Rhizoremediation of hydrocarbon contaminated soil using Australian native grasses

A Thesis submitted for the degree of
Doctor of Philosophy

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DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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SUMMARY

The breakdown of contaminants in soil resulting from microbial activity that is enhanced in the presence of the plant root zone (rhizosphere) has been termed *rhizoremediation*. To date, Australian native plants have not been assessed for their hydrocarbon rhizoremediation potential. The use of native plants offers an economically feasible and environmentally sustainable cleanup option for the rehabilitation and restoration of hydrocarbon contaminated sites in Australia. The aim of the study was to evaluate the potential of Australian native grass species for the rhizoremediation of aliphatic hydrocarbon contaminated soil from a mine site.

Candidate Australian native grass species (*Poaceae*) were selected following the development of essential and desirable growth criteria. Nine perennial Australian grasses were evaluated for seedling emergence in sandy loam soil sourced from a mine site which was artificially contaminated with a 60:40 diesel/oil mix at concentrations of 30 000 mg/kg, 10 000 mg/kg, 5 000 mg/kg and 0 mg/kg (control). Seedling emergence was not adversely affected by the presence of hydrocarbon contamination at the exposed concentrations for eight of the nine species studied ($p > 0.05$). Three promising species were assessed for relative growth performance in diesel/oil contaminated (10 000 mg/kg, 5 000 mg/kg) and uncontaminated (control) soils in greenhouse studies to assess their tolerance of aliphatic hydrocarbon contaminated soil.

Cymbopogon ambiguus (Lemon Scented grass) is a summer growing perennial with widespread distribution throughout Australia including the region where the mine site is situated. *Brachiaria decumbens* (Signal grass) (naturalised) is adapted to humid tropical areas of Australia and is native to the site and sourced from seed banks. *Microlaena stipoides* (Weeping grass var. Griffin) is a cool season grass, widely distributed throughout Australia in moister regions. The three evaluated species survived for 120 days in the diesel/oil contaminated soil at the exposed concentrations without adverse growth affect ($p > 0.05$). In some instances (e.g. *C. ambiguus*) growth stimulation occurred in the presence of

contamination producing significantly more root biomass compared with the control ($p < 0.0001$).

Most hydrocarbon degradation is believed to occur through microbial processes, and so the plant-associated microbial community was examined in the three tolerant species. The assessment of the influence of grass on the abundance and activity of microorganisms in the rhizosphere revealed species-specific plant-induced changes in the soil microbial community. Selective enrichment of hydrocarbon degrading microorganisms was demonstrated in the rhizosphere soil of the Australian grasses tested, to varying degrees. *C. ambiguus* appeared to have the greatest influence on stimulation of hydrocarbon degrading microorganisms, followed by the cool season grass *M. stipoides*. *B. decumbens* showed consistently lower numbers of hydrocarbon degrading microorganisms in rhizosphere soil over time compared to the other two species ($p < 0.01$). The influence of grasses on microbial community structure (defined as community DNA fingerprint) in diesel/oil contaminated soil suggested no new microbial population was favoured by the grasses (qualitative shift), rather there were relative quantitative changes in existing members of the microbial population. Soil lipase activity did not appear to be an optimal bioindicator of rhizoremediation and may encompass total soil microbial activity not exclusively the hydrocarbon degrading microorganisms of interest.

The assessment of biodegradation of hydrocarbons in soil is essential to characterise the effectiveness of plant species in rhizoremediation. Residual diesel and oil concentrations (as total petroleum hydrocarbons, TPH) were measured using Gas Chromatography. The presence of single species successfully enhanced the removal of hydrocarbons from soil (for all species). All showed significantly lower residual hydrocarbon concentrations than those in unplanted soil after 100 days ($p < 0.01$). Significantly, it was not necessary to add N and P to achieve up to 90% reduction in hydrocarbon concentrations in the soil. The relative performance of each grass species varied. In soil planted with *C. ambiguus* hydrocarbon concentrations were reduced faster and to a greater extent than the other species studied, from 10 000 mg/kg to approximately 1 100 mg/kg TPH (88% removal). Similar endpoint success was recorded for

M. stipoides which facilitated 80% reduction in hydrocarbon concentrations. Interestingly, *B. decumbens* (the only naturalised species) did not perform as well as the other species (although still significantly better compared to unplanted controls), with hydrocarbon concentrations reduced to approximately 4 500 mg/kg (49%). Hydrocarbon concentrations in unplanted (control) soil were reduced by 45% through natural biodegradation processes. Plant root and shoot tissue was periodically assessed for hydrocarbon accumulation and was shown to be negligible. A multispecies planted trial using *C. ambiguus* plus *B. decumbens* had no additional influence on total TPH removal. The final TPH removal efficiency in the multispecies trial was not significantly different ($p > 0.05$) from that of the best single species performer of the two i.e. *C. ambiguus*. In a field application the planting of multiple species may still be desirable in order to preserve site biodiversity and assist rehabilitation of the area.

A strong relationship between abundance of hydrocarbon degrading microorganisms in the rhizosphere and hydrocarbon biodegradation was demonstrated for all species ($p < 0.01$). Those species which showed greatest stimulation of the microbial population resulted in enhanced TPH removal from soil. These species were the summer grass *C. ambiguus* and the winter species *M. stipoides*. This may allow for broader application both seasonally and geographically across Australia. *B. decumbens* showed successful rhizoremediation to a lesser degree, but may still be an option in multiple planting strategies.

This investigation identified three Australian grass species (from the nine evaluated) that are candidates for further investigation for *in situ* rhizoremediation potential at field scale.

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Editorial features

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CHAPTER ONE

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Global industrialisation over the past two centuries has resulted in widespread contamination of the environment with persistent organic and inorganic wastes. Contaminated land has generally resulted from past industrial activities where awareness of the environmental health effects connected with the production, use, and disposal of hazardous substances were less recognised than today. The problem is worldwide, and the estimated number of contaminated sites is significant and increasing (Mougin 2002; Kaimi *et al* 2006). Accurate detail regarding the extent of hydrocarbon contamination in the terrestrial environment has been difficult to quantify because of the unintentional nature of the contamination (largely through accidental spillage or around factories and petrol stations). Hydrocarbon pollution is ubiquitous in the environment, and for example, in the United Kingdom accounts for over 15% of all pollution incidents (Stroud *et al* 2007).

There have been increasing international efforts to remediate contaminated sites using "green" technologies, either as a response to the risk of adverse health or environmental effects or to enable site redevelopment (Vidali 2001). An examination of global markets for remediation services (United States International Trade Commission [USITC] 2004) reported steady growth in recent years, with value increasing from US$25.7 billion in 1996 to US$29.9 billion in 2002. The annual Australian market size was valued at an estimated US$675 million in 2000. Growth in this market is expected to continue due to increased environmental awareness, public pressure, and emerging legislation (USITC 2004).

This introductory chapter reviews some of the common land remediation and restoration techniques used for soil contaminated with petroleum hydrocarbon pollutants. Specific attention is paid to aliphatic hydrocarbon pollutants and to

technologies utilising plants as enhancers of the biodegradation and site remediation process.

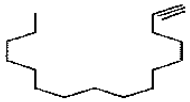
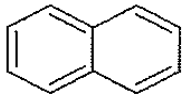
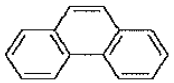
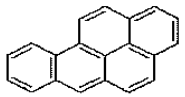
1.2 Aliphatic hydrocarbons in soil: Physicochemical properties

Aliphatic hydrocarbons make up a substantial proportion of organic contamination in the terrestrial environment (Stroud *et al* 2007). This single class of contaminant is subject to physicochemical processes which can affect the fate and behaviour in soil such as degree of loss, sequestration and interaction with microflora (Germida *et al* 2002). The production of aliphatic hydrocarbons is understandably associated with the petroleum industry. Aliphatic hydrocarbons are major components of crude oil and petroleum products (Merkl *et al* 2004a). Unlike aromatic hydrocarbons which contain one or more benzene rings, aliphatic hydrocarbons are saturated and unsaturated linear or branched open-chain structures. Aliphatic hydrocarbons are defined as open-chain methane derivatives, which are both non-aromatic and non-cyclic organic compounds, containing carbon and hydrogen (Stroud *et al* 2007). **Table 1.1** shows the members of aliphatic hydrocarbon groups and their properties. Collectively these physicochemical properties mean that mid-length aliphatic contaminants are not readily volatilised or leached from soil. Hydrophobicity has been determined as a critical property controlling hydrocarbon behaviour in soil, affecting sequestration and biological availability. For example, as shown in Table 1.1 the aliphatic hexadecane is a very hydrophobic hydrocarbon (high octanol-water partition coefficient), and several orders of magnitude more insoluble than the polycyclic aromatic hydrocarbon (PAH) phenanthrene (Stroud *et al* 2007).

Diesel fuel is produced by refining crude oils and is a complex mixture of petroleum hydrocarbons with a carbon chain length of between C8 to C26. Engine oil is a petroleum distillate and highly refined mineral oil. Chemical constituents of engine oil include non-volatile mixture of long chain aliphatic, saturated and unsaturated hydrocarbons (C20-C50). Diesel fuel has a high content of normal, branched, cyclic and unsaturated alkanes (60 to > 90% by volume) (ATSDR 1996). It also contains recalcitrant aromatic hydrocarbons (5-

40% by volume), and small amounts of alkenes (0-10% by volume) obtained from the middle-distillate, gas-oil fraction during petroleum separation. Benzene, toluene, ethylbenzene, xylenes and PAHs (especially naphthalene) may be present at levels of parts per million in diesel fuel (ATSDR 1996). Owing to the complexity of the diesel fuel and engine oil mixtures, analytical techniques used in most environmental assessments measure the total petroleum hydrocarbon mixture (Adam and Duncan 1999).

Table 1.1 Physicochemical properties of selected hydrocarbons (taken from Stroud *et al* 2007).

Hydrocarbon group		Name	Formula	Molecular weight (g mol^{-1})	Structure	Melting point (°C)	Boiling point (°C)	Solubility (mg l^{-1})	log K_{ow}
Aliphatic	Alkane	Tetradecane	$C_{14}H_{30}$	198·38		5·5	253	0·000 282	7·2
	MODEL Alkane	Hexadecane	$C_{16}H_{34}$	226·44		18	287	0·0009	9·1
	Alkene	Hexadecene	$C_{16}H_{32}$	224·43		3–5	274	N/A	N/A
	Alkyne	Hexadecyne	$C_{16}H_{30}$	222·42		15	148	N/A	N/A
Aromatic	PAH	Naphthalene	$C_{10}H_8$	128·18		79–83	217·9	30	3·36
	MODEL PAH	Phenanthrene	$C_{14}H_{10}$	178·22		97–101	340	1·1	4·16
	PAH	Pyrene	$C_{16}H_{10}$	202·6		156	404	0·135	5·19
	PAH	Benzo[a]Pyrene	$C_{20}H_{12}$	252·31		175–179	495	0·0038	6·06

N/A, Data not available.

1.3 Bioremediation: The microbial degradation of organic pollutants in soil

Bioremediation is defined as the use of microorganisms to degrade or detoxify environmental pollutants (Bamforth and Singleton 2005). It is a cleanup technology that offers the possibility to eliminate or render harmless various organic contaminants using natural biological activity in soil (Vidali 2001; Jorgensen 2007). The most commonly targeted organic pollutants for bioremediation include petroleum, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorinated phenols, and aliphatics (Lynch and Moffat 2005).The microorganisms involved in bioremediation are often indigenous to the contaminated area or isolated from elsewhere and brought to the site. The process involves microorganisms able to degrade the contaminant that increase in numbers when the contaminant is present (Mougin 2002); when the contaminant is degraded, the biodegrading population declines. If bioremediation is effective in completely mineralising the pollutant, the residues of treatment are harmless products including carbon dioxide, water and cell biomass (Jorgensen 2007).

The control and optimisation of bioremediation processes is a complex system involving many factors (Vidali 2001; Bamforth and Singleton 2005). These factors include: the existence of a microbial population capable of degrading the pollutants; the bioavailability of contaminants to microbial attack; the environmental factors contributing to microbial growth (type of soil, temperature, soil pH, the presence of oxygen or other electron acceptors, and nutrient content). Bioremediation promotes the microbial metabolism of contaminants by adjusting the water, air and nutrient supply in the soil (Bamforth and Singleton 2005).

Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process for contaminated soil. It can also be carried out *in situ* (onsite) eliminating the need to transport quantities of waste offsite. The potential threats to human health and the environment that arise from

transportation are also eliminated by *in situ* bioremediation. There are some limitations to bioremediation, including its restriction to compounds that are susceptible to biodegradation, and regulatory uncertainty regarding acceptable performance criteria and endpoints for bioremediation treatments (Vidali 2001). Bioremediation has had increasing success in the restoration of sites contaminated with petroleum hydrocarbons. The *Exxon Valdez* oil spill in Alaska is an important example (Pritchard *et al* 1992), though this was a 'test site' and not a full-scale remediation. Many critical reviews on bioremediation technologies exist, and the reader is directed to these for further information as it is outside the scope of this thesis (Allard and Neilson 1997; Vidali 2001; Mougin 2002; Bamforth and Singleton 2005; Jorgensen 2007).

1.4 Phytoremediation: Plant-assisted bioremediation

Phytoremediation is the *in situ* use of plants and their associated microorganisms to "extract, sequester or detoxify pollutants for the treatment of contaminated soils, sediments and water" (Frick *et al* 1999). It is a technology applicable to sites containing organic, nutrient or metal pollutants. This definition applies to all plant-influenced biological, chemical, and physical processes that aid in remediation of contaminated substrates. A description of key phytoremediation processes and mechanisms is given in Section 1.5. In the majority of cases for organic pollutants, phytoremediation works by accelerating natural biodegradation rates (bioremediation). In the last decade phytoremediation has gained increasing acceptance as an area of research and equally as a viable cleanup technology particularly for organic pollutants (Alkorta and Garbisu 2001; van der Lelie *et al* 2001; Arthur *et al* 2005).

The concept of phytoremediation is not new. The use of plants in wastewater treatment technology is over 300 years old (Cunningham and Berti 1993). Historically, much of the early research on phytoremediation of organic contaminants focused on agricultural chemicals such as pesticides (see reviews Anderson *et al* 1993; Sicilano and Germida 1998). More recently, there has been increasing interest in the phytoremediation of petroleum hydrocarbons as

widespread and recalcitrant pollutants (Schnoor 2002). In particular, the application of phytoremediation for PAHs and aliphatic petroleum hydrocarbon contamination is being explored (Frick *et al* 1999; Hutchinson *et al* 2003; Arthur *et al* 2005). This is due to the increased international awareness of the adverse impacts of hydrocarbon contamination on human health and the environment.

1.4.1 Influence of environmental factors on phytoremediation

The 'greening' of a site confers additional benefits other than just remediation. These include protection against wind erosion, reduction of surface run-off, reinforcement of soil by roots, protection of biodiversity, and enhancement of the site generally in terms of aesthetics (Smith *et al* 2006). The control and optimisation of phytoremediation processes is influenced by a complex system of many environmental factors. These factors include: type of soil, temperature, pH, organic matter content, water and oxygen availability, and nutrients. Some of the factors affect degradation processes directly while others impact phytoremediation by altering the bioavailability of the pollutant (see also Section 1.4.2). Overall there are numerous soil and environmental factors that influence the fate of a contaminant in the plant root zone. **Table 1.2** outlines optimum environmental conditions for the degradation of hydrocarbon contaminants in soil.

Contamination with hydrocarbon based compounds affects the carbon:nitrogen (C:N) ratio in soil and can lead to nitrogen immobilisation (Newman and Reynolds 2004). The inorganic mineral nutrients that are most often reported to limit the breakdown on petroleum hydrocarbons in soil are nitrogen and phosphorus (Hutchinson *et al* 2003).

Soil type, structure and organic matter can limit the bioavailability of petroleum contaminants (Germida *et al* 2002). A proportion of petroleum hydrocarbon contaminants can be strongly adsorbed on organic matter in the soil system. Plant root exudates increase the organic matter content in contaminated soils, and this may affect contaminant bioavailability through sorption (Hutchinson *et al* 2003). In some cases, petroleum hydrocarbons are not readily desorbed, and are therefore not available for phytoremediation.

Table 1.2 Environmental conditions affecting phytoremediation[a]

Parameter	Optimum value for hydrocarbon degradation	Condition required for microbial activity
Soil moisture	30-90%	25-28% water holding capacity
Soil pH	6.5-8.0	5.5-8.8
Oxygen content	10-40%	Aerobic
Temperature (°C)	20-30	15-45
Nutrient content	C:N:P = 100:10:1	N and P for microbial growth
Type of soil		Low clay or silt content

[a] adapted from Frick *et al* 1999; Vidali 2001; Hutchinson *et al* 2003; Bamforth and Singleton 2005.

1.4.2 Special considerations in phytoremediation

Contaminant tolerance is an important consideration if phytoremediation is to be effective. Elevated concentrations of contaminants may result in a toxic response, which may include the death of the plant or soil microorganisms (Germida *et al* 2002). The successful implementation of any phytoremediation system will require the establishment of tolerant plants in the contaminated soil. Factors to consider include: selection of an appropriate (ideally native) plant species, the influence of contaminants on seed germination, and the ability to tolerate the presence of contamination with minimal adverse growth effect (Germida *et al* 2002; Hutchinson *et al* 2003). Further discussion of the 'ideal candidate' plant is given in Section 1.6.1.

Mixtures of contaminants in soil may cause difficulties in phytoremediation. Hydrocarbon-contaminated sites may also be polluted with metals, salts, pesticides, and/or other petroleum mixtures, thus complicating phytoremediation efforts. In these situations soil microbes may selectively degrade organic

compounds that are easiest to assimilate or provide the most energy, a phenomenon termed *diauxy* (Frick *et al* 1999). Mixtures of organic compounds can promote the microbial degradation of petroleum hydrocarbons, particularly if one or more components of the mixture is a co-metabolite of others (Nocentini *et al* 2000). In this instance, microbes primarily degrading one type of organic compound may also fortuitously degrade a second compound present at concentrations too low to independently support bacterial growth or not recognised as a substrate.

The bioavailability of a pollutant is important for its phytoremediation. For plants and their associated microorganisms to remediate pollutants in soil, they must be in contact with them and able to act on them. Pollutant bioavailability depends on the chemical properties of the contaminant, soil properties, environmental conditions, and biological activity (Pilon-Smits 2005). The most important chemical property of a pollutant used in the phytoremediation industry is the octanol-water partitioning coefficient (K_{ow}), a measure of its hydrophobicity and potential for movement in soil. Contaminants with low log K_{ow} (≤ 1) are considered very water soluble and mobile and can be accumulated in plants (Cunningham and Berti 1993). Pollutants with intermediate log K_{ow} (1-4) are also taken up by the roots. Compounds in this range would be considered reasonable targets for phytoremediation. Contaminants with log K_{ow} greater than 4 are generally bound to soil organic matter or adsorbed to roots and not substantially translocated to the shoot (Pilon-Smits 2005). Aliphatic hydrocarbons have high K_{ow}, and therefore uptake into plant tissue is considered a minor pathway for their removal from soil systems (see further discussion in Section 1.5).

Amendments may be added to soil that make contaminants more bioavailable for plant uptake or microbial attack. For example, the addition of natural organic acids such as citrate or malate will lower the pH and chelate metals such as cadmium and lead from soil particles, making them more available for plant uptake (Pilon-Smits 2005; Santos *et al* 2006). Similarly, adding surfactants (e.g. Tween 80) to soil may also increase the bioavailability of hydrophobic organic contaminants (Gao *et al* 2007).

Certain petroleum hydrocarbons are easier to phytoremediate than others and the inherent biodegradability of a hydrocarbon ultimately depends on its chemical nature (Stroud *et al* 2007). The physical properties of aliphatic hydrocarbons (see Section 1.2) determine their susceptibility to microbial attack and the potential for degradation. On entering the terrestrial environment the downward migration of diesel fuel through the soil profile is limited by the physical properties of the fuel, and is often adsorbed in the organic rich surface soil (Adam and Duncan 2002). This makes diesel-contaminated soil a likely candidate for phytoremediation as the pollutant is held in the surface soil and within the root zone of many plant species. The individual constituents of diesel fuel are inherently biodegradable, but the rates of degradation depend heavily on physical and climatic conditions and on soil microbial composition (Adam and Duncan 1999).

Measuring the success of a phytoremediation project is a discussion point in the literature. Several suggestions are offered as summarised by Hutchinson *et al* (2003). One recommendation is that success could be determined based on whether the technology achieved certain endpoints (i.e. risk-based levels, or state and federal standards). Others suggest that success could be measured based on the percent of hydrocarbon reduction achieved during a short period of time. This approach may underestimate the long-term benefits of phytoremediation as a sustainable technology which restores the soil ecosystem over a prolonged period (Hutchinson *et al* 2003).

1.4.3 Comparison of phytoremediation to alternative remediation strategies

Phytoremediation has made significant gains in market and community acceptance in recent times. In addition to its favourable economics, according to various authors (Frick *et al* 1999; Macek *et al* 2000; Glick 2003) the main advantages of phytoremediation in comparison with classical remediation approaches can be summarised as follows:

1. Less disruptive to the environment;
2. Can be applied *in situ*;
3. Economically competitive;

4. Avoids excavation and heavy traffic;
5. No need for disposal sites;
6. Has potential versatility to treat a diverse range of hazardous materials;
7. Is well-suited to large areas of surface contamination;
8. Has the potential to be rapid;
9. Plants act as indicators of contamination;
10. Plants help contain contaminants;
11. Plants transfer oxygen and nutrients to rhizosphere;
12. Improves soil quality and prevents erosion;
13. Preserves the natural structure and texture of the soil;
14. Is relatively easy to apply;
15. Has favourable public perception.

A cost comparison by Frick *et al* (1999) of phytoremediation to alternative remediation methods including physical/chemical, engineering and bioremediation reveal a clear overall advantage. The cost of applying phytoremediation to a site was estimated at US$17-$100 per cubic metre (based on a cost of US$2500-$15000 per hectare to a depth of 15 cm), which is several orders of magnitude less than the costs associated with physical/chemical remediation technologies. The authors suggested costs associated with the use of engineering techniques to remediate contaminated soil could range from US$10 to over US$1000 per cubic metre, and may ultimately depend on the technique employed and *in situ* or *ex situ* design. For example, *ex situ* techniques, although faster, are more costly ranging from US$60-$1180 per cubic metre. Costs associated with bioremediation of petroleum hydrocarbons generally range from US$98-$400 per cubic metre. These costs can quickly escalate depending on the level of monitoring, analytical costs, and degree of security and safety that are required. Other factors also contribute to the generally lower cost of phytoremediation, including the comparative costs associated with administration, site management, regulatory reporting, and analysis of data incurred by other remediation techniques (Frick *et al* 1999; USITC 2004).

1.5 Mechanisms of hydrocarbon phytoremediation

There are four primary mechanisms by which plants and their associated microorganisms phytoremediate hydrocarbon-contaminated soil (**Figure 1.1**). These mechanisms include phytostabilisation, phytodegradation, phytovolatilisation, and rhizoremediation (also referred to as rhizodegradation) (Pilon-Smits 2005). Plants can also uptake inorganic contaminants such as metals in a process called phytoextraction. This is outside the focus of this study and readers are directed to reviews by Lasat (2002) and Arthur *et al* (2005) for further information. Factors such as the type of plant used, and importantly the physical properties of the contaminant, can determine which mechanism occurs (Newman and Reynolds 2004).

For each of these mechanisms a large variety of plant species have been assessed for their phytoremediation potential. There are numerous literature reviews which summarise information on plant species that play a role in the phytoremediation of petroleum hydrocarbons (for example Frick *et al* 1999; Schnoor 2002; Arthur *et al* 2005; Pilon-Smits 2005). Based on published studies, the phytoremediation potential of a plant species can be classified from hydrocarbon tolerant only, to suspected ability to phytoremediate, and ultimately demonstrated ability to phytoremediate.

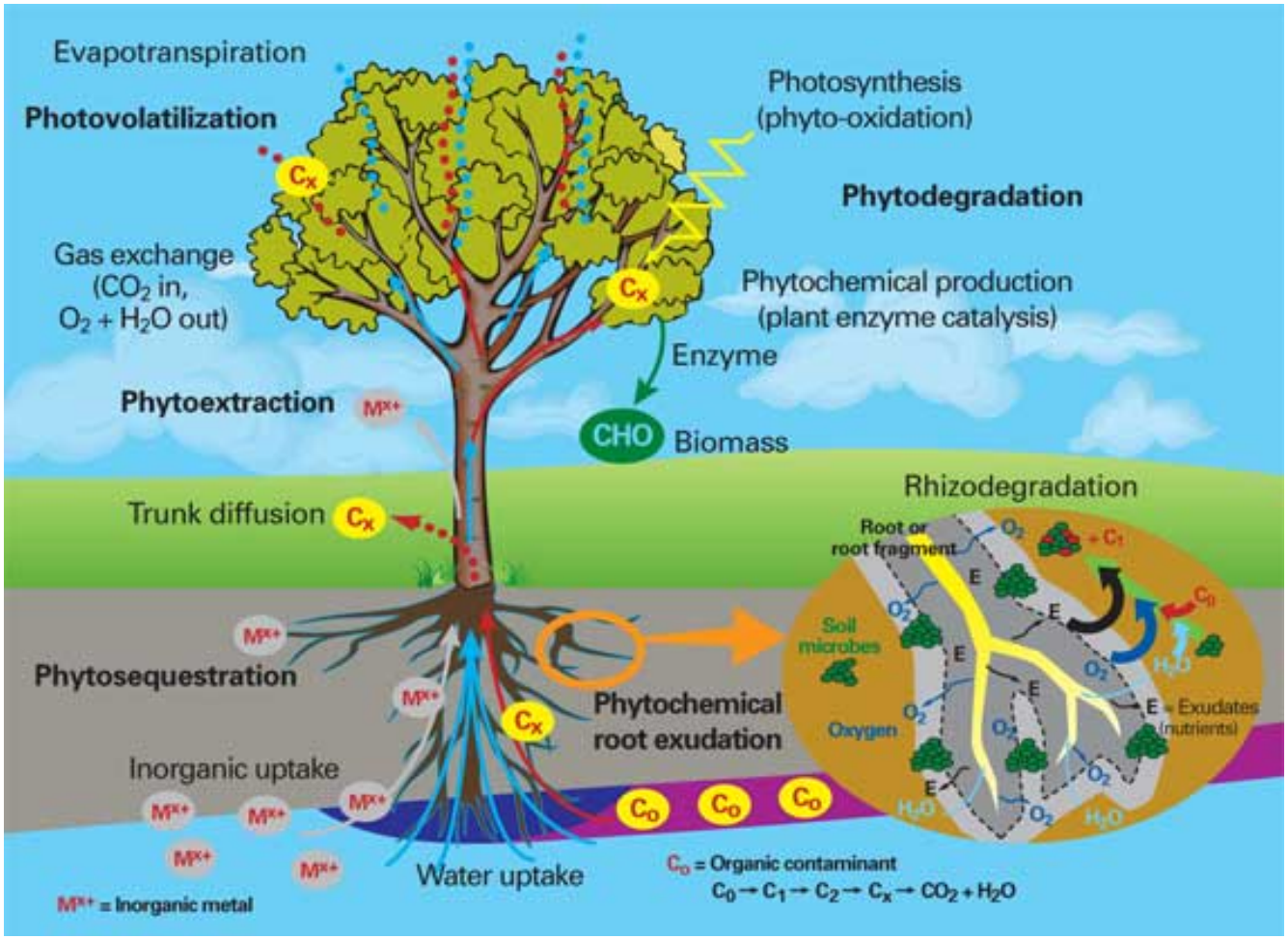


Figure 1.1 Mechanisms of phytoremediation (Source: Seslar 2005)

1.5.1 Phytostabilisation

Phytostabilisation is the immobilisation or containment of a pollutant in soil. It involves the use of plants to reduce the bioavailability and migration of contaminants in soil (Germida *et al* 2002). Contaminants are not necessarily degraded when they are phytostabilised. The goal is simply to contain and isolate contaminants *in situ*. Direct mechanisms of phytostabilisation by plants include adsorption of the contaminants on the root surface, accumulation by the roots, or isolation within the root zone using plants as organic pumps (Adam and Duncan 1999; Pilon-Smits 2005). Schnoor (2002) suggests organic chemicals with log K_{ow} values greater than 3.0 are strongly sorbed to plant roots. Studies examining the fate of PAHs suggest that adsorption onto roots may be significant (Schwab *et al* 1998; Banks *et al* 1999). The hydrophobicity and strong sorption to soil particles of such compounds tends to preclude vegetative uptake. There are also indirect mechanisms to phytostabilise contaminants. This involves the plant supplying enzymes that bind contaminants into soil organic matter (or humus) in a process called humification (Frick *et al* 1999).

There are demonstrated reports in the literature of phytostabilisation of hydrocarbon contaminants using a variety of plant species. Schwab *et al* (1998) reported adsorption of naphthalene (PAHs) to the roots of alfalfa (*Medicago sativa* L.) and tall fescue (*Festuca arundinacea* Schreber). In a laboratory study using detached roots, up to 30% of naphthalene adsorbed to the roots of alfalfa, while 15% adsorbed to roots of tall fescue. Results indicated that adsorption of naphthalene onto root segments increased with the age of the plant. The authors concluded that adsorption of lipophilic compounds onto the surface of roots may be an important sink for PAHs in soils and an initial step in phytoremediation.

Binet *et al* (2000a) reported limited phytostabilisation of a mixture of eight PAHs by ryegrass (*Lolium perenne*). Between 0.006 and 0.11% of extractable PAHs were adsorbed onto the roots of the plant as determined by GC-MS. Accumulation of PAHs was found to be limited in the root tissue, and negligible in shoot tissue. The authors noted that the majority of PAHs dissipation (33-66%) was likely to be due to biodegradation or biotransformation in the rhizosphere soil.

It appears in the case of petroleum hydrocarbons, phytostabilisation may simply involve the establishment of a vegetative cover to minimise potential migration of the contaminant through soil erosion or leaching (Germida *et al* 2002).

1.5.2 Phytodegradation

Phytodegradation is also referred to in the literature as phytotransformation, which indicates the role of internal plant mechanisms and processes to breakdown the contaminant (Newman and Reynolds 2004). Plants possess many of the enzymes such as cytochrome P450s and glutathione-s-transferase present in other eukaryotic systems for the breakdown of toxic chemicals (Mougin 2002). Once an organic contaminant is taken up and translocated by a plant, it undergoes one or more phases of transformation: conversion (oxidations, reductions), conjugation (with glutathione, sugars), and compartmentation (deposition in vacuoles or bound to cell wall) (Schnoor 2002; Newman and Reynolds 2004). It is important to identify, quantify, and understand the significance of metabolites formed during phytodegradation. Often transformation products are less toxic and/or less available than the parent compounds, but this is not always the case (Arthur *et al* 2005). Phytodegradation can also occur through the exudation of plant-derived enzymes into the soil.

To date, there have been limited studies in which plants have demonstrated hydrocarbon degradation directly by phytodegradation mechanism (Mougin 2002; Newman and Reynolds 2004). One example of direct hydrocarbon degradation by a plant is described by Edwards *et al* (1982). In that study, the uptake and metabolism of ^{14}C-anthracene by soybeans (*Glycine max*) was assessed. Soybeans grown in soil containing ^{14}C-anthracene assimilated and translocated 2.1% of the contaminant to its stems and 0.4% to its leaves. The majority of anthracene remained in the soil (93%), with small amounts found in the soybean roots (2.3%). Evidence of metabolism of ^{14}C-anthracene by the soybean plants was detected through $^{14}CO_2$ efflux measurements, and accounted for 6% of the total ^{14}C activity of the plant. The authors concluded that anthracene could be catabolized by soybean plants, although rates and metabolites were not determined in the study.

Recent attention has been given to the potential role of symbiotic endophytic microorganisms in phytodegradation (see review Doty 2008). The term 'endophytic' refers to bacteria living within plant tissues in contrast to rhizosphere bacteria living on or around the plant roots (Newman and Reynolds 2005). Work by van Aken and colleagues (2004) showed that a plant endophyte *Methylobacterium* sp. strain BJ001 isolated from hybrid poplar was involved in the degradation of explosives such as TNT (2,4,6-trinitrotoluene) and HMX (hexahydro-1,3,5-trinitro-1,3,5-triazine). The bacterium was involved in the mineralisation of approximately 60% of the compounds to carbon dioxide in two months. The associations of endophytic organisms with their hosts are varied and complex and are only beginning to be explored, and may reveal them to be important contributors in rhizodegradation of recalcitrant pollutants in the environment.

For compounds such as PAHs, petroleum compounds and PCBs, the role of the rhizosphere appears to be much more critical than direct uptake and metabolism by the plant or its associated symbionts (Hutchinson *et al* 2003).

1.5.3 Phytovolatilisation

Phytovolatilisation refers to the uptake and transpiration of a contaminant by a plant. In this process, the contaminant or its metabolite is released into the atmosphere (Pilon-Smits 2005). This mechanism of contaminant removal may have implications regarding contamination of the atmosphere, and consequently, regulatory compliance issues with air quality guidelines (Schnoor 2002).

Watkins *et al* (1994) provided an example of phytovolatilisation as a mechanism of hydrocarbon removal from soil. In this case the effect of Bell Rhodesgrass (*Chloris gayana*) on the fate of naphthalene in soil microcosms was assessed with respect to volatilisation and biological mineralisation. With the use of radiolabelled [7-^{14}C]naphthalene the authors demonstrated enhanced volatilisation in the presence of vegetation. However losses of more than 50% of naphthalene or its degradation products prevented a mass balance calculation. The authors concluded that the pollutant was taken up by the grass roots, translocated within the plant, and volatilised through the above ground biomass.

Interestingly, naphthalene mineralisation was shown to decrease in vegetated microcosms in comparison to those without vegetation.

Overall, plant uptake and accumulation of hydrocarbons from contaminated soil is quite small and limited to low molecular weight compounds (Chaineau *et al* 1997). Many petroleum hydrocarbons are large, high molecular weight molecules which are also lipophilic, thus excluding them from the plant root (Qui *et al* 1997). Phytodegradation and phytovolatilisation are therefore considered minor pathways of hydrocarbon removal from soil systems.

In light of this, hydrocarbon removal in phytoremediation efforts is generally attributed to enhanced microbial degradation in the rhizosphere or plant root zone. This is the fourth primary mechanism of phytoremediation and is known as rhizoremediation (or rhizodegradation). The processes of rhizoremediation are a focus of this study, and thus discussed in more depth in the section below.

1.6 Rhizoremediation: Bioremediation in the rhizosphere

The rhizosphere has been defined as the volume of soil adjacent to and influenced by plant roots (Yateem *et al* 2007), and is characterised by intense microbial activity. Rhizoremediation involves the breakdown of contaminants in soil as a result of microbial activity that is enhanced within the rhizosphere (Kuiper *et al* 2004). During rhizoremediation, plant root exudates (e.g. organic acids, carbohydrates) can help stimulate the survival and action of microorganisms in the soil, that may subsequently degrade pollutants in the plant rhizosphere. This form of phytoremediation, involving the interaction between plant and microorganisms, has been referred to in the literature as the *rhizosphere effect* (Anderson *et al* 1993; Olson *et al* 2003). The objective of rhizoremediation is increased microbial numbers and activity, and the exploitation of that increased microbial activity to enhance biotreatment. Rhizoremediation is suggested to be the primary mechanism responsible for hydrocarbon degradation in phytoremediation efforts (Frick *et al* 1999;

Hutchinson *et al* 2003). Microbial densities in the rhizosphere are suggested to be 1 to 4 orders of magnitude higher than in bulk soil (Pilon-Smits 2005).

In the current study, the rhizosphere and its role in phytoremediation is the primary mechanism investigated for aliphatic hydrocarbon removal and is the focus of the remainder of this introductory review. The literature suggests that plants and microorganisms are involved both directly and indirectly in the degradation of contaminants (Germida *et al* 2002). An example of direct degradation of contaminants is described earlier in the section on phytodegradation (Section 1.5.2). There is a considerable body of information on the indirect roles played by plants and their associated microorganisms in the degradation of hydrocarbons (Frick *et al* 1999; Walker *et al* 2003; Kuiper *et al* 2004). Some of these indirect roles are considered here. Plant-produced compounds may serve as co-substrates facilitating microbial degradation of the more recalcitrant compounds (Walker *et al* 2003). This has been referred to as co-metabolism and is a process in which compounds not utilised for microbial growth or energy are transformed to other products (Pilon-Smits 2005). Co-metabolism relies on the presence of lower molecular weight structural analogues that trigger enzyme induction (Heitkamp and Cerniglia 1988; Pilon-Smits 2005).The co-metabolic process may result in lower contaminant concentration endpoints than can be obtained without plants (see Reynolds and Wolf 1999). The products of rhizoremediation (e.g. alcohols, acids, carbon dioxide and water) are generally less toxic and less persistent in the environment than the parent compounds (Kuiper *et al* 2004).

Hydrocarbon contaminants in soil are potentially phytotoxic to plants and can interfere with plant establishment and growth (Adam and Duncan 1999). Some plant species have been shown to thrive in low levels of diesel and other aliphatic contaminants in soil, and subsequently enhance their removal from the soil environment (Adam and Duncan 1999; Dominguez-Rosado and Pichtel 2004).

1.6.1 Selection of plant species for rhizoremediation – What is an ideal candidate?

The selection of suitable plant species for rhizoremediation is an important consideration. Not all species will tolerate the presence of contamination, or be able to effectively enhance remediation of hydrocarbons from the soil. This is due to variation in plant morphology (e.g. roots), physiology (e.g. root exudates), and microbial interactions in the rhizosphere (Walker *et al* 2003). For example, monocotyledonous plants such as grasses typically have highly branched fibrous roots that are more likely to cover a large surface area compared to the tap root systems of trees and shrubs (Yateem *et al* 2007). Similarly, the rhizosphere of certain trees (e.g. hybrid poplars) has been reported to enrich for fewer hydrocarbon degrading microorganisms than soil outside the root zone (Hutchinson *et al* 2003). These traits make some species less applicable to rhizoremediation technologies than others.

To achieve maximum hydrocarbon reduction in soil and to successfully establish a stable vegetation cover, various criteria must be considered. The development of site-specific selection criteria for plant species is discussed in further depth in Chapter 2. A brief description is given here of an ideal plant candidate for the rhizoremediation of hydrocarbon contaminated soil. The plants should be chosen carefully so that they provide a maximum surface area per unit volume of soil (Aprill and Sims 1990; Smith *et al* 2006). This would enable greatest rhizosphere-contaminant-microbe interactions. Plants should be native to the area for which they are being used. There can be ecological risks associated with introduced species to an area, and the use of native species protects local biodiversity. Due to the frequent poor nutrient availability in contaminated sites (Kirkpatrick *et al* 2006) they should be able to tolerate low N and P availability. Much research has been carried out on the use of legumes in this regard as they are able to fix nitrogen (Nichols *et al* 1997; Dzantor *et al* 2000).

The most extensively characterised fibrous root systems belong to the grass family *Poaceae*. Grass root systems possess an extensive surface area compared to other plant types, and have been recognised in many studies for their potential for remediation of hydrocarbon contaminated sites (Gunther *et al* 1996; Qui *et al*

1997; Xia 2004). The sod-forming grasses show particular potential as they produce horizontal stems that may grow above (stolons) or below (rhizomes) the surface of the soil. This growth characteristic means they have the ability to stabilise soil surfaces against erosive forces (Aprill and Sims 1990). Grasses also have an inherent genetic diversity which may provide a competitive advantage in adapting to unfavourable environmental conditions (Pichtel and Liskanen 2001; Tesar *et al* 2002). The morphological characteristics of grasses (e.g. fibrous root systems), coupled with their growth characteristics and physiology, make them an ideal vegetative cover for the *in situ* treatment of hydrocarbon contaminated soil.

1.6.2 The use of grasses for rhizoremediation of hydrocarbons

Tolerance of plants to hydrocarbon contamination is the prerequisite for successful rhizoremediation (Tesar *et al* 2002). Numerous studies have investigated the effect of hydrocarbons on growth and development of grass species (Adam and Duncan 1999; Kulakow *et al* 2000; Robson *et al* 2003; Huang *et al* 2004). Plant screening experiments have shown grasses to be tolerant to various hydrocarbons, in particular aliphatics. **Table 1.3** outlines grass species grown around the world which have been shown to tolerate hydrocarbon contamination in soil.

Table 1.3 Grass species shown to have hydrocarbon tolerance.

Grass species	Contaminant	Results/Comment	Source
11 grass species	Diesel (spiked 25 and 50 g diesel kg^{-1} soil)	Broad range of diesel tolerance observed in germination experiments. Some species thrived in low levels of contamination (e.g. Creeping bent, *Agrostis stolonifera*), while others were diesel intolerant. Overall plant height reduced in contaminated soil compared with uncontaminated.	Adam and Duncan 1999
Fescue grass (*Cyndon dactylon*)	Diesel (spiked range 100 to 8000 mg diesel kg^{-1})	Seed germination percentage 97% in uncontaminated conditions, and decreased by less than 8% even at diesel concentration of 8000 $mgkg^{-1}$. Note: grown on filter paper.	Al-Ghazawi *et al* 2005
Tall fescue (*Festuca arundinacea*) Kentucky bluegrass (*Poa pratensis*) Wild rye (*Elymus Canadensis*)	Creosote (spiked 0.5, 1, 2 and 3 g kg^{-1})	Plant tolerance to creosote varied among species (order of tolerance Tall fescue > Kentucky bluegrass > Wild rye). For example, at 1 gkg^{-1} Tall fescue not affected (100% germination relative to control); Kentucky bluegrass germination only to 80%; Wild rye 10% germination. All species had ≤ 50% germination in 3 gkg^{-1}.	Huang *et al* 2004

Table 1.3 continued…

Perennial ryegrass (*Lolium perenne*)	Weathered crude oil (spiked 1%, 5% and 10%)	Crude oil concentration up to 5% had no significant effect on seedling emergence by 28 days after planting. Plant height significantly reduced at all concentrations. Root biomass not significantly affected at any concentration.	Issoufi *et al* 2006
26 grass species	Weathered PAHs contaminated sediment	Mean germination percentages not statistically different between contaminated and uncontaminated soil. Plant growth generally stunted in contaminated soil compared with uncontaminated. Tolerance varied among species; Tall fescue and Perennial ryegrass showed high tolerance.	Kulakow *et al* 2000
Brachiaria brizantha *Panicum maximum*	Crude oil (spiked 3%, 5%)	Contaminant level had no significant influence on seedling emergence. *Brachiaria brizantha* promising as showed high seedling emergence and least affected biomass production in contaminated soil.	Merkl *et al* 2004b

Table 1.3 continued…

Mixed grasses: Hard fescue (*Festuca ovina*) Red fescue (*Festuca rubra*) Perennial ryegrass (*Lolium perenne*)	Diesel (spiked 2% w/w)	All grasses grew at rates comparable to uncontaminated soil, with no toxicity symptoms apparent.	Pichtel and Liskanen 2001
11 grass species	Crude oil (spiked 0.5, 1, and 5%)	All grasses showed phytoremediation potential based on survival and growth performance indicators.	Robson *et al* 2003
4 grasses: Cocks-foot (*Dactylis glomerata*) Tall fescue (*Festuca arundinacea*) Red fescue (*Festuca rubra*) Perennial ryegrass (*Lolium perenne*)	Mixture of 7 PAHs (spiked 1000 $mgkg^{-1}$). Coal tar (spiked 1600 $mgkg^{-1}$ total PAHs). Weathered coking plant soil containing 5326 $mgkg^{-1}$ total PAHs.	PAHs (mixture and coal tar) caused no significant reduction in % germination of any grass species after 10 days, nor did aged coking soil after 14 days (compared with control). Foliage yields in spiked (mixture and coal tar) soil significantly less than control for 3 species. In coking soil only *L. perenne* not significantly reduced.	Smith *et al* 2006
Annual ryegrass (*Lolium multiflorum* cv. Lolita)	Diesel (spiked 5, 10 and 25 g diesel kg^{-1} soil)	25 g diesel kg^{-1} soil shown to be critical contamination level for cultivation. Even at 5 g diesel kg^{-1} biomass reduced by 82%, but produced more biomass compared to control soil.	Tesar *et al* 2002

The screening of a plant species for the ability to successfully grow and establish in contaminated soil is one of the first steps in the process of species selection for rhizoremediation. This is followed by evaluation of the plant's influence on the degradation of hydrocarbons in soil. A successful candidate should show enhanced removal of the contaminant from soil. **Table 1.4** outlines grass species shown to facilitate enhanced rhizoremediation of hydrocarbons in soil.

Aprill and Sims (1990) were among the first to demonstrate rhizoremediation of hydrocarbons in the presence of plants. The authors found a statistically significant increase in removal of a range of PAHs from soils vegetated with eight different prairie grasses compared to unvegetated soils. They suggested the fibrous root system of the perennial grasses may be more effective in microbial stimulation in the rhizosphere. The authors noted that the order of removal among the PAHs correlated with the water solubility of the compound. That is, the most water soluble PAHs showed the greatest extent of degradation in the rhizosphere.

In another seminal paper, pyrene was significantly degraded in the rhizosphere of fescue (*Festuca arundinacea* Schreb.), sudan grass (*Sorghum vulgare* Pres.), and switchgrass (*Panicum virgatum* L.) after 24 weeks compared to the unplanted control (Reilley *et al* 1996). Interestingly, pyrene removal in the rhizosphere was further enhanced by the addition of simple organic acids (e.g. formic and succinic acid). This highlights the importance of root exudates and rhizosphere microorganisms in the rhizoremediation of PAHs. Miya and Firestone (2000) also noted the importance of microbial stimulation in the rhizosphere for successful PAHs removal. They noted the total number of phenanthrene degrading microorganisms in the rhizosphere of slender oatgrass (*Avena barbata* Pott ex Link) was approximately three-fold that of unplanted, bulk soil. The higher microbial numbers corresponded to a significant decrease in contaminant concentration, suggesting the stimulatory effect of vegetation on pollutant degradation.

Table 1.4 Grass species shown to facilitate rhizoremediation of hydrocarbon pollutants.

Grass species	Contaminant	Results/Comments	Source
Westerwold's ryegrass (*Lolium multiflorum* L.)	Diesel	Enhanced degradation of diesel in planted treatment (3% remaining) compared with unplanted soil (4 months).	Adam and Duncan 2003
8 prairie grasses: Big bluestem (*Andropogon gerardi*) Indiangrass (*Sorghastrum nutans*) Switchgrass (*Panicum virgatum)* Canadian wild-rye (*Elymus canadensis*) Little bluestem (*Schizachyrium scoparius*) Side oats grama (*Bouteloua curtipendula*) Western wheatgrass (*Agropyron smithii*) Blue grama (*Bouteloua graciis*)	Chrysene, Benzo[a]pyrene, Benz[a]anthracene, Dibenz[a,h)]anthracene.	Greater degradation of contaminants in planted compared to unplanted systems. Degradation greatest to least: Benz[a]Anthracene > chrysene > Benzo[a]pyrene > dibenz(a,h)anthracene (219 days).	Aprill and Sims 1990

Table 1.4 continued…

Tall fescue (*Festuca arundinacea* Schreber)	Benzo[a]pyrene	Enhanced degradation of benzo[a]pyrene in presence of plants: Residual benzo[a]pyrene lower in soil with plants (44.2%), compared to without plants (53%) (185 days).	Banks *et al* 1999
Ryegrass (*Lolium perenne* L.)	Mix of 8 PAHs (3-6 rings)	Enhanced degradation where extractable concentrations of all PAHs were lower in planted than unplanted systems (180 days).	Binet *et al* 2000
Tall fescue (*Fetusca arundinacea*) Switchgrass (*Panicum virgatum* L.)	Pyrene	Greater mineralisation of ^{14}C-pyrene in planted compared with unplanted soils (190 days): 37.7% in tall fescue systems 30.4% in switchgrass systems 4.3% in unplanted controls.	Chen *et al* 2003
Grass/maize mixture Grasses: Creeping red fescue (*Festuca rubra*) Fawn tall fescue (*Festuca arundinacea*) Perennial ryegrass (*Lolium perenne*)	Used motor oil	A 38% degradation of oil shown in planted soil (increased to 67% with fertilizer added), compared with unplanted (150 days).	Dominguez-Rosado and Pichtel 2004

Table 1.4 continued…

Tall fescue (*Festuca arundinacea Screb.)*	Benzo[a]pyrene	Mineralisation of benzo[a]pyrene marginally higher in planted soil than unplanted control: 4.1% mineralisation in planted, compared with 4.0% for unplanted control (180 days).	Epuri and Sorensen 1997
Ryegrass (*Lolium perenne L.)*	Hydrocarbon mixture of saturated, unsaturated and branched chain aliphatics, and PAHs.	Increased degradation of hydrocarbon concentrations in planted soils compared with unplanted: Residual hydrocarbon concentrations 3% in planted soils (4330 $mgkg^{-1}$ to120 $mgkg^{-1}$), compared with 18% in unplanted soils (790 $mgkg^{-1}$) (22weeks). Note: Aliphatics disappeared faster in vegetated systems.	Gunther *et al* 1996
Perennial ryegrass (*Lolium perenne* L.)	Diesel	Ryegrass enhanced the degradation of total petroleum hydrocarbons (TPH) over unplanted controls: 57% TPH removal in planted soil, compared with 36% in unplanted soil (102 days).	Hou *et al* 2001
Ryegrass (*Lolium multiflorum*)	Diesel (spiked 1.8% w/w)	Enhanced degradation - Percentage of TPH remaining in planted soil 55% lower than in unplanted soil by 152 days (13% remaining in planted soil versus 27% remaining in unplanted control).	Kaimi *et al* 2006

Table 1.4 continued…

Italian ryegrass (*Lolium multiflorum*) Bermuda grass (*Cynodon dactylon*) Southern crabgrass (*Digitaria ciliaris*) Red clover (*Trifolium pratense*)	Diesel (spiked 2% w/w)	By mature growth stage of plants significantly lower TPH concentration in soils planted with Italian ryegrass (4410 $mgkg^{-1}$) and Bermuda grass (3640 $mgkg^{-1}$) than in unplanted controls (5790 $mgkg^{-1}$).	Kaimi *et al* 2007a
Brachiaria brizantha *Cyperus aggregatus* *Eleusine indica*	Crude oil	Soils planted with grasses showed significantly lower total oil and grease concentrations than the unplanted controls (180 days). Levels of saturated hydrocarbons lower in planted than in unplanted soil for all species. *Brachiaria brizantha* also removed significant aromatics.	Merkl *et al* 2005a
Slender oat grass (*Avena barbata* Pott ex Link)	Phenanthrene	Enhanced rate of removal in rhizosphere: 17.2 mg/kg/d in planted soil compared with 12.4 mg/kg/d in unplanted bulk soil controls (45 days).	Miya and Firestone 2000
Switchgrass (*Panicum virgatum*) Little Bluestem grass (*Schizachyrium scoparium*)	PAHs	Enhanced degradation of total PAHs in planted soil compared with unplanted (180 days): 57% reduction with switchgrass 47% reduction with bluestem grass 26% reduction in unplanted control.	Pradhan *et al* 1998

Table 1.4 continued…

Prairie Buffalograss (*Buchloe dactyloides* var. 'Prairie') Kleingrass (*Panicum coloratum* var. 'Verde') Mixture of 12 other warm- and cool-season grasses	PAHs	Field study. Significant reductions in both low and high-molecular weight PAHs in soils planted with buffalograss and kleingrass compared to unplanted control soils (3 years). Example: Residual naphthalene concentration 2 $mgkg^{-1}$ in soil with Kleingrass, 100 $mgkg^{-1}$ in unplanted soil control.	Qui *et al* 1997
Sudangrass (*Sorghum vulgare* Pres.) Switchgrass (*Panicum virgatum* L.) Tall fescue (*Festuca arundinacea* Schreb.)	Anthracene, Pyrene	Planted soils showed significantly lower concentrations of PAHs than unplanted soils: Range from 30% to 40% more degradation in planted soils than in unplanted controls (24 weeks).	Reilley *et al* 1996
Annual ryegrass (*Lolium multiflorum* Lam.) Red fescue (*Festuca rubra* L.)	Diesel, crude oil	Field site. Soil planted with ryegrass and red fescue showed significantly lower TPH concentrations than in unplanted controls (640 days). Crude oil: reduction from 6200 $mgkg^{-1}$ to 1400 $mgkg^{-1}$ in planted soil, compared with 2500 $mgkg^{-1}$ in unplanted control. Diesel: reduction from 8350 $mgkg^{-1}$ to 700 $mgkg^{-1}$in planted soil, compared with 2200 $mgkg^{-1}$ in unplanted controls.	Reynolds and Wolf 1999a

Table 1.4 continued…

4 grass mix: Perennial ryegrass (*Lolium perenne*) Kentucky bluegrass (*Poa pratensis*) Red fescue (*Festuca rubra*) Blue fescue (*Festuca ovina*)	Oil shale	Field study. A range of 83%-98% reduction of oil products and phenols observed in top 10 cm of planted soil compared with unplanted (16 months)	Truu *et al* 2003
Ryegrass (*Lolium perenne*)	Diesel	TPH degradation in planted soil (83%) greater than in unplanted (12 weeks).	Vouillamoz and Milke 2001

Recently, Merkl *et al* (2004a; 2005a) assessed tropical pasture grasses for the rhizoremediation of petroleum contaminated soils in Venezuela. In greenhouse experiments, three grasses (*Brachiaria brizantha, Cyperus aggregatus, Eleusine indica*) were analysed for crude oil dissipation (total oil and grease, and fraction composition) in the rhizosphere. In these studies, the grass species were shown to tolerate various levels of contamination without significant affect on plant growth. In addition, soils planted with a tropical grass consistently showed significantly lower total oil and grease concentrations than unplanted soil controls. The authors highlighted the importance of using native plant species in rhizoremediation efforts since they are often adapted to the prevailing environmental conditions.

Research has also shown that grass species can be effective in removing a range of petroleum hydrocarbons from industrially contaminated soil (see also Table 1.4). Pradhan *et al* (1998) determined that switchgrass (*Panicum virgatum*) and little bluestem grass (*Schizachyrium scoparium*) were effective in enhancing the degradation of PAHs in soil from a manufactured gas plant. The results of a greenhouse study showed switchgrass stimulated the removal of 57%, and little bluestem grass 47% of total PAHs in soil. PAHs removal in control soil without vegetation was only 26%. Reynolds and Wolf (1999a) assessed rhizosphere-enhanced remediation of crude oil and aliphatic hydrocarbons such as diesel fuel contamination in soil at a U.S. Department of Defence installation. Results indicated that soils planted with annual ryegrass (*Lolium multiflorum* Lam.) and red fescue (*Festuca rubra* L.) showed significantly lower diesel and crude oil concentrations than in unplanted controls. After 640 days, crude oil contaminated soil planted with both species was reduced by 77%, while the unplanted control showed a reduction of 60%. Likewise, diesel contaminated soil planted with both species showed a 92% reduction over the same period, compared to just 74% reduction noted for the unplanted control. Recently, Phillips *et al* (2006) reported enhanced remediation in the presence of grasses of a weathered hydrocarbon-polluted soil from a decommissioned flare-pit in Canada. The site had a 30 year history of storage and burning of liquid waste hydrocarbons, and the soil showed total petroleum hydrocarbon (TPH) concentration of 5300 mg kg^{-1} (primarily fractions C16-C50). Soil planted with the grass creeping red fescue (*Festuca*

rubra) resulted in a TPH reduction of 50% after 4.5 months compared with only 28% reduction in the unplanted soil.

A complicating factor in rhizoremediation studies is that some hydrocarbons are more susceptible to microbial degradation than others. The study by Reynolds and Wolf (1999a) described above showed that diesel (an aliphatic hydrocarbon) was reduced in soil to a greater extent than crude oil (a mixture of aliphatic and more recalcitrant aromatic hydrocarbons). Similarly, Gunther *et al* (1996) noted that the growth of ryegrass (*Lolium perene* L.) reduced aliphatic hydrocarbons in soil to a greater extent than PAHs. After 22 weeks, the initial extractable hydrocarbon concentration of 4330 mg total hydrocarbon per kg soil decreased to less than 120 mg per kg soil (a 97% reduction) in soils planted with ryegrass, but to only 790 mg per kg soil (82% reduction) in unplanted soil. Thus some petroleum hydrocarbons can be readily rhizoremediated, whereas others are not. This may be linked to contaminant bioavailability, the capability of the rhizosphere microbial community, and the chemical nature of root exudates (Siciliano and Germida 1998).

While much of the research on rhizoremediation has been limited to studies in the greenhouse, there has been increasing demand for evaluation of the technology under field conditions. Qui *et al* (1997) conducted a 3-year field study to evaluate the rhizoremediation of PAHs in soil at a Union Carbide Seadrift Plant in the United States. The growth of prairie Buffalograss (*Buchloe dactyloides* var. 'Prairie') in aged, contaminated soils resulted in accelerated and significant reductions in PAH levels in the rhizosphere compared to unplanted, control soil. In a parallel experiment assessing the performance of 12 warm season grasses, the authors determined that kleingrass (*Panicum coloratum* var. 'Verde') showed the most potential for growth in contaminated soils and PAH removal in the rhizosphere. In all test plots grass tissue analysis found no evidence of PAH uptake and bioaccumulation. The authors concluded that microbial metabolism was the primary mechanism for PAH removal in these field studies.

Similarly, a field study was conducted at a U.S. Navy Fuel Terminal on soils contaminated with aged diesel fuel (Hutchinson *et al* 2003). Vegetated treatments were planted with a mixture of cool-season and warm-season grasses, and evaluated against an unplanted control. Higher microbial numbers and specific contaminant degrading microorganisms were recorded in the vegetated soil. After 2 years, vegetated plots showed significantly higher removal of total petroleum hydrocarbons and PAHs compared to unplanted controls. These studies reveal enormous potential for the effective use of grasses in enhanced rhizoremediation of hydrocarbon contaminated soils.

1.7 Australian perspective on phytoremediation

The extent of site contamination in Australia is still largely uncertain and depends somewhat on the criteria used to define contamination. There have been widely varying estimates of contaminated sites Australia wide, with some reports including landfills and mining activities in the total sum of contaminated sites. A 1999 report by the National Environment Protection Council (NEPC 1999) is the most commonly sourced statistic and proposes that there are an estimated 80 000 contaminated sites in Australia, many of which have been impacted by aliphatic petroleum hydrocarbon pollutants. Often site contamination occurs in areas where land values may not economically justify the enormous cost of conventional cleanup techniques such as incineration and land-filling (NEPC 1999).

A market overview for global remediation services (USITC 2004) states that the most commonly used techniques for site remediation in Australia include bioremediation, thermal treatment, chemical treatment, *in situ* reactive walls, and containment. While these methods represent varying degrees of technological advancement, the report suggests the most common remediation method in Australia remains removal and storage of contaminated soil in hazardous waste facilities, a method that the Australian Government is increasingly trying to discourage. There is currently no overarching environmental legislation in Australia with respect to site contamination, rather disparate bills which vary

between states that address specific environmental issues. The Environment Protection Authority establishes guidelines at the national level but individual states and territories enforce their own legislation (NEPC 1999).

Research into phytoremediation in Australia is relatively new, with interest increasing only in the last decade and focussing on the remediation of mining-affected lands (Robinson and Anderson 2007). Phytoremediation technology that has been developed overseas cannot be readily transferred to Australian conditions due to significant differences in climate, soil types, endemic plant species and environmental regulation performance criteria (Michael *et al* 2007). The application of phytoremediation in Australia has been limited to metal-contaminated soils, mostly from industrial activities. **Table 1.5** outlines examples of phytoremediation application to Australian contaminated soils. Much of the work has been on the identification of 'hyperaccumulating' plant species, or those with the capacity to tolerate and accumulate very high concentrations of heavy metals in plant biomass without showing phytotoxicities (Archer and Caldwell 2004; Hayes *et al* 2003; Robinson and Anderson 2007). Other studies have focussed on plant species already growing on the contaminated sites as a form of phytocover (Michael *et al* 2007).

Table 1.5 Application of phytoremediation to Australian soil contamination.

Plant species	Contaminant	Results/Comments	Source
Plant species dominant on derelict silver mine site: Common rush (*Juncus usitatus*) Spiny-headed mat rush (*Lomandra longifolia*) Couch (*Cynodon dactylon*) Bracken fern (*Pteridium esculentum*) Black wattle (*Acacia decurrens*) Teatree (*Melaleuca alternifolia*)	Mixture of heavy metals including As, Mn, Fe, Cd, Pb, Zn, and Ni at high concentrations caused by acid mine drainage. Soil low pH (2.9-5.6).	Couch, common rush and spiny-headed mat rush identified as suitable for phytostabilisation on site due to tolerance of acid soil and accumulation of significant high concentrations of Pb and Cu.	Archer and Caldwell 2004
Plant species abundant on cattle dip site: Kikuyu grass (*Pennisetum cladenstinum*) Rainbow fern (*Calochlaena dubia*)	Arsenic from cattle dipping at concentration range 0.15-0.21%	Rhizosphere microbes promoted arsenic accumulation by the grass species (45% increase in arsenic content in shoots).	Chopra *et al* 2007

Table 1.5 continued…

Dominant plant species on an abandoned metal mine site: Trees – *Acacia melanoxylon, Eucalyptus dalrympleana, Pinus radiate.* Shrubs and grasses – *Baeckea utilis, Lomandra longifolia, Poa labillardieri.*	Mixture of trace metals (Cd, Cu, Pb and Zn). Large proportion in bioavailable forms (up to 34%).	All species shown to be efficient trace metal accumulators, with the grass (*Poa labillardieri*) identified as a hyperaccumulator of lead (0.097% w/w in roots).	Hayes *et al* 2003
Native Australian perennial shrub *Hybanthus floribundus* (known nickel hyperaccumulator)	Nickel. Potting mix experimentally contaminated with 26mM kg^{-1} Ni.	After 20 weeks, significant hyperaccumulation reported where stem sections contained lower Ni than leaf tissues (1800 mg kg^{-1} versus 7800 mg kg^{-1}, respectively).	Kachenko *et al* 2008
A salt-tolerant drought-hardy grass (introduced exotic species in Australia): Rhodes grass (*Chloris gayana*)	Mixture of heavy metals from mine tailings (Ag, As, Cd, Pb, Sb and Zn) at concentrations 5-50% by weight.	Rhodes grass tolerated range of metal concentrations, showing no adverse growth affect up to 25% (wt) by 3 months growth (considered hypertolerant). Plant tissue accumulated low levels of all elements tested (< 250 µg/g).	Keeling and Werren 2005
Crop species: Coriander (*Coriandrum sativum*) Canola (*Brassica napus*) Linseed (*Linum usitatissimum*) Indian mustard (*Brassica juncea*) Safflower (*Carthamus tinctorius*)	Cadmium	Indian mustard produced the greatest biomass and removed the greatest amount of Cd from soil.	McLaughlin *et al* 1998

Table 1.5 continued…

Native Australian perennial plants indigenous to landfill and surrounding community: 6 tree species (including a legume) 6 grass species	Landfill site for mixed waste disposal, including basal quarry scalpings.	Plants assessed as phytocover and hydraulic control of site. Relative growth performance (survival and growth) showed high tolerance to the site contamination, with few mortalities and no visible signs of plant stress. Note: addition of nutrient amendments had detrimental effect on plant growth of native species.	Michael *et al* 2007

In a local review on phytoremediation by Chaudhry *et al* (1998), attention is given to identifying accumulator plants that remediate metal contaminated soils. The authors highlight findings of major studies for the Australasian region, including two trace metal surveys (Cd, Cu, Pb and Zn) of a steelworks and abandoned mine contaminated sites. Of particular interest, the Australian native grass *Poa labillardieri* (Common tussock) was highlighted as an especially viable option due to its impressive ability to hyperaccumulate lead. This was expanded upon in a later publication by the same group (Hayes *et al* 2003) where dominant plant species on an abandoned metal mine site (including native trees, shrubs and grasses) were shown to be efficient trace metal accumulators. Similarly, Keeling and Werren (2005) describe the use of Rhodes grass (*Chloris gayana*) for phytoremediation of metal contaminated soil from mining operations in Northern Australia. Rhodes grass tolerated a range of metal concentrations up to 25% (wt), showing no adverse affect on growth by 3 months. Plant tissue was found to accumulate low levels of all metal and trace elements tested (< 250 µg/g). The authors concluded that the species was hypertolerant to the presence of heavy metals in the soil. Whilst the study appears to demonstrate the suitability of Rhodes grass to phytoremediation of metal contaminated mine sites, precautions should be taken for its wider application. The limitations of such findings are based on Rhodes grass being considered an exotic introduced species and a high-risk plant for conservation of native plant communities (Mallett 2002). This is because the species is considered an inherently aggressive competitor with native species and can rapidly invade native ecosystems. Caution should therefore be applied in selecting even demonstrably suitable plant species for phytoremediation when they are not native, particularly in or near areas where conservation of native biodiversity is a desired outcome.

1.8 Research objectives

The potential for Australian native plants in phytoremediation of hydrocarbon contaminated soil has not previously been explored. The provision of a viable phytoremediation technology would offer an economically feasible and environmentally sustainable option for the remediation of hydrocarbon contaminated sites in Australia. The development of this technology may have extensive application to the nationwide problems associated with hydrocarbon contaminated sites.

The aim of this study was to evaluate the potential of Australian native grasses (*Poaceae*) in the rhizoremediation of aliphatic hydrocarbon contaminated soil sourced from a mine site. The selection of new plant species for rhizoremediation purposes is important. A multi-faceted approach can help identify potential candidate species for this application.

Therefore, the specific objectives of the study were to:

1. Develop and apply plant selection criteria and a screening protocol for plant species to determine ideal candidates for the rhizoremediation of hydrocarbon contaminated soil in Australia.
2. Characterise selected grass species for tolerance to hydrocarbon contaminated soil in germination and growth performance trials in diesel and oil contaminated soil from a mine site.
3. Determine the capability of Australian grass species to enhance hydrocarbon removal from mine site soil.
4. Assess the quantitative and qualitative changes in microbial community dynamics in the rhizosphere of Australian grasses during the rhizoremediation process.

CHAPTER TWO

2 PLANT SELECTION AND SOIL ANALYSIS

2.1 Introduction

The selection of plant species is critical to the success of rhizoremediation. A growing body of literature reports that not all plant species have the same potential for enhancing remediation. The general plant type (e.g. monocots, legumes) can influence parameters that affect hydrocarbon degradation in the rhizosphere (Pichtel and Liskanen 2001).

Research on suitable plant species for rhizoremediation of petroleum hydrocarbons has shown that various grasses and leguminous plants are promising candidates (Aprill and Sims 1990; Adam and Duncan 1999; Merkl *et al* 2004b; 2005a). This may in part be due to their highly branched, fibrous root systems which can harbour large microbial numbers (Anderson *et al* 1993), and can stabilise soil and provide larger surface area for root-soil contact (Kulakow *et al* 2000). Warm season grasses are especially suited to rhizoremediation application due to their inherent characteristics such as deep fibrous roots, and tolerance of drought and low nutrient availability (Waters *et al* 2001; Tesar *et al* 2002). Hardy grasses are already commonly employed as vegetative covers for erosion control as remediated sites move toward ‘closure’ (Cunningham and Berti 1993).

Rapid and cost effective techniques are needed to select plant species for use in phytoremediation, vegetative capping, or revegetation at hazardous waste sites (Kulakow *et al* 2000). A simple screening procedure to aid in the selection of plants would help increase the success and decrease the cost. Plant species selection is a critical management decision for phytoremediation (Frick *et al* 1999).

To achieve maximum hydrocarbon reduction and to establish stable vegetation cover, various criteria must be considered (Smith *et al* 2006). Plants should be chosen carefully so that they provide a maximum root zone influence. Selected

species should be native to the area and tolerant to the conditions of the soil. As cost is an important factor, plants that require little attention e.g. fertilisers are preferable (Kulakow *et al* 2000).

A screening procedure to select plants for use in phytoremediation should have several aspects (Aprill and Sims 1990; Dzantor *et al* 2000; Kulakow *et al* 2000; Hutchinson *et al* 2003; Merkl *et al* 2005a):

- Information should be gathered on the local adaptation, growth requirements, special attributes (such as salt tolerance), and life history characteristics (e.g. perennial vs. annual) for each species under consideration.
- Candidates should be grown in a seedling growth trial, using the contaminated soil and (if available) an uncontaminated control soil to compare for relative growth. Environmental conditions for the screening trial should simulate the temperature and photo-period conditions in the field during the growing season.
- A list of species should be developed for use in the field, based on screening trial results and interpretation of published information about candidate species.

A database could be developed from numerous screening trials to create guidelines for selection of plants for a range of soil conditions and climates. Screening of plants for use at any site should take place first at the family, genus, and species levels before selection of genotypes within a species is warranted (Kulakow *et al* 2000).

The potential for any Australian native plants in rhizoremediation of hydrocarbon contaminated soil has not been explored. A major hurdle to the application of rhizoremediation in Australia is the identification and availability of suitable plant species.

This chapter outlines the initial phase of the study; the development of plant selection criteria and a screening protocol for identification of species as ‘ideal candidates’ for the rhizoremediation of hydrocarbon contaminated soil in

Australia. Adopting this approach, Australian native grass species (*Poaceae*) were selected for use in the current study. Soil was collected from a mine site and analysed for physical and chemical properties, and results are presented.

2.2 Materials and methods

2.2.1 Soil collection

Rio Tinto Alcan operates an open-cut bauxite mine at Weipa on the western coast of Cape York Peninsula in Queensland, Australia. A major long-term land regeneration objective is in place at the site with a focus on the re-establishment of native species (Schwenke *et al* 1999). Soil at the site has been impacted over time with hydrocarbons, particularly aliphatics such as diesel and lube oils, from machinery used in the mining process. Uncontaminated soil representative of the overall site was collected from the Weipa mine site and sent to Flinders University of South Australia for use in this study. By artificially contaminating 'clean' site soil with known hydrocarbon concentrations, a greater experimental control was ensured. That is, the ability to assess growth and hydrocarbon tolerance at a range of contaminant levels for individual plant species. Data could then be compared with growth performance in uncontaminated controls. Understanding the hydrocarbon tolerance range of a plant aids decision-making in sowing strategies for different areas of the site depending on level on contamination.

2.2.2 Soil analysis

Selected physical and chemical characteristics of the uncontaminated mine site soil were assessed in triplicate. Composite soil samples were used in analysis from collected randomised soil samples provided.

2.2.2.1 Maximum water holding capacity

The maximum water holding capacity of the Weipa soil was determined as described by Rayment and Higginson (1992). Briefly, 50 g of soil was placed in a funnel with a Whatman No. 1 filter paper (185 mm). A hose with a closed clip was attached and 50 mL deionised water added. The soil/water slurry was allowed to stand for 30 min, and the clip was opened and allowed to drain for 30 min. Maximum water holding capacity (expressed as percentage) was calculated as the difference between the volume of water added and the volume of water

drained, minus the water held in the filter paper, plus the water previously in the soil.

2.2.2.2 Soil pH

The pH value of the soil was determined using a soil/water suspension ratio of 1:5 (Rayment and Higginson 1992). Twenty grams of air-dried, sieved (1 mm) soil was added to 100 mL of deionised water, and mechanically shaken end-over-end for 1 h. Soil/water suspensions were prepared in triplicate. The suspension was allowed to settle for 30 min, and pH determined by stirring during measurement using a calibrated Hanna (HI9025) pH meter (Hanna Instruments Ltd, United Kingdom).

2.2.2.3 Electrical conductivity

The electrical conductivity indicates the amount of soluble (salt) ions in soil. The determination of electrical conductivity (EC) was made using a 1:5 soil:water suspension (Rayment and Higginson 1992). The suspension was prepared by weighing 10 g air dried, sieved (1.5 mm) soil and adding 50 mL deionised water. The suspension was mechanically shaken at 150 RPM on an orbital mixer for 1 h to dissolve the soluble salts. Electrical conductivity was measured using a calibrated Hanna (HI9033) conductivity meter (Hanna Instruments Ltd, United Kingdom).

2.2.2.4 Soil organic carbon

Soil organic carbon content was determined using the wet oxidation-redox titration method as outlined in Soil Sampling and Methods of Analysis (Tiessen and Moir 1993). The procedure involves the oxidation of organic carbon using a dichromate solution and titration with a ferrous ammonium sulfate solution to determine the amount of dichromate remaining.

In digestion tubes 1 g sieved (1 mm) air-dried soil sample (in triplicate) was added to 7.5 mL potassium dichromate digestion mixture. Tubes were placed in a 150°C preheated block digester for 45 min. Digested samples were allowed to cool and transferred to 250 mL conical flasks, where 25 mL water, 2.5 mL 85% phosphoric acid (H_3PO_4), and two drops of ferroin indicator solution were added. Samples were then titrated with ferrous ammonium sulfate (~0.2 *M*) indicator

solution to a colour change from green to brown. Two heated and two unheated method blanks were included in the analysis to precisely ensure the molarity of ferrous ammonium sulfate solution. Organic carbon was calculated stoichiometrically from the titre of ferrous ammonium sulfate used in the back titration of unused dichromate. Organic carbon content was expressed as total milligrams carbon per gram soil, and as a percentage carbon (w/w) of total composition.

2.2.2.5 Particle size distribution

Soil particle size analysis was carried out in accordance with the pipette method of Sheldrick and Wang (1993) in triplicate. Ten grams of sieved (1.5 mm), air-dried soil was pretreated with 10 mL of 30% H_2O_2 in 250 mL conical flasks to remove organic matter. Samples were gently heated to 80°C for 45 min to remove residual peroxide. A sodium hexametaphosphate dispersing agent (50 mL) was added and flasks placed on an orbital mixer (Ratek Instruments Pty Ltd, Australia) for 24 h at 100 RPM.

Suspension was poured into 1 L measuring cylinder and made up to 1 L with deionised water, followed by thorough mixing by manually inverting to homogenise. Using a volumetric pipette, 20 mL samples were taken from the cylinder at predetermined depth and time intervals in accordance with temperature and respective settling velocities of silt (20 µm) and clay (2 µm) fractions. That is, 8 cm depth at time intervals of 3 min 25 sec (silt) and 5 h 41 min (clay) at 23°C. The samples were transferred to predried and weighed glass scintillation vials, then dried at 105°C for 24 h, cooled in a desiccator and reweighed. A method blank was also analysed using the dispersing agent in deionised water for correction. Total sand retained underwent repeated washing/settling to remove silt and clay and oven dried under same conditions, then reweighed.

Particle size distribution was expressed as the weight of individual oven dried fractions as a percentage of the dry weight of the original 10 g soil sample. Soil texture class was determined using the triangular diagram of texture classes

according to the International Scheme (see http://www.uwsp.edu/geo/faculty/ritter/glossary/s_u/soil_texture_triangle.html).

2.2.2.6 Inorganic nitrogen

Soil nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N) and ammonium nitrogen (NH_4-N) were determined as described by Keeney and Nelson (1982). This method involves an equilibrium extraction of the soil sample with 2 N KCl and determination of nitrate, nitrite and ammonium using colorimetry and spectrophotometry techniques.

The extraction procedure involved two dilutions for comparison: a 1:10 dilution of 5 g soil (1 mm) in 50 mL 2 N KCl, and a 1:2 dilution of 25 g (1 mm) soil in 50 mL 2 N KCl. Triplicate suspensions were placed on an orbital mixer (Ratek Instruments Pty Ltd, Australia) at 180 RPM for 30 min. Triplicate method blanks of 50 mL 2 N KCl were also prepared. Samples were then filtered gravimetrically through 90 mm Whatman glass microfibre filters and extract analysed.

For analysis of nitrate, 500 µL NaOH reagent was added to 5 mL filter extract and vortex mixed. A reducing agent was added (420 µL) and left for 4 min before adding 730 µL colour reagent and vortex mixing. The mixture was left for 30 min for colour development, then absorbance was read at 543 nm against a 2 N KCl blank using a HACH DR/2000 spectrophotometer (HACH Company, Colorado). Analysis of nitrite was by addition of 200 µL colour reagent to 5 mL filter extract and vortex mixing, followed by 20 min settling for colour development. Absorbance was then read on the spectrophotometer at 543 nm against a 2 N KCl blank. Analysis of ammonium was also determined by colorimetry. One drop of mineral stabiliser was added to 5 mL filter extract and vortex mixed. One drop of polyvinyl alcohol and 200 µL Nessler reagent was added and 30 min allowed for colour development. Absorbance was read at 420 nm against a 2 N KCl blank.

Standard calibration lines were prepared for NO_3-N, NO_2-N and NH_4-N. The equations from the standard lines were used to determine by interpolation the

NO_3-N, NO_2-N and NH_4-N concentrations of the soil samples, multiplied by the dilution factor. Results were reported as soil nitrate concentration (NO_3-N) in mg/kg, soil nitrite concentration (NO_2-N) in mg/kg, and soil ammonium concentration (NH_4-N) in mg/kg.

2.2.2.7 Phosphorus

The estimation of available soil phosphorus was determined by the sodium bicarbonate method, also known as the 'Olsen *et al* method' (1982). This method estimates the relative bioavailability of ortho-phosphate (PO_4-P) using 0.5 N $NaHCO_3$ adjusted to pH 8.50. The extraction process involves 2 g of air dried, sieved (1.5 mm) soil added to 40 mL of 0.5 N $NaHCO_3$ extraction solution. The suspension and a method blank is placed on an orbital mixer (Ratek Instruments Pty Ltd, Australia) for 30 min and then gravimetrically filtered. Phosphorus content is determined spectrophotometrically at 882 nm by reacting with ammonium molybdate using ascorbic acid as a reductant in the presence of antimony. Phosphorus concentration is calculated for blank and unknown samples from a calibration standard curve. Soil bicarbonate available phosphorus (PO_4-P) was expressed in mg/kg.

2.2.2.8 Aluminium

Triplicate soil samples were sent to Amdel Analytical Laboratories Ltd (Adelaide, Australia) for determination of soil extractable Al_2O_3 using Inductively Coupled Plasma-Optical Emission Spectrometry. Results were expressed as mg Al_2O_3 in 100 g soil.

2.3 Results and discussion

2.3.1 Soil analysis

Selected physical and chemical characteristics of the mine site soil are shown in **Table 2.1**. Soil was determined to be slightly acidic (pH 6.3) sandy loam. This value falls within the optimum soil pH range (5.0-6.5) for growth of most Australian plant species (Peverill *et al* 1999). At low pH values (<5.5), the soil bioavailable aluminium increases so that its concentration can become toxic to plants and soil organisms (Peverill *et al* 1999). The measured concentration of soil extractable Al_2O_3 (47.6 mg/100 g soil) would not be bioavailable given the high pH of the soil (Wenzl *et al* 2001).

The measured soil organic carbon content was low 0.26%, although most Australian soils are said to contain <5% organic carbon (Rayment and Higginson 1992). The sandy loam soil showed limited plant-available nutrient levels (N, P) as could be expected at this geographic location (Schwenke *et al* 1999). By world standards, nutrient content of the majority of Australian soils is low (Peverill *et al* 1999). The measured plant-available nitrogen (NO_3-N, NO_2-N, NH_4-N) and phosphorus (PO_4-P) in the mine site soil were below critical concentrations for optimum growth of most cropable species (Peverill *et al* 1999).

Table 2.1 Physical and chemical properties of Weipa mine site soil.

Property	*Value*
Maximum Water Holding Capacity	46 %
Moisture Content (Field capacity)	14 %
pH	6.3
Electrical conductivity	35.2 µS/cm
Soil Texture	Sandy loam
% Sand	60
% Silt	25
% Clay	15
Organic Carbon content	0.26 %
Nitrate-N (NO_3-N)	2.51 mg/kg
Nitrite-N (NO_2-N)	< 0.02 mg/kg
Ammonia-N (NH_4-N)	0.30 mg/kg
Phosphate-P (PO_4-P)	1.16 mg/kg
Al_2O_3	47.6 mg/100 g soil

2.3.2 Plant selection

The Rio Tinto Alcan mine site has an established seed bank consisting of plant species native to the area. The seed bank list of plant species was considered first to establish if any would be suitable for the rhizoremediation application. Plant species available in seed banks were dominated by large tree species such as Eucalyptus and Melaleuca (*Myrtaceae*), and small to medium bushes such as Acacia (*Mimosaceae*) and Grevillea (*Proteaceae*). A single grass species (*Brachiaria decumbens; Poaceae*) was available in seed banks and native to the site, though considered naturalised in Australia (Mallett 2002). The requirement for dense plant coverage coupled with rapid growth rate in successful rhizoremediation disqualified the use of higher plants. The grass species available in the seed bank was selected for inclusion in the study.

The next step in plant selection involved a desk-top survey using evidence in the literature combined with geographical and botanical information. This enabled the development of selection criteria for 'ideal candidates' for the rhizoremediation of hydrocarbon contaminated soil. Adopting this approach, Australian native grass species (*Poaceae*) were selected for the current study based on the following desirable criteria:

- Dense and fibrous root system for maximum surface area and enhanced microbial activity,
- Native (see further discussion below),
- Rapid growth,
- Dense coverage to provide good soil cover and prevent soil erosion,
- A perennial plant, where management can be reduced considerably due to their long growing season,
- Aggressive root systems (common in grasses) which can disrupt soil aggregates and enhance access of trapped hydrophobic contaminants,
- Easy to establish and maintain,
- Hardy and drought tolerant,
- Suitable as site restoration species (long term stability),
- Tolerance of low nitrogen and phosphorus availability, as is commonly found with hydrocarbon contaminated soil,

- Capacity to excrete organic and/or nitrogenous compounds into the rhizosphere,
- Suitable for livestock feeding,
- Wide growth distribution across the continent,
- High hydrocarbon tolerance, as determined by screening procedures in contaminated soil.

There is some discussion in the literature regarding the use of native versus non-native plants for the rhizoremediation of contaminated sites (Frick *et al* 1999; Huxtable and Whalley 1999). In most situations, plants that are native to the region of contamination have been shown to be most appropriate for rhizoremediation (Merkl *et al* 2004b; 2005a). Differences in environmental conditions and restrictions on species importation mean that each country may need to identify indigenous plants to use for phytoremediation (Robson *et al* 2003). The introduction of non-native plants into any agricultural ecosystem should not be taken lightly. Species chosen for rhizoremediation must be well adapted to the soil and climatic conditions of the region. This means that average temperature, annual rainfall, and length of growing season are important considerations in rhizoremediation planning (Frick *et al* 1999). Practical considerations such as cost and availability of seed are also important. Observation of natural revegetation at the site can provide additional information on potential plant species.

Based on the developed selection criteria outlined above, 26 Australian native grass species (*Poaceae*) (including one naturalised species native to the Weipa site) were selected for the study (see full list in **Appendix I**). Based on initial seed availability, nine perennial grass species (of these 26) were ultimately evaluated.

Eight warm season grasses:

- *Alloteropsis semialata* (R.Br.) Hitchc. (Cockatoo grass),
- *Bothriochloa bladhii* (Retz.) S.T. Blake (Forest bluegrass),
- *Brachiaria decumbens* Stapf (Signal grass) (naturalised; native to site),
- *Cymbopogon ambiguus* (Hack.) A. Camus (Lemon Scented grass),

- *Dichanthium sericeum* (R.Br.) A. Camus (Queensland bluegrass),
- *Heteropogon contortus* (L.) Roem & Schult. (Black speargrass),
- *Sarga plumosum* (R.Br.) Spangler (Plume sorghum), and
- *Themeda triandra* Forssk. (Kangaroo grass);

and one cool season grass:

- *Microlaena stipoides* var. Griffin (Labill.) R.Br. (Weeping grass).

Native grass seeds were obtained commercially with the exception of *B. decumbens* which was sourced courtesy of Rio Tinto Alcan from seed banks at the Weipa mine site.

The selected species of *Poaceae* used in the current study including their growth habit and distribution across Australia are described in **Table 2.2**.

2.4 Conclusion

The success of phytoremediation depends in part on establishing a plant cover at a site that has sufficient root-soil contact to produce the desired contaminant degradation. The development of selection criteria as a screening procedure to select plants for inclusion in phytoremediation studies is the critical first step. A simple screening protocol followed by a greenhouse study can help identify candidate species that survive and thrive through the initial establishment period (Kulakow *et al* 2000). Species with vigorous growth and tolerance to the contaminated conditions will have the best chance of producing the initial plant community that will enhance long-term ecosystem processes, including contaminant degradation.

Table 2.2 Selected *Poaceae* screened for hydrocarbon tolerance and rhizoremediation potential.

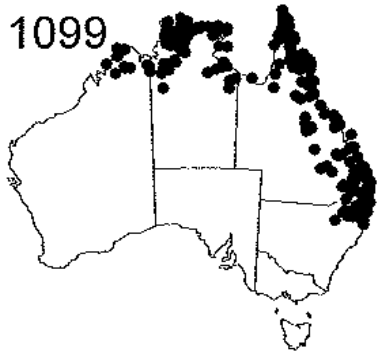
Species (Common name)	Growth habit	Distribution
Alloteropsis semialata (Cockatoo grass)	Erect, rhizomatous, tufted perennial. Growth to 1 m high. Considered good grazing grass with high herbage production. 	Tropical and sub-tropical Australia, widespread in east-central Cape York Peninsula. 

Table 2.2 continued…

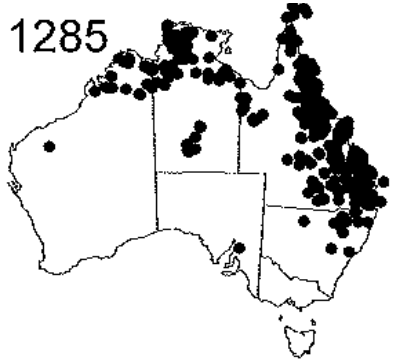
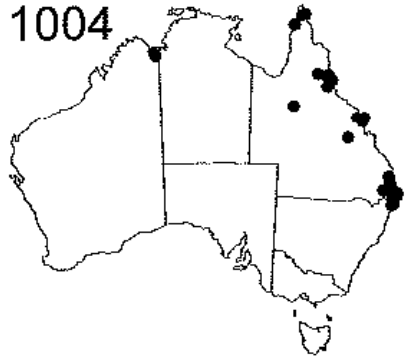
Bothriochloa bladhii (Forest bluegrass)	Dense strongly tufted, erect perennial to 1 m high. Summer growing, hardy and frost tolerant. 	Widespread throughout tropical and sub-tropical Australia. 
Brachiaria decumbens (Signal grass)	Low growing perennial. Aggressive growth habit forming dense soil cover. Well adapted to wide range of soils in humid and sub-humid tropics. 	Adapted to humid tropical areas of Australia. Native to Weipa site and seed banks. 

Table 2.2 continued…

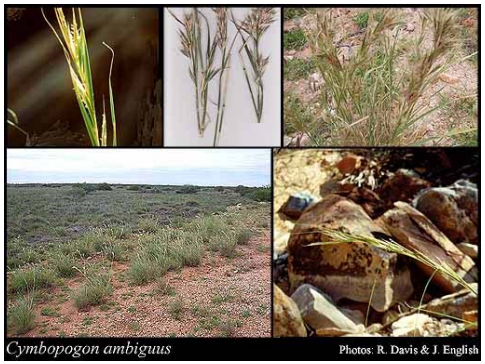
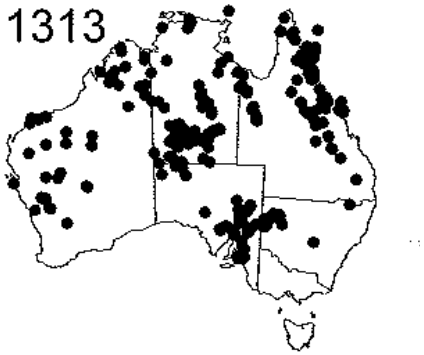
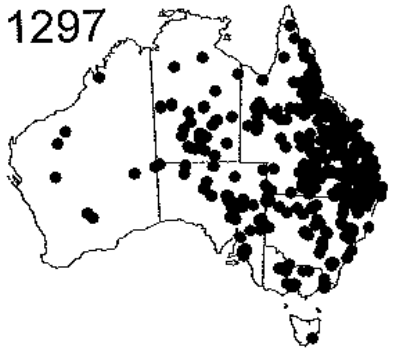
Cymbopogon ambiguus (Lemon Scented grass)	Dense tufted perennial 0.3-1.8 m high. Used in Aboriginal medicine and as a medicinal herb. Strong citron scent when foliage crushed. Very adaptable to different soil types. Drought and frost hardy. 	Widespread throughout Australia except in cool temperate regions. 
Dichanthium sericeum (Queensland bluegrass)	Summer growing tussocky perennial 0.4-0.8 m tall. Moderately drought tolerant and palatable to livestock. 	Widespread in eastern and central Australia. 

Table 2.2 continued…

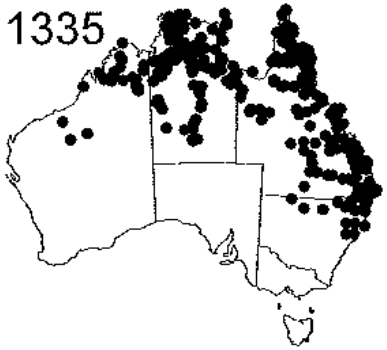
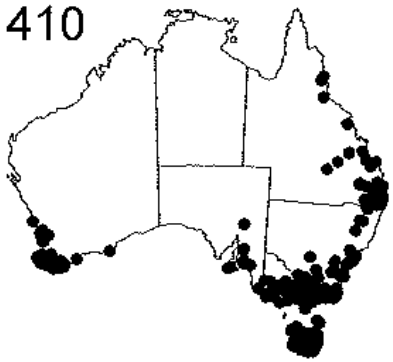
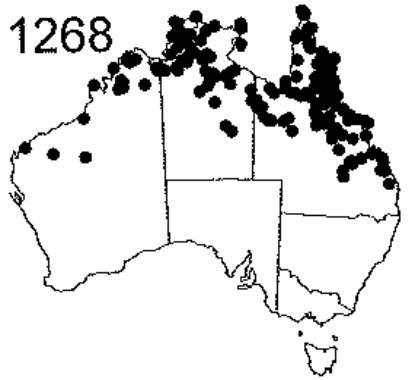
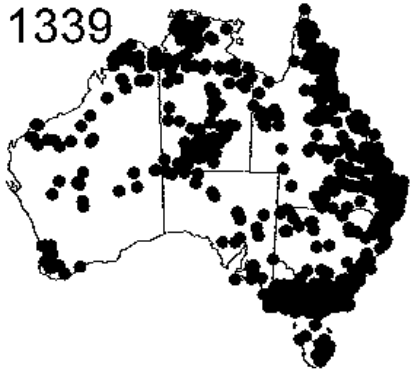
Heteropogon contortus (Black speargrass)	Native vigorous summer growing grass. Perennial, tufted, erect, densely growing tussock to 1.5 m high. Used as a medicinal in Aboriginal medicine.  	Tropical and sub-tropical commonly found as understory in *Eucalyptus* woodlands. 
Microlaena stipoides (Weeping grass)	Slender, tufted perennial to 0.7 m high, with contracted rhizomes. Cool season grower. Soil binder and dense cover; produces fine, soft turf used for landscaping. Tolerant to acid soils, drought and frost. 	Distributed throughout Australia in moister regions. 

Table 2.2 continued…

Sarga plumosum (Plume sorghum)	Tufted native fodder perennial 0.7-3 m high. Short rhizomes.  	Native pastures of dry grasslands in northern Australia. 
Themeda triandra (Kangaroo grass)	Summer fast growing native perennial with dense tussock 0.3-1.5 m high. Fire and drought resistant.  	Widely distributed throughout Australia – all states and territories. 

Sources: (Waters *et al* 2001); Maps (Mallett 2002); Images (Department of Environment and Conservation 1998; Australian National Botanic Gardens 2004)

CHAPTER THREE

3 GERMINATION AND PLANT GROWTH IN DIESEL/OIL CONTAMINATED SOIL

3.1 Introduction

Tolerance of plants to hydrocarbon contamination is a basic criterion for successful rhizoremediation. The screening of a plant species for the ability to successfully germinate and grow in contaminated soil is one of the initial steps in the process of species selection for successful rhizoremediation. This is prior to evaluation of the plant's influence on the degradation of hydrocarbons in soil. A successful candidate should show minimal if any adverse growth effect in the presence of contamination (Tesar *et al* 2002; Merkl *et al* 2004a).

Several protocols have already been suggested to screen plants for hydrocarbon tolerance (Liste and Alexander 1999; Kulakow *et al* 2000; Robson *et al* 2003). Kulakow *et al* (2000) selected candidate plants based on literature searches, and then screened the 29 species of grasses and legumes for growth performance on weathered hydrocarbon contaminated soil. Robson *et al* (2003) assessed the relative growth rate (RGR) of plants as a way of identifying potential phytoremediators. It was hypothesized that plants with low RGR would be more successful for land reclamation because fewer nutrients and water are required than for higher RGR.

The ability of species to germinate in hydrocarbon contaminated soil is often the first step in screening for tolerance. Smith *et al* (2006) demonstrated that germination tests alone do not predict growth of plants on hydrocarbon contaminated soil. The authors noted that germination of some plants may be unaffected by hydrocarbon contaminated soil, but subsequent growth may be significantly reduced. Consequently, germination testing does not provide a short cut to assessing the suitability of species for soil remediation.

It has been shown that seedling growth and size generally correlate with survival and reproduction (Issoufi *et al* 2006), and these parameters have been used to evaluate the potential of plants to grow in contaminated soil. In particular, the physical morphology of plant roots becomes an important factor when designing rhizoremediation treatment (Yateem *et al* 2007). Well developed fibrous roots (biomass) provide a larger surface area for colonisation by soil microorganisms (Kaimi *et al* 2007a; 2007b), thereby maximising the rhizoremediation potential. Similarly, root growing depth is important and influences the depth of soil that can be effectively treated (Vidali 2001). Root growth also disrupts soil aggregates to improve water infiltration and oxygen diffusion (Kaimi *et al* 2006).

If plants can be successfully established on polluted soils, then the plant-microbial interaction in the rhizosphere may provide enhanced breakdown of hydrocarbons in vegetated soils as opposed to non-vegetated soils (Adam and Duncan 2002). It is apparent that a multi-faceted approach to plant screening is most favourable for characterising species and their rhizoremediation potential in hydrocarbon contaminated soil.

This chapter outlines the first stage in a multi-faceted screening approach of selected Australian grass species for potential rhizoremediation of diesel/oil contaminated soil. Within the framework of research on diesel/oil rhizoremediation, attention was given to the effect of diesel/oil on plant growth performance. This included the effect of diesel/oil on seed germination, biomass production (root and shoot), root to shoot ratio of biomass, and relative growth rates. The most successful plant species (showing high tolerance and minimal adverse growth affect) would then be selected for a subsequent rhizoremediation study of hydrocarbon contaminated soil.

3.2 Materials and methods

3.2.1 Screening experiments – Seed viability and germination in soil

Seed viability was assessed in accordance with International Rules for Seed Testing procedures (ISTA 1985). The test measures the level of dehydrogenase (DHA) activity which is an indicator of viability. Seeds were stained using 0.5% tetrazolium chloride solution and evaluated; living cells stained a red colour whilst dead cells did not stain. Due to limited seed availability, viability tests were performed on four replicates of 25 seeds per species. Results were expressed as % viable and % non-viable.

Diesel fuel and 20-50W engine oil were obtained commercially. Sieved mine site soil (2 mm) was experimentally contaminated with a 60:40 diesel/oil mix at concentrations of 3% (w/w) (30 000 mg/kg), 1% (w/w) (10 000 mg/kg), 0.5% (w/w) (5 000 mg/kg) and 0% (control) and mixed thoroughly to achieve a homogeneity. These concentrations represent levels likely to be encountered at the mine site. Germination pots were filled with 200 g of freshly contaminated or uncontaminated (control) soil. Fifty seeds were planted for each replicate (4 replicate pots, 200 seeds in total) for treatments (3%, 1%, 0.5% w/w) and control (0%), for each grass species. Seeds were planted to a shallow depth (5 mm), and soil moisture content maintained at approximately 60% (w/w) soil water holding capacity (see also Section 2.2.2.1) throughout the experiment.

Germination experiments were conducted in a greenhouse during the warm season with natural light conditions. Average maximum and minimum temperatures recorded throughout the experiment were 31.5°C (±3.6) and 20.6°C (±3.3), respectively. Germination was assessed by seedling emergence and monitored daily. After 60 days, plants from each treatment and control were counted for final germination results. Germination of each species for treatments and control was expressed as the percentage of total seeds planted.

3.2.2 Plant growth experiments

Based on germination performance, three promising grass species were assessed for growth characterisation in diesel/oil contaminated (1%, 0.5% w/w) and uncontaminated (control) soil; *B. decumbens* (Signal grass), *C. ambiguus* (Lemon Scented grass), and *M. stipoides* var. Griffin (Weeping grass). One hundred seeds were planted per replicate (3 replicate pots, 140 mm; 1 kg soil each) for treatments and control, for each grass species. Final *n* values for each treatment and species were dependent on germination (Mean *n* = 44, Range = 29-59). Plant growth experiments were conducted over the warm season. The two summer growing species *B. decumbens* and *C. ambiguus* were held in the greenhouse with average maximum and minimum recorded temperatures 34.8°C (±3.9) and 21.1°C (±4.6), respectively. *M. stipoides* is a cool season grass with a well established optimum growth temperature range of 15°C to 25°C (Crawford and Wilkens 1998; Waters *et al* 2001). Plant growth experiments for this species were conducted in a plant growth cabinet set at 25°C for 16 h (light) and 18°C for 8 h (dark) (Crawford and Wilkens 1998).

After 4, 8 and 12 weeks of growth grasses were harvested. Plant roots were gently washed in distilled water to remove soil particles. Plant growth variables were recorded for each treatment and control for each species. Shoot length was determined by measuring the longest leaf (mm), and root length by measuring the longest root (mm). Root and shoot biomass (g dry weight, DW) were determined for each plant after drying at 70°C for 48 h. Root/shoot ratios were calculated as the dry mass of roots divided by the dry mass of shoots (g). Relative growth rate (RGR) was calculated using the formula below (Equation 3.1).

Equation 3.1

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

Where W_1, W_2 are the dry weight of roots (g)

At t_1, t_2 growth times (4 weeks and 12 weeks respectively)

3.2.3 Statistical analysis

Prior to statistical analyses, data were tested for normality and homogeneity of variance (Levene Test) using SPSS v.14 for Windows. Analysis of variance was

conducted at $\alpha = 0.05$ to determine treatment significance for germination, root and shoot length and biomass, relative growth rate and root/shoot ratio, for each species. Factored into the analysis of variance model was inter-pot variation of replicates within treatments (see Equation 3.2). This allowed for analysis of 'pot' as a random factor to be clustered within treatments (e.g. 1%) for each dependent variable (e.g. root length). Treatment mean values which showed significance were separated using Bonferroni Multiple Comparisons test at 5% level of significance.

Equation 3.2

$$Y = \mu + \text{Treatment}_i + \text{Random error} + \text{Pot}_j$$

Where,

Y is dependent variable (e.g. root length)

μ is mean recorded value

i is treatment variation (e.g. 1% diesel/oil)

Random error is variation between plants

j is pot variation within treatment (variation between replicate pots within the same treatment group)

3.3 Results

3.3.1 Seed viability and germination in soil

Results for seed viability differed between species, ranging from 35% viable (*B. bladhii, T. triandra*) to 52% viable (*C. ambiguus*).

The ability of seeds to germinate in diesel/oil contaminated and control soil varied greatly depending on grass species (**Table 3.1**). Some species did not respond at all showing little or no germination in any of the soils including the control (e.g. *A. semialata*, *H. contortus*). Of the species that did respond, only *B. bladhii* showed a statistically significant decrease in germination (by 83%) in diesel/oil contaminated soil (0.5% w/w) compared to the control soil (ANOVA, $p = 0.009$). This was not observed for soil contaminated with 1% (w/w) diesel/oil compared to control soil for this species. For all other grass species the presence of diesel/oil contamination had no significant adverse effect on germination after 60 days when compared to the uncontaminated control.

B. decumbens had the greatest percentage germination (38.5%) of all species tested, and this was observed in the 1% (w/w) diesel/oil contaminated soil. Interestingly, germination at 3% (w/w) diesel/oil for *B. decumbens* (11.5%) was significantly lower than at 1% (w/w) treatment (38.5%) (ANOVA, $p = 0.01$), although no significant difference between other treatments were noted for this species. *C. ambiguus* and *M. stipoides* also showed higher percentage germination in 3% (w/w) and 1% (w/w) contaminated soil compared with other treatments, although these were not shown to be significant. A delay in germination (by five days) was noted for *M. stipoides* in all contaminated soils when compared with control, although no significant difference was found in final germination results between treatments for this species.

Of importance, two of the grass species (*B. decumbens* and *C. ambiguus*) demonstrated lower % germination when sown in clean high-nutrient potting mix compared with the control (0%) mine site soil (see **Appendix II** for more details).

Table 3.1 Germination of Australian grasses in control soil (0%) and diesel/oil contaminated soil (0.5%, 1% and 3%) (w/w). Mean values ± standard deviation, n = 200. * indicates significantly different to control (0%) at $p = 0.01$ (shading).

	Germination (%) ± SD			
	Diesel/oil concentration			
Grass species (Common name)	**0%**	**0.5%**	**1%**	**3%**
Alloteropsis semialata (Cockatoo grass)	0	0	0	nd
Bothriochloa bladhii (Forest bluegrass)	24.8 ± 12.6	4.0 ± 4.9 *	10.0 ± 1.6	nd
Brachiaria decumbens (Signal grass)	24.8 ± 12.9	21.5 ± 11.5	38.5 ± 3.4	11.5 ± 5.7 [a]
Cymbopogon ambiguus (Lemon scented grass)	12.3 ± 8.8	12.5 ± 13.4	22.5 ± 11.8	22.0 ± 11.0
Dichanthium sericeum (Queensland bluegrass)	4.8 ± 6.7	0.5 ± 1	0	nd
Heteropogon contortus (Black speargrass)	0	1 ± 1.2	0	nd
Microlaena stipoides (Weeping grass)	21.8 ± 10.2	20.0 ± 8.2	28.0 ± 4.9	35.0 ± 9.9
Sarga plumosum (Plume sorghum)	5.0 ± 4.7	2.5 ± 1.9	4.0 ± 0	nd
Themeda triandra (Kangaroo grass)	6.0 ± 4.3	7.0 ± 5.3	3.0 ± 4.8	nd

nd not determined

[a] significantly different to 1% treatment for this species ($p = 0.01$)

3.3.2 Plant growth characterisation in diesel/oil contaminated soil

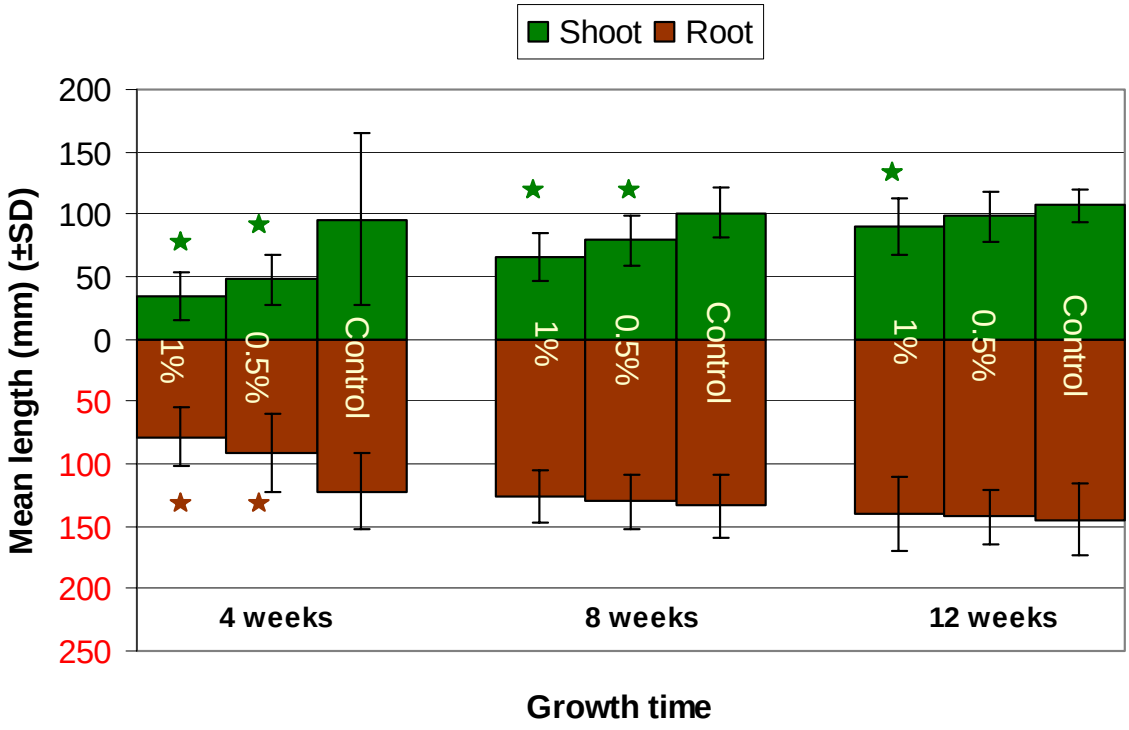
3.3.2.1 Root and shoot length

Seedling growth and size in the diesel/oil contaminated and uncontaminated (control) soils showed that the growth response to the presence of contamination varied between grass species. The three evaluated species (*B. decumbens*, *C. ambiguus* and *M. stipoides*) survived for the duration of the experiment (12 weeks) in the diesel/oil contaminated soil, and in some instances produced longer roots when grown in contaminated soil than in control soil.

Root and shoot length of *B. decumbens* grown in diesel/oil contaminated and control soil is presented in **Figure 3.1**. At early stage of development (4 weeks), diesel/oil produced a delay in root and shoot growth for this species, showing significantly lower root and shoot lengths in diesel/oil contaminated soil (0.5% and 1%) compared with the control (ANOVA, $p \leq 0.001$). Shoot lengths continued to be significantly reduced in 1% diesel/oil contaminated soil compared with the control for the duration of the growth period (ANOVA, $p \leq 0.01$). For root length, this adverse affect was not sustained for the remainder of the growth period, and no significant difference between root lengths in diesel/oil contaminated and control soil was noted at 8 or 12 weeks growth.

C. ambiguus showed an interesting growth response to the presence of diesel/oil contamination (**Figure 3.2**). Similar to *B. decumbens*, an initial delay in root growth (length) in the presence of diesel/oil (0.5% and 1%) was observed for *C. ambiguus* (ANOVA, $p = 0.01$). This adverse affect continued to 8 weeks growth in 1% diesel/oil for root length of this species (ANOVA, $p = 0.009$). By the end of the growth period (12 weeks) root length in diesel/oil contaminated soil (0.5% and 1%) had surpassed that of the control, showing significantly greater root lengths (ANOVA, $p \leq 0.03$). Shoot length was not adversely affected by the presence of diesel/oil at any stage of development for this species.

(a)



(b)

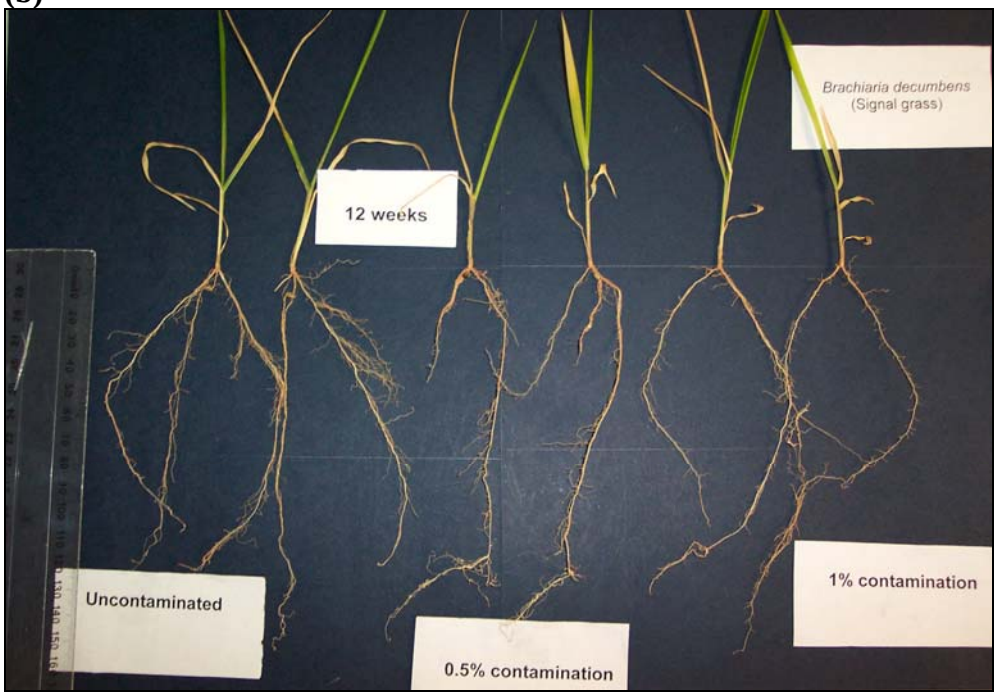
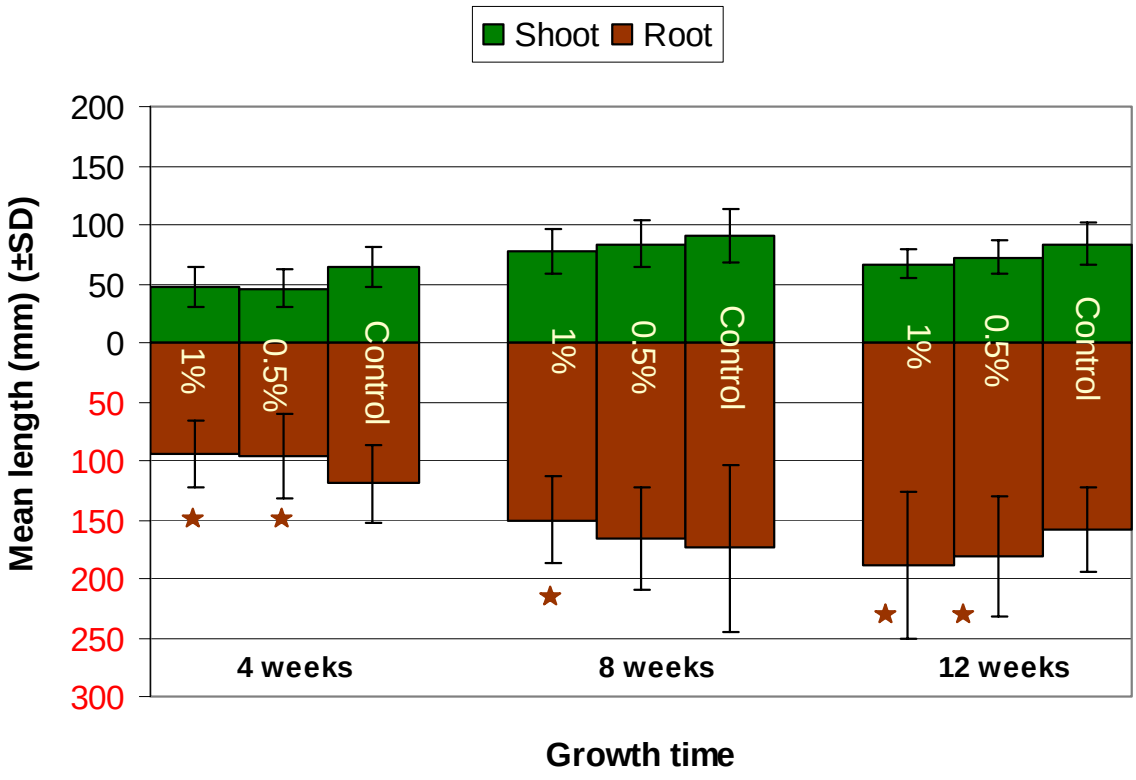


Figure 3.1 (a) Root and shoot lengths (mm) of *B. decumbens* (Signal grass) grown in uncontaminated control soil and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p \leq 0.01$.
(b) Image of *B. decumbens* at 12 weeks growth across all treatments.

(a)



(b)

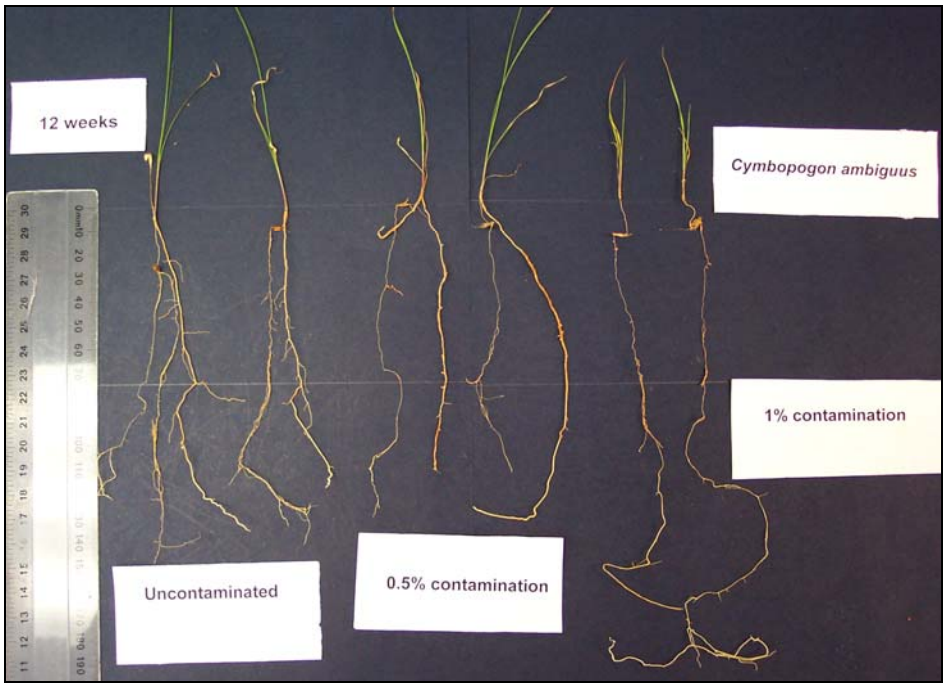
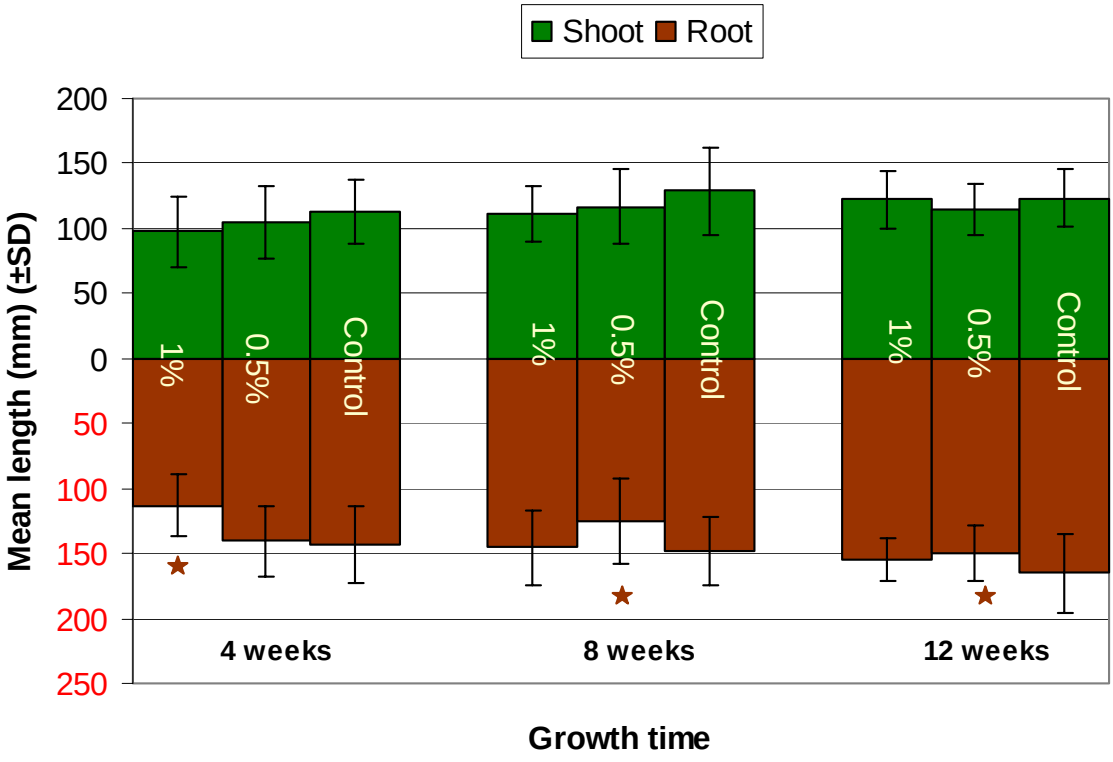


Figure 3.2 (a) Root and shoot lengths (mm) of *C. ambiguus* (Lemon scented grass) grown in uncontaminated control soil and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p \leq 0.01$. **(b) Image of *C. ambiguus* at 12 weeks growth across all treatments.**

(a)



(b)

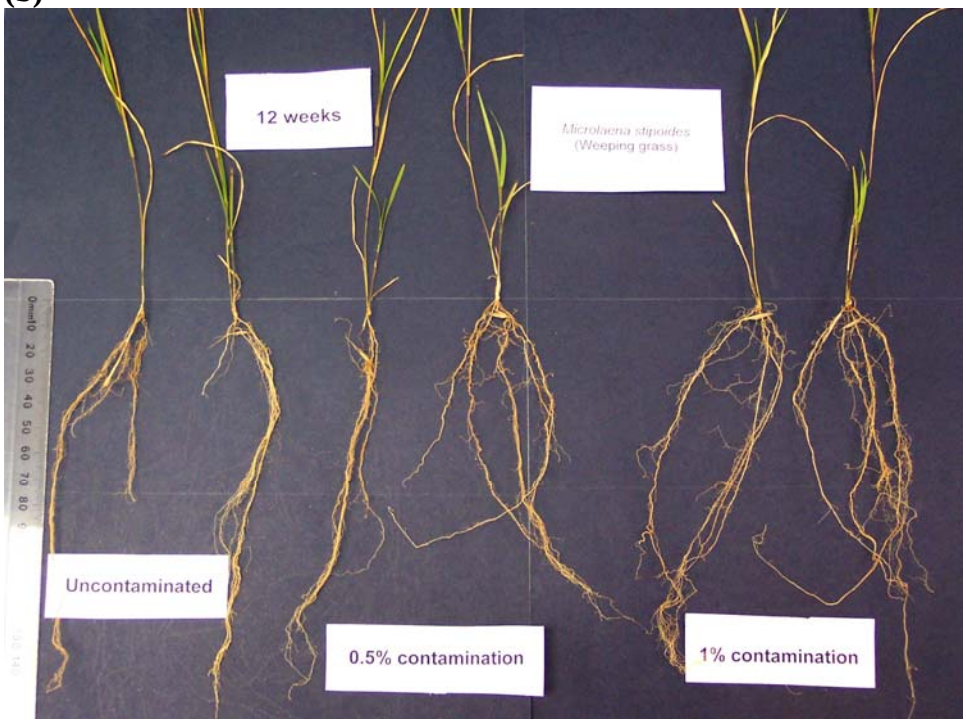


Figure 3.3 (a) Root and shoot lengths (mm) of *M. stipoides* (Weeping grass) grown in uncontaminated control soil and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p \leq 0.01$.
(b) Image of *M. stipoides* at 12 weeks growth across all treatments.

M. stipoides showed a variable response to the presence of diesel/oil on root and shoot length over the growth period (**Figure 3.3**). As with the other grasses tested, *M. stipoides* showed early reduced root length in 1% diesel/oil contaminated soil compared with control (ANOVA, $p = 0.001$). This was not observed for the 0.5% treatment at 4 weeks growth, showing no significant difference to control. By 8 weeks growth, the adverse affect of diesel/oil was no longer observed in the 1% treatment, showing no significant difference to the control. In contrast, a reduced root length was seen in the 0.5% diesel/oil treatment compared with control (ANOVA, $p = 0.001$). This continued for the remainder of the growth period, and at 12 weeks root length was lowest in the 0.5% diesel/oil (ANOVA, $p = 0.02$) but no significant difference was noted between other 1% diesel/oil and control. Shoot length was not adversely affected by diesel/oil throughout the growth period, showing no significant difference to control.

3.3.2.2 Root and shoot biomass

Root and shoot biomass recorded over the 12 week growth period provided evidence of high tolerance to the presence of diesel/oil by this grass species. In some instances considerably more root biomass was produced in the presence of diesel/oil contamination.

B. decumbens showed tolerance to the presence of diesel/oil contamination at the exposed concentrations (see **Figure 3.4**). Root biomass for this species was not adversely affected by diesel/oil exhibiting no significant difference in root production between contaminated and control soils at any stage of the growth period. This is in contrast to shoot development, where reduced biomass production was recorded in diesel/oil contaminated treatments at all stages of growth compared with control (ANOVA, $p \leq 0.003$).

Of particular interest, the presence of diesel/oil had an apparent stimulatory effect on the root biomass production of *C. ambiguus* over the growth period (see **Figure 3.5**). This species demonstrated significantly increased root biomass production in the presence of diesel/oil contamination (1% and 0.5% w/w) compared with control by 12 weeks growth (ANOVA, $p = 0.0001$). No

significant difference in shoot biomass was recorded between treatments at any stage of the growth period for this species.

Similarly, *M. stipoides* showed some evidence of a stimulatory effect of diesel/oil on root biomass production (see **Figure 3.6**). Significantly increased root biomass production was recorded at 8 weeks growth for *M. stipoides* in soil with 0.5% diesel/oil (w/w) compared to uncontaminated control (ANOVA, p = 0.04). This result was not observed in 1% (w/w) contamination plantings. By 12 weeks growth, *M. stipoides* showed no significant difference in root biomass production between contaminated and uncontaminated soil treatments. This is in contrast to shoot development, where reduced biomass production was recorded in diesel/oil contaminated treatments compared with control (ANOVA, $p \leq 0.04$). These data suggest *M. stipoides* showed tolerance to the presence of diesel/oil contamination at the exposed concentrations, and final root growth performance was not adversely affected.

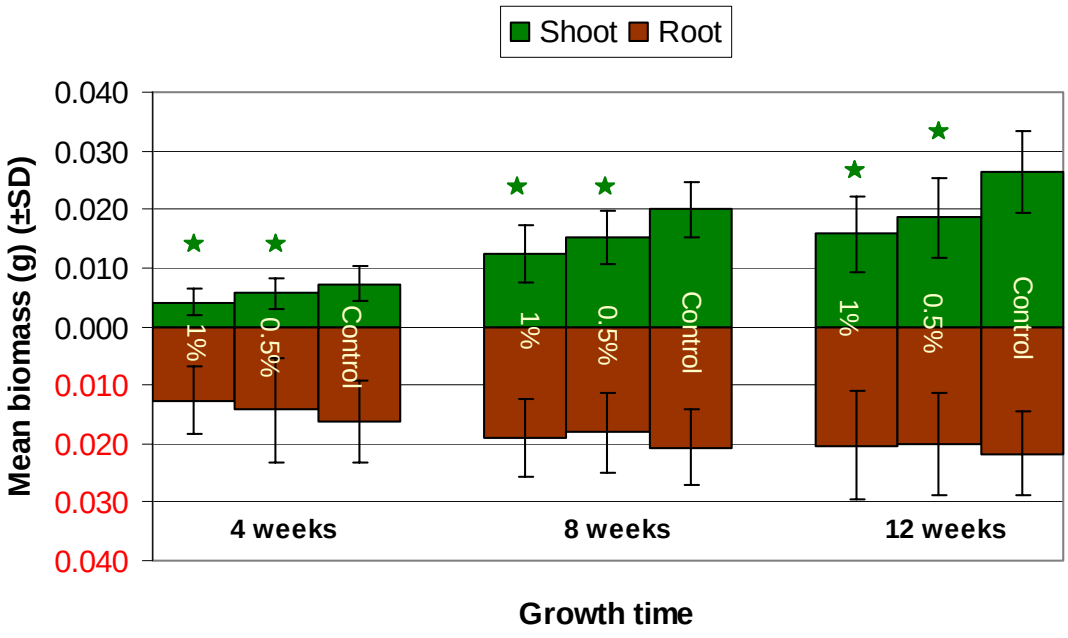


Figure 3.4 Root and shoot biomass production (g) of *B. decumbens* (Signal grass) grown in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p \leq 0.003$.

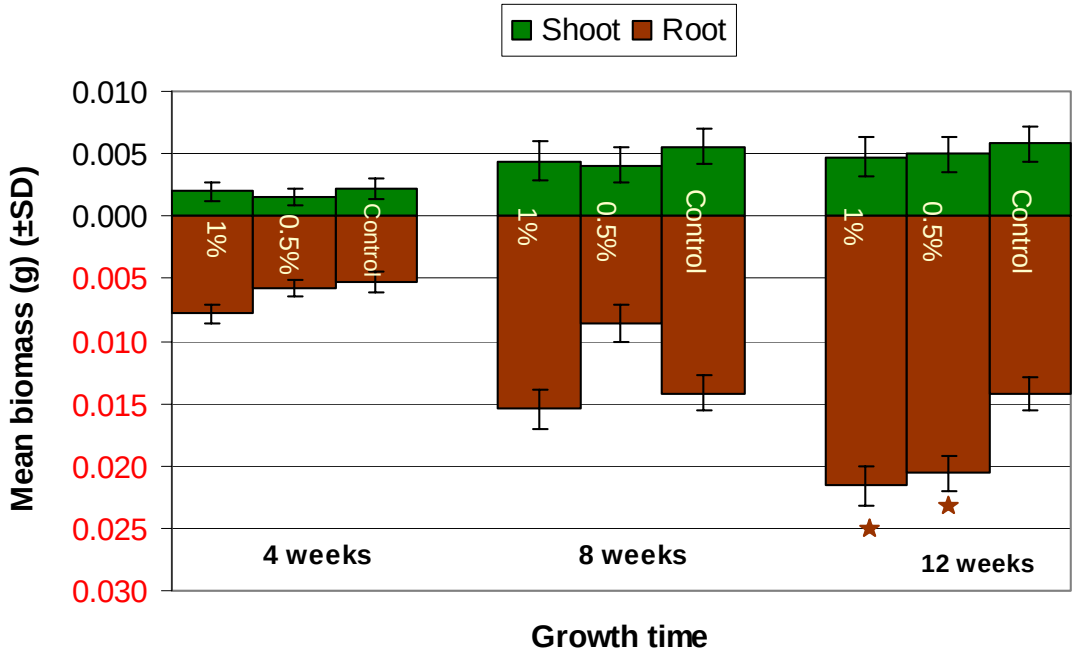


Figure 3.5 Root and shoot biomass production (g) of *C. ambiguus* (Lemon Scented grass) grown in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p = 0.001$.

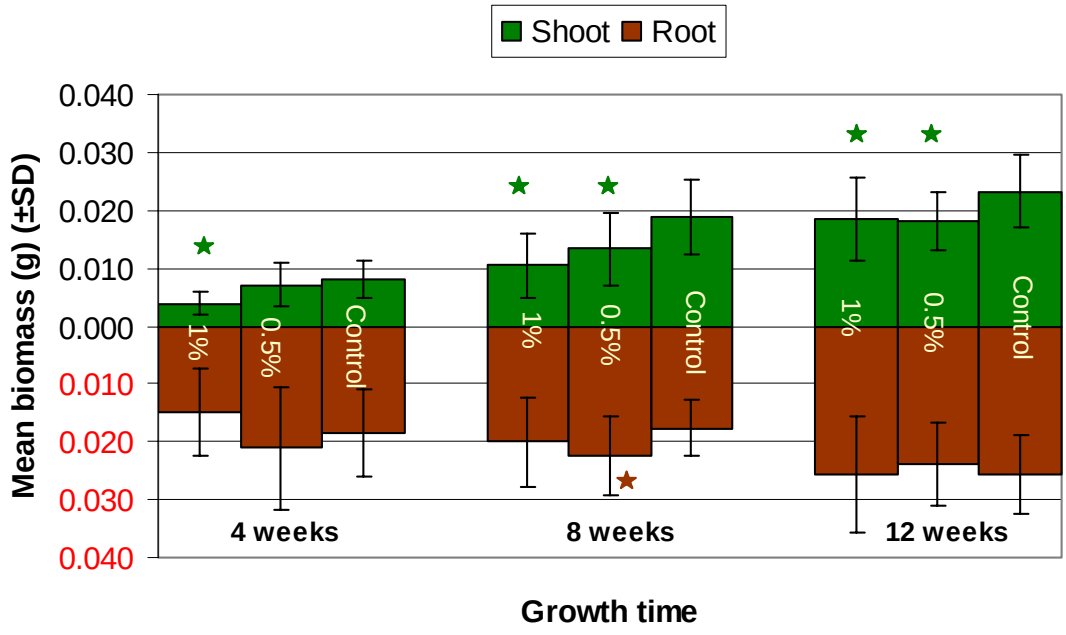


Figure 3.6 Root and shoot biomass production (g) of *M. stipoides* (Weeping grass) grown in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w) at 4, 8 and 12 weeks growth. Mean values ± standard deviation, n = 29-59. * indicates significant difference to control at $p \leq 0.04$.

3.3.2.3 Relative growth rates (RGR) of roots and shoots

Mean relative growth rates of roots (biomass) were consistently higher in diesel/oil contaminated plantings than in control plantings for all species tested (**Figure 3.7**), although these were not shown to be significant due to large variation (SD) between individuals within species. *C. ambiguus* was notable in this regard showing highest mean RGR of roots in diesel/oil contaminated soil (0.174, 0.5% w/w) of all the species tested. *B. decumbens* and *M. stipoides* exhibited similar (although lower) mean RGR of roots in diesel/oil contaminated soil.

Comparable relative growth rates of shoots (biomass) were observed for all grass species across all treatments (**Figure 3.8**). No significant differences were found between treatments. *M. stipoides* recorded the highest mean RGR of shoots in diesel/oil contaminated soil (0.195, 1% w/w) of all the grasses tested.

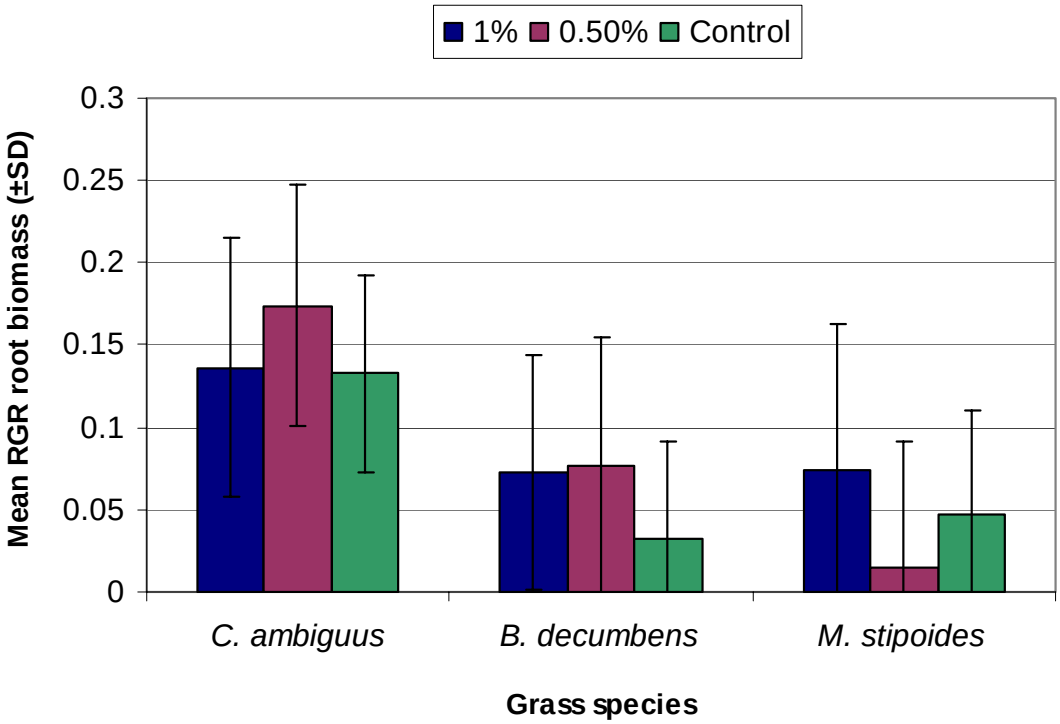


Figure 3.7 Relative growth rates (RGR) of grass roots (biomass) in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w). Mean values ± standard deviation, n = 29-59.

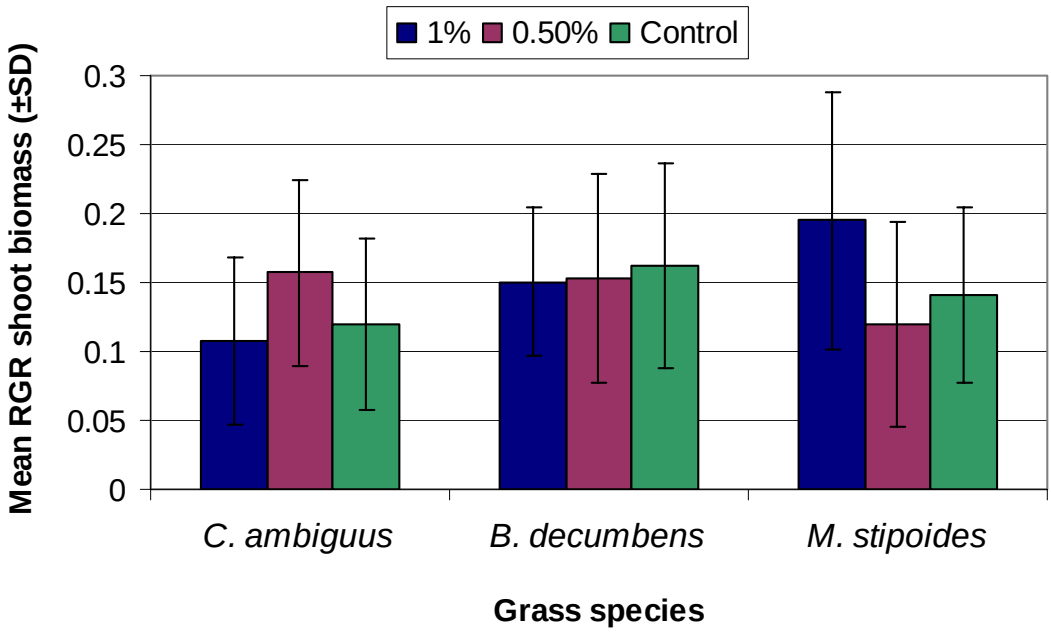


Figure 3.8 Relative growth rate (RGR) of grass shoots (biomass) in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w). Mean values ± standard deviation, n = 29-59.

3.3.2.4 Root to shoot ratio of biomass

The root/shoot ratio reflects the balance between belowground and aboveground plant growth. The higher the ratio, the more of the plant biomass is taken up by the roots. For rhizoremediation application, a high root/shoot ratio would be of significant benefit. The mean root/shoot ratios for the three grass species were assessed (**Table 3.2**).

After 4 weeks growth all species demonstrated significantly increased root/shoot ratios in diesel/oil contaminated soil compared with control (ANOVA, $p \leq 0.01$). Thereafter, *B. decumbens* displayed no significant difference in root/shoot ratio between contaminated and control soils for the remainder of the growth period. In contrast, *C. ambiguus* continued the shift towards root biomass production in contaminated soil (resulting in significantly increased root/shoot ratios), compared with the control at 8 and 12 weeks growth (ANOVA, $p \leq 0.01$). Similarly, at 8 weeks growth, *M. stipoides* showed increased root production compared with shoot production (i.e. higher root/shoot ratio) in diesel/oil contaminated soil compared with uncontaminated (ANOVA, $p \leq 0.01$). By 12 weeks growth there was no significant difference in root/shoot ratio between treatments for this species.

Table 3.2 Root to shoot ratios of biomass in uncontaminated soil (control) and diesel/oil contaminated soil (0.5% and 1% w/w). Mean values ± standard deviation, n = 29-59. * indicates significantly different to control at $p = 0.01$ (light shading), ** at $p \leq 0.001$ (dark shading).

	Root to Shoot ratios								
	4 weeks growth			8 weeks growth			12 weeks growth		
	Diesel/oil concentration								
Grass species (common name)	1%	0.5%	control	1%	0.5%	control	1%	0.5%	control
Brachiaria decumbens (Signal grass)	4.12 ± 3.19 **	3.75 ± 3.21 **	2.66 ± 1.59	1.64 ± 0.54	1.26 ± 0.52	1.09 ± 0.49	1.37 ± 0.49	1.17 ± 0.57	0.88 ± 0.38
Cymbopogon ambiguus (Lemon scented grass)	4.88 ± 3.56 **	4.09 ± 2.08 *	2.85 ± 1.86	3.69 ± 1.63 *	2.24 ± 0.85	2.63 ± 1.14	5.00 ± 2.38 **	4.54 ± 1.67 **	2.78 ± 1.44
Microlaena stipoides (Weeping grass)	4.34 ± 2.61 **	3.39 ± 1.67 **	2.49 ± 1.27	2.25 ± 1.09 **	2.01 ± 1.09 *	1.06 ± 0.53	1.47 ± 0.49	1.36 ± 0.37	1.15 ± 0.38

3.3.3 Comparison between species for growth performance

Final recorded root depths in diesel/oil contaminated soil varied between three grass species screened (range 140-188 mm). *C. ambiguus* demonstrated the highest potential for root depth penetration in diesel/oil contaminated soil of all grasses screened. Significantly longer root length in the presence of contamination after 12 weeks growth was demonstrated for this species compared with the control, and compared with the other species screened (ANOVA, $p = 0.001$). Furthermore, *C. ambiguus* consistently demonstrated significantly greater root/shoot ratios (biomass) in diesel/oil contaminated soil compared with the other two species (ANOVA, $p = 0.001$), and greater relative growth rates (roots) in diesel/oil contaminated soil compared with the other two species (ANOVA, $p \leq 0.02$).

In terms of root biomass production in the presence of diesel/oil contamination, no significant difference between species was found by the end of the growth period. This suggests comparable tolerance to the presence of contamination for all species screened.

Overall, growth performance indicators suggest *C. ambiguus* to be an ideal candidate for rhizoremediation application based on highest potential for root depth penetration, biomass production, relative growth rate (root biomass) and root to shoot ratio when grown in diesel/oil contaminated soil. *B. decumbens* and *M. stipoides* also warrant merit since the presence of diesel/oil had no adverse affect on growth of these species at the root level, despite shoot growth for these species being adversely affected.

3.4 Discussion

3.4.1 Grass seed viability and germination in diesel/oil contaminated soil

Low germination rates were observed for all nine grass species across all treatments (highest 38.5%) (section 3.3.1). This could be due in part to the low seed viability found for all species (35%-52%). This highlights the importance of plant screening prior to rhizoremediation to confirm species suitability and determine seed quality. Low germination can be attributed to grasses not being as easy to establish from seeds as other plants (e.g. legumes). This is due to inherent problems related to poor seed quality, seed dormancy or short seed lifespan associated with the *Poaceae* (Waters *et al* 2001; Adkins *et al* 2002). For example, Adkins *et al* (2002) discussed evidence of seed dormancy mechanisms in Australian warm season grasses including members of *Poaceae* screened in the current study. Species within the genera *Brachiaria, Bothriochloa* and *Themeda* showed evidence of seed coat-imposed barriers to germination acting as a permeability barrier to oxygen uptake, or a mechanical barrier to embryo expansion (Adkins *et al* 2002). Seed pre-treatments such as plant-derived smoke exposure or seed scarification have been shown to break some of these dormancy mechanisms in Australian grasses and improve germination percentages (Read and Bellairs 1999; Adkins *et al* 2002). This may be an option for future optimisation of germination performance in rhizoremediation field trials using the selected species, although the results shown here represent the worse-case scenario.

Soil quality is another major determinant of seedling emergence and viability that must be considered. It is important to note that two grass species (*B. decumbens* and *C. ambiguus*) showed poorer % germination in a high-nutrient potting mix compared to the low nutrient mine site soil (control, 0%). This highlights the adapted trait found in many Australian native species where low soil fertility and low nutrient conditions are optimum for growth (Huxtable 1997; Neumann and Martinoi 2002). It also suggests that the addition of fertiliser etc commonly practiced in phytoremediation and bioremediation may not be suitable, and in fact that conventional bioremediation practices may be harmful

to native vegetation. A similar finding was reported by Michael *et al* (2007) who assessed the use of Australian native trees and other plant species as a phytocover for the hydraulic control of a landfill. Growth trials showed that the addition of mulch and nutrients to the system was of detriment to plant growth during the growing season.

Successful germination of seeds in diesel fuel contaminated soil has been shown to be highly dependent on plant species. Some species are notably tolerant (Smith *et al* 2006) whilst other species are completely intolerant of diesel fuel contamination (Adam and Duncan 2002). This variability in tolerance was evident in the current study. Eight of the nine species tested showed no significant adverse affect on seed germination in any contaminated soil treatments when compared with the control. This demonstrates a range of tolerance to diesel/oil by these species at the exposed concentrations. Of note, *B. decumbens* exhibited relative tolerance to diesel/oil up to 1% (w/w) without adverse affect on germination. Significantly lower germination percentage at 3% (w/w) was observed for this species (compared with 1% treatment), although not to the extent below performance in the uncontaminated soil. This suggests a mild inhibitory effect on germination at higher diesel/oil concentrations for *B. decumbens*.

Al-Ghazawi *et al* (2005) reported some variability between species in tolerance to diesel fuel contamination. A decline in alfalfa (*Medicago sativa*) germination at 500 mg/kg diesel or higher was noted, whereas under the same contaminated conditions fescue grass (*Cyndon dactylon*) was relatively tolerant and exhibited a higher % germination than alfalfa. Germination was assessed on filter papers so results of the study would only be a preliminary indication of diesel inhibition of seeds under real soil conditions. Smith *et al* (2006) reported high hydrocarbon tolerance when four grass and three legume species were assessed for germination in soil contaminated with 1000 mg/kg PAH mixture. After ten days, PAHs caused no significant difference in % germination of any of the plant species tested.

An inhibitory effect of diesel fuel on germination may be attributed to the physico-chemical constraints induced by diesel fuel (Kroening *et al* 2001). The coating of the seed and surrounding soil particles may act as a physical barrier preventing or reducing both water and oxygen transfer (Adam and Duncan 2002). The adverse effect of diesel/oil on germination of *B. bladhii* may have been due to such physical barriers, combined with possible seed dormancy issues discussed above.

The evaluation of germination in field soil samples in this study provided conditions closer to those that would be experienced in reality. Typically, germination trials have been carried out under closed conditions in containers such as petri dishes (Al-Ghazawi *et al* 2005; Smith *et al* 2006). This can result in build up of vapour from the lighter fractions and delay or reduce germination (Smith *et al* 2006). Assessment in soil is therefore important when using germination testing as a screening tool for species for subsequent field studies.

The germination results in this study suggest several Australian grass species have potential as candidates for rhizoremediation of aliphatic hydrocarbon contaminated soil. The required ability to germinate in the presence of diesel/oil contamination was demonstrated for eight of the nine species tested. *B. decumbens, C. ambiguus* and *M. stipoides* showed the most promise in this regard, as higher percentage germination was noted for these species in diesel/oil contaminated soil (at 1% w/w) compared to the uncontaminated control.

3.4.2 Plant growth performance in diesel/oil contaminated soil

Growth performance of all three grass species suggests high tolerance to the presence of diesel/oil contamination in soil at the exposed concentrations. Species that exhibit high germination and subsequent survival and growth in contaminated soil are more suitable for rhizoremediation. In particular, root biomass production is one of the most important descriptors of a plant's suitability. Banks *et al* (2003b) demonstrated that the greatest removal of total petroleum hydrocarbon concentration occurred in the period with greatest root growth. No single trait can completely describe plant performance and thus a multi-factorial assessment approach such as the one adopted here is warranted.

Results provide evidence that *C. ambiguus* (and to a lesser extent *M. stipoides*) are ideal candidates for investigation of rhizoremediation technology showing increased growth (root depth and biomass) in the presence of contamination (sections 3.3.2.1 and 3.3.2.2). This suggests these species may be capable of switching their carbon allocation to roots under stressful conditions (Robson *et al* 2003). Plant root depth becomes important since it is generally accepted that the physical and biological conditions favouring degradation of organic pollutants decreases with increasing soil depth (Olson *et al* 2001). Vidali (2001) suggested that in many soils effective oxygen diffusion for desirable rates of biodegradation extend to a range of only a few centimetres to 30 cm into the soil profile. The average root depths recorded for the Australian grasses in this study (~14 – 19 cm) are therefore ideal for application to rhizoremediation. In addition, *C. ambiguus* is a warm season grass while *M. stipoides* is suited to cool season growth, allowing for climate variation and extended active period during a growing season in rhizoremediation application. *B. decumbens* (the only naturalised species included in the study) showed no adverse growth effect on roots in the presence of diesel/oil contamination suggesting it was also suitable for inclusion in further rhizoremediation study.

M. stipoides and *B. decumbens* recorded reduced shoot biomass production in the presence of diesel/oil contamination compared with controls. In terms of assessing growth performance, this was not ideal since a preferred candidate for rhizoremediation (as outlined in Chapter 2) should show tolerance to contamination in all aspects of growth. Ideally, the shoots should grow well enough to set seed, and to do that a plant needs well established aerial parts. Despite this, these species were still suitable candidates for further investigation into their rhizoremediation potential since root growth, ultimately the site of biodegradation, was not adversely affected by the presence of diesel/oil.

Previous reports in the literature show the presence of petroleum hydrocarbons can significantly reduce plant biomass (Kulakow *et al* 2000; Tesar *et al* 2002; Robson *et al* 2003). When evaluating species for phytoremediation, the decrease in plant growth and especially root biomass should be as low as possible (Merkl *et al* 2005a). Kulakow *et al* (2000) screened 26 native and introduced grass

species in the United States for growth in petroleum hydrocarbon contaminated sediments. Although grass species were not affected equally by the presence of contamination, the authors reported considerable reductions in plant growth in the contaminated soil after 180 days compared to the control for all species tested. An average of 77% reduction in root biomass in contaminated soil compared with control was reported.

Similar adverse affects on plant growth in the presence of hydrocarbon contamination was observed by Robson *et al* (2003). Thirty nine plants (grasses and legumes) native or naturalised in Canada were assessed for their ability to survive in crude oil contaminated soil. The authors reported the addition of 0.5%, 1% and 5% (w/w) crude oil to soil significantly decreased both the root biomass and total plant biomass by at least 22% of the control for all but three species. It is important to note that the composition of crude oil is different to diesel fuel and lube oils, and includes more phytotoxic components such as polycyclic aromatic hydrocarbons (PAHs) (ATSDR 1996). Based on these reports, a reduced plant growth performance might have been the expected outcome in the presence of diesel/oil contamination for the Australian grasses.

In contrast to these reports, and as observed in the current study, oil contamination may sometimes have no adverse affect on growth, or can cause growth stimulation (Adam and Duncan 1999; Reynolds *et al* 1999; Merkl *et al* 2004a). Smith *et al* (2006) report the growth (total dry biomass) of perennial ryegrass (*Lolium perenne*) was unaffected when grown in an aged coking works soil measuring >5000 mg kg^{-1} PAH mixture. In a laboratory study, Reynolds *et al* (1999) reported the effect of amending silt loam soil with a model organic contaminant (including diesel and pyrene) on the growth of four grass species in the United States. The authors noted that at 1000 mg TPH kg^{-1} (0.1%) three of the four grass species assessed showed increased root biomass production compared with uncontaminated controls after six weeks growth.

Similarly, a study by Merkl *et al* (2004a) noted enhanced growth in the presence of crude oil contamination. Two grass species (including a *Brachiaria* sp.) and six legumes naturalised in eastern Venezuela were evaluated for tolerance to

crude oil added at 3% and 5% to savannah soil. The authors observed increased shoot length and biomass, and increased root/shoot ratios in four of the legumes. Of interest, the *Brachiaria* sp. evaluated in the study showed high seedling emergence and least affected (though reduced) biomass production of the grasses assessed. It also exhibited no adverse affect on root/shoot ratio in the presence of 5% (w/w) crude oil. The Australian naturalised grass *Brachiaria decumbens* evaluated in the current study showed no adverse growth affect in the presence of diesel/oil contamination on root biomass production or root/shoot ratio.

The root/shoot ratio is known to increase under insufficient nutrient conditions (Kaimi *et al* 2007b). This can be because plants allocate photosynthates to the roots rather than to the vegetative parts to obtain nutrients (Kaimi *et al* 2007b). An increase root/shoot ratio was observed in diesel/oil contaminated soil for all grasses screened in the current study at some stage of the growth period (section 3.3.2.4). A similar effect was noted by Kaimi *et al* (2007b) when Italian ryegrass (*Lolium multiflorum* L.) was grown in soil contaminated with 0.8% diesel. The authors reported higher root/shoot ratio in plants grown with 0.8% diesel compared with uncontaminated controls.

Merkl *et al* (2005a) reported higher RGR for roots (and shoots) of the grass *Brachiaria brizantha* when grown in 5% (w/w) crude oil compared with uncontaminated controls. In the current study, higher (although not significant) RGR for roots were observed in diesel/oil contaminated soil for all species tested compared with the uncontaminated controls (section 3.3.2.3). Comparable relative growth rates of shoots (biomass) were recorded between Australian grasses, revealing no significant differences. Since any size advantage of a plant can increase through time due to exponential growth, the high recorded RGR (root biomass) of the grasses must be a favourable characteristic. Results here are in contrast to the hypothesis offered by Robson *et al* (2003), where low RGR was seen indicative of stress tolerance (as high root biomass production was maintained). The three grasses screened in the current study exhibited high RGR and were able to maintain normal growth rates when faced with a stressful habitat (i.e. diesel/oil contaminated soil).

In this study, a significant stimulatory effect of diesel/oil contamination on growth of *C. ambiguus* at relatively high hydrocarbon concentrations (1% w/w) was noted. *M. stipoides* also demonstrated root growth stimulation in the presence of 0.5% (w/w) diesel/oil concentration. Reasons for enhanced growth may include an hormonally influenced stress response as suggested by Merkl *et al* (2004a). Alternatively, stress responses by some plants facing nutrient limitation may also result in growth stimulation. Increased root biomass may also be a strategy to stimulate water, nitrogen, or phosphate uptake in the plant (Frick *et al* 1999).

It is important to note that all growth performance experiments conducted in the current study were without the addition of soil amendments. By not adding nitrogen or phosphorus fertiliser in these experiments a worst-case scenario for plant growth is presented. This is because oil is composed of approximately 85% carbon and when added to soil may cause immobilisation and depletion of essential nutrients such as N and P (Issoufi *et al* 2006) and consequently microbial depletion. Measurement of available N and P in the soil was not performed during the experiment in the current study, and may be valuable to demonstrate immobilisation of nutrients by microorganisms during transformation.

This is the first report of Australian plant species exhibiting growth stimulation effect in the presence of diesel/oil contamination. In light of this, screening of other native species may be of value. Encouraging growth performance outcomes of the three Australian grasses suggests their potential as candidates for rhizoremediation of aliphatic hydrocarbon contaminated soil. The ability to tolerate diesel/oil without adverse growth affect was demonstrated for all species. *C. ambiguus* was most promising as significant growth stimulation (in particular roots) was demonstrated in diesel/oil contaminated soil compared with controls.

3.5 Conclusion

The success of rhizoremediation will depend on establishing a plant cover at a site that has sufficient root-soil contact to produce the desired contaminant degradation. The multi-faceted assessment of plant performance used here has confirmed three Australian grass species as suitable candidates for further investigation of their rhizoremediation potential. Grasses were able to germinate from seed and tolerate the presence of diesel/oil contamination at the exposed concentrations. In some instances the presence of diesel/oil was stimulatory to plant growth (in particular root development). In the next stage of this multi-faceted approach, studies relating to hydrocarbon degradation and microbial dynamics in the rhizosphere soil and the root zone are undertaken using these species.

CHAPTER FOUR

4 THE EFFECT OF AUSTRALIAN GRASSES ON MICROBIAL COMMUNITY DYNAMICS IN DIESEL/OIL CONTAMINATED SOIL

4.1 Introduction

The primary factor influencing the rhizoremediation of petroleum hydrocarbons in soil is microbial activity. Their role includes the biotransformation of petroleum compounds into harmless compounds. Microbial populations and activity in the soil are strongly associated with the type of contaminant, the plant species, and the water and nutrient contents of the soil (Hutchinson *et al* 2003). The extensive rhizosphere of Australian grasses (see Section 3.3.2) creates an optimum environment for contaminant-degrading microorganisms. Microbial densities of hydrocarbon degraders have been shown to be consistently greater in the rhizosphere soil than in soil without vegetation (see review Kuiper *et al* 2004). Plant roots and their exudates increase microbial numbers in the rhizosphere by one to four orders of magnitude, thus increasing microbial activity (Olson *et al* 2003; Pilon-Smits 2005). This enhanced microbial biomass and activity is known as the *rhizosphere effect*. The success of rhizoremediation must not only focus on tolerant plant species (see Chapter 3), but also on the formation of rhizosphere-specific associations between plant roots and pollutant-degrading microorganisms.

To understand how the composition of the microbial community affects the process of rhizoremediation, it is necessary to analyse the microbial response to hydrocarbon pollution both quantitatively and at a metabolic level. Enumeration of hydrocarbon degrading microorganisms in soil is accomplished most reliably by Most Probable Number (MPN) procedures, because other bacteria may grow on impurities present in even highly purified agars (Wrenn and Venosa 1996). MPN enumerations allow for the use of selective growth substrates, and estimation of population density of specific contaminant-degrading bacteria. Only viable organisms are enumerated by the MPN determination. The MPN

technique is based on determination of the presence or absence of microbes in several consecutive dilutions of soil (Torstensson 1997). Theoretically a positive reading means that at least one organism was present initially in the portion used for inoculation.

Soil biological activities are vital for the restoration of soil contaminated with hydrocarbons. The use of these activities as bioindicators may be informative monitoring tools during the rhizoremediation of hydrocarbon contaminated soils (Maila and Cloete 2005). The use of bioindicators provides a direct, inexpensive and uncomplicated methodology compared with traditional monitoring techniques (e.g. GC-MS). Soil biological activities have been used as bioindicators of pollution with hydrocarbons (Margesin *et al* 1999; Riffaldi *et al* 2006), heavy metals (Saviozzi *et al* 2006) and pesticides (Top *et al* 1999).

Due to their central role in the soil environment, soil enzyme activities are attractive as bioindicators for monitoring impacts on soils. Enzymes in soil are the catalysts of important metabolic process functions including the decomposition of organic inputs and the detoxification of xenobiotics (Maila and Cloete 2005). Enzymes that have been tested for their potential to monitor hydrocarbon removal include soil lipases, dehydrogenases, catalases and ureases. Products released from hydrocarbon biodegradation are substrates for hydrolases including esterases-lipases (Margesin *et al* 2007). Therefore, soil lipase activity may be a valuable tool to monitor biodegradation of petroleum hydrocarbons, such as diesel oil, in freshly contaminated soil. The induction of soil lipase activity has been shown to be a valuable indicator of oil biodegradation in contaminated soils (Margesin *et al* 2000; Lee *et al* 2008).

This chapter outlines the next stage in a multi-faceted approach and investigates the ability of Australian grasses to stimulate the rhizosphere microbial community for the biodegradation of hydrocarbon contaminants in soil. Quantitative changes in rhizosphere microbial communities caused by the planting of different grass species in a soil contaminated with diesel/oil were measured. Also measured were the changes in microbial enzyme activity (lipase) as a bioindicator in the monitoring of hydrocarbon removal.

4.2 Materials and methods

4.2.1 Grass rhizosphere soil sampling

Three grass species showing best tolerance to diesel/oil (of the nine originally tested in the germination trials) were assessed for influence on microbial community dynamics in the rhizosphere; *B. decumbens, C. ambiguus and M. stipoides*. For the purpose of this investigation *rhizosphere soil* was defined as soil adherent to roots of grasses following gentle shaking by hand. This is recognised in the literature as an accepted definition and collection method for rhizosphere soil (Smalla *et al* 2001; Costa *et al* 2005; Kim *et al* 2006).

Sieved (2 mm) mine site soil was artificially contaminated with 60/40 diesel/oil mixture at 1% (w/w) concentration, which represents the level likely to be found at the mine site. Diesel/oil contaminated soil was then placed into pots (140 mm; 1 kg) and either planted with a single grass species (20 seeds) for treatment, or left unplanted (control). Pots were placed under growth conditions described in Section 3.2.2 for 100 days. Destructive (pot) sampling was performed periodically and generally followed day zero (sowing), germination, and weekly thereafter until 100 days after planting (DAP).

In planted treatments, one composite rhizosphere soil sample per pot was collected from the roots of six randomly selected plants. For unplanted controls six random soil cores (~5 g) per pot were collected and composited. Rhizosphere and control (unplanted) composite soil samples were used for analysis of microbial community dynamics (MPN and lipase activity) in the presence of diesel/oil contamination.

4.2.2 Most Probable Number (MPN) assay for enumeration of diesel-degrading organisms in rhizosphere soil

A most probable number (MPN) method was adapted from Gaskin and Bentham (2005) for the enumeration of microorganisms in soil (rhizosphere and non-rhizosphere) capable of utilising diesel as a sole source of carbon and energy. MPN assay of diesel degrading organisms was performed in six replicates for

planted treatments and unplanted controls, for each species at each sampling time. Planted treatments and unplanted control were also compared to unvegetated bulk clean mine site soil.

Rhizosphere (or control) soil samples (1 g) were suspended in 9 mL phosphate buffered saline (NaCl 8 gL^{-1}; K_2HPO_4 1.21 gL^{-1}; KH_2PO_4 0.34 gL^{-1}) for inoculation into 24-well pre-sterilised cell culture trays (Becton-Dickinson Labware, USA). Each well in the tray contained 1.1 mL Bushnell-Haas liquid media (KH_2PO_4 1 gL^{-1}; K_2HPO_4 1 gL^{-1}; NH_4NO_3 1 gL^{-1}; $MgSO_4.7H_2O$ 0.2 gL^{-1}; $FeCl_3$ 0.05 gL^{-1}; $CaCl_2.2H_2O$ 0.02 gL^{-1}) and 100 µL filter sterilised diesel. 150 µL of soil suspension (rhizosphere or control) was then added to the first five consecutive wells (top row) in a tray. Serial dilutions of the inoculum (10^{-1} to 10^{-4}) were then prepared for each row of wells in the tray. Replicate trays were then incubated at 28°C for seven days for microbial growth.

Following seven days incubation 100 µL of 2 mg fluorescein diacetate/mL acetone was added to each well and trays were re-incubated for twenty minutes to allow for colour development. Wells with observable release of fluorescein (yellow) and fluorescence under ultraviolet light were scored as positive for microbial activity (see **Figure 4.1**). Final MPN values per gram of soil in the initial inoculum were obtained using tenfold dilution five tube MPN published tables (de Man 1983).

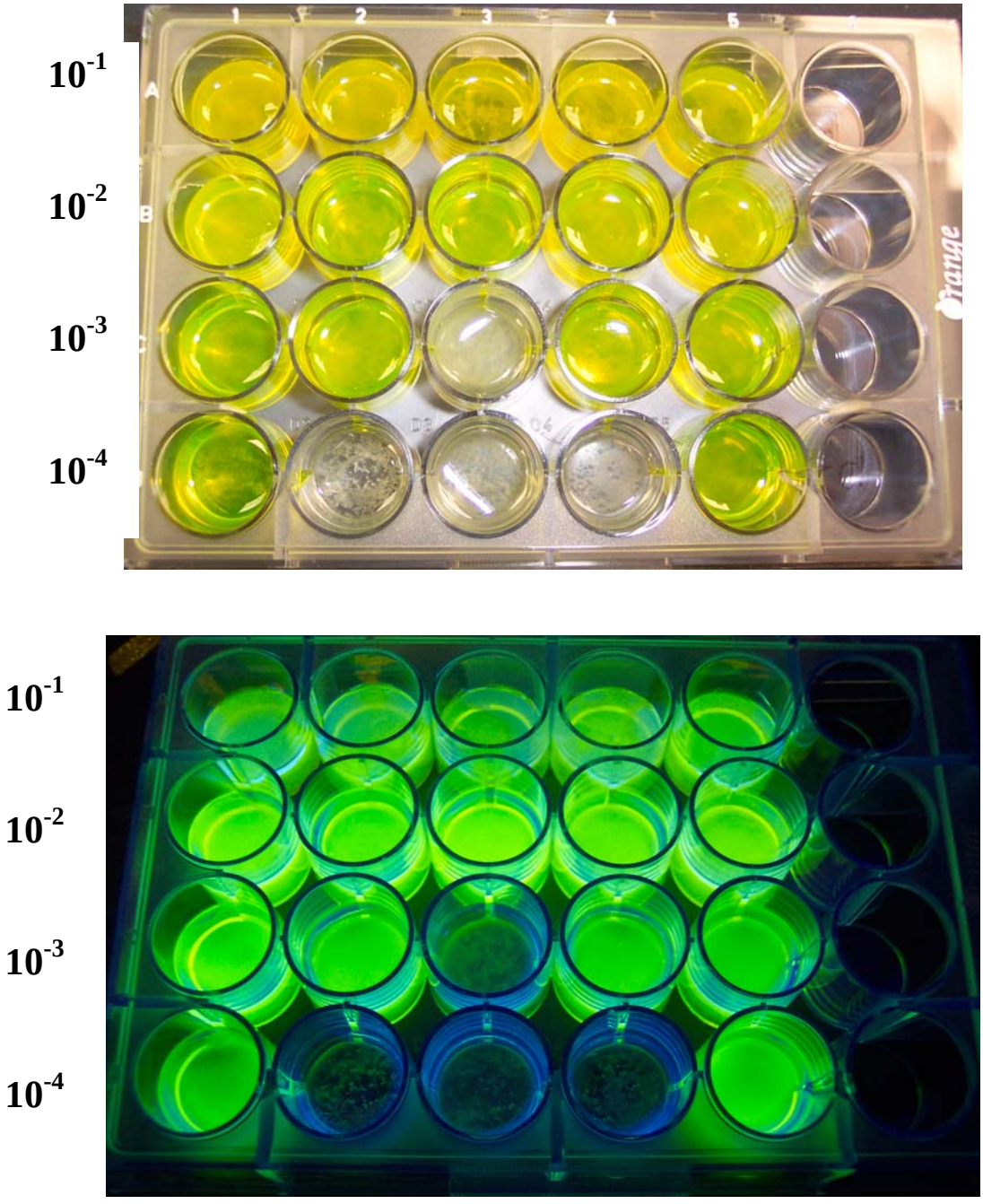


Figure 4.1 Example MPN assay showing release of fluorescein (yellow wells) and fluorescence (UV exposed) as positive for microbial growth of hydrocarbon degrading microorganisms. Shows five positive wells in the 10^{-1} and 10^{-2} dilutions, four positive in 10^{-3} dilution, and two positive in the 10^{-4} dilution.

4.2.3 Soil lipase assay as a measure of biological enzyme activity in the rhizosphere

A colorimetric assay as described by Margesin *et al* (2002) was used for the rapid quantification of lipase activity in soil. This method measures the conversion of *p*-nitrophenyl butyrate (pNPB) as substrate to *p*-nitrophenol (pNP) in a colorimetric reaction.

Sampled rhizosphere or unplanted control soil (0.1 g) was weighed into four replicate centrifuge tubes. A 100 mM NaH_2PO_4 buffer solution adjusted to pH 7.25 using NaOH was pre-warmed to 30°C for ten minutes in a water bath. Five mL of the buffer solution was added to each tube and gently mixed. Then 50 µL of substrate solution (100 mM pNPB diluted in 2-propanol and stored at -20°C) was added to each tube. The contents were mixed and the tubes were incubated in the water bath at 30°C for ten minutes. In order to measure the pNP release from the substrate, a method control (four replicates) was prepared without soil.

To stop the reaction, the tubes were cooled for ten minutes on ice. Thereafter the tubes were centrifuged at 2000 *g* at 4°C for five minutes, and then held on ice. The extinction of released pNP was measured using a spectrophotometer (Shimadzu UV-1700 spectrophotometer, Japan) at 400 nm against the reagent blank.

To prepare a calibration curve, standards containing 0 (reagent blank), 25, 50, 75, 100 and 125 µg pNP were made by adjusting volumes of 0 to 1.25 mL of a working standard solution (100 µg pNP/ mL phosphate buffer) to 5 mL with buffer. After subtracting the control reading (hydrolysis in the absence of soil) from the sample reading (hydrolysis in presence of soil), soil lipase activity was expressed as µg pNP/(g soil dry wt. x 10 min).

4.2.4 Statistical analysis

Prior to statistical analysis, data were tested for normality and homogeneity of variance (Levene Test) using SPSS v.14 for Windows and GraphPad Prism v.4.0. Independent samples t-test (2-tailed) and analysis of variance (ANOVA) at $\alpha = 0.05$ were conducted to determine treatment significance at each sampling time for MPN and lipase activity, for each species. Treatment mean values which showed significance using ANOVA were separated using Bonferroni Multiple Comparisons test at 0.05 level of significance.

4.3 Results

4.3.1 Quantitative hydrocarbon-degrader community changes measured by MPN assay

Results of MPN analyses of hydrocarbon degrading microbial populations over time in rhizosphere and unplanted (control) soil treatments were obtained for each grass species.

Figure 4.2 presents hydrocarbon degrading population sizes over time in rhizosphere and control treatments of *B. decumbens*. Initial hydrocarbon degrading populations (t = 0 DAP) in planted and unplanted diesel/oil treatments were similar to those measured in clean bulk soil (representing background numbers). After seven days exposure to the contaminant, hydrocarbon degrading population numbers increased significantly in rhizosphere soil relative to background numbers found in bulk clean soil (ANOVA, $p = 0.01$), but interestingly in unplanted soil population size remained similar to background levels.

Most importantly, within diesel/oil contaminated treatments the rhizosphere environment created by *B. decumbens* supported substantially higher numbers of hydrocarbon degrading microorganisms than unplanted (control) soil from the time of germination (t = 7 DAP) onward (T-test, $p \leq 0.03$) (with exceptions 78 and 89 DAP). This provides evidence of a rhizosphere effect created by *B. decumbens*, although perhaps a weak effect since the stimulation in microbial numbers was within one order of magnitude compared with control.

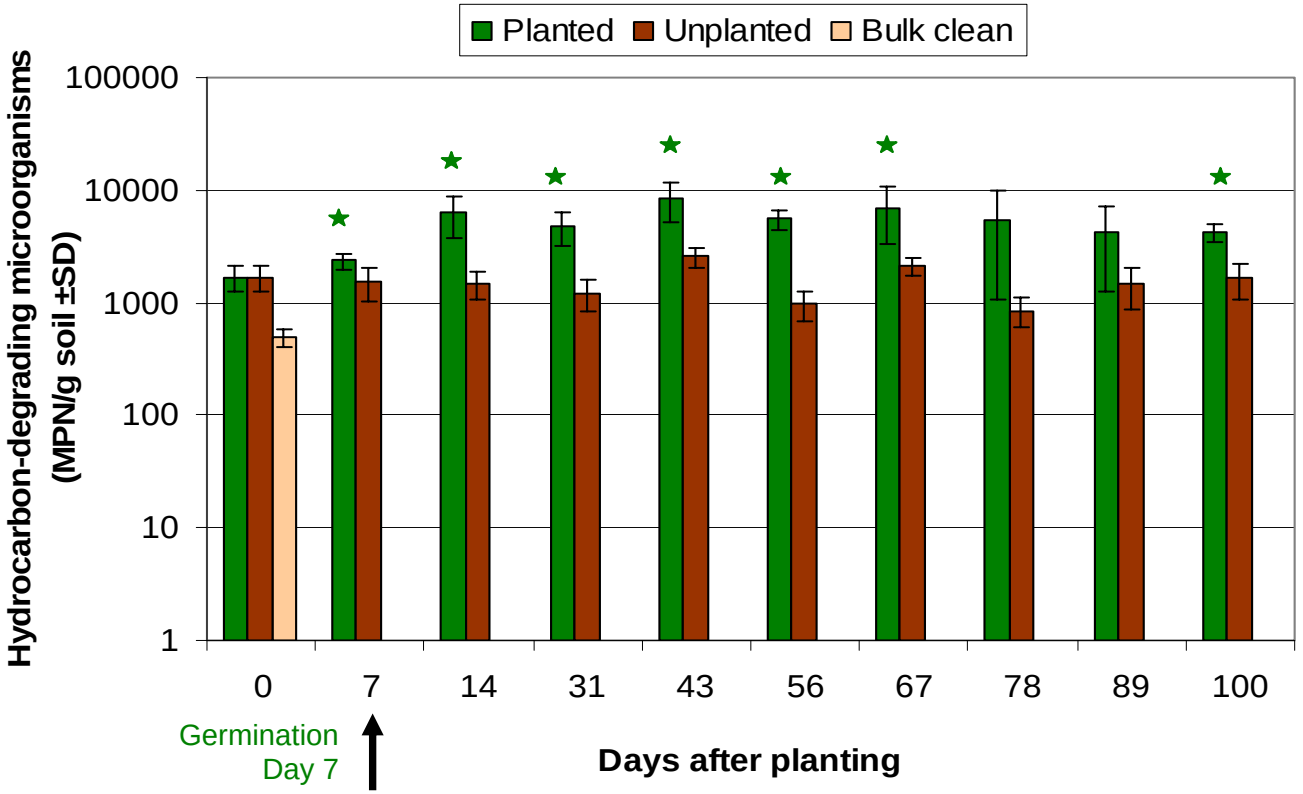


Figure 4.2 Hydrocarbon-degrading microbial populations over time in diesel/oil contaminated soil (1% w/w) from the rhizosphere of *B. decumbens* (planted) and unplanted control as determined by MPN assay. A bulk clean soil control is shown at t = 0 DAP representing background levels. Data are means ± standard deviation (n = 6). * indicates significant difference to unplanted control at $p < 0.05$.

The roots of *C. ambiguus* were also found to increase numbers of hydrocarbon degrading microbial population above numbers measured in unplanted soil (**Figure 4.3**). Initially (t = 0 DAP), when compared to bulk clean soil, numbers of hydrocarbon degrading microorganisms in contaminated planted and unplanted treatments were not significantly different. Predictably, the addition of diesel/oil to the soil significantly increased numbers of hydrocarbon degrading microorganisms one hundred-fold in both the presence and absence of plants after nine days exposure (ANOVA, $p = 0.0001$). In the planted treatment, hydrocarbon-degrader abundance remained two orders of magnitude above background levels (clean bulk soil) for the entire experimental period (ANOVA, $p \leq 0.001$). In unplanted soil numbers of hydrocarbon degrading microorganisms were only significantly greater than background levels until 17 DAP (ANOVA, $p = 0.0001$), after which no significant difference was noted. This can be linked with the pattern of hydrocarbon removal noted for this species (see Section 6.3.1).

Irrespective of background levels, the hydrocarbon degrading microbial population was consistently greater in contaminated planted soil than in unplanted (control) soil by at least one hundred-fold for the duration of the experiment (T-test, $p \leq 0.01$) (exceptions 9 and 28 DAP). This demonstrates *C. ambiguus* significantly stimulated the microbial community in the rhizosphere capable of degrading aliphatic hydrocarbons.

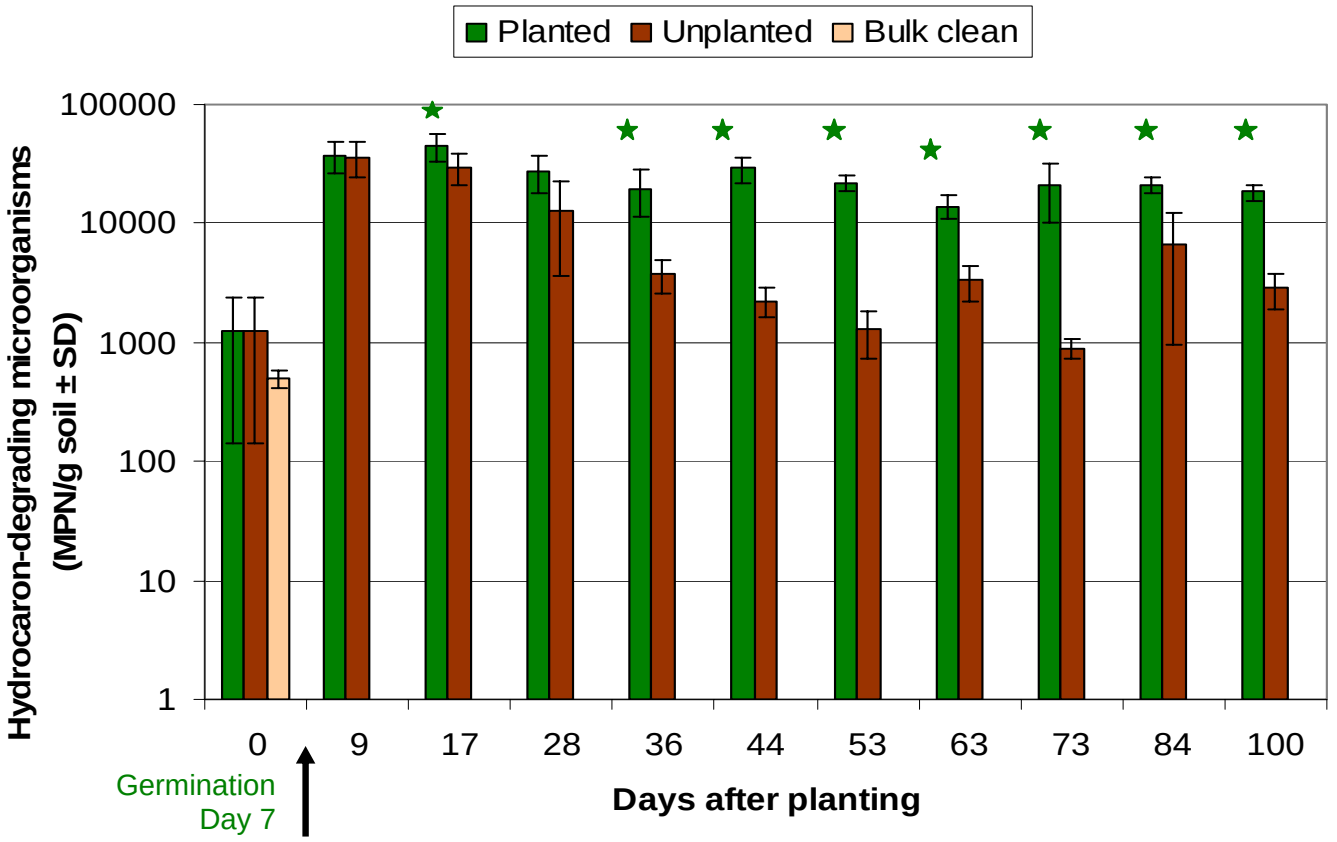


Figure 4.3 Hydrocarbon-degrading microbial populations over time in diesel/oil contaminated soil (1% w/w) from the rhizosphere of *C. ambiguus* (planted) and unplanted control as determined by MPN assay. A bulk clean soil control is shown at t = 0 DAP representing background levels. Data are means ± standard deviation (n = 6). * indicates significant difference to unplanted control at $p \leq 0.01$.

Microbial numbers and activity in the rhizosphere of *M. stipoides* is of particular interest since it is a cool season grass in comparison to the warm season growth of the other two species used in this study (see also Section 4.3.2). This meant that the growth of *M. stipoides* was maintained at an average maximum temperature 10°C cooler than the other two species. Despite the temperature difference, patterns in microbial population growth in the rhizosphere of *M. stipoides* capable of degrading hydrocarbon (**Figure 4.4**) were found to be comparable to the other grasses screened. Initial microbial population sizes (t = 0 DAP) in planted and unplanted contaminated soil were similar to background levels in bulk clean soil. Consistent with the experiment using *B. decumbens*, numbers of hydrocarbon degrading microorganisms in unplanted soil exposed to diesel/oil remained at low background levels for the duration of the test period. That is, the expected increase in hydrocarbon-degrader abundance following exposure to the contaminant was not observed in the unplanted treatments for either trial (but was observed in trial with *C. ambiguus*). In soil planted with *M. stipoides* numbers of hydrocarbon degrading microorganisms remained two orders of magnitude greater than background levels in clean bulk soil for the entire experiment (ANOVA, $p \leq 0.01$).

Of most interest in this quantitative analysis is the comparison of hydrocarbon degrading population growth between planted and unplanted soil exposed to diesel/oil. In this regard, the rhizosphere of *M. stipoides* significantly stimulated the microbial population capable of degrading hydrocarbon by one hundred fold increase relative to unplanted control from the time of germination onward (t = 11 DAP) (T-test, $p \leq 0.01$) (exception 67 DAP). This is consistent with microbial population changes of hydrocarbon degrading microorganisms observed for the other grasses screened.

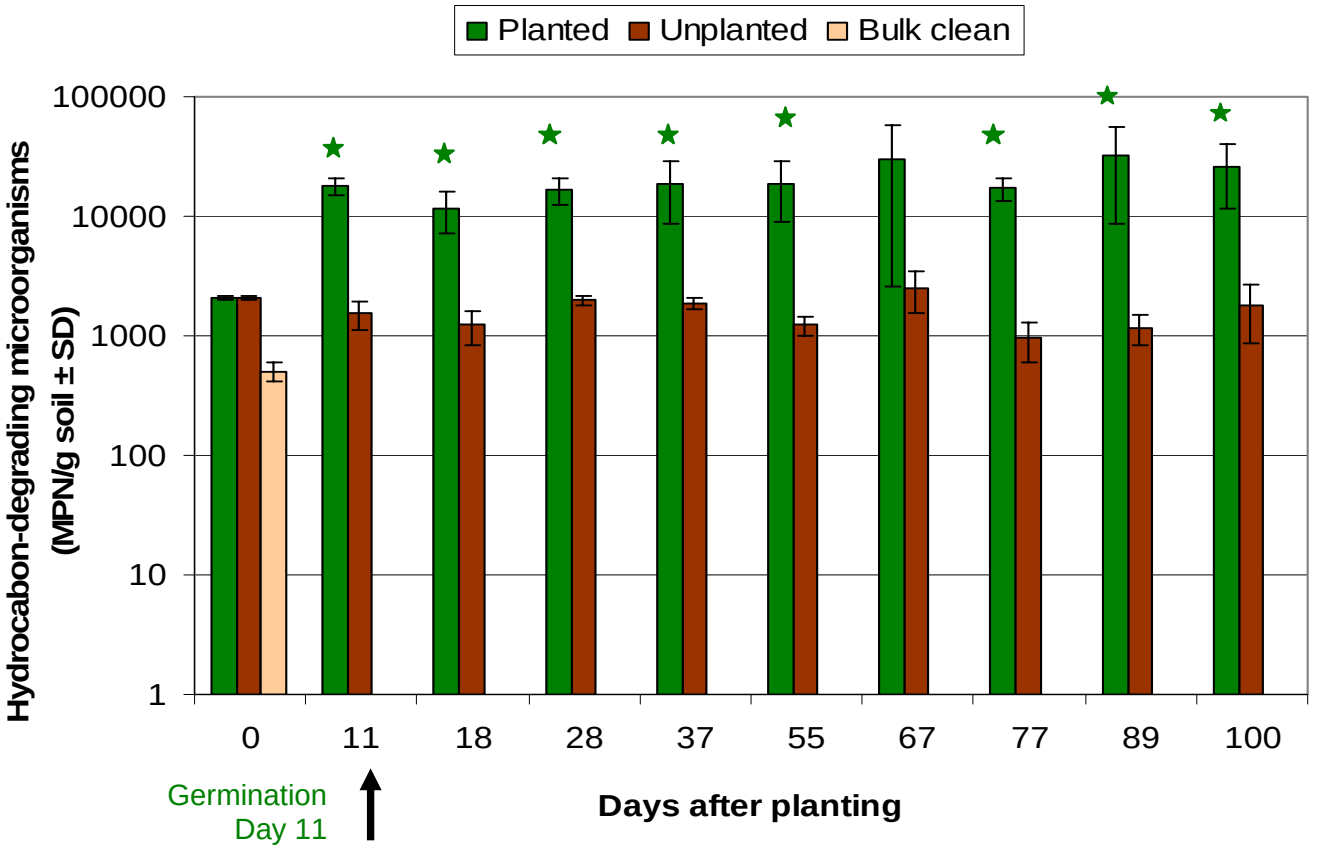


Figure 4.4 Hydrocarbon-degrading microbial populations over time in diesel/oil contaminated soil (1% w/w) from the rhizosphere of *M. stipoides* (planted) and unplanted control as determined by MPN assay. A bulk clean soil control is shown at t = 0 DAP representing background levels. Data are means ± standard deviation (n = 6). * indicates significant difference to unplanted control at $p \leq 0.01$.

4.3.2 Soil lipase activity - a bioindicator to monitor changes in rhizosphere microbial populations during rhizoremediation

Changes in soil lipase activity in the rhizosphere of Australian grasses were consistent with the patterns observed for numbers of hydrocarbon degrading microorganisms (Section 4.3.1), and also hydrocarbon degradation rates (Section 6.3.2). In particular, an increase in soil lipase activity during the initial stage matched the increase in hydrocarbon-degrader abundance for all grass species.

Figure 4.5 presents soil lipase activity over time in rhizosphere and unplanted control treatments of *B. decumbens*. Initial soil lipase activity (t = 0 DAP) in planted and unplanted diesel/oil treatments were similar to those measured in clean bulk soil (representing background level). From point of germination onward (t = 7 DAP), soil lipase activity was consistently greater in the rhizosphere of *B. decumbens* compared with the unplanted control (T-test, $p < 0.05$). For the first 30 days of the experiment, soil lipase activity steadily increased in both planted and unplanted systems. This was followed by a brief plateau in activity in both planted and unplanted treatments. The half way point of the experiment (~50 DAP) corresponded to a decrease in soil lipase activity in planted and unplanted systems, which was followed by a second (and lower) plateau for the remainder of the experiment in both treatments. Final lipase activity (t = 100 DAP) in unplanted soil returned to initial low levels, while in planted soil remained significantly higher than control (T-test, $p = 0.005$) and background levels.

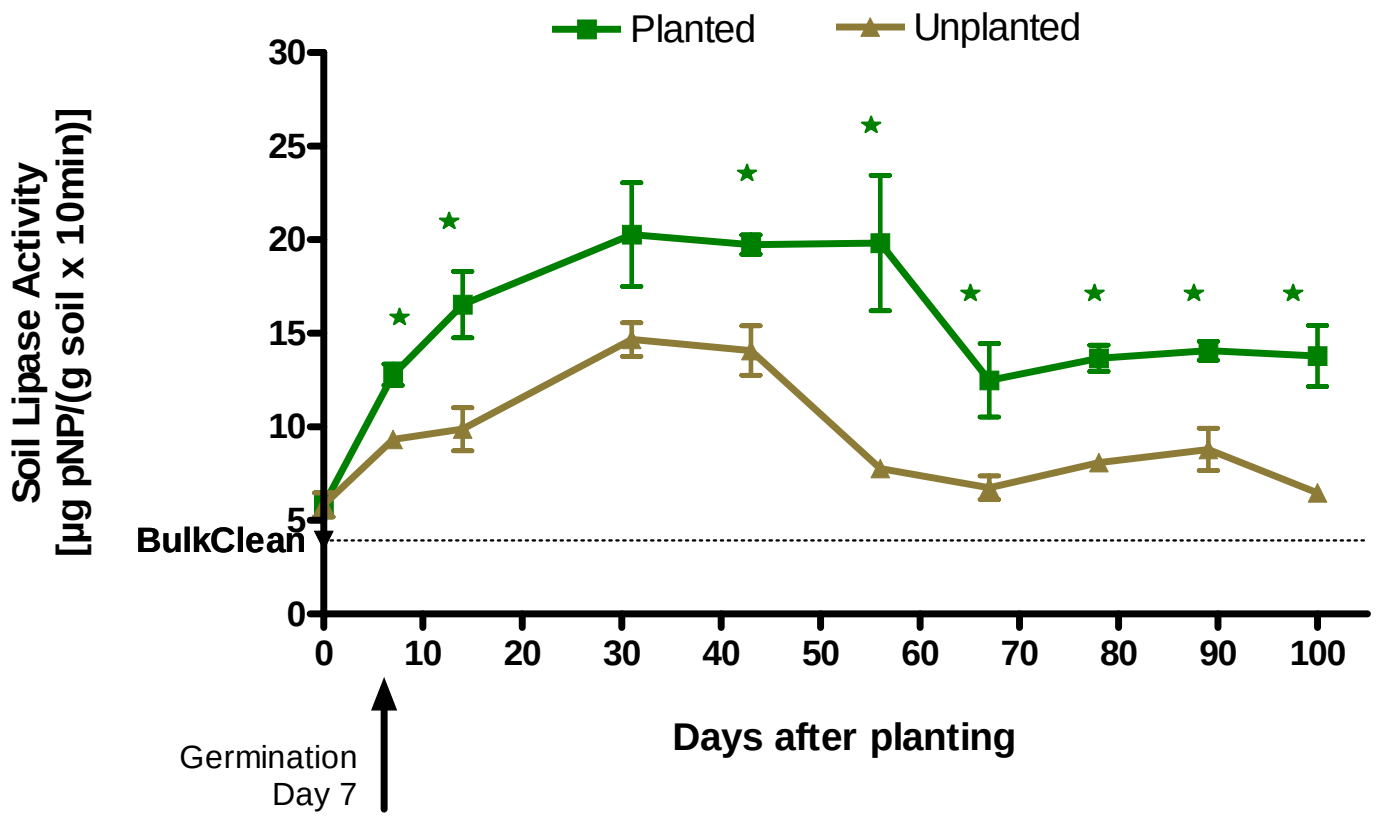


Figure 4.5 Time course lipase activity in diesel/oil (1% w/w) contaminated rhizosphere soil of *B. decumbens* (planted) and unplanted (control) soil. A bulk clean soil control is shown representing background level. Data are means ± standard deviation (n = 4). * indicates significant difference to unplanted control at $p < 0.05$.

Figure 4.6 presents soil lipase activity over time in rhizosphere and unplanted control treatments of *C. ambiguus*. A significant initial increase (over the first 14 days) in soil lipase activity was observed in the rhizosphere of *C. ambiguus* (T-test, $p = 0.009$) relative to the control. This is similar to that observed for *B. decumbens*. The increase in soil lipase activity also coincided with germination of *C. ambiguus* in the planted treatment. Thereafter for this species, soil lipase activity fluctuated in both planted and unplanted treatments until approximately 70 DAP. At this time, a marked and sustained decrease in soil lipase activity was noted in both planted and unplanted treatments. General enzyme activity pattern showed from germination (t = 7 DAP) onward, significantly higher soil lipase activity was measured in the rhizosphere of *C. ambiguus* compared to the unplanted control (T-test, $p < 0.05$) (exceptions 9, 28, 44, 53 DAP).

Changes in soil lipase activity in the rhizosphere of *M. stipoides* (see **Figure 4.7**) were in line with that found for the other species, in particular at the beginning and end of the experimental period. An initial marked increase in soil lipase activity, which corresponded to germination of the grass, was observed. This high lipase activity was generally maintained until 55 DAP, after which a decrease in activity was measured in both planted and unplanted treatments. A plateau in soil enzyme activity was observed for the remainder of the experimental period. From germination (t = 11 DAP) onward, soil lipase activity was consistently higher in the rhizosphere of *M. stipoides* compared with the unplanted control (T-test, $p \leq 0.02$). Final soil lipase activity (t = 100 DAP) in unplanted control returned to background levels (bulk clean), but remained significantly higher in the rhizosphere soil of *M. stipoides* (T-test, $p = 0.014$).

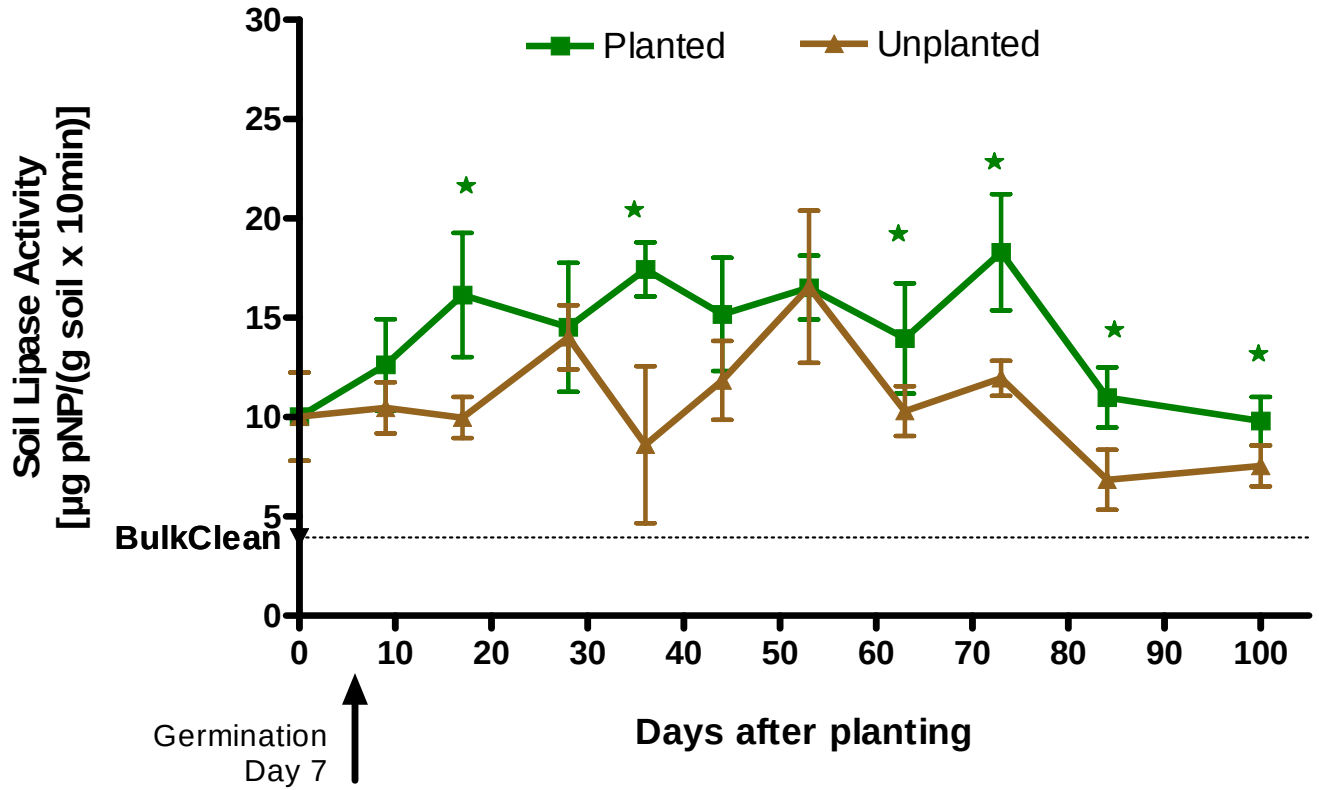


Figure 4.6 Time course lipase activity in diesel/oil (1% w/w) contaminated rhizosphere soil of *C. ambiguus* (planted) and unplanted (control) soil. A bulk clean soil control is shown representing background level. Data are means ± standard deviation (n = 4). * indicates significant difference to unplanted control at $p < 0.05$.

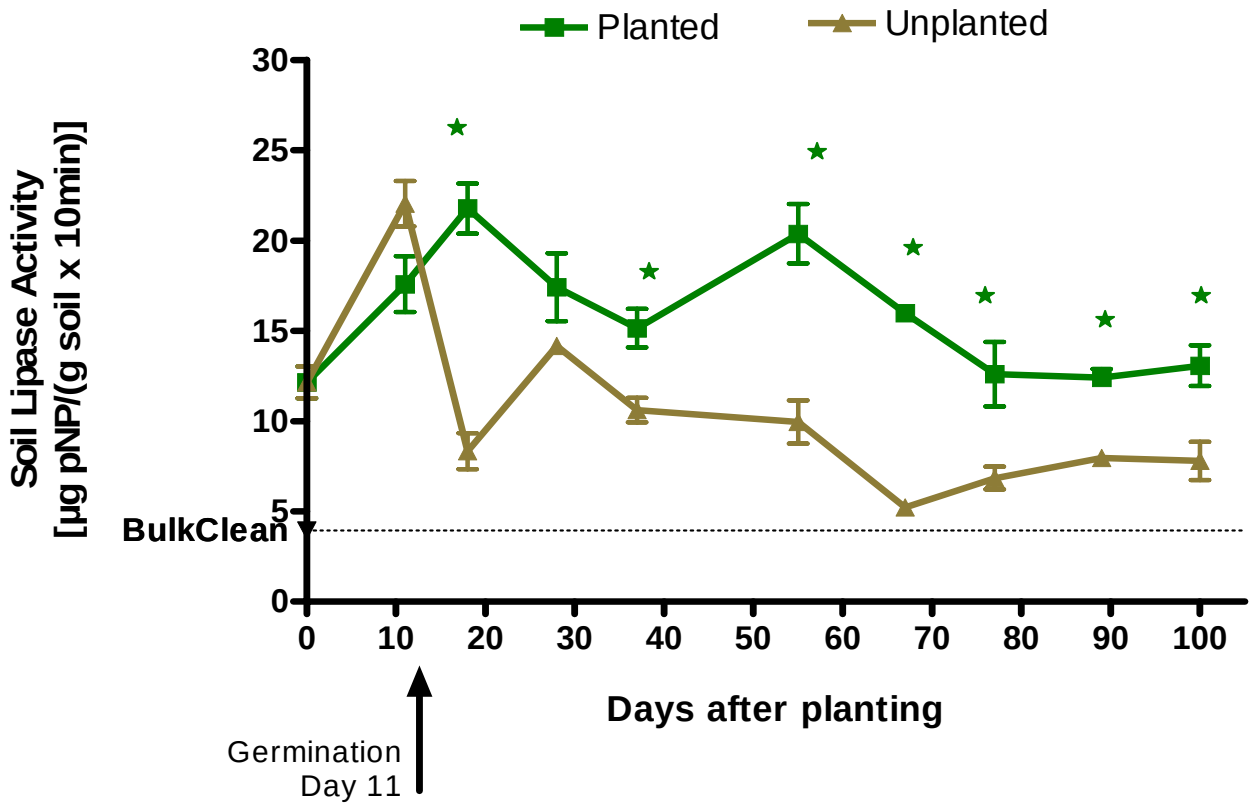


Figure 4.7 Time course lipase activity in diesel/oil (1% w/w) contaminated rhizosphere soil of *M. stipoides* (planted) and unplanted (control) soil. A bulk clean soil control is shown representing background level. Data are means ± standard deviation (n = 4). * indicates significant difference to unplanted control at $p \leq 0.02$.

4.3.3 Comparison between grass species for influence on rhizosphere microbial community

4.3.3.1 Quantitative hydrocarbon-degrading community changes

Overall, selective enrichment of hydrocarbon degrading microorganisms was demonstrated in the rhizosphere soil of all Australian grasses tested (see **Figure 4.8**). Although total abundance of hydrocarbon-degraders in the rhizosphere soil of all species was low (10^4 to 10^5 MPN organisms/g soil), these results were significantly higher (by at least one order of magnitude) relative to the unplanted controls (ANOVA, $p < 0.05$). *C. ambiguus* appeared to have the greatest influence on promotion of hydrocarbon degrading microorganisms, showing higher relative population of organisms over time compared with the other species tested. This was followed by the cool season grass *M. stipoides*, although no statistically significant difference was found between these two species (ANOVA, $p > 0.05$). The only significant difference found between species was for *B. decumbens*, which consistently showed lower abundance of hydrocarbon degrading microorganisms in rhizosphere soil over time than the other two species (ANOVA, $p < 0.01$). The unplanted controls from the three trials were comparable for the majority of the experimental period (ANOVA, $p > 0.05$), and consistently lower than the planted treatments for all species (ANOVA, $p < 0.05$).

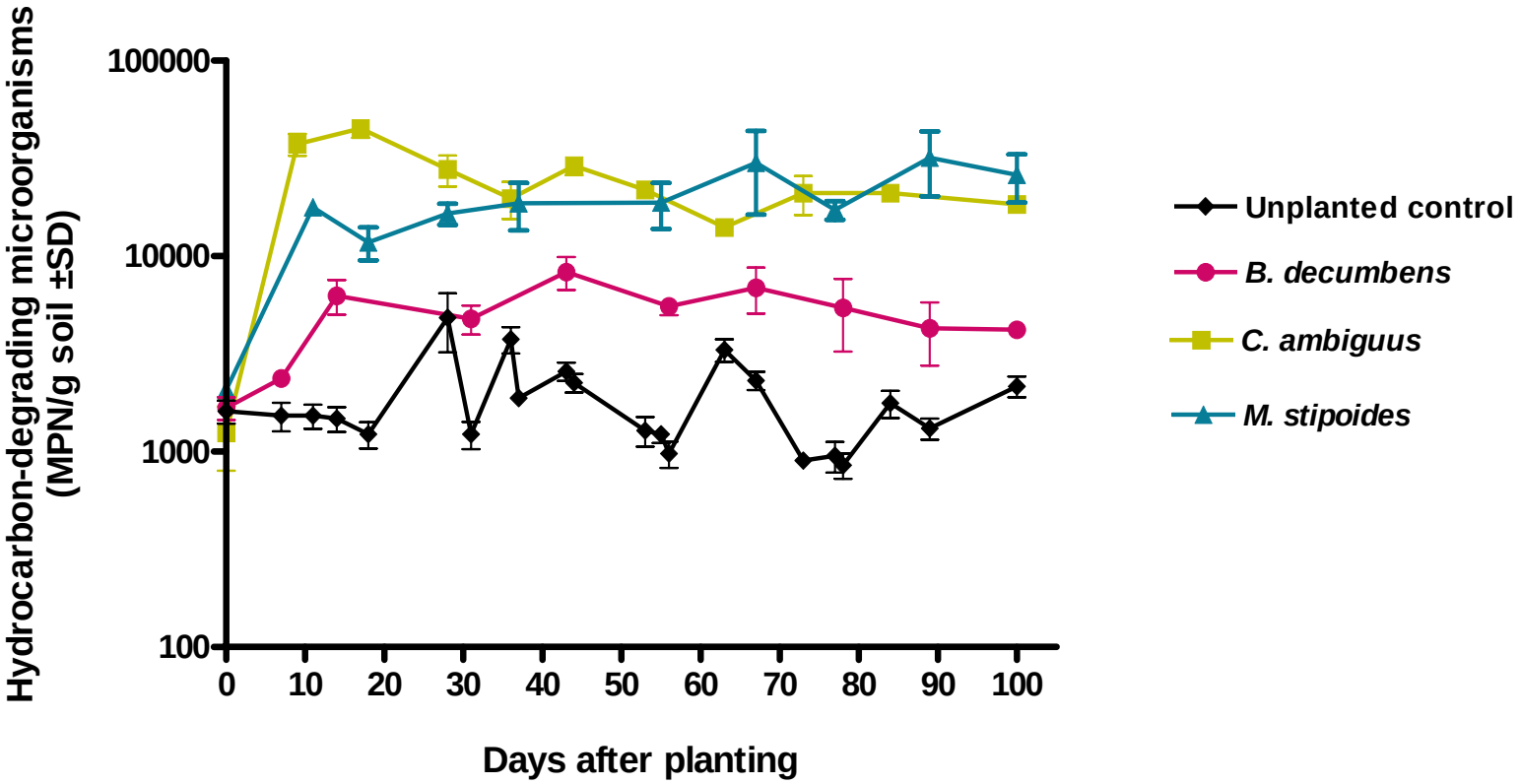


Figure 4.8 Hydrocarbon-degrading microbial populations over time in diesel/oil contaminated soil (1% w/w) from the rhizosphere of Australian grasses (*B. decumbens*, *C. ambiguus* and *M. stipoides*) and unplanted (control) soil. Data are means ± standard deviation (n = 6, except control n = 18 combining data from three trials).

4.3.3.2 Soil lipase activity changes in rhizosphere soil

The presence of Australian grasses significantly stimulated soil lipase activity in the rhizosphere relative to the unplanted control soil (ANOVA, $p \leq 0.01$), and background levels represented by bulk clean soil (ANOVA, $p < 0.01$) (see **Figure 4.9**). When comparing between species for soil lipase activity, no significant difference was observed overall (ANOVA, $p > 0.05$). This pattern of soil lipase activity between species does not match what was predicted, given the variation in hydrocarbon–degrader abundance (see Section 4.3.3.1) and total petroleum hydrocarbon removal (see Section 6.3.1) between species. Expected changes in soil lipase activity were observed at the beginning and end of the experiment for each species. In all planted treatments an initial marked increase in soil lipase activity was measured relative to the control. This also corresponded to increased numbers of hydrocarbon degrading microorganisms at early stage of the experiments for all species (see Figure 4.7 and Section 4.3.2). Towards the end of the experimental period a decrease in soil lipase activity was noted for all species (while abundance of hydrocarbon-degraders held steady).

4.3.3.3 Correlations between MPN hydrocarbon-degraders and lipase activity

Pearson correlations between all parameters for each species are shown in Section 7.3: including plant growth parameters, microbial numbers (MPN), soil lipase activity and total petroleum hydrocarbon concentration. Interactions/relationships between these parameters will be revisited and discussed in Chapter 7.

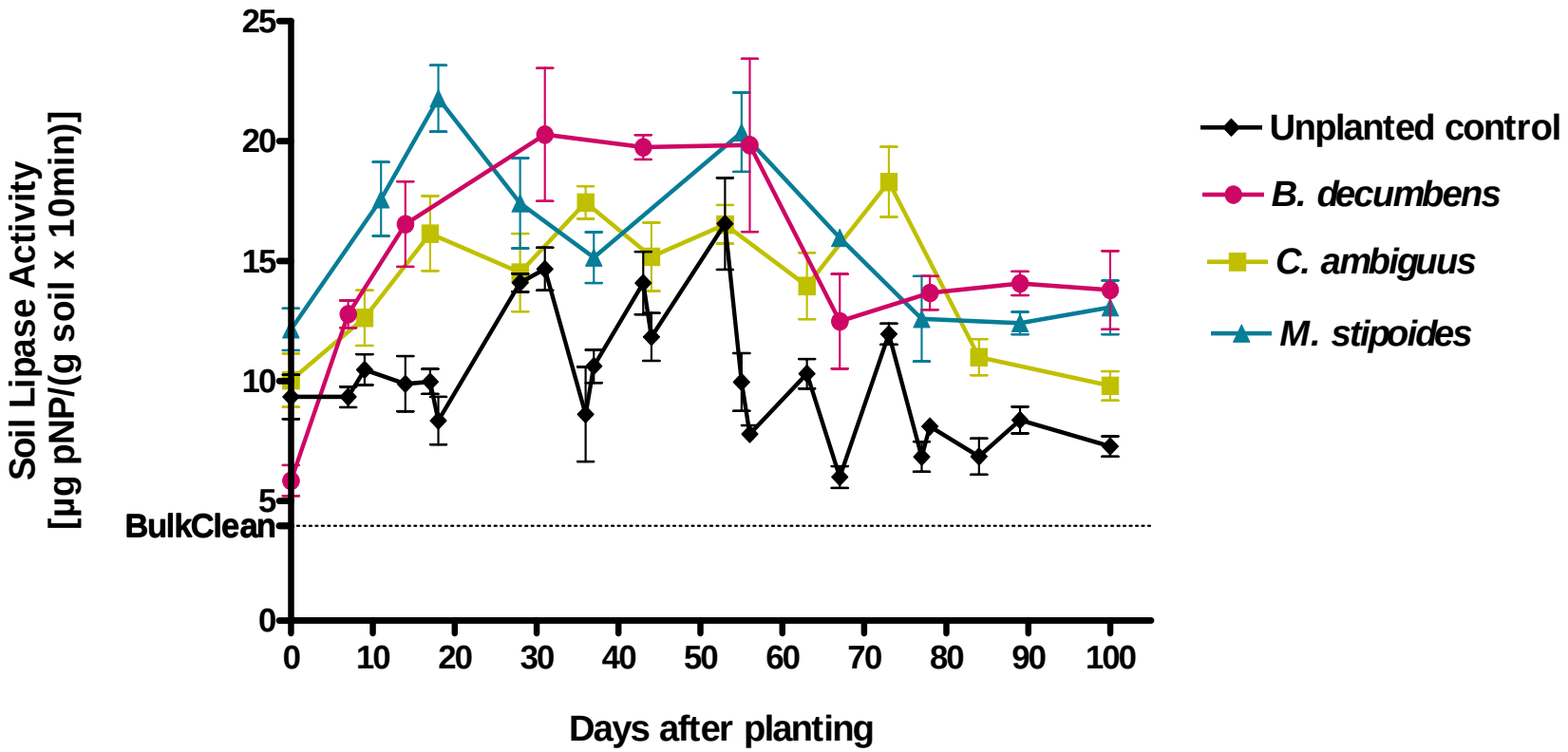


Figure 4.9 Soil lipase activity over time in diesel/oil contaminated soil (1% w/w) from the rhizosphere of Australian grasses (*B. decumbens*, *C. ambiguus* and *M. stipoides*) and unplanted (control) soil. A bulk clean soil control is shown representing background level. Data are means ± standard deviation (n = 4, except control n = 12 combining data from three trials).

4.4 Discussion

This chapter aimed to assess the ability of Australian grasses to stimulate, both quantitatively and metabolically, the rhizosphere microbial community able to degrade aliphatic hydrocarbons in soil. The presence of hydrocarbons in soil has been shown to stimulate microbial populations (Gaskin and Bentham 2005), but plants appear to add additional influence. Plants have been shown to increase the microbial numbers in the rhizosphere, a phenomenon termed the *rhizosphere effect* (Kirk *et al* 2005).

Quantitative changes were measured in rhizosphere microbial communities in response to the planting of different grass species in a soil contaminated with diesel/oil. All grass species were shown to promote the growth of hydrocarbon degrading populations by one order of magnitude or greater than the unplanted controls. This provided evidence for the *rhizosphere effect*, by demonstrating stimulation of microorganisms in the rhizosphere of grasses capable of degrading diesel/oil. The rhizosphere effect observed in the current study appears to be due to a metabolic influence from the plant and not related directly to the presence of hydrocarbon.

Microbial consortia are known to quickly respond to environmental changes by adjusting the community composition, that is, by favoured growth of those members able to survive the modified conditions (Cappello *et al* 2007). The addition of diesel/oil to soil could be predicted to enrich for petroleum degrading microorganisms, usually found in low abundance (Vidali 2001; Kim *et al* 2006). This was demonstrated in the current study in some cases. For two of the three trials (*B. decumbens* and *M. stipoides*), the addition of diesel/oil to unplanted soil did not correspond to significant increase in hydrocarbon degrading microorganism numbers relative to background levels in clean soil. Whereas, in diesel/oil soil treatments planted with a grass, significant selective enrichment of hydrocarbon degrading microorganisms was measured relative to unplanted and background numbers in all cases. This suggests the presence of a grass species had additional influence on the quantitative changes in the soil microbial

community capable of degrading hydrocarbon. It appears plant roots provide degrading organisms with a variety of conditions that help to selectively support their growth in this environment. The presence of vegetation may affect many physical conditions in the soil, including structure, porosity, hydraulic conductivity and infiltration rate (Hutchinson *et al* 2003). These properties, in turn, influence the microbial activity by regulating the transport of required water and nutrients through the soil profile and by controlling soil aeration. Potential nutrients reported as supplied by plant roots include amino acids, organic acids, carbohydrates, growth factors, and soluble proteins (Miya and Firestone 2000; Lee *et al* 2008). For example, in the nitrogen limited soil used in the current study (see Section 2.3.1), such root exudates may have been provided by the grasses and used by the hydrocarbon degrading microorganisms as a source of N.

Although stimulation of hydrocarbon degrading microorganisms was noted in the presence of all grasses, the degree of influence on microbial abundance between species was not equal. Species-specific responses were noted, and comparative estimates show *C. ambiguus* and *M. stipoides* had the greatest influence on the promotion of hydrocarbon degrading microorganism numbers in the rhizosphere. For all species, a significant rapid increase in abundance of hydrocarbon degrading microorganisms was noted in the first ten days, which is indicative of a stimulated biodegradation process (see Section 6.3.1). Thereafter, the hydrocarbon degrading population in the rhizosphere of grasses remained relatively unchanged for the duration of the experiment, i.e. reaching steady-state growth. Changing stages of plant growth over time did not appear to affect the rhizosphere hydrocarbon degrading populations. These results indicate that the microbial community was not reproducing, but had reached stationary growth phase. The resulting removal rates of diesel/oil from the soil could perhaps then be attributed to the metabolic activity of the stable population size, where for example, no further increase in microbial numbers was noted but more of the existing population became metabolically active and thus higher hydrocarbon removal rates are seen. Notably, metabolic activity in the soil was comparable where removal rates were varied (see further discussion below).

Selective enrichment of hydrocarbon degrading microorganisms in the rhizosphere environment as demonstrated for the Australian grasses is not a unique finding. Plant-specific alterations to the rhizosphere microbial community as observed in the current study have been reported elsewhere in the literature (see Reynolds *et al* 1999; Kirk *et al* 2005). Grasses in particular have been shown to exhibit a rhizosphere effect (increased microbial numbers) in the presence of hydrocarbon contamination (Miya and Firestone 2000; Kirk *et al* 2005; Merkl *et al* 2006).

Miya and Firestone (2000) found the rhizosphere of a common annual grass in the United States slender oat (*Avena barbata*) selectively enriched the phenanthrene degrading population over time by as much as an order of magnitude compared with unvegetated soil. Interestingly, the greatest enrichment was observed during the latter stages of the experiment (between 24 and 32 DAP). This is in contrast to the current study, where the greatest enrichment in the rhizosphere occurred in the initial ten days. This may be due to the more bioavailable composition of aliphatic hydrocarbons such as diesel fuel and engine oils compared with the less bioavailable polycyclic aromatic hydrocarbon phenanthrene causing longer lag phases in microbial growth. In addition, soil type and organic content can influence contaminant bioavailability through adsorption capacity etc, and the sandy loam mine site soil with its low organic content used in the current study would allow for high bioavailability (Germida *et al* 2002).

Similarly, Kirk *et al* (2005) reported increased diesel degrading bacteria in the rhizosphere of the perennial ryegrass *Lolium perenne*. The authors used an MPN method to estimate bacteria capable of degrading petroleum hydrocarbons including diesel. Ryegrass altered the rhizosphere by supporting 37 times more petroleum degrading bacteria after seven weeks compared to the unplanted soil. A comparable hydrocarbon degrading population enhancement was noted in the current study for the Australian grasses. All grasses supported at least an order of magnitude higher numbers of hydrocarbon degrading microorganisms in the rhizosphere relative to the unplanted soil. Shifts in microbial population in the rhizosphere of ryegrass, as reported by Kirk *et al* (2005), were more gradual over

time compared with results found for grasses in the current study. Again, this could be ascribed to the weathered hydrocarbon contamination of the soil used in the ryegrass report, affecting the bioavailability of the pollutant. Temperature is also an important environmental factor affecting microbial growth. The experimental temperature employed in the current study for the growth of Australian grasses was more than 5°C warmer than that used to grow ryegrass by Kirk *et al* (2005). This may account for the more rapid growth of the microbial population observed in the rhizosphere of Australian grasses.

Recently, Merkl *et al* (2006) assessed the Venezuelan tropical grass *Brachiaria brizantha* for its influence on microbial population and degradation activity in petroleum contaminated soil. The authors reported after 22 weeks the presence of the plant had a clear positive effect on growth of oil and alkane degrading microorganisms, but for aromatic and cycloalkane degrading microorganisms no difference was found to unplanted control. This is particularly interesting since the Australian grass *Brachiaria decumbens* used in the current study showed significant stimulation of the aliphatic- (diesel/oil) degrading microbial community relative to unplanted controls, a comparable result with that observed by Merkl *et al* (2006).

Overall, the Australian grasses used in the current study demonstrated stimulation of hydrocarbon degrading microorganisms in the rhizosphere (relative to unplanted controls). This was reflected in lower residual hydrocarbon concentrations (see Section 6.3.1).

Soil biological investigations, such as microbial counts and enzyme activities, provide information on the presence and activity of viable microorganisms and on the impact of the effects of environmental stress, such as contamination, on the metabolic activity of soil (Margesin *et al* 2007). In order for optimal rhizoremediation to occur, soil environmental conditions should permit microbial growth as well as activity of specific pollutant degrading communities (Vidali 2001). Changes in nutrient availability to below critical levels can create conditions not energetically viable for microorganisms to continue reproduction or metabolic activity and consequently arrest the biodegradation process. Soil

enzyme activities (e.g. dehydrogenase, catalase or phosphatase) have been shown to increase significantly after contamination, but decrease after a certain (enzyme-specific) period (Margesin *et al* 2002). Contrary to this decline following the most active phase of oil biodegradation, lipase activity tends to remain at an elevated level for a long period of time (Lee *et al* 2008). The increase of lipase activity after oil contamination may be attributed to the formation of products released during the hydrocarbon biodegradation process. Dehydrogenases, catalases and urease have been found only to be useful for indicating the onset of biodegradation process, as their activities decline rapidly after the rate of biodegradation has decreased (Margesin *et al* 1999; Maila and Cloete 2005).

In the current study, after an initial increase in the number of hydrocarbon degrading microorganisms an equilibrium in microbial abundance was observed for all species. However metabolic activity (soil lipase) continued to fluctuate throughout the experimental period. There are four possible explanations for this observation. First, soil conditions (nutrient) no longer permitted the proliferation of the hydrocarbon degrading microorganisms, but supported the metabolic activity of the existing population. Second, lipase (which degrades lipids in glycerine and fatty acids) is produced by a large variety of microorganisms including hydrocarbon degrading microorganisms (Riffaldi *et al* 2006), and assay results would encompasses total microbial load in terms of its metabolic activity not just hydrocarbon degrading organisms. Third, changes in plant activity with shifting growth phase (such as exudation patterns) may have influenced the metabolic activity of the rhizosphere microbial population. Finally, changes in enzyme activity may be a response to the depletion of short-chain aliphatics and then degradation of long-chain more hydrophobic compounds. The lipase assay may also be sensitive to other non-specific esterase enzymes. These explanations would account for the fluctuating enzyme activities in the rhizosphere and little difference in levels between species, despite steady-state being reached in terms of both microbial numbers and hydrocarbon concentration (see also Section 6.3.1). Further, it has been suggested that most enzymatic tests are artificial and refer to the potential activity of soil enzymes (Maila and Cloete 2005).

Margesin *et al* (1999; 2000; 2007) demonstrated that measuring lipase activity is a valuable tool for monitoring oil biodegradation in freshly diesel oil contaminated soil. The residual hydrocarbon content correlated significantly negatively with soil lipase activity independent of fertiliser addition. The authors reported in all studies that soil lipase activity increased with time (and decreasing residual hydrocarbon concentration) and remained level after reaching its maximum activity. Maximum activity was reached rapidly within the first two weeks of experiment (Margesin *et al* 1999; 2000), and decreased in the presence of low hydrocarbon concentrations (2 500 – 5 000 mg TPH/kg soil) but remained almost unchanged in highly contaminated soil (10 000 – 20 000 mg TPH/kg soil) (Margesin *et al* 2007). The induction of enzyme activity was attributed to the appearance of products released from hydrocarbon biodegradation, which are the substrate for hydrolases including esterases-lipases.

Recently, Lee *et al* (2008) reported fluctuating lipase activity during a 105 day experiment assessing soil microbial activity and the effect of various amendments on heavy mineral oil bioremediation. The authors noted a marked increase after 30 days in amended soils, followed by a sharp decrease after 45 days. Lipase activity remained mostly unchanged in non-amended soils during the experiment. As with previous studies, Lee *et al* (2008) reported lipase activity was strongly negatively correlated to remaining hydrocarbon, particularly saturated and aromatic fractions. Of particular interest, in hay-amended soil a low rate of hydrocarbon degradation was recorded despite high levels of lipase activity. The authors attributed this to plant-residue derived lipid compounds rather than mineral hydrocarbons being preferentially used by soil microorganisms in this treatment. Naturally occurring lipids and various anthropogenic lipids are degraded by soil microorganisms, this process is closely related to soil lipase (Lee *et al* 2008). A similar phenomenon could account for results found for the Australian grass *M. stipoides* in the current study, where high lipase activity was recorded that did not correlate to hydrocarbon degradation directly (see further discussion in Chapter 7).

Riffaldi *et al* (2006) also found lipase activity to be the most valuable biological parameter for testing hydrocarbon degradation in soil. A strong negative

correlation between residual hydrocarbon and soil lipase activity after 100 days was reported. The authors also reported a general increase in lipase activity in soil over time for all treatments (contaminated, uncontaminated, and with/without fertiliser). Interestingly, the pattern of lipase activity and in particular the reaching of maximum values revealed differences with Margesin *et al* (1999; 2000). Riffaldi *et al* (2006) reported an initial stasis (10-35 days), followed by a sharp increase on the 55th day, and a consequent stabilisation towards constant values. The authors presumed the sharp increase was attributable to a late response by the microbial community after a period of stress following addition of hydrocarbons.

It is important to note that the reports of Margesin *et al* (1999; 2000) and Riffaldi *et al* (2006) were conducted in the absence of plants. This makes comparison with lipase activities found in the current study difficult. If comparison of these previous studies is made with the unplanted control in the current study, the patterns appear to match that found by Riffaldi *et al* (2006). This is not surprising as the unplanted control in the current study mimics the bioremediation conditions reported by Riffaldi *et al* (2006). As shown in Figure 4.8 in this chapter, lipase activity in the unplanted control reveals an initial stasis (20 days), followed by fluctuating peaks in activity, and a consequent stabilisation towards constant values in the final 30 days (similar to that found by Riffaldi *et al* 2006). This is in contrast to the lipase activity patterns observed in grass planted treatments, in particular during early stages of the experiment. Significant initial increases were observed in rhizosphere soil of all grass treatments during the first 20 days, similar to that observed by Margesin *et al* (1999; 2000). Thereafter, lipase activity patterns appear to match that of the unplanted control, and those reported elsewhere; namely, fluctuation in lipase activity and consequent stabilisation towards constant values towards the end of the experimental period. Further, it is important to highlight that in all grass planted treatments, lipase activity was consistently greater than in unplanted controls irrespective of pattern of activity over time. This suggests that the presence of Australian grasses had a significant stimulatory effect on the metabolic activity of the microbial community in the mine site soil. Variation between species in terms of their individual influence is discussed later in Chapter 7.

To the author's knowledge, this is the first report in hydrocarbon remediation studies of stimulated soil lipase activity enhanced in the presence of a plant rhizosphere. This outcome did not necessarily subsequently correlate to enhanced hydrocarbon biodegradation (see further discussion in Sections 6.3 and 7.3). Bioremediation studies as discussed above have shown soil lipase activity to be a useful indicator to monitor changes in microbial populations caused by petroleum hydrocarbons (Margesin *et al* 1999; 2000; Riffaldi *et al* 2006; Lee *et al* 2008). It has subsequently been proposed as a useful bioindicator of hydrocarbon biodegradation. It appears findings in the current study highlighted the weakness of the lipase assay in terms of predicting hydrocarbon removal when plants were present. Enumeration of hydrocarbon degrading microbial abundance appears to be a more accurate indicator of remediation potential. In rhizoremediation the plant adds substrates to the soil that influence the microbial population, whereas in bioremediation studies the hydrocarbon is sole substrate and stimulant of microbial activity.

4.5 Conclusion

The Australian grasses assessed in the current study altered the microbial community i.e. caused plant-induced changes in the soil microbial abundance and activity. These changes were species-specific and could contribute toward biodegradation of petroleum hydrocarbons in contaminated soil. It appears changes in microbial numbers and lipase activity are indicative of a stimulated process, but do not necessarily represent an accurate measurement of the actual biodegradation, nor the species-specific influence.

CHAPTER FIVE

5 MOLECULAR ECOLOGY: DNA FINGERPRINTS TO INVESTIGATE SOIL MICROBIAL COMMUNITY STRUCTURE

5.1 Introduction

The study of microbial diversity and community dynamics is rapidly growing in microbial ecology. Interest in this area has been catalysed by the rapid advancement of molecular ecology methodologies. In particular, analysis of microbial communities that take part in hydrocarbon biodegradation activities has been a focus (MacNaughton *et al* 1999). The use of nucleic acid based molecular techniques for fingerprinting the 16S ribosomal DNA (rDNA) component of microbial cells is a powerful technique for elucidating shifts in microbial ecology. It has been applied to assess the impacts of actively bioremediating (MacNaughton *et al* 1999; Kasai *et al* 2005; Labbe *et al* 2007) and phytoremediating microbial communities (Chen and Banks 2004; Kirk *et al* 2005).

Microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilising and surviving toxic contamination. As a result, these communities are typically less diverse than those in non-stressed systems (Kirk *et al* 2005), although diversity may be influenced by the length of time the population has been exposed. The shift in such a microbial community structure in response to a contaminant can be monitored in greater detail using Denaturing Gradient Gel Electrophoresis (DGGE) – a general community-based approach (Labbe *et al* 2007).

A community-based approach to investigations of soil microbial structure can give useful information on diversity and influence of environmental conditions (e.g. presence of pollutants) on the microbial community as a whole. It does not provide information on functionality of specific members within the community or allow easy elucidation of species. Functionality of specific members of the

microbial community (e.g. hydrocarbon degrading microorganisms) was assessed as presented in Chapter 4. In this regard, the microbial community in the rhizosphere of Australian grasses was assessed over time both numerically (using MPN, Section 4.3.1) and metabolically (using enzyme activity, Section 4.3.2). By also examining the influence of hydrocarbon contamination on the structure of the whole microbial community in the rhizosphere (using DGGE), a valuable insight can be gained into the role of different species and the influence on microbial populations in contaminated soil. DNA profiling also provides a more complete picture of microbial ecology than culture based methods which may only show ~1% of a soil bacterial community (Muyzer *et al* 1993; Ogram 2000).

DGGE has been increasingly used as a tool for exploring microbial community structure in the rhizosphere (Smalla *et al* 2001; Costa *et al* 2005). In particular, the technique has elucidated valuable information on plant-dependent shifts in rhizosphere microbial composition including seasonal shifts and relative abundance of microorganisms in the rhizosphere (the *rhizosphere effect*) (Smalla *et al* 2001). DGGE analysis reveals the biocomplexity of a habitat with respect to a specific region of ribosomal DNA. The 16S rDNA is a highly conserved region of ribosomal DNA which is present in all bacteria, but which has species level sequence differences (Ogram 2000). Primers can also be used to target eukaryotes or 18S rDNA for fungi. DGGE has numerous advantages including reproducibility, high sensitivity, and the ability to detect many species simultaneously (Kirk *et al* 2004). The partial community level fingerprints derived from DGGE banding patterns have been analysed for diversity studies based on the number and intensity of DNA bands as well as similarity between treatments (Kirk *et al* 2004).

This chapter outlines the application of DGGE to determine the influence of grasses on microbial community structure (defined as community DNA fingerprint) in diesel/oil contaminated soil. The hypothesis was that the presence of native grasses alters the microbial community structure in the rhizosphere of contaminated soil, and that the extent of this effect is dependent on grass species.

5.2 Materials and methods

5.2.1 DNA extraction from soil

Rhizosphere and unplanted (control) diesel/oil contaminated soil samples were collected as outlined in Section 4.2.1. Soil samples from three time points for each species were used for molecular analysis: *B. decumbens* 7, 56 and 100 days after planting (DAP), *C. ambiguus* 9, 53 and 100 DAP, and *M. stipoides* 11, 55 and 100 DAP. For each species and treatment and control, a 500 mg soil sample was used for molecular analysis. DNA was extracted directly from rhizosphere and unplanted soil samples using the BIO 101 FastDNA® SPIN Kit for Soil according to the manufacturer's instructions (Qbiogene, Carlsbad, USA). A bead beater (Bio101 Fast Prep, Holbrook, USA) was used for 30 sec at speed setting 5.5 (m/s) instead of shaking for ten minutes.

5.2.2 PCR

Bacterial 16S rDNA was amplified using the primer pair 341F, 5'-GC clamp-CCTACGGGAGGCAGCAG-3' and 534R, 5'-ATTACCGCGGCTGCTGG-3' (Invitrogen, Carlsbad, USA). These primers have been used previously for bacterial community analysis using DGGE (Kirk *et al* 2005; MacNaughton *et al* 1999). A 40 bp GC clamp at the 5' end of the forward primer (341F) had the sequence: 5'-GGA GCC CGG CGA CCG GCG CGC GCG GCG GCG GCA CGG GGG C. The GC clamp ensures the DNA fragment is not completely separated into single strands which would migrate through the gel too rapidly compared with non-denatured DNA (Kirk *et al* 2005).

For PCR, 2 µL of extracted DNA was added to 23 µL of PCR reaction master mix composed of 16.4 µL ultra-pure PCR water (MOBIO, Carlsbad, USA) 2.5 µL PCR-buffer (10X) (Fisher Scientific, Suwanee, USA), 2.0 µL $MgCl_2$ (25 mM) solution (Fisher Scientific, Suwanee, USA), 0.4 µL dNTPs (2 mM per base), 0.8 µL of each primer (500 µM) (Invitrogen, Carlsbad, USA), and 0.1 µL Taq polymerase (5 U/µL) (Qbiogene, Illkirch, France). For each PCR reaction a negative control (ultra-pure DNA-free water) and a positive marker (standard bacterial mix) was prepared. The standard positive marker consisted of a mixture

of pure culture DNA extracts of *Pseudomonas fluorescens*, *Bacillus amyloliquifaciens* and *Bacillus subtilis*.

DNA was amplified in an Eppendorf Mastercycler (Biolab, Victoria, Australia) using the following program: denaturation for 4 min at 94°C, and 40 cycles of primer annealing and extension (94°C for 30 s, 63°C for 1 min and 72°C for 30 s) followed by 10 min at 72°C for final primer extension. Reaction mixtures where then cooled and held at 4°C. Successful amplification was verified by electrophoresis in 1.8% (w/v) agarose gels with GelRed™ (Biotium, Hayward, USA).

5.2.3 DGGE

DGGE polyacrylamide gels were used with a denaturing gradient of 35% to 55% (also 55% to 80% was used and showed poor resolution) with 100% of denaturant corresponding to 40 mL of formamide and 42 g urea in 100 mL. The cast gels were allowed to polymerise overnight. DGGE was performed using 20 µL of PCR product in 1X TAE buffer at 60°C, at a constant voltage of 150 V for five hours (BIO-RAD Dcode™ systems, München, Germany). Eighteen samples and two standard bacterial mixes were loaded per DGGE gel. The standard consisted of a mixture of pure culture DNA extracts of *Pseudomonas fluorescens*, *Bacillus amyloliquifaciens* and *Bacillus subtilis*.

After electrophoresis, the DGGE gels were stained for 30 minutes in the dark with *SYBR Gold* (Invitrogen, Carlsbad, USA). Gels were then photographed on a UV dark table using video imaging.

5.3 Results

Successful amplification was achieved for the 16S rDNA region of soil microbial population obtained from the rhizosphere of Australian grasses and unplanted controls. DNA extracted from all soils gave the expected PCR product for the bacterial primers with GC-clamp. Visualisation by agarose gel electrophoresis of the successfully amplified region of DNA is shown in **Figure 5.1** confirmed by the presence of a PCR product.

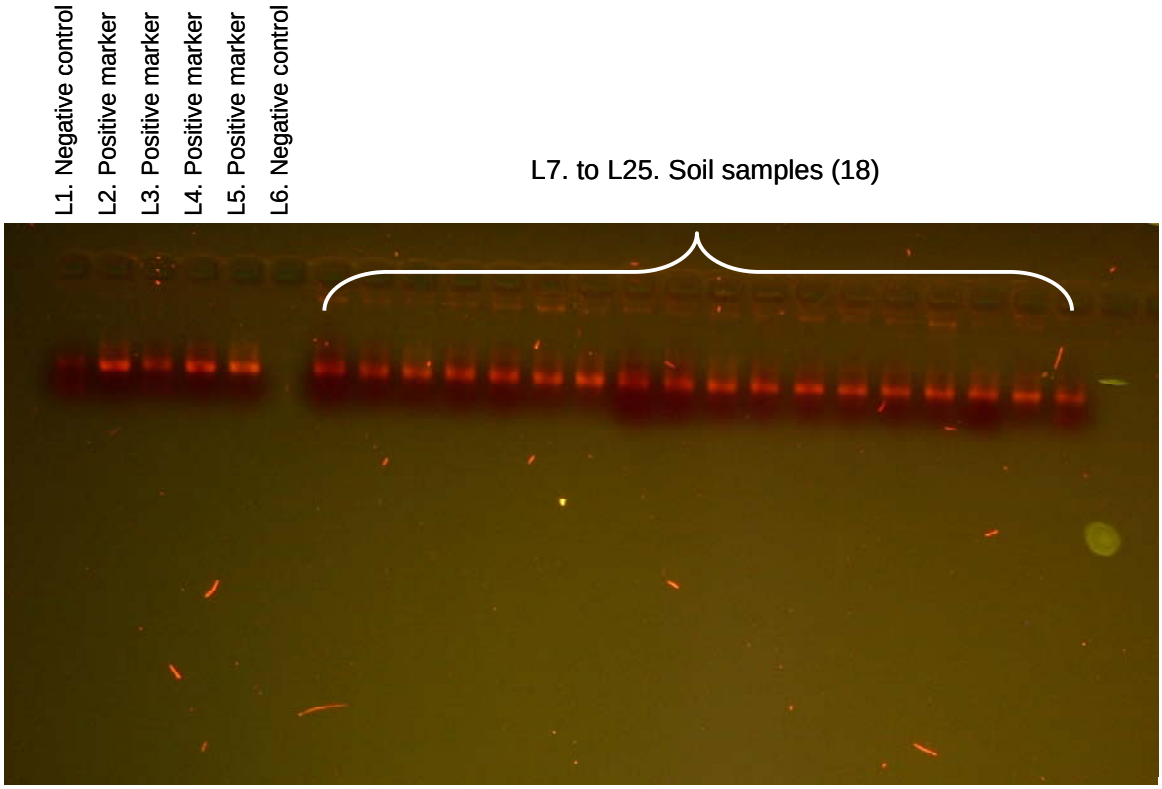


Figure 5.1 PCR amplification of 16S rDNA of soil samples obtained from rhizosphere of grasses and unplanted controls (Lane 7 to Lane 25). All soil samples show positive PCR product. Negative PCR controls (Lane 1 and Lane 6) and positive markers (standard bacterial mix) (Lane 2 to Lane 5) are also shown to confirm PCR success.

Phylogenetic comparison of planted and unplanted diesel/oil contaminated soils, using DGGE fingerprinting of PCR-amplified 16S rDNA genes, was used to describe the different microbial communities. Separation of the PCR product by DGGE to assess banding patterns which reflect the microbial diversity present in the samples proved difficult. Limited success was achieved for the DGGE profiles of rhizosphere and unplanted control soils. **Figure 5.2** shows representative DGGE profiles for soil microbial communities in the rhizosphere of Australian grass species and unplanted controls in diesel/oil contaminated soil over three time points.

In general the DGGE profiles from planted soils showed similar band profiles to their corresponding unplanted soils, and were predominantly characterised by a number of faint bands. For example, a clear band was evident in the profile of *C. ambiguus* planting at 9 DAP (lane 9) which was also found in the unplanted treatment of the same trial at 53 DAP (lane 12). Similarly, the planted (lane 2) and unplanted (lane 3) profiles of *M. stipoides* at 11 DAP show very similar banding patterns. The appearance of blurred or smeared bands was evident in all species banding profiles. Some clear bands were observed in treatments across different grass species. For example, a dominant band in the planted *B. decumbens* profile at 56 DAP (lane 17) was also seen in the unplanted control at 100 DAP in the *M. stipoides* trial (lane 7), although not seen elsewhere within each species profiles.

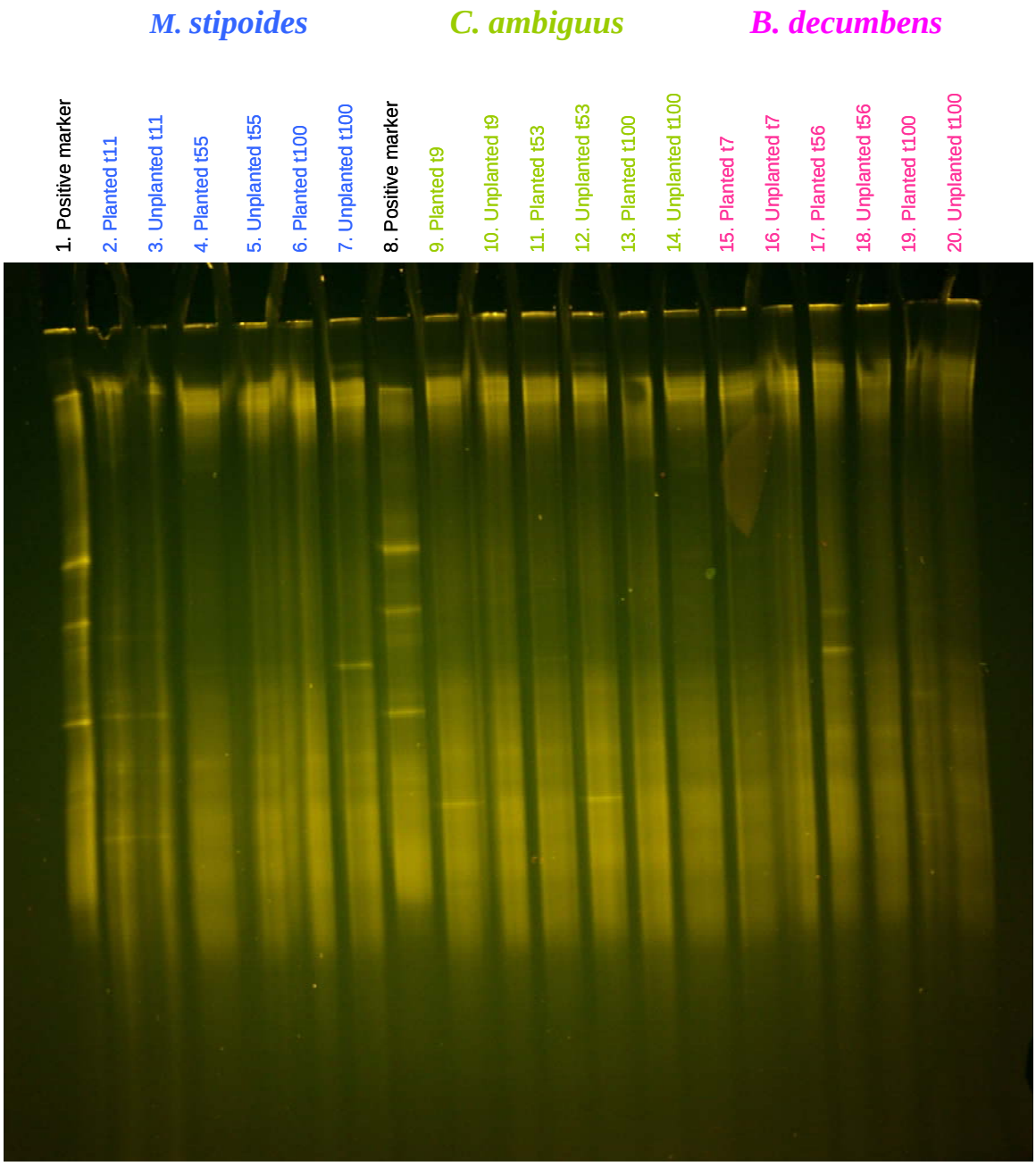


Figure 5.2 Comparison of DGGE profiles of PCR-amplified 16S rDNA gene fragments from rhizosphere (planted) and unplanted diesel/oil contaminated soils over time. Lanes: 1 and 8, positive markers (standard bacterial mix); 2 to 7, *M. stipoides* at time 11, 55 and 100 days after planting (DAP); 9 to 14, *C. ambiguus* at 9, 53 and 100 DAP; 15 to 20, *B. decumbens* at 7, 56 and 100 DAP.

5.4 Discussion

Results obtained from DGGE analysis do not conclusively indicate that the grasses altered the composition of the soil microbial community. A possible inference from this could be that the native grasses did not change community structure (qualitative shift), rather favoured existing members (quantitative shift). This is supported by results obtained on functionality and relative quantitative changes in numbers of specific community members (hydrocarbon degrading microorganisms) (see Section 4.3.1) and metabolic activity (see Section 4.3.2) in the rhizosphere.

Problems performing DGGE analysis experienced in the current study have been reported elsewhere, particularly for high diversity environments such as soils (Kisand and Wikner 2003; Green 2006). When general bacterial primers have been applied to the systems, with subsequent DGGE analysis, gel profiles can look 'smeared' as observed in the current study. This can be in part due to the very high number of bands, many of them weak and indistinct (Wieland *et al* 2001). Molecular analysis using DGGE can be troublesome with high microbial diversity such as found in soil, where the technique can reach its limitations. One recommendation when dealing with high microbial diversity could be to design more specific primers to apply to the system (Wu *et al* 1998). A focus could then be on specific groups of bacteria e.g. Actinomycetes, for which there are highly specific primers. Specific primers could then be used directly for DGGE or nested with general bacterial primers.

Blurry, fuzzy or smeared bands in DGGE can also be a sign of PCR problems. They can indicate issues with gel quality, old reagents, an improper gradient, irregular current, old buffer, or temperature control issues (Green 2006). Bands in the top of the gel are most commonly fuzzy and indistinct, which may be an indication that these are artifacts of the PCR/DGGE analysis. In particular, if a band is present in all the samples, very high in the gel, and fuzzy, it is indicative of heteroduplex bands which denature rapidly, or perhaps single stranded DNA (Green 2006). These simple explanations could account for the banding profiles recorded in the current study, especially since it was also observed for the

positive controls. Such information may be helpful for future optimisation of DGGE analysis.

Alternatively, Kisand and Wikner (2003) suggest that extended fuzzy bands in the migration direction which hamper band resolution may be a result of multiple melting domains (MMD). A distinct band is obtained only if a particular sequence has a single melting site. The authors reported that marked fuzzy DGGE band morphology, due to multiple melting domains, occurred in aquatic isolates and clones in the *Flavobacterium* group. The authors concluded that mixed communities from environmental samples may contain species with MMD and thus would not be possible to resolve using currently available universal primer pairs. A similar phenomenon was reported by Wieland *et al* (2001) describing formation of "cloudy bands" during denaturation and subsequent migration of DGGE profiles for a soil bacterial community.

Despite the difficulties of performing DGGE analysis on high diversity environments such as soils, the technique has been successfully used to characterise rhizosphere microbial community structure and dynamics (Kasai *et al* 2005; Kim *et al* 2006). The characterisation of rhizosphere microbial community diversity and composition, and in particular shifts in population, has been demonstrated in phytoremediation of polluted soils (Kirk *et al* 2005, Phillips *et al* 2006).

Mahmood *et al* (2005) characterised the native bacterial community in a pristine grassland soil capable of degrading the pesticide pentachlorophenol (PCP) using DGGE of amplified 16S rRNA genes (DNA-DGGE). The soil had no prior history of exposure to PCP or other organic pollutants. PCP degradation was found to be associated with significant changes in bacterial community structure, leading to the appearance of seven bands in the DGGE profile which provided evidence of qualitative shifts in community structure. Interestingly, the majority of bands increased in relative intensity during the first 35 days of the experiment, which also coincided with the greatest removal of PCP (a total of 39% of measurable PCP was degraded after 63 days).

Similarly, Kirk *et al* (2005) and Phillips *et al* (2006) demonstrated shifts in the bacterial community structure in the presence of plants, and that these changes were plant-specific. Kirk *et al* (2005) assessed the DGGE community fingerprint in the rhizosphere of perennial ryegrass (*Lolium perenne*) and alfalfa (*Medicago sativa*), and both species grown together in petroleum contaminated soil. Results from DGGE analysis showed the plants altered the composition of the microbial community. The cluster analysis indicated that the microbial composition in plant rhizospheres were different from the unplanted (bulk) soils. Interestingly, the mixed planting (perennial ryegrass and alfalfa) showed similar DNA banding patterns to alfalfa alone. This suggests alfalfa had a greater influence on the composition of the dominant rhizosphere bacterial population. Of all plant treatments, the DGGE fingerprint of perennial ryegrass differed the most from the original bulk soil. Phillips *et al* (2006) reported a similar finding, when DGGE analysis revealed alfalfa had a dominant effect on the structure of the rhizosphere microbial community in mixed planted treatments with ryegrass. Of note in the case of Phillips *et al* (2006), total petroleum hydrocarbon reduction was greatest in single-species treatments.

Kim *et al* (2006) reported complex profiles of microbial community structure when characterising the rhizosphere of alfalfa (*Medicago sativa*) during phytoremediation of diesel contaminated soil. DGGE analysis revealed that the microbial community structure was most highly influenced by the combined presence of diesel contamination and plant roots, but that diesel contamination alone had a higher influence than the rhizosphere. The authors also reported total microbial activity and abundance of hydrocarbon degrading microorganisms was highest in the diesel contaminated rhizosphere soil compared with unplanted and clean control soils. Alfalfa facilitated removal efficiency of 82% after seven weeks, compared with only 59% in unplanted soil.

In contrast to these reports, and as observed in the current work (Section 6.3.1), studies have reported no shift in community fingerprint in the presence of the plant rhizosphere, despite enhanced rhizoremediation. Siciliano *et al* (2003) assessed changes in rhizosphere microbial community composition and function during a PAH phytoremediation field trial. No detectable shifts in the 16S rDNA

composition (as determined by DGGE) of soil microbial community between planted and unplanted treatments was found, suggesting a highly similar and relatively stable community over the study period. Planted treatments included a native grass mixture with tall fescue (*Festuca arundinacea*) and Rose clover (*Trifolium hirtum*), and was successful in decreasing TPH concentrations by 30% after two years. The authors concluded that the observed decrease in TPH was attributable to increased bacterial numbers in the rhizosphere containing hydrocarbon catabolic genes. A similar explanation could be inferred from results in the current study. Soil community DGGE profiles did not appear to change, but relative numbers of existing members did as examined by MPN analysis of hydrocarbon degrading microorganisms (Section 4.3.1). The resultant decrease in TPH (Section 6.3.1) could then be attributed to the quantitative shifts in specific hydrocarbon degrading microbial community members, rather than qualitative shifts in community structure.

Chen and Banks (2004) also found no correlation between pyrene concentration and microbial community shifts in the rhizosphere of tall fescue (*Festuca arundinacea*) using PCR-DGGE analysis. Even though the presence of grass significantly enhanced pryene degradation relative to unplanted controls (undetectable after 91 days in planted treatment and 11% remaining in unplanted control), the authors suggest biodegradation may have been accomplished by the pre-existing microbial community. It is also possible that the method was unable to detect subtle community shifts (Wieland *et al* 2001). The authors noted that the significant pyrene mineralisation in the presence of tall fescue suggested that a rhizosphere effect was occurring, but may not have been associated with enhanced growth of specific bacterial pyrene degraders. The authors did not enumerate specific contaminant degrading microorganisms in the rhizosphere (as done in the current study). This may have provided further explanation of the observed effects.

It is clear that there are a wide range of methods available to study soil microbial diversity, including biochemical and molecular-based techniques. Each method has its limitations and only provides a partial picture of soil microbial diversity.

Since current knowledge cannot evaluate the effectiveness of each method (Kirk *et al* 2004), the study of microbial populations should be researched on as many different levels as possible. This would provide a more complete assessment of changes in microbial structure and function in a habitat.

5.5 Conclusion

The influence of Australian grasses on microbial community structure (defined as community DNA fingerprint) in diesel/oil contaminated soil remains unclear. Results from DGGE profiles suggest no new population was favoured by the grasses (qualitative shift), rather relative quantitative changes in existing members of the microbial population (as confirmed by MPN analysis, Section 4.3.1). Further work is needed to determine more clearly how the rhizosphere exerts a positive effect on the number (MPN), activity (lipase), and potential diversity (DGGE) of indigenous bacterial populations in hydrocarbon contaminated soils.

CHAPTER SIX

6 ENHANCED DEGRADATION OF ALIPHATIC HYDROCARBONS IN SOIL PLANTED WITH AUSTRALIAN GRASSES

6.1 Introduction

It is well established that the use of indigenous soil microorganisms for site remediation of hydrocarbons holds potential (Reynolds *et al* 1999; Pichtel and Liskanen 2001; Jorgensen 2007). The process is sometimes slow and with restricted endpoint outcomes due to limited substrate availability or other inhibitory effects. Technologies are needed to accelerate or stimulate natural decomposition processes to treat fuel and oil contaminated soil, thus reducing risks to public health and ecosystems and rehabilitating affected sites. Plants such as grasses hold clear potential as a means to enhance microbial growth (as demonstrated in Chapter 4 and elsewhere in the literature) and associated contaminant degrading processes.

The study of hydrocarbon rhizoremediation in soil environments aims to determine the additional degradative influence of plant species and their associated microbial community on natural microbial-based decomposition outcomes (Kuiper *et al* 2004). Plants have been shown to encourage hydrocarbon contaminant reduction principally by providing an optimum environment for microbial proliferation in the rhizosphere (Adam and Duncan 1999). Vegetation has been shown to influence degradation of hydrocarbons via root exudation of organic compounds that stimulate the activity of microorganisms in the rhizosphere, thus increasing rates of biodegradation (Kaimi *et al* 2006). Root exudates might act as structural analogues to contaminant molecules and alter the microbial community by enriching populations of hydrocarbon degrading microorganisms (Pichtel and Liskanen 2001).

Chemical analysis plays a crucial role in evaluating hydrocarbon contaminated soils, and extraction procedures are a critical step. Extraction of hydrocarbons

from soil for monitoring biodegradation has posed its share of analytical challenges (Dominguez-Rosado and Pichtel 2004). Owing to the complexity of diesel fuel and engine oil mixtures, analytical techniques used in most environmental assessments measure the total petroleum hydrocarbon (TPH) mixture (Adam and Duncan 1999). Soil texture, contaminant complexity, and aging (weathered contamination) are all important factors influencing extraction efficiency. Schwab *et al* (1999) found that mechanical shaking of soil with a dichloromethane or acetone treatment was equivalent to the Soxhlet extraction method for TPH and PAH determination. Soxhlet extraction is an US EPA-approved method for volatile and semivolatile organic contaminants from soil materials, but it has many disadvantages including long extraction periods, potential for loss of volatile compounds, and less efficient extraction from field-moist soils. Mechanical shaking of diesel contaminated soil with hexane was also found to be effective for extraction and analysis (Pichtel and Liskanen 2001). The shaking method was designed to provide simpler and more efficient extraction of petroleum hydrocarbons from soil.

In previous chapters, work has been presented confirming three Australian grass species as suitable candidates for further investigation of their rhizoremediation potential. All species were able to germinate and grow successfully in diesel/oil contaminated soil at the exposed concentrations (Chapter 3). Further, all species influenced the microbial community in the rhizosphere to varying degrees (Chapters 4 and 5). Enrichment of hydrocarbon degrading microorganisms in the rhizosphere was demonstrated for all grass species.

This chapter outlines the final stage in a multi-faceted approach, the evaluation of diesel/oil biodegradation over time in the rhizosphere of Australian grasses. The aim was to assess the extent to which Australian grasses enhanced the biodegradation of aliphatic hydrocarbons in soil relative to natural decomposition in the absence of vegetation.

6.2 Materials and methods

6.2.1 Grass rhizosphere soil sampling

Three separate single species planted trials (*B. decumbens*, *C. ambiguus*, or *M. stipoides*) and a multispecies planted trial (*B. decumbens* plus *C. ambiguus*) were conducted. Each trial had unplanted control soils, this is particularly important as *M. stipoides* has cool season growth where *B. decumbens* and *C. ambiguus* are summer growing species. Specific growth conditions and soil sample collection (rhizosphere and unplanted) were as outlined in Section 4.2.1. The multispecies planted trial was sampled in the same way as the single-species trials (Section 4.2.1), except that rhizosphere soil samples included plants representing both species. No additional nutrients were used in the experiments, which provided a worse-case scenario for degradation potential.

Time-course analysis was performed periodically and generally followed day zero (sowing), germination, and weekly thereafter until 100 days after planting (DAP). The length of experimental period was determined by conventional literature which suggested the bulk of aliphatics would be biodegraded within this timeframe (Hou *et al* 2001; Pichtel and Liskanen 2001; Kaimi *et al* 2007b). Six replicate soil samples (rhizosphere or unplanted control) for each treatment at each time point were used for analysis of total petroleum hydrocarbon (TPH) concentration.

Root and shoot tissue were separately assessed for TPH accumulation periodically, for all grass species. Harvested plant tissue was washed thoroughly in distilled water and separated into roots and shoots.

6.2.2 Extraction of total petroleum hydrocarbons (TPH) from soil

Recovery of total petroleum hydrocarbons (TPH) from soil was achieved using a mechanical shaking solvent extraction method as described by Schwab *et al* (1999). A 1:1 ratio of weight to volume (g sample to mL solvent; 5 g:5 mL) was used for the extraction of TPH from soil samples. Fresh plant tissue (root and shoot) was first placed in centrifuge tubes and ground using a small pestle. Then

extraction of TPH from plant tissue was performed in the same way as for soil samples. The extraction solvent was dichloromethane (DCM, chromatography grade), with 100 mg/L internal standard naphthalene (puriss p.a. standard for GC, Fluka, Australia) added to each sample with the addition of DCM. Sample vials were shaken vigorously by hand for 2 min, and then placed on an orbital platform shaker (~300 RPM) for 20 min. Sample vials were then centrifuged at 3000 RPM for 5 min (Phoenix Clements 400 Orbital, Australia) to pellet the soil and separate the supernatant. Approximately 1 mL of the decanted supernatant (DCM with internal standard sample extracts) were added to GC vials. Single, double and triple extractions were assessed for optimal recovery, with single extractions reproducibly demonstrating > 98% recovery. Subsequently a single extraction procedure was used for all analyses.

6.2.3 Determination of TPH concentration by gas chromatography

Total petroleum hydrocarbon (TPH) concentration in soil extracts was determined using a Varian Star 3600CX Gas Chromatograph equipped with a flame ionisation detector (GC-FID) and splitless injector. The following conditions were standard for all analyses:

Column:	DB-5 capillary column (30 m x 0.25 mm ID x 0.25µm)
Carrier gas:	Nitrogen
Injector temperature:	275°C
Detector temperature:	300°C
Column flow rate:	1.5 mL/min

For analysis of TPH extracts, the oven temperature program was as follows: 40°C for 2 min after injection, followed by an increase of 9°C per minute to 275°C and held for 15 min.

The concentration of TPH was calculated using the internal standard. Standard solutions (10 mL) were prepared using the internal standard, and TPH concentrations ranging from 1000 – 10000 mg/L (7 total). Standards were analysed by GC-FID and the peak areas and peak ratios of TPH to the internal standard were calculated. Peak areas (as total chromatogram area) were

integrated using Varian Star™ v. 4.5 software, and encompassed diesel range chain hydrocarbons from C_{10} to C_{28} as well as motor oil range chain hydrocarbons C_{16} to C_{34}. A standard line was obtained ($R^2 = 0.9907$) using the concentration of TPH standards versus the peak area. The concentration of TPH (mg/kg soil) was calculated using the ratio between TPH and internal standard peak areas and the respective TPH standard line. The lower limit of quantitation (LOQ) was determined to be 500 mg/kg TPH. Quality control steps were included by running a single dichloromethane blank every six samples.

6.2.4 Statistical analysis

Prior to statistical analysis, data were tested for normality and homogeneity of variance (Levene Test) using SPSS v.14 for Windows and GraphPad Prism v.4.0. Independent samples T-tests (2-tailed) and analysis of variance (ANOVA) at $\alpha = 0.05$ were conducted to determine treatment significance at each sampling time for TPH concentration, for each species. Treatment mean values which showed significance using ANOVA were separated using Bonferroni Multiple Comparisons test at 0.05 level of significance. Non-linear regressions (curve fit) where performed on rates of TPH removal in planted and unplanted (control) treatments. **Equation 6.1** shows the non-linear regression model used for analysis, i.e. top-to-bottom exponential decay. This model is a modification of the standard One Phase Exponential Decay model (where Y approaches zero at high values of X), and incorporates where the curve plateaus at some value other than zero. This allows for comparison between k values as a rate, where higher k values denote faster rates of removal in this model.

Equation 6.1

Equation: Top to Bottom

$$Y = (Top - Bottom)*\exp(-K*X) + Bottom$$

6.3 Results

6.3.1 TPH removal in the rhizosphere of Australian grasses and unplanted soil

Periodic assessment of TPH concentration revealed the influence of the rhizosphere on diesel/oil removal, for each individual species compared with corresponding unplanted controls.

Figure 6.1 shows the pattern of removal of TPH from soil planted with *B. decumbens* and unplanted control soil over time. TPH concentrations were consistently lower in rhizosphere soil compared with unplanted control soil from 14 DAP onward (T-test, $p < 0.01$). Germination for *B. decumbens* was seven days after planting (DAP). Final mean TPH concentration (at 100 DAP) was significantly lower in the rhizosphere soil (4510 ± 506 mg/kg) compared with the unplanted control soil (5966 ± 546 mg/kg) (T-test, $p = 0.001$). This corresponded to 49% total removal in the presence of the grass from the initial concentration, compared with only 33% total removal in the unplanted control soil. Within the planted treatment over time, the influence of *B. decumbens* on TPH removal was dramatic and rapid. A significant proportion (approximately 90%) of the total TPH loss occurred in the first 14 DAP, in particular during the seven days following germination.

TPH removal over time in the rhizosphere of *C. ambiguus* and unplanted control soil is presented in **Figure 6.2**. Germination for this species was 7 DAP. Significantly lower residual TPH concentrations were recorded in the rhizosphere of *C. ambiguus* compared with the unplanted control from 17 DAP onward (T-test, $p < 0.01$). At 100 DAP, significantly lower residual TPH concentration (1132 ± 550 mg/kg) was recorded in the planted treatment with *C. ambiguus* than in the unplanted control (4743 ± 334 mg/kg) (T-test, $p < 0.0001$). This corresponded to 88% total TPH removal in the presence of the grass from initial concentration. Large variability in removal pattern over time was noted for both planted and unplanted treatments, as well as between replicates within a treatment. Despite this variability, the majority (approximately 95%) of total

TPH removal in the rhizosphere took place in the initial two weeks of the experiment.

M. stipoides is a cool season grass in comparison to the warm season growth of the other two species used in this study, meaning that the growth and experimental conditions were maintained at an average maximum temperature 10°C cooler. The pattern of TPH removal from the rhizosphere of *M. stipoides* and unplanted control soil over time is presented in **Figure 6.3**. Residual TPH concentrations were consistently lower in rhizosphere soil compared with unplanted control soil from 11 DAP onward (T-test, $p < 0.05$), coinciding with germination. TPH concentration steadily decreased over time in the rhizosphere soil following germination. The total TPH removal from soil was 80% (from initial concentration) recorded in the presence of the grass rhizosphere, which was noted in the first half of the experimental period (55 DAP). Thereafter, no significant further reduction in TPH concentration was noted in the planted treatment (ANOVA, $p > 0.05$). Final mean TPH concentration (at 100 DAP) was 1781 ± 455 mg/kg in the rhizosphere of *M. stipoides*, which was significantly lower than residual TPH concentration recorded in the unplanted control soil (4363 ± 411 mg/kg) (T-test, $p < 0.0001$).

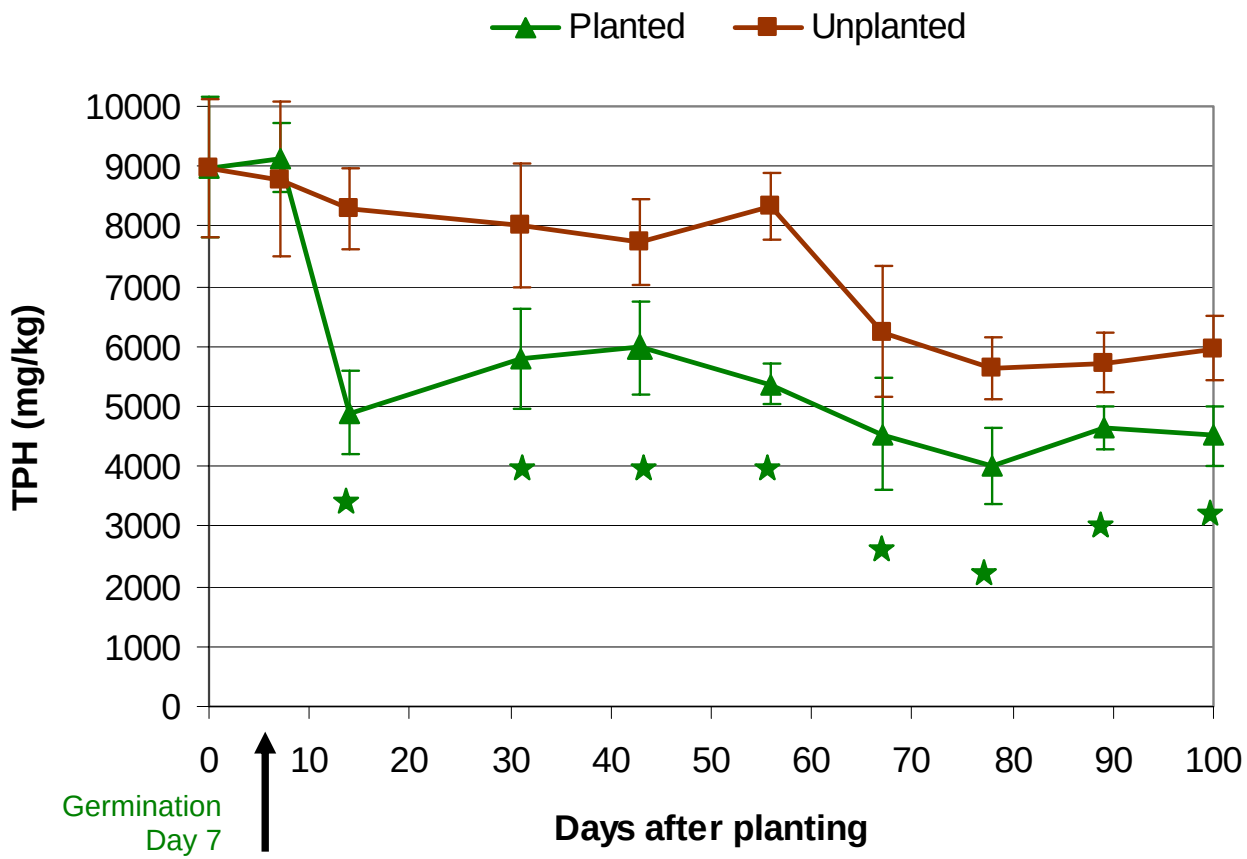


Figure 6.1 Removal of total petroleum hydrocarbons (TPH) over time in the rhizosphere of *B. decumbens* (planted) and unplanted control soil. Data are mean values ± SD (n = 6). * indicates significant difference to unplanted control at $p < 0.01$.

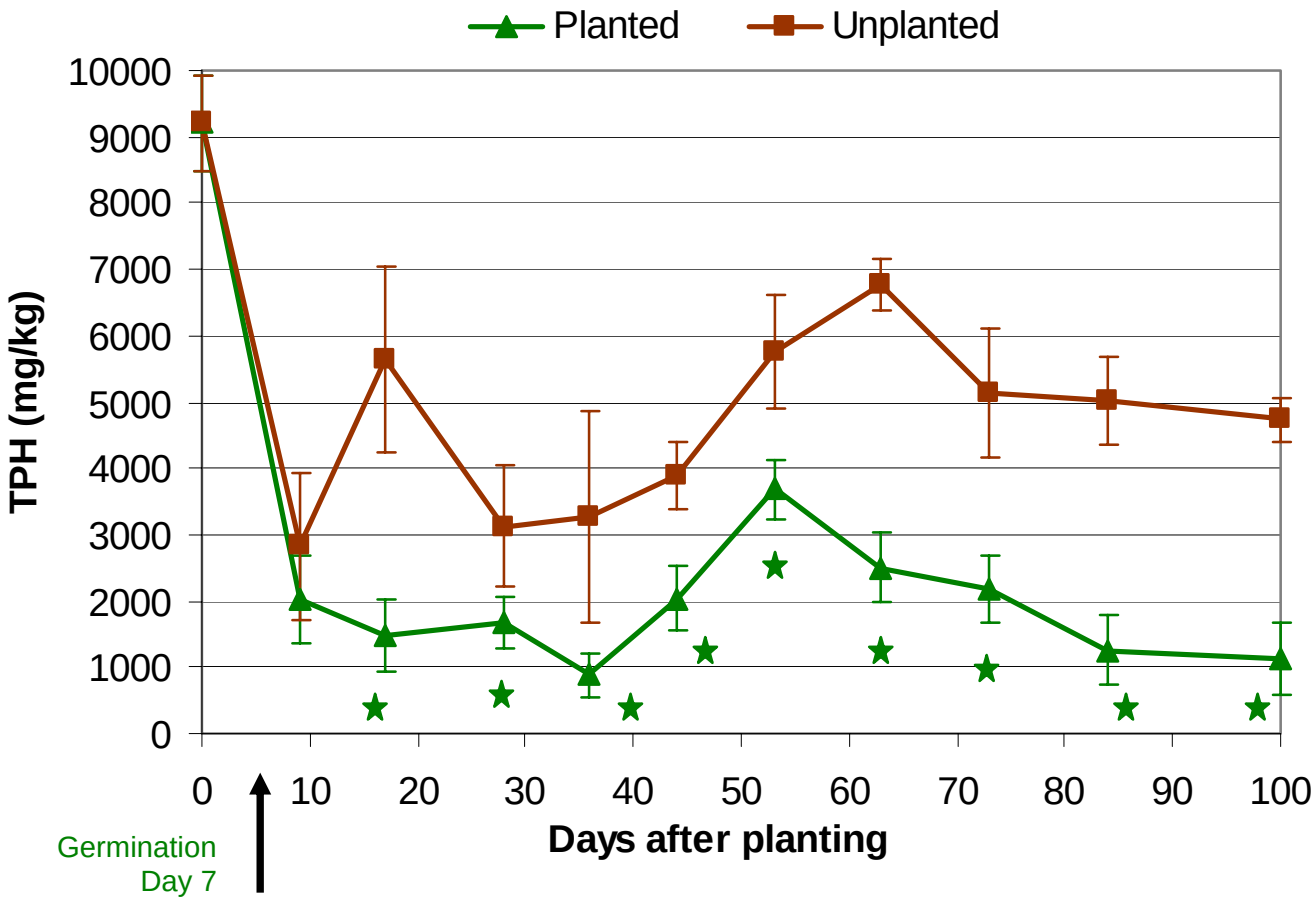


Figure 6.2 Removal of total petroleum hydrocarbons (TPH) over time in the rhizosphere of *C. ambiguus* (planted) and unplanted control soil. Data are mean values ± SD (n = 6). * indicates significant difference to unplanted control at $p < 0.01$.

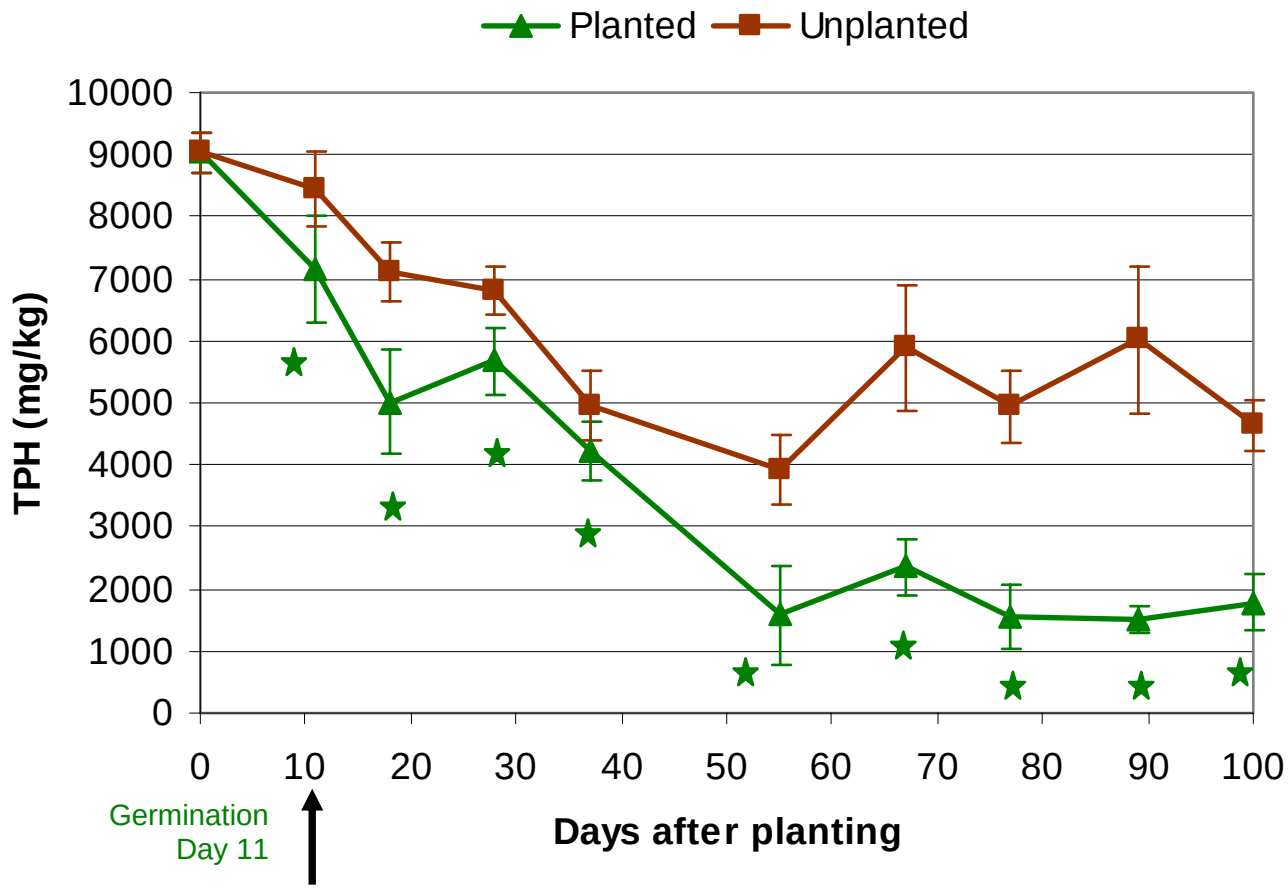


Figure 6.3 Removal of total petroleum hydrocarbons (TPH) over time in the rhizosphere of *M. stipoides* (planted) and unplanted control soil. Data are mean values ± SD (n = 6). * indicates significant difference to unplanted control at $p < 0.05$.

The multispecies trial planted with *B. decumbens* plus *C. ambiguus* provided interesting comparison in total TPH removal with the single species trials. The pattern of TPH removal from the rhizosphere of a mixed planting with *B. decumbens* and *C. ambiguus*, and unplanted control soil over time is presented in **Figure 6.4**. TPH concentrations were consistently lower in rhizosphere soil compared with unplanted control soil from the point of germination (7 DAP for both species) onward (T-test, $p < 0.01$), consistent with findings in the single species trials. Thereafter, gradual decreases in TPH concentration was seen in the planted treatment over time, reaching a maximum TPH removal of 85% from the initial concentration at 100 DAP. Final mean TPH concentration (at 100 DAP) was 1353 ± 474 mg/kg in the rhizosphere soil, which was significantly lower than residual TPH concentration recorded in the unplanted control soil (4085 ± 612 mg/kg) (T-test, $p < 0.0001$). Further comparison between single and multispecies influences on total removal of TPH is discussed in Section 6.3.2.

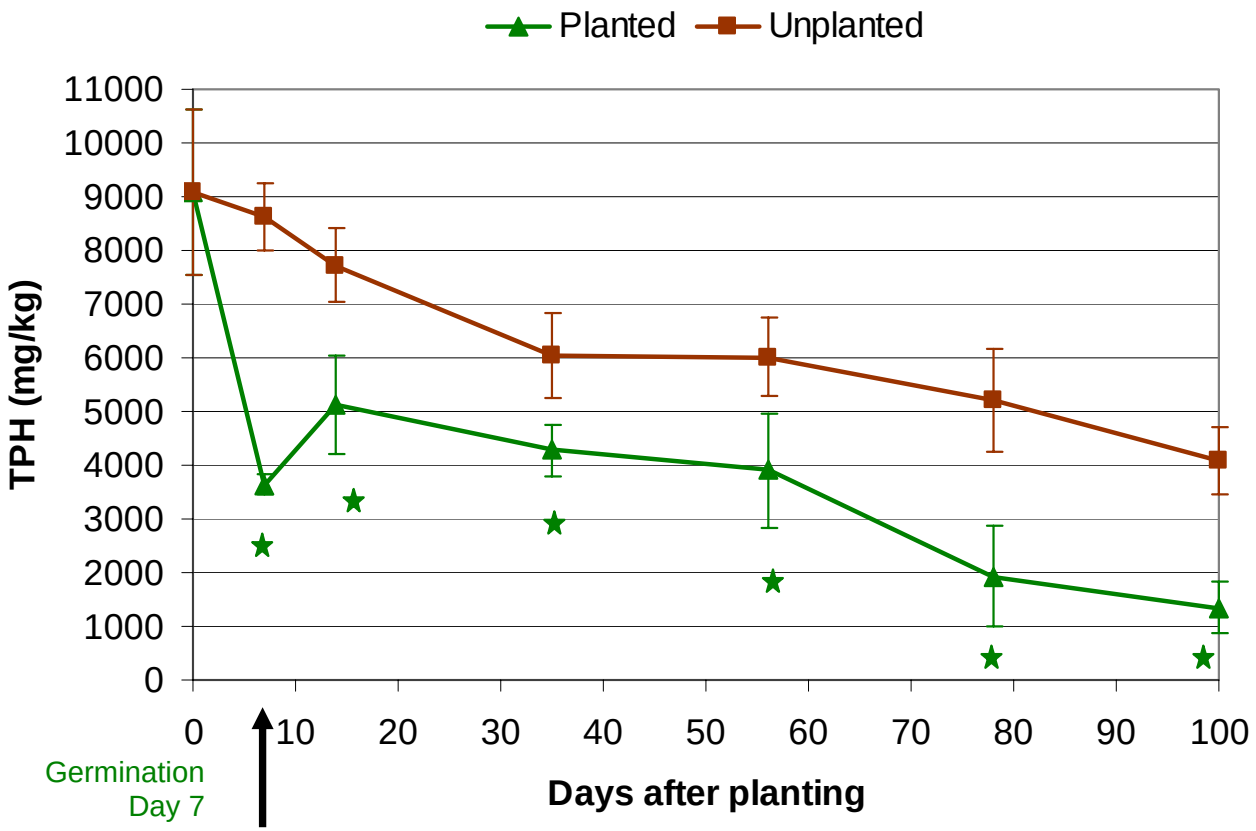


Figure 6.4 Removal of total petroleum hydrocarbons (TPH) over time in the rhizosphere of a mixed planting with *B. decumbens* and *C. ambiguus* (planted), and unplanted control soil. Data are mean values ± SD (n = 6). * indicates significant difference to unplanted control at $p < 0.01$.

Figure 6.5 presents examples of typical chromatograms obtained over time for TPH profiles in a planted treatment (*M. stipoides*). Comparison of the chromatograms showing TPH traces in planted treatments typically revealed relatively uniform loss across all fractions of TPH over time. This can be seen by the overall reduction in total peak area (relative to internal standard) over time, rather than greater loss of lighter fractions (e.g. C_{10}-C_{16}) with the heavier fractions remaining.

Periodic assessment of plant tissue for TPH accumulation showed no recorded concentrations in roots or shoots for any grass species, at any time point (see typical example chromatogram **Appendix III**).

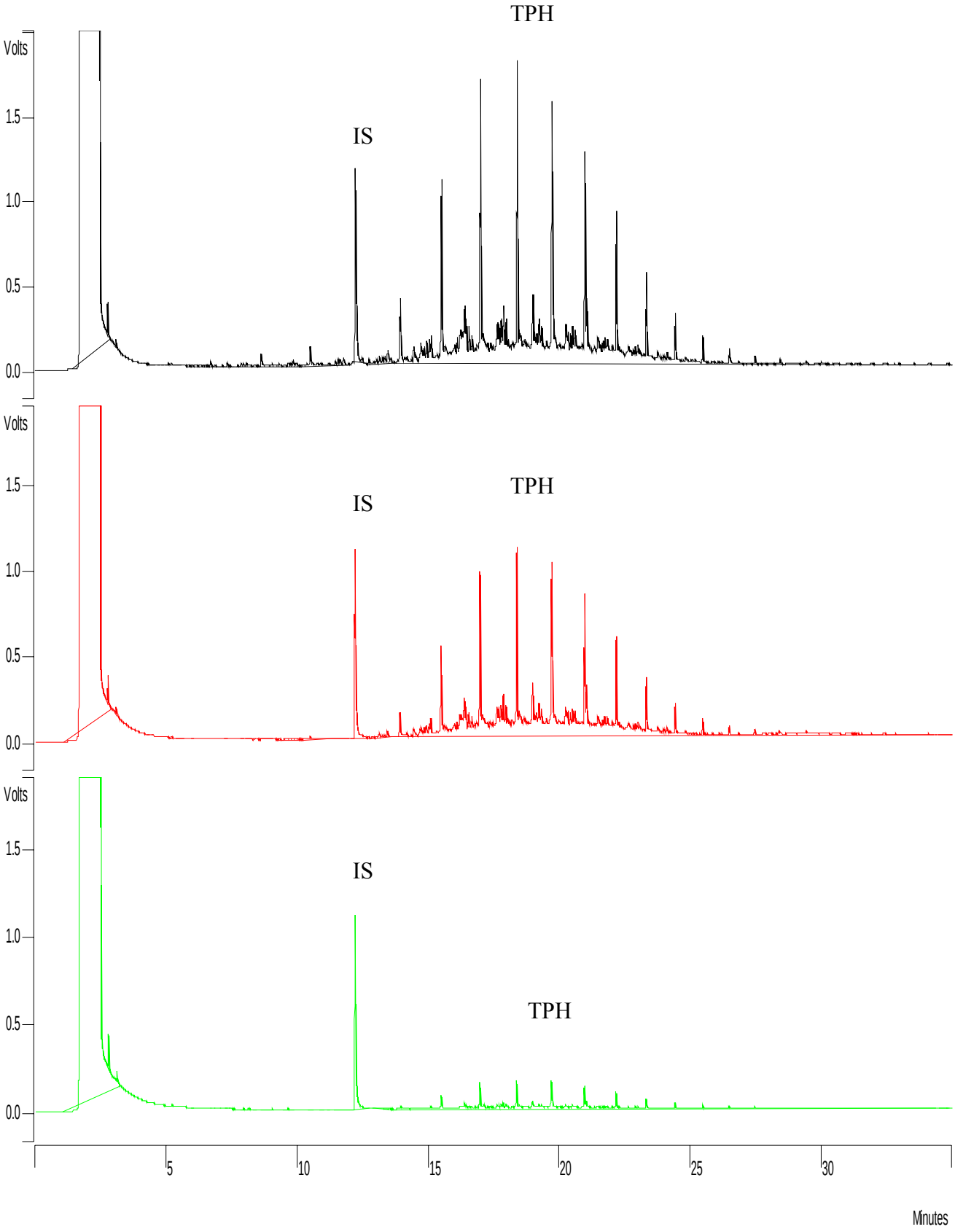
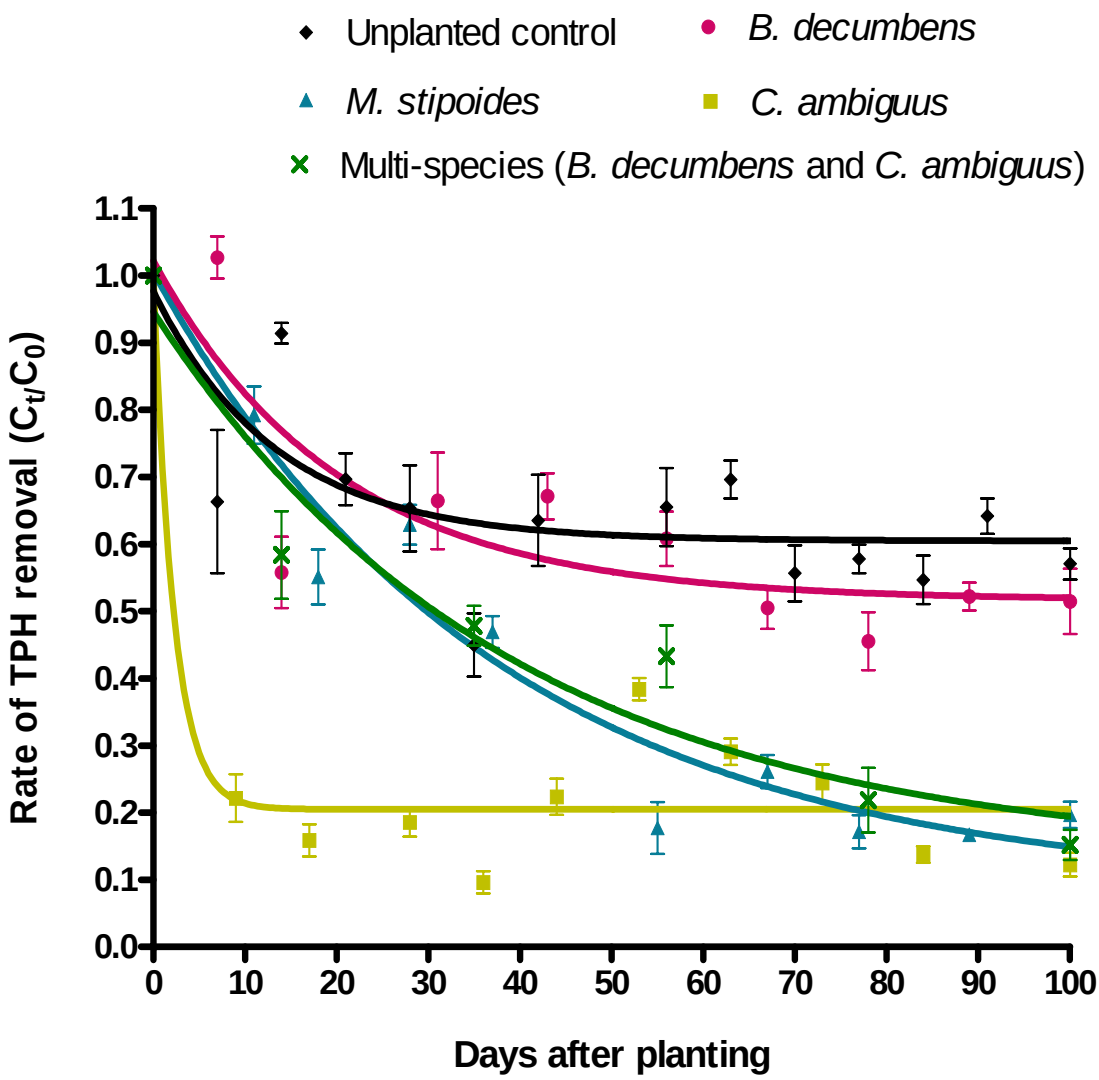


Figure 6.5 Examples of typical recorded chromatograms showing changes in TPH profiles relative to the internal standard (IS) over three experimental time points in a planted treatment. Chromatograms are from *M. stipoides* planted treatments at 0 DAP (black, top), 37 DAP (red, middle) and 100 DAP (green, bottom).

6.3.2 Comparison between species for extent and rate of TPH removal

The total extent of TPH removal in the presence of grasses by 100 DAP varied between species (see Figures 6.1 to 6.4). TPH endpoints in soil planted with *C. ambiguus* or *M. stipoides* were significantly lower than recorded for *B. decumbens* (ANOVA, $p = 0.0001$). No significant difference was found in final TPH concentrations in the rhizosphere soil of *C. ambiguus* or *M. stipoides* (ANOVA, $p > 0.05$). Interestingly, the multispecies trial planted with *C. ambiguus* plus *B. decumbens* had no additional influence on total TPH removal. The final TPH concentration recorded in the multispecies trial was not significantly different (ANOVA, $p > 0.05$) from that for the best single species performer i.e. *C. ambiguus*. However, significantly lower final TPH concentration was found in the multispecies planting compared with the trial of *B. decumbens* planted alone (ANOVA, $p = 0.001$).

The rate of TPH removal in planted treatments and unplanted control soil is presented in **Figure 6.6**. While overall endpoints in TPH concentrations demonstrated enhanced removal in the presence of plants (Section 6.3.1), the rate of removal was not equal between species. A non-linear regression model allows for comparison of k values (see Section 6.2.4), where larger k denotes faster rate of removal. In this respect, *C. ambiguus* demonstrated the fastest removal efficiency of TPH ($k = 0.4478$) compared with other grass species and the unplanted control soil. *B. decumbens* showed overall a similar rate of removal to the unplanted control ($k = 0.0495$ and 0.0747, respectively). The cool season grass *M. stipoides* showed the slowest rate of removal ($k = 0.0267$) for all single species trials. The multispecies trial which comprised two summer growing species (*B. decumbens* and *C. ambiguus*) was slower than might have been predicted, showing a rate of removal ($k = 0.0259$) similar to that calculated for *M. stipoides* ($k = 0.0267$).



	Unplanted control	*B. decumbens*	*C. ambiguus*	*M. stipoides*	Multispecies
K	0.0747	0.0495	0.4478	0.0267	0.0259
R^2	0.2674	0.6269	0.8670	0.9049	0.8412

Figure 6.6 Rates of TPH removal in planted soil (single grass species and multispecies treatments) and unplanted control soil. Mean values ± standard deviation, n = 6 (except control n = 18). Smoothed lines show non-linear regression curves with corresponding K and R^2 values presented in the table.

6.4 Discussion

The majority of studies on rhizoremediation of hydrocarbons have focused on the assessment of individual species compared to unplanted soil (see reviews Hutchinson *et al* 2003; Kuiper *et al* 2004 and also literature summary in Table 1.4). In these studies, the extent of organic pollutant removal in planted soil has been significantly greater than in unplanted soil. The results of the current study demonstrated enhanced removal of hydrocarbons in soil planted with native grasses compared with unplanted soil. This finding is in line with others, although, some interesting species-specific difference were noted.

C. ambiguus recorded the greatest overall removal efficiency (88%) and fastest rate of removal (k = 0.4478) of all native grasses assessed. The total TPH removal in the presence of *M. stipoides* (80%) was not significantly different to *C. ambiguus*, but the rate of removal was considerably slower for this cool season species (k = 0.0267). *B. decumbens* facilitated greater removal of TPH compared with the unplanted control (49% and 33%, respectively), but did not perform as well in terms of TPH endpoint as the other two species. Rate of TPH removal by *B. decumbens* was not largely different from the unplanted control (k = 0.0495 and 0.0747, respectively). Outcome for the multispecies trial planted with the two summer growing species *B. decumbens* and *C. ambiguus*, showed no added removal efficiency beyond the best single species outcome. The rate of removal in this case, however, was slower than that recorded for the individual species (multispecies k = 0.0259), and closer to that observed for the cool season grass *M. stipoides* (k = 0.0267). Overall the results demonstrate enhanced rhizoremediation of aliphatic hydrocarbons in the presence of native grasses, without any requirement for nutrient addition.

What factors might be contributing to the species-specific induced responses observed between native grasses? This may be clarified by examining the species-specific influences on the growth and activity of microbial communities inhabiting the rhizosphere (see also Section 4.3). Evidence of the rhizosphere effect, or stimulation of microbial community in the presence of the root zone, was demonstrated for all grasses. However, similar to the variation in TPH

removal efficiency between species, the extent of rhizosphere effect for each species differed. In this regard, *C. ambiguus* demonstrated the greatest stimulation of hydrocarbon degrading microorganisms in the rhizosphere compared with the other grasses (Section 4.3.1). This was followed by *M. stipoides* and then *B. decumbens*. Further examination of correlations between all plant and microbial parameters is discussed in Chapter 7 which examines the relative rhizoremediation performances of the native grasses.

There are several documented aspects of plant contribution to the removal of pollutants from soil. Firstly, the direct interaction of plant roots with hydrocarbons in the soil by sorption, uptake and transport (Gunther *et al* 1996). Uptake and transport of diesel/oil into the roots and shoots of native grasses was shown to be negligible in the current study. Secondly, the stimulation of microbial pollutant degradation due to rhizosphere interactions (Glick 2003) as discussed above. This is likely to be the primary role influencing the fate of diesel/oil in soil in the current study (and is discussed in more detail in Chapter 7). Thirdly, a more general effect of vegetation cover on environmental conditions and soil properties (Germida *et al* 2002). Environmental factors influenced by the grass species may have contributed to rhizoremediation outcomes above that recorded in unplanted soil in the current study. Some of these factors are discussed below.

The importance of oxygen in the biological remediation of petroleum contaminants, especially saturated aliphatics (i.e. diesel), is well documented (Frick *et al* 1999; Olson *et al* 2003; Bamforth and Singleton 2005). Plants may enhance the oxygenation of contaminated soils improving remediation potential. Roots can act as physical channels which transport oxygen to the root zone, enhancing aerobic conditions for biological degradation. Roots also increase the soil porosity allowing increased diffusion of atmospheric oxygen (Rentz *et al* 2003). Vegetation cover can also moderate temperature and moisture conditions, which influences availability of oxygen (Gunther *et al* 1996). Plant root systems may increase the moisture content of soil by promoting an effective circuit for water movement (Jing *et al* 2008). Jing *et al* (2008) showed soil moisture content increased by 5% in petroleum polluted soil planted with grasses compared with

unplanted soil. The plant root system prevented water from flowing out by absorption and fixation of water, thus improving the moisture content of the soil. This in turn satisfies the demand of microorganisms and becomes favourable to the degradation of petroleum hydrocarbons.

Besides the impacts on moisture and oxygen availability, plants can influence nutrient cycling and the supply of nutrients in the soil. The release of organic substrates from roots (and seasonal root dieback) is a key process influencing nutrient availability in the rhizosphere (Grayston *et al* 1996). Some organic compounds in root exudates (i.e. phenolics, organic acids, alcohols, proteins) may serve as carbon and nitrogen sources for growth and long-term survival of microorganisms that are capable of degrading contaminants (Alkorta and Garbisu 2001). The chemical composition of root exudates and rates of exudation differ considerably among plant species and between different stages of plant development (Germida *et al* 2002). Adam and Duncan (2003) discussed how the carbon:nitrogen (C:N) ratio of the soil is altered when contaminated with hydrocarbons. They proposed that the added carbon may stimulate microbial numbers but can cause an imbalance in the C:N ratio. This can result in immobilisation of soil N and subsequent N-limitation for plant growth and remediation potential.

The inorganic mineral nutrients that are most often reported to limit biological remediation of hydrocarbon contaminants in soil are nitrogen and phosphorus (Germida *et al* 2002; Hutchinson *et al* 2003). The addition of nitrogen fertiliser has been observed to enhance the remediation of hydrocarbons in soil (Dakora and Phillips 2002; Merkl *et al* 2005). Not all phytoremediation systems respond to the addition of nutrients (Kirkpatrick *et al* 2006). Some researchers have observed diminished rates of microbial degradation after the addition of nitrogen and phosphorus amendments (Rentz *et al* 2003), and attributed this phenomenon to inhibition of oligotrophic degraders or the stimulation of noncompetent bacteria (Olson *et al* 2003). In addition, there is an adaptive trait found in many Australian native plants where low soil fertility and low nutrient conditions are optimal for growth (Huxtable 1997; Neumann and Martinoi 2002). This suggests that the addition of fertiliser commonly practiced in phytoremediation may not

be suitable, and in fact may be harmful to native vegetation. By not adding nutrients in the current study, a worse-case scenario for diesel/oil remediation in the presence of native grasses is presented. Other studies have assessed grasses for their rhizoremediation potential of aliphatic hydrocarbon contaminated soils (see summary Table 1.4). These have demonstrated a range efficiencies in hydrocarbon (as TPH) removal in both nutrient amended and unamended conditions.

In studies assessing unamended soils, the reported extent of TPH removal in the presence of plants ranges from 38% (Dominguez-Rosado and Pichtel 2004) or 40% (Jing *et al* 2008), up to 83% (Vouillamoz and Milke 2001). Dominguez-Rosado and Pichtel (2004) conducted a phytoremediation study of soil contaminated with used motor oil spiked at 1.5% (w/w) (15000 mg/kg). Plants were assessed for their rhizoremediation potential with and without fertiliser addition (commercial fertiliser reported at 10-10-10 pre-planting). A grass/maize mixture was used comprising the grasses creeping red fescue (*Festuca rubra*), fawn tall fescue (*F. arundinacea*) and ryegrass (*Lolium perenne*), plus maize (*Zea mays*). Plant species were not assessed individually for their rhizoremediation potential. The grass/maize mix reduced total oil/grease concentration in the soil by 38% at 150 days in the absence of amendment. Removal of the contaminant was increased to 67% in the planted system with fertiliser application. Unplanted control soil (with fertiliser) showed 47% reduction of total oil/grease concentration by 150 days. This provides interesting comparison to performance of individual Australian grasses and the multispecies trial in the current study. The worst outcome in the grass/maize study (planted soil without fertiliser addition) achieved only 38% reduction in grease/oil concentration, whereas a greater efficiency by all the Australian grasses including multispecies trial was recorded in the absence of nutrient addition (range 49-88% individual trials and 85% for the multispecies trial). The higher initial concentration of oil contamination in the Dominguez-Rosado and Pichtel (2004) study, combined with the weathered nature of the contaminant could account for the poorer outcome in removal efficiency by the mixed species.

Recently, Jing *et al* (2008) conducted a phytoremediation trial on petroleum polluted soil from an oil field site in East China. Oil concentrations in the soil were measured at 5000 mg/kg. Three grasses were assessed for their rhizosphere effects, Pannicum, *Eleusine indica*, and Tall fescue (no further Latin species information provided). No fertiliser or amendment details were mentioned, so it is assumed none were used. Oil degradation in the rhizosphere of grasses was shown to be 3-4 times higher than in unplanted soil after 150 days. Individual grasses achieved 30-40% removal (Tall Fescue demonstrated the highest removal efficiency 40%), compared with 10% in unplanted control. Again these removal efficiencies are lower than that recorded for any Australian native species in the current study. Although no details are given, it is likely that the contamination in the oil field soil was aged or more complex to some degree, thus potentially influencing its bioavailability for rhizoremediation. Soil used in the current study was freshly spiked with diesel/oil mix, and at double the concentration reported by Jing *et al* (2008). As for the Dominguez-Rosardo and Pichtel (2004) study, these factors of weathered pollutant and initial concentration influence the suitability of comparison with outcomes in the current work. The studies provide some context to assess performance of the native grasses in conditions without nutrient addition.

A study reported by Vouillamoz and Milke (2001) examined the relative rhizoremediation performance in the presence and absence of compost amendment. Ryegrass (*Lolium perenne*) was assessed in soil experimentally contaminated with diesel at 0.5% (5000 mg/kg). The effect of compost addition at three levels on TPH removal was measured. The addition of compost aided degradation in both the planted and unplanted soil, but the combination of grass and compost lead to lowest TPH values after 12 weeks. The authors reported total TPH removal in treatments with grass but no compost was 83%, which was not significantly different from either the unplanted amended soil treatment or the unplanted unamended soil treatment (both recording ~88% removal). Soil treatment with ryegrass and compost achieved the 94% TPH removal. It is important to consider that the addition of compost may have provided a dilution effect of contamination, or potentially a microbial inoculum, thereby affecting the relative rhizoremediation performances. The presented outcome for ryegrass

without compost (83% removal) was similar to that found for two individual grass species in the current study, namely *C. ambiguus* (88% removal) and *M. stipoides* (80% removal). This is despite the fact that the initial concentration in the current study was double that used by Vouillamoz and Milke (2001) and included motor oil in the mixture. Since the soils were freshly contaminated in both instances it provides a good point of comparison and shows the favourable performance of Australian grasses.

The majority of studies assessing grasses for aliphatic hydrocarbon rhizoremediation have incorporated nutrient amendments. In these studies the reported extent of TPH removal in the presence of plants ranges from approximately 60% [55-62% (Kaimi *et al* 2007a); 57% (Hou *et al* 2001); 68% (Dominguez-Rosado and Pichtel 2004)], up to 97% (Gunther *et al* 1996).

In a pioneer study, Gunther *et al* (1996) assessed ryegrass (*Lolium perenne*) for its influence on biodegradation of a mixture of hydrocarbons in soil. Saturated, unsaturated and branched chain aliphatics, and PAHs were spiked at 5000 mg/kg total hydrocarbon in an agricultural soil. Soil columns with and without vegetation were fertilised at initial stage of the experiment and every 8 weeks thereafter for a total of 22 weeks (154 days) (type and amount of fertiliser not reported). Hydrocarbons dissipated faster and to a greater extent in ryegrass planted soil than in unplanted control soil. By 22 weeks, 97% removal (reported as ~3% remaining) was demonstrated in the planted system, compared with 82% removal in unplanted controls (~18% remaining). In terms of removal of fractions within the mixture, aliphatics reportedly disappeared faster in the planted system, whereas PAH rates between planted and unplanted soils were not significantly different. In the current study, *C. ambiguus* demonstrated the greatest extent (88%) and fastest rate of aliphatic hydrocarbon removal after 100 days at comparable levels to that reported by Gunther *et al* (1996), without the requirement for nutrient addition. Corresponding unplanted control soil recorded only 50% hydrocarbon removal. This indicates nutrient limitation to TPH biodegradation in the absence of plants, notwithstanding the affects outlined above associated with plant roots as oxygen channels and influence on soil porosity.

Hou *et al* (2001) also assessed ryegrass (*Lolium perenne*) for rhizoremediation performance in hydrocarbon contaminated soil. In their study clean garden soil was spiked with diesel fuel to an initial concentration of 6400 mg/kg. Nutrients (NH_4NO_3, K_2HPO_4) were dissolved in water and added to soil based on a C:N:P of 100:5:1. TPH was analysed periodically for 102 days. Degradation of diesel contaminated soil was stimulated by the presence of ryegrass, demonstrating 57% TPH removal in planted soil compared with 36% in unplanted soil. The soil microbial population was not assessed, but soil gas CO_2 did not correlate with TPH loss rates. The reported efficiency of TPH removal is considerably lower than that recorded in the current study for two of the three Australian grasses assessed. Correlations between microbial parameters and TPH degradation by Australian grasses is discussed in Chapter 7. Interestingly, the authors reported that ryegrass improved TPH removal only after full root establishment (~40 days). Pre-germinated plants were sown in contaminated soil and the authors concluded that full development of the root system was a prerequisite to see additional benefits for rhizoremediation. In contrast, enhanced TPH removal was consistently demonstrated from the point of germination (defined as seedling emergence) onward for Australian grasses used in the current study. The planting of a seedling which has an established rhizosphere and associated microbial population into contaminated soil may influence rhizosphere dynamics compared with plants grown from seed. That is, the use of seedlings may introduce an established microbial population into another established population which exists in the soil. This could affect the rhizoremediation process and efficiency through competition and other microbial interactions in the rhizosphere.

Kaimi *et al* (2007a) screened twelve common plant species in Japan including three grasses for rhizoremediation of diesel contaminated soil. Italian ryegrass (*Lolium multiflorum*), Bermuda grass (*Cyndon dactylon*) and Southern crabgrass (*Digitaria ciliaris*) were assessed in soil spiked at a relatively high concentration of diesel (2% w/w; 20000 mg/kg). Liquid fertiliser was added weekly to the soil (N:P:K = 5%:10%:5%). After 140 days, TPH concentrations in soil planted with ryegrass or Bermuda grass were significantly lower than in unplanted controls. There was no significant difference between crabgrass and unplanted control outcomes. Percentage removal of TPH was 55% for ryegrass, 62% for Bermuda

grass and 40% unplanted control. In the current study, variation between diesel/oil removal efficiencies by individual grasses compared with unplanted controls was also observed (range 49-88%). This highlights the importance of species screening to optimise rhizoremediation performance outcomes.

Merkl *et al* (2005a) assessed tropical grasses and legumes naturalised in Venezuela for the rhizoremediation of soil spiked with heavy crude oil to 5% (w/w) (50000 mg/kg). Three grasses were examined individually and included the species *Brachiaria brizantha*, *Cyperus aggregatus* and *Eleusine indica* (common names not provided). The Australian grass species *Brachiaria decumbens* was assessed in the current study, which provides an interesting point of comparison. In the Merkl *et al* (2005a) research, NPK fertiliser (reported as 20:20:20) was applied every two weeks to all treatments. Soil planted with *B. brizantha* and *C. aggregatus* showed significantly lower total oil and grease (TOG) concentration after 180 days compared with non-vegetated soil. *B. brizantha* was the best performer (most effective) showing 22% less TOG than unplanted control by 180 days. Fraction analyses revealed soil planted with *B. brizantha* or *C. aggregatus* had 50% and 30% less saturates (respectively) than the unplanted control. Furthermore, *B. brizantha* caused 15% greater dissipation of aromatics than unplanted controls. In the current study, *B. decumbens* successfully enhanced removal of aliphatic hydrocarbons from soil (relative to control), showing 49% total TPH removal by 100 days. This is comparable to the removal of saturated fractions of TOG reported in the study by Merkl *et al* (2005a).

Information about the effectiveness of multispecies rhizoremediation of petroleum hydrocarbons is limited (Dominguez-Rosado and Pichtel 2004; Phillips *et al* 2006). In a field application the planting of multiple species may be desirable in order to re-establish site biodiversity. A thorough understanding of the effect of individual species, as well as species planted together, is important for the application of rhizoremediation in field situations. Results from the multispecies trial in the current study demonstrated that dual planting is important to assess since interactions can alter the outcome. In this instance a multispecies planting could still be an option for the current site since TPH

endpoints were as good as for best individual performer despite the overall removal rate being slower. In addition, the planting of multiple species may be beneficial even when some plants are not assisting in the remediation process. This is because *B. decumbens* is naturalised to the mine site and may assist biodiversity and rehabilitation of the area.

Maila and Randima (2005) assessed a multispecies grass trial of Velvet signal grass (*Brachiaria serrata*) and African millett (*Eleusine coracana*) for the rhizoremediation of a sandy loam soil artificially contaminated with a PAH mixture at 1% (naphthalene, fluorene and pyrene). Dissipation of PAH was compared with single species trials and unplanted controls. PAH dissipation was found to be higher in planted soils compared to unplanted soil after ten weeks of incubation. No significant difference in PAH removal was found between single species treatments with the different grasses. However, naphthalene removal was higher in the multispecies soil compared to the single species and unplanted soils. Reported removal of naphthalene was to undetectable levels for the multispecies, 96% reduction in the single species and 63% reduction in the unplanted soils. Similar removal efficiencies were found for fluorene. Reductions of 96% from the mulitspecies treatment, 81% in the best single species treatment (*E. corocana*) and 47% in the unplanted control soil were recorded. For pyrene (a higher molecular weight PAH), no significant difference in removal was found between single and multispecies planted treatments. This report is in contrast to outcomes in the current study where the multispecies trial (which included a *Brachiaria* species) showed no added removal efficiency beyond the best single species outcome by the end of the experimental period (100 days). This may be due to the difference in rhizoremediation potential of the individual species in the current study, whereby one species may have contributed more to the remediation process. The presence of the second and less effective grass species (*B. decumbens*) may have contributed to the overall slower rate of removal recorded for the multispecies trial via interspecies competition than seen for the *C. ambiguus* single species treatment. This may be due to competitive interactions between rhizosphere microbial populations associated with each species. Microbial dynamics were not assessed in the multispecies trial, but warrants further study to clarify the role of populations associated with each

grass species. Alternatively, the presence of two species may have affected nutrient cycling and availability for microorganisms in the rhizosphere thereby slowing the rate of removal.

Phillips *et al* (2006) assessed the potential of six different prairie plants naturalised in Canada for the remediation of a weathered hydrocarbon contaminated flare-pit soil. The study assessed a range of single species planted treatments including grass species and a legume, and a mixed planted treatment (comprising all individual species). By the end of the 4.5 month trial, TPH concentrations were reduced between 4% and 49% in planted soils. Hydrocarbon degradation was distributed relatively evenly across F3 and F4 fractions for all treatments except one. The F3 and F4 hydrocarbon range are defined as carbon chain lengths between C16 to C30 and C34 to C50, respectively (ATSDR 1996). Typical petroleum products that are found in these fractions include diesel fuel, motor oil and lubricating oils (ATSDR 1996). Two grasses (creeping red fescue and perennial ryegrass) facilitated the highest and lowest overall reductions in TPH concentration (49% and 4%, respectively) when grown individually. Mixed plant treatments and alfalfa treatments facilitated the next lowest overall reduction of approximately 10%. This was attributed to the difference in relative numbers of hydrocarbon degrading microorganisms supported by individual species. Creeping red fescue rhizosphere soil had the highest number of degraders, while perennial ryegrass had the lowest. Further examination of links between TPH removal efficiencies by Australian grasses and microbial parameters such as relative numbers of hydrocarbon degrading microorganisms supported in the rhizosphere are discussed in Chapter 7.

6.5 Conclusion

Published studies and the current work demonstrate the varying influence of grass species on hydrocarbon rhizoremediation in the presence and absence of nutrient addition. In the current study, Australian grasses enhanced diesel/oil removal relative to unplanted controls without the need for nutrient addition. Addition of N and P to the system may improve the removal efficiencies further. Alternatively, nutrient addition to a rhizoremediation system involving native species which prefer low soil nutrients for growth may inhibit biodegradation efficiencies. The optimisation of rhizoremediation of diesel/oil in soil by the Australian native grasses with respect to nutrients would benefit from further investigation.

CHAPTER SEVEN

7 RELATIVE RHIZOREMEDIATION PERFORMANCES OF AUSTRALIAN GRASSES IN DIESEL/OIL CONTAMINATED SOIL

7.1 Introduction

Accurate assessment of the relative performance of new plant species in rhizoremediation technologies requires some essential considerations. A demonstrated tolerance of candidate plants to hydrocarbon contamination in soil is an important parameter. Chapter 3 detailed the individual germination and growth performance outcomes of Australian grasses in diesel/oil contaminated versus clean soil. Eight out of nine grasses were shown to successfully germinate from seed and tolerate the presence of diesel/oil contamination at the exposed concentrations. In some instances the presence of diesel/oil was stimulatory to plant growth, in particular root development (e.g. *C. ambiguus*). Since most hydrocarbon degradation is believed to occur through a rhizosphere effect (Joner *et al* 2001; Glick 2003) the associated microbial community must also be examined. This is particularly important since plant-microbe interactions and subsequent rhizosphere effect reportedly vary between plant species (Kuiper *et al* 2004; Arthur *et al* 2005). Chapter 4 described each grass' influence on the abundance and activity of hydrocarbon degrading microorganisms in the rhizosphere. Species-specific plant-induced changes in the soil microbial community were observed for all grasses. Changes in microbial numbers and lipase activity were indicative of a stimulated process and differed between species. Results from DGGE profiles outlined in Chapter 5 were inconclusive but suggest no new microbial population was favoured by the grasses (qualitative shift), rather relative quantitative changes in existing members of the microbial population. Ultimately the assessment of biodegradation of hydrocarbons in soil is essential to characterise the effectiveness of rhizoremediation. Chapter 6 revealed the Australian grasses successfully enhanced diesel/oil removal relative to unplanted controls without the need for nutrient addition.

Some researchers have reported solely on survivability and growth of plants in hydrocarbon contaminated soil (Adam and Duncan 2003). Others assess the indigenous soil microbial communities in order to determine whether rhizoremediation is a viable cleanup option (Phillips *et al* 2006; Yateem *et al* 2007). To fully exploit this technology, understanding the complexity of rhizosphere dynamics that result in the reduction of petroleum hydrocarbons is required. The use of multiple parameters for the assessment of rhizoremediation ability is desirable in order to accurately determine the potential application of new species.

There is a paucity of data that have encompassed the relationship between plant growth, microbial activity and degradation of contaminants (Kaimi *et al* 2006). For example, Gunther *et al* (1996) showed that enhanced hydrocarbon degradation was caused by an increased rhizosphere microbial community (in comparison to unplanted soil), but root growth was not studied. Hou *et al* (2001) found an increase in biodegradation subsequent to the formation of higher ryegrass root intensity, but did not evaluate time-course change. Banks *et al* (2003a) demonstrated that the greatest decrease in petroleum hydrocarbon (TPH) concentrations occurred in the period with greatest root growth, but did not evaluate concurrent microbial activity.

The purpose of this chapter is to review all parameters investigated during the course of this multi-faceted approach for the assessment of rhizoremediation potential of native grasses in a diesel/oil contaminated mine site soil.

7.2 Materials and methods

7.2.1 Statistical analysis

Data from multiple variables presented in previous chapters were statistically analysed to identify and describe relationships between parameters for each grass species. These included TPH concentration in soil (Chapter 6), MPN data and soil lipase activity (Chapter 4), and plant growth data in the presence of contamination (Chapter 3). Plant growth performance was obtained at three time points only (4, 8 and 12 weeks after planting), thus statistical comparison with other variables can only be made at matching time points.

Prior to statistical analysis, data were tested for normality and homogeneity of variance using SPSS v.14 for Windows and GraphPad Prism v.4.0. Pearson correlations (r_p) (2-tailed) were preformed to describe the relationship between measured variables. It is important to note that much of the important changes in soil variables occurred in the initial stages of experiments prior to first plant growth assessment (4 weeks). This infers that correlations at later time points may not reflect the true relationships between these parameters as they relate to degradation and growth performance.

7.3 Results

Data from multiple variables were statistically analysed to reveal relationships between parameters for each grass species. **Figure 7.1** presents combined data on residual TPH concentration, numbers of hydrocarbon degrading microorganisms (MPN), and soil lipase activity in the rhizosphere of *B. decumbens* measured over time (see also Chapters 4 and 6). In the initial 20 days after planting (DAP) an inverse relationship was observed between residual TPH concentration, and the number hydrocarbon degrading microorganisms (MPN) and soil lipase activity for this species. Thereafter a gradual decrease in abundance of hydrocarbon degrading microorganisms in the rhizosphere was recorded once TPH concentration had reached a plateau.

Table 7.1 outlines Pearson correlations (r_p) between soil parameters in the rhizosphere measured for *B. decumbens*, including their significance, correlation coefficients and the number of measurements available per parameter. A significant negative correlation was found between residual TPH concentration and number of hydrocarbon degrading microorganisms (MPN) in the rhizosphere of *B. decumbens* (r_p = -0.504, p = 0.001). A significant (although weaker) correlation was also observed between TPH concentration and soil lipase activity in the presence of the grass (r_p = -0.350, p = 0.027). Also in the rhizosphere, the number of hydrocarbon degrading microorganisms (MPN) and soil lipase activity were shown to be significantly positively correlated (r_p = 0.323, p = 0.042). In the unplanted control soil, no significant correlation between parameters (TPH, MPN or lipase activity) was found ($p > 0.05$). Only a relationship between TPH concentration and time was found for the unplanted control soil (r_p = -0.794, $p < 0.001$). At 30, 60 and 90 DAP (4, 8 and 12 weeks plant growth) no significant correlation was found between *B. decumbens* growth parameters (e.g. root biomass, root:shoot ratio, see Chapter 3) and other measured parameters (TPH, MPN, lipase activity) ($p > 0.05$) at these corresponding time points. The Pearson correlation summaries for unplanted controls and plant growth parameters for *B. decumbens* are given in **Appendix IV** - Tables 1 and 2.

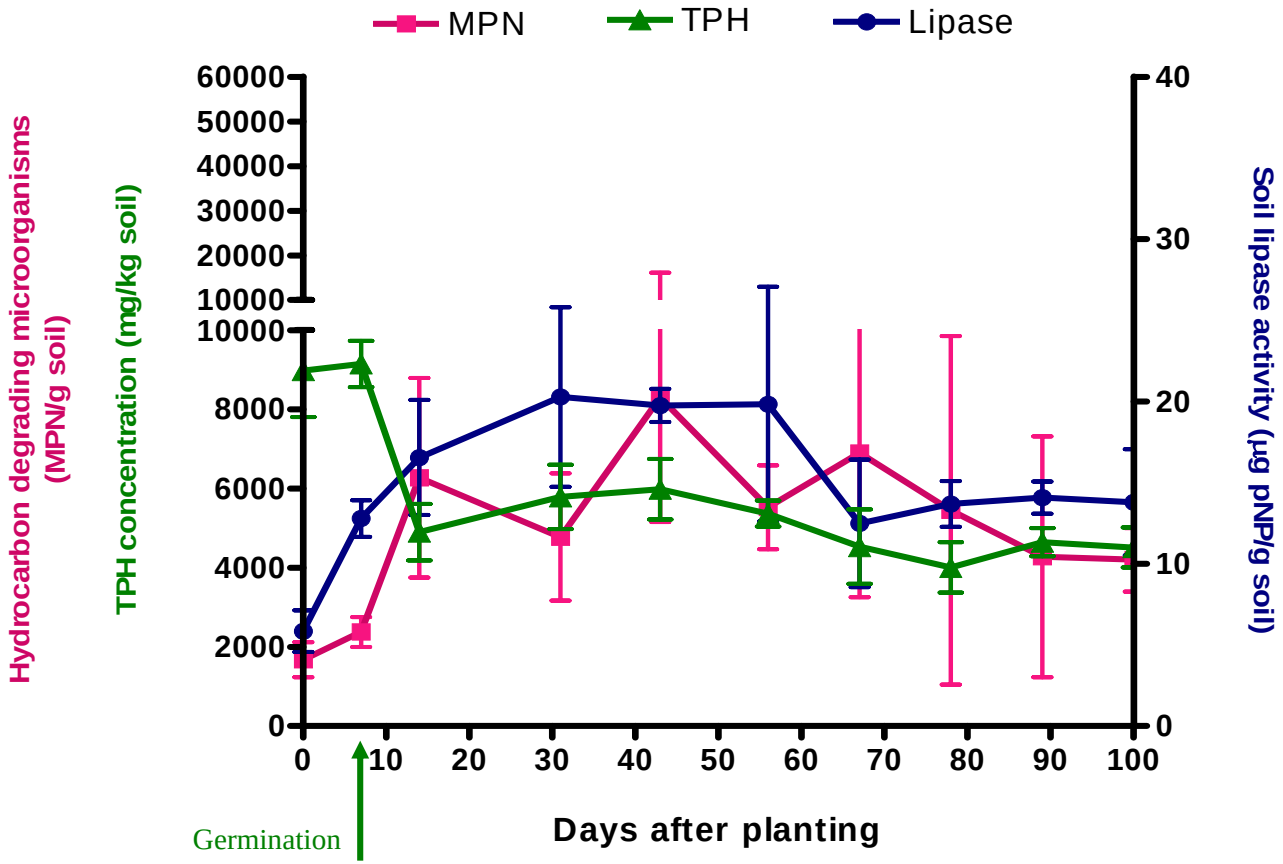


Figure 7.1 Pattern of TPH degradation and changes in number of hydrocarbon degrading microorganisms (MPN) and lipase activity over time in soil planted with *B. decumbens*. Residual TPH concentration and MPN data on left Y-axis. Soil lipase activity on right Y-axis. Values are means ± SD.

Table 7.1 Pearson correlation coefficients for measured parameters in the rhizosphere of *B. decumbens*. * Correlation is significant at the 0.05 level (2-tailed) (light shading). ** Correlation is significant at the 0.01 level (2-tailed) (dark shading).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/g soil	Lipase activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	59			
TPH (mg/kg soil)	Pearson Correlation	-.737(**)	1		
	Sig. (2-tailed)	.000			
	N	59	59		
MPN/g soil	Pearson Correlation	.201	-.504(**)	1	
	Sig. (2-tailed)	.213	.001		
	N	40	40	40	
Lipase activity (µg pNP/g soil)	Pearson Correlation	.124	-.350(*)	.323(*)	1
	Sig. (2-tailed)	.447	.027	.042	
	N	40	40	40	40

Figure 7.2 presents combined data on residual TPH concentration, numbers of hydrocarbon degrading microorganisms (MPN), and soil lipase activity in the rhizosphere of *C. ambiguus* measured over time. In the initial 14 days after planting (DAP) a marked inverse relationship was observed between residual TPH concentration and the number of hydrocarbon degrading microorganisms (MPN). This was also seen to a lesser extent between TPH concentration and soil lipase activity for this species during the same period. Subsequently a slow decrease in number of hydrocarbon degrading microorganisms was measured as the TPH concentration reached a plateau (as observed for *B. decumbens*). Soil lipase activity remained relatively constant throughout the remainder of the experimental period.

Table 7.2 outlines Pearson correlations (r_p) between soil parameters in the rhizosphere measured for *C. ambiguus*. Similar relationships were observed between parameters for this species as seen for *B. decumbens*. A significant negative correlation was noted between residual TPH concentration and number of hydrocarbon degrading microorganisms (MPN) in the rhizosphere of *C. ambiguus* (r_p = -0.592, $p < 0.001$). A significant correlation was also observed for residual TPH concentration and soil lipase activity in the rhizosphere of this species (r_p = -0.346, $p = 0.020$). The abundance of hydrocarbon degrading microorganisms (MPN) in the rhizosphere was shown to significantly positively correlate to soil lipase activity (r_p = 0.324, $p = 0.030$). In corresponding unplanted soil (controls) for this trial, no significant correlations were recorded for any measured parameters (TPH, MPN or soil lipase activity) ($p > 0.05$). Growth parameters recorded for *C. ambiguus* (see Chapter 3) over three time points (30, 60 and 90 DAP) did not show significant correlations to any other measured parameter in the rhizosphere ($p > 0.05$) (see summary correlation Tables 3 and 4 in **Appendix IV**).

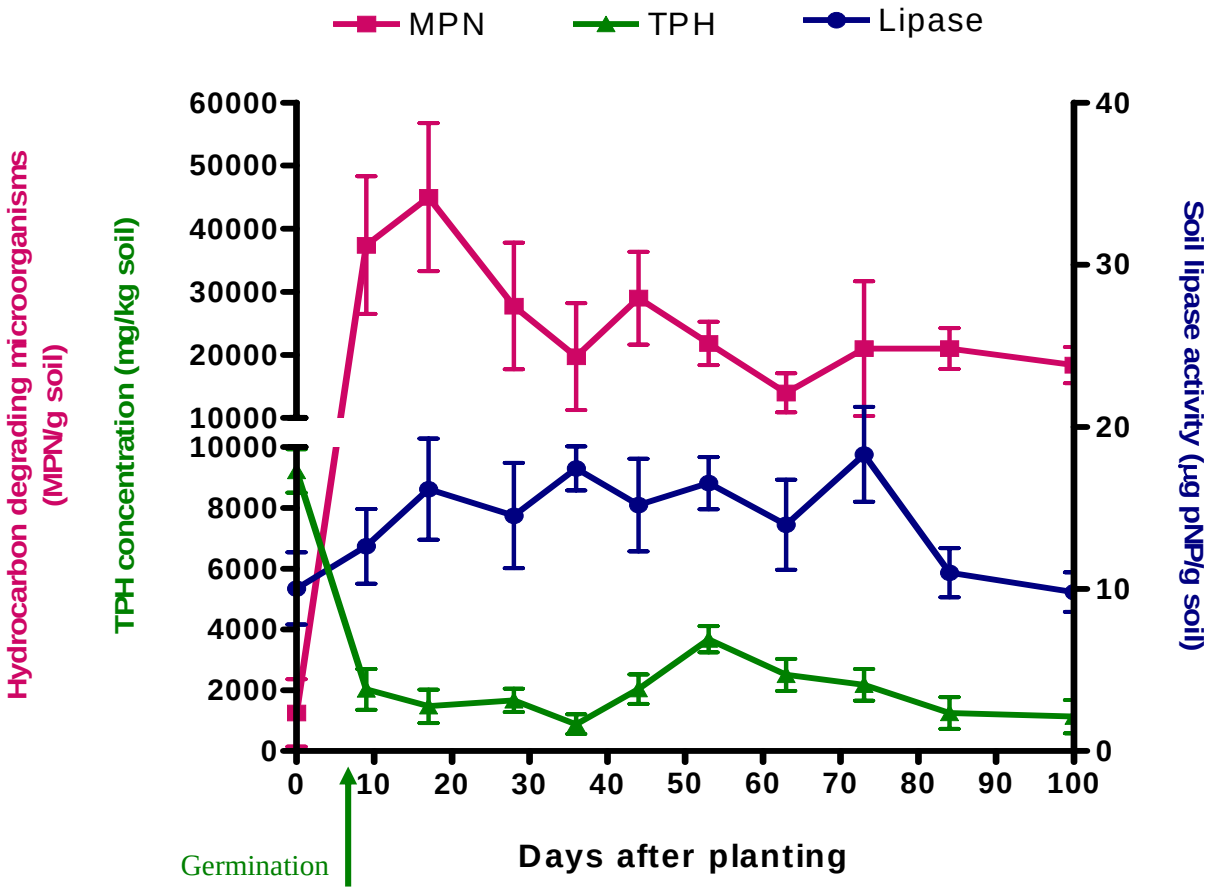


Figure 7.2 Pattern of TPH degradation and changes in number of hydrocarbon degrading microorganisms (MPN) and lipase activity over time in soil planted with *C. ambiguus*. Residual TPH concentration and MPN data on left Y-axis. Soil lipase activity on right Y-axis. Values are means ± SD.

Table 7.2 Pearson correlation coefficients for measured parameters in the rhizosphere of *C. ambiguus*. * Correlation is significant at the 0.05 level (2-tailed) (light shading). ** Correlation is significant at the 0.01 level (2-tailed) (dark shading).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/ g soil	Lipase activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	63			
TPH (mg/kg soil)	Pearson Correlation	-.477(**)	1		
	Sig. (2-tailed)	.000			
	N	61	61		
MPN/ g soil	Pearson Correlation	-.155	-.592(**)	1	
	Sig. (2-tailed)	.245	.000		
	N	58	56	58	
Lipase activity (µg pNP/g soil)	Pearson Correlation	-.016	-.346(*)	.324(*)	1
	Sig. (2-tailed)	.916	.020	.030	
	N	45	45	45	45

Correlations between parameters in the rhizosphere of *M. stipoides* provided interesting contrast to the other grass species, particularly with respect to soil lipase activity. **Figure 7.3** presents combined data on residual TPH concentration, numbers of hydrocarbon degrading microorganisms (MPN), and soil lipase activity in the rhizosphere of *M. stipoides* measured over time. In the initial 20 DAP a strong inverse relationship was observed between TPH concentration and microbial parameters (MPN and lipase activity) for this cool season grass species. This pattern is then sustained to some degree between TPH concentration and numbers of hydrocarbon degrading microorganisms (MPN).

Table 7.3 outlines Pearson correlations (r_p) between soil parameters in the rhizosphere measured for *M. stipoides*. Correlation analysis for this species reveals interesting contrasts to relationships found for the other two grasses, particularly with respect to microbial data. A significant negative correlation was found between residual TPH concentration and number of hydrocarbon degrading microorganisms (MPN) (r_p = -0.449, p = 0.004). This is a similar relationship to that observed for the other grass species. With respect to soil lipase activity and its relationship to other soil parameters, correlations found for *M. stipoides* are in contrast to that observed for the other species. No significant correlation was found between residual TPH concentration and soil lipase activity for *M. stipoides* ($p > 0.05$). Nor was a significant relationship found between number of hydrocarbon degrading microorganisms (MPN) and soil lipase activity for this species ($p > 0.05$). Another point of contrast not observed for other species was the relationship found between microbial data and time (as days after planting, DAP). For *M. stipoides,* time (DAP) was shown to positively correlation with MPN data (r_p = 0.469, p = 0.002) and negatively correlate with soil lipase activity (r_p = -0.335, p = 0.035) in the rhizosphere.

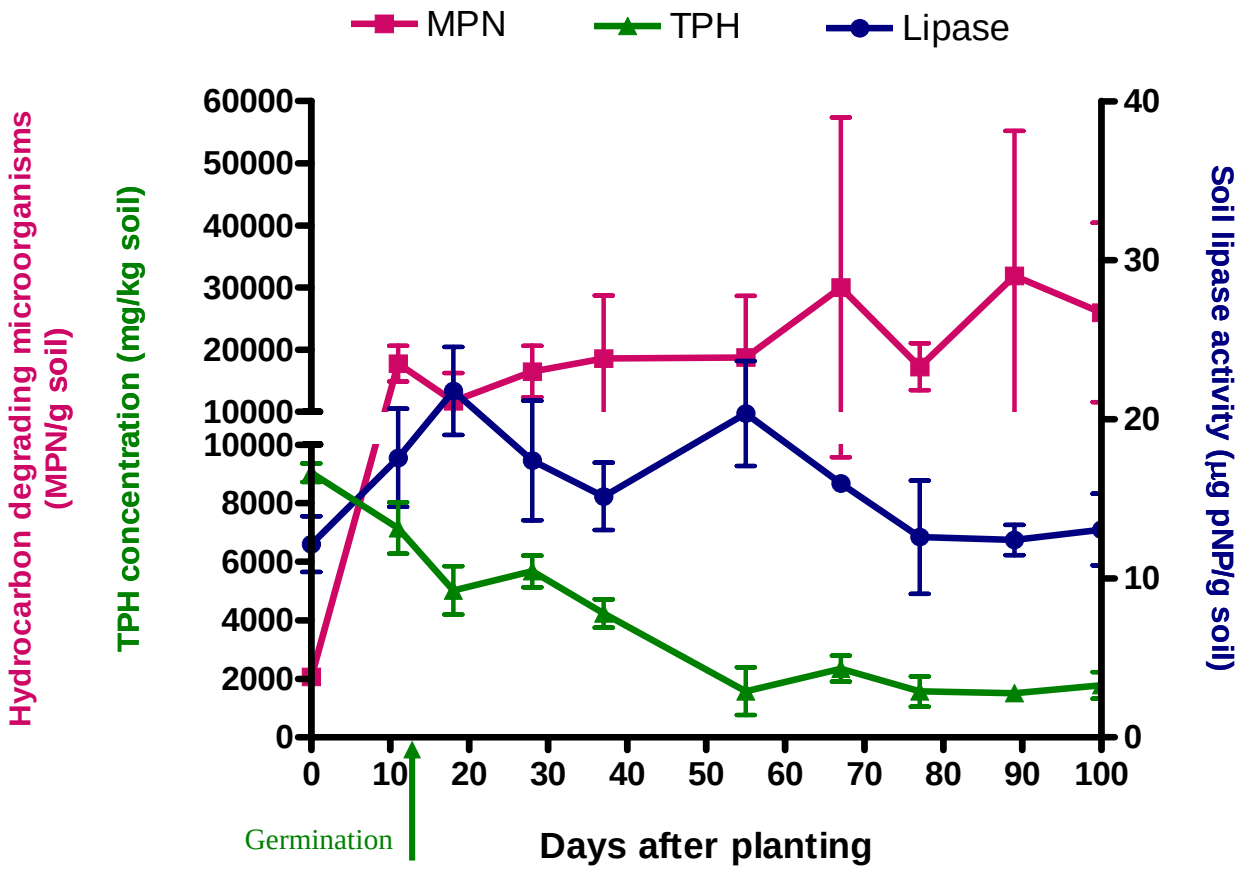


Figure 7.3 Pattern of TPH degradation and changes in number of hydrocarbon degrading microorganisms (MPN) and lipase activity over time in soil planted with *M. stipoides*. Residual TPH concentration and MPN data on left Y-axis. Soil lipase activity on right Y-axis. Values are means ± SD.

Table 7.3 Pearson correlation coefficients for measured parameters in the rhizosphere of *M. stipoides*. * Correlation is significant at the 0.05 level (2-tailed) (light shading). ** Correlation is significant at the 0.01 level (2-tailed) (dark shading).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/g soil	Lipase activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	59			
TPH (mg/kg soil)	Pearson Correlation	-.894(**)	1		
	Sig. (2-tailed)	.000			
	N	58	58		
MPN/g soil	Pearson Correlation	.469(**)	-.449(**)	1	
	Sig. (2-tailed)	.002	.004		
	N	40	40	40	
Lipase activity (µg pNP/ g soil)	Pearson Correlation	-.335(*)	-.008	-.059	1
	Sig. (2-tailed)	.035	.963	.718	
	N	40	40	40	40

In unplanted control soils for the *M. stipoides* trial, a significant positive correlation was found between residual TPH concentration and soil lipase activity ($r_p = 0.483$, $p = 0.002$). This was not observed in the unplanted soil controls used in the trials for warm season grasses *B. decumbens* and *C. ambiguus*, nor in the planted systems of any species. When relationships between growth parameters of *M. stipoides* (see also Chapter 3) and rhizosphere soil variables were assessed, no significant relationships were noted (see correlation summary Tables 5 and 6 in **Appendix IV**).

7.4 Discussion

The Australian grasses assessed in the current study were all shown to effectively enhance remediation of diesel/oil contaminated soil. The species-specific rhizoremediation outcomes, however, were not uniform.

By examining individual plant influence on microbial parameters in the soil, a clearer understanding of species-specific induced responses can be gained. Evidence in the current work supports microbially-facilitated remediation as a major pathway of removal from soil, enhanced by the presence of grasses (the rhizosphere effect). As shown earlier in this chapter (Section 7.3), a significant (negative) correlation was found between number of hydrocarbon degrading microorganisms in the rhizosphere of grasses and residual concentration of diesel/oil contamination in soil for all plant species. This relationship was not observed in the unplanted control soils, suggesting evidence of the rhizosphere effect. In unplanted control soils the only significant relationships found were between measured soil parameters and time (for example TPH concentration negatively correlated with time $p < 0.0001$).

In contrast, a clear relationship was not demonstrated in all cases with respect to soil lipase activity and diesel/oil removal in the presence of grasses. Lipase activity has been previously utilised in bioremediation studies as a measure of soil biological activity and as a bioindicator of remediation (Margesin *et al* 1999; Lee *et al* 2008). The induction of soil lipase activity was shown to be a valuable indicator of biodegradation in freshly diesel contaminated soils (Margesin *et al* 1999) and aged heavy mineral oil contaminated soil (Lee *et al* 2008). In this chapter, soil lipase activity was shown to significantly (negatively) correlate with residual diesel/oil concentration for *B. decumbens* and *C. ambiguus* but not *M. stipoides*. However, *M. stipodes* performed better in terms of rhizoremediation outcome than *B. decumbens*. Overall soil lipase activity was shown to be similar over time and between species (see Figures 7.1-7.3). This suggests that the soil lipase assay encompasses total soil microbial activity rather than exclusively hydrocarbon degrading microorganisms of interest. Margesin *et al* (2000) also

reported relatively constant lipase activity following an initial rapid increase in a bioremediation study of diesel and PAH contaminated soil. In the current work, a variable relationship was also found between soil lipase activity and number of hydrocarbon degrading microorganisms (MPN) in the rhizosphere of grasses. A significant (positive) relationship between these parameters was only demonstrated for two of the grasses (*B. decumbens* and *C. ambiguus*). Again this points to the value of assessing multiple variables in screening species for remediation potential. Soil lipase activity does not appear to be an ideal bioindicator of remediation if used in isolation. This is demonstrated using results for *B. decumbens* which would have indicated it to be the most effective in remediation showing the greatest soil lipase activity overall compared with the other two grasses (Section 4.3.3.2). In fact this species was shown to be the least effective when compared with the other grasses for the rhizoremediation of diesel/oil from soil (Section 6.3.1).

It appears that monitoring the abundance of hydrocarbon degrading microorganisms in the rhizosphere may be a more appropriate bioindicator for rhizoremediation potential than soil lipase activity. The pattern of diesel/oil removal from soil for each species was found to be strongly related to the stimulation of hydrocarbon degrading microorganisms in the rhizosphere, as demonstrated by the significant (negative) correlations.

Plant root development has been shown to be an important parameter to include when assessing the rhizoremediation potential of a species (Merkl *et al* 2005a; Kaimi *et al* 2006). Well developed fibrous roots (biomass) are thought to provide a larger surface area for colonisation by soil microorganisms (Kaimi *et al* 2007a; 2007b), thereby maximising the rhizoremediation potential. For example, Merkl *et al* (2005a) showed a significant positive correlation between root biomass production of two Venezuelan grasses and subsequent oil degradation in soil. In the current study, no significant relationship between plant growth parameters and diesel/oil removal was observed. This may be due to the limitation in time points available for statistical comparison between parameters. The assessment of plant growth variables began at four weeks growth (30 DAP), by which time much of the rhizoremediation had already taken place (Section 6.3.1).

Monitoring root growth more closely immediately after germination may allow for clearer elucidation of the role of biomass production in microbial stimulation and hydrocarbon biodegradation for these Australian grasses. It appears plant growth parameters may not always reflect (correlate with) hydrocarbon degradation potential. Plants have even been shown to be adversely affected in growth but still facilitate enhanced removal of the contaminant (Kulakow *et al* 2000; Merkl *et al* 2005a; Liste and Prutz 2006). Alternatively, species can show high tolerance in terms of growth performance that may not correspond to greatly enhanced remediation, as observed in the current work with *B. decumbens* compared to the other grasses. Parrish *et al* (2005) provided a clear example of this in a 12 month greenhouse study to evaluate rhizoremediation of PAHs in soil using Tall fescue and yellow sweet clover. Although tall fescue had the highest root and shoot biomass and root surface area, the species did not result in the highest contaminant degradation rates. This is despite having significantly greater number of PAH degrading microorganisms in the rhizosphere compared with unvegetated controls. These results combined with those obtained in the current work indicate that there is not always a clear link between the degradation of hydrocarbons in soil and root growth parameters. In a multi stage approach it remains important to screen plants for growth performance in order to select for species least adversely affected by the presence of contamination. The important role of the plant should include factors other than rhizoremediation endpoints, such as impact on site biodiversity and overall restoration.

The use of a multi-faceted approach has enabled accurate characterisation of rhizoremediation potential of native grasses in diesel/oil contaminated soil. In this regard, relative overall performance demonstrates *C. ambiguus* to be the best candidate. This species was shown to stimulate the rhizosphere microbial community capable of degrading diesel/oil to the greatest degree (Sections 4.3.1 and 4.3.2), and subsequently demonstrated greatest TPH removal from soil (Section 6.3.1). Results revealed *M. stipoides* to be equally successful as *C. ambiguus* in the rhizoremediation of diesel/oil from soil. Despite the variable results obtained for soil lipase activity as it related to residual TPH

concentrations and MPN data, overall rhizoremediation performance was equal to that found for the warm season species *C. ambiguus*.

The overall relative performance of *B. decumbens* again highlights the desirability of using a multi-faceted approach in screening species for their rhizoremediation potential. For example, if a single parameter were used to characterise this species' potential, a very different conclusion may have been drawn. This species grew well in diesel/oil contaminated soil at the exposed concentrations (Section 3.3.2). Soil lipase activity also suggested it could be successful at mediating microbially-enhanced remediation (Section 4.3.2). Ultimately, *B. decumbens* demonstrated significant stimulation of hydrocarbon degrading microorganisms in the rhizosphere and subsequently enhanced removal of diesel/oil relative to unplanted control soil. It is also clear that the relative performance of this species in relation to the other grasses was not as good. The outcomes of individual parameters measured for *B. decumbens* provide conflicting conclusions. The information as a whole more accurately characterises the potential application of this species to rhizoremediation of diesel/oil contaminated mine site soil.

7.5 Conclusion

Mechanisms of rhizoremediation are still not well understood. By using a multi-faceted approach to screen new species for their rhizoremediation potential a more complete characterisation of relative performance can be gained. Employing this strategy, the relative performance of Australian grasses for the rhizoremediation of diesel/oil contaminated soil was assessed by analysing all previously presented data (Chapters 3, 4 and 6). All species demonstrated high tolerance to the presence of diesel/oil contamination without adverse growth affect (Chapter 3). However, root growth performance was not shown to significantly correlate to biodegradation outcomes or changes in soil microbial populations in the rhizosphere (see Section 7.3). Despite this, assessment of plant growth is still important in order to select species able to tolerate the contaminated soil conditions and help in site restoration and rehabilitation. All grass species were shown to stimulate the rhizosphere microbial community capable of degrading diesel/oil, to varying degrees (as shown in Chapter 4). Statistical analysis presented in this chapter demonstrated a strong relationship (correlation) between microbial numbers in the rhizosphere and TPH biodegradation for all species. Those species which showed greatest stimulation of the microbial population resulted in enhanced TPH removal from soil (see also Chapter 6). These were the summer grass *C. ambiguus* and the winter species *M. stipoides*. *B. decumbens* showed successful rhizoremediation to a lesser degree, but may still be an option in multiple planting strategies with *C. ambiguus*.

CHAPTER EIGHT

8 GENERAL DISCUSSION

The work described in this thesis has centred on the evaluation of Australian native grasses in the rhizoremediation of aliphatic hydrocarbon contaminated soil from a mine site. The major outcomes of this research have been:

1. Development and application of plant selection criteria and screening protocol for plant species to determine ideal candidates for the rhizoremediation of hydrocarbon contaminated soil in Australia (Chapter 2).
2. Characterisation of selected grass species for tolerance to hydrocarbon contaminated soil in germination and growth performance trials in diesel and oil contaminated soil from a mine site (Chapter 3).
3. Assessment of quantitative and qualitative changes in microbial community dynamics in the rhizosphere of Australian grasses during the rhizoremediation process (Chapters 4 and 5).
4. Evaluation of the capability of Australian grass species to enhance hydrocarbon removal from mine site soil (Chapter 6).
5. Review of the relative performances of Australian grasses investigated during the course of this multi-faceted approach for the assessment of rhizoremediation potential of native grasses in a diesel/oil contaminated mine site soil (Chapter 7).

From the results of the current study, it is clear that degradation of diesel/oil in soil is stimulated by the presence of Australian grasses (Chapter 6). Plant-specific changes in microbial populations in the rhizosphere (Chapter 4) affected degradation of petroleum hydrocarbons in contaminated soil. Greater bacterial counts in the rhizosphere including specific organisms capable of metabolising a contaminant of concern are considered significant to plant-fostered biodegradation (Liste and Prutz 2006). For example, Kamath *et al* (2004) found that plant facilitated PAH disappearance correlated well with the abundance of root-associated bacteria populations capable of degrading PAH. Uptake of diesel/oil into plant tissue is considered limited due to the high lipophilicity of hydrocarbons (Siciliano *et al* 2003), and was confirmed to be negligible in the current work (Section 6.3.1).

The use of a multi stage approach adopted in the current research allowed for a more accurate characterisation of individual species' rhizoremediation potential. This was demonstrated in a number of ways. The initial stage of species inclusion in the study was based on development and application of selection criteria (Chapter 2). Based on these criteria, all species included in the current research were considered ideal candidates for rhizoremediation application. Subsequently, growth performance in diesel/oil contaminated soil revealed that all screened grasses had high tolerance to the presence of contamination at the exposed concentrations (Chapter 3). If selection criteria and growth performance were used in isolation to assess rhizoremediation potential, all screened grass species could be considered equally suitable.

The species-specific rhizoremediation outcomes, however, were not uniform. In fact the resultant rhizoremediation outcome of diesel/oil contamination in soil was not equal between species. *C. ambiguus* and *M. stipoides* demonstrated significantly greater diesel/oil removal efficiencies than *B. decumbens* in single species trials (Chapter 6). This is particularly advantageous since *M. stipoides* has cool season growth whereas *C. ambiguus* is a summer growing species. These optimal growth differences allow for greater application throughout the seasons and potentially at other sites in temperate and tropical regions.

The multispecies planting with *B. decumbens* and *C. ambiguus* (Chapter 6) had no additional influence on total TPH removal. Final TPH concentration was not significantly different from that found for the best single species performer (i.e. *C. ambiguus*) however the rate of TPH removal was slower when both species were present. The underlying assumption has been that the effects of mixed plant populations would be synergistic, with a greater positive total effect than the sum of each individual species effect (Phillips *et al* 2006). This was not demonstrated in the current work using two summer growing Australian species. Nevertheless, in a field application the planting of multiple species may still be desirable in order to preserve site biodiversity. This is particularly applicable in the current work since one species (*B. decumbens*) is native to the mine site and sourced from seed banks on site.

The desirability of using multiple stages in the assessment of species for rhizoremediation potential is further highlighted using data obtained for *C. ambiguus*.

Results suggest that assessing single factors may not necessarily be predictive of rhizoremediation outcomes, whereas multiple factors can clarify the true extent of species potential. For example, in plant growth performance experiments *C. ambiguus* was shown to significantly stimulate root growth (in particular biomass) in the presence of contamination (Chapter 3). This may have provided a greater area for microbial colonisation as is suggested in the literature (Gunther *et al* 1996; Germida *et al* 2002; Merkl *et al* 2005a). In fact, root growth parameters were not shown to significantly correlate to numbers of hydrocarbon degrading microorganisms, nor residual TPH concentrations for this or any species studied (Chapter 7). These statistical outcomes may have been due to the limitation in comparison between these parameters for clear relationships. That is, much of the microbial stimulation in the rhizosphere (Chapter 4) was recorded prior to the first root growth measurements (Chapter 3). Alternatively, it may point to species-specific root exudation patterns at early stages of growth in response to the presence of contamination.

The observed *rhizosphere effect* may be due to plant root exudates that provide a source of carbon and energy for microorganisms in the root zone. Selective enrichment of degrading microbial populations may be achieved through altering exudation, ultimately resulting in enhanced biodegradation of hydrocarbons (Kirk *et al* 2005). Plants may respond to chemical stresses by increasing or changing exudation, which modifies the rhizosphere microflora (Miya and Firestone 2000; Parrish *et al* 2005). Reilley *et al* (1996) reported that pyrene degradation rates increased in rhizosphere soil and were highest when rhizosphere organic acids were added to the soil. Root exudate patterns are known to be dependent upon plant species and the stage of plant development (Germida *et al* 2002; Kuiper *et al* 2004), as well as physicochemical environment and presence of environmental stresses (Jones 1998). Hegde and Fletcher (1996) found that the release of phenolics by the roots of red mulberry (*Morus rubra* L.) increased continuously from an early vegetative stage to leaf senescence, with a massive release at the end of the season accompanying leaf senescence. Similarly, Leigh *et al* (2002) reported a two-fold increase in the concentration of phenolic compounds in fine roots of mulberry during the later stages of the growth season. This in turn promoted the growth and activity of bacteria capable of degrading organic pollutants such as PCB.

Exudation patterns in Australian grasses were not assessed in the current study. While the Australian grasses may release exudates into the rhizosphere (Crawford and Wilkens 1998; Roelofs *et al* 2001), they may differ or be species-specific in terms of molecules and the quantity secreted. Since this is the first time these Australian grasses have been screened for rhizoremediation of hydrocarbon contaminated soil, limited information exists on the individual responses to contamination in terms of exudation patterns. One example reported in the literature suggests *B. decumbens* can hyper-excrete organic acids such as citrate under aluminium contamination stress (Wenzl *et al* 2001). Wenzl *et al* (2001) measured organic acid exudation by whole roots of *Brachiaria decumbens* cultivated in nutrient solution containing aluminium (Al^{3+} at 115µM). Citrate exudation was found to be stimulated by approximately five times under aluminium stress. The authors suggested that this mechanism allowed for aluminium resistance in the species through external detoxification and formation of non-toxic aluminium chelates. The mine site soil used in the current research showed trace levels of aluminium (Section 2.3.1) however at the recorded pH (6.3) this was not likely to be in a bioavailable form for plants. Nevertheless, such a mechanism may have contributed in part to the stimulation of rhizosphere microbial community observed for this species (Chapter 4). Furthermore, citric acid is a compound thought to induce hydrocarbon degrading enzymes and increased hydrocarbon mineralisation (Liste and Prutz 2006). Increased lipase activity and diesel/oil biodegradation was recorded for *B. decumbens* relative to unplanted control soil (Chapters 4 and 6). This is despite *B. decumbens* demonstrating poorer overall relative performance compared with the other grasses screened (Chapter 7). Further investigation may help elucidate the role of grass exudates in microbial stimulation within the rhizosphere, as well as contaminant availability to microbial degraders enhancing hydrocarbon biodegradation.

Other researchers have found variable relative performances between species for rhizoremediation of hydrocarbons from soil particularly with respect to relationships between parameters (e.g. microbe abundance and biodegradation) (see also Chapter 7). Recent work by Kaimi *et al* (2007a) encompassed the relationship between plant growth, microbial activity and degradation of petroleum contaminants. Twelve plant species including several grasses were screened for their rhizoremediation ability for the cleanup of diesel contaminated soil (spiked at 2% w/w) in Japan. During the 141

day greenhouse study, changes in TPH concentration, soil dehydrogenase activity (DHA) and the number of aerobic bacteria were evaluated along with general plant growth. The grasses were shown to have longer roots than other plant types, and at mature stage of growth several species demonstrated significantly lower TPH concentration in soil than unplanted controls. A strong relationship between TPH concentration and soil DHA was found for all species screened, but the degree of correlation differed between species. In the current research, a significant relationship between residual TPH and soil lipase activity was found for two of the three grasses studied. Kaimi *et al* (2007a) found microbial counts were not correlated with the TPH concentration in soil. In contrast, the number of hydrocarbon degrading microorganisms was significantly (negatively) correlated to residual TPH for all grass species in the current study. DHA is a measure of total microbial activity in the soil and can indicate the onset of biodegradation processes (Margesin *et al* 1999; Maila and Cloete 2005). It is also a surrogate marker for mineralisation as it is linked to CO_2 production via the respiratory chain. Results of Kaimi *et al* (2007a) suggest DHA may be a better index of degradation activity than lipase enzyme used in the current study.

Lee *et al* (2008) also assessed multiple parameters when characterising the degradation ability of four native Korean plant species (2 grasses, 2 legumes) in soil experimentally contaminated with pyrene and phenanthrene (PAH). During the 80 day greenhouse experiment, periodic assessment was made of plant growth, residual PAH content in soil and microbial activity (number of microorganisms and DHA). In most cases, plant growth was adversely affected showing root and shoot yields consistently lower in PAH contaminated soil than in control soil. This is in contrast to the growth performance of native grasses in the current work, which showed high tolerance to the presence of diesel/oil contamination (Chapter 3). Numbers of soil microbes (total viable population) fluctuated during the Lee *et al* (2008) experiment, but there were no significant differences in the abundance of microorganisms between planted and unplanted soil. Despite this, biological parameters measured during the experiment were shown to be significantly correlated with PAH concentration. The number of microorganisms were positively correlated with PAH concentration, whereas DHA was strongly negatively correlated with PAH concentration. Again this is in contrast to the relationship found in the current work where the number of hydrocarbon degrading microorganisms was seen to strongly negatively correlate with

residual TPH concentration (Chapter 7). This suggests total microbial counts as monitored by Lee *et al* (2008) may not reflect stimulation of specific hydrocarbon degrading microorganisms in the rhizosphere which contribute to the overall rhizoremediation outcomes.

Clear relationships between plant growth performance and microbial parameters in rhizoremediation also vary in the literature (Merkl *et al* 2005a; Phillips *et al* 2006). Merkl *et al* (2005a) assessed tropical plant species from Venezuela for the rhizoremediation of soil contaminated with 5% (w/w) heavy crude oil. In greenhouse experiments, three legumes and three grasses were tested for plant biomass production and ability to stimulate oil dissipation through enhanced microbial activity. The two perennial grass species (*Brachiaria brizantha* and *Cyperus aggregatus*) showed a significant relationship between root biomass and oil degradation in soil. The grasses showed reduced biomass production under the influence of the contaminant, but facilitated significantly lower residual oil concentration than unplanted soil. This is in contrast to the findings of the current study where root biomass was not seen to be significantly related to TPH concentration (including the perennial *Brachiaria decumbens*) (Chapter 7). This may be due to the high growth performance of the Australian grasses in comparison to the adverse growth effect noted in the previous study. Nevertheless, both the current work and previous report (Merkl *et al* 2005a) demonstrated reduced TPH concentrations and increased microbial activity in the presence of grasses.

Phillips *et al* (2006) similarly found no correlation between TPH concentration in soil and root or shoot biomass production in two grass species (perennial ryegrass and creeping red fescue). Both species significantly enhanced degradation of TPH in soil relative to unplanted controls, although to varying degrees. Perennial ryegrass demonstrated 4% reduction in TPH concentration by 4.5 months, while creeping red fescue resulted in 49% reduction. The authors attributed the difference to relative numbers of hydrocarbon degrading microorganisms supported by individual species. Creeping red fescue rhizosphere soil had the highest number of degrading organisms, while perennial ryegrass had the lowest. Therefore while stimulation of soil microorganisms has been assumed to be higher with increasing root biomass (i.e. larger rhizosphere for microbial population) (Gunther *et al* 1996; Germida *et al* 2002;

Merkl *et al* 2005a), it is not always required to see increased degradation. This again highlights the potential role of other factors such as exudation patterns in individual species under contaminated conditions, and its affect on the soil microbial community (rhizosphere effect).

Another microbial factor potentially contributing toward rhizoremediation is the role of mycorrhizae (Robertson *et al* 2007). Interactions between plant roots and their symbionts such as arbuscular mycorrhizal (AM) fungi may contribute to the survival and growth of plants in soil contaminated with hydrocarbons (Binet *et al* 2000a). Further, mycorrhizae have been shown to modify root physiology (enzyme activity, exudation) in a manner that stimulates the degradation of PAH (Joner *et al* 2001; Joner and Leyval 2003). Joner *et al* (2001) were among the first to demonstrate enhanced PAH degradation in the presence of AM fungi associated with the rhizosphere of clover and ryegrass. Mycorrhizal colonisation of roots can also result in increased root surface area for nutrient acquisition (Khan *et al* 2000). This could be of significant benefit since inputs of large quantities of carbon sources (i.e. hydrocarbons) tend to result in rapid depletion of the available pools of inorganic nutrients such as nitrogen and phosphorus (Margesin *et al* 2007). Nutrient limitation can be a rate limiting step in the rhizoremediation process (Germida *et al* 2002). The Australian grasses used in the current research were not studied for AM fungi colonisation. There is a paucity of information in the literature regarding the potential incidence of AM fungi associated with native grasses used in the current study. A generally held view is that mycorrhizae are ubiquitous and are present in the majority of plants growing under natural conditions (Khan *et al* 2000; Joner and Leyval 2003; Robertson *et al* 2007). The potential impact of mycorrhizas in the context of diesel/oil rhizoremediation using native grasses may benefit from further investigation.

This study sought to determine whether the presence of selected Australian grass species enhanced the removal of aliphatic hydrocarbons from a mine site soil. The rhizosphere of grasses was shown to exert a positive effect on the abundance and activity of indigenous microbial populations capable of degrading diesel and oil in soil (Chapter 4). The influence of Australian grasses on microbial community structure (defined as community DNA fingerprint) in diesel/oil contaminated soil remains unclear. Results from DGGE profiles suggest no new population was

favoured by the grasses (qualitative shift) (Chapter 5), rather relative quantitative changes in existing members of the microbial population (Chapter 4). Consistent with previous reports (Dominguez-Rosado and Pichtel 2004; Kim *et al* 2006; Kaimi *et al* 2007b) the outcomes of the current work demonstrated significantly increased biodegradation of aliphatic hydrocarbons in planted soil compared with unplanted soil for all species studied (Chapter 6). This resulted in subsequent lower endpoint diesel/oil concentrations in the presence of grasses compared with unplanted control soil (Chapter 6). Additionally, increased rates of diesel/oil degradation were observed in the presence of grasses (particularly *C. ambiguus*) compared with unplanted soil (Chapter 6). A strong relationship was found between microbial characteristics in the rhizosphere of grasses (particularly abundance of diesel/oil degrading microorganisms) and contaminant biodegradation efficiency (Chapter 7). Overall results highlight the primary role of microbial degradation in the fate of hydrocarbon pollutants in the soil. Further investigations should help to elucidate the complex processes at the soil-plant interface involved in the fate of hydrocarbons in soil with native grasses.

To date, Australian native plants have not been assessed for their hydrocarbon rhizoremediation potential. The use of native grasses offers an economically feasible and environmentally sustainable cleanup option for the rehabilitation and restoration of hydrocarbon contaminated sites in Australia. The aim of the study was to evaluate the potential of Australian native grass species for the rhizoremediation of aliphatic hydrocarbon contaminated soil from a mine site. This investigation has successfully identified three Australian grass species that are candidates for further investigation for *in situ* field scale rhizoremediation at the mine site.

There is scope for future work particularly regarding underlying mechanisms responsible for observed species-specific rhizoremediation outcomes. These future directions can be summarised as follows:

- What are the species-specific induced responses observed during the rhizoremediation process? Not all species were shown to be successful candidates, and of those that were, each achieved enhanced remediation differently.

- What plant-specific exudates are being deposited into the rhizosphere during the remediation process? Are plant roots in fact critical to the observed effects? What is the effect of the contaminant on the pattern and quantity of root exudation?

- Nutrient availability can be a rate limiting step in the rhizoremediation process. What is occurring in nutrient cycling within the rhizosphere? Where do the N and P come from in the presence of plants to allow for improved rhizoremediation compared with unplanted soil? Could the possible role of mycorrhizae explain nutrient acquisition?

- Ultimately in remediation studies it is one aspect to assess freshly contaminated soil, compared to aged or weathered contaminated soils. What rhizoremediation outcomes could be achieved using the Australian grasses under conditions of aged soil contamination where residual pollutant bioavailability may be reduced?

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APPENDIX I

Candidate Australian native grass species based on selection criteria
(**bold** indicates species that were evaluated in the study)

Species (Common name)

1. ***Alloteropsis semialata*** **(Cockatoo grass)**
2. *Aristida holathera var. holathera* (Erect kerosene grass)
3. *Aristida latifolia* (Feathertop wiregrass)
4. ***Bothriochloa bladhii subsp. Bladhii*** **(Forest bluegrass)**
5. *Brachiaria piligera* (Hairy arm grass)
6. *Brachiaria subquadripara* (Two finger grass)
7. *Chrysopogon fallax* (Ribbon grass or Golden beard grass)
8. ***Cymbopogon ambiguus*** **(Lemon scented grass)**
9. ***Dichanthium sericeum*** **(Queensland bluegrass)**
10. *Digitaria bicornis* (Finger grass)
11. *Elytrophorus spicatus* (Spike grass)
12. *Eragrostis cumingii* (Cuming's love grass)
13. *Eragrostis fallax* (Love grass)
14. *Eriochloa procera* (Cup grass)
15. *Eriochloa pseudocrotricha* (Perennial cup grass)
16. ***Heteropogon contortus*** **(Black speargrass)**
17. *Imperata cylindrical* (Kunai grass)
18. ***Microlaena stipoides*** **(Weeping grass)**
19. *Paspalum scrobiculatum* (Kodo millet)
20. *Perotis rara* (Comet grass)
21. ***Sarga plumosum*** **(Plume sorghum)**
22. *Schizachyrium fragile* (Senale red grass)
23. *Setaria surgens* (Pigeon grass)
24. ***Themeda triandra*** **(Kangaroo grass)**
25. *Tripogon loliiformis* (Five minute grass)
26. ***Brachiaria decumbens*** **(Signal grass)** (naturalised; native to mine site and seed available from local seed banks)

APPENDIX II

Germination of native grasses in high-nutrient potting mix.
Data based on single trial, n = 60 seeds per species.

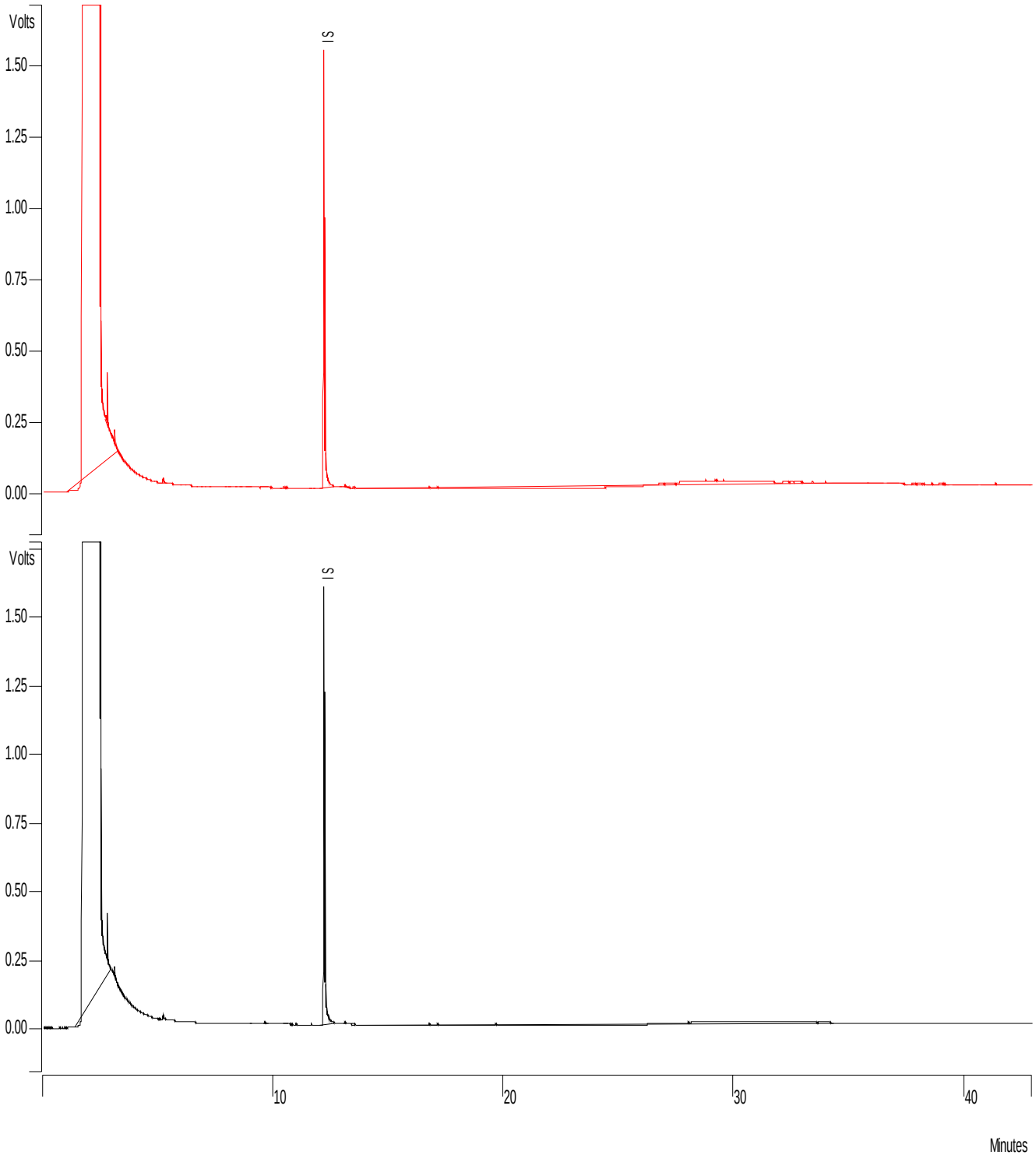
Potting mix composition: coco peat, sand, lime, gypsum, superphosphate, iron sulphate, iron chelate, micromax, calcium nitrate, osmocote.

Grass Species	**% Germination in potting mix**
Alloteropsis semialata	*nd*
Bothriochloa bladhii	*nd*
Brachiaria decumbens	0.0 *
Cymbopogon ambiguus	6.7 *
Dichanthium sericeum	15.0
Heteropogon contortus	3.3
Microlaena stipoides	28.3
Sarga plumosum	*nd*
Themeda triandra	13.3

nd not determined
* lower % germination than in clean mine site soil control (see also Section 3.3.1)

APPENDIX III

Examples of typical recorded chromatograms showing no detected TPH profiles relative to the internal standard (IS) in plant tissue. Chromatograms are from *B. decumbens* at 43 DAP for shoots (red, top) and roots (black, bottom).



APPENDIX IV

Table 1 Pearson correlation coefficients for measured parameters in the unplanted control soil used in the *B. decumbens* trial. ** Correlation is significant at the 0.01 level (2-tailed).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/g soil	Lipase Activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	58			
TPH (mg/kg soil)	Pearson Correlation	-.794(**)	1		
	Sig. (2-tailed)	.000			
	N	58	58		
MPN/g soil	Pearson Correlation	-.075	.031	1	
	Sig. (2-tailed)	.646	.851		
	N	40	40	40	
Lipase Activity (µg pNP/g soil)	Pearson Correlation	-.219	.103	.055	1
	Sig. (2-tailed)	.174	.525	.738	
	N	40	40	40	40

Table 2 Pearson correlation coefficients for measured plant growth variables and soil parameters in the rhizosphere of *B. decumbens* at three time points (30, 60 and 90 DAP). * Correlation is significant at the 0.05 level (2-tailed).

		Mean TPH mg/kg	Mean Root biomass (g) @1%	Mean Root/Shoot ratio @ 1%	MPN/g soil	Mean Lipase activity (µg pNP/g soil)
Mean TPH mg/kg	Pearson Correlation	1				
	Sig. (2-tailed)					
	N	3				
Mean Root biomass (g) @ 1%	Pearson Correlation	-.865	1			
	Sig. (2-tailed)	.335				
	N	3	3			
Mean Root/Shoot ratio @ 1%	Pearson Correlation	.834	-.998(*)	1		
	Sig. (2-tailed)	.372	.037			
	N	3	3	3		
MPN/g soil	Pearson Correlation	.529	-.031	-.028	1	
	Sig. (2-tailed)	.645	.980	.982		
	N	3	3	3	3	
Mean Lipase activity (µg pNP/g soil)	Pearson Correlation	.952	-.669	.624	.763	1
	Sig. (2-tailed)	.198	.533	.571	.447	
	N	3	3	3	3	3

Table 3 Pearson correlation coefficients for measured parameters in the unplanted control soil used in the *C. ambiguus* trial. * Correlation is significant at the 0.05 level (2-tailed).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/g soil	Lipase activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	65			
TPH (mg/kg soil)	Pearson Correlation	-.036	1		
	Sig. (2-tailed)	.778			
	N	63	63		
MPN/g soil	Pearson Correlation	-.288(*)	.014	1	
	Sig. (2-tailed)	.029	.917		
	N	58	56	58	
Lipase activity (µg pNP/g soil)	Pearson Correlation	-.181	.012	-.079	1
	Sig. (2-tailed)	.239	.938	.608	
	N	44	44	44	44

Table 4 Pearson correlation coefficients for measured plant growth variables and corresponding soil parameters in the rhizosphere of *C. ambiguus* at three time points (30, 60 and 90 DAP).

		Mean TPH mg/kg	Mean Root biomass (g) @ 1%	Mean Root/Shoot ratio @ 1%	MPN/g soil	Mean Lipase activity (µg pNP/g soil)
Mean TPH mg/kg	Pearson Correlation	1				
	Sig. (2-tailed)					
	N	3				
Mean Root biomass (g) @ 1%	Pearson Correlation	-.268	1			
	Sig. (2-tailed)	.827				
	N	3	3			
Mean Root/Shoot ratio @ 1%	Pearson Correlation	-.969	.023	1		
	Sig. (2-tailed)	.158	.985			
	N	3	3	3		
MPN/g soil	Pearson Correlation	-.665	-.542	.828	1	
	Sig. (2-tailed)	.537	.636	.379		
	N	3	3	3	3	
Mean Lipase activity (µg pNP/g soil)	Pearson Correlation	.648	-.908	-.440	.139	1
	Sig. (2-tailed)	.551	.276	.710	.911	
	N	3	3	3	3	3

Table 5 Pearson correlation coefficients for measured parameters in the unplanted control soil used in the *M. stipoides* trial. ** Correlation is significant at the 0.01 level (2-tailed).

		Days after planting (DAP)	TPH (mg/kg soil)	MPN/g soil	Lipase activity (µg pNP/g soil)
Days after planting (DAP)	Pearson Correlation	1			
	Sig. (2-tailed)				
	N	60			
TPH (mg/kg soil)	Pearson Correlation	-.706(**)	1		
	Sig. (2-tailed)	.000			
	N	60	60		
MPN/g soil	Pearson Correlation	-.149	.110	1	
	Sig. (2-tailed)	.359	.501		
	N	40	40	40	
Lipase activity (µg pNP/g soil)	Pearson Correlation	-.618(**)	.483(**)	-.061	1
	Sig. (2-tailed)	.000	.002	.709	
	N	40	40	40	40

Table 6 Pearson correlation coefficients for measured plant growth variables and soil parameters in the rhizosphere of *M. stipoides* at three time points (30, 60 and 90 DAP).

		Mean TPH (mg/kg)	Mean Root biomass (g) @ 1%	Mean Root/Shoot ratio @ 1%	MPN/g soil	Mean Lipase activity (µg pNP/g soil)
Mean TPH (mg/kg)	Pearson Correlation	1				
	Sig. (2-tailed)					
	N	3				
Mean Root biomass (g) @ 1%	Pearson Correlation	-.860	1			
	Sig. (2-tailed)	.341				
	N	3	3			
Mean Root/Shoot ratio @ 1%	Pearson Correlation	.969	-.960	1		
	Sig. (2-tailed)	.160	.181			
	N	3	3	3		
MPN/g soil	Pearson Correlation	-.625	.936	-.799	1	
	Sig. (2-tailed)	.571	.230	.411		
	N	3	3	3	3	
Mean Lipase activity (µg pNP/g soil)	Pearson Correlation	.161	-.642	.401	-.871	1
	Sig. (2-tailed)	.897	.556	.737	.326	
	N	3	3	3	3	3

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Screening of Australian Native Grasses for Rhizoremediation of Aliphatic Hydrocarbon-Contaminated Soil

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SCREENING OF AUSTRALIAN NATIVE GRASSES FOR RHIZOREMEDIATION OF ALIPHATIC HYDROCARBON-CONTAMINATED SOIL

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Rhizoremediation involves the breakdown of contaminants in soil resulting from microbial activity that is enhanced in the plant root zone. The objective of this study was to identify Australian native grass species as suitable candidates for rhizoremediation application. Seeds of nine perennial Australian native grasses were sown in soil from a mine site and artificially contaminated with a 60:40 diesel/oil mixture at concentrations of 1% (w/w), 0.5% (w/w), and 0% (control). Seedling emergence was not adversely affected by the presence of hydrocarbon contamination for all but one grass species. Three promising species (*Brachiaria decumbens*, Cymbopogon ambiguus*, and* Microlaena stipoides *var. Griffin) were assessed for growth characterization in contaminated and uncontaminated soils. The evaluated species survived for 120 days in the contaminated soil and, in some instances, produced considerably more root biomass in the presence of contamination.* C. ambiguus *showed growth stimulation in the presence of contamination (1% and 0.5% w/w) with significantly increased root biomass production compared with the control (*$p = 0.0001$*).* B. decumbens *and* M. stipoides *showed tolerance, without adverse growth effects in the presence of diesel/oil at the exposed concentrations. Stimulation of the rhizosphere microbial population that is capable of degrading diesel/oil was found for all of the species tested, using a most probable number method for enumeration. This investigation has identified suitable candidates for further investigation of their rhizoremediation potential.

KEY WORDS: phytoremediation, rhizosphere, native grass, germination, mine site rehabilitation

INTRODUCTION

The breakdown of contaminants in soil resulting from microbial activity that is enhanced in the presence of the plant root zone (rhizosphere) has been termed *rhizoremediation* (Kuiper *et al*., 2004). During rhizoremediation, it is believed that plant root exudates (*e.g*., organic acids, carbohydrates) may help to stimulate the survival and action of microorganisms in the soil, which subsequently degrade pollutants in the plant

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rhizosphere. This process has been proposed as the primary mechanism responsible for hydrocarbon degradation in plant-assisted remediation efforts (Frick, Farrell, and Germida, 1999; Hutchinson, Schwab, and Banks, 2003).

The selection of plant species may have a substantial impact on the success of rhizoremediation. A growing body of literature reports that plant species do not all have the same potential for enhanced remediation (Frick *et al*., 1999; Hutchinson *et al*., 2003). The general plant type (*e.g*., monocotyledonous, leguminous) may determine the influence on soil parameters that affect hydrocarbon degradation in the rhizosphere (Pichtel and Liskanen, 2001). Notwithstanding this, plant type cannot be used to predict the likely influence of individual species. Variation in the extent of these influences within plant types and between genera should be expected. These variations may be a result of plant morphology (*e.g*., roots), physiology (*e.g*., root exudates), and microbial interactions in the rhizosphere (Pichtel and Liskanen, 2001; Walker *et al*., 2003).

Research has shown that various grasses and leguminous plants are potential candidates for the rhizoremediation of hydrocarbons (Aprill and Sims, 1990; Adam and Duncan, 1999; Merkl, Schultze-Kraft, and Infante, 2004a, 2005). This may be due to their highly branched, fibrous root systems, which can harbor large microbial numbers and exert a greater influence on the soil environment (Anderson, Guthrie, and Walton, 1993). Warm-season grasses have been reported to be especially applicable to rhizoremediation due to inherent characteristics such as deep fibrous roots, and tolerance of drought and low nutrient availability (Aprill and Sims, 1990; Dzantor, Chekol, and Vough, 2000; Waters, Whalley, and Huxtable, 2001).

Screening plants for tolerance to hydrocarbon contamination is the first step in any selection process for rhizoremedation (Tesar, Reichenauer, and Sessitsch, 2002). A plant's ability to successfully grow and establish in contaminated soil is a basic prerequisite for successful rhizoremediation. Numerous studies have investigated the effect of hydrocarbons on the growth and development of grass species (Adam and Duncan, 1999; Kulakow, Schwab, and Banks, 2000; Robson *et al*., 2003; Huang *et al*., 2004). Plant-screening experiments have shown grasses to be tolerant to various hydrocarbons, particularly aliphatic hydrocarbons (Adam and Duncan, 1999; Pichtel and Liskanen, 2001; Dominguez-Rosado and Pichtel, 2004).

The potential for Australian native plants in the rhizoremediation of hydrocarbon-contaminated soil has not been explored. A major hurdle to the application of rhizoremediation in Australia is the identification and availability of suitable native plant species: the introduction of exotic species may result in adverse ecological outcomes. The provision of an economically feasible and environmentally sustainable option for the remediation of hydrocarbon-contaminated sites in Australia would be of significant benefit. An attractive feature of rhizoremediation is the low-cost, low-technology approach, which makes the strategy economically viable for site rehabilitation and restoration (Frick *et al*., 1999).

The objective of this study was to identify Australian native grass species that may be suitable candidates for rhizoremediation application. Identified species were evaluated for seedling emergence and growth (biomass) in diesel/oil-contaminated and uncontaminated soils. Stimulation of hydrocarbon-degrading bacterial populations in the rhizosphere was also assessed. This study helped to determine which species may be best suited for further study as candidates for rhizoremediation of aliphatic hydrocarbon-contaminated soil.

METHODS

Soil and Plant Selection

Rio Tinto Aluminium Limited operates an open-cut bauxite mine at Weipa on the western coast of Cape York Peninsula in Queensland, Australia. A major long-term land-regeneration objective is in place, with a focus on the re-establishment of native species. Areas of the site have been impacted with hydrocarbons, particularly aliphatics such as diesel and lube oils, from machinery used in the mining process. Uncontaminated soil was collected from the Weipa mine site and sent to Flinders University of South Australia, Adelaide, Australia, for use in the study. Selected physical and chemical characteristics of the soil were assessed in triplicate.

Australian native grass species were selected for the study (including one naturalized species native to the Weipa site) based on criteria including morphological characteristics (*e.g.*, fibrous root systems), coupled with their growth characteristics and physiology, tolerance of low nitrogen and phosphorus availability, and wide growth distribution across the continent. A perennial plant is of particular importance where management can be reduced considerably due to their long growing season. Nine perennial grass species were evaluated in the study: eight warm-season grasses—*Alloteropsis semialata* (Cockatoo grass), *Bothriochloa bladhii* (Forest bluegrass), *Brachiaria decumbens* (Signal grass; naturalized, native to site), *Cymbopogon ambiguus* (Lemon Scented grass), *Dichanthium sericeum* (Queensland bluegrass), *Heteropogon contortus* (Black speargrass), *Sarga plumosum* (Plume sorghum), and *Themeda triandra* (Kangaroo grass)—and one cool-season grass—*Microlaena stipoides* var. Griffin (Weeping grass). Native grass seeds were obtained commercially with the exception of *B. decumbens*, which was sourced from seed banks at the Weipa mine site.

Screening Experiments—Seedling Emergence in Soil

Diesel fuel and 20–50W engine oil were obtained commercially. Sieved mine site soil (2 mm) was artificially contaminated with a 60:40 diesel/oil mix at concentrations of 1% (w/w) (10,000 mg/kg), 0.5% (w/w; 5,000 mg/kg), and 0% (control) and was mixed thoroughly to achieve a homogeneity. Germination pots were filled with 200 g of contaminated or uncontaminated (control) soil. Fifty seeds were planted for each replicate (four replicate pots, 200 seeds in total) for treatments (1%, 0.5% w/w) and control (0%), for each grass species. Seeds were planted upright to a shallow depth (5 mm) and soil moisture content was maintained at approximately 60% (w/w) of the soil's water-holding capacity throughout the experiment.

Germination experiments were conducted in a greenhouse during the warm season with natural light conditions. Average maximum and minimum temperatures recorded throughout the experiment were 31.5°C (±3.6) and 20.6°C (±3.3), respectively. Germination was assessed by seedling emergence and monitored daily. After 60 days, plants from each treatment and control were counted for final seedling emergence results. Seedling emergence of each species for treatments and the control was expressed as the percentage of total seeds planted.

Plant Growth Experiments

Based on germination performance, three grass species were assessed for growth characterization in diesel/oil contaminated (1%, 0.5% w/w) and uncontaminated (control)

soil; *B. decumbens* (Signal grass), *C. ambiguus* (Lemon Scented grass), and *M. stipoides* var. Griffin (Weeping grass). One hundred seeds were planted per replicate (three replicate pots; 140 mm, 1 kg soil) for treatments and control, for each grass species. Final n values for each treatment and species were dependent on germination (Mean $n = 44$, range 29–59). Plant growth experiments were conducted over the warm season. The two summer-growing species, *B. decumbens* and *C. ambiguus*, were held in the greenhouse with average maximum and minimum recorded temperatures 34.8°C (±3.9) and 21.1°C (±4.6), respectively. *M. stipoides* is a cool-season grass with a well-established optimum growth temperature range of 15–25°C (Crawford and Wilkens, 1998; Waters *et al*., 2001). Plant growth experiments for this species were, therefore, conducted in a plant growth cabinet set at 25°C for 16 hours (light) and 18°C for 8 hours (dark) (Crawford and Wilkens, 1998).

At 60 and 120 days after planting (DAP), grasses were carefully harvested. Plant roots were gently washed in distilled water to remove soil particles. Root and shoot biomass (gram dry weight, DW) were determined for each plant after drying to constant mass at 70°C for 48 hours. Root/shoot ratios were calculated as the dry mass of roots divided by the dry mass of shoots (grams).

Most Probable Number Assay

A five-well most probable number (MPN) method was used as described previously (Gaskin and Bentham, 2005), with two adjustments; diesel was used as the sole hydrocarbon substrate and MPN multi-well trays were incubated at 28°C for 10 days prior to the addition of fluorescein diacetate. This method enables enumeration of microorganisms in the rhizosphere that are capable of utilizing hydrocarbon as a source of carbon and energy. For the purpose of this investigation, rhizosphere soil was defined as soil adherent to roots following vigorous shaking by hand. One composite rhizosphere soil sample per pot was collected from the roots of six randomly selected plants. The MPN assay of diesel-degrading organisms in rhizosphere soil was performed in four replicates for each treatment and species: 0%, 0.5% and 1% (w/w) planted (each species), at sampling times 60 and 120 DAP. Treatments were compared to clean (0%) unplanted and 1% (w/w) unplanted soils.

Statistical Analysis

Prior to statistical analysis, data were tested for normality and homogeneity using SPSS (Statistical Package for the Social Sciences, Illinois) v.12 for Windows. Analysis of variance was conducted at $\alpha = 0.05$ to determine the treatment significance for emergence, root biomass, and root/shoot ratio. Treatment means showing significance were separated using Bonferroni Multiple Comparisons test at 5% level of significance.

RESULTS AND DISCUSSION

Seedling Emergence

Selected physical and chemical characteristics of the mine site soil are shown in Table 1. The sandy loam soil showed low nutrient levels available to plants, as could be expected at this geographical location (Schwenke, Mulligan, and Bell, 1999).

The ability of seeds to germinate in diesel/oil-contaminated soil varied greatly depending on the grass species (Table 2). Some species did not respond at all, showing little

Table 1 Physical and chemical properties of Weipa mine site soil

Property	*Value*
Maximum water-holding capacity	46%
Moisture content (field capacity)	14%
pH[a]	6.3
Electrical conductivity[a]	35.2 μS/cm
Soil texture[b]	Sandy loam
Percent sand	60
Percent silt	25
Percent clay	15
Organic carbon content[c]	0.26%
Nitrate-N (NO_3-N)[d]	2.51 mg/kg
Nitrite-N (NO_2-N)[d]	<0.02 mg/kg
Ammonia-N (NH_4-N)[d]	0.30 mg/kg
Phosphate-P (PO_4-P)[e]	1.16 mg/kg
Al_2O_3[f]	47.6 mg/100 g

[a]1:5 H_20.; [b]Pipette method.; [c]Wet oxidation redox titration.; [d]KCL extraction.; [e]Olsen sodium bicarbonate.; [f]Analysed by Amdel Ltd (Adelaide, Australia).

or no seedling emergence in any of the soils (*e.g.*, *A. semialata*, *H. contortus*). Of the species that did respond, only *B. bladhii* showed a statistically significant decrease in seedling emergence (by 83%) in soil contaminated with 0.5% (w/w) hydrocarbon compared to the control soil ($p = 0.009$). No significant difference was found in seedling emergence for soil contaminated with 1% (w/w) diesel/oil compared to the control soil for this species, which highlights the importance of plant screening prior to rhizoremediation efforts to confirm suitability. For all other grass species, the presence of hydrocarbon contamination had no significant negative effect on seedling emergence. In the 1% (w/w) diesel/oil-contaminated soil, *B. decumbens* had the greatest percentage seedling emergence (38.5%) of all species tested. *C. ambiguus* and *M. stipoides* also showed higher percentage seedling emergence in 1% (w/w) contaminated soil compared with other treatments, although these differences were not shown to be significant.

Overall, low emergence rates were observed for all grass species across all treatments (highest 38.5%), which can be attributed to the fact that many grasses are not as easy to establish from seeds as other plants (*e.g.*, legumes) due to inherent problems related to poor seed quality, seed dormancy, or short seed lifespan associated with the *Poaceae* (Adkins, Bellairs, and Loch, 2002; Merkl, Schultze-Kraft, and Infante, 2004b). Soil quality is another major determinant of seed emergence and viability that must be considered. The germination of seeds in diesel-fuel–contaminated soil has been shown to be highly dependent on plant species. Some species are notably tolerant while other species are completely intolerant of diesel-fuel contamination (Adam and Duncan, 2002). This variability in tolerance was also evident in the current study. An inhibitory effect of diesel fuel on germination may also be attributed to the physico-chemical constraints induced by diesel fuel (Adam and Duncan, 2002). This coating of the seed and surrounding soil particles may act as a physical barrier preventing or reducing both water and oxygen transfer (Adam and Duncan, 2002).

Table 2 Seedling emergence of Australian grasses in control soil (0%) and diesel/oil contaminated soil (0.5% and 1% w/w). Mean values ± standard deviation.

	% Seedling emergence ± SD		
	Diesel/oil concentration		
Grass species (Common name)	0%	0.5%	1%
Alloteropsis semialata (Cockatoo grass)	0	0	0
Bothriochloa bladhii (Forest bluegrass)	24.8 ± 12.6	4.0 ± 4.9*	10.0 ± 1.6
Brachiaria decumbens (Signal grass)	24.8 ± 12.9	21.5 ± 11.5	38.5 ± 3.4
Cymbopogon ambiguus (Lemon scented grass)	12.3 ± 8.8	12.5 ± 13.4	22.5 ± 11.8
Dichanthium sericeum (Queensland bluegrass)	4.8 ± 6.7	0.5 ± 1	0
Heteropogon contortus (Black speargrass)	0	1 ± 1.2	0
Microlaena stipoides (Weeping grass)	21.8 ± 10.2	20.0 ± 8.2	28.0 ± 4.9
Sarga plumosum (Plume sorghum)	5.0 ± 4.7	2.5 ± 1.9	4.0 ± 0
Themeda triandra (Kangaroo grass)	6.0 ± 4.3	7.0 ± 5.3	3.0 ± 4.8

*Significantly different to control (0%) at $p < 0.05$.

The data in the current study suggest that several Australian grass species have potential as candidates for rhizoremediation of aliphatic hydrocarbon-contaminated soil. The required ability of some species to germinate in the presence of diesel/oil contamination was demonstrated. Of the species screened, *B. decumbens, C. ambiguus*, and *M. stipoides* showed the most promise in this regard, as a higher percentage of seedling emergence was noted for these species in 1% (w/w) diesel/oil-contaminated soil compared to the uncontaminated control.

Plant Growth Characterization

Plant growth characteristics in the diesel/oil-contaminated and uncontaminated soils showed that the grass species growth response to the presence of contamination varied. The three evaluated species survived for 120 days in the contaminated soil and, in some instances, produced considerably more root biomass in the presence of contamination (Figure 1). *B. decumbens* showed tolerance to the presence of diesel/oil contamination at the exposed concentrations. Growth for this species was not adversely affected in the presence of contamination, with no significant difference in root biomass production found between contaminated and uncontaminated soils at 60 and 120 DAP. Of particular interest, *C. ambiguus* showed significantly increased root biomass production in the presence of contamination (1% and 0.5% w/w) as compared with the control ($p = 0.0001$) at 120 DAP. That is, the presence of diesel/oil had an apparent stimulatory effect on the root biomass production over the growth period. *M. stipoides* initially showed significantly increased root biomass production when grown in soil with hydrocarbon (0.5% w/w) as compared to uncontaminated control ($p = 0.045$) 60 DAP, although this result was not observed in 1% (w/w) contamination plantings. At 120 DAP, *M. stipoides* showed no significant difference in root biomass production between contaminated and uncontaminated soil, suggesting that *M. stipoides* showed tolerance to the presence of diesel/oil contamination at the exposed concentrations as growth was not adversely affected.

The root/shoot ratio reflects the balance between belowground and aboveground plant growth. The higher the ratio, the more of the plant biomass is taken up by the roots.

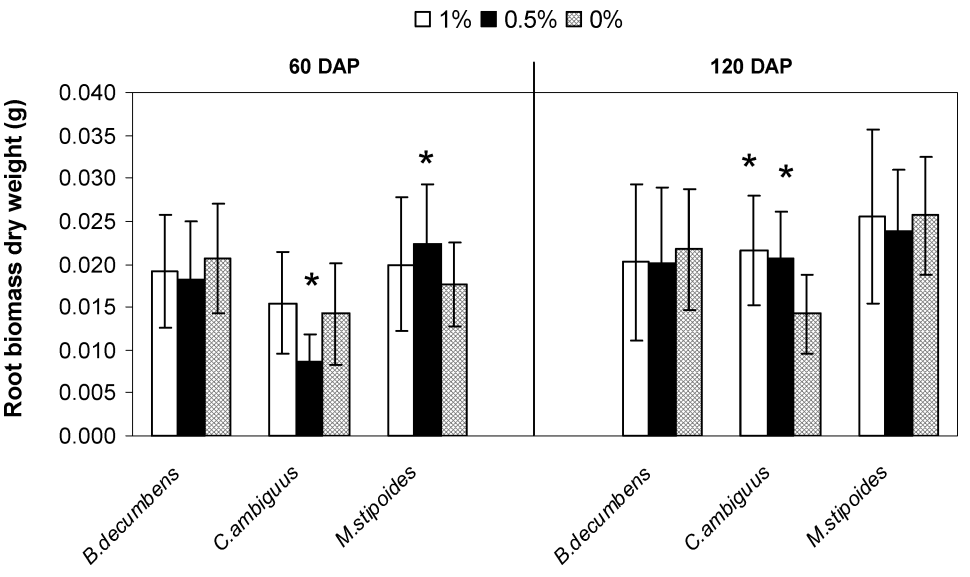

Figure 1 Root biomass production (g DW) of grasses grown in uncontaminated soil (0%) and diesel/oil-contaminated soil (0.5% and 1% w/w) at 60 and 120 DAP. Values are means ± standard deviation. *Significantly different to control (0%) at $p < 0.05$.

For rhizoremediation application, a high root/shoot ratio would be of significant benefit. The mean root/shoot ratios for the three grass species were assessed (Figure 2) and show similar patterns to the root biomass production data. *B. decumbens* showed no significant difference in root/shoot ratio between contaminated and control soils over the growth period. In contrast, *C. ambiguus* showed a shift toward root biomass production in contaminated soil compared with uncontaminated from 60 to 120 DAP, which resulted in significantly increased root/shoot ratios ($p = 0.001$) in the presence of contamination. Similarly, at 60 DAP, *M. stipoides* showed increased root production compared with shoot production (*i.e.*, higher root/shoot ratio) in contaminated soil as compared with uncontaminated soil ($p = 0.008$). By 120 DAP there was no significant difference in the root/shoot ratio between treatments for this species. Relative growth rates were lower in control plantings than in contaminated plantings for all species tested, which demonstrated tolerance to hydrocarbon contamination (data not shown).

Overall, the growth data for all three species suggest high tolerance to the presence of diesel/oil contamination in soil at the exposed concentrations. In particular, *C. ambiguus* and *M. stipoides* are ideal candidates for investigation of rhizoremediation technology, showing increased growth in the presence of contamination. In addition, *C. ambiguus* is a warm-season grass while *M. stipoides* is suited to cool-season growth, thereby allowing for climate variation in rhizoremediation application.

Previous reports in the literature show that the presence of petroleum hydrocarbons can significantly reduce plant biomass (Kulakow *et al.*, 2000; Tesar *et al.*, 2002; Robson *et al.*, 2003). Kulakow *et al.* (2000) screened 26 native and introduced grass species in the United States for growth in petroleum hydrocarbon-contaminated sediments. Although grass species were not affected equally by the presence of contamination, the authors reported considerable reductions in plant growth in the contaminated soil after 180 days

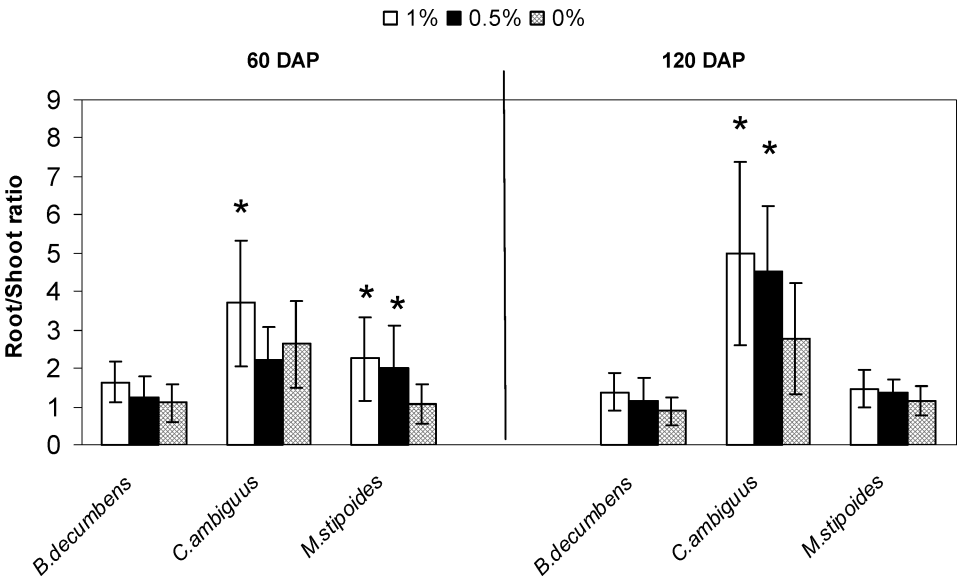

Figure 2 Root/shoot ratio of grasses grown in uncontaminated soil (0%) and diesel/oil-contaminated soil (0.5% and 1% w/w) at 60 and 120 DAP. Values are means ± standard deviation. *Significantly different to control (0%) at $p < 0.05$.

as compared to the control for all species tested. An average of 77% reduction in root biomass in contaminated soil as compared with the control was reported. Similar adverse affects on plant growth in the presence of hydrocarbon contamination were observed by Robson *et al*. (2003). Thirty-nine plants (grasses and legumes) native or naturalized in Canada were assessed for their ability to survive in crude-oil–contaminated soil. The authors reported the addition of 0.5%, 1%, and 5% (w/w) crude oil to soil significantly decreased both the root biomass and total plant biomass by at least 22% of the control for all but three species.

In contrast to these reports, and as observed in the current study, oil contamination may also cause growth stimulation (Adam and Duncan, 1999; Reynolds *et al*., 1999; Merkl *et al*., 2004a). In a laboratory study, Reynolds *et al*. (1999) reported on the effect of amending silt loam soil with a model organic contaminant (including diesel and pyrene) on the growth of four grass species in the United States. The authors noted that at 1000 mg TPH kg^{-1} (0.1%), three of the four grass species assessed showed increased root biomass production as compared with uncontaminated controls. Similarly, a study by Merkl *et al*. (2004a) noted enhanced growth in the presence of crude-oil contamination. Merkl *et al*. (2004a) evaluated two grass species (including a *Brachiaria* sp.) and six legumes naturalized in eastern Venezuela for tolerance to crude oil added at 3% and 5% to savannah soil. The authors observed increased shoot length and biomass, as well as increased root/shoot ratios in four of the legumes. Of interest, the *Brachiaria* sp. evaluated in the study showed high seedling emergence and least affected (though reduced) biomass production of the grasses assessed. The Australian naturalized grass *Brachiaria decumbens* evaluated in the current study showed no adverse growth affect in the presence of diesel/oil contamination.

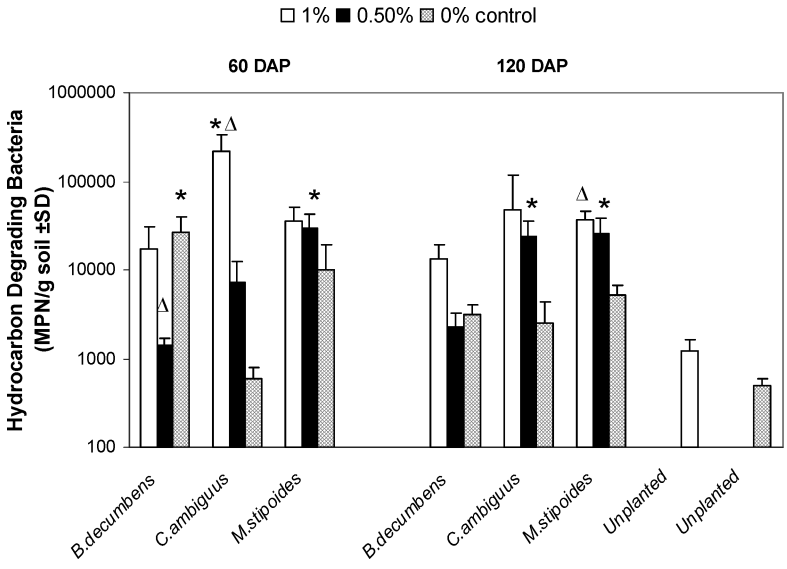

Figure 3 Hydrocarbon-degrading bacteria from rhizosphere soil of three plant treatments at 60 and 120 DAP and unplanted bulk soil as determined by MPN assay. Soils are uncontaminated (0% control) or contaminated with diesel/oil (0.5% and 1% w/w). Values are means ± standard deviation. *Significantly different to unplanted control (0%, 1%) at $p < 0.05$. Δ Significantly different to planted control (0%) at $p < 0.05$.

A significant stimulatory effect of diesel/oil contamination on growth of the two native species (*C. ambiguus* and *M. stipoides*) assessed at high hydrocarbon concentrations (1% w/w) was noted. This is the first report of this effect associated with Australian species. In light of this, screening other native species may be of value. The reasons for enhanced growth may include an hormonally influenced stress response as suggested by Merkl *et al.* (2004a). Alternatively, stress responses by some plants facing nutrient limitation may also result in growth stimulation. Increased root biomass may also be a strategy to stimulate water, nitrogen, or phosphate uptake in the plant (Frick *et al.*, 1999).

Bacterial Stimulation in the Rhizosphere

The presence of hydrocarbons in soil has been shown to stimulate microbial populations (Gaskin and Bentham, 2005), but plants appear to add additional influence. Plants have been shown to increase the microbial numbers in the rhizosphere, a phenomenon termed the *rhizosphere effect* (Kirk *et al.*, 2005). In this study, the stimulation of the rhizosphere microbial population capable of degrading diesel/oil mix was found in some treatments for all of the species tested at 60 DAP and for two species (*C. ambiguus* and *M. stipoides*) at 120 DAP, relative to unplanted controls (Figure 3).

The numbers of hydrocarbon-degrading bacteria in the rhizosphere soil were elevated in plants showing increased root biomass. For example, *C. ambiguus* showed root biomass stimulation in the presence of diesel/oil at 120 DAP relative to the control, which corresponded to significantly increased numbers of hydrocarbon-degrading bacteria in the rhizosphere soil ($p = 0.035$ 120 DAP). This result provides evidence of the rhizosphere effect with this grass species, where increased root biomass allowed for increased microbial colonization, and vice versa.

For *M. stipoides*, significantly increased root growth in 0.5% (w/w) contaminated soil at 60 DAP also corresponded to stimulation of microbial numbers in the rhizosphere for this treatment as compared with unplanted contaminated systems ($p = 0.003$). This suggests that for this species the presence of a low concentration of diesel/oil had a rhizosphere effect on the microbial community that was not evident at higher concentrations. The reason for this disparity is not currently understood; further work is needed to clarify the true extent of rhizosphere effect in this species.

For *B. decumbens*, no significant difference was found between treatments for root growth (biomass), nor was there a difference found between numbers of hydrocarbon-degrading bacteria in contaminated and uncontaminated planted systems. However, for this species when clean (bulk) soil was measured with and without the presence of plants, increased microbial numbers were noted in the planted systems at 60 DAP ($p = 0.03$). This rhizosphere effect may be due to an exudation effect from the plant and not related directly to the presence of hydrocarbon.

Plant-specific alterations to the rhizosphere microbial community as observed in the current study have been reported elsewhere in the literature (see Reynolds *et al*., 1999; Kirk *et al*., 2005). Grasses in particular have been shown to exhibit a rhizosphere effect (increased microbial numbers) in the presence of hydrocarbon contamination (Miya and Firestone 2000; Kirk *et al*., 2005; Merkl *et al*., 2006). Miya and Firestone (2000) found selective enrichment of phenanthrene-degrading bacteria an order of magnitude greater in rhizosphere soil of slender oat grass (*Avena barbata*) as compared with unplanted bulk soil, indicating that the rhizosphere environment created by slender oat supported a substantial population of organisms capable of degrading phenanthrene. Similarly, Kirk *et al*. (2005) reported increased diesel-degrading bacteria in the rhizosphere of perennial ryegrass (*Lolium perenne*) using an MPN method. Over a 7-week experiment, the numbers of diesel-degrading bacteria increased by 230-fold in the perennial ryegrass rhizosphere as compared with only a 51-fold increase in bulk soil. A comparable population enhancement was noted in the current study for the grass *C. ambiguus*, where the rhizosphere effect generated several orders of magnitude greater microbial numbers when compared to unplanted bulk soil (see Figure 3).

In contrast, some studies using grasses have shown no significant difference in rhizosphere and non-rhizosphere microbial numbers. Binet *et al*. (2000) reported no significant difference in the number of PAH-degrading bacteria in the rhizosphere soil as compared with non-rhizosphere soil, although decreased concentrations of freshly spiked PAHs were found. Merkl *et al*. (2006) studied the effect of the tropical grass *Brachiaria brizantha* on the microbial population in petroleum-contaminated loamy sand of Venezuela. After 14 weeks, no significant difference in microbial numbers was found between rhizosphere and non-rhizosphere soil, which is consistent with the results found in the current study for the Australian naturalized grass *Brachiaria decumbens*. While an initial increase in rhizosphere microbial numbers was found relative to non-rhizosphere soil, at a later sampling date (120 DAP) no significant difference in diesel/oil-degrading bacteria was noted.

This investigation reports on Australian plant species that can elevate hydrocarbon-degrading microbial numbers in the rhizosphere. This effect was demonstrated even in the absence of hydrocarbon. Potentially useful species for rhizoremediation of contaminated soils in Australia have been identified using this multi-faceted screening approach. The influence of these plants on hydrocarbon degradation in the root zone needs to be established.

CONCLUSION

This investigation has identified Australian native grass species that are suitable candidates for further investigation of their rhizoremediation potential. In some instances the presence of hydrocarbon was stimulatory to plant growth and hydrocarbon-degrading microbial numbers. Studies relating to hydrocarbon degradation and microbial dynamics in the rhizosphere soil and the root zone are proceeding using these species.

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