis* sp. DG892 **AY258083**	100
	α-Proteobacteria	*Ochrobactrum* sp. Pb4 **AY310304**	99.7
	γ-Proteobacteria	*Pseudomonas aeruginosa* **AY268175**	100
	γ-Proteobacteria	*Xanthomonas* sp. LCSAOTU19 **AF506043**	99.4
	γ-Proteobacteria	*Hydrocarboniphaga effusa* strain AP10 **AY363245**	99.7
Oleander -HC	*α-Proteobacteria*	*Mesorhizobium* sp. LCSAOTU23 **AF506052**	100
	γ-Proteobacteria	*Pseudomonas* sp. 12C_10 **AY689083**	100
	γ-Proteobacteria	*Pseudomonas* sp. BCNU171 **DQ229316**	100
Buffelgrass -HC	*α-Proteobacteria*	*Stappia alba* strain 5OM30 **AJ889010**	99.4
	α-Proteobacteria	*Stappia marina* strain mano18 **AY628423**	97.8
	β-Proteobacteria	*Alcaligenes* sp. O-1 **AY753188**	100
	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	99.7
	γ-Proteobacteria	*Xanthomonas* sp. LCSAOTU19 **AF506043**	100

Table 3.9 (Contd.). BLASTn analysis of the 16S rDNA V6-8 sequence from DGGE bands of dominant hexadecane-degrading bacteria

Origin	Phylogenetic assignment	Nearest neighbor and GenBank Accession No.	Identity (%)
Day 114			
Soil +HC	*α-Proteobacteria*	*Sphingomonas* sp. MBIC1965 **AB025720**	98.8
	α-Proteobacteria	*Rhizobium etli* strain S1 **AY509210**	98.6
	γ-Proteobacteria	*Pseudomonas indica* strain IMT40 **AF302796**	97.5
	γ-Proteobacteria	*Achromatium minus* **AMAJ10596**	95.5
Milo +HC	*α-Proteobacteria*	*Sphingopyxis* sp. DG892 **AY258083**	98.8
	γ-Proteobacteria	*Pseudomonas* sp. SB1 **AY683883**	98.8
	γ-Proteobacteria	*Achromatium* sp. JD13 **AF129551**	94.7
	γ-Proteobacteria	*Alcanivorax* sp. CBF L5 **AB166953**	93.8
	γ-Proteobacteria	*Hydrocarboniphaga effusa* strain AP10 **AY363245**	98.8
Oleander +HC	*α-Proteobacteria*	*Sinorhizobium* sp. FJ38 32-1-A **AF364857**	96.4
	α-Proteobacteria	*Prosthecomicrobium pneumaticum* **AB017203**	97.2
	γ-Proteobacteria	*Achromatium* sp. JD13 **AF129551**	94.7
	γ-Proteobacteria	*Pseudomonas stutzeri* **AJ270454**	100
	Actinobacteria	*Nocardioides* sp. EN47 **AY148082**	98.1
Buffelgrass +HC	*α-Proteobacteria*	*Rhizobium* sp. CHNTR26 **DQ337571**	99.7
	α-Proteobacteria	*Sinorhizobium* sp. S005 **AF285963**	97.8
	α-Proteobacteria	*Agrobacterium tumefaciens* strain UP-3 **AY364329**	99.1
	α-Proteobacteria	*Devosia* sp. PIC-C22 **DQ227786**	96.9
	α-Proteobacteria	*Prosthecomicrobium pneumaticum* **AB017203**	97.5
	γ-Proteobacteria	*Pseudomonas aeruginosa* **AM087130**	100

In future experiments *Alcanivorax* could be used as a model bacterium to monitor bioremediation at the site on Hickam Air Force Base or at similar sites where this bacterium may dominate alkane degradation. It is expected to be undetectable where the contaminant is absent, or to be present in low numbers where there is a low concentration of alkanes, or where N and P limit contaminant degradation.

3.5.2.5 Impact of contamination on species richness

Environmental stresses usually decrease species diversity. Upon contamination of soil with petroleum hydrocarbons and during bioremediation of the polluted soil, biodiversity decreases (Head et al., 2006; Röling et al., 2002). Cheung and Kinkle (2001) showed a decrease in *Mycobacterium* phenotypes in soil heavily contaminated with petroleum hydrocarbon, when compared to a less contaminated soil. Contamination with petroleum hydrocarbons may negatively affect microbial diversity at one site, while at another site it may not be affected or increased (Juck et al., 2000).

In this experiment, species richness was calculated as the number of different genotypes (species per genus) per phylogenetic group and totaled for each contaminated and uncontaminated treatment (Table 3.10). Since on day 0 the contaminant conditions were virtually the same for uncontaminated and contaminated soil, the total number of genotypes was the same for the two treatments. By day 56, species richness decreased across all contaminated treatments (6 types of different genotypes) compared to the

uncontaminated treatments (15 types of different genotypes). By day 114, the contaminant was virtually gone and the number of different genotypes increased in the originally contaminated treatments to 18, which is similar once more to the number in the uncontaminated treatments (19). These observations are in agreement with those in the literature presented above.

Table 3.10. Changes in dominant hexadecane-degrading bacteria richness (genotype numbers) during the course of the experiment

Phylogenetic assignment	Number of genotypes					
	Day 0		Day 56		Day 114	
	-HC	+HC	-HC	+HC	-HC	+HC
α-Proteobacteria	4	6	6	1	8	9
β-Proteobacteria	0	1	0	0	1	0
γ-Proteobacteria	2	0	7	5	7	8
Actinobacteria	1	1	2	0	2	1
Firmicutes	0	0	0	0	1	0
Total	7	8	15	6	19	18

3.6 Diversity of functional genes in hexadecane-degraders determined by PCR-DGGE

3.6.1 *alkB* and *alkM* genes for hexadecane degradation

From the alkane hydroxylase primers described in table 2.2, the primer set targeting the *alkM* gene seemed to be too specific for *Acinetobacter* spp. and although Kohno et al. (2002) reported DNA amplification for 44 strains from at least 7 genera, it did not result in amplification products with any of the isolates from this work (Table 3.4). Since no *Acinetobacter* spp. were found in our samples, this primer set was not used any further.

The second set of primers from Kohno et al. (2002) for the *alkB* gene (Table 2.2) seems to be of a broadly specific and the amplified DNA produced multiple banding patterns on the agarose gel electrophoresis for several samples (results not shown). Moreover, the product size of 185 bp was not considered appropriate for DGGE or sequencing analysis and therefore this primer set was not used either.

Primers designed by Chénier et al. (2003) were more useful. The size of the product (549bp) was suitable for DGGE analysis. The Chénier et al. (2003) primer set produced amplicons with our two isolates of *Mycobacterium* sp. (418 and 428) as shown in Table 3.11. This may be expected as the primers were designed from the *alkB* gene of

Rhodococcus sp. Q15. However, inconsistent results were obtained with the PCR products of some samples (Table 3.11) most likely due to the degeneracy of these primers (Fjellbirkeland et al., 2001).

3.6.2 Re-designed primers from the Chénier DNA segments for *Pseudomonas* and *Alcanivorax*

The *alkB* primers re-designed for *Alcanivorax* sp. and *Pseudomonas* sp. resulted in successful amplification of DNA samples containing strains determined by 16S rDNA analysis to be *Alcanivorax* sp. (Table 3.7).

Amplification with our primers specific for *Alcanivorax borkumensis* and *Pseudomonas* sp. was obtained for the expected samples (Table 3.11) and the products were subjected to DGGE. Amplicons for our putative *P. indica* isolate, PBN606, were too weak and could not be used in the DGGE analysis. The 40-bp GC-clamp (Nübel et al., 1996) also affected the amplification efficiency of the primers and therefore was not used. All amplicons produced one band on the DGGE gel except for MD3, which produced two bands (gel not shown).

Table 3.11. Bacterial isolates and samples used for PCR amplifications of *alkB* gene with different primer sets

Isolate # or Sample code [a]	Possible ID from 16S rDNA partial sequences [b]	Primer set [c]		
		CF:CR	AbF:AbR	NdF:NdR
Isolates				
418	*Mycobacterium* sp.	+	-	+
428	*Mycobacterium* sp.	+	-	+
PBN606	*Pseudomonas indica IMT40*	-	w	-
PFSW607	*Pseudomonas* sp.	-	+	-
805	*Nocardioides* sp.	nd	nd	+
808	*Mycobacterium* sp.	nd	nd	w
Samples				
Day 0				
SC3		nd	-	+
Day 56				
SC3	*Mycobacterium* sp.	nd	-	+
NOC1	*Nocardioides* sp.	-	-	+
NOC2		-	-	+
NOC3		+	nd	nd
BGC1		nd	w	nd
BGC4	*P. indica* IMT40	-	w	nd
SD1	*Alcanivorax* sp.	-	+	-
SD2		+	nd	nd
SD3		+	nd	nd
SD4	*Alcanivorax* sp.	nd	+	-
MD1	*Alcanivorax* sp.	nd	+	nd
MD3	*Alcanivorax* sp.	nd	+	nd
NOD1	*Alcanivorax* sp.	nd	+	-
NOD2		i	nd	nd
NOD3	*Alcanivorax* sp.	nd	+	-
BGD2	*P. indica* IMT40	nd	w	nd
Day 114				
MC3	*Pseudomonas* sp.	i	-	nd
NOC3	*Pseudomonas* sp.	i	-	+
SD2		nd	+	+
SD4		i	nd	nd
NOD1		-	+	nd
NOD2	*Nocardioides* sp.	-	-	+

[a] S, soil; M, milo; NO, oleander; BG, buffelgrass; C, uncontaminated; D, contaminated

[b] for the samples, names represent possible targets of *alkB* primers from the bacteria identified by 16S rDNA analysis (tables 3.6, 3.7 and 3.9)

[c] CF:CR, primers from Chénier et al. (2003); AbF:AbR, primers for *Alcanivorax* sp. and *Pseudomonas* sp.; NdF:NdR, primers for *Nocardioides* sp. and *Mycobacterium* sp.; +, visible band of expected size; w, weak band of expected size; i, inconsistent results; nd, not determined

The *alkB* sequences from environmental samples for day 56 and our *Pseudomonas* sp. isolate, PFSW607, were compared to sequences of known hexadecane degraders in the NCBI database in a tree based on maximum likelihood analysis (Fig. 3.11). The same tree topography was obtained with kitch and neighbor-joining distance-based methods. Sequences from *Burkholderia cenocepacia* H124 (gi 13591531), *Acinetobacter venetianus* (gi 63020530) and *Pseudomonas fluorescens* CHA0 (gi 13445192) (gi: GenInfo identifier from the GenBank) clustered away from those in our samples (Fig. 3.11). A cluster with high identity to that of *Alcanivorax borkumensis* AP1 (gi 33186768) and another to *Pseudomonas aeruginosa* PAO1 (gi 9947482) can be clearly distinguished (Fig. 3.11). Our results from the PCR-DGGE analysis of the partial 16S rDNA sequences on day 56 (Table 3.7) show that the unplanted soil, as well as the milo and oleander rhizospheres samples contained a dominant *Alcanivorax* sp. population. This was confirmed by our *alkB* sequences for the milo and oleander rhizospheres seen by the branch closest to *A. borkumensis*. A sequence from a milo sample (MD2) that had no degrader detected by 16S rDNA analysis, clusters with the other samples with *alkB* gene homologous to that in *Alcanivorax borkumensis*.

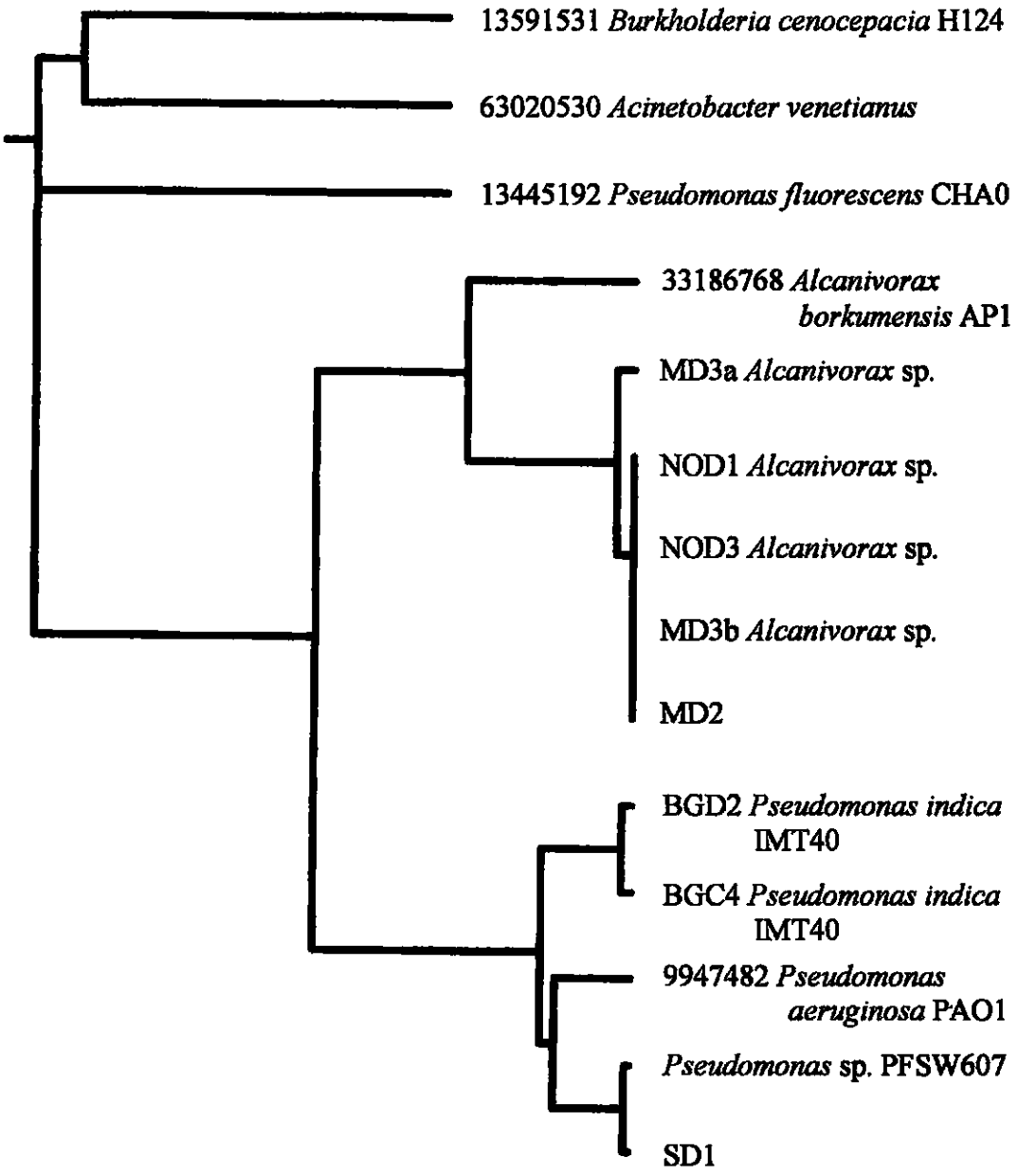


Figure 3.11. Phylogram for *alkB* re-designed primers (targeting *Alcanivorax* and *Pseudomonas*) based on partial gene fragments (491 bp). The tree is based on a maximum likelihood analysis. GI numbers are given for the sequences obtained from the NCBI database: *Burkholderia cenocepacia* H124 (gi 13591531); *Acinetobacter venetianus* (gi 63020530); *Pseudomonas fluorescens* CHA0 (gi 13445192); *Alcanivorax borkumensis* AP1 (gi 33186768); *Pseudomonas aeruginosa* PAO1 (gi 9947482). All samples are from day 56 (See Table 3.11 footnotes for samples origin.)

The bottom portion of the tree shows a cluster of Pseudomonads (Fig. 3.11). A branch within this cluster contains the BGD2 and BGD4 samples from day 56 which were identified as *P. indica* IMT40 (Table 3.7). *P. indica* is a butane degrader which alkane hydroxylase system had no similarity to any other systems studied (Padda et al., 2001; van Beilen et al., 2003). The distribution of the BGD2 and BGD4 *alkB* sequences in the tree confirms that they are different from those of the other *Pseudomonas* spp. Our *Pseudomonas* sp. isolate, PFSW607 branched close to the reference strain *Pseudomonas aeruginosa* PAO1. Amplification of *alkB* sequences from soil (SD1 in Fig. 3.11) indicated a possible *Pseudomonas* sp. alkane degrader not detected by the 16S rDNA analysis. Thus targeting catabolic genes may provide a useful tool for identifying hydrocarbon-degrading populations such as *Alcanivorax* sp. and *Pseudomonas* sp.

3.6.3 Re-designed primers for *Nocardioides* and *Mycobacterium*

Degenerate primers designed by Chénier et al. (2003) were modified to target *Nocardioides* spp. and *Mycobacterium* spp. detected in our samples by PCR-DGGE analysis of the partial 16S rDNA sequences (section 3.5.2.4). Successful amplifications were obtained from samples and isolates containing *Mycobacterium* sp. or *Nocardioides* sp. (Table 6). Amplification efficiency was also reduced with the attachment of the 40-bp GC-clamp to the forward primer (Nübel et al., 1996), and DGGE was performed without the clamp. A DGGE gel with amplicons of *alkB* partial sequences of Gram-positive bacteria from the environmental samples and isolates is presented in Fig. 3.12.

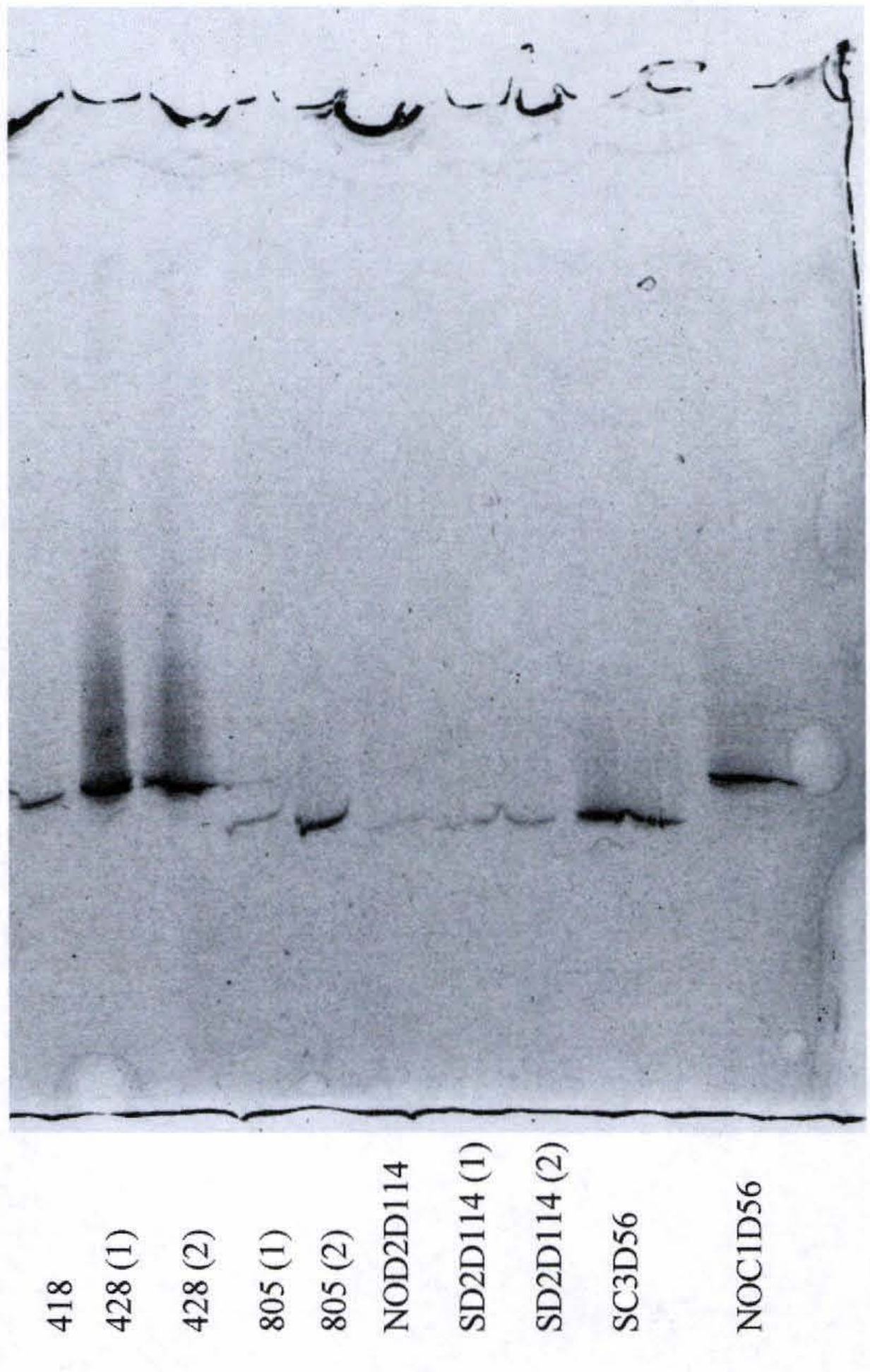


Figure 3.12. DGGE fingerprints of *alkB* gene fragments amplified with re-designed primers for *Nocardioides* and *Mycobacterium* using DNA from isolates and MPN samples (Table 3.11). Lack of sharpness of DGGE is due to the use of primers without a GC-clamp.

Comparison with available sequences of Gram-positive degraders of long chain alkanes [*Gordonia* sp. TF6 (gi 54649959), *Nocardia* sp. H17-1 (gi 48686657), *Prauserella rugosa* (gi 13872674), *Rhodococcus erytrhopolis* (gi 13750756) and *Rhodococcus* sp. Q15 (gi 15420774); gi: GenInfo identifier from the GenBank], and our samples and isolates (Table 3.11) was done in a phylogram based on maximum likelihood analysis (Fig. 3.13). Kitch and neighbor-joining distance-based methods, shared the same tree topography as the maximum likelihood tree.

Sequences related to *Prauserella rugosa* (gi 13872674) are in the top cluster (Fig 3.13). The alkane hydroxylase of *P. rugosa* is closely related to the *Nocardioides* sp. CF8 AlkB (van Beilen et al., 2003). Our *Nocardioides* isolate (805) and *Nocardioides* sp. in uncontaminated oleander samples on day 56 (NOC1) identified by partial 16S rDNA sequence analysis (Table 3.9), were found to contain an *alkB* gene that clustered with that of *Prauserella rugosa*.

The partial *alkB* gene from one contaminated oleander rhizosphere from day 114 produced two bands on the DGGE gel (NOD2a and NOD2b). The sequences of the two bands branched separately within the *Prauserella-Nocardioides* cluster (Fig 3.13). The two sequences could belong to two different strains or to two homologous *alkB* genes within the same bacterium.

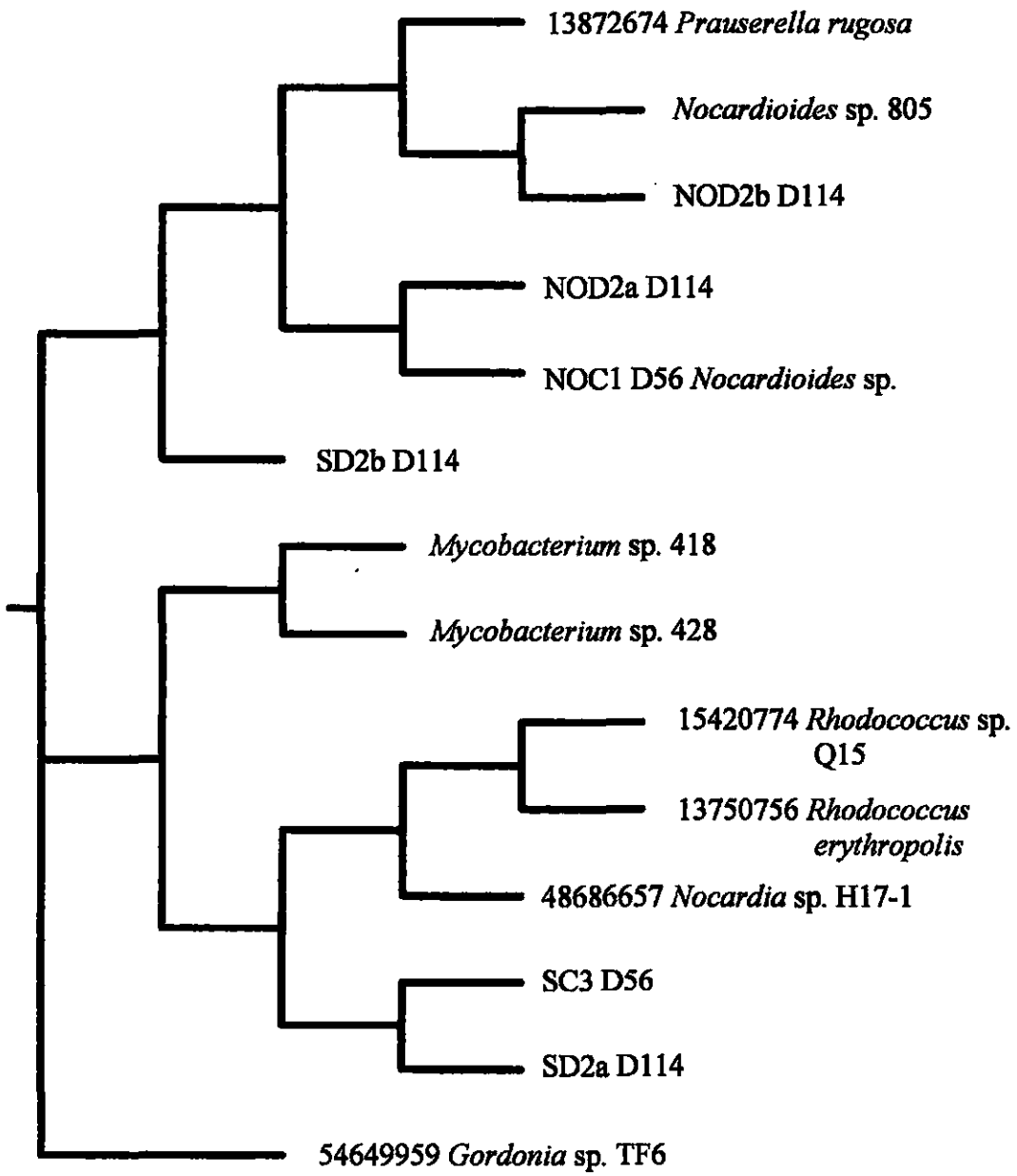


Figure 3.13 Phylogram for *alkB* (re-designed primers for *Mycobacterium* and *Nocardioides*) based on partial gene fragments (549 bp). The tree is based on a maximum likelihood analysis. GI numbers are given for the sequences obtained from the NCBI database: *Gordonia* sp. TF6 (gi 54649959); *Nocardia* sp. H17-1 (gi 48686657); *Rhodococcus erythropolis* (gi 13750756); *Rhodococcus* sp. Q15 (gi 15420774); *Prauserella rugosa* (gi 13872674). (See table 3.11 footnotes for sample origin).

The *Mycobacterium-Rhodococcus-Nocardia* cluster (Fig. 3.13), had a branch with two isolates of *Mycobacterium* sp. (418 and 428) (Table 3.4). Another branch has the *Rhodococcus* and *Nocardia* reference strains. Close to this branch are two alkB gene from soil samples (SC3, an uncontaminated sample from day 56, and SD2a, a contaminated sample from day 114). On the basis of 16S rDNA analysis, *Mycobacterium* sp. was identified in sample SC3 (Table 3.7), but no degrader bacteria was identified from sample SD2a. Note that sample SD2 from day 114 produced two DGGE bands (SD2a114 and SD2b114). The second band of the soil sample of day 114 did not cluster with any other sample or degrader strain, but branched near the *Prauserella-Nocardioides* cluster. It is possible that there are two different Gram-positive alkane degraders present in this sample. Given the limited number of sequences of degrader strains in the database, we were unable to obtain a potential identity for these degraders based solely on DNA sequencing.

The primer set for the *alkB* region chosen by Chénier et al. (2003) and the two primer sets modified for this study seem promising for community surveys of Gram-positive alkane-degraders. For instance, *Nocardioides* sp. 805 (Table 3.11) was isolated on the basis of its ability to degrade phenanthrene. In this study it was revealed that it also possessed an *alkB* gene, which confers the ability to degrade alkanes. The presence of multiple genes for the degradation of a variety of hydrocarbons has been found previously for other bacterial strains (Churchill et al., 1999; Whyte et al., 1997).

3.7 Identity of some phenanthrene-degrading bacteria isolates as determined by PCR-DGGE analysis of partial sequences of 16S rDNA.

Scrapings of phenanthrene-degrading bacteria plates could not be used to determine the identity of these bacteria by PCR-DGGE because a lot of bacteria that do not produce halos grow in the agar, presumably on acetone leftover or impurities in the agar. Some presumed phenanthrene degraders, i.e., those that produced clear halos on the plates, were isolated and tested again for degradation of phenanthrene in liquid cultures and then identified by PCR-DGGE analysis of partial 16S rDNA sequences.

Table 3.12 shows that all the isolates from either milo or oleander rhizosphere samples, that reliably degraded phenanthrene, were Gram-positive bacteria belonging in the *Arthrobacter* and *Mycobacterium* genera. Daane et al. (2001) obtained from the rhizosphere of *Spartina alterniflora*, a salt marsh plant, *Arthrobacter oxydans* PR-P3 (as determined by FAME), which was capable of degrading both phenanthrene and pyrene. Several *Mycobacterium* spp. are known to degrade PAHs (Churchill et al., 1999). *Rhizobium* and *Sinorhizobium,* although producing large halos on phenanthrene plates, did not consistently produce yellow products from the degradation of phenanthrene crystals. *Sinorhizobium* did not grow on the crystals. It is possible that more than one bacteria species was growing at the center of the halo and that a non-degrader was picked during isolation procedures.

Table 3.12. Identity and phenanthrene degrading ability of some putative phenanthrene degrading bacteria isolated from plates prepared with day 56 samples

Isolate or strain name[a]	Isolate #	Possible ID from partial 16S rDNA	Halo size	Yellow products from phenanthrene[d]
PNOD1 (3) 10^{-4}	800	*Arthrobacter sp.*	Small	+/-
PNOD2 (1) 10^{-4}	801	*Arthrobacter sp.*	Large	+
PMD1 (2) 10^{-4}A	802	*Arthrobacter sp.*	Large	+
PNOD1 (2) 10^{-4}	803	*Arthrobacter sp.*	Small	-
PMD1 (2) 10^{-4}B	804	*Arthrobacter sp.*	Large	+
PNOD2 (1) 10^{-4}	805	*Nocardioides sp.*	Large	+/-
PNOC3 (3) 10^{-3}	806	*Rhizobium sp.*	Large	+/-
PNOD2 (3) 10^{-4}	807	*Sinorhizobium sp.*	Large	-
PMD1 (1) 10^{-4}	808A	*Mycobacterium sp.*	ND	+
Reference strains				
PM600[b]		*Sphingomonas* sp.	Large	+
Pseudomonas putida F1[c]			ND	-

[a] PNOD, isolate from oleander growing in contaminated soil; PNOC, isolate from oleander growing in uncontaminated soil; PMD, isolate from milo growing in contaminated soil
[b] Positive control
[c] Negative control
[d] Evaluated by yellow products from growth on phenanthrene crystals in liquid Bushnell Haas medium (Wrenn and Venosa, 1996); for both experiments, there was no color for any strain without phenanthrene (even for yellow PM600) nor for control medium alone
[e] No data

3.8 Development of primers for a gene involved in phenanthrene degradation (phdI*)* in Gram-positive bacteria

Despite considerable effort to amplify a region of the *phdI* gene, no amplicon was obtained with the various primers that were designed in this study. A very weak amplification product was obtained when an annealing temperature gradient was performed with one of the primer sets, but this was not reproducible and the DNA could not be concentrated sufficiently for further optimization of the design. Further work needs to be done to find a better region for amplification of the *phdI* gene.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

1. The duration of the experiment described here allowed for the virtually complete degradation of the alkane contaminant (1000 mg hexadecane/ kg of coastal soil from Hickam Air Force Base) in unplanted soil by day 114. Contrary to previous results in the same soil contaminated with 10,000 mg of diesel/ kg of soil (Sun et al., 2004), none of the plants enhanced the degradation of hexadecane in this experiment. These results were expected for oleander, an ineffective shrub at diesel decontamination in a previous experiment (Sun et al., 2004). However, milo, which had been an efficient tree in the past experiment, was shown to slightly retard the degradation of hexadecane. Buffelgrass, which had not been thoroughly tested by Sun et al. (2004) also retarded hexadecane depletion. The major factor contributing to differences in results is probably the watering regime. In this experiment an effort was made to maintain soil moisture content at approximately 50% water holding capacity. In the previous experiment, the unplanted soil moisture was higher than the planted soil moisture. The higher soil moisture may have hampered the degradation of diesel in unplanted soil because less oxygen was available if more pores were filled with water. Thus the efficiency of milo in the previous experiment may have been due, in part, to a higher transpiration rate. Differences in the morphology of the leaves of the plants support this hypothesis. Milo has large leaves whereas oleander, which is resistant to drought, has narrow leaves with a

thick cuticle as a protection against evaporation. Other factors that may have contributed to differences in plant performance in the two experiments are differences in the nature of the contaminant (diesel vs. hexadecane) and the contaminant load (10,000 mg diesel/ kg of soil vs 1,000 mg of hexadecane and 200 mg of phenanthrene/ kg of soil). These results underline the complexity of the role of plants in bioremediation and the need for more studies to determine critical factors involved in phytoremediation at each site.

The degradation of phenanthrene (200 mg/kg of soil) was complete in all treatments by day 56, precluding the determination of the role of plants in the depletion process. Shorter-term experiments will be needed in future.

2. In general, the effects of the contaminant on plant height and biomass were negligible and short-lived. By day 114, differences between contaminated and uncontaminated treatments were not significant.

3. The response of bacterial populations to the hexadecane and phenanthrene contaminants and to plant roots was, in general, similar to the response to diesel fuel seen in a previous experiment (Jones et al., 2004).

The heterotrophic bacteria (total aerobic bacteria) preferred the root exudates and dead root cells to the hydrocarbons (Table 3). Regardless of contamination, throughout the experiment these bacteria responded more to the rhizosphere of the woody plants than to the rhizosphere of buffelgrass. No high numbers of starvation-surviving heterotrophic

bacteria were generated in the contaminated soil after depletion of the contaminant (Fig. 3.3A).

The hexadecane-degrading bacteria showed no preference for root exudates and dead root cells or hexadecane (Table 3.3).

On day 56 the rhizosphere of buffelgrass favored populations of hexadecane degraders to a higher extent than the rhizospheres of the two woody plants under uncontaminated conditions (Fig. 3.3B). In the rhizosphere of contaminated plants, an effect of hexadecane occurred only in the two woody plant pots. These results suggest different populations of hexadecane degraders inhabit the rhizospheres of buffelgrass and woody plants.

By day 114 the average concentration of hexadecane in all treatments was very low (Fig. 3.1) and there was no response of hexadecane degraders to the hydrocarbons in the planted treatments (Fig. 3.3B). Unexpectedly, high levels of hexadecane degraders remained in the contaminated unplanted treatments although the carbon sources available in soil organic matter were insufficient to allow growth of any of the microorganisms examined in this study (Fig. 3.3B, section 3.3). Presumably the unplanted-soil hydrocarbon degraders may have produced some triacylglycerides as storage material while growing on hexadecane (Alvarez et al., 2000; Alvarez, 2003) for use during starvation periods.

Phenanthrene-degrading bacteria were undetectable when hydrocarbons or plant roots were absent. They appear to have preferred phenanthrene to the root exudates and dead root cells throughout the experiment (Fig. 3.3C). Under uncontaminated conditions on day 56 they favored the rhizosphere of milo followed by that of oleander and then that of buffelgrass. In contrast, the "phenanthrene effect" was significantly higher in the rhizosphere of buffelgrass than in that of oleander and in the rhizosphere of oleander than that of milo.

On day 114, high levels of phenanthrene degraders persisted in all contaminated treatments although the phenanthrene had been gone since day 56 at the latest, and no "rhizosphere effect" existed except for oleander. Phenanthrene degraders, like hexadecane degraders, seem to be able to persist at high levels in the environment in the absence of a carbon source for long periods of time after growing on phenanthrene. The mechanism responsible for the survival of the phenanthrene degraders is still to be elucidated.

4. Soil lipase activity measurements to monitor alkane (hexadecane) degradation were shown to be feasible in the presence of plants. All increases in lipase activity, presumably due to the degradation of naturally occurring plant alkanes or derivatives, were significantly lower than the lipase activity due to the hexadecane contaminant degradation in planted and unplanted treatments.

Lipase activity increased in all treatments while the major part of the hexadecane was degraded (day 0 to 56) (Fig. 3.4). When the levels of hexadecane were low and the rate of degradation was slower (day 56 to 114) lipase activity decreased for all treatments except buffelgrass, for which it remained stationary. The lipase activity could not differentiate between the two treatments that retarded hexadecane degradation (milo and buffelgrass) and the other treatments (unplanted soil and oleander), presumably because the variability was high. It may be possible to decrease the variability of the measurements, and therefore increase the discriminatory power of the assay, by making more replicates and using more soil in the assay. However, the plot of lipase activity measurements indicated an almost complete hexadecane degradation that coincided with a decrease in lipase activity or the maintenance of a constant level. Thus monitoring lipase activity in planted or unplanted soil contaminated with alkanes could provide useful information regarding the progress of biodegradation. Lipase activity could be used in lieu of the more lengthy and costly GC analyses to monitor the bioremediation process. A final GC analysis would then suffice.

5. The diversity of the dominant hexadecane degraders obtained from positive MPN wells at the highest soil dilution was examined by PCR-DGGE of the V6-8 region of the 16S rDNA. Sequencing of the resulting bands allowed identification of the dominant hexadecane degraders in planted and unplanted treatments in contaminated and uncontaminated conditions. Major shifts in the dominant populations of hexadecane degraders occurred during the hexadecane degradation process.

On day 0 a large proportion of the genotypes of the dominant hexadecane degraders were *Alphaproteobacteria*. A few *Betaproteobacteria* and *Gammaproteobacteria* as well as *Actinobacteria* of the genus *Nocardioides* were also found. None of the bacteria detected on day 0 were specialized hydrocarbon degraders.

In uncontaminated treatments on day 56, near equal numbers of *Alphaproteobacteria* and *Gammaproteobacteria* genotypes occurred across planted and unplanted treatments, along with the *Actinobacteria*, *Nocardioides* and *Mycobacterium*, and *Hydrocarboniphaga*, a specialized hydrocarbon degrader. In contaminated treatments, *Gammaproteobacteria* dominated. *Alcanivorax*, a cosmopolitan gammaproteobacterium associated with the bioremediation of oil spills in coastal areas (Yakimov et al., 1998; Head et al., 2006; Kasai et al., 2002), was among the dominant hexadecane degraders in all planted and unplanted treatments except buffelgrass. Buffelgrass 'selected' one single genotype of hydrocarbon degrader in contaminated rhizospheres, *Pseudomonas indica* IMT40, an alkane degrader (Pandey et al., 2002). The retardation of hexadecane degradation in soil planted to milo on day 56 (Fig. 3.1) may be due to a lower proportion of hexadecane degraders (presumably of the *Alcanivorax* type) among total bacteria (Table 3.7) in the milo samples than in unplanted soil and soil planted to oleander. A high proportion of hexadecane degraders (*P. indica* IMT40) among the total bacteria in the buffelgrass samples may have decreased the capacity of this treatment to degrade hexadecane because *P. indica* may preferably feed on root exudates because it can use a large number of simple carbon sources (Pandey et al., 2002). In contrast *Alcanivorax* feeds almost exclusively on alkanes (Yakimov et al., 1998).

On day 114 in both contaminated and uncontaminated conditions there was almost equal number of *Alphaproteobacteria* and *Gammaproteobacteria* genotypes and a few Gram-positive bacteria among the hexadecane degraders. Thus a major switch in populations of hexadecane-degrading bacteria had occurred in the contaminated treatments. *Alcanivorax*, the alkane-degrader specialist, was virtually absent when the hydrocarbon was gone. The populations of alkane degraders had reverted to bacteria that were more versatile in terms of the C sources they were capable of using.

These results indicate that in this soil, changes in the ratio of *Alphaproteobacteria* to *Gammaproteobacteria* may be an indicator of the progress of bioremediation. Also, the presence of *Alcanivorax* populations could be an excellent indicator of the progress of remediation since *Alcanivorax* disappeared rapidly when the alkanes were gone (Kasai et al., 2002; Head et al., 2006). *Alcanivorax* could be used to screen for efficient plants for field experiments. Plants that select populations of *Alcanivorax* in their rhizospheres under contaminated conditions would be good candidates for phytoremediation.

6. Once the major players in the degradation of hexadecane had been identified by partial sequencing of their 16S rRNA genes, the confirmation of the presence of a gene coding for an alkane hydroxylase, the first enzyme in the oxidation pathway of alkanes was sought. Due to the diversity in the sequences of this gene among different alkane degraders, few of the samples and isolates produced amplicons in polymerase chain reactions with primers for *alkB* from Chénier et al. (2003). These primers were

originatelly designed using *Rhodococcus* but were capable of amplifying some *Pseudomonas* sequences (Table 3.11). The redesigned primers for *alkB* targeting *Alcanivorax* and *Pseudomonas* produced amplicons in all tested samples that contained *Alcanivorax* and some that contained *Pseudomonas*, as determined on the basis of 16S rDNA sequencing. The redesigned primers for *alkB* targeting *Nocardioides* and *Mycobacterium* produced amplicons with the DNA of 3 isolates of either of those genera and one sample in which *Nocardioides* had been detected by 16S rDNA sequencing. Other strains of unknown identity produced amplicons in PCR with these primers.

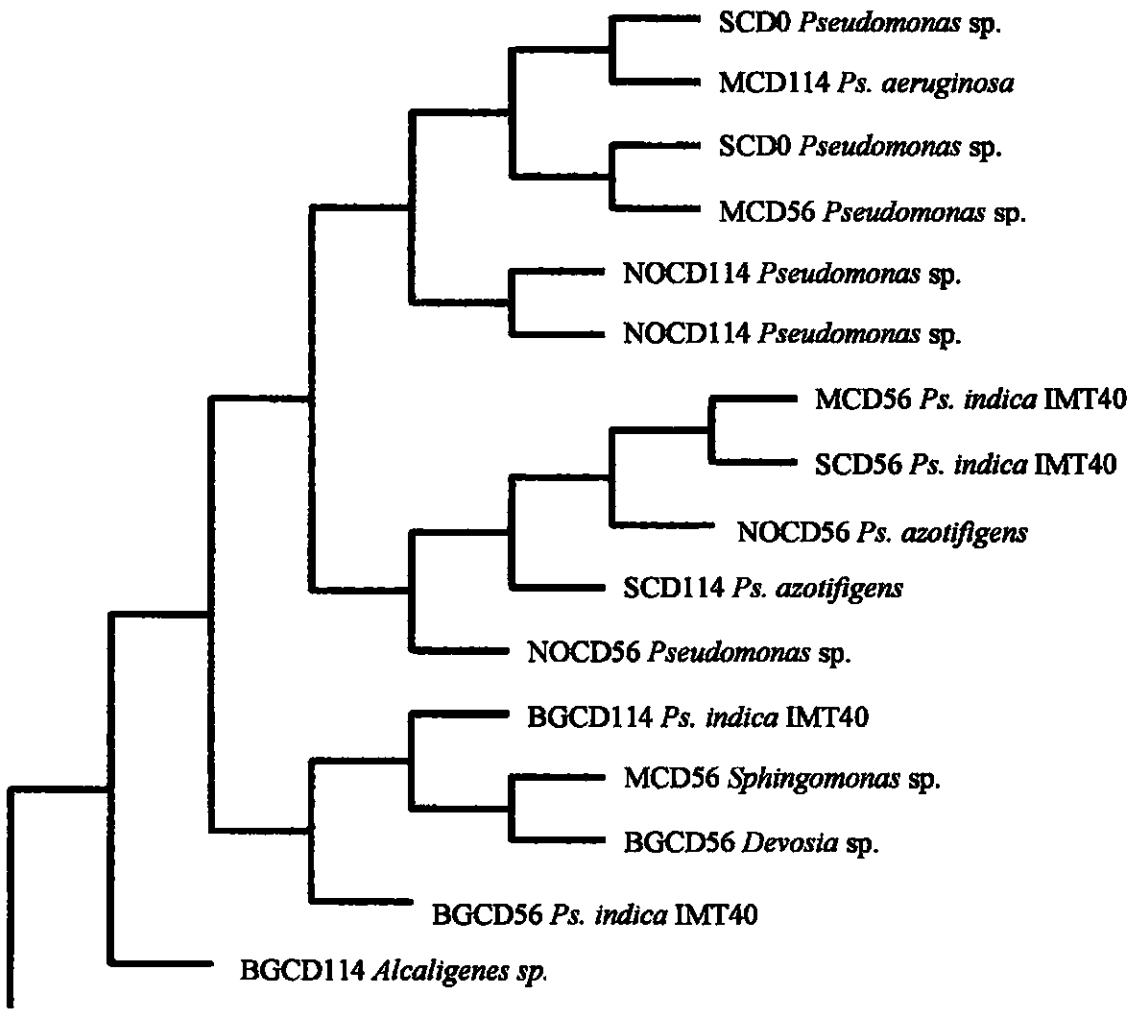
7. Mostly Gram-positive bacteria were isolated from phenanthrene plates prepared with dilutions of rhizosphere soil from milo and oleander. Most of these were confirmed as phenanthrene degraders by testing for benzene-ring fission. *Arthrobacter* isolates were the most numerous. *Nocardioides* and *Mycobacterium* were also detected.

8. Attempts at designing primers to amplify the *phdI* gene from Gram-positive phenanthrene degraders were unsuccessful. This gene codes for the 1-hydroxy-2-naphthoate dioxygenase, which is unique to the degradation of phenanthrene, whereas most other enzymes are also involved in the degradation of naphthalene. The major hindrance in this part of the work was the scarcity of known gene sequences.

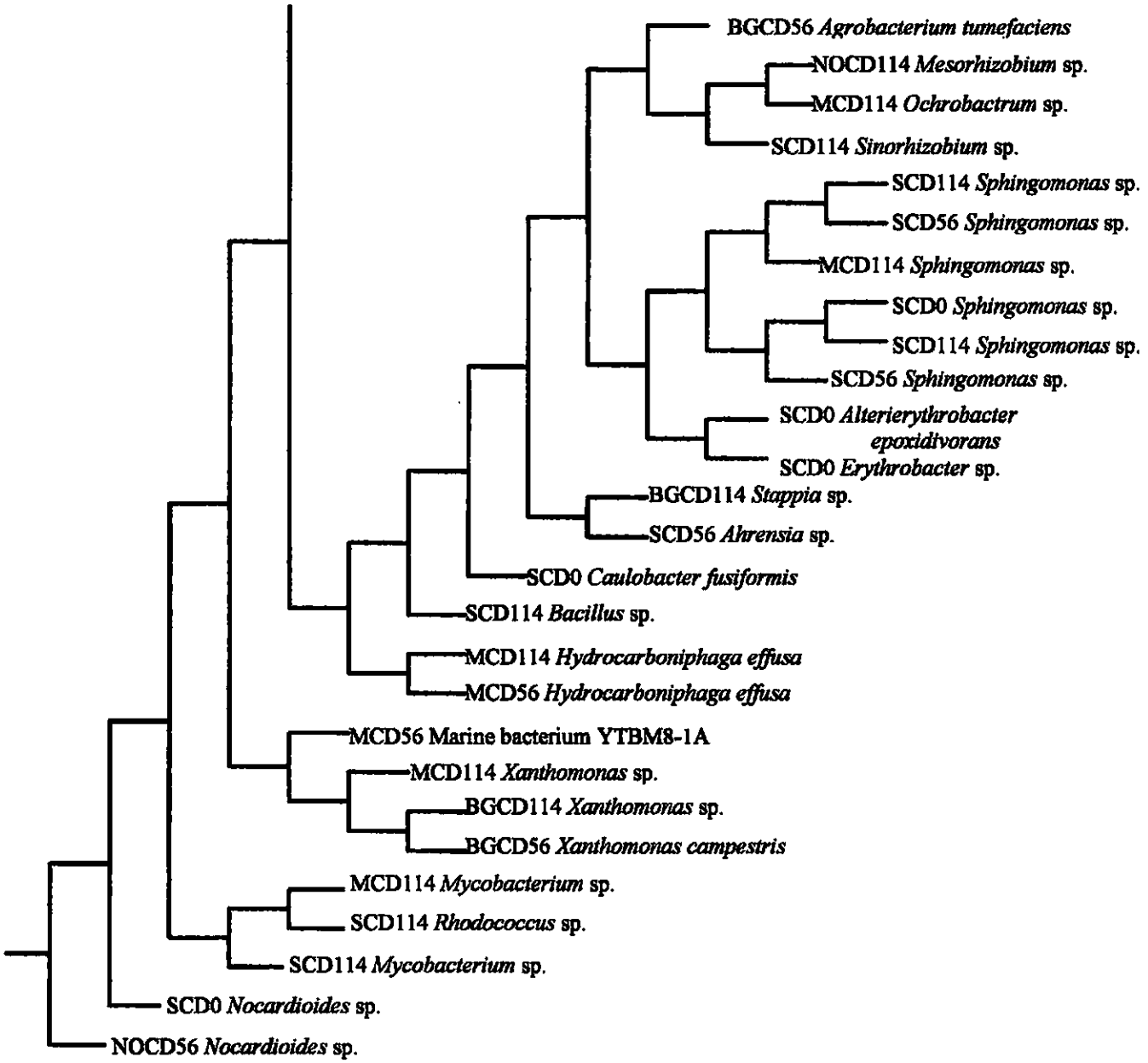
APPENDICES

APPENDIX A. Sample code origin used throughout the study and number of hexadecane-degrader genotypes obtained from pooled MPN wells at the highest dilution (First trial)

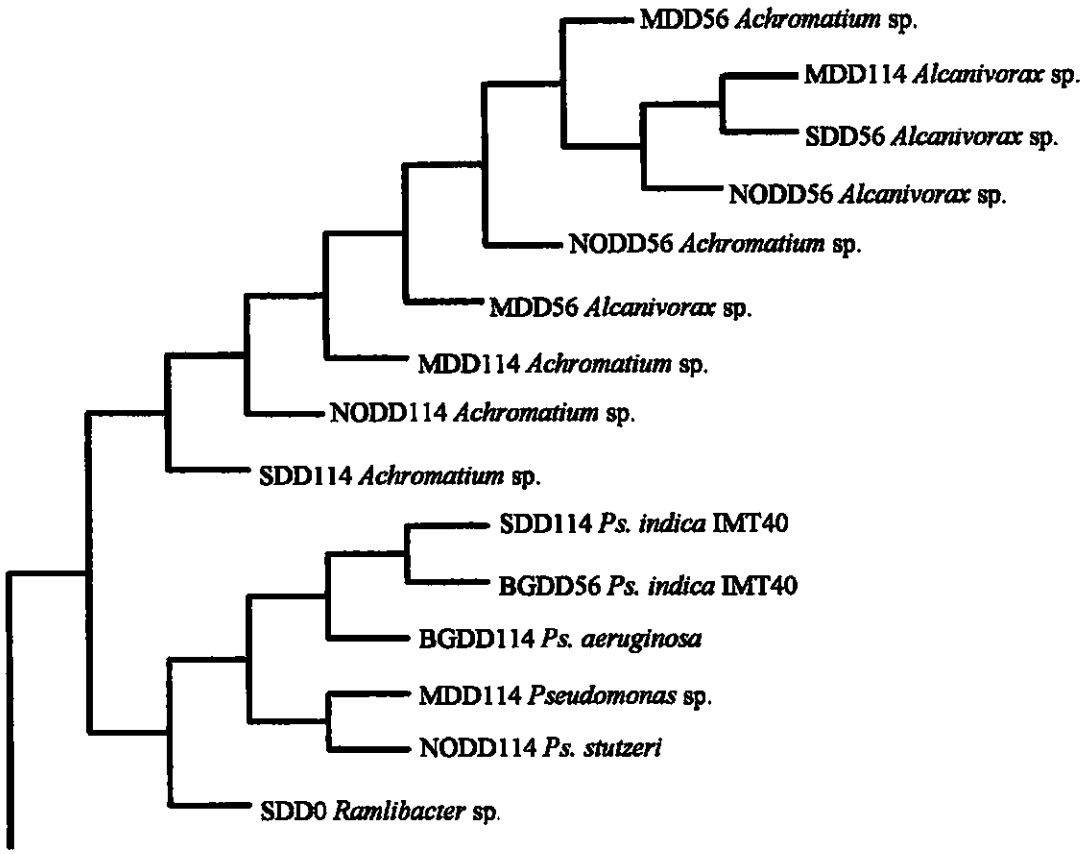
Sample code	Sample origin	Hydrocarbon treatment	MPN Dilution used	Number of positive wells	Number of genotypes	
					Predicted	Actual
Day 0						
SC2	Soil	-HC	10^{-5}	2	2	4
SC3	Soil	-HC	10^{-5}	1	1	2
SD2	Soil	+HC	10^{-5}	1	1	2
SD3	Soil	+HC	10^{-5}	1	1	3
Day 56						
SC1	Soil	-HC	10^{-5}	1	1	3
SC2	Soil	-HC	10^{-4}	4	≤4	2
SC3	Soil	-HC	10^{-5}	1	1	1
SC4	Soil	-HC	10^{-4}	5	≤5	1
MC2	Milo	-HC	10^{-6}	1	1	1
MC4	Milo	-HC	10^{-6}	2	≤2	2
NOC1	Oleander	-HC	10^{-5}	2	≤2	1
NOC2	Oleander	-HC	10^{-5}	2	≤2	1
BGC1	Buffelgrass	-HC	10^{-7}	2	≤2	2
BGC1	Buffelgrass	-HC	10^{-8}	1	1	3
BGC4	Buffelgrass	-HC	10^{-8}	1	1	1
SD1	Soil	+HC	10^{-7}	4	≤4	1
SD4	Soil	+HC	10^{-7}	2	≤2	2
MD1	Milo	+HC	10^{-6}	5	≤5	1
MD3	Milo	+HC	10^{-7}	2	≤2	1
NOD1	Oleander	+HC	10^{-6}	5	≤5	2
NOD3	Oleander	+HC	10^{-6}	4	≤4	3
BGD2	Buffelgrass	+HC	10^{-9}	1	1	1
BGD4	Buffelgrass	+HC	10^{-9}	1	1	1
Day 114						
SC2	Soil	-HC	10^{-4}	1	1	3
SC4	Soil	-HC	10^{-4}	2	≤2	5
MC3	Milo	-HC	10^{-6}	5	≤5	2
MC4	Milo	-HC	10^{-6}	5	≤5	2
NOC2	Oleander	-HC	10^{-6}	1	1	1
NOC3	Oleander	-HC	10^{-6}	2	≤2	1
BGC1	Buffelgrass	-HC	10^{-6}	1	1	1
BGC3	Buffelgrass	-HC	10^{-7}	3	≤3	4
SD1	Soil	+HC	10^{-6}	2	≤2	2
SD4	Soil	+HC	10^{-6}	2	≤2	3
MD2	Milo	+HC	10^{-6}	1	1	3
MD3	Milo	+HC	10^{-6}	1	1	2
NOD2	Oleander	+HC	10^{-6}	2	≤2	2
NOD3	Oleander	+HC	10^{-6}	2	≤2	2
BGD3	Buffelgrass	+HC	10^{-6}	4	≤4	3
BGD4	Buffelgrass	+HC	10^{-7}	3	≤3	3



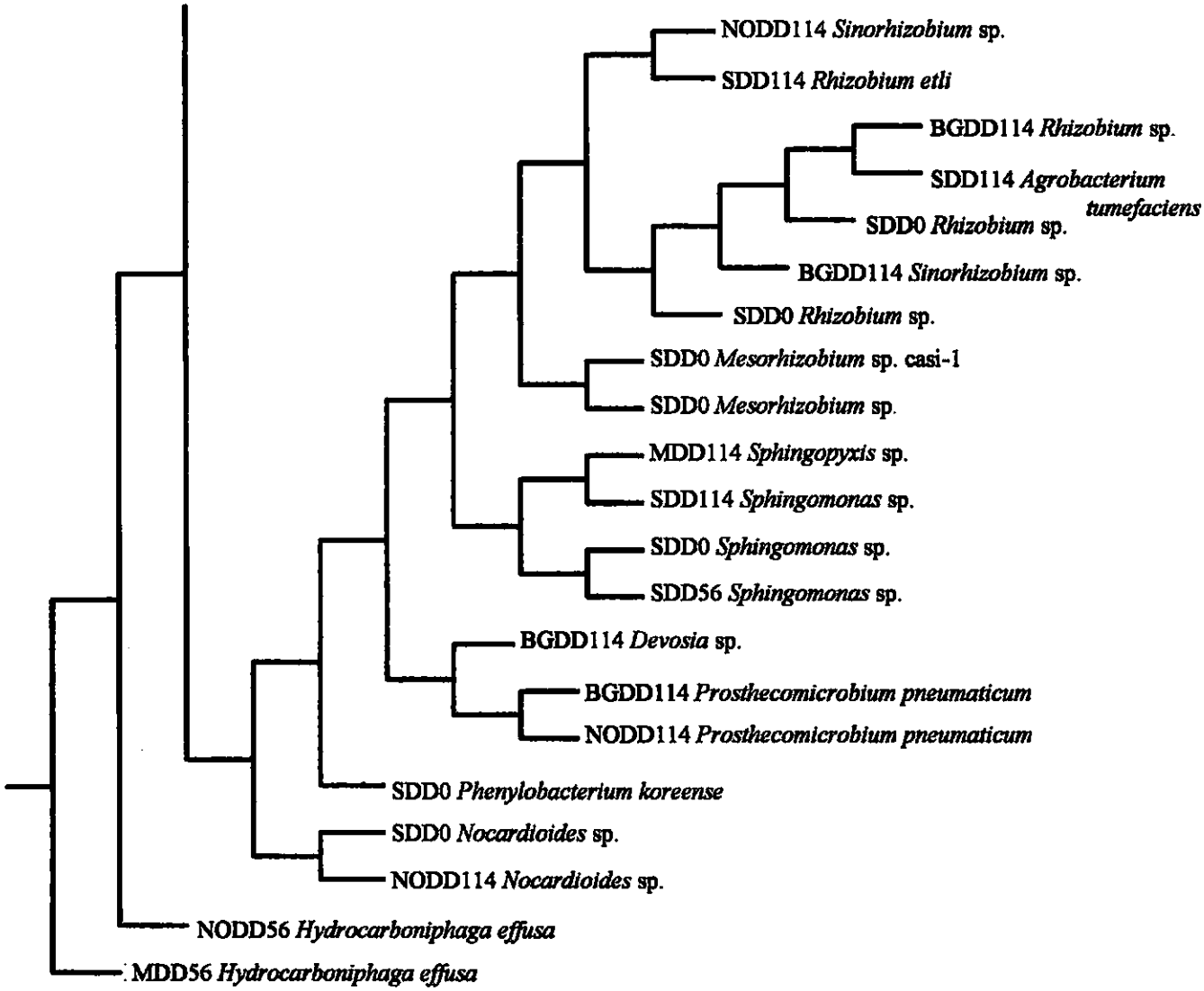
Appendix B. Phylogram for 16S rDNA based on partial gene fragments from uncontaminated samples (419bp). The tree is based on neighbor-joining distance method. (See Appendix A for sample codes)



Appendix B (Contd.). Phylogram for 16S rDNA based on partial gene fragments from uncontaminated samples (419bp). The tree is based on neighbor-joining distance method. (See Appendix A for sample codes)



Appendix C. Phylogram for 16S rDNA based on partial gene fragments from contaminated samples (419bp). The tree is based on neighbor-joining distance method. (See Appendix A for sample codes)



Appendix C (Contd.). Phylogram for 16S rDNA based on partial gene fragments from contaminated samples (419bp). The tree is based on neighbor-joining distance method. (See Appendix A for sample codes)

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