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# Phytoremediation of hydrocarbon contaminants in sub-Antarctic soils

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# Phytoremediation of hydrocarbon contaminants in sub-Antarctic soils

Thesis submitted in partial fulfilment of a Bachelor of

Advanced Environmental Science Honours

King penguins amongst the *Poa foliosa* on Macquarie Island. Photo: N. Grant

By

**Jessica Bramley-Alves**

University of Wollongong

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## Abstract

Accidental fuel spills on sub-Antarctic Macquarie Island have caused considerable contamination. Due to the island's high latitude position, its climate, and the fragility of its ecosystems, traditional methods of remediation are unsuitable for onsite clean up. However, if left untreated, even minor to moderate fuel spills could take decades before natural attenuation reduces the petroleum to environmentally acceptable concentrations. Currently, low cost, low disturbance *in-situ* methods to enhance biodegradation of fuel products, such as nutrient additions and air sparging, are under examination on Macquarie Island. This study investigated the potential of the sub-Antarctic native tussock grass, *Poa foliosa*, to contribute to such remediation efforts. This species was selected as it is common in areas of contamination and displays criteria which enhance phytoremediation efficiency. Growth trials were conducted with seedlings of *P. foliosa* in soil artificially spiked with Special Antarctic Blend (SAB) diesel at concentrations of 0, 1 000, 5 000, 10 000, 20 000 or 40 000 mg/kg. Replicate pots, containing single seedlings, were compared with paired unplanted pots at each SAB soil concentration. Pots were kept under controlled conditions (8°C; photoperiod of 8.75/13.25 hours) to simulate the growth environment on Macquarie Island. Plants were harvested destructively at 0, 2, 4 and 8 months. Tolerance of *P. foliosa* to SAB, and the effects of fuel contaminants on plant health and productivity (biomass production, plant morphology, pigments and photosynthetic health) were assessed. The rate of SAB degradation and the microbial communities within the rhizosphere (total heterotrophs and hydrocarbon degraders) were compared between planted and unplanted treatments. This study found *P. foliosa* to be highly tolerant across all SAB concentrations tested with respect to biomass, although higher concentrations of 20 000 and 40 000 mg/kg caused slight reductions in leaf length, width and area. Total Petroleum Hydrocarbons (TPH) were degraded 35 - 48% faster in planted soils compared to unplanted soil and were approaching soil background levels within four months. Although *P. foliosa* significantly stimulated the growth of both total heterotrophs and hydrocarbon degraders at low concentrations of 0 and 1 000 mg/kg, the presence of microbes in the root zone did not appear to be the sole driving force behind TPH degradation. This study provides persuasive evidence that phytoremediation using *P. foliosa* is a valuable technology in the suite of current *in-situ* remediation methodologies being adopted at these sites, and may be applicable to the remediation of spills in other cold climate regions.

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## Abbreviations

| | |
|---|---|
| AAD | Australian Antarctic Division |
| BH | Bushnell-Haas media |
| Chl *a/b* | Chlorophyll *a/b* ratio |
| DW | Dry weight |
| $F_v/F_m$ | Optimal quantum efficiency |
| HPLC | High pressure liquid chromatography |
| INT | Iodonitrotetrazolium chloride solution |
| LED's | Light-emitting diodes |
| MPN | Most probable number |
| MRGR | Mean relative growth rate |
| PGPR | Plant growth-promoting rhizobacteria |
| PAH | Poly-aromatic hydrocarbon |
| SAB | Special Antarctic blend |
| SLA | Shoot to leaf area |
| Tchl | Total chlorophyll content |
| TPH | Total petroleum hydrocarbon |
| UCM | Unresolved complex mixture |
| WW | Wet weight |

# Chapter 1

# Introduction

## 1.1 Sub-Antarctic Macquarie Island

### Environment

Sub-Antarctic Macquarie Island lies in isolated waters midway between Tasmania, New Zealand and the Antarctic continent (54°38'S and 158°53'E; Selkirk *et al*. 1986; Figure 1.1). The island stretches north to a length of 34 km, and has a maximum width of only 5.5 km (Selkirk *et al.* 1990). The greater part of Macquarie Island is dominated by a large undulating plateau that rises from the coastline to a height of 240-250 m above sea level, with peaks that reach 433 m in the southern region (Kantvilas and Seppelt 1992). The plateau surface is littered with numerous lakes and streams, and is bounded by scarps which fall steeply to a narrow, low-lying coastal fringe (Bunt and Rovira 1988; Medek 2008). The steepness of the slopes, combined with the island's height, creates an environment which is highly threatened by slippage and greatly exposed to the natural elements.

The island is a Tasmanian State Reserve and an important terrestrial habitat for migrating animals (Selkirk *et al.* 1990). In 1997 the island was World Heritage listed for its unique geological and biological wealth and significance (Bergstrom *et al.* 2009).

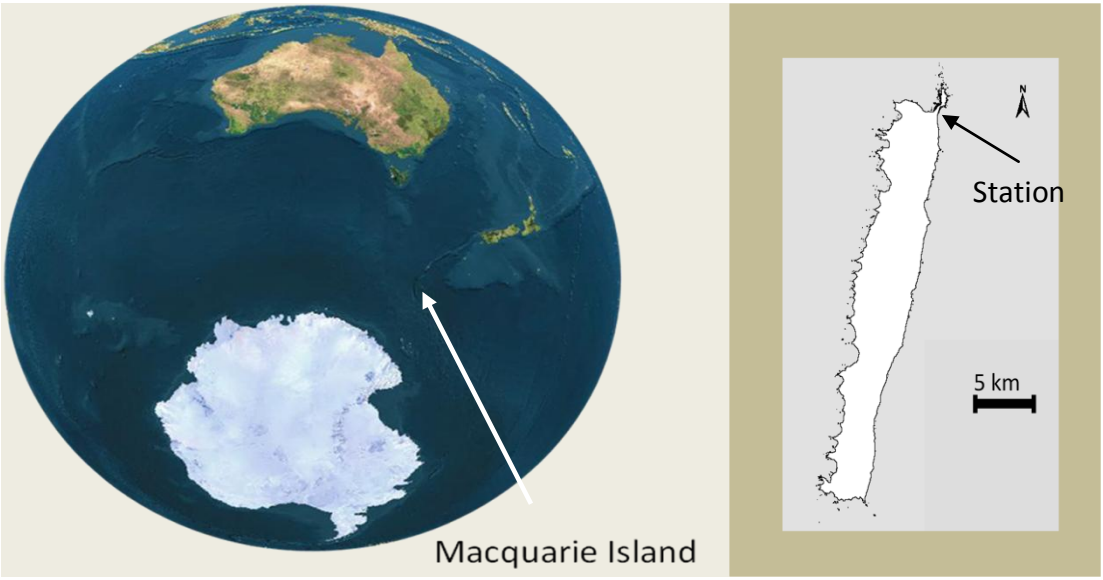


**Figure 1.1** Location of Macquarie Island in the southern ocean, and the placement of the Australian research station (adapted from Wasley *et al.* 2009).

## Climate

The climate of Macquarie Island is heavily influenced by the surrounding Southern Ocean (Kantvilas and Seppelt 1992). Due to the island's position near the Antarctic convergence region, the diurnal and seasonal climate remains relatively uniform (Selkirk *et al.* 1990). The island is cold, moist and windy, with almost constant cloud cover and precipitation, resulting in a mean of only 2.2 hours of sunshine per day (Bunt and Rovira 1988). Air temperatures range from a mean maximum of 8.8°C in January to a minimum of 1.6°C in July. The average wind speed is 34 km/h, but can reach up to 185 km/h (Australian Bureau of Meteorology 2008). The climatic factor with greatest annual variation is day length, ranging from 7 hours in winter to 17 hours in summer (Selkirk *et al.* 1990; Kantvilas and Seppelt 1992).

## Vegetation

Macquarie Island is an emergent proportion of the Macquarie Ridge and was formed by crustal accretion during sea floor spreading at the Antarctic-Australian spreading ridge (Christodoulou *et al.* 1984; Crohn 1986). As a result, Macquarie Island has never been connected to an adjacent land mass and all plants have arrived on the island via long-distance transoceanic dispersal or human introduction of seeds and spores (Seppelt 2004). Macquarie Island's closest neighbours are the Auckland and Campbell Islands, over 640 km away. The island's remoteness

from other land masses creates a barrier to propagule importation, and consequently there is low genetic diversity within the island's vegetation (Taylor 1955). Those species which successfully cross the ocean barrier are exposed to harsh environmental conditions that limit their survival potential. As a result the vegetation of Macquarie Island is poor and lacks diversity with only 46 species of native vascular flora, three of which are endemic to the island – *Azorella macquariensis* Orchard, *Corybas dienemus* (D.L. Jones), and *Puccinellia macquariensis* (Cheeseman; Copson 1984).

Macquarie Island is devoid of trees and shrubs due to the prevailing climate, but supports a wealth of grass and herb species that occur mostly around the lower slopes and narrow coastline, where they are protected from the wind (Spiegel and Stephenson 2000; Seppelt 2004). The vegetation is classified into five main formations, grassland, herbfield, fen, bog and feldmark (Taylor 1955).

**Grasslands** consist of two main species, a large tussock grass *Poa foliosa* (Hook.f.), and the mega herb *Stilbocarpa polaris* (Homb. et Jacq.) Gray, as well as the lesser species, *Poa cookii* (Hook.f.) Hook.f., and *Poa litorosa* (Cheeseman) and a few bryophytes (Taylor 1955; Seppelt 2004). Grasslands dominate well drained areas of the island where the water table is more than one foot below the surface, generally on the steep coastal slopes and inland valleys (Bunt and Rovira 1988). Short herb communities are often found in conjunction with *P. foliosa*, particularly in areas of disturbance, such as former land slips and in areas heavily grazed by rabbits (Kantvilas and Seppelt 1992).

In the higher areas of the plateau, in areas of moderate winds or in sheltered valleys, or in areas where the water table is close to the surface, **herbfields** prevail (Ashton 1965; Seppelt 2004). Herbfields contain greater species diversity than grasslands, and species include the dominant button moss, *Dicranoweisia antarctica* (C.M.) Par., as well as cushions of *Azorella selago* Hook., and the associate species *Pleurophyllum hookeri* and *Stilbocarpa polaris* A. Grey. (Clifford 1953; Spiegel and Stephenson 2000).

**Fen** communities occur in small patches on the raised beach terrace and in the valley floors of the plateau in areas where the water level is at or slightly above ground level and in contact with basaltic rocks and mineral soils (Taylor 1955). Similarly, **bog** formation requires the

same level of water, but is found in association with peat soils and only occurs in one area of the island, at Handspike point.

**Feldmark**, generally an open community of dwarf plants, mosses and lichens, is found in areas of high wind velocity and covers the greater part of the island above altitudes of 180 m (Kantvilas and Seppelt 1992). The vegetation cover varies depending on underlying rock substratum, drainage and exposure, and is composed predominantly of bryophytes such as *Ditrichum strictum* (Clifford 1953; Seppelt 2004).

**Fuel contamination and the impacts of human activity**

The relative isolation of Macquarie Island from population centres, as well as its extreme climatic and physical conditions, has greatly aided in its protection and conservation (Hall and Waulters 1994). However, like most other ecosystems on the earth, the environment of Macquarie Island has been modified by anthropogenic activities (Convey 2006; Tin *et al.* 2008). These activities have included: direct exploitation of resources such as penguin oil (Selkirk 1985; Bergstrom and Selkirk 2007), species introductions such as cats, rabbits and rodents (Copson and Whinam 2001; Whinam *et al.* 2005; Bergstrom *et al.* 2009), nature tourism (Hall and Waulters 1994), and accidental contamination and spills associated with research expeditions (Leszkiewicz 2001; Rayner *et al.* 2007; Schafer *et al.* 2007; Delille *et al.* 2007a). As a result, Macquarie Island has been left with a considerable legacy of marine and terrestrial contamination.

Fuel spill contamination is one of the most prevalent and damaging environmental issues within Antarctic and sub-Antarctic regions (Margesin and Schinner 1999; Snape *et al.* 2006; Rayner *et al.* 2007; Schafer *et al.* 2007). Petroleum hydrocarbons persist in the environment, are toxic and sometimes carcinogenic and can present significant health risks if they enter the food chain (Maila and Cloete 2002; Roy *et al.* 2005). Petroleum hydrocarbons are toxic to many soil invertebrates, and also impact plant growth and development, dissolving biological membranes and inhibiting seed germination (Chaudhry *et al.* 2005; Peng *et al.* 2009).

Several studies suggest that spills in higher latitude ecosystems are significantly more damaging, and have greater long term effects, than spills at lower latitudes, e.g. the *Exon Valdez*

oil tanker spill in Alaska (Simpson *et al.* 1995; Delille *et al.* 2002a; Braddock *et al.* 2003; Snape *et al.* 2006). This is largely due to the reduced temperature, water availability, nitrogen and oxygen levels in soil, all of which reduce natural degradation rates and the processing rates and efficiency of indigenous hydrocarbon degrading micro-organisms (Aislabie *et al.* 2001; 2006). Low temperatures also decrease the viscosity of oil and fuels, reducing the volatilisation of toxic short-chain alkanes and increasing their water solubility (Margesin and Schinner 1999). Volatile oil fractions in high latitude environments therefore persist for longer and the offsite migration of fuel or oil is relatively fast. Low temperatures also limit the activity of hydrocarbon degrading micro-organisms which leads to a reduced rate of natural attenuation (intrinsic bioremediation; Delille and Pelletier 2002b; Zekri and Chaalal 2005). In contrast to some sites closer to the poles (Ferguson *et al.* 2004), subpolar sites are limited by excess, rather than lack of, water (Rayner *et al.* 2007). This can lead to soils that are highly water-logged, and thus oxygen deficient. Furthermore, high levels of rainfall leach nitrogen from already poor sub-Antarctic soil (Walworth *et al.* 2007b), making nitrogen most often the limiting factor for hydrocarbon biodegradation in cold region soils (Mohn and Stewart 2000; Braddock *et al.* 2003). As a result, higher latitude regions impacted by hydrocarbon contamination experience a prolonged ecosystem recovery.

Several studies have investigated the effects of fuel spills at Macquarie Island. Impacts on marine communities were still seen one year after an oil spill from a tanker (*Nella Dan*) that ran aground on the island in 1987 (Simpson *et al.* 1995; Smith and Simpson 1998). In terrestrial habitats, Rayner *et al*. (2007) estimated that the rate of natural biodegradation of hydrocarbons from a spill on Macquarie Island was as low as 10 – 20 mg/kg soil per day. This rate is significantly lower than hydrocarbon degradation rates reported for soils in tropical and temperate regions (Merkl *et al.* 2005b; Wang *et al.* 2008). This research suggests that, if left untreated, even minor to moderate fuel spills could take tens to hundreds of years before natural attenuation can reduce the petroleum to environmentally acceptable concentrations (< 500 mg fuel per kg/soil; Rayner *et al.* 2007; Walworth *et al*. 2007a).

Human activities associated with the research station on Macquarie Island are heavily reliant on fossil fuels. During the cycle of transportation, storage and usage of the fuel, several accidental spills have occurred (Bergstrom and Selkirk 2007; Tin *et al*. 2008). Special Antarctic

Blend (SAB) is the main type of fuel used in on the Island as it allows vehicles and machinery to function at low temperatures. The majority of fuel contamination is associated with the storage of fuel, or occurs during the station's refuelling activities (Smith 2000; Bergstrom and Selkirk 2007; Rayner *et al*. 2007).

Fuel spills have occurred in the range of approximately 100 – 10 000 L, but three substantial plumes near the station on Macquarie Island have been identified as the sites of most concern (Rayner *et al.* 2007). Two of these plumes are located at the Main Power House, and one at the Fuel Farm (Figure 1.2).

An initial assessment of the soil contamination at Macquarie Island station was conducted by Deprez *et al*. in 1994. This study identified both spills at the Main Power House and the Fuel Farm, where fuel is stored, as highly contaminated with petroleum hydrocarbons. Eight 35 000 L bulk fuel tanks are situated at the Fuel Farm and while no spills at this site have been recorded, it is postulated that a spill may have occurred in the vicinity during 1988 (Australian Antarctic Division 2007). Deprez *et al*. (1994) recorded total petroleum hydrocarbons (TPH) up to 3 500 mg/kg at the soil surface. In a more recent study Rayner *et al*. (2007) showed that soil at the site had an average concentration of 800 mg/kg over an affected area of 760 $m^2$, and contained approximately 600 metric tons of low to moderately contaminated sandy soil.

The Main Power House was first contaminated in 1975 by an overflow of the settling tanks (Australian Antarctic Division 2007). No record of the volume of fuel spilt was recorded, but Deprez *et al.* (1994) reported that, similar to the Fuel Farm, the site contained up to 3 500 mg/kg of TPH in the soil. Concentrations of 2 800 mg/kg were recorded by Rayner *et al.* in 2007, over an area of 110 $m^2$ resulting in approximately 100 metric tons of contaminated soil.

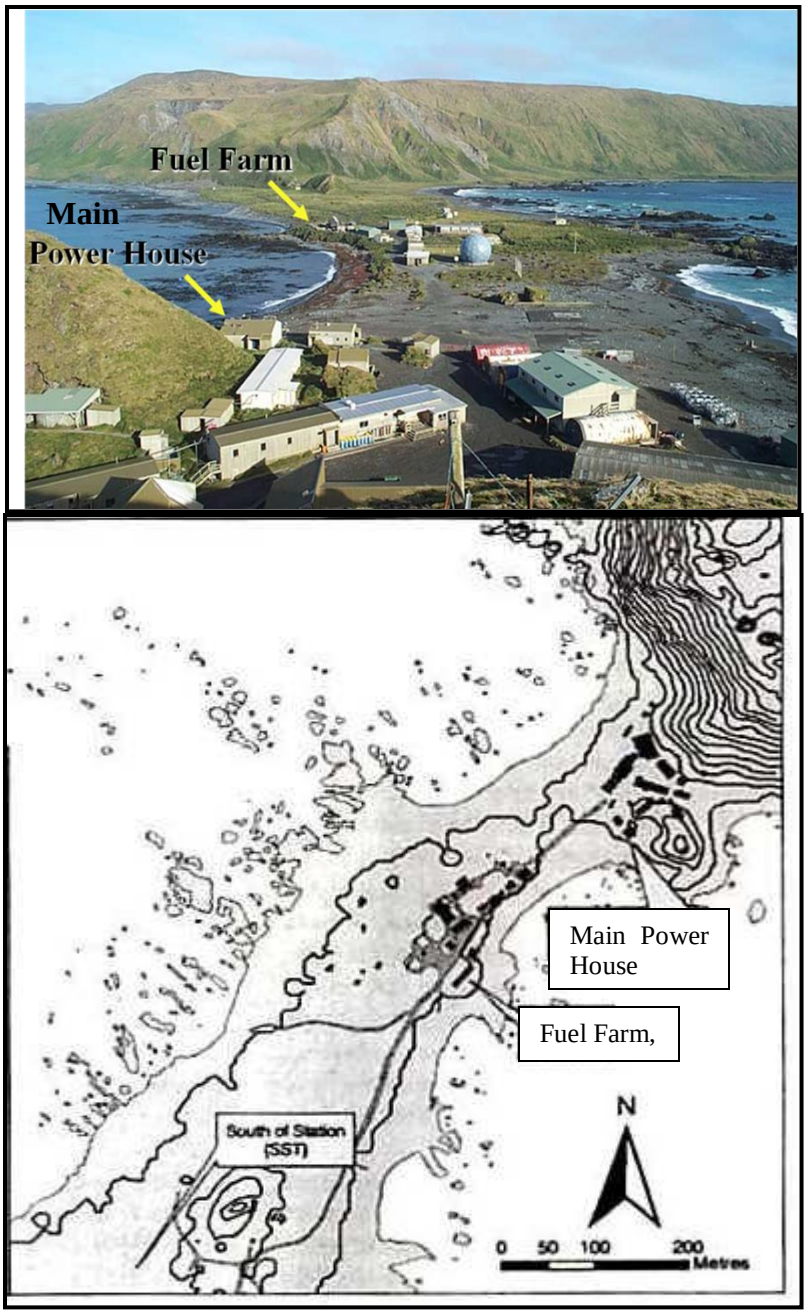


**Figure 1.2** Location of the two main contaminated areas on Macquarie Island. Adapted from Wasley *et al.* (2009) and Rayner *et al.* (2008).

The most recent spill at Macquarie Island occurred at the Main Power House in 2002. This area has an average fuel concentration of 7 000 mg/kg over an area of 220 $m^2$, resulting in approximately 180 metric tons of contaminated soil (Rayner *et al.* 2007). This is classified as a high level of contamination (Riser-Roberts 1998). Remediation of these sites is necessary as past

studies have shown the inadequacy of natural attenuation in areas at such low latitudes (Snape *et al*. 2006; Schafer *et al*. 2007; Delille *et al*. 2007b).

## 1.2 Environmental Remediation

The constant increase of environmental contamination by anthropogenic wastes and human activities has resulted in a global effort to devise methods of remediating sites which pose a threat to human health or to the environment. Although there have been many success stories in relation to environmental remediation, the process can sometimes be costly, time consuming and has, on occasion, caused more harm than good (Paine *et al.* 1996).

Traditional remediation techniques work to either 'decontaminate' the soil or to 'stabilise' the contaminants within it (Cunningham *et al*. 1995). This is achieved by a range of techniques based on physical, chemical or biological processes that can be broadly classified as either *in situ* or *ex situ*. *In situ* techniques include those in which the soil is remediated on site without the need for excavation or re-disposal. In contrast *ex situ* techniques require the contaminated soil to be excavated for treatment either on or off site (Table 1.1; Riser-Roberts 1998; Khan *et al*. 2000; Boulding and Ginn 2004). *Ex situ* techniques are, in general, less favourable than *in situ* techniques due to their invasive impact on the environment and their high cost (Cunningham *et al*. 1995).

**Table 1.1** Examples of traditional treatment methods for contaminated soil (Riser-Roberts 1998).

| ***In situ* techniques** | ***Ex situ* techniques** |
|---|---|
| Physical/Chemical processes<br>• air sparging (*currently under investigation at Macquarie Island*)<br>• hydrolysis<br>• steam injection<br>Biological processes<br>• bioventing<br>• bioremediation | Physical/Chemical processes<br>• soil washing<br>• thermal treatment<br>• chemical extraction<br>Biological processes<br>• bioreactors<br>• bio piles |

**Remediation in cold climates**

The success of remediation is heavily reliant on the nature of the contaminant(s) and on the environmental conditions in which it is applied (Chaudhry *et al.* 2005). In the case of cold regions, such as Macquarie Island, the need for active remediation has been demonstrated because of the slow natural attenuation rates which result in contaminant concentrations remaining high in the environment for many years (Snape *et al*. 2006; Delille *et al.* 2007; Rayner *et al.* 2007).

Due to the environmental conditions on Macquarie Island, and restrictions on disturbance and changes to the environment, the application of a number of traditional *ex situ* techniques for soils contaminated with petroleum hydrocarbons, such as bioslurry reactors, 'bio piles' and chemical extractions (Riser-Roberts 1998; Aislabie *et al.* 2001; Boulding and Ginn 2004), are not possible. In addition, the success of these methods is limited, due to low temperatures and

variable water distributions on Macquarie Island (Simpson *et al*. 1995; Schafer *et al.* 2007; Snape *et al.* 2008).

The selection of a remediation strategy to use at a site is usually based on a balance between the cost of the treatment and the time required for the remediation to occur (Snape *et al.* 2008). Both treatment cost and duration increase in cold regions as compared to temperate regions (Figure 1.3).

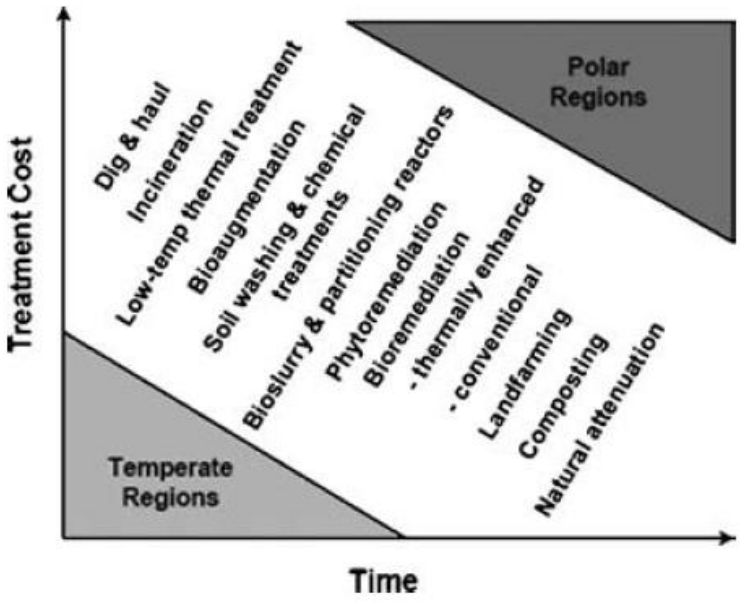


**Figure 1.3** Cost-time relationship for soil remediation options suitable for petroleum contaminants (Modified after Reynolds *et al*. 1998).

The most common, and quickest, remediation strategy used in Antarctic regions is the *ex situ* 'dig and haul' method where the contaminated soil is excavated mechanically and shipped back to the home country for treatment and disposal (Aislabie *et al.* 2001). In Antarctic and sub-Antarctic regions however, this technique is expensive, with estimated costs from Antarctica being approximately US$4 000/metric ton (Rayner *et al*. 2007; Snape *et al.* 2008). In addition, the ecological consequences and effects on terrestrial sub-Antarctic ecosystems of this method, which removes vast amounts of soil, may be significant and has not been assessed scientifically (Aislabie *et al.* 2001). For example, 'dig and haul' techniques can generate extensive changes to the chemical, physical and biological characteristics of the soil and may cause significantly more damage to fragile sub-Antarctic environments than if the spill were left undisturbed. This is

evident in an assessment of hydrocarbon contamination on Heard Island by Stark *et al.* (2003), which deemed 'dig and haul' remediation techniques inappropriate with reference to the obligations of the Madrid Protocol. Under Annex III (Waste Disposal and Waste Management) Article 1.5 of Madrid Protocol, Australia is legally bound not to dispose of wastes in such a way that:

*'the removal of any structure or waste material in circumstances where the removal by any practical portion would result in greater adverse environmental impacts than leaving the structure or waste material in its existing place'*

With this goal in mind, more sophisticated response options are needed in order to successfully remediate the three petroleum spills identified on Macquarie Island.

**Possible remediation strategies for sub-Antarctica**

There are numerous sites contaminated by petroleum hydrocarbons in the sub-Antarctic (Aislabie *et al.* 2001). Studies by Stark *et al.* (2003) at Heard Island suggest *in situ* procedures as the most appropriate clean up option for sub-Antarctic islands. Possible *in situ* strategies for cold climate soils have been explored in studies by Snape *et al*. (2008), and include bioremediation.

Bioremediation involves the use of micro-organisms (mainly bacteria) to destroy or transform hazardous contaminants through their metabolism (Riser-Roberts 1998). Field experiments by Delille *et al*. (2002a; 2003; 2007a), and Coulon *et al.* (2004) on the sub-Antarctic Kerguelen Archipelago found evidence of indigenous hydrocarbon-degrading micro-organisms with the potential for rapid biodegradation of hydrocarbons in soil. The presence of indigenous hydrocarbon-degrading species is essential as the Antarctic Treaty prohibits the importation of foreign organisms into the Antarctic region (Bunt and Rovira 1988; Aislabie *et al.* 2001). Coulon *et al.* (2004) also found that the efficacy of bioremediation increased significantly when the soil was treated with slow release fertilizer or fish composts, however, some remaining product residues had relatively high toxicity.

The success of *in situ* bioremediation in the sub-Antarctic is predominately dependent on contaminant type and substratum characteristics. While Coulon and Delille (2006) reported successful remediation, Walworth *et al*. (2007a) found bioremediation was inhibited in sub-polar

regions where nitrogen levels were below 1 200 mg N/kg $H_2O$. This poses a potential problem for many contaminated cold region soils, which are often nitrogen deficient (Rayner *et al.* 2007), except in areas that receive substantial inputs from animal sources (Erskine *et al.* 1998). Furthermore, the biodegradation of certain organic petroleum compounds is restricted due to their recalcitrant nature and, with the exception of naphthalene, by their low solubility and strong absorption potential (Banks *et al.* 2000; Smith *et al.* 2006; Euliss *et al.* 2008). Once in soil, hydrocarbons show little to no volatilisation, are generally immobile, and are very slow to degrade (McNicoll and Baweja 1995; Smith *et al.* 2006; Schröder *et al.* 2008). Studies have shown that microbial bioremediation alone can take up to four years to degrade relatively small concentrations of contaminant and is not sufficient to obtain a complete return to near pristine conditions in sub-Antarctic soil (Delille *et al.* 2007a).

Other *in situ* remedial techniques considered for the sub-Antarctic include bioventing, which is routinely used in sub-Arctic North America, including Alaska and Canada (Filler *et al.* 2006). Bioventing involves oxygenation and warming of the contaminated soil through the vertical injection of air into the sub surface (Riser-Roberts 1998; Dupont 2006). Available hydrocarbons are volatilised and removed via strategically placed vertical extraction wells. The advantages of bioventing include a 10 000 times higher rate of gas diffusion than that of solutes, an increased rate of air diffusion providing oxygen to micro-organisms, and an increased rate of volatilisation of oil which improves and unifies the distribution of oil on the surface and in the aggregates (Riser-Roberts 1998). However, a number of disadvantages of this treatment restrict its application. Bioventing can alter ground freezing and is usually limited to the thaw season. The continuous airflow can also lead to desiccation of the soil, reducing moisture and nutrients that are vital for the stimulation of micro-organisms (Hoeppel *et al.* 1991).

Landfarming has also been used extensively as a remedial treatment in cold regions such as Alaska, Canada, Sweden, The Netherlands and Denmark (Riser-Roberts 1998; Snape *et al.* 2008). Several studies, however, have shown mixed results in Antarctic and Arctic climates (McCarthy *et al.* 2004). Landfarming involves spreading out contaminated soil to a thin layer (between 0.3-1.0 m thick) and regularly tilling (ventilating) the soil, as well as mixing the soil with a range of nutrients contained in fertilizers (Sanscartier *et al.* 2009). During this process, hydrocarbons may be lost via volatilisation or bioremediation (Paudyn *et al.* 2008). The

technique is relatively simple and low cost, however, it is encumbered by its sensitivity to weather conditions, low rates of degradation in cold conditions and its potential for contaminating ground water through leachate (Riser-Roberts 1998).

While detailed studies of petroleum contamination at Macquarie Island have been presented (Deprezer *et al.* 1994 and Rayner *et al.* 2008) only one study has evaluated possible remedial treatments for the island (Australian Antarctic Division 2007). The strategies differed between the three spill locations and included methods such as permeable reactive barriers, nutrient addition, air sparging and generating biopiles. Past attempts to remediate the spills at Macquarie Island have included the use of aeration in the form of both air sparging and 'microbioventing" (Rayner *et al.* 2008). Air sparging was unsuccessful as it penetrated the shallow water table which led to channel development. In contrast, microbioventing successfully aerated a wide area of soil and removed considerable quantities of hydrocarbon from the water fraction. This method however is costly and invasive, and the technical equipment used requires constant monitoring and servicing. Further research is therefore needed to devise a model of remediation that is both cost effective and environmentally sensitive.

## 1.3 Phytoremediation

Over recent years, scientists have increasingly looked to plants as a means of counterbalancing the negative anthropogenic impacts of contamination and assimilation of contaminants in the environment (Cunningham and Ow 1996; Harvey *et al*. 2002; Roy *et al.* 2005; Wang *et al.* 2008). The presence of vegetation has been shown to have positive effects on contaminated soil, leading to a greater rate of degradation, removal, and mineralisation of wastes than in non-vegetated soils (Gaskin *et al.* 2008; Liste and Prutz 2006; Banks *et al.* 2003; Delille *et al.* 2003; Macek *et al.* 2000). This advance toward environmental restoration has been termed phytoremediation (*phyto* – plant, *remediation* – to recover).

Phytoremediation involves the use of vascular plants, fungi, and algae to remove and control wastes or to augment waste breakdown through micro-organisms in the plant's rhizosphere (McCutcheon and Schnoor 2003; Chen *et al.* 2004). The technique was originally used as a method to treat waste water and contaminated ground water via a series of constructed wetlands, reed bed and floating plant systems (Cunningham *et al.* 1995). More recently the use

of phytoremediation has been expanded to address a larger range of contaminants in soils, such as heavy metals, hydrocarbons and atmospherically transported pollutants (Khan *et al.* 2000).

**Advantages and disadvantages of phytoremediation**

Phytoremediation is an attractive alternative to physical and chemical remediation techniques. Phytoremediation is a "clean" technology that has the ability to operate without the aid of an external power source. Plants can act as self-engineers, working independently to remediate a site by controlling the local biochemistry, rhizosphere and microclimate in order to continually remove or break down contaminants from the soil (Ensley 2000; McCutcheon and Schnoor 2003). Phytoremediation is therefore both low maintenance and low cost (Ensley 2000) and can be used on a much larger scale than other treatment technologies (Macek *et al.* 2000). It may prove ideal for sub-Antarctic locations where remoteness restricts access and ongoing monitoring and maintenance of equipment. Currently, up to $50 billion per year is spent on environmental clean-up worldwide (Pilon-Smits 2005). Phytoremediation is on average tenfold cheaper than traditional techniques such as soil excavation and soil washing or burning (Pilon-Smits 2005). Additionally, phytoremediation carries little risk to the environment and human health. The *in situ* application of phytoremediation creates minimal disturbance to the contaminated site (Ensley 2000) and increases public acceptance due to plant aesthetics (Tucker and Shaw 2000). Non-intrusive remedial techniques such as phytoremediation are paramount in sub-Antarctic areas, including Macquarie Island, where traditional techniques are deemed too invasive to the fragile ecosystem.

Nevertheless, phytoremediation has a number of disadvantages that may constrain its application on Macquarie Island. While cost is phytoremediation's greatest advantage, time is its greatest disadvantage (see Figure 1.3; Table 1.2). Phytoremediation is habitually slower than other technologies and is generally regarded as a long term remediation strategy to be used in combination with others (Cunningham *et al.* 1995; Khan 2005). Estimates have predicted that 18 – 60 months may be needed for full site recovery in some cases (Ensley 2000) and in cold climates such as the sub-Antarctic, duration is likely to be increased. In addition, plants used for phytoremediation must be able to tolerate exposure to high levels of contaminants without showing any toxic effects (Macek *et al.* 2000; Khan *et al*. 2000). As the introduction of potentially suitable plant species is prohibited on Macquarie Island, appropriate species must be

found within the native flora. Finally, phytoremediation is limited to the depths of roots, and hence may need to be coupled with other remedial technologies in cases where contaminant filtration is deeper than can be reached by the plant (Pilon-Smits 2005).

**Table 1.2** Advantages and disadvantages of phytoremediation**.**

| **Advantages** | **Disadvantages** |
|---|---|
| • Low cost<br>• Low maintenance<br>• Minimal disruption to the environment<br>• Independent of external energy supply<br>• Public acceptance: aesthetically pleasing<br>• *In situ* application avoids excavation<br>• Applicable to a variety of contaminants<br>• There is no need for disposal sites<br>• Can be used during site investigation or after closure | • It may take longer than other technologies<br>• May not be applicable to mixed wastes<br>• Only applicable to surface soils<br>• Lack of recognised economic performance data<br>• Formation of vegetation may be limited by extremes of environmental toxicity and climate |

**Phytoremediation of petroleum hydrocarbons**

The application of phytoremediation is governed by the environment and the class of contaminant in the soil (Allard *et al.* 2000; Parrish *et al.* 2005; Alarcon *et al.* 2008). Organic and inorganic contaminants have fundamentally different chemical and physical properties and are therefore removed via different phytoremediation strategies (Figure 1.4). The chemical characteristics of organic contaminants, such as petroleum hydrocarbons, means that they are less readily incorporated by plants and therefore mechanisms for removal are usually less understood than for inorganic compounds (Hou *et al.* 2001; Chen *et al*. 2004). The presence of vegetation generally accelerates the biodegradation of organic compounds via three techniques: phytodegradation, phytovolatilization or rhizodegradation. All three systems rely on modifying the contaminant within the soil, and usually achieve this via a synergistic relationship between the plants and their allied microbiological communities (Sicilliano and Germida 1998; Alkorta and Garbisu 2001). This is in contrast to inorganic contaminants, as they are unchallengeable at an elemental level and plants must therefore work to either phytoextract the contaminant, rhizofiltrate it, or phytostabalize the contaminant within the soil (Cunningham and Ow 1996;

Philips *et al.* 2008). Detailed descriptions of these processes are outside the scope of this study, see reviews by McCutcheon and Schnoor (2000), Pilon-Smits (2005) and Wensel (2008) for further details.

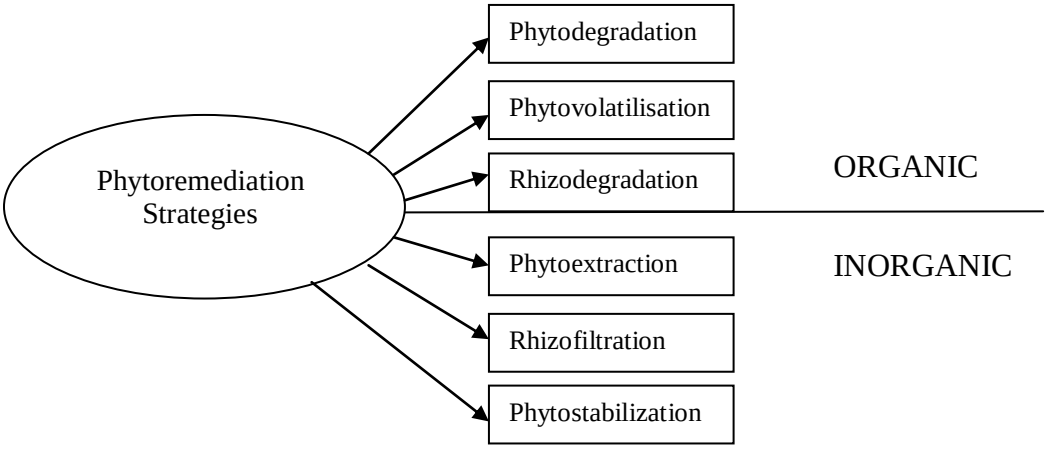


**Figure 1.4** Phytoremediation strategies employed for various contaminant types. Refer to Wensel (2008) for further details.

Over the past decade, substantial progress has been made in applying phytoremediation to inorganic pollutants such as heavy metals (Khan *et al*. 2000; Chen *et al*. 2004; Fischerova *et al.* 2006) and organic pollutants such as poly-aromatic hydrocarbons (PAH), nutrients and landfill leachate (Maila and Cloete 2002; Roy *et al.* 2005; Schröder *et al.* 2008). Most studies have focused on the ability of plants to hyperaccumulate inorganic heavy metals from soils (Macek *et al.* 2000; Ensley 2000; Khan 2005; Kvesitadze *et al.* 2006), with relatively few exploring the potential of plants to remediate soils contaminated with petroleum hydrocarbons, especially in cold climates (Alkorta and Garbisu 2001).

From a detailed study of the literature, rhizodegradation is predicted to be the most likely strategy used by plants on Macquarie Island to clean up sub-Antarctic sites contaminated with SAB. This form of phytoremediation involves an interaction between plants and micro-organisms and is referred to as 'Rhizoremediation', 'Rhizodegradation' or the 'Rhizosphere effect' (Anderson *et al.* 1993; Newman and Reynolds 2004). The 'rhizosphere' is the zone where roots exert a strong influence on the soil and it extends approximately 1 mm from the plant roots (Banks *et al*. 2003; Olson *et al.* 2003). This area of the soil has increased micro-organism populations and a considerably higher number of metabolically active micro-organisms

compared to any other region of the surrounding soil (Nichols *et al.* 1996; Phillips *et al.* 2008). The presence of such large microbial numbers are due, in part, to plant exudates which serve as a source of energy, carbon and nitrogen (Siciliano *et al.* 2003; Euliss *et al.* 2008; Kovar 2009). During rhizodegradation the plant provides these root exudates (such as enzymes, simple sugars, amino acids, aliphatics and aromatics) to stimulate the growth of root-associated micro-organisms (Khan 2005; Gaskin *et al.* 2008; Phillips *et al.* 2008). Root growth can also perturb deeper soil, causing better oxygen diffusion and water infiltration, changing the carbon dioxide concentration, pH, redox potential, osmotic potential, moisture content and oxygen concentration of the soil which leads to an environment better able to support high microbial biomass (Lin *et al.* 2008). In return, microbes can reduce the phytotoxicity of the contaminants in the soil or augment the degradative capacity of the plant (Sicilliano and Germida 1998). This mutualistic relationship between plants and microbes in the rhizosphere therefore leads to accelerated degradation of petroleum hydrocarbons in the presence of plants (Maila and Cloete 2002; Newman and Reynolds 2004; Zhuang *et al.* 2007; Peng *et al.* 2009).

Phytoremediation of petroleum contaminants has proven to be a feasible method for the removal of petroleum hydrocarbons from surface soil in both growth room studies and field trials in natural conditions (Hou *et al.* 2001; Lalande *et al.* 2003; White *et al.* 2006; Liste and Pruz 2006; Gaskin *et al.* 2008). In a growth room study, Peng *et al.* (2009) observed generally greater remediation efficiency in soil planted with *Mirabilis jalapa* L. than in un-vegetated soils. Euliss *et al*. (2008) found that sediments planted with sedge (*Carex stricta*), switchgrass (*Panicum virgatum*) and gamagrass (*Tripsacum dactyloides*) had significantly less residual total petroleum hydrocarbons (TPH) after one year of growth (70% reduction) than soils containing no plants (20% reduction). Similarly, Chen *et al.* (2003) demonstrated that switchgrass (*Panicum virgatum*) and tall fescue (*Festuca arundinacea*) mineralised hydrocarbons by 30% and 38% respectively, compared to 4.3% in the unplanted control. Palmroth *et al*. (2002) found phytoremediation of diesel fuel using a legume mixture (White clover, *Trifolium repens* and Pea, *Pisum sativum*) to be successful in sub-Arctic soils, and concluded that phytoremediation is a viable, low-cost remedial technology for diesel contaminated soils in sub-Arctic regions.

Coupling phytoremediation with other physical and chemical processes already in place on Macquarie Island (such as nutrient additions and air sparging) may be an effective solution to

the issue of SAB contamination within this region. There is evidence to suggest that a multi-technique approach, involving phyto-oxidation, volatilisation, and microbial remediation coupled with phytoremediation, can be twice as effective in reducing TPH levels as landfarming, 50% more effective than bioremediation, and 45% more effective than phytoremediation treatments alone (Huang *et al.* 2004; 2005; Lin *et al.* 2008). However, before this option can be explored, the identification of a suitable species for phytoremediation, and an assessment of its ability to reduce SAB concentrations above natural microbial and mechanical processes, needs to be undertaken.

## 1.4 Aims and thesis structure

This thesis presents the first experimental investigation into the viability of phytoremediation in the sub-Antarctic. Phytoremediation of Macquarie Island fuel spills using the native grass *Poa foliosa* was assessed in a laboratory trial. The aims of this thesis were to (1) investigate the tolerance of *P. foliosa* to hydrocarbons, (2) evaluate if the presence of *P. foliosa* significantly increases the microbial populations within the soil and (3) assess the ability of *P. foliosa* to assist with biodegradation of hydrocarbons in the soil. The thesis is divided into 4 chapters as follows:

**Chapter 1:** Introduction to Macquarie Island, possible remediation techniques for petroleum contamination in cold climates and an overview of phytoremediation.

**Chapter 2:** The tolerance of *P. foliosa* to a range of hydrocarbons is paramount if this species is to be used in phytoremediation strategies. A number of physical parameters were evaluated to assess plant health over time in plants exposed to a range of hydrocarbon concentrations. These include non-destructive techniques such as chlorophyll florescence measurements and destructive techniques such as leaf length, width and area, shoot and root biomass, and chlorophyll pigment extraction.

**Chapter 3:** The rhizosphere of plants harbours populations of microbes. This chapter describes investigations to determine whether the presence of *P. foliosa* increases microbial abundance in the soil. Most Probable Number assays were used to compare planted and unplanted replicates across a range of hydrocarbon concentrations through time. The effect of *P. foliosa* on

hydrocarbons in the soil was assessed by measuring the total petroleum hydrocarbon (TPH) concentration of both planted and unplanted replicates over time and between concentrations.

**Chapter 4:** This chapter provides a synthesis of the major findings of this study and makes recommendations for future research.

# Chapter 2

## The tolerance of the sub-Antarctic grass *Poa foliosa* to Special Antarctic Blend (SAB) fuel, and the effects of petroleum hydrocarbons on plant health and productivity

### 2.1 Introduction

Phytoremediation has been found to be a feasible approach for the *in situ* cleanup of surface soils contaminated with petroleum hydrocarbons (Fiorenza *et al*. 2000; Banks *et al*. 2003; Wang *et al.* 2008). Plant physiology, its tolerance to the physico-chemical nature of the contaminant, and its ability to thrive in the given environment, all greatly influence a plant's remedial efficiency (Siciliano *et al.* 2003; Gaskin *et al.* 2008). Precursor studies to identify plant tolerance to hydrocarbon contamination in sub-Antarctic conditions are therefore necessary in the selection of appropriate species for the successful application of phytoremediation strategies in this region.

A growing body of literature suggests that the basic composition of a plant dictates its ability to promote the degradation of petroleum hydrocarbons (Hutchinson *et al.* 2003; Liste and Prutz 2006; Phillips *et al.* 2009). Certain plant characteristics, such as a large fibrous root system, fast growth, large biomass both above and below ground, the presence of root exudates and numerous microbial interactions enhance a plant's phytoremediation potential (April and Sims 1990; Cunningham and Ow 1996; Hou *et al.* 2001). Families of plants that displayed these characteristics, such as the Poaceae family, were found to be the most effective at reducing hydrocarbon concentrations in soils in a growth room study that compared the remedial potential of eight plant families (Olson *et al.* 2007). Similarly, a recent study by Euliss *et al.* (2008) found a significant reduction in petroleum hydrocarbon concentration in soils planted with sedge (*Carex stricta*), switchgrass (*Panicum virgatum*) and gamagrass (*Tripsacum dactyloides*). Whereas, willow (*Salix exigua*) and poplar (*Populus spp*.) species did not significantly reduce soil hydrocarbon concentrations. The plant species used can therefore greatly influence the effectiveness of phytoremediation. This being said, the basic precondition for any successful

remediation species includes the plants ability to proliferate in the presence of high levels of petroleum contaminants (Frick *et al.* 1999; Huang *et al.* 2004).

In general, the existence of petroleum hydrocarbons in soil has a negative impact on plant growth and development (Joner *et al.* 2004; Gaskin *et al.* 2008). Growth is inhibited with increasing hydrocarbon concentration (Maila and Cloete 2002; Smith *et al.* 2006), or slowed to the point that plants do not produce meaningful biomass for successful remediation (McNicoll and Baweja 1995; Kvesitadze *et al.* 2006). Numerous studies have investigated the response of an assortment of species to various concentrations of hydrocarbons, with the general consensus that grass species display a certain level of resistance (Gunther *et al*. 1996; Banks *et al.* 2003; Huang *et al.* 2004; Liste and Prutz 2006; Gaskin *et al.* 2008; Wang *et al.* 2008). However, no research has explored hydrocarbon tolerance of grass species endemic to Macquarie Island.

The potential to use phytoremediation on Macquarie Island is compromised by the vast comparative climatic difference of sub-Antarctica compared to lower latitudes, coupled with stringent restrictions on species importations. Hence suitable climate and contaminant tolerant native species must be identified and, to my knowledge, no studies have been conducted on phytoremediation in the sub-Antarctic (Palmroth *et al.* 2002). In a study in which the phytoremediation potential of 39 sub-Arctic cold-tolerant plants was investigated, *Psoralea esculentu* (a legume species) and *Agropyron perctiniforme* (a grass species) were found to be the most tolerant to hydrocarbons, and the most promising for use in phytoremediation (Robson *et al.* 2003). These findings, coupled with the previously identified favourable physiological features, imply that Macquarie Island's largest endemic grass species, *Poa foliosa*, maybe a suitable candidate for phytoremediation in the sub-Antarctic region.

The object of this study was to determine the tolerance of *P. foliosa* to petroleum hydrocarbon contamination. The effects of hydrocarbons (SAB) on the health and growth of *P. foliosa* were examined by experimentally exposing plants to a range of concentration treatments over eight months. Biomass production, plant morphology, and maximal photosynthetic efficiency were all used as indicators of plant health. Maximal photosynthetic efficiency of PSII is a chlorophyll florescence signal that provides a useful gauge of plant stress (Krause and Weis 1991; Maxwell and Johnson 2000). The majority of healthy plants have a maximal

photosynthetic efficiency, measured as the variable over the maximal fluorescence $F_v/F_m$, of between 0.80 – 0.83 (Bjorkman and Demmig 1987).

The specific aims of this study were to 1) observe the sensitivity of *P. foliosa* to SAB, and 2) to determine the effects of exposure to different concentrations of hydrocarbons on the health, morphology and biomass of *P. foliosa*. It was hypothesised that exposure to higher concentrations of hydrocarbons in the soil would significantly decrease both the health and growth potential of *P. foliosa*. The null hypothesis was that hydrocarbon concentration would not affect the health and growth potential of *P. foliosa*.

## 2.2 Materials and Methods

### 2.2.1 Study species

The sub-Antarctic native tussock grass *Poa foliosa* (Hook. f.; Figure 2.1) is widely distributed across the coastal zones of Macquarie Island and is most abundant on the upper beach slopes and raised coastal terraces (Seppelt 2004). It grows readily on the Isthmus (where Australia's research station is located; Figure 2.2), and surrounds the sites of SAB contamination. This species was selected for this study as it displays a number of the criteria of a potentially successful plant for phytoremediation with a deep (~0.5 m) fibrous root system, a large biomass and a fast growth rate (Clayton *et al*. 2002).

**Figure 2.1** *Poa foliosa* in its natural habitat at Macquarie Island. Photograph taken by N. Grant.

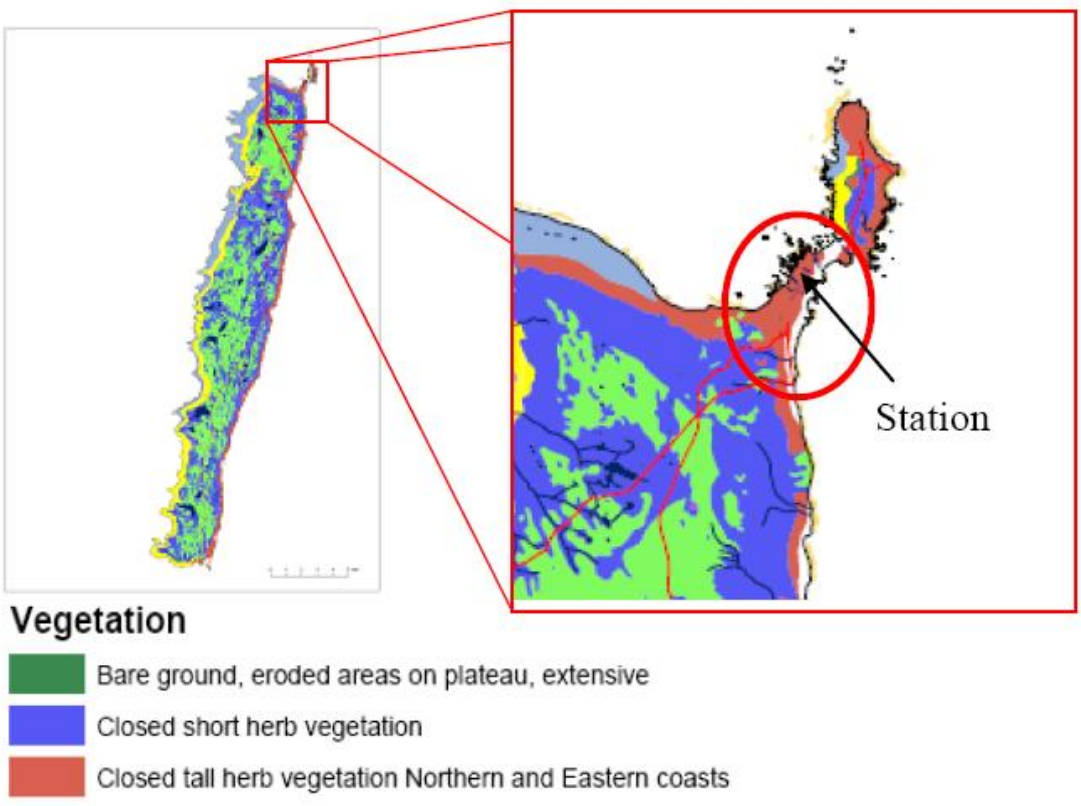


**Figure 2.2** Distribution of vegetation on the Isthmus of Macquarie Island. Adapted from Macquarie Island vegetation. Edition: 1. Australian Antarctic Division.

*Poa foliosa* can reach a height of 1.5 m with numerous leaf-blades, generally 15 – 40 cm long and 3 – 6 mm wide. It develops and spreads almost entirely by its complex rhizome system, resulting in the accumulation of an extremely dense mass of dead and living roots beneath the ground's surface, creating a pedestal of fibrous peat that supports the large crown of the plant (Ashton 1965). As much as one-third of the weight of the top of the plant is replaced in the first year following defoliation, indicating that the growth rate of *P. foliosa* is high despite the cold climatic conditions of Macquarie Island (Ashton 1965).

The life of the plant is cyclic, with four stages of development (Ashton 1965): 1) Pioneer Phase which includes seedlings; 2) Building Phase in which tussocks are beginning to form; 3) Mature Phase when the maximum height is reached; and 4) Degenerate Phase when the leaves become shorter and there is a substantial increase in the proportion of dead to living leaves. *Poa foliosa* can develop to the building phase in less than five to six years and can remain a mature or degenerate plant for approximately 50 years.

### 2.2.2 Sampling procedure and experimental design

An eight-month laboratory study was done to evaluate the applicability of phytoremediation using *P. foliosa* to remediate petroleum spills in cold climates. Special Antarctic Blend (SAB) fuel was obtained from the Australian Antarctic Division (AAD) and used to spike the soil (Tasmanian Devils Dirt potting mix) at nominal concentrations of 1 000, 5 000, 10 000, 20 000 and 40 000 mg/kg in addition to the control. SAB was applied to the soil in 50 ml increments, during which the soil was continuously mixed in a large mechanical mixer. Following the addition of SAB, each treatment was mixed thoroughly for ten minutes to achieve homogeneity.

Two sets of five replicate pots, each containing approximately 1 L of soil, were prepared for each treatment. Five replicates at each concentration were planted with a single transplanted seedling, and the remaining five replicates were left unplanted (Figure 2.3). Seedlings of *P. foliosa* (Pioneer Phase) were obtained from the Royal Tasmanian Botanic Gardens on the 17$^{th}$ of October 2007. These plants germinated from the Botanical Gardens seed bank on the 3$^{rd}$ of June 2007 and were grown in conditions similar to a sub-Antarctic climate. The seedlings were randomly selected from the stock of plants and randomly assigned to pots using a random number generator table.

**Figure 2.3** Planted and unplanted replicate pots.

All pots were kept in a temperature controlled growth room at 8°C (± 1°C). The room was lit by eight incandescent lights and four high intensity growth lights which were programmed to a day/night photoperiod of 8.75/13.25 hours to simulate the average yearly natural daylight cycle on Macquarie Island. Pots of the same SAB concentration were stored together in group trays to prevent the volatiles released from affecting the concentration of different treatments (Figure 2.4). Planted and unplanted pots of the same concentration were randomly arranged in each tray and each tray was randomly placed on shelves within the growth room. Every three days, trays were rotated clockwise on the shelves and each pot was given ~30 ml of water. Foliar spray was also applied to ensure leaves maintained maximum health. Pots were allowed free drainage into individual pans, and the water collected was used to re-water the plant to minimise the loss of hydrocarbons from the soil.

**Figure 2.4** Planted and unplanted replicates in grouped SAB concentration trays within the growth room.

An initial harvest was conducted at the start of the experiment and at two, four and eight months thereafter, for the 0, 1 000, 5 000, and 10 000 mg/kg treatments. Pots at concentrations of 20 000 and 40 000 mg/kg were only harvested at four and eight months. During each harvest, plant material was separated from the soil and kept in a refrigerator (4°C) prior to analysis. Soil

from each pot was homogenised and sub-sampled into samples which were kept in a refrigerator (4°C) until analysed.

### 2.2.3 Soil organic content

A 10 g soil sample of uncontaminated Tasmanian Devils Dirt potting mix was sieved to obtain the <2 mm fraction and milled for three minutes at 500 rpm. Five 500 mg sub-samples were then weighed into a pre-cleaned crucible and analysed for total carbon using a Shimadzu TOC – VCSH analyser (Shimadzu TOC analyser, Kyoto Japan) with solid state module attachment (SSM - 5000A).

### 2.2.4 Chlorophyll fluorescence

Chlorophyll *a* fluorescence measurements were performed throughout the experiment at approximately bi-weekly (first two months) then monthly intervals (from two to eight months). Measurements were taken using a diving pulse-amplitude-modulated photosynthesis yield analyser (Diving-PAM, H. Walz Effeltrich, Germany). All plants were dark adapted for 20 minutes prior to measurements of maximum photosynthetic efficiency. After 10 minutes the PAM settings were optimised to the *P. foliosa* leaves to ensure maximum signal resolution for the species. All measurements were collected within two hours of midday. Four replicate measurements were taken per plant and, due to the leaf morphology of the species, five leaves adjacently banded together were required to gain a representative reading. The youngest fully expanded leaves were used, and measurements were taken approximately four cm from the base of the leaf blades.

### 2.2.5 Leaf morphology

Prior to the destruction of plants for biomass estimates, the physical parameters of leaf morphology, including leaf length, width and area, were measured and recorded. Once separated from the soil, plants were placed inside individual plastic zip lock bags to retain moisture, and were kept in a refrigerator (4°C) for a maximum of one day before analyses were performed. Due to the extensive number of leaves per plant, a sub-sample of 30 leaves was measured. Once separated from the roots, shoots were mixed and laid out in a flat line on a bench surface. The first 30 leaves were then taken from the left hand side of the line. Leaves were separated from

the stems at the axil, and the length and width of each leaf was measured to the nearest millimetre.

The leaf surface area was determined by running each leaf through a Portable Leaf Area Meter (LI-COR Model LI-3000A Portable Leaf Area Meter, Lincoln, Na., USA). This system utilises a flat-bed digital scanner with light-emitting diodes (LED's) which are sequentially pulsed to examine the leaf. Each individual leaf was placed between two clear transparent sheets and spread out to maximise leaf surface area. The sheets were then placed in the scanner, and pulled through twice to obtain an average reading for surface area.

### 2.2.6 Plant biomass

The above ground and below ground biomass was measured destructively. The total shoots and leaves from each plant were weighed to obtain a shoot wet biomass estimate. Roots were carefully washed over a 0.25 mm sieve using tap water to remove soil. Any displaced roots were extracted from the sieve. Roots were then blotted dry with paper towel, and weighed to obtain a root wet biomass. Shoot and root samples were then dried at 60°C for 72 hours, and reweighed to acquire dry biomass estimates which were used to calculate shoot to root ratios for each plant. The Mean Relative Growth Rate (MRGR) of shoots and roots was calculated based on the following equation (taken from South 1995):

$$MRGR = (\ln W_2 - \ln W_1)/(T_2 - T_1)$$

Where $W_1$ and $W_2$ are the dry weight of shoot or root at $T_1$ (two months) and $T_2$ (four months) respectively, ln is the natural logarithm.

### 2.2.7 Photosynthetic pigment extraction

Three leaves per plant were randomly selected for pigment extraction. Between 10 and 20 mg (wet weight) of photosynthetically active plant tissue, per leaf, was weighed, and instantly frozen at -80°C for a maximum of two months until further analysis. Each sample was ground to a fine powder using acid washed sand and liquid nitrogen in a mortar and pestle. A 0.5 ml aliquot of buffered 80% acetone (pH 7.8) was then added and the sample was ground further to achieve homogeneity. Samples were transferred to 2 ml Eppendorf tubes and combined with three further washings of the mortar and pestle (each of 0.5 ml) with the same solvent. Tubes were allowed to

stand in the freezer for a period of 24 hours, after which they were centrifuged for 10 minutes at 2500 rpm (in an Eppendorf 54153 D bench centrifuge). The supernatant was made up to 1.5 ml. Absorbance at wavelengths of 750, 646.6 and 663.6 nm was determined using a UV-Visible spectrophometer (Shimadzu UV-1691, UV-Vis Spectrophometer, Kyoto Japan). Total chlorophyll concentrations and chlorophyll *a/b* were determined using the equations of Porra *et al*. (1989).

### 2.2.8 Statistical analysis

Raw data for each measured parameter from each treatment were analysed for significant differences. Prior to analysis, data were tested for normality using the Shapiro-Wilk W Test, and for homogeneity of variance using Cochran's *C* test. Square root transformations were used where needed to normalise the distribution of the data. Two extreme outliers were excluded from the chlorophyll *a/b* data. Outlier exclusion did not change the outcome of results.

Data were analysed using a general linear model in the statistical program JMP (Vol. 5.1 SAS Institute Inc., U.S.). Where the general linear models revealed significant interactions, Tukey HD *post hoc* tests were used to identify significantly different means. Trial analyses were run to test the power of a Tukey HD test on the selected data. Very few situations were found to have marginally failed to reject the null hypothesis which suggests that there are no problems from insufficient power.

For data sets larger than 900 observations, a Shapiro-Wilks W Test was often over sensitive in detecting data as non normal, even after data transformation. In these cases non-parametric Kruskal-Wallis test were run, followed by pair wise Wilcoxon tests. The Wilcoxon *p* values were compared to sigma values obtained from a Bonferroni test.

Two-way ANOVA analysis between concentration treatments and time were not carried out due to uneven sampling numbers. However, in situations where no significant differences were found between concentration treatments 0 to 10 000 mg/kg (such as biomass), values were pooled and t-tests were used to examine the change between two and four months.

As a result of the plants extremely root bound state (Figure 2.5), data from the eight month harvest were not analysed since this state likely had a major influence on plant health.

**Figure 2.5** Examples of the root bound state of plants at the eight month harvest.

## 2.3 Results

### 2.3.1 Soil organic content

Triplicate measurements gave an average soil organic content of 13.6 ± 0.2% (mean ± SD).

### 2.3.2 Morphology

**Leaf length**

There was no difference in leaf length between SAB treatments at two months (Figure 2.6a). After four months exposure concentrations of SAB from 1 000 to 10 000 mg/kg were intermediate between the control and higher SAB treatments but not significantly lower than the control. At higher concentration treatments, 20 000 to 40 000 mg/kg, however, leaves were significantly shorter than the control (0 mg/kg; $p = 0.0001$; Table 2.1).

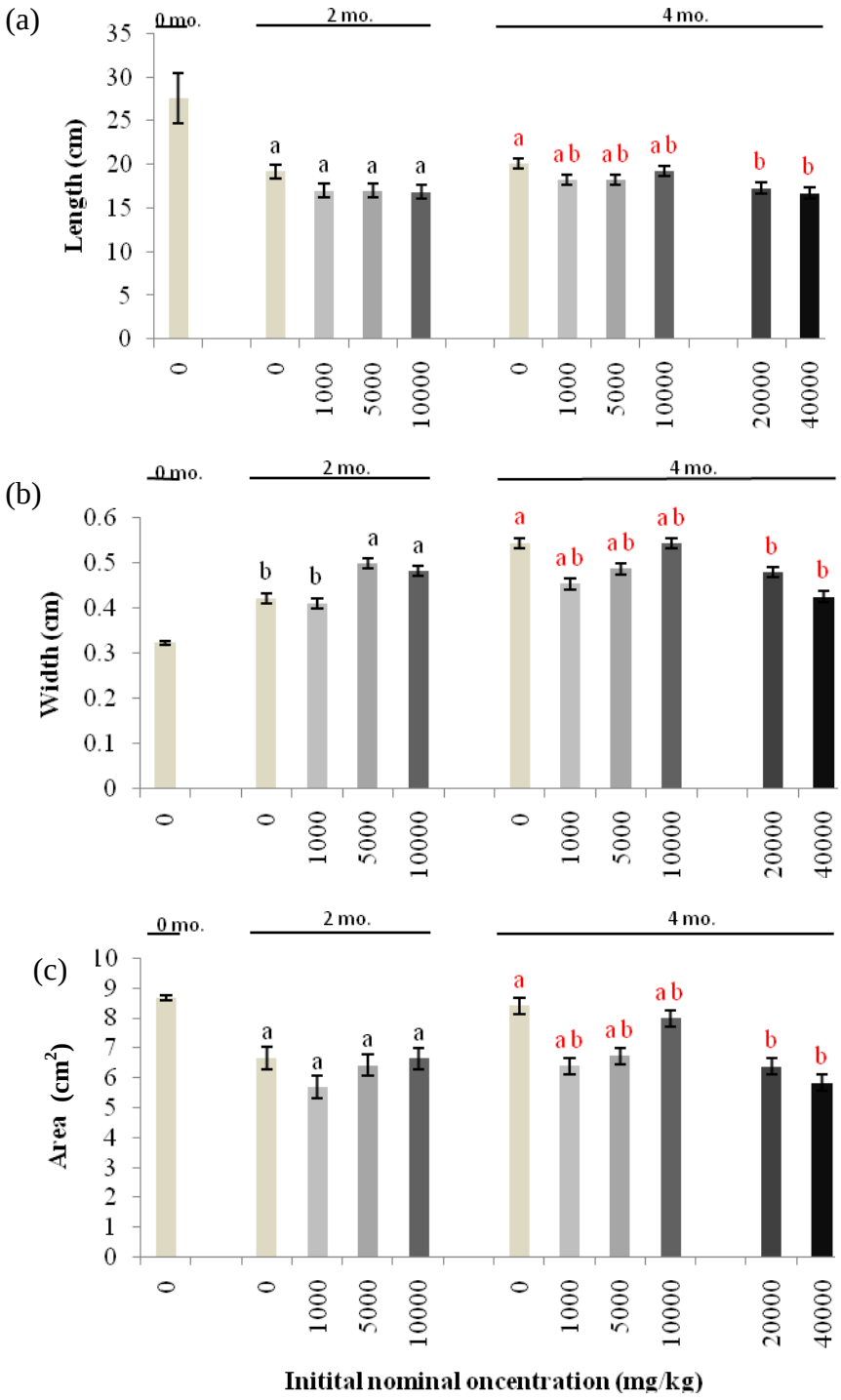


**Figure 2.6** Changes in the mean (a) leaf length, (b) leaf width and (c) leaf area of *P. foliosa* in response to increasing hydrocarbon concentrations for time intervals 0, 2 and 4 months. Data are means ± SEM, n = 5. Columns with the same letter are not significantly different at p = 0.05. Different coloured letters above bars represent separate analyses.

**Table 2.1** Effect of SAB fuel on the morphological characteristics of the species *P. foliosa* following 2 and 4 months exposure. One-way ANOVA and Kruskal-Wallis ($p < 0.05$), * indicates Kruskal-Wallis test.

| 2 months | | | | 4 months | | | |
|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F ratio** | **p value** | **Source** | **df** | **F ratio** | **p value** |
| **Length** | 3 | 2.156 | 0.092 | **Length*** | 5 | 26.8 | 0.0001 |
| **Width*** | 3 | 42.35 | 0.0001 | **Width*** | 5 | 94.5 | 0.0001 |
| **Area** | 3 | 1.48 | 0.218 | **Area*** | 5 | 66.7 | 0.0001 |
| **Error** | 596 | | | **Error** | 924 | | |
| **Total** | 599 | | | **Total** | 929 | | |

### Leaf width

At two months, leaf width was significantly wider at concentration treatments of 5 000 and 10 000 mg/kg, when compared to 0 and 1 000 mg/kg (Figure 2.6b; $p = 0.0001$; Table 2.1). After a four months exposure, leaf width was variable between treatments, similar to the results found for leaf length at the same time interval (Figure 2.6a,b). Again, leaf widths at concentration treatments between 1 000 to 10 000 mg/kg were intermediate between the control and higher SAB treatments but not significantly lower than the control. At higher concentration treatments, 20 000 to 40 000 mg/kg, however, leaves were significantly thinner than the control (0 mg/kg; $p = 0.0001$; Table 2.1).

### Leaf area

No significant change was detected in regard to the average leaf area of *P. foliosa* across differing hydrocarbon treatments at two months (Figure 2.6c), however after four months, leaf area, displayed a similar pattern as found for leaf length and width at the same time interval, where leaf area at concentrations between 1 000 to 10 000 mg/kg were intermediate between the control and higher SAB treatments but not significantly lower than the control. Leaf area significantly declined ($p = 0.0001$) at concentration treatments higher than 10 000 mg/kg when compared to the control (Figure 2.6c; Table 2.1).

**Root structure**

Although no quantitative analyses were undertaken on root structure, root structure differed qualitatively between concentration treatments after four and again after eight months exposure. The roots of plants in the control were composed of a distinctly larger quantity of fine fibrous roots and fewer coarse roots than those in the hydrocarbon treatments, especially in the highest concentrations tested of 20 000 and 40 000 mg/kg after eight months (Figure 2.7).

**Figure 2.7** Root structure of *P. foliosa* exposed to (a) 0 and (b) 40 000 mg/kg SAB after eight months.

### 2.3.3 Biomass

**Shoot and root dry weight biomass**

Root and shoot dry weight comparisons between treatments indicated that the presence of hydrocarbons in the soil did not impair the normal growth of *P. foliosa* (Figure 2.8a,b). At both two and four months, no significant change was detected in regard to the mean above ground dry biomass of *P. foliosa* across differing hydrocarbon treatments (Figure 2.8a; Table 2.2). Similarly, the below ground biomass data showed no significant difference between SAB concentrations at either time interval (Figure 2.8b; Table 2.2). Both root and shoot dry biomass increased significantly from two to four months between treatments 0 to 10 000 mg/kg ($p = 0.05$; Appendix A).

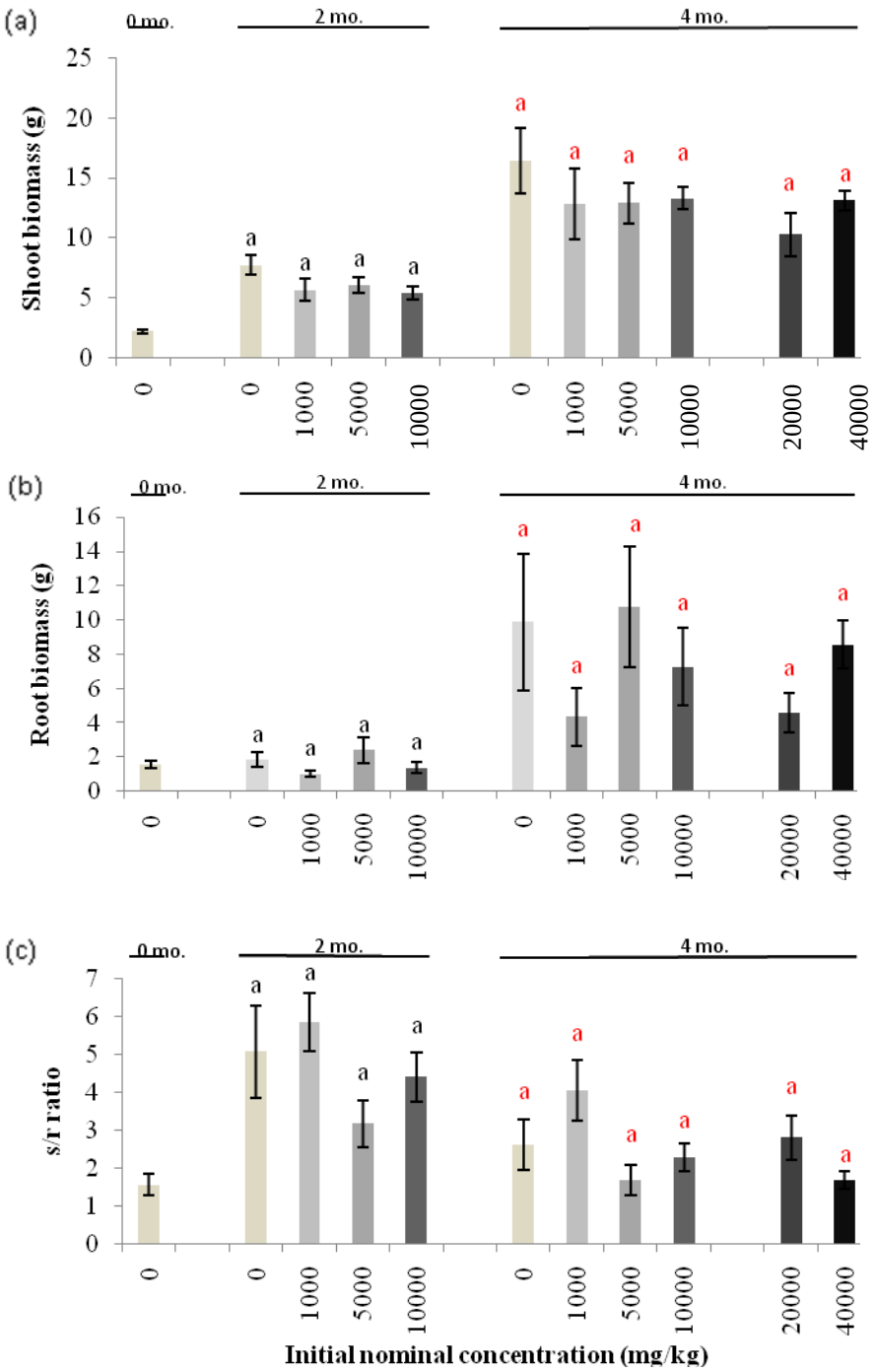


**Figure 2.8** Changes in the mean (a) shoot dry biomass, (b) root dry biomass, and (c) shoot/root ratio of *P. foliosa* in response to increasing hydrocarbon concentrations, for time intervals 0, 2 and 4 months. Data are means ± SEM, n = 5. Columns with the same letter are not significantly different at p = 0.05. Different coloured letters above bars represent separate analyses.

**Table 2.2** Effect of SAB fuel on shoot and root biomass of *P. foliosa* following 2 and 4 months exposure. One-way ANOVA ($p < 0.05$).

| 2 months | | | | 4 months | | | |
|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F ratio** | **p value** | **Source** | **df** | **F ratio** | **p value** |
| **Shoots** | 3 | 2.017 | 0.152 | **Shoots** | 5 | 0.949 | 0.467 |
| **Roots** | 3 | 1.578 | 0.233 | **Roots** | 5 | 1.09 | 0.391 |
| **s/r ratio** | 3 | 1.80 | 0.187 | **s/r ratio** | 5 | 1.96 | 0.12 |
| **Error** | 16 | | | **Error** | 24 | | |
| **Total** | 19 | | | **Total** | 29 | | |

## Shoot/Root Ratio

No significant differences between treatments were detected in the shoot to root ratio of plants exposed to different hydrocarbon concentration at either two or four months (Table 2.2). However, the ratio of shoots to roots between treatments 0 to 10 000 mg/kg decreased significantly from two to four months ($p = 0.05$; Appendix A).

## Mean Relative Growth Rate

Both the shoots and roots displayed a positive increase in mean relative growth rate (MRGR) from the control to the highest treatment concentration of 10 000 mg/kg of SAB, however this change was not significant (Table 2.3; Appendix B). The MRGR for plant shoots and roots was highest in the 10 000 mg/kg treatment. The shoot MRGR was lowest in soil contaminated at 1 000 mg/kg, whereas root MRGR was lowest at 0 and 5 000 mg/kg.

**Table 2.3** Means (±SE) of the relative growth rates (MRGR) for shoot and root of *P. foliosa* at different levels of SAB (petroleum) soil contamination, using data from the two and four month harvest.

| | **Mean relative growth rate (g/month)** | |
|---|---|---|
| **Concentration (mg/kg)** | **Shoot (n = 5)** | **Root (n = 5)** |
| 0 | 0.74 (± 0.14) | 0.37 (± 0.08) |
| 1000 | 0.60 (± 0.25) | 0.39 (± 0.12) |
| 5000 | 0.70 (± 0.30) | 0.37 (± 0.10) |
| 10000 | 0.80 (± 0.09) | 0.46 (± 0.06) |

### 2.3.4 Photosynthetic efficiency ($F_v/F_m$)

In general the photosynthetic efficiency ($F_v/F_m$) of *P. foliosa* was high throughout the experiment (Figure 2.9) and showed no significant difference between hydrocarbon treatments at either the two (60 day) or four month (120 day) sampling interval (Figure 2.10; Table 2.4). However, Fv/Fm did vary over time, declining to 0.6 in all treatment between 50 and 65 days, after which it increased to a level higher than the initial control (Figure 2.9).

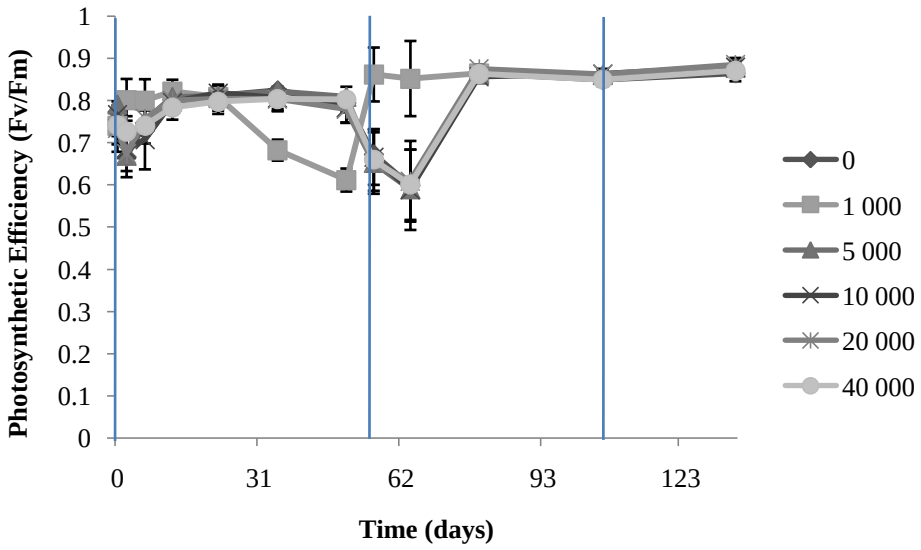


**Figure 2.9** Changes in the mean $F_v/F_m$ over time, n = 35 and error bars represent one standard error about the mean. Vertical lines represent harvest periods at 0, 2 and 4 months.

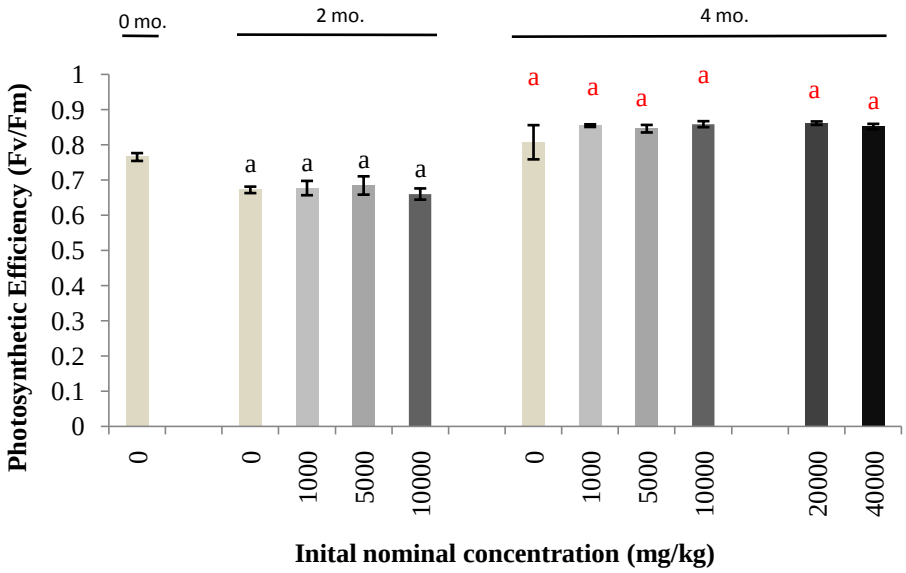


**Figure 2.10** Changes in the mean $F_v/F_m$ of *P. foliosa* replicates in response to increasing hydrocarbon concentrations, for time intervals 0, 2 and 4 months. Data are means ± SEM, n = 5. Columns with the same letter are not significantly different at p = 0.05. Different coloured letters above bars represent separate analyses.

**Table 2.4** Effect of SAB fuel on shoot and root biomass of *P. foliosa* following 2 and 4 months exposure. One-way ANOVA (p < 0.05).

| 2 months | | | | 4 months | | | |
|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F ratio** | **p value** | **Source** | **df** | **F ratio** | **p value** |
| **$F_v/F_m$** | 3 | 0.570 | 0.640 | **$F_v/F_m$** | 5 | 0.293 | 0.911 |
| **Error** | 17 | | | **Error** | 24 | | |
| **Total** | 20 | | | **Total** | 29 | | |

### 2.3.5 Photosynthetic pigment results

#### Total chlorophyll content

The total chlorophyll content of *P. foliosa* at SAB concentrations of 5 000 mg/kg was significantly lower than 0 treatments at both two (p = 0.037) and four (p = 0.02) months (Figure 2.11a; Table 2.5). All other concentration treatments were intermittent between 0 and 5 000 mg/kg.

### Chlorophyll *a/b* ratio

The mean chlorophyll *a/b* ratio (chl *a/b*) of *P. foliosa* showed a tendency to increase with increasing SAB concentration at two months (Figure 2.11b), however, no significant differences in chl *a/b* between concentrations were detected at this time. At the four month harvest, no difference in the mean chl *a/b* ratio was observed between treatments (Figure 2.11b, Table 2.5).

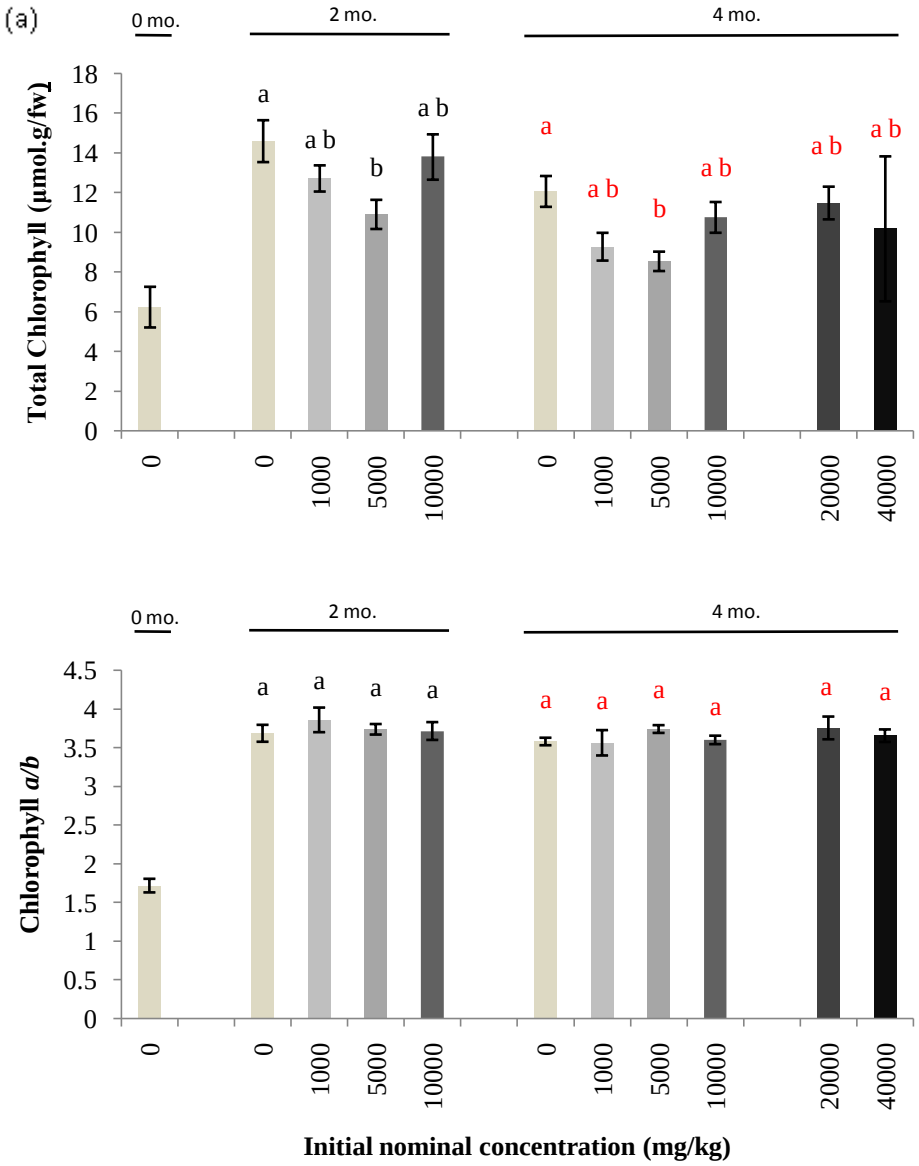


**Figure 2.11** Changes in the mean (a) total chlorophyll content (µmol/gfw) and (b) chlorophyll *a/b* ratio of *P. foliosa* in response to increasing hydrocarbon concentrations, for time intervals 0, 2 and 4 months. Data are means ± SEM, n = 5. Columns with the same letter are not significantly different at p = 0.05. Different coloured letters above bars represent separate analyses.

**Table 2.5** Effect of SAB fuel on total chlorophyll and chlorophyll *a/b* of *P. foliosa* following 2 and 4 months exposure. One-way ANOVA ($p < 0.05$).

| | 2 months | | | | 4 months | | |
|---|---|---|---|---|---|---|---|
| **Source** | **df** | **F ratio** | **p value** | **Source** | **df** | **F ratio** | **p value** |
| **Total Chl** | 3 | 3.008 | 0.037 | **Total Chl** | 5 | 2.824 | 0.02 |
| **Chl *a/b*** | 3 | 0.78 | 0.509 | **Chl *a/b*** | 5 | 0.652 | 0.66 |
| **Error** | 56 | | | **Error** | 84 | | |
| **Total** | 59 | | | **Total** | 89 | | |

## 2.4 Discussion

Give that the overall impact of SAB on the biomass production of this species is negligible, the hypothesis that growth would be affected was rejected for all concentrations. Since there were some minor adverse physiological and morphological effects, the hypothesis regarding adverse health is accepted. This was particularly the case for leaf morphology impacts at higher concentrations of SAB (20 000 – 40 000 mg/kg) and for total chlorophyll at 5 000. However, it is important to note that these impacts were minimal and did not affect overall plant yields.

Petroleum hydrocarbons have been found to have an inhibiting effect on general plant health and to suppress plant growth (especially roots), due to acute toxicity of light oil fractions as well as unfavourably altered soil conditions (DeLaune *et al.* 2003; Merkl *et al.* 2005b; Thompson *et al.* 2008). Brandt *et al.* (2006) investigated the potential of *Veiveria zizaniodes* for phytoremediation of petroleum hydrocarbon contaminated soils in Venezuela. Although this plant was found to tolerate the contaminant in a concentration of 50 000 mg/kg, biomass and plant height were significantly reduced. Similar adverse effects on plant growth in the presence of hydrocarbon contamination were observed by Liste and Prutz (2006). Thirteen plant species from four groups (grasses, cruciferes, legumes, herbs) were assessed for their ability to thrive in a long-term contaminated soil from a former manufactured gas plant site (Liste and Prutz 2006). The authors reported a general reduction in seed germination, plant survival, and shoot yield as a result of the contaminant. More relevant to research presented in this thesis, plants growing in cold conditions also displayed this negative reaction to petroleum contamination. In a study in sub-Arctic Canada, Robson *et al.* (2003) found that the ability of thirty-nine cold tolerant plants

to grow in hydrocarbon-contaminated soils, was negatively affected by the addition of 5 000, 10 000 and 50 000 mg/kg of crude oil. Both the root biomass and total plant biomass significantly decreased by at least 22% relative to the control for all but two species, *Psoralea esculenta* (a native legume) and *Artemisia frigida* (a native forb).

In contrast to these reports, and as observed in the present study on *P. foliosa*, oil contamination may have little to no effect on plant species and may even cause growth stimulation (Merkl *et al.* 2005a; Liste and Prutz 2006; Gaskin *et al.* 2008; Peng *et al.* 2009). The causes of growth stimulation are thought to include hormonal effects or the release of nutrients from the hydrocarbons (Baker 1970). A number of studies, including Chiapusio *et al*. (2007) have reported a similar effect in certain plant species. Liste and Prutz (2006), for example, found that seed germination (lupin) and shoot biomass production (oat, mustard, pea) was improved in the presence of petroleum hydrocarbons within soil. Likewise, Gaskin *et al*. (2008) reported that seedling emergence, in all but one native Australian grass species, was unaffected by the presence of 10 000 and 50 000 mg/kg of petroleum hydrocarbons and some species (*Cymbopogon ambiguus*) produced considerably more root biomass in the presence of contamination. The remaining species in the study by Gaskin *et al.* (2008) showed no adverse growth effects on root or shoot biomass in the presence of the contaminant at all the exposed concentration treatments, as also found within the current study at both harvest periods for shoot and root biomass of *P. foliosa*.

Although root biomass was not significantly affected by the presence of hydrocarbons in this study, changes in the general root morphology were observed in qualitative visual assessments. Root morphology of the control and lower concentrations, especially after four months exposure, were distinctly more fibrous with a finer rooting system than plants exposed to higher concentrations (20 000 and 40 000 mg/kg). This finding is not uncommon within the literature, and is not surprising as fine roots tend to develop in response to nutrients ($NO_3^-$) within soil, a condition that is suppressed in the presence of contaminants such as petroleum hydrocarbons (Harvey *et al.* 2002). In a study on the influence of heavy crude oil on root morphological characteristics of graminoids, Merkl *et al*. (2005a) reported a similar trend which indicated that crude oil causes a coarser root growth. Roots of *Brachiaria brizantha* and *Cyperus aggregates* were found to have shorter and thicker roots when exposed to contaminants, and both

expressed a decrease in specific root length and an increase in average root diameter. Thompson *et al.* (2008) also found that part of *Cynodon dactylons* physiological response to hydrocarbon contamination was an increase in the mean root diameter. However, their findings suggest that the larger mean diameter was a result of an increase in diameter of the existing roots, rather than a decrease in the number of very fine roots. During unfavourable environmental conditions, such as when exposed to contamination, *P. foliosa* root cortical tissues may become strongly lignified, limiting the development of fine roots. Therefore, further studies to collect quantitative data on the effect of hydrocarbons on *P. foliosa* are recommended, as fine roots (<0.8 mm) play the most important role in general nutrition and water uptake, and comprise most of the root surface area of a plant (Harvey *et al.* 2002).

From the two to four month period of the experiment shoot to root ratio of *P. foliosa* decreased significantly, indicating a flux of carbon to the roots. Generally this process occurs when there is a reduction in soil-derived resources such as nutrient supply, water, oxygen and temperature (Merkl *et al.* 2005b). It is also an indicator that the plant is a suitable species for use in phytoremediation as it displays the ability to change carbon fluxes in response to altered environmental conditions. Furthermore, *P. foliosa* displays a low mean relative growth rate (MRGR; 0.74 for shoots and 0.37 for roots) when compared to other species in the literature such as *B. brizantha* and *C. aggregates* (Merkl *et al.* 2005b). A comparative analysis of sub-Antarctic and alpine grasses by Medek *et al*. (2007) found sub-Antarctic grasses, including *P. foliosa* have inherently low MRGR's. Plants with low MRGR are known to be more widespread on infertile soils and have been physiologically linked to stress resistance (Elias and Chadwick 1979). A study by Robson *et al*. (2003) showed that plants with low MRGR are more likely to be successful for reclamation of infertile disturbed lands than those species with higher MRGR due to their suppressed demand for nutrients and water. Robson *et al*. (2003) found that out of thirty-nine cold tolerant plants (grasses and legumes) in hydrocarbon-contaminated conditions those species with the lowest MRGR in uncontaminated soil were the ones that exhibited the least change in biomass with the addition of crude oil and vice versa.

The general pattern of growth through time shows that *P. foliosa* has the ability to proliferate and flourish regardless of the presence of SAB contamination. Since growth conditions simulated the conditions of Macquarie Island, this suggest this result would be

transferable to a field situation, with soil analysis showing that the organic content within Tasmanian Devils Dirt potting mix (13%) is in the range of Macquarie Island soils which can contain up to 30% organic content. For certain parameters, such as maximal photosynthetic efficiency ($F_v/F_m$), shoot biomass, leaf length and area, there was an initial decrease after two months exposure, followed by an increase at four months. This trend is particularly prevalent in mean photosynthetic efficiency, which ranges from an average of 0.76 at the commencement of the experiment, to 0.67 at two months and to 0.84 at four months. The typical value of healthy plants, according to Björkman & Demmig (1987), ranges between 0.80 - 0.83. This indicates that plant stress levels in the current experiment were increased to a mild level after two months, but chlorophyll health increased to pre-exposure (healthy) levels by four months. Possible reasons for this could be that the hydrocarbons were in greater abundance in the soil at two months than at four months due to the presence of microbes associated with the plant, actively degrading contaminants and remediating the soil. It also shows the potential of *P. foliosa* to recover from exposure to contamination, even after experiencing moderate levels of stress ($F_v/F_m$ = 0.67), similar to results found in growth room studies with other grasses (DeLaune *et al*. 2003).

Further analysis into growth parameters of *P. foliosa*, including calculating the Shoot to Leaf Area (SLA) ratio and examining leaf pigments (via High Pressure Liquid Chromatography (HPLC)), may be useful in gaining a broader understanding of the effects of fuel on the grass. A detailed quantitative root study, including measuring parameters such as root width, length and the ratio of fine roots to coarse roots, would also benefit this research. These and other recommendations are discussed in chapter 4.

In conclusion, *P. foliosa* displays a good tolerance to petroleum hydrocarbons, and should be considered as a potential candidate for phytoremediation of SAB fuel contaminated soils on Macquarie Island. The following chapter investigated whether the presence of *P. foliosa* affects levels of TPH in the soil and if it influences the microbial flora in SAB contaminated soil.

# Chapter 3

## Phytoremediation of Special Antarctic Blend (SAB) fuel using sub-Antarctic grass *Poa foliosa*

### 3.1 Introduction

*Poa foliosa* may be a potential candidate for phytoremediation of soils contaminated with fuels under sub-Antarctic conditions. The grass displays the necessary resilience to petroleum hydrocarbons (Chapter 2), appropriate physiological characteristics which enhance hydrocarbon degradation, and resides in areas of Macquarie Island where major spills have occurred. However, the crux of phytoremediation lies in the plants' ability to accelerate the removal of total petroleum hydrocarbons (TPHs) above natural microbial and mechanical levels. Therefore, comparative studies of soil TPH dissipation in the presence and absence of *P. foliosa* are paramount to gauge the success of this species for phytoremediation in sub-Antarctic regions.

Plants have been shown to augment hydrocarbon degradation in soil (Macek *et al.* 2000; Delille *et al.* 2003; Newman and Reynolds 2004; Liste and Prutz 2006; Gaskin *et al.* 2008). There is however, limited research into the potential of phytoremediation in high latitudes (Gibson 2000; Filler *et al.* 2008). Since it is acknowledged that micro-organisms play a major role in the degradation of hydrocarbons during the phytoremediation process (Sicilliano and Germida 1998; Siciliano *et al.* 2003; Pilon-Smits 2005; Merkl *et al.* 2006), microbial populations associated with *P. foliosa* must be examined. The mutualistic relationship of plants with rhizosphere microbes can vary between plant species, subsequently influencing the rhizosphere effect on hydrocarbon degradation (Chaudhry *et al.* 2005; Khan 2005; Gaskin *et al.* 2008; Phillips *et al.* 2008). The greater the number of specific microbes in the rhizosphere able to metabolise a contaminant, along with greater total microbial counts, significantly improves plant fostered biodegradation (Olsen *et al.* 2003; Liste and Prutz 2006). For example, plant-facilitated oil dissipation was found to correlate well with the abundance of root-associated microbes populations in a greenhouse study using Italian ryegrass (*Lolium multiflorum*; Alarcon *et al.* 2008). Similarly, a study by Lin *et al.* (2008) found 13 - 30% higher removal of diesel fuel in plant-microorganism synergy treatments than in treatments with plants alone. Therefore, quantifying the micro-organisms within the rhizosphere of *P. foliosa* is important in characterising the species potential for phytoremediation.

The object of this thesis was to determine the ability of *P. foliosa* to phytoremediate hydrocarbons in sub-Antarctic soils. The plants' capacity to degrade petroleum hydrocarbons from the soil, as well as the plants' ability to stimulate microbial populations in the rhizosphere, were assessed by experimentally comparing planted versus unplanted replicates, at different SAB concentrations, over an eight month study.

It was hypothesised that planted treatments would have significantly larger populations of both total heterotrophs and hydrocarbon degrading micro-organisms, and significantly reduced contaminant concentrations, compared to unplanted treatments. The null hypothesis was that the presence of *P. foliosa* would make no difference to the number of micro-organisms or the concentration of TPH within the soil.

## 3.2 Materials and Methods

### 3.2.1 Sampling procedure and experimental design

As reported in Chapter 2 section 2.2.2.

### 3.2.2 SAB diesel fuel analysis

Total petroleum hydrocarbons were recovered from the soil using methods adapted from Snape *et al.* (2005). The amount of sub-sample was modified to ensure the most accurate reading for each concentration. For concentrations 0 to 10 000 mg/kg, 10 g of soil was placed into a pre-weighed 40 ml head space vial with a Teflon septa, whereas 5 and 2.5 g of soil was used for the 20 000 and 40 000 mg/kg treatments respectively. Sub-samples were spiked with 1 ml of an internal standard mixture containing 250 mg/L bromoeicosane; 25.9 mg/L d10-anthracene; 49.8 mg/L d10-ethylbenzene; 62.4 mg/L fluoroheptane; and 250 mg/L cyclo-octane. Following this, 10 ml of hexane and 10 ml of Milli Q water were added. Sub-samples were then tumbled end over end for 12 hours at room temperature to ensure petroleum hydrocarbon separation.

The hexane layer was extracted and transferred into a 2 ml vial and analysed on an Agilent 6890 Gas Chromatography-Flame Ionisation Detector (GC-FID) fitted with an auto-sampler (Agilent 7683 ALS) and using helium as the carrier gas. Separation was achieved using an SGE BP-1 column (35 m x 0.22 mm Internal Diameter, 0.25 µm film thickness). The extract (1 µL) was injected at 310°C and at a pulsed split of 1:15. Extracts were cross calibrated with an

in-house SAB standard. The rate of flow of carrier gas was 1.3 mL/min for the duration of the oven program. The initial oven temperature of 50°C was held for three minutes prior to a ramp up to 320°C at 18°C/min. Detector temperature was 340°C. TPH concentrations were determined using a calibration curve, generated from the internal standard, to integrate the combined areas under resolved peaks and the Unresolved Complex Mixture (UCM). TPH was measured within the range of C9-18 to the internal standard peak response.

### 3.2.3 Most Probable Number Assay

Enumeration of hydrocarbon degrading microbes was carried out using a Most Probable Number (MPN) protocol as described by Powell *et al*. (2006). Each replicate was assessed for SAB degraders in separate sterile 96-well microtitre trays. Each tray was divided into three sections (A, B and C) for triplicate analyses and all wells were filled with 160 μL of Bushnell-Haas (BH) mineral salt medium (3.27 g/L). Initial dilutions of approximately 0.5 g of soil (wet weight) to 4.5 ml of BH broth were vortexed for 30 seconds (repeated twice) to achieve homogeneity. Each initial dilution of 40 μL was used to inoculate the appropriate first four wells on the tiered tray. Serial dilutions of 5 μL were undertaken with the last 5 μL discarded (refer to Figure 3.1). Five μL of filtered-sterilized SAB was then applied to the surface of each well. The last row of wells were not inoculated and served as a negative control for growth. After five days of incubation at 10ºC, 50 μl of iodonitrotetrazolium chloride solution (INT) (3.0 g/l) was added to each well and trays were incubated for 24 hours. Wells were scored as positive on the formation of a pink precipitate.

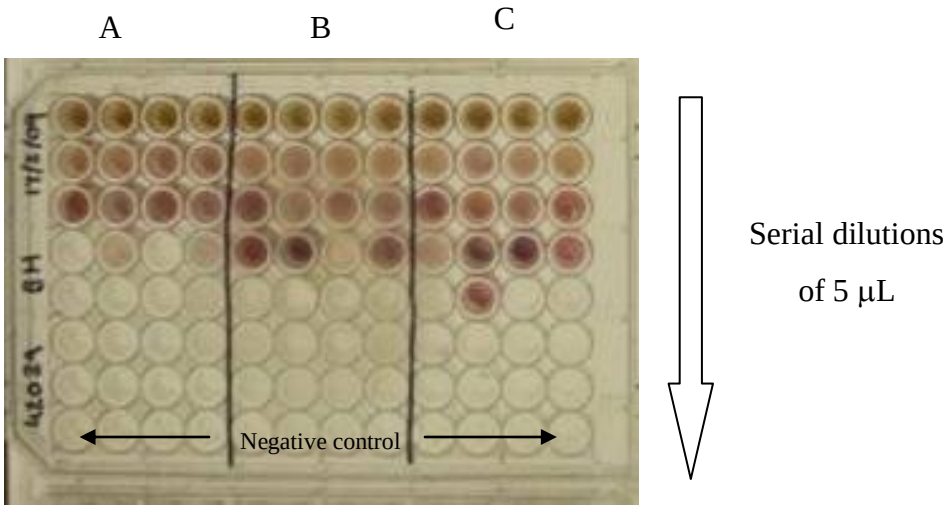


**Figure 3.1** Most Probable Number assay showing 47 positive (pink) readings for hydrocarbon degrading microbial growth.

Total heterotrophs were identified in a similar manner to hydrocarbon degrading microbes except that half-strength tryptone soya broth (15.0 g/L) was used instead of BH. Positive wells were indicated by an opaque reading after seven (rather than five) days incubation. For the initial harvest and the first harvest (two months) all wells were filled with 180 µL of tryptone soya broth and 20 µL of initial sample dilutions as counts of total heterotrophs were anticipated to be higher than hydrocarbon degraders. This resulted in serial dilutions of 10 µL. At the second harvest (four months) the ratio of media to initial sample dilution (40 µL) was adjusted back to those used for hydrocarbon degrading microbes.

### 3.2.4 Statistical Analysis

Prior to analysis, data were tested for normality using the Shapiro-Wilks W Test, and homogeneity of variance using Cochran's *C* test. $Log_{10}$ transformations were used to normalise the distribution of the data for the relative increase in total heterotrophs and hydrocarbon degrading microbes, while square root transformations were used to normalise TPH data. A single outlier was excluded from the hydrocarbon degrading data as well as the TPH data to achieve a normal data set. This outlier exclusion did not change the outcome of results. MPN results for the 20 000 and 40 000 mg/kg treatments were not included in analysis as data was only available for planted replicates.

All data were analysed using the statistical program JMP (Vol. 5.1 SAS Institute Inc., U.S.). A three-way analysis of variance (ANOVA) was done with the significance level set at p = 0.05. Where significant interactions were observed, Tukey HD *post hoc* tests were used to identify significantly different means. Single regression analyses were also performed at p = 0.05.

## 3.3 Results

### 3.3.1 Total petroleum hydrocarbon degradation

The presence of *P. foliosa* significantly increased the removal of Total Petroleum Hydrocarbons (TPH) from contaminated soil (p = 0.0076; Figure 3.2; Table 3.1). After two months of exposure, concentration of TPH in planted treatments decreased by 41, 45 and 48% more than their unplanted counterparts, at concentrations of 1 000, 5 000, and 10 000 mg/kg respectively. Similarly, after four months, concentrations of TPH were 35, 39 and 41% of the respective controls 1 000, 5 000, and 10 000 mg/kg. Initial nominal concentrations of TPHs (1 000, 5 000 and 10 000 mg/kg) declined across all treatments over four months (Appendix C). The mean concentration of TPH in soil continued to decrease from two to four months at higher contaminant concentrations, with a significant decline at 5 000 mg/kg (p = 0.0032; Figure 3.2; Table 3.1). At the two month stage, as the concentration of SAB increased from 0 to 10 000 mg/kg, the TPH remaining in the soil increased significantly in both planted and unplanted treatments (p = 0.0032; Table 3.1). Likewise, after four months, as the concentration of SAB increased from 0 to 10 000 mg/kg, the soil TPH increased significantly (p = 0.0032), with the exception of 1 000 mg/kg which was statistically similar to the control (Table 3.1).

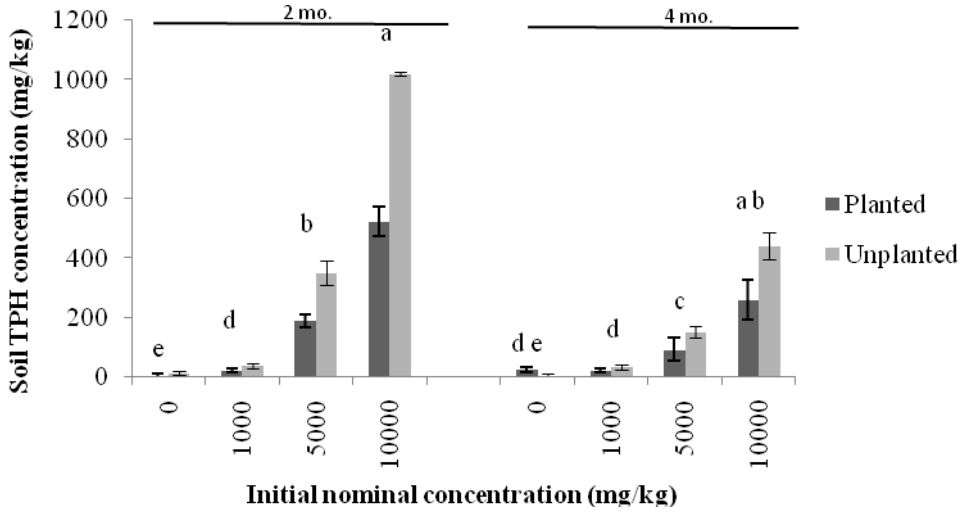


**Figure 3.2** Changes in the TPH concentration in planted (*P. foliosa*) and unplanted soil in relation to increasing hydrocarbon concentrations (mg/kg), for time intervals 2 and 4 months. Data are means ± SEM, n = 5. All planted versus unplanted pairs are significantly different. Letters **a** to **e** represent differences between the two time interval groups across concentrations. Columns with the same letter are not significantly different.

**Table 3.1** Three-way ANOVA table: effect of time, concentration, planted/unplanted and interactions on the level of TPH in soil (n.s. indicates a non significant value).

| | Source | df | Chi-Squared | p value |
|---|---|---|---|---|
| **Level of TPH in soil** | **Whole model** | **15** | **24.97** | **0.0001** |
| | **Time** | 1 | n.s. | n.s. |
| | **Concentration** | 3 | 109.4 | 0.0001 |
| | **Planted/Unplanted** | 1 | 7.65 | 0.0076 |
| | **Time x Concentration** | 3 | 5.16 | 0.0032 |
| | **Time x Planted/Unplanted** | 1 | n.s. | n.s. |
| | **Concentration x Planted/Unplanted** | 3 | n.s. | n.s. |
| | **Time x Concentration x Planted/Unplanted** | 3 | n.s. | n.s. |

### 3.3.2 Microbial populations in the rhizosphere

The composition of microbial populations within the rhizosphere changed through time. In the first two months after SAB exposure, total heterotrophs increased by a factor of 15, from $2xE^{06}$ up to $2.5xE^{07}$, before declining significantly to pre-experiment levels by four months (see Appendix D; Figure 3.3a). The number of heterotrophic micro-organisms increased with increasing hydrocarbon concentrations in unplanted treatments with significant increases at 5 000 mg/kg and again at 10 000 mg/kg ($p = 0.0001$; Figure 3.3a; Table 3.2). In contrast, for

planted treatments total heterotrophs peaked at 1 000 mg/kg but no significant differences were detected across all treatments (Figure 3.3a). In comparison with unplanted treatments, the presence of *P. foliosa* significantly augmented the number of total heterotrophs in the root zone for treatment concentrations of 0 and 1 000 mg/kg ($p = 0.0001$; Table 3.2). At 5 000 mg/kg total heterotroph numbers were similar across both planted and unplanted treatments, but at 10 000 mg/kg unplanted treatments displayed twice the number of microbes. This trend was still apparent at four months even though population numbers had declined significantly compared to the two month time interval ($p = 0.0001$; Table 3.2).

The presence of hydrocarbon degrading microbes in the rhizosphere also changed over time and these microbes were most abundant at the two month harvest, although the pattern was somewhat different to the total microbial counts (Figure 3.3b). As with total heterotrophs, the levels of hydrocarbon degrading microbes fell significantly between the two and four month time interval ($p = 0.001$; Figure 3.3b; Table 3.2). Hydrocarbon degraders increased proportionally with increasing hydrocarbon concentrations for unplanted treatments with significant increases at each concentration ($p = 0.019$; Table 3.2). For planted treatments, hydrocarbon degraders increased significantly with concentration between 0 and 5 000 mg/kg but remained constant above this concentration ($p = 0.019$; Figure 3.3b; Table 3.2). Hydrocarbon degrader levels were significantly higher in the planted, compared to the unplanted 0 mg/kg treatment ($p = 0.0001$; Table 3.2), for both harvests, but did not differ in all other treatment concentrations.

Although not significant, after two months of exposure there was a trend for the proportion of hydrocarbon degrading microbes, to total heterotrophs, to increase with increasing fuel concentration for unplanted treatments. In the planted treatments this trend occurred up to the 5 000 mg/kg treatment (Figure 3.3c). Planted treatments at two months contained greater numbers of hydrocarbon degrading microbes than did unplanted treatments at concentrations of 0 and 5 000 mg/kg, but not at 1 000 or 10 000 mg/kg. At four months the trend was more established with a clear proportional increase in hydrocarbon degrading microbes with increasing fuel concentration. Hydrocarbon degraders were proportionally greater for planted treatments across all fuel concentrations, with the exception of 10 000 mg/kg in which the two treatments were similar.

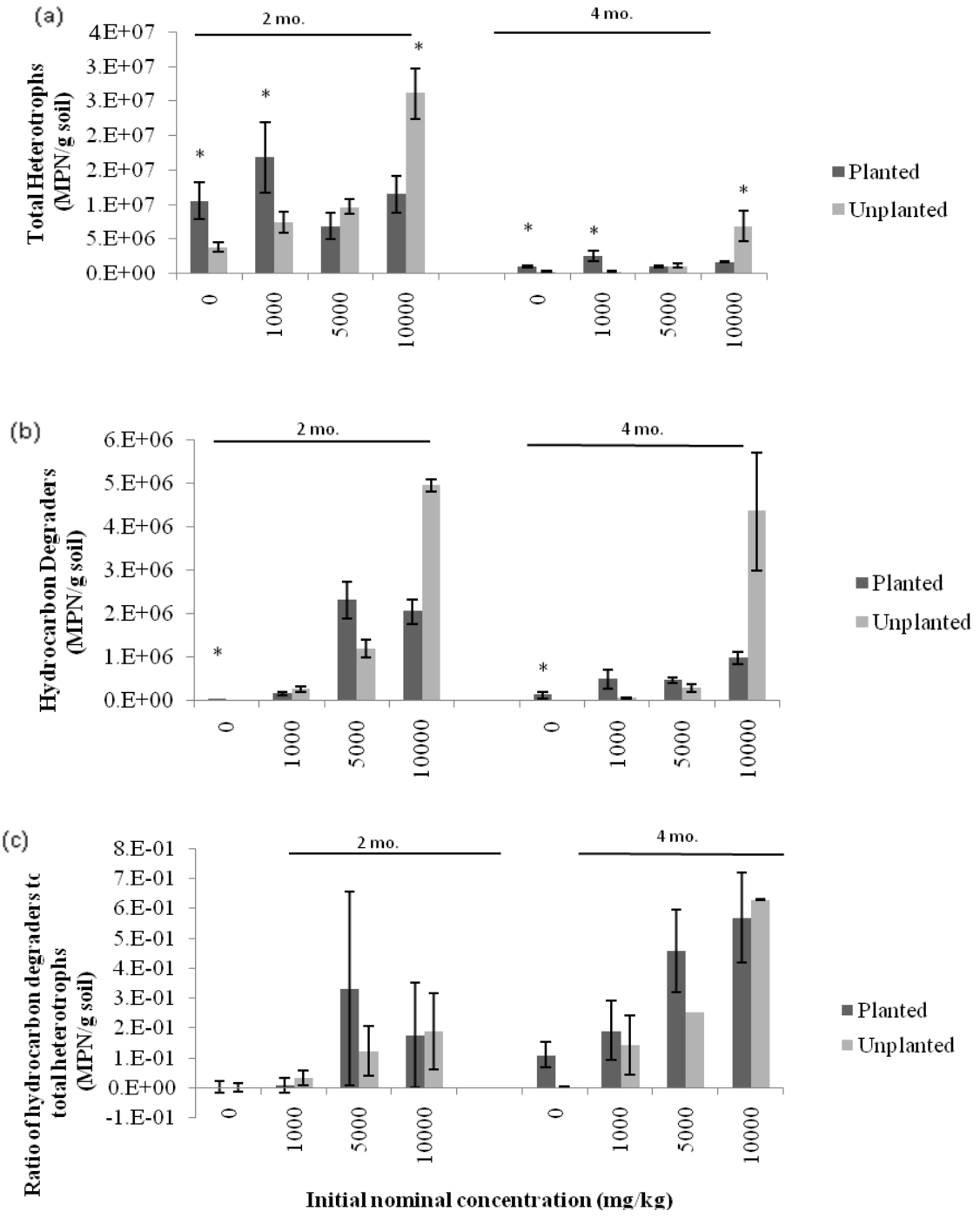


**Figure 3.3** Change in the number of (a) total heterotrophs, (b) hydrocarbon degrading microbes and (c) the ratio of hydrocarbon degrading microbes to total heterotrophs, from the rhizosphere of *P. foliosa* and unplanted soil in relation to increasing hydrocarbon concentrations (mg/kg), for time intervals 2 and 4 months. Data are means ± SEM, n = 5. * indicates significant differences between planted and unplanted replicates across concentrations.

**Table 3.2** Three-way ANOVA table: effect of time, concentration, planted/unplanted and interactions on levels of total heterotrophs and hydrocarbon degrading microbes (n.s. indicates a non significant value).

| | Source | df | Chi-Squared | p value |
|---|---|---|---|---|
| **Total heterotrophs** | **Whole model** | **15** | **19.34** | **0.0001** |
| | **Time** | 1 | 193.21 | 0.0001 |
| | **Concentration** | 3 | 16.77 | 0.0001 |
| | **Planted/Unplanted** | 1 | n.s. | n.s. |
| | **Time x Concentration** | 3 | n.s. | n.s |
| | **Time x Planted/Unplanted** | 1 | n.s. | n.s. |
| | **Concentration x Planted/Unplanted** | 3 | 12.34 | 0.0001 |
| | **Time x Concentration x Planted/Unplanted** | 3 | n.s. | n.s. |
| **Hydrocarbon degrading microbes** | **Whole model** | **15** | **37.75** | **0.0001** |
| | **Time** | 1 | 28.37 | 0.0001 |
| | **Concentration** | 3 | 163.83 | 0.0001 |
| | **Planted/Unplanted** | 1 | 8.41 | 0.004 |
| | **Time x Concentration** | 3 | n.s. | n.s. |
| | **Time x Planted/Unplanted** | 1 | 5.64 | 0.019 |
| | **Concentration x Planted/Unplanted** | 3 | 8.77 | 0.0001 |
| | **Time x Concentration x Planted/Unplanted** | 3 | n.s. | n.s. |

### 3.3.3 Relationships between hydrocarbon degradation and microbes

A significant positive association between the number of total heterotrophs in the sample and the TPH concentration in the soil was found for unplanted treatments (linear regression; $R^2$ = 0.654; p = 0.0001). This relationship was not found for planted treatments ($R^2$ = 0.026; p = 0.321; Figure 3.4a). Sixty-five percent of variation in concentrations of TPH in unplanted treatments can be explained by variations in total microbial populations. The number of hydrocarbon degrading microbes significantly influenced TPH concentrations for both unplanted ($R^2$ = 0.682; p = 0.0001) and planted ($R^2$ = 0.192; p = 0.0046) treatments, explaining 68 and 19% of the variation within the data respectively (Figure 3.4b). The ratio of hydrocarbon degrading microbes to total heterotrophs did not have a significant relationship to TPH concentrations in the soil for either unplanted ($R^2$ = 0.045; p = 0.184), or planted ($R^2$ = 0.045; p = 0.188; Figure 3.4c) treatments.

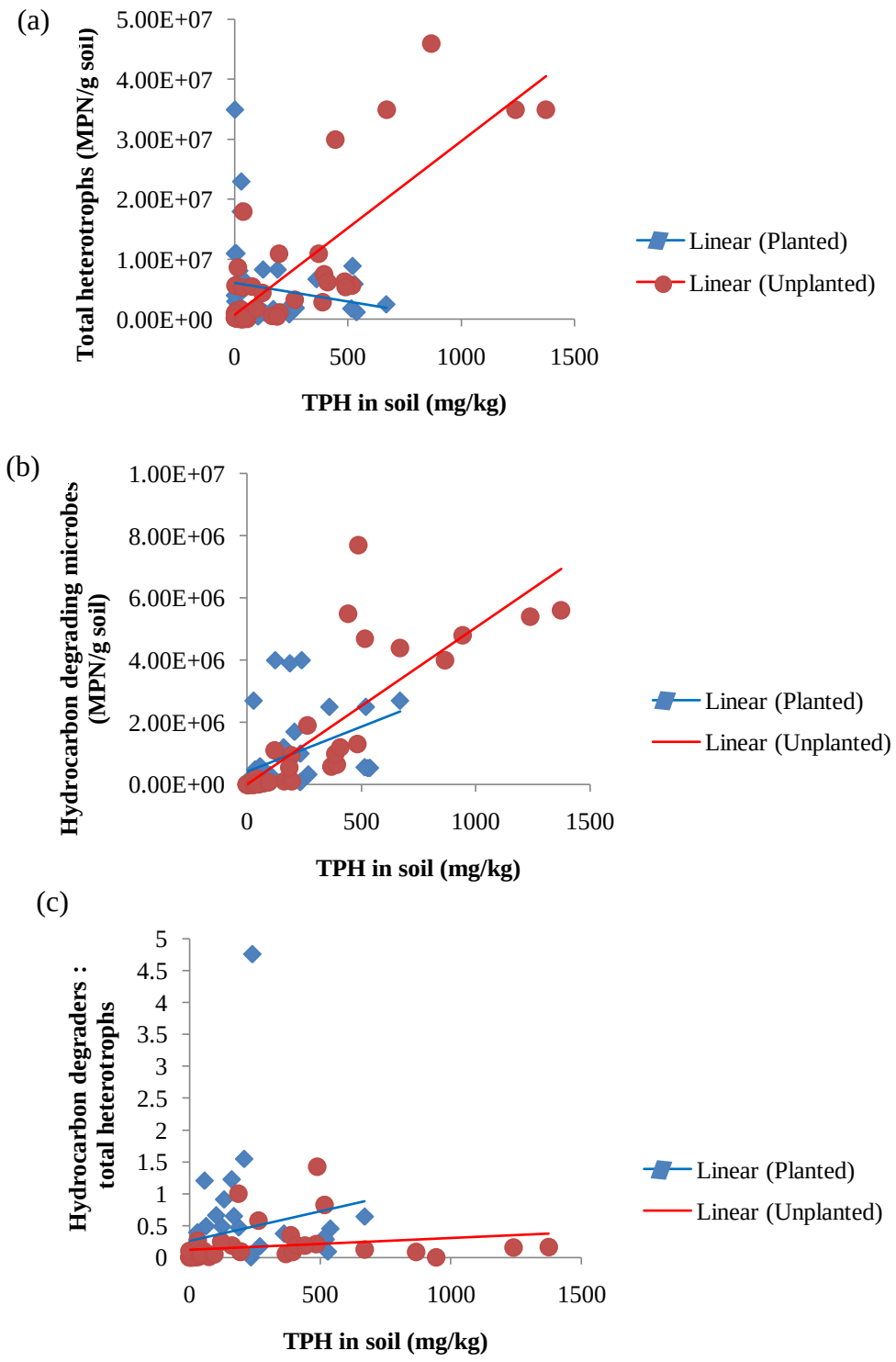


**Figure 3.4** Relationship between (a) total heterotrophs, (b) hydrocarbon degrading microbes and (c) the ratio of hydrocarbon degraders to total heterotrophs, to the level of soil petroleum hydrocarbons (TPH) in planted (*P. foliosa*) and unplanted soil, for time intervals 2 and 4 months.

## 3.4 Discussion

The aim of this study was to determine whether *P. foliosa* has the potential to facilitate the degradation of hydrocarbons in soils under sub-Antarctic conditions, in order to assess the utility of phytoremediation as an effective soil treatment option for contaminated sites at Macquarie Island. The presence of *P. foliosa* was found to significantly decrease concentrations of hydrocarbons in soil by up to 48% more than unplanted soils, indicating that phytoremediation, using this species, is a highly successful technique. The presence of *P. foliosa* also modified microbial community structure (total heterotrophs and hydrocarbon degrading microbes) within the soil, but only significantly increased microbial levels at lower treatment concentrations.

The presence of vegetation is reported to have an enhancing effect on microbial populations within soil (see reviews by Hutchinson 2003 and Wenzel 2008), and accordingly to improve degradation of soil contaminants due to co-metabolic processes (Chaudhry *et al.* 2005; Gaskin *et al.* 2008). For example, Liste and Felgentreu (2006) found higher counts of culturable microbes and actinomycetes in vegetated soil compared to un-vegetated soil, coupled with a 15.6% decrease in final TPH concentrations. Similarly, Philips *et al.* (2009) reported Altai wild rye (*Elymus angustus* Trin.) supported up to 100 times more endophytic hexane degraders than the unplanted control, promoting rates of TPH degradation that were up to 50% higher than in unplanted treatments. In keeping with these findings, *P. foliosa* significantly decreased TPH levels across all concentration treatments by up to 48% more than in unplanted soil. Additionally, final TPH concentrations in planted treatments (at four months) were reduced to below 300 mg/kg, which was the average soil background level in initial uncontaminated samples (Appendix C). By this time interval planted treatments had reduced soil TPH concentrations by 99.5, 98.0 and 97.3% of the initial nominal concentrations of 1 000, 5 000 and 10 000 mg/kg respectively. This rate of TPH reduction was unexpectedly fast when compared to studies such as Hutchinson *et al.* (2001) where a decrease of 49% was observed after 6 months and 57 to 68% after 12 months; Pradhan *et al.* (1998) where degradation was reported to be 57% after 6 months; and Chen *et al.* (2003) who reported losses of 35% after 6 months.

Potential reasons for enhanced degradation in the current study may include direct uptake of the contaminant by *P. foliosa*. Although organic compounds are not usually incorporated by

plants, due to their chemical structure (Cunningham and Ow 1996) it is possible that in this situation *P. foliosa* played an active role in absorbing SAB and accumulating it in its roots and leaves. While studies in regard to phytoextraction of organics are scant within the literature, research by Parrish *et al.* (2006) using *Cucurbita pepo* ssp. *pepo* (zucchini), *Cucumis sativus* (cucumber) and *Cucurbita pepo* ssp. *ovifera* (squash) to extract weathered hydrocarbons from soil, and Gao and Zhu (2004) using 12 plants species to extract pyrene from contaminated soils, have shown that phytoextraction of organics is a feasible pathway. Therefore, it is possible that uptake may have occurred in the current study.

Furthermore, the unique composition of SAB (which has been treated to remove heavy oil fractions that prevent it functioning in cold climates) may have made the contaminant more amenable, in both planted and unplanted treatments, to biodegradation and abiotic processes such as volatilisation due to its low molecular weight (Parrish *et al*. 2006). Also, soil leachate could potentially be a method of SAB loss in this experiment, as low temperatures can increase the water solubility of fuels (Margesin and Schinner 1999), however all runoff was recycled back into the pots to ensure against this. Further investigations are needed to gain a more comprehensive understanding of both the abiotic processes, and plant induced processes, affecting the rate of SAB degradation under cold conditions.

Although concentrations of TPH were reduced in the presence of *P. foliosa*, as microbial populations were not significantly higher in planted treatments, factors other than microbial populations are influencing the degradation of the fuel. This is in contrast to findings of Banks *et al.* (2003), Siciliano *et al.* (2003), Chiapusio *et al.* (2007) and Gaskin *et al.* (2008), who observed significantly higher numbers of microbes in planted soil compared to the unplanted control. Contrary to the hypothesis, and similar to studies by Radwan *et al.* (1998), Kudjo Dzantor *et al.* (2000), Chaiapusio *et al*. (2007) and Cofield *et al.* (2007), this study generally observed no difference in microbial populations between planted and unplanted treatments (with the exception of 0 and 1 000 mg/kg). The current study also found even lower levels of both, total heterotrophs and hydrocarbon degrading microbes, in planted treatments than in unplanted treatments at 10 000 mg/kg, similar to reports from Merkl *et al.* (2006). Moreover, this experiment found that higher numbers of total heterotrophs or hydrocarbon degraders in the soil did not correspond to increased levels of hydrocarbon loss across concentrations for planted soil,

only unplanted soil (c.f Figures 3.2 and 3.3a and b). For example, maximum levels of TPH degradation in planted treatments occurred at 10 000 mg/kg of SAB. At this treatment, levels of total heterotrophs and hydrocarbon degraders were over 50% lower than in the unplanted soil, yet measured TPH degradation was 41 to 48% higher. Also, the regression analysis between microbes and soil TPH suggests that in unplanted soil, TPH concentration is strongly associated with presence of microbes, with 65 and 68% of the variation in TPH levels explained by total heterotrophs and hydrocarbon degrading microbes respectively. Conversely, in planted treatments total heterotrophs showed no association with soil TPH concentration, whilst hydrocarbon degraders showed a weak association of 19%. It therefore appears that, while *P. foliosa* was highly successful at remediating SAB contaminated soil, the plants' effectiveness is not solely linked to the presence of microbial populations as initially hypothesised, or perhaps it is in this case also linked to other plant associated mechanisms. This being said, the levels of micro-organism observed in the current experiment were considerably higher than expected for cold regions (Leszkiewicz 2001; Delille *et al.* 2003; Rayner *et al*. 2007), thereby supporting bioremediation as a potential option for the sub-Antarctic. This contrasts with expectations that it would be inadequate due to low microbial longevity (Delille *et al.* 2007a).

The lack of correlation between the breakdown of SAB and microbial populations in soil in this study suggests that *P. foliosa* invokes the use of 'phytodegradation' rather than 'rhizoremediation' to reduce concentrations of TPH in the soil. Phytodegradation refers to the use of internal plant mechanisms and processes to degrade organic pollutants (Wenzel 2008). Parameters known to aid contaminant breakdown, reported in Wensel *et al.* (2008) and Chaudlhry *et al*. (2005), include plant specific root enzyme exudates, volatilisation, changes to the physical and chemical nature of the soil and increasing the supply of oxygen to the root zone (essential for oxidation of organic contaminants). For example, a study by Olsen *et al.* (2003) proposed that certain root-released compounds act as biosurfactants making contaminants more bioavailable. This means that the pollutant can be readily taken up from the soil, or easily transformed by living organisms. Furthermore, Banks *et al.* (2000) reported root turnover of soil may change the soil's chemical and physical properties to favour contaminant degradation and assist in contaminant volatilisation. Margesin and Schimmer (1999) suggested that plant induced changes in the moisture content and water availability within soils can also significantly affect the degradation of organics. However, to date, there have been limited studies in which plants

have demonstrated the degradation of organics, such as petroleum hydrocarbons, by phytodegradation mechanisms alone (Newman and Reynolds 2004). It is therefore plausible that microbes may have been misrepresented in this experiment, masking their actual influence on TPH degradation.

Microbes at higher TPH concentrations have a faster metabolic rate due to the presence of excess carbon as a food source (Nichols *et al.* 1996). This high level of metabolic activity may have influenced the numbers of microbes catalogued in this experiment in two ways. Firstly, the presence of *P. foliosa* may have accelerated the degradation process to such an extent that the microbes were less plentiful in the planted treatment by the two and four month time intervals (when counted), since a reduction in the availability of the contaminant diminishes microbial fecundity (Alkorta and Garbisu 2001; Liste and Prutz 2006). Secondly, the bi-products of highly active microbial overturn can lead to a direct decrease in levels of soil pH, causing microbial inhibition at later stages of hydrocarbon remediation (Merkl *et al.* 2006). For example, Merkl *et al.* (2005) found that when planted with *B. brizantha*, petroleum hydrocarbons were degraded at a faster rate than in unplanted soil causing a higher metabolic turnover. This led to an accumulation of organic acids in the soil which subsequently decreased the pH causing microbial inhibition towards the end of the experiment in planted treatments (Merkl *et al*. 2005). Most microbes favour, and develop best under, relatively neutral pH conditions (Huang and Chen 2003). Thus, in order to understand if microbial affects are being 'masked' by either of these possibilities, further studies are needed to examine weekly fluctuations of microbes between zero and two months and to analyse the level of organic acids in planted and unplanted treatments.

If microbes are in fact the driving force of phytoremediation using *P. foliosa*, but are simply becoming less plentiful in the latter stages of contaminant degradation, then stimulating populations of microbes, for example by the addition of plant growth-promoting rhizobacteria (PGPR; Saleh *et al.* 2004), nitrogen fertilizer (Thompson *et al.* 2008; Ayotamuno *et al.* 2009) or phosphorus fertilizer (Alarcon *et al.* 2008), may further enhance SAB degradation. It is important to remember that, apart from the many beneficial interactions, plants and microbes also compete for the same resources (Wenzel 2008). In soils where nutrients are low, such as those polluted with hydrocarbons (Harvey *et al.* 2002), resource competition may be a limiting factor of microbial growth and biodegradation (Joner *et al.* 2004). For example, the promotion of

hydrocarbon degrading microbes has been shown by Siciliano *et al.* (2003) to be strongly modulated by abiotic factors. This study found the influence of plants on microbial communities to be dependent on available nutrients, and suggested that phytoremediation requires other site management techniques, such as increased fertilization, to exploit it to its full potential. Furthermore, studies on sub-Antarctic soils by Coulon *et al.* (2004) and Delillie *et al*. (2004) showed that the addition of fertilizer improves petroleum hydrocarbon degradation, however the adverse toxic residues that remain in low temperature soils after fertilization was noted as a considerable drawback. Conversely, Thompson *et al.* (2008) reported mixed results in relation to the benefits of additional nitrogen fertilizer to promote contaminant degradation. They reported that although nitrogen aided in the promotion of root growth (creating a better environment for micro-organisms) it also decreased the bioavailability and bioaccessibility of the contaminant, thus reducing biodegradation potential. Similarly, hydrocarbon degradation in a crude-oil contaminated soil was inhibited following nutrient addition (Chaineau *et al*. 2005). However, other studies have suggested that this repression may be soil specific (Phillips *et al.* 2006; 2009). Macquarie Island soils vary in nitrogen content depending on their vicinity to areas heavily occupied by animals (Erksine *et al.* 1998). Nevertheless, as the field application of this work is expected to have lower nutrient levels than the potting mix used in this experiment, nutrient additions may be an important factor to on site phytoremediation success.

A number of reports in the literature favour the addition of PGPR rather than fertilizers (Wenzel 2008). Zhuang *et al.* (2007), for example, reported that PGPR significantly aids in increasing plant tolerance to hydrocarbons, by prompting plant growth to create a better microbial environment and the PGPR also directly degrade the contaminant themselves. Specifically, the bacteria *Enterobactor cloacae* was shown to be most effective under total petroleum hydrocarbon contamination (Huang *et al.* 2005). Nevertheless, introducing this particular strain of bacteria on to Macquarie Island is unlikely to be approved due to laws regarding species importations (Bergstrom *et al.* 2009). Further studies on possible endemic PGPRs would be needed before this option can be considered as a possible aid to phytoremediation on Macquarie Island.

Suggestions for future research include a more detailed study into how *P. foliosa* influences SAB removal, particularly at early stages following fresh contamination. Measuring

parameters such as soil organic acid levels, pH, oxygen, moisture content and the level of plant exudates could also provide important insights. Testing plant shoots and roots for TPH accumulation would clarify whether *P. foliosa* can phytoextract SAB from the soil via absorption, uptake and transportation, although octanol/water partition coefficient (Kow) information available for SAB tends to exclude this as a major mechanism. These and other limitations are discussed in chapter 4.

In conclusion, these findings provide persuasive evidence that phytoremediation using *P. foliosa* is an effective treatment selection to reduce SAB levels under sub-Antarctic conditions. It does not appear that the species uses micro-organisms as its sole form of SAB removal, however, further studies are needed in this area.

# Chapter 4

## General Discussion and Conclusions

### 4.1 Overview

This thesis aimed to evaluate the native Macquarie Island grass species, *P. foliosa*, as a potential candidate for the phytoremediation of fuel contaminated soils under sub-Antarctic conditions. The major findings of this research include:

1. *Poa foliosa* is highly tolerant to soils contaminated with SAB between concentrations 0 to 10 000 mg/kg and can proliferate in SAB contaminated soils of up to 40 000 mg/kg.

2. The presence of *P. foliosa* significantly increases rates of TPH degradation in soils contaminated with SAB resulting in soils with nearly 50% lower contaminants than unplanted treatments. Concentrations of TPH in soil planted with *P. foliosa* approached background levels within four months.

3. The presence of *P. foliosa* changes microbial compositions in soils contaminated with SAB and significantly stimulates the growth of both total heterotrophs and hydrocarbon degraders in soils containing low SAB concentrations (up to 1 000 mg/kg).

4. The presence of microbes in the root zone does not appear to be the sole force of TPH degradation in soil planted with *P. foliosa*.

From the results of the current study, it is clear that *P. foliosa* has a highly significant positive effect on the degradation of SAB within soil and would be recommended as a useful method in the suite of *in-situ* remediation technologies that are being adopted at Macquarie Island. *Poa foliosa* plants showed only a slight reduction in health when exposed to concentrations of 5 000, 20 000 and 40 000 mg/kg. Whilst plants significantly encouraged the growth of microbial populations at lower concentrations, the stimulation of TPH degradation by plants was not solely due to increased microbial levels. *Poa foliosa* also has the potential to be used on other sub-Antarctic islands or in similar cold climates where the plant natively resides.

## 4.2 Discussion, limitations and recommendations for future work

While the results of this study are promising, additional studies conducted in the field to assess the response and effectiveness of *P. foliosa* in the remediation of soils under natural conditions, would further complement and validate this work. A number of studies, including those by DeLaune *et al*. (2003), Robson *et al.* (2003) and Chiapusio *et al.* (2007), have highlighted differences between responses observed in growth room experiments, versus those noted in the field. Growth room experiments can sometimes restrict the ability of plants to carry out phytoremediation. For example, experiments by DeLaune *et al.* (2003) found *Spartina patens* in the field had a better rooting system than their growth room counterparts, as root growth was not confined to a small volume such as occurs within pots. When contaminated with oil, growth room plants mostly died or did not regenerate many new shoots, whereas plants in the field had the ability to regenerate from underground rhizomes and showed substantial recovery to exposure. Root confinement is likely to have had a negative effect on the health of *P. foliosa* within this experiment. *Poa foliosa* is known to develop and spread almost entirely by its complex rhizome system, resulting in an accumulation of a dense mass of roots beneath the ground's surface (Ashton 1965). By the end of the study (eight months) potted plants were extremely root bound with the rhizome system severely restricted by pot confinement (Figure 2.5). Thus, it maybe anticipated that *P. foliosa* would produce a larger root biomass in the field, leading to a greater rhizosphere surface area and as a result, plants would potentially be more effective at phytoremediation.

Although Macquarie Island conditions were simulated as best as possible in this study, the controlled conditions of the growth room did not allow *P. foliosa* to experience the unpredictable environment of the field. Not only were the plants protected from dramatic fluctuations in weather (such as wind speed, cloud cover, temperature and water availability) but they were potted in commercially available soil, rather than in soil from Macquarie Island, to which fresh hydrocarbons were added. Plant toxicity of hydrocarbons varies widely with soil characteristics (Smith *et al.* 2006; Liste and Prutz 2006) and using only one contaminated soil type in a screening experiment may prevent an accurate identification of species tolerance under a range of naturally occurring soil types and conditions (Robson *et al.* 2003). Furthermore, the use of freshly contaminated soil in this study does not replicate the current conditions of fuel

contamination that *P. foliosa* would be exposed to if planted for remediation of current contaminated sites on Macquarie Island. The most recent spill encountered at Macquarie Island occurred at the Main Power House in 2002 (Rayner *et al.* 2007), meaning the minimum age of contamination within Macquarie Island soils is seven years. It is well-known that bioavailability is one of the most limiting factors to petroleum hydrocarbon degradation (Mohn and Stewart 2000; Mohan *et al.* 2006; Olson *et al.* 2007). A study by Allard *et al.* (2000), using aged creosote-contaminated soil, found that age of the contamination was the limiting factor in its bioavailability and consequently its degradation. Over time, interactions with soil organic matter cause a decline in hydrocarbon degradation through adsorption, diffusion and dissolution, and covalent binding, which result in bound hydrocarbon molecules which are not readily biodegraded (Parrish *et al.* 2005). Therefore, hydrocarbon properties along with soil organic matter can strongly influence the bioavailability of compounds in soil and thus phytoremediation success (Smith *et al.* 2006; Chiapusio *et al.* 2007). Our experiment is not conducive to a real field situation, where soil can vary in total organic matter content by up to 30%, hydrocarbons are not homogenously mixed through soil, and are also recalcitrant. A field trial of *P. foliosa*, in a range of soils contaminated with various aged hydrocarbons is consequently essential to more thoroughly assess this applicability of the species for phytoremediation.

It was noted that, by the four month harvest the TPH concentrations in planted soil were approaching those found in initial uncontaminated samples (~300 mg/kg dw). This result was unforeseen as it was anticipated that the cold temperature (7 - 9°C) would slow the rate of hydrocarbon degradation. The temperatures of Macquarie Island are unfavourable for plant growth, microbial activity and thus phytoremediation of organics, which have been found to biodegrade most efficiently in conditions of intense light and humidity and at high temperatures between 30 and 40°C (Yeung *et al.* 1997; Merkl *et al.* 2005a). Because of this, it was estimated that TPH degradation could take up to twelve months, and that little activity would occur within the first two months of planting. However, this was not the case, and research on the changes in TPH concentrations and microbial community response based on weekly observation and measurements within the first two months of exposure to contamination would be useful. Our study has shown this is the period in which biodegradation is greatest and the largest differences between the planted and unplanted treatments are expected to occur. It is important to note that

this result may vary across studies depending on the age of the hydrocarbons, the soil type and characteristics including organic content. Soils with a high organic content may decrease contaminant bioavailability and therefore influence lag phases in microbial growth (Gaskin 2008). Macquarie Island soils generally have a higher organic content (up to 30%) than the soils used in the current study (13%), and as a result may have a more pronounced lag time.

Experimentally analysing the success of *P. foliosa* plants at different stages of maturity may be advantageous in achieving the fastest rate of phytoremediation. The greater the rooting system, the larger the amount of contaminated soil that is exposed to remedial rhizosphere effects (Liste and Felgentreu 2006). The current study used transplanted species in the pioneer phase, however, the life history of the species suggests that plants in the mature phase may be best for phytoremediation. During the mature phase *P. foliosa* forms a dense mass of dead and living roots beneath the ground's surface, creating a pedestal of fibrous peat that supports the large crown of the plant (Ashton 1965). Full root development has been found to be a prerequisite for the benefits of phytoremediation (Hou *et al.* 2001), therefore, targeting a time in the life cycle of *P. foliosa* where roots are most developed could prove beneficial. Plant root exudates (the main stimulator of degrading micro-organisms) also change with plant age (Gunther *et al.* 1996) and may be greater at a later stage of plant development (Merkl *et al.* 2005b).

An important point to consider is the high level of variability in the microbial data, especially at 10 000 mg/kg. Accurate enumeration of soil micro-organism is difficult in any situation and there are limitations to the traditional methods of microbial inventory (plate counts) that were used in this experiment (Banks *et al.* 2003). Factors that may have caused this variation include the constant state of flux of microbes and the fact that only a small proportion of the total soil biomass is comprised of microbial cells. Although attempts were made to standardise procedures and the site of collection of soil samples wherever possible, samples were not always able to be consistently sampled from the soil surrounding the rhizosphere. This may account for such discrepancies within the data and may also have led to an under-representation of the actual densities of microbes within planted treatments. Furthermore, microbial samples were calculated on a wet weight, rather than on a dry weight basis, and differences in soil moisture content at the time of sampling were highly evident. Recalculating the MPN data based on dry weights could

potentially reduce variation. Further studies could also carry out functional diversity assessments as a more comprehensive method of analysing changes in microbial communities (Banks *et al*. 2003). Although hydrocarbon degrading micro-organisms did not appear to correlate strongly with TPH degradation in planted treatments (Figure 3.4c), no information was gathered in regard to which microbes within these populations were actually increasing or decreasing. It may be that specific microbes within hydrocarbon degrading populations are more apt at degrading SAB, and as a result smaller numbers of these are required to substantially reduce large TPH quantities.

As previously mentioned, a number of *in situ* mechanisms such as micro-bioventing have been successfully applied on Macquarie Island (Rayner *et al.* 2007) and the purpose of this study was to identify if phytoremediation would be a useful method to compliment these already established techniques. Huang *et al*. (2004) found that a multi-process approach to phytoremediation, which comprises different aspects of contaminant removal from soils, can increase efficiency of contaminant removal compared to phytoremediation alone by up to 45%. Both Huang *et al.* (2005) and Lin *et al.* (2008) have reported TPH removal of up to 88% by using a multi-process system including bioremediation, phytoremediation and land-farming to remediate soil within eight months. Similarly, field studies in cold climates have also reported multi-process phytoremediation systems success (Greenberg *et al.* 2008). A relevant area of future research would be to investigate the efficiency of the techniques already in place at Macquarie Island in conjunction with phytoremediation, and to devise a model of the most complementary *in situ* techniques to achieve the fastest and most effective remedial design for fuels in the sub-Antarctic environment. This could be extended to examine the effects of mixing *P. foliosa* with other grass species that already inhabit Macquarie Island such as *Poa cooki* and *Poa annua* similar to mixed plant methods carried out by Philips *et al*. (2006; 2009).

A question remains in regard to the anticipated remedial efficiency of *P. foliosa* when planted in differing levels of SAB contamination in the field. If applied on Macquarie Island, *P. foliosa* would be required to remediate soils ranging from 800 to 7 000 mg/kg. It is within this zone (5 000 mg/kg) that a number of health parameters indicated plant stress. The question is, will this detrimentally affect the plants ability to degrade THP? From this study's findings it appears that it would not. Even though *P. foliosa* plant health seemed slightly compromised for

some parameters such as chlorophyll content, the plant still degraded SAB at a rate 35 to 45% more than its unplanted counterpart. Similar results were found in both a long-term field study on the degradation of petroleum hydrocarbons via crop growth (Liste and Felgentreu 2006) and a study involving Altai wild rye (Philips *et al.* 2009). Contaminant degradation was found to be higher in plants that showed signs of stress and growth depression. Reasons for this particular pattern can be explained by findings from Kamath *et al.* (2004) who concluded that under stressful conditions plants are particularly effective in augmenting contaminant biodegradation. This is because stress stimulates a greater release of chemicals inductive to microbial hydrocarbon degradation from the plant roots, thus increasing microbial populations and subsequently increasing the level of degradation (Chaudhry *et al.* 2005). Phytoremediation can consequently be seen as a defence mechanism against the phytotoxic effects of soil contaminants, and provides further evidence to its usefulness as an *in situ* method for the remediation of sub-Antarctic soils.

## 4.3 Conclusion

In conclusion, this study describes the first investigation into the potential of *P. foliosa* to phytoremediate soils contaminated with SAB fuel under sub-Antarctic conditions. Experiments have successfully shown that, not only can the species tolerate high levels of SAB, but its presence significantly reduces levels of SAB in soil within two months. The use of *P. foliosa* to phytoremediate sites on Macquarie Island is an environmentally friendly, economical, sustainable and feasible option within the suite of other remedial strategies already in place. This study provides the foundations for further applied research aimed at reducing the human impacts within the sub-Antarctic region.

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# Appendix

## Appendix A

**Table A.** The effect of time on shoot, root biomass and shoot/root ratio of *P. foliosa*. *t*-test ($p < 0.05$).

| | Mean (± SE) | | *t*-test | | |
|---|---|---|---|---|---|
| | **2 months** | **4 months** | ***t*** | **c.v.** | **0.05** |
| Shoots | 6.17(0.72) | 13.83(2.08) | 20.430 | 2.101 | significant |
| Roots | 1.65(0.42) | 8.04(2.86) | 15.767 | 2.101 | significant |
| s/r ratio | 4.62(0.81) | 2.64(0.55) | 7.542 | 2.101 | significant |

## Appendix B

**Table B.** The effect of SAB concentration of the relative growth rates (MRGR) for shoots and roots of *P. foliosa* using data from the two and four month harvest. One-way ANOVA ($p < 0.05$).

| | | **Shoot** | |
|---|---|---|---|
| **Source** | **df** | **F ratio** | **p value** |
| **Shoots** | 3 | 0.202 | 0.893 |
| **Roots** | 3 | 0.164 | 0.918 |
| **Error** | 16 | | |
| **Total** | 19 | | |

## Appendix C

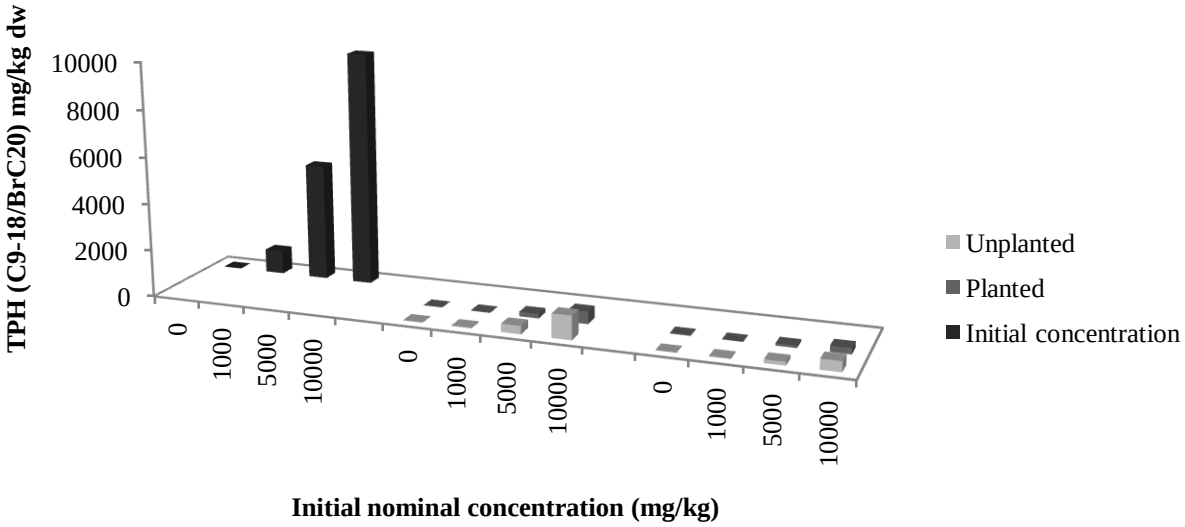


**Figure C.** Changes in the TPH concentration of planted (*P. foliosa*) and unplanted soil in relation to increasing hydrocarbon concentrations (mg/kg), for time intervals 0, 2 and 4 months (n= 5).

## Appendix D

**Table D.** Initial soil microbial counts of total heterotrophs and hydrocarbon degraders, n = 5.

| | **Initial number (MPN/g soil)** |
|---|---|
| **Total heterotrophs** | $16 \times 10^5$ |
| **Hydrocarbon degraders** | 10144.4 |