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Phytoremediation of hydrocarbon contaminants in subantarctic soils: an effective management option

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Keywords
Remediation, Macquarie island, Diesel, Petroleum hydrocarbon, Poa foliosa, Toxicity

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Phytoremediation of hydrocarbon contaminants in subantarctic soils: an effective management option

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Abstract

Accidental fuel spills on world heritage subantarctic Macquarie Island have caused considerable contamination. Due to the island's high latitude position, its climate, and its fragile ecosystem, traditional methods of remediation are unsuitable for on-site clean up. We investigated the tolerance of a subantarctic native tussock grass, *Poa foliosa* (Hook. f.), to Special Antarctic Blend (SAB) diesel fuel and its potential to reduce SAB fuel contamination via phytoremediation. Toxicity of SAB fuel to *P. foliosa* was assessed in an 8 month laboratory growth trial under growth conditions which simulated the island's environment. Single seedlings were planted into 1 L pots of soil spiked with SAB fuel at concentrations of 1 000, 5 000, 10 000, 20 00 and 40 000 mg/kg (plus control). Plants were harvested at 0, 2, 4 and 8 months and a range of plant productivity endpoints were measured (biomass production, plant morphology and photosynthetic efficiency). *Poa foliosa* was highly tolerant across all SAB fuel concentrations tested with respect to biomass, although higher concentrations of 20 000 and 40 000 mg SAB/kg soil caused slight reductions in leaf length, width and area. To assess the phytoremediation potential of *P. foliosa* (to 10 000 mg/kg), soil from the planted pots was compared with that from paired unplanted pots at each SAB fuel concentration. The effect of the plant on SAB fuel concentrations and the associated microbial communities found within the soil (total heterotrophs and hydrocarbon degraders) were compared between planted and unplanted treatments at the 0, 2, 4 and 8 month harvest periods. The presence of plants resulted in significantly less SAB fuel in soils at 2 months and a return to background concentration by 8 months. Microbes did not appear to be the sole driving force behind the observed hydrocarbon loss. This study provides evidence that phytoremediation using *P. foliosa* is a valuable remediation option for use at Macquarie Island, and may be applicable to the management of fuel spills in other cold climate regions.

Key words: remediation, Macquarie Island, diesel, petroleum hydrocarbon, *Poa foliosa,* toxicity.

1. Introduction

Petroleum hydrocarbon contamination is increasingly becoming a global problem, with spills reported across every ecosystem, including the sparsely populated high latitude polar-regions. Petroleum is highly persistent in the environment, toxic in nature and presents significant health risks to organisms once it enters the food chain (Stark et al., 2003; Hentati et al., 2013). Several studies suggest that spills in higher latitude ecosystems are significantly more harmful, with greater long-term effects, than spills at lower latitudes (Delille et al., 2002; Braddock et al.,

2003). This is largely due to the reduced temperature, which along with low nitrogen and oxygen levels in soil, lessens both natural degradation rates and the processing rates and efficiency of indigenous hydrocarbon degrading microorganisms (Coulon et al., 2005; Snape et al., 2006). As a result the rate of natural biodegradation of total petroleum hydrocarbons (TPH) in the subantarctic is estimated to be as low as 10 – 20 mg TPH/kg soil per day (Rayner et al., 2007), a figure which is significantly lower than hydrocarbon degradation rates reported for soils in tropical and temperate regions (Wang et al., 2008). This suggests that, if left untreated, even minor to moderate fuel spills in these regions could take tens to hundreds of years before natural attenuation would reduce the petroleum to environmentally acceptable concentrations (~ 220 mg/kg soil; Australian Antarctic Division, 2013).

An extensive history of polar exploration and exploitation, together with present research activities, has left subantarctic islands, such as Macquarie Island, with a legacy of petroleum pollution as a result of accidental fuel spills (Aislabie et al., 2001; Schafer et al., 2007; Tin et al., 2008). Australia's research station on Macquarie Island is heavily reliant on a light range diesel fuel known as Special Antarctic Blend (SAB), which is comprised mainly of petroleum hydrocarbons in the range C9-18. During the cycle of transportation, storage and usage of SAB fuel, a number of spills have occurred (Rayner et al., 2007). These spills typically range from between 100 – 10 000 L, but three substantial plumes near the station have been identified as the sites of most concern (Rayner et al., 2007). Australia is committed to the clean-up of these sites, with active remediation starting in 2009. Traditional *ex situ* remediation strategies in subantarctic environments are expensive (approximately US$4 000/metric ton; Rayner et al., 2007; Snape et al., 2008), hence sophisticated response options for the remediation of the spills identified on Macquarie Island and in other subantarctic ecosystems are being investigated.

One potentially attractive, non-invasive and inexpensive, yet unexplored, option of remediation on Macquarie Island is phytoremediation. 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; Gaskin and Benthan, 2010; Zhang et al., 2010). Over the past decade substantial progress has been made in applying phytoremediation to inorganic pollutants such as heavy metals (Bhargava et al., 2012; Asensio et al., 2013) and organic pollutants such as poly-aromatic hydrocarbons (PAH), nutrients and landfill leachate (Schröder et al., 2008; Souza et al., 2013). Most studies have focused on the ability of plants to hyperaccumulate inorganic heavy metals from soils (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). However, sub-Arctic studies by Palmroth et al., (2002) and Robson et al., (2003) suggest phytoremediation to be feasible, and point to rhizodegradation as the most likely strategy employed by the plant to remove contaminants. The breakdown of hydrocarbons through rhizodegradation is a two-part process. Firstly the plant provides root exudates (such as enzymes, simple sugars, amino acids, aliphatics and aromatics) to stimulate the growth of root-associated micro-organisms (Khan et al., 2013). Root growth can also extend into deeper soil, allowing access to air and water, and thus 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 capacity of the plant to degrade contaminants (Khan et al., 2013). This mutualistic relationship between plants and microbes in

the soil has been found to accelerate loss of petroleum hydrocarbons in soil when plants are present (Hutchinson, 2003; Wenzel, 2008).

The prospect of using phytoremediation on Macquarie Island is potentially complicated by the vast comparative climatic difference compared to lower latitudes, coupled with stringent restrictions on species importations. Hence suitable climate and contaminant-tolerant native species must be identified. In general, the existence of petroleum hydrocarbons in soil has a negative impact on plant growth and development (Joner et al., 2004). 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 (Kvesitadze et al., 2006). From the numerous studies that have investigated the response of different species to contamination, the general consensus is that grass species display the highest level of resistance (Olsen et al., 2007; Gaskin et al., 2008; Wang et al., 2008; Barrutia et al., 2011; Cook and Hesterberg, 2013). Furthermore, out of 39 cold-tolerant test species and under comparable sub-Arctic conditions, *Psoralea esculenta* (Pursh) Rydb. (a legume) and *Agropyron pectiniforme* Roem. & Schult. (a grass) were found to be the most tolerant species to hydrocarbons, and the most promising for use in phytoremediation (Robson et al., 2003). These findings suggest that Macquarie Island's largest endemic grass species, *Poa foliosa* (Hook. f.), may be a suitable candidate for phytoremediation in the subantarctic region. The grass is widely distributed across the coastal zones of Macquarie Island, grows readily on the isthmus (where Australia's research station is located) and surrounds the three sites where SAB fuel contamination has occurred. Moreover, it has a relatively deep (~0.5 m) fibrous root system, a large biomass and a fast growth rate. To date, no research has explored hydrocarbon tolerance of any grass species or of any flora endemic to Macquarie Island.

Pairing 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 managing petroleum hydrocarbon contamination within this region. There is evidence to suggest that a multi-technique approach, involving phyto-oxidation, volatilisation and microbial remediation together with phytoremediation, can be twice as effective in reducing petroleum hydrocarbon levels as is 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 fuel concentrations more effectively than natural microbial and mechanical processes alone must be undertaken. Therefore the aims of this study are twofold: firstly to test the toxicity of SAB fuel to the native tussock grass, *Poa foliosa* and secondly, to assess the phytoremediation potential of this species. This study uses an Australia-based growth trial experiment approach, which is an important precursor to field trials. Due to the extremely remote nature of the field site (three days sail from nearest port), access is restricted, difficult and expensive. Field trials at this site therefore require an especially rigorous evidence-base before the methods can be considered for deployment in the field. This paper presents the first experimental investigation into the viability of phytoremediation in the subantarctic using the native tussock grass, *P. foliosa*.

2. Materials and Methods

2.1 Experimental design and test conditions

An 8-month pot-based laboratory growth trial (harvested at zero, two, four and eight months) was conducted to evaluate (1.) the tolerance of *Poa foliosa* to SAB fuel and (2.) the applicability of using this species to remediate petroleum spills in cold climates.

The soil media used in the experiment was a commercially available potting mix (Tasmanian Devils Dirt potting mix). Total organic carbon (TOC) content of the mix was 13.6 ± 0.2% (mean ± SD, n=5), determined for five 500 mg sub-samples from a 10 g sample of the mix, for which the 2 mm sieved fraction had been milled for three minutes at 500 rpm before TOC was determined using a Shimadzu TOC–VCSH analyser (Kyoto, Japan) with solid-state module attachment (SSM - 5000A). Special Antarctic Blend (SAB) diesel fuel was obtained from the Australian Antarctic Division (AAD) and used to spike the soil to six nominal concentrations of 1 000, 5 000, 10 000, 20 000 and 40 000 mg SAB/kg soil (plus control). The SAB fuel 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 fuel, each treatment was mixed thoroughly for ten minutes to achieve homogeneity before being transferred to 1 L pots. Concentration of TPH was measured for initial spike quantities of SAB fuel (initial target concentrations are referred to throughout).

Poa foliosa plants used were three-month old seedlings, germinated at the Royal Tasmania Botanic Gardens (Hobart), from Tasmanian Seed Conservation Centre stock collected from Macquarie Island.

Poa foliosa's tolerance to SAB fuel was conducted over the full concentration range, to 40 000 mg/kg. Five replicate pots of spiked soil per concentration and harvest interval were prepared as described and each planted with a *P. foliosa* seedling. All concentrations were harvested at four

and eight months, concentrations up to 10 000 mg/kg were also harvested at two months, making a total of 80 planted pots, plus five plants for harvest at the start of the experiment. At each harvest, plants were measured for a range of growth and productivity measures, described below.

Phytoremediation potential of *P. foliosa* was tested over four concentrations (from 0 to 10 000 mg/kg SAB). This test measured fuel concentrations in soil in planted versus matched unplanted pots, sampled at the two, four and eight month harvest periods. For the matched unplanted pots, five additional replicate pots of spiked soil per concentration and harvest interval were prepared as described and left unplanted (60 unplanted pots). Soils for the planted treatment were collected from the planted pots used in the toxicity test, making a total of 120 pots. At each harvest, soil samples were measured for concentration of TPH and a range of microbial community characteristics, as described below.

All pots were maintained in a temperature controlled growth room at 8 $^{\circ}$C (± 1 $^{\circ}$C) at the AAD, Kingston, Tasmania. The room was lit by 8 incandescent and 4 high-intensity growth lights, which were programmed to a day/night photoperiod of 10/14 hours to simulate the annual mean daily light exposure on Macquarie Island. Pots of the same treatment concentration were stored together in trays to prevent any pot leachate from affecting the SAB fuel concentration in different treatments. Planted and unplanted pots of the same concentration were randomly arranged in each tray and trays were randomly placed on shelves within the growth room. Every 3 days, trays were rotated on the shelves and each pot was given adequate water to maintain moist soil without producing excess leachate. Foliar nutrient spray (Maxicrop seaweed plant food concentrate (Multicrop, Australia) applied at a ratio of 2 to 2.5 mL per L of water) was also applied fortnightly to promote plant growth and to better represent field conditions, which

experience high nutrient inputs from animal sources (Erskine et al. 1998). Any leachate produced, drained into individual pans under each pot and the water collected was used to re-water the pot to minimise loss of hydrocarbons from the system via this pathway.

At each harvest period, plant material was separated from the soil and both were kept at 4 °C prior to analysis. Soil from each replicate pot, for each treatment, was homogenized, and sub-samples taken and kept at 4 °C until analysed.

2.2 Plant toxicity endpoints

Optimum photosynthetic efficiency was assessed as the chlorophyll fluorescence parameter (Fv/Fm) using a pulse-amplitude-modulated (PAM) chlorophyll fluorometer (H. Walz Effeltrich, Germany). Plants were dark-adapted prior to measurements of maximum photosynthetic efficiency and PAM settings were optimised. All measurements were collected within 2 hours of midday. Up to 5 fully expanded leaves were grouped together to obtain a sufficient leaf area for each measurement and 4 replicate measures were made per plant. The youngest fully expanded leaves were used, and measurements were taken approximately 4 cm from the base of the leaf blades. Measurements were performed throughout the experiment at approximately bi-weekly (first 2 months) then monthly intervals (from 2 to 8 months).

Prior to the destruction of plants for biomass estimates, plant morphology measures, including leaf length, width and area, were determined. Once separated from the soil, plants were placed inside individual plastic zip lock bags to retain moisture and were kept at 4 °C for a maximum of 1 day before analysis. Due to the extensive number of leaves on plants, a random sub-sample of 30 leaves per plant was measured. Leaves were separated from the stems at the axil and the

length and width of each leaf measured. The leaf surface area was determined using a Portable Leaf Area Meter (LI-COR Model LI-3000A Portable Leaf Area Meter, Lincoln, Na., USA). Leaves were scanned twice to obtain an average reading for surface area.

The above-ground and below-ground biomass were measured destructively. Roots were carefully washed over a 0.25 mm sieve using tap water to remove soil. Any displaced roots were extracted from the sieve. Shoot and root samples were then dried at 60 ˚C for 72 hours, and weighed to obtain dry biomass estimates. These were then 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):

$$MRGR = (\ln W2 - \ln W1) / (T2 - T1)$$

where W1 and W2 are the dry weight of shoot or root at T1 (2 months) and T2 (4 months) respectively.

2.3 Phytoremediation endpoints

2.3.1 Soil fuel chemistry

Total petroleum hydrocarbons (TPH) were recovered from the soil using methods adapted from Snape et al., (2005) with the amount of sub-sample modified to ensure an accurate measurement for each concentration (10 g of soil for 0 to 10 000 mg SAB/kg soil, 5 and 2.5 g for 20 000 and 40 000 mg SAB/kg soil 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 mixed by tumbling end over end for 12 hours at room temperature to ensure extraction of petroleum hydrocarbons and were centrifuged at 1 000 r.p.m. for 5 minutes.

Approximately 2 mL of the hexane layer containing the petroleum hydrocarbons was transferred into 2 mL vials and analysed on an Agilent 6890 Gas Chromatography-Flame Ionisation Detector (GC-FID) fitted with an auto-sampler (Agilent 7683 ALS) using helium as the carrier gas. Separation was achieved using an SGE BP-1 column (35 m x 0.22 mm ID, 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 fuel 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 then ramped up to 320 °C at 18 °C/min. Detector temperature was 340 °C. Concentrations of TPH 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. To examine pathways for TPH loss, biomarker ratios were calculated from signals detected for individual compounds (as described by Snape et al. 2005 and 2006) for soils harvested at 2 months for the 10 000 mg/kg treatment.

2.3.2 Microbial responses

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 in separate sterile 96-well microtitre trays. Each tray was divided into three sections 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 medium were vortexed for 30 seconds (twice) to achieve homogeneity. Each initial dilution of 40 μL was used to inoculate the appropriate first four wells on the titre tray. Serial dilutions at 1:5 were performed. Five μL of filtered-sterilized SAB fuel was then added to the surface of each well. The last row of wells were not inoculated and served as a negative control for growth. After 5 days incubation at 10 ºC, 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.

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 7 days incubation. Serial dilutions of 1:10 were performed for the initial and 2-month harvests and 1:5 serial dilutions were performed at other time points.

Microbial diversity for a subset of samples was assessed by community level physiological profiling (details are provided in Supplementary information).

2.4 Statistical Analysis

Fuel biomarkers were analysed using two-tailed two-sample t-tests to compare 1. Freshly spiked soil vs unplanted soil at 2 months (n=3) and 2. Soil at 2 months for planted vs unplanted (n=5). All other data were analysed using a general linear model in the program JMP 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. Linear regressions were used to establish relationships in microbial data. Prior to analysis raw data were tested for normality

using the Shapiro-Wilk W Test, and for homogeneity of variance using Cochran's *C* test. Log10 transformations were required to normalise microbial data, while square root transformations were used to normalise TPH and plant physiology data. A single outlier was excluded from the hydrocarbon degrading microbial data as well as the TPH data to achieve a normal data set. This outlier exclusion did not change the overall outcome of results. In cases where normality was not detected by the Shapiro-Wilks W Test, non-parametric Kruskal-Wallis tests were run, followed by pair wise Wilcoxon tests. The Wilcoxon *p* values were compared to sigma values obtained from a Bonferroni test to establish significance.

3. Results

3.1 Toxicity of SAB fuel to plants

Poa foliosa was found to be highly tolerant to SAB fuel, with no severe physiological effects detected in response to fuel exposure. Throughout the experiment leaves generally lengthened and increased in width, resulting in an increased area, especially by 8 months (Figure 1). There was no difference in leaf length or area between SAB fuel treatments at 2 months, however leaves were wider at higher concentrations compared to lower concentrations (Figure 1b; $p = 0.0001$). After 4 months' exposure, the two highest concentration treatments (20 000 to 40 000 mg SAB/kg soil) resulted in plants that had significantly shorter, narrower leaves with less leaf area than plants in the control (Figure 1c; $p = 0.0001$). The plants growing at lower SAB fuel concentrations were not significantly different from control plants apart from leaf width, which was significantly narrower than the control at the 1 000 and 5 000 mg SAB/kg soil treatments ($p = 0.001$), but significantly wider in the 10 000 mg SAB/kg soil treatment ($p = 0.0001$). At the final 8-month harvest plants grown in 10 000 mg SAB/kg soil had significantly wider leaves,

with a greater leaf area, than in any other treatment (p = 0.0001). The highest concentrations of SAB fuel only had a significant impact on leaf width (p = 0.0001).

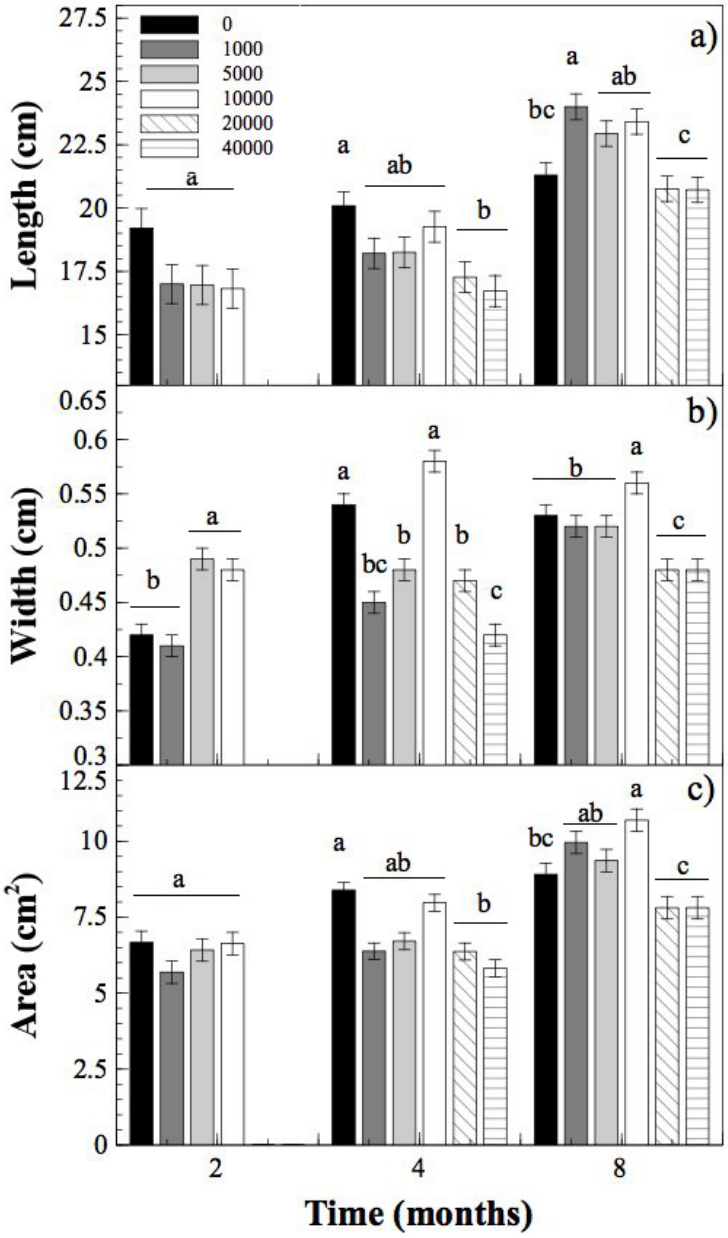

Figure 1: Leaf morphology measures: (a) length, (b) width and (c) area for *P. foliosa* in response to initial nominal SAB fuel concentrations up to 40 000 mg/kg, measured at time intervals 2, 4 and 8 months. Initial harvests were 27.6±0.87 cm, 0.32±0.0 cm and 8.66±0.07 cm^2 respectively. Data are means ± SEM, n = 30. ***** indicates a significant difference from the control at p = 0.05 within each harvest interval. No data collected for 20 00 and 40 000 mg/kg treatments at the 2 month harvest.

Comparisons between treatments indicated that the presence of hydrocarbons in soil did not impair the normal growth of *P. foliosa* (Table 1). No significant change was detected in regard to the mean above ground dry biomass of *P. foliosa* across differing SAB fuel treatments at 2 ($p = 0.152$), 4 ($p = 0.467$) or 8 ($p=0.822$) months. Similarly, the below ground biomass data showed no significant difference between SAB fuel concentrations at 2 ($p = 0.233$), 4 ($p = 0.391$) or 8 months ($p = 0.377$).

Table 1: Mean shoot and root biomass, shoot to root ratio and photosynthetic efficiency (Fv/Fm) for *P. foliosa* exposed to initial nominal hydrocarbon concentrations up to 40 000 mg/kg, harvested at 0, 2, 4 and 8 months. Data are means ± SEM, n = 5. Means were not significantly different from the control at p = 0.05.

Time (months)	SAB concentration (mg/kg)	Shoot biomass	Root biomass	Shoot/Root ratio	Fv/Fm
0	**0**	2.17±0.17	1.56±0.22	1.54±0.28	0.76±0.01
2	**0**	7.70±0.83	1.83±0.43	5.06±1.21	0.67±0.09
	1000	5.64±0.91	1.00±0.17	5.85±0.75	0.67±0.02
	5000	6.00±0.62	2.40±0.77	3.16±0.61	0.68±0.02
	10 000	5.34±0.52	1.37±0.31	4.40±0.65	0.65±0.01
4	**0**	16.39±2.72	9.86±3.95	2.61±0.66	0.80±0.04
	1000	12.81±2.99	4.33±1.67	4.04±0.8	0.85±0.01
	5000	12.88±1.68	10.73±3.53	1.67±0.38	0.84±0.01
	10 000	13.25±0.93	7.26±2.28	2.26±0.36	0.85±0.02
	20 000	10.27±1.79	4.56±1.13	2.79±0.59	0.86±0.01
	40 000	13.08±0.79	8.56±1.37	1.67±0.23	0.85±0.01
8	**0**	31.78±3.84	119.86±34.29	3.53±0.86	0.85±0.01
	1000	35.79±4.44	173.54±37.76	4.75±0.60	0.86±0.01
	5000	39.00±5.00	113.54±25.12	2.28±0.55	0.88±0.01
	10000	40.75±2.42	137.54±15.28	3.41±0.36	0.85±0.01
	20 000	34.61±2.91	144.20±22.79	4.62±1.21	0.88±0.01
	40 000	29.57±3.96	135.43±30.39	4.49±0.88	0.85±0.01

Shoot to root ratio remained unchanged by the presence of hydrocarbons in each treatment (Table 1). No significant differences were detected between treatments at 2 (p = 0.187), 4 (p = 0.120) or 8 (p = 0.597) months.

In general the Fv/Fm of *P. foliosa* was high throughout the experiment and showed no significant difference between hydrocarbon treatments at the 2, 4 or 8 month sampling intervals (p=0.641, 0.911, 0.848 respectively) (Table 1).

3.2 Changes in soils in response to phytoremediation

Measured concentrations of TPH obtained in spiked soils are presented in Table 2 and were

generally 70-80% of the target nominal value. By 2 months, TPH concentrations in planted treatments were 41, 45 and 48% lower than their unplanted counterparts, at concentrations of 1 000, 5 000, and 10 000 mg SAB/kg soil respectively. This difference was significant for the 10 000 mg SAB/kg soil treatment ($p = 0.007$; Figure 2). After 4 months, the difference in concentration of TPH had declined to 35, 39 and 41% respectively and were no longer significant ($p = 0.07$; Figure 2). By 8 months, all treatments were approaching background levels (under 30 mg TPH/kg soil) with the exception the 10 000 mg SAB/kg soil unplanted treatment, which maintained an average of 109 mg TPH/kg soil per sample, and was significantly higher than the 10 000 mg SAB/kg soil planted treatment ($p = 0.03$; Figure 2).

Table 2: Initial spike concentration for SAB fuel in soil media. Measured as TPH (C_9 to C_{18}) mg/kg calculated on a dry mass basis for 3 replicate samples.

Target Concentration TPH (mg/kg)	**TPH C9-18 (mg/kg)**	
	Mean	**SD**
0	<50	-
1 000	831	88
5 000	3 581	78
10 000	6 872	296
20 000	13 856	1 076
40 000	32 002	2 620

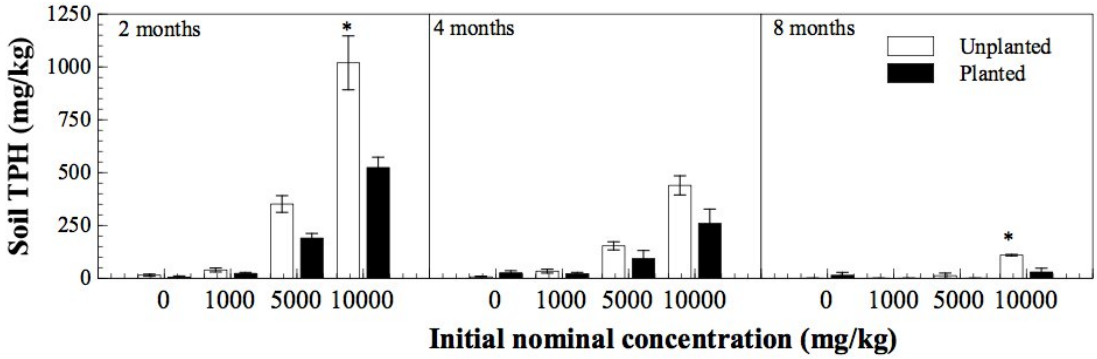

Figure 2: Soil TPH concentration (mg/kg) in planted (*P. foliosa*) and unplanted soil at time intervals 2, 4 and 8 months for the range of initial nominal SAB fuel concentrations. Data are mean ± SEM, n = 5. * indicates significant difference between planted and unplanted treatments at p = 0.05 across concentrations, within each sample interval.

Soil hydrocarbon biomarkers show that the soils have undergone substantial evaporation and biodegradation (Table 3) over the 2-month period. TPH losses observed are therefore due to a combination of both processes. Whilst the presence of plants resulted in reduced TPH concentrations, they appeared to have little effect on the proportion of mass loss via evaporation or biodegradation (Table 3).

Table 3: Biodegradation and evaporation biomarker ratios (mean ± SD) for soil samples of target concentration 10 000 mg SAB/kg for freshly spiked soil and for samples harvested at 2 months after spiking for planted and unplanted treatment.

Chemical fuel indices	Freshly spiked Soil (n=3)		Soil 2 months after spike			
			Unplanted (n=5)		Planted (n=5)	
TPH (C9 - C18)	6651	42	1048	298	509	100
Biodegradation						
n-C_{12}/*i*-C_{13}	5.09	0.05	2.03	0.90	1.91	0.21
n-C_e	3.17	0.01	0.68	0.62	0.22	0.11
n-C_{14}/*i*-C_{15}	3.20	0.04	0.45	0.52	0.04	0.09
n-C_{15}/*i*-C_{16}	1.02	0.00	0.12	0.18	0.00	0.00
n-C_{10}/$(R+UCM)_{9.5\text{-}10.5}$	0.17	0.00	0.02	0.03	0.00	0.00
n-C_{11}/$(R+UCM)_{10.5\text{-}11.5}$	0.22	0.00	0.03	0.03	0.01	0.00
Evaporation						
n-C_{12}/*i*-C_{14}	4.68	0.01	1.13	0.67	0.81	0.20
i-C_{15}/*i*-C_{16}	3.52	0.02	3.49	0.09	3.86	0.29
i-C_{14}/*i*-C_{16}	13.15	0.07	7.04	1.02	5.98	0.82
i-C_{13}/*i*-C_{16}	12.08	0.11	3.81	1.11	2.52	0.62
n-C_{11}/*i*-C_{16}	70.13	0.21	4.08	4.63	1.11	0.42
n-C_{10}/*i*-C_{16}	61.31	0.20	1.21	1.99	0.00	0.00
n-C_9/*i*-C_{16}	40.59	0.15	0.18	0.30	0.00	0.00

The number of microbes within the soil changed through time. Total heterotrophs (TH) were highest across all treatments at the 2-month time interval, after which they declined to pre-experiment levels by 4 months, with numbers negligible by 8 months (Figure 3a). The presence of *P. foliosa* significantly augmented the number of total heterotrophs in the soil for treatment concentrations of 0 and 1 000 mg SAB/kg soil at both 2 and 4 months ($p = 0.0001$; Figure 3a). At 5 000 mg SAB/kg soil total heterotroph numbers were similar across both planted and unplanted treatments, but at 10 000 mg SAB/kg soil the unplanted treatment contained twice the number of TH microbes. This trend was still apparent at 4 months even though population numbers had declined significantly compared to the 2-month time interval ($p = 0.0001$). There

were no significant differences between planted and unplanted treatments at the 8-month harvest interval ($p = 0.09$).

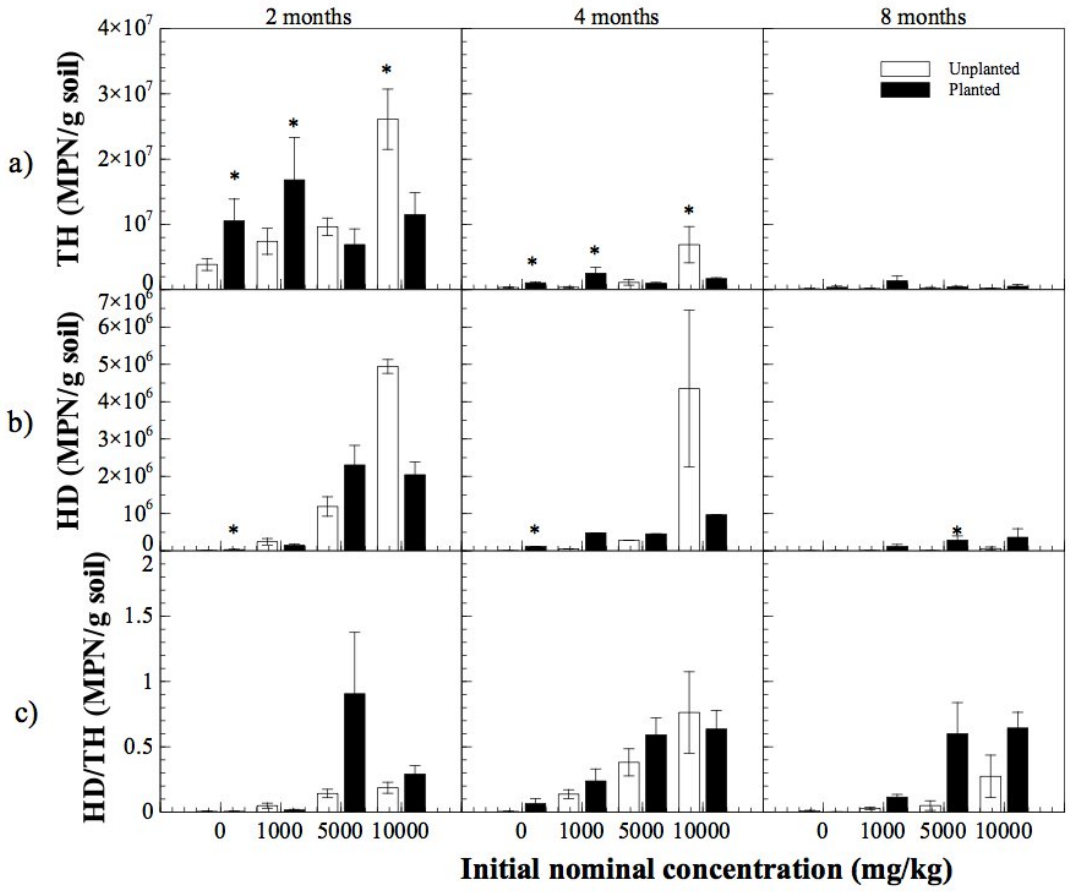

Figure 3: Number of (a) total heterotrophs (TH), (b) hydrocarbon degrading (HD) microbes and (c) the ratio of HD/TH, in soils with initial nominal concentrations up to 10 000 mg/kg SAB fuel, sampled at time intervals of 2, 4 and 8 months for treatment pots planted with *P. foliosa* or left unplanted. Data are means ± SEM, n = 5. * indicates significant differences between planted and unplanted treatments at $p = 0.05$ across concentrations, within each sample interval.

Hydrocarbon degrading microbes in the soil were singularly most abundant at the 2-month harvest, with levels declining by 4 months and reaching uniformly low levels by 8 months (Figure 3b). In planted soil, hydrocarbon degrader abundance increased with concentration

between 0 and 5 000 mg SAB/kg soil but plateaued above this concentration, resulting in populations that were less than half of the comparative unplanted treatment at 10 000 mg SAB/kg soil ($p = 0.001$). Although at very low levels, hydrocarbon degraders in unspiked control soils for both the 2 and 4 month harvests, were significantly higher in the planted treatment than in the unplanted treatment ($p = 0.0001$). They did not differ at any other treatment concentration. After 8 months, however, soil planted with *P. foliosa* showed significantly higher levels of hydrocarbon degrading microbes at the 5 000 mg SAB/kg soil contamination level ($p = 0.001$).

At 2 months there was a trend towards a higher proportion of hydrocarbon degrading microbes to total heterotrophs, with increasing fuel concentration for unplanted treatments, although this trend was not significant. In the planted treatments this trend occurred up to the 5 000 mg SAB/kg soil treatment, whilst in unplanted treatments it continued to 10 000 mg SAB/kg soil treatment (Figure 3c). Planted treatments at 2 months contained proportionally more hydrocarbon degrading microbes than did unplanted treatments at concentrations of 0, 5 000 and 10 000 mg SAB/kg soil, but not at 1 000 mg SAB/kg soil. At 4 months the trend was better 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 SAB/kg soil in which the two treatments were similar. After 8 months the presence of hydrocarbon degraders declined by over 50% in unplanted treatments compared to the 4-month time interval proportions, whilst numbers in the planted treatment remained high.

For unplanted treatments there was a significant positive association between the number of total soil heterotrophs and the TPH concentration in the soil ($R^2 = 0.678$; $p = 0.0001$; Figure 4a). This relationship was not found for planted treatments ($R^2 = 0.003$; $p = 0.723$). Approximately 65% of variation in concentrations of TPH in unplanted treatments can be explained by variations in total microbial populations. The number of hydrocarbon degrading microbes was significantly correlated with TPH concentrations for both unplanted ($R^2 = 0.704$; $p = 0.0001$) and planted ($R^2 = 0.226$; $p = 0.0004$) treatments, explaining 68 and 19% of the variation within the data respectively (Figure 4b). The ratio of hydrocarbon degrading microbes to total heterotrophs was not correlated with TPH concentrations in the soil for either unplanted ($R^2 = 0.058$; $p = 0.085$), or planted ($R^2 = 0.060$; $p = 0.07$) treatments (Figure 4c).

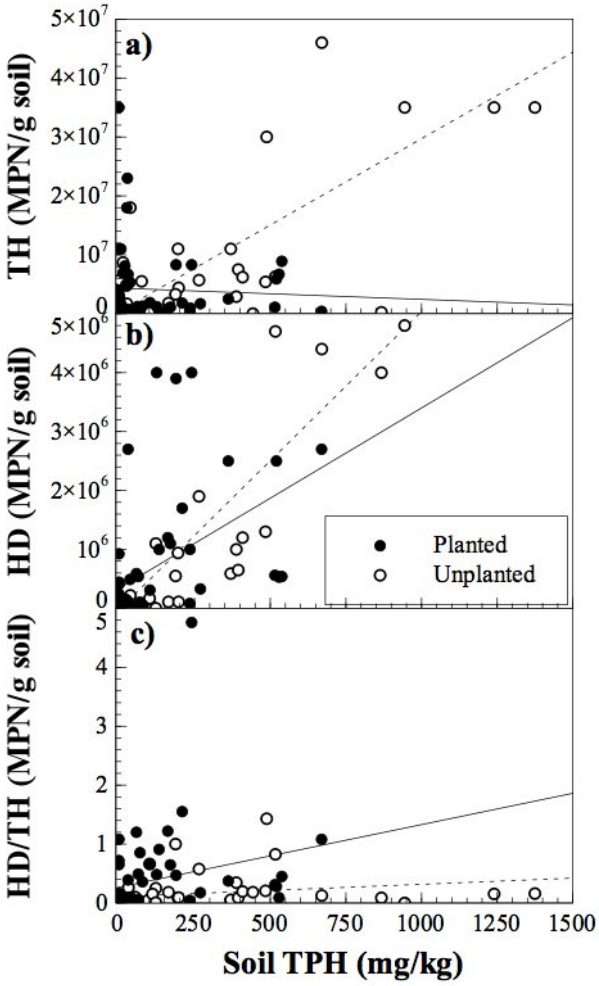

Figure 4: The relationship between microbes (MPN/g soil) and concentration of soil TPH (mg/kg) for (a) total heterotrophs (TH), (b) hydrocarbon degrading (HD) microbes and (c) the ratio of HD/TH, in planted (*P. foliosa*) and unplanted soil, for time intervals 2, 4 and 8 months (n = 5). Lines represent results of linear regression, dashed lines represent unplanted pots and solid lines represent planted pots.

Community level physiological profiling of microbial communities showed a significant shift in response to both the presence of fuel (control versus 5 000 mg/kg) and over time (2 versus 4 months; see Supplementary Information for details).

4. Discussion

This study confirms that *P. foliosa* is a strong candidate for phytoremediation of current fuel spills on Macquarie Island. Not only is it highly tolerant to SAB, but also its presence in highly contaminated soil results in significantly lower concentrations of hydrocarbons when compared to unplanted soil. *Poa foliosa* was also found to modify microbial community structure within the soil, resulting in augmented populations of total heterotrophs in the control and in the 1 000 mg SAB/kg soil treatment at both 2 and 4 months.

Plant tolerance to soil contamination is crucial for successful remediation (Barrutia et al., 2011), however the acute toxicity of petroleum hydrocarbons often results in suppressed plant growth (especially in roots) (DeLaune et al., 2003; Merkl et al., 2005b; Thompson et al., 2008). The present study suggests that *P. foliosa* belongs to the group of plants that are either unaffected or show a stimulated growth in the presence of hydrocarbon contamination (Merkl et al., 2005a; Liste and Prutz, 2006; Gaskin et al., 2008; Peng et al., 2009). For example, seed germination and biomass production of species of oat, mustard and pea was improved in the presence of petroleum hydrocarbons within soil (Liste and Prutz, 2006). Likewise, 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* A. Camus) produced considerably more root biomass in the presence of contamination (Gaskin et al., 2008).

Between 2 and 4 months the 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 also suggests that the species is suitable for phytoremediation as it displays the ability to

change carbon fluxes in response to altered environmental conditions. Furthermore, *P. foliosa* has a low mean relative growth rate (MRGR; 0.74 for shoots and 0.37 for roots) when compared to other species (Merkl et al., 2005b). A comparative analysis of subantarctic and alpine grasses found subantarctic grasses, including *P. foliosa*, have inherently low MRGRs (Medek et al., 2007). Plants with a low MRGR are known to be more widespread on infertile soils and this trait has been linked to stress resistance (Elias and Chadwick, 1979) making such plants more likely to be successful for reclamation of infertile disturbed lands than those species with higher MRGRs and higher demands for nutrients and water. Out of 39 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 impact (biomass change) with the addition of crude oil (Robson et al., 2003).

The presence of vegetation is known to enhance microbial populations within soil (see reviews by Hutchinson, 2003, Wenzel, 2008 and Khan et al., 2013) and accordingly to improve degradation of soil contaminants due to co-metabolic processes (Chaudhry et al., 2005; Gaskin et al., 2008). For example, higher counts of culturable microbes and actinomycetes were found in vegetated soil when compared to un-vegetated soil, coupled with a 15.6% decrease in final TPH concentrations (Liste and Felgentreu, 2006). Similarly, Altai wild rye (*Elymus angustus* Trin.) supported up to a hundred times more endophytic hexane degraders than the unplanted control, promoting rates of TPH degradation that were up to 50% higher than in unplanted treatments (Philips et al., 2009). In the current study, soils planted with *P. foliosa* had significantly reduced TPH levels, to 48% of the soil concentration of unplanted soil in the 10 000 mg SAB/kg soil treatment at the two month harvest. Significant decreases in TPH concentration across all treatments within such a short timeframe was surprising, particularly when compared to studies

on other related species (Hutchinson et al., 2001 and Pradhan et al., 1998). This may have been due, in part, to the evaporative effects of refrigeration on soil volatiles. Nevertheless, as phytoremediation in subantarctic environments would be implemented as a long-term management option we continued the experiment to the 8-month time interval to examine the return of soil TPH to background levels. Once again, the effects of phytoremediation after 8 months were evident, with planted soils displaying significantly lower concentrations of fuel contaminants when compared to unplanted soil at 10 000 mg TPH/kg soil.

The majority of studies report an increase in microbes within planted soil across a range of contamination levels, relative to unplanted soil (Siciliano et al., 2003; Chiapusio et al., 2007; Gaskin et al., 2008; Gaskin and Bentham 2010; Barrutia et al., 2011) and comparatively few studies report no difference (Radwan et al., 1998; Kudjo Dzantor et al., 2000; Chaiapusio et al., 2007; Cofield et al., 2007). However, an increase in culturable microbial populations in planted soils was only seen at low concentrations of TPH in this study. In addition, 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 for unplanted soil. Suggesting that in the unplanted soil, hydrocarbon loss was due, at least in part, to microbial activity. Maximum levels of TPH degradation in planted treatments occurred at 10 000 mg SAB/kg soil, where levels of total heterotrophs and hydrocarbon degraders were over 50% lower than in the unplanted soil, yet measured TPH concentrations were 41 to 48% higher. In unplanted soil, TPH concentration was strongly associated with the 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 only a weak association (19%). It

therefore appears that whilst *P. foliosa* was successful at remediating SAB contaminated soil, the plants' effectiveness is not solely linked to the presence of microbial populations and may be linked to other plant-associated mechanisms.

It is possible that *P. foliosa* uses 'phytodegradation' as well as '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 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, which is essential for oxidation of organic contaminants (Wensel, 2008; Chaudlhry et al., 2005). It is hypothesised that certain root-released compounds act as biosurfactants making contaminants more bioavailable and thus facilitating pollutant uptake from the soil, or transformation by living organisms (Olsen et al., 2003; Shahsavari et al., 2013). Furthermore, root turnover of soil may change the soil's chemical and physical properties to favour contaminant degradation and assist in contaminant volatilization (Banks et al., 2000). Plant-induced changes in the moisture content and water availability within soils could also significantly affect the loss of organics (Margesin and Schimmer, 1999).

Whilst the plants undoubtedly facilitated bioremediation, it is also possible that the effect of microbiological degradation may have been 'masked' in this experiment. At higher TPH concentrations microbes 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 2, 4 and 8 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 by-products of highly active microbial overturn can lead to a direct decrease in soil pH, causing microbial inhibition at later stages of hydrocarbon remediation (Merkl et al., 2006). This was demonstrated in a study with *Brachiaria brizantha* (Hochst. Ex A. Rich.), where petroleum hydrocarbons were degraded at a faster rate in planted versus unplanted soil resulting in a higher metabolic turnover (Merkl et al., 2005a). The resulting accumulation of organic acids in the soil subsequently decreased the pH causing microbial inhibition towards the end of the experiment in planted treatments (Merkl et al., 2006). Most microbes favour and develop best under relatively neutral pH conditions (Huang and Chen, 2003). Furthermore, the presence of exudates in planted treatments also provide hydrocarbon degrading bacteria with an alternative source of carbon, which may account for the low correlation of this type of bacteria to TPH in planted treatments in this study. In order to understand if microbial effects were 'masked' in this study, weekly measurements of soil pH and microbe concentration between 0 and 2 months would be required. Additionally, the culture-based methods used are unable to detect some bacteria and other, non culture-based methods would provide a greater insight into the microbial structure and changes of this particular contaminated environment (Zhang et al., 2012).

If microbes were found to be facilitating phytoremediation by *P. foliosa*, but were 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), or soil fertilizer (Thompson et al., 2008; Ayotamuno et al., 2009; Alarcon et al., 2008) may further enhance SAB fuel degradation. Despite their 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). The promotion of hydrocarbon degrading microbes is strongly modulated by abiotic factors (Powell et al., 2010), with the influence of plants on microbial communities dependent on available nutrients. Successful phytoremediation requires other site management practices, such as increased fertilization, to ensure it reaches its full potential (Siciliano et al., 2003). However, studies on subantarctic soils have shown that whilst the addition of fertilizer improves petroleum hydrocarbon degradation, adverse toxic residues that remain in low temperature soils after fertilization can present considerable drawbacks (Coulon et al., 2004; Delillie et al., 2002). There have been mixed results in relation to the benefits of additional nitrogen fertilizer to promote contaminant degradation. Nitrogen can promote root growth (creating a better environment for micro-organisms) and also decrease the bioavailability and bioaccessibility of the contaminant, thus reducing biodegradation potential (Thompson et al., 2008; Chaineau et al., 2005). However, other studies have suggested that this repression may be soil specific (Phillips et al., 2006; 2009). Whilst the organic content (13%) of the potting mix used in this study was within the range reported for Macquarie Island soils, soils at Macquarie Island can contain up to 30% organic content. Hence field application of this work would require consideration of appropriate fertilisation regimes to ensure on site phytoremediation success, especially as aged hydrocarbons could potentially be harder to remove from the soil than freshly spiked soil (Chigbo and Batty, 2013).

This study has shown that *P. foliosa* may be suitable to remediate the contaminated sites on Macquarie Island, which are contaminated with concentrations of up to 7 000 mg TPH/kg soil.

This species is well able to tolerate this level of contamination, and the experimental pot system tested in this study indicates the presence of *P. foliosa* can result in significantly less TPH. As in other studies, contaminant degradation was found to be higher in plants that showed slight signs of stress and growth depression (Philips et al., 2009; Liste and Felgentreu, 2006). It appears that, for at least some species, moderately stressful conditions augment effective phytoremediation due to stress stimulated chemical release, which increases microbial populations and promotes the level of hydrocarbon degradation (Kamath et al., 2004; Chaudhry et al., 2005). Phytoremediation can consequently be seen as a defense mechanism against the phytotoxic effects of soil contaminants and provides further evidence of its usefulness as an *in situ* method for the remediation of subantarctic soils.

Conclusion

This study describes the first investigation into the potential of *P. foliosa* to phytoremediate soils contaminated with SAB fuel under subantarctic conditions. These experiments demonstrate that *P. foliosa* tolerates high levels of SAB fuel, and that, within 2 months, its presence significantly reduces levels of SAB fuel in soil compared to unplanted soil and reduces the time for soil to return to background contaminant levels. The use of *P. foliosa* to remediate contaminated soil on Macquarie Island is an environmentally friendly, economical, sustainable and feasible option. Future studies exploring the potential of this species to facilitate hydrocarbon removal and enhance microbial biodiversity under current and projected future field conditions would be valuable especially if integrated into the suite of remediation strategies (*in-situ* air and nutrient additions) already in place on Macquarie Island.

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