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Environmental Impact Statement

To derive remediation targets and environmental quality guidelines, Species Sensitivity Distribution (SSD) models require toxicity data from a minimum of eight species from at least four taxonomic group. Prior to this study there was no toxicity data available for sensitive early life stages of native subantarctic plants exposed to total petroleum hydrocarbons (TPH) from diesel fuel. The TPH concentrations of contaminated soils required to inhibit germination and root and shoot growth in early life stages was high, but due to the climate of subantarctic regions, the hydrocarbon concentrations at spill sites may persist over time, so these high concentrations remain environmentally relevant. Therefore the data obtained here makes a significant contribution to the SSD model currently being developed to guide remediation activities at fuel contaminated sites at Macquarie Island and in subantarctic regions more generally.

Impact of hydrocarbons from a diesel fuel on the germination and early growth of subantarctic plants

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ABSTRACT

Special Antarctic Blend (SAB) is a diesel fuel dominated by aliphatic hydrocarbons that is commonly used in Antarctic and subantarctic regions. The past and present use of SAB fuel at Australia's scientific research stations has resulted in multiple spills, contaminating soils in these pristine areas. Despite this, no soil quality guidelines or remediation targets have been developed for the region, primarily due to the lack of established indigenous test species and subsequent biological effects data. In this study, twelve plant species native to subantarctic regions were collected from Macquarie Island and evaluated to determine their suitably for use in laboratory-based toxicity testing, using germination success and seedling growth (shoot and root length) as end points. Two soil types (low and high organic carbon (OC)) were investigated to reflect the variable OC content found in soils on Macquarie Island. These soils were spiked with SAB fuel and aged for 14 d to generate a concentration series of SAB-contaminated soils. Exposure doses were quantified as the concentration of total petroleum hydrocarbons (TPH, nC9-nC18) on a soil dry mass basis. Seven species successfully germinated on control soils under laboratory conditions, and four of these species (*Colobanthus muscoides* Hook.f., *Deschampsia chapmanii*, *Epilobium pendunculare* A.Cunn. and *Luzula crinita* Hook.f.) showed a dose-dependent inhibition of germination when exposed to SAB contaminated soils. Contaminated soils with low OC were generally more toxic to plants than high organic carbon soils. Increasing soil-TPH concentrations significantly inhibited shoot and root growth, and root length was identified as the most sensitive endpoint. Although the test species were tolerant to SAB contaminated soils in germination assays, development of early life stages (up to 28 d) were generally more sensitive indicator of exposure effects, and may be more useful endpoints for future testing.

Keywords: total petroleum hydrocarbons, total organic carbon, monocots, dicots, germination, root and shoot growth.

INTRODUCTION

One of the greatest challenges in protecting Antarctic and subantarctic regions is the effective management of the widespread and damaging effects of petroleum hydrocarbons arising from fuel spills [1-3]. Investigations of hydrocarbon contamination and degradation under polar conditions commenced more than 30 years ago, and have mostly been limited to the Arctic region [4, 5]. However, Snape and colleagues [6] estimated up to 10 million cubic meters of hydrocarbon contaminated soils in the Antarctic, with concentrations up to 59,000 mg/kg hydrocarbons reported in soils at Casey's Station [7]. In contrast to the relatively rapid recovery of oil spills in tropical climates [8, 9], cold climates have a number of characteristics that reduce the natural attenuation of petroleum hydrocarbons to negligible rates. Low temperatures, intrinsic nutrient limitations, and the anaerobic nature of soils caused by water saturation, combine to significantly inhibit the metabolic activity of hydrocarbon-degrading microbes; low temperatures will also reduce rates of hydrocarbon volatilisation and evaporation [1, 3, 10].

While numerous fuel spills have occurred in Antarctic and subantarctic regions there have been relatively few attempts to remediate contaminated sites [2]. Remediation to pristine levels in Antarctica and subantarctic regions is prohibitively difficult and costly. The negative environmental impacts and high costs associated with excavating and transporting contaminated soils for off-site processing and treatment have led the Australian Antarctic Division (AAD) to abandon dig-and-haul methods in favour of implementing alternative on-site *in situ* remediation techniques [2, 3]. The *in situ* techniques utilised on subantarctic Macquarie Island include injection of nutrients and aeration into the soils, both of which promote natural attenuation by encouraging hydrocarbon-degrading microbes [3, 10-14].

In order to set practical remediation targets for fuels, a comprehensive understanding of the availability of petroleum hydrocarbons in fuel-contaminated soils is required. Contaminant availability may be evaluated by surrogate chemical approaches, however, biological assays more clearly represent the potential risk contaminants pose to their local environment. Plant-based bioassays are well established, with standard protocols developed for temperate climates and plant species by the Organisation for Economic Co-operation and Development [15] and International Organization for Standardization [16, 17]. These protocols have subsequently been adapted for various Arctic and cold-climate species [18], however, there is still limited information available on the effects of hydrocarbons on cold-climate plants, and no established methods to assess hydrocarbon effects on native

subantarctic plants. Consequently, there is insufficient evidence to establish robust remediation guidelines, and site-specific testing is required for an accurate assessment of the biological effects of fuel-contaminated soils in Antarctica and subantarctic regions.

For comprehensive site remediation guidelines to be established, contemporary best practices recommend that toxicity data be obtained for a minimum of eight species across at least four taxonomic groups that reflect the range of those present in the natural community [19, 20]. This study is focused on subantarctic Macquarie Island, for which there is data for biological responses to total petroleum hydrocarbons (TPH) exposure for earthworms [21], soil microbes [13, 14] and the grass *Poa foliosa* [22]. This is insufficient information to enable guideline development to assess the risk of fuel-contaminated soils on native subantarctic species. This study evaluated seeds from twelve native plant species for germination success on control soils under laboratory conditions. Germinating species were then assessed in a 28-d bioassay using a concentration series of soils contaminated with Special Antarctic Blend (SAB) fuel. To account for the variable organic carbon (OC) content in subantarctic soils, parallel bioassays were performed using both low and high OC soils. Effects were measured using germination rates, and seedling growth (root and shoot length), reported against mean soil TPH concentration.

METHODS

General procedures. Glassware used for bioassays and hydrocarbon extraction and analyses were new or cleaned before use by washing with phosphate free detergent and rinsing with acetone, dichloromethane (DCM; Suprasolv, Merck) and Milli-Q deionised water (18 MΩ/cm; Merck Millipore).

Soil preparation and characterisation. Artificial soils were used to provide a standardised soil matrix. Two soil matrices were prepared: the low OC soil consisted of (w/w) 80% propagating sand (sieved to <1 mm) and 20% kaolin clay (<40 μm); and the high OC soil had (w/w) 70% propagating sand, 20% kaolin clay, and 10% *Sphagnum* moss (sieved to <2 mm).

Test soils were characterised for physical and chemical properties (Table 1). Soil pH was measured using a combined pH-mV-temperature meter (TPG Pty Ltd) calibrated as per manufacturer's instructions. Soil moisture was determined after heating to 110°C for 24 h, and dry bulk density and porosity determined after drying for 48 h at 60°C [23]. Particle size distribution was determined using a Mastersizer 2000 particle size analyser (Malvern

Instruments Ltd, Worcestershire, United Kingdom), calculated using Mie and Fraunhofer scattering. Total organic carbon (TOC) was determined using the loss on ignition (LOI) method [24], where soils (<2 g) were heated (105°C, 24 h), combined with HCl, heated (450°C, 24 h), and mass loss determined. Total Kjeldahl nitrogen (TKN) was determined using macro-Kjeldahl digestion (4500-N_{org} B.) and titrimetric (4500-NH_3 C.) methods adapted from Eaton et al. [25]. Total available phosphorous was determined following persulphate digestion (4500-P B.5) and ascorbic colorimetric detection (4500-P E.) as per Eaton et al. [25].

Soils were spiked with fuel at nominal concentrations of 20,000 and 40,000 mg SAB/kg soil (dry mass) in amber Winchester bottles. Bottles were immediately capped and homogenised (20±1°C, 24 h, dark) using a mechanical tumbler (Environmental Express®). Spiked and control soils were aged in uncapped bottles (15±1°C, 14 d, ventilated, dark) to allow the most volatile hydrocarbons to evaporate, reflecting a more ecologically relevant soil contamination composition. Aged soils were diluted with control soils to produce a concentration series between 1,250 and 40,000 mg SAB/kg soil, and toxicity testing was commenced within 48 h. Soil subsamples for TPH analysis were collected on Day 0 and Day 28 (test commencement and termination) and stored at -20°C (dark) until analysed.

Total petroleum hydrocarbon (TPH) analysis. Subsamples of soils (12 g) were extracted with 1:1 Milli-Q:hexane for TPH analysis (n-C9 to n-C18), measured by gas chromatography with flame ionisation detection (GC-FID, Agilent GC 6890N, SGE BP1 column (35 m x 0.22 mm ID, 0.25 µm film thickness)). Carrier gas (helium) velocity at the injector was 23.9 mL/min, and on column was 1.3 mL/min for 17 min increased to 3.0 mL/min for 7 min. Samples (3 µL) were injected with a split ratio of 15:1 (30 psi) at 310°C. The samples were cross calibrated with an in-house SAB fuel internal standard composed of 250, 50, 50 and 250 µg/mL of bromoeicosane, *p*-terphenyl, tetracosane and cyclooctane, respectively. Oven temperature was maintained at 50°C for several minutes and then ramped to 320°C at 18°C/min, with the FID heated to 330°C. Combined areas under resolved peaks and the unresolved complex mixture (UCM) were integrated relative to internal standards. For quality assurance/quality control (QA/QC) purposes, method blanks, duplicates and spike recoveries were performed on at least 10% of samples. All blanks were below the limit of detection (≤ 20 mg/kg). Spiked recoveries (equivalent to 5,000 mg SAB /kg for 10 g soil, extracted into 10 mL hexane) were between 95-113% (mean ± SD, 103 ± 6%) and the mean

Table 1. Physico-chemical properties of test soils in comparison to reported values for standardised organic carbon soils [18] (mean ± SE, n≥3)

Soil properties	Low organic carbon soil	High organic carbon soil	Standardised organic carbon soil [18]
Particle size distribution (%)			
Sand, 63 - 1000 µm	85.1±0.7	66±3	77.3
Silt, 4.0 - < 63 µm	7.5±0.3	3.3±0.4	7.8
Clay, < 4 µm	7.4±0.4	15±2	14.9
Added Organic matter (*Sphagnum* moss) 1- 2 mm	Not added	15.6±0.7	9
Moisture - storage (%)	6.0±0.1	18.6±0.5	~20
Moisture - testing (%)	~70-80	~70-80	~70-80
Wet bulk density (g/cm^3)	1.23±0.03	0.24±0.02	0.98
Water-holding capacity (%)	35±11	171±14	71.5
Porosity (%)	54±1	90.8±0.6	-
pH	6.5±0.5	6.5±0.5	6
Total organic carbon (%)	2.3±0.6	6.5±0.6	4.46
Phosphorous (mg/kg)	9.1±0.9	30±5	23
Kjeldahl Nitrogen (mg/kg)	54.2	345	500

relative standard deviations (RSDs) of site and method duplicates were typically within 20-30%. The certified reference material analysed (CRM-560, Diesel Soil 4, 448-522 mg/kg nC12-nC28) were within the predicted intervals for the nC9-nC18 component. Analysis of control samples detected no TPH compounds within the nC9-nC18 and associated UCM hydrocarbon range, therefore no interference from the components of the soil matrix was observed in the spiked samples.

Test species, bioassay conditions, and seed viability bioassay. Seeds from twelve terrestrial plant species (detailed in Table 2) were collected from Macquarie Island in January-February 2013. Most of the twelve species occur throughout the subantarctic region, including New Zealand's subantarctic islands, with two of the species also occurring on Australia's Heard and McDonald Islands. A description and history of the seed collection site are provided in the supplementary information. Seeds from each species were stored separately in paper bags under ambient laboratory conditions until returned to Australia where they were kept at 2±2°C in sealed plastic bags containing silica beads (Silica Gel Australia) to reduce moisture

and prevent fungal infestation. Mature, healthy seeds were selected for testing based on seed size and colour. To assess seed viability and suitability for laboratory-based bioassays, 20 seeds of each of the twelve species were planted on unspiked (control) high organic soils (n=3) and were observed daily using microscopy (Dino-Lite digital microscope and accompanying DinoXcope software) for up to 28 d. Germination was defined as the rupture of the testa (seed coat) by the epicotyl.

All seed germination bioassays were conducted in Normax glass petri dishes (diameter 90 mm). Each dish contained one soil type at a single test concentration and was divided into quadrants with twenty seeds of a single species evenly planted in each quadrant. Petri dishes spiked with the same SAB concentration were sealed in plastic containers (Sistema©, 5 L, 21.0 x 24.2 x 10.5 cm). A systemic fungicide (Garden King Fongarid®) was used as a preventative and treatment for fungal infections. Seeds were pre-soaked in fungicide for 20–30 min and if fungus appeared during testing, the test units were sprayed with fungicide. Seeds were incubated in plastic containers at 15±1ºC which is the maximum average daily temperature in surface soils at Macquarie Island on sunny summer days, reflecting field-realistic and optimal temperatures for minimising germination time and maximising germination success. Maximum light intensity inside the plastic containers was 55 µmol/m^2/s. Observations of seeds throughout bioassays were performed using a Dino-Lite digital microscope and accompanying DinoXcope software.

Exposure bioassays. To determine a suitable dose–response concentration range of SAB for each species, a 28-d rangefinder bioassay was performed. The seven species that germinated on at least one of the soil types in the viability bioassay underwent further testing on SAB contaminated soils. Twenty seeds from each species were tested on a soil concentration series (1,250 to 40,000 mg SAB/kg) on both the low and high OC soils.

The four species that successfully germinated during the rangefinder assay were subsequently tested in a definitive toxicity test. In definitive bioassays, twenty seeds were used per test, and tests were performed in triplicate over 28 d on the low and high OC soils. Germination was recorded daily, and at test completion, early plant growth (root and shoot lengths) was measured using the program ImageJ (public domain Java image processing).

Table 2. Native subantarctic plant species investigated to determine the sensitivity of seeds to SAB contaminated soils. Description is adapted from George et al. [27].

Species	Family	Description	Distribution
Monocots			
Agrostis magellanica Lam.	Poaceae	Tufted grass	Circumpolar. Wide range of subantarctic islands, New Zealand and South America
Deschampsia chapmanii Petrie	Poaceae	Tufted grass	Macquarie Is., New Zealand's subantarctic islands, plus New Zealand
Festuca contracta Kirk	Poaceae	Tufted grass	Wide range of subantarctic islands and South America
Luzula crinita Hook.f.	Juncaceae	Tufted grass-like herb	Macquarie Is. and New Zealand's subantarctic islands
Dicots			
Acaena minor (Hook.f.) Allan	Rosaceae	Prostrate herb	Macquarie Is. and New Zealand's subantarctic islands
Cardamine corymbosa Hook.f.	Brassicaceae	Low herb	Macquarie Is. and New Zealand's subantarctic islands
Colobanthus muscoides Hook.f.*	Caryophyllaceae	Cushion-forming herb	Macquarie Is., plus New Zealand's subantarctic and Chatham islands
Colobanthus sp. (*C. affinis* (Hook.) Hook.f. or *C. apetalus* var. *alpinus* (Kirk) L.B.Moore	Caryophyllaceae	Cushion-forming herb	Macquarie Is. and New Zealand. *C. affinis*: also Australia *C. apetalus* var. *alpinus*: also New Zealand's subantarctic islands
Epilobium brunnescens (Cockayne) P.H.Raven & Engelhorn subsp. *brunnescens*	Onagraceae	Mat-forming herb	Macquarie Is., New Zealand's subantarctic islands and New Zealand
Epilobium pendunculare A.Cunn.	Onagraceae	Mat-forming herb	Macquarie Is., New Zealand subantarctic islands and New Zealand
Montia fontana L. subsp. *fontana* *	Portulacaceae	Mat-forming, herb	Almost cosmopolitan in cool regions. Macquarie Is., Heard Is., New Zealand's subantarctic islands, New Zealand, and Australia (Tasmania)
Pleurophyllum hookeri Buchan.	Asteraceae	Large rosette-forming herb	Macquarie Is. and New Zealand's subantarctic islands

* These species were observed growing in areas of known fuel spills at time of seed collection

Data analysis. All data represents the actual soil-TPH concentrations that were not normalised to soil-TOC. Two-tailed t-tests were used to identify differences in soil TPH over time, at 0.05 significance levels. Dose-response curves were constructed and point estimates calculated by linear interpolation using ToxCalc for Microsoft Excel (Version 5.0.23, TidePool Scientific Software, California, 1994). Data was tested for normality using the Shapiro-Wilk test, and for homogeneity of variance using Bartlett's Test. Point estimates including the concentrations at which 50% of the population response was inhibited (IC_{50}) were determined using Dunnett's Multiple Comparison Test for parametric data or Wilcoxon Rank Sum test with Bonferroni correction for non-parametric data. In cases where the IC_{50} was calculated to be greater than the highest treatment concentration, confidence intervals were not calculated. No observed effective concentrations (NOEC) and lowest observable effective concentrations (LOEC) values were determined using Steel's Many-One Rank test. Equality of variance and normality were tested at 0.01 and hypothesis tests and control comparisons were tested at 0.05 significance levels.

RESULTS

Soil characteristics. The low OC soil was marginally sandier than the high OC soil (62.5 µm - 1 mm at 85.1±0.7 and 66±2%, respectively), and the high OC soil contained added organic matter (15.6±0.7% in 1-2 mm particle range). Therefore the high OC soil had almost three times more TOC than the low OC soil (6.5±0.6 and 2.3±0.6%, respectively), which increased the soil's water-holding capacity by almost five times (171±14 and 35±11%, respectively). The high OC content also produced a higher porosity, a lower wet-bulk density, and total phosphorus and total Kjeldahl nitrogen concentrations that were over three and four times higher, respectively, than in the low OC soil. Therefore the *Sphagnum* moss contributed substantial nutrients to the high OC soil (Table 1).

Total petroleum hydrocarbons. Both the low and high OC control soils contained <20 mg TPH/kg ($n \geq 8$), and thus had a minimal contribution of hydrocarbons to the total TPH measured in test soils. For all test soils, there was a significant decrease ($p < 0.05$) in TPH concentrations over the 28-d test period (Table 3). Greater losses occurred in the high OC soils, with average 28-d losses of 70 and 38% for TPH from the high and low OC soils, respectively. Soils dosed with lower fuel concentrations also lost a larger percentage of their initial TPH over time than did soils at higher concentrations. For example, initial TPH

concentrations decreased in high OC soils by 91% for treatments starting with ≤ 2,500 ± 600 mg TPH/kg and by 48% for soils ≥ 3,000 ± 750 mg TPH/kg. Time-averaged mean exposure concentrations for each treatment were used in all statistical analyses and data interpretations.

Germination success on control soils (seed viability). Seven of the twelve species examined were able to germinate on control soils and were identified as potentially suitable for laboratory-based bioassays. These were *C. corymbosa, C. muscoides, Colobanthus* sp., *D. chapmanii*, *E. pendunculare, L. crinita,* and *M. fontana* (Figure 1). Germination on the high OC soil was generally more successful than on low OC soil, particularly for *C. muscoides* in which 80% of the seeds germinated within 21 d on high OC soil, compared to 5% of seeds on low OC soil. In the low OC soil, only four species germinated (Figure 1); *L crinita* and *E. pendunculare* were most successful, with 55% and 40% of the seeds germinating, respectively. Other species were less successful with between 10-30% of the seeds germinating on either soil.

Table 3. Nominal-SAB exposure doses and measured total petroleum hydrocarbon (TPH) concentrations in aged soils at the start and termination of the subantarctic plant germination bioassays (Days 0 and 28), and as the mean time-averaged exposure concentration (mean ± SD, n=3). Percentage TPH losses from soils over exposure period are also presented.

Nominal-SAB fuel spike (mg/kg)	TPH in soil (mg/kg) § Day 0	Day 28	Time averaged exposure	Total Loss (D_0-D_{28})	% Loss of TPH over 28 d
High organic carbon soil					
1,250	190 ± 50	0 ± 25	100 ± 20	190 ± 50	100 ± 25
2,500	1,100 ± 260	230 ± 60	640 ± 160	800 ± 200	78 ± 20
5,000	2,500 ± 600	100 ± 20	1,300 ± 300	2,400 ± 600	96 ± 24
10,000	3,000 ± 750	1,300 ± 300	2,200 ± 540	1,700 ± 400	56 ± 14
20,000	7,100 ± 1,800	3,800 ± 900	5,400 ± 1,400	3,300 ± 800	47 ± 12
40,000	13,000 ± 3,000	7,600 ± 1,900	10,000 ± 2,600	5,600 ± 1,400	42 ± 11
Low organic carbon soil					
1,250	400 ± 100	80 ± 20	240 ± 60	320 ± 80	80 ± 20
2,500	1,200 ± 300	1,200 ± 300	1,200 ± 300	*	*
5,000	2,600 ± 650	2,200 ± 550	2,400 ± 600	400 ± 100	16 ± 4
10,000	8,500 ± 2,100	6,000 ± 1,500	7,300 ± 1,800	2,500 ± 600	30 ± 8
20,000	16,000 ± 4,000	11,000 ± 2,700	13,000 ± 3,300	5,300 ± 1,300	33 ± 8
40,000	35,000 ± 9,000	25,000 ± 6,000	30,000 ± 7,400	10,500 ± 2,600	30 ± 8

* Measurement error occurred
§ High and low organic carbon control soils had <20 mg TPH/kg

Germination response to contaminated soils. Rangefinder bioassays showed that SAB-contaminated soils reduced germination success, and the degree of inhibition varied across species and soil types. Four species (*C. muscoides*, *D. chapmanii*, *E. pendunculare,* and *L. crinita*) showed dose-dependent responses to fuel-contaminated soils at concentrations up to 10,000 and 30,000 mg SAB/kg for high and low OC soils, respectively, and as such these concentrations and species were used in the definitive bioassay. Again there was greater germination success on the high OC fuel contaminated soils, which is consistent with the trend observed in seed viability assays. However, a clearer and less variable dose-dependent response was observed on the low OC soils (Supplementary Information Figure S3). Three species showed no or insignificant germination in SAB contaminated soils across the entire concentration series investigated; *C. corymbosa*, *Colobanthus* sp. and *M. fontana*.

Definitive toxicity tests. Definitive bioassays were performed in triplicate for 28 d in a concentration series of soils ranging from 0-10,000 and 0-30,000 mg SAB/kg in high and low OC soils, respectively. In these bioassays the suitability of germination and early-life growth (root and shoot length) as toxicity endpoints were evaluated. The contaminated soils had a pronounced inhibitory effect on the germination and subsequent growth of early-life stages, with the degree of inhibition varying with species, endpoints and soil types (Figure 2, Table 4). Germination success was the least variable endpoint, with most species having the same or similar IC_{50} values (~5,000 and >10,400 mg TPH/kg for low and high OC soils, respectively). The exception to this was *E. pendunculare*, which was particularly tolerant, with an IC_{50} of 28,000 mg TPH/kg on low OC soil. Germination was generally less sensitive to SAB fuel exposure on the high OC soils, again with the exception of *E. pendunculare*.

For the early life stages, the response to contaminated soils in terms of root growth was similar between species. Root growth was inhibited by 50% in all species at concentrations >2,300 mg TPH/kg, except for *L. crinita* on low OC soils and *E. pendunculare* on high OC soils (both with estimated IC_{50} of 190 mg TPH/kg, which was below the lowest tested exposure concentration of 240 mg TPH/kg). The effect of soil OC content on the IC_{50} root growth in contaminated soils was not consistent across the species, with the high OC soils resulting in less tolerance to fuel in *E. pendunculare* (190 mg TPH/kg), but more tolerance in *C. muscoides* and *L. crinita* (5,400 and 4,100 mg TPH/kg, respectively). Soil type did not affect the toxicity of SAB contaminated soils on root growth of *D. chapmanii* (IC_{50} of ~2,300 mg TPH/kg).

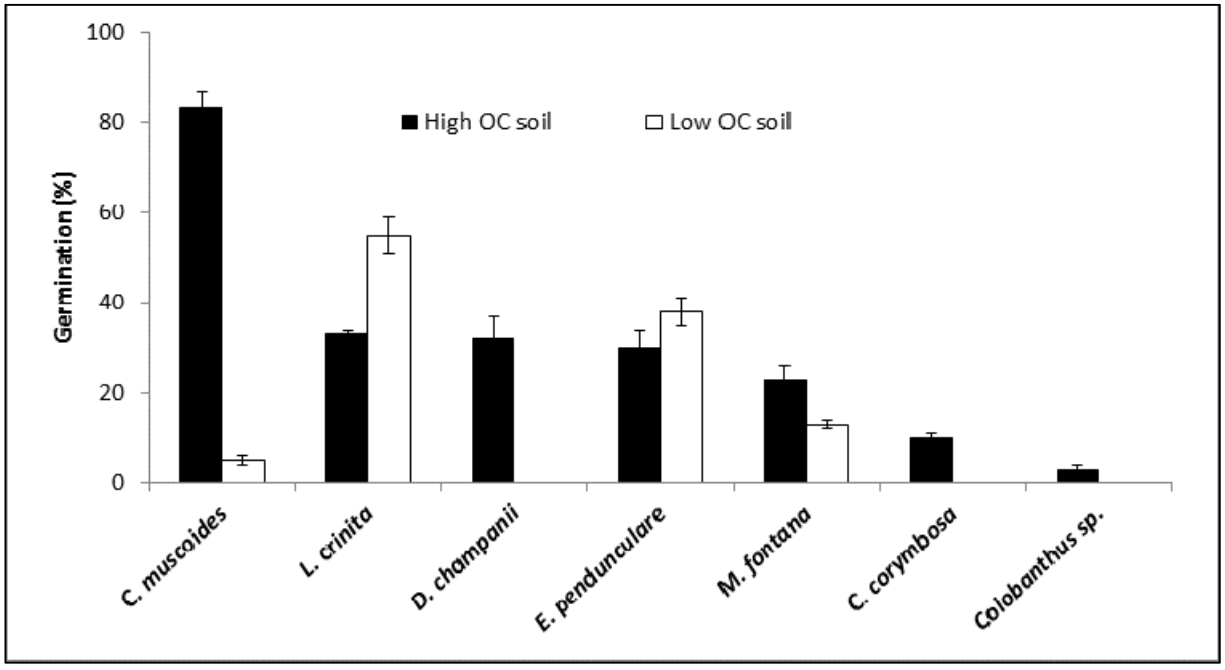

Figure 1. Germination success of native subantarctic plants seeds in low and high organic carbon control soils after a minimum of 21 d (mean ± SD, n=3; 20 seeds per replicate). Data not presented for non-germinating plant species (*A. magellanica, A. minor, E. brunnescens, F. contracta* and *P. hookeri*).

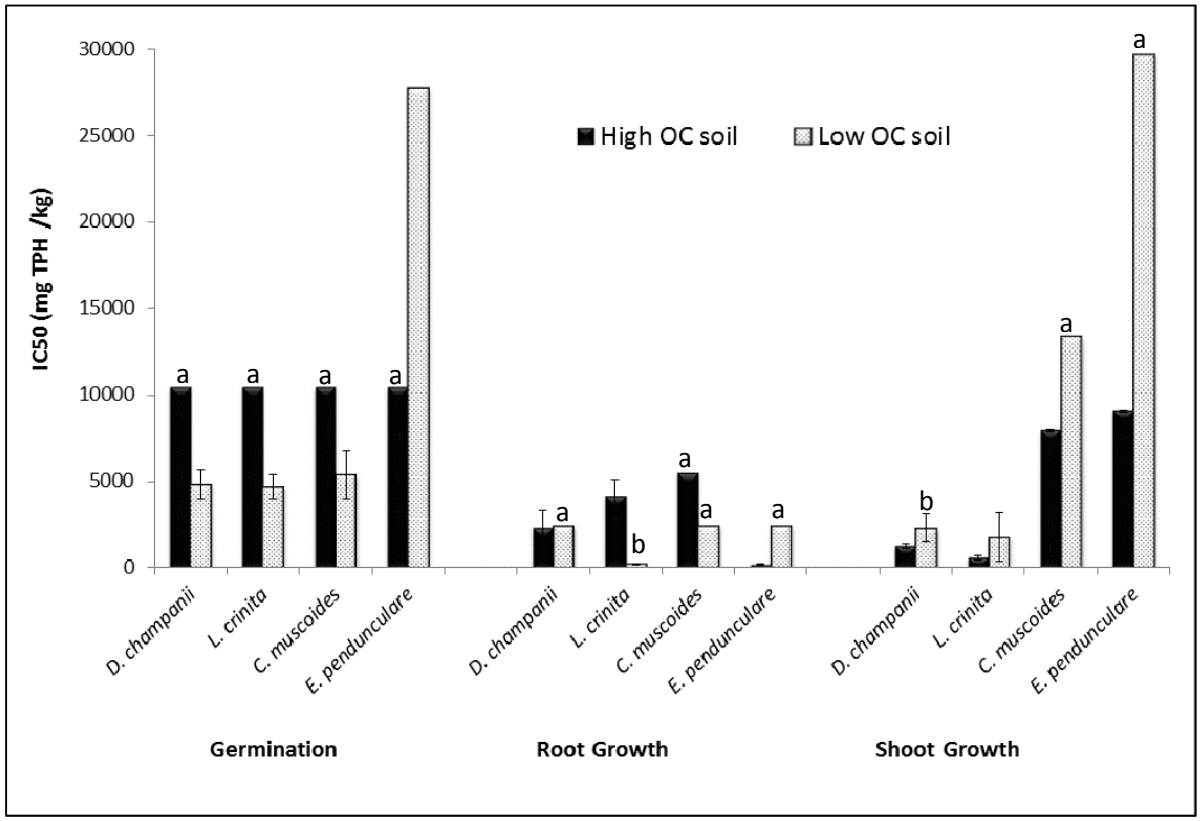

Figure 2. TPH-IC_{50} values generated in toxicity tests with subantarctic plants. Tests were performed using two soil types (high and low organic carbon soils) for germination success and subsequent early-life stage growth (root and shoot length). Mean ± standard deviation, n $\geq$ 3. Where [a] indicates that the IC_{50} estimate is greater than highest test concentration, and [b] that the IC_{50} estimate is less than the lowest test concentration.

Shoot length was the most variable endpoint with the four test species dividing into two groups: *D. chapmanii* and *L. crinita* were significantly more sensitive to SAB contaminated soils than *C. muscoides* and *E. pendunculare*. The level of total OC in soils did not affect IC_{50} values for *D. chapmanii*, *L. crinita*, and *C. muscoides* with both low and high OC soil matrices having similar responses (570-1,200 and 1,300-2,300 mg TPH/kg, respectively, Figure 2, Table 4). Interestingly for shoot length the high OC content in soils enhanced the effect of SAB contaminated soils, with all species having lower IC_{50} values in the high OC assays. This interaction was most pronounced with *E. pendunculare*, as it was more sensitive to all three endpoints on the high OC soils (germination success, root length and shoot length, Figure 2).

Discussion

Soil characteristics. Organic carbon content in the soil had a significant impact on the toxicity of the SAB contaminated soil to the plant species tested, with clear differences between the low and high OC soil types across all three endpoints (Figure 2). Arguably two of the most important factors in determining the toxicity of petroleum hydrocarbons in soil are the level of organic matter in the soil and the duration or age of contamination. The ameliorating effect of OC on hydrocarbon bioavailability is well established, with hydrocarbon content generally normalised to the OC concentration in sediment toxicity assessments [28]. Lowered hydrocarbon bioavailability is attributed to increased hydrocarbon sequestration onto organic matter and therefore, decreased bioavailability, which is consistent with equilibrium partitioning for neutral organics based on sorption coefficients [29-32].

A significant effect of higher OC content was the greater losses of TPH from soils that occurred during the bioassay, which is consistent with previous studies [12, 22, 33]. Hydrocarbons are volatile compounds that chemically transform and degrade at different rates depending on the physicochemical characteristics of the soil and environmental factors such as light intensity, temperature, oxygen availability and microbial activity. The oil spill itself can increase soil temperatures by up to 10°C due to decreased soil surface albedo [49], which in turn increases rates of hydrocarbon volatilisation. In addition, petroleum hydrocarbons undergo complex physical and chemical interactions with soils, strongly sorbing to OC, making it less available for biological uptake [4]. Organic matter provides more nutrients in soils (Table 1) that will facilitate not only seedling growth, but also higher microbial activity, and this microbial activity will contribute to the higher TPH losses in high

Table 4. 28-d IC_{50} values for germination success, root and shoot length of seedlings of subantarctic plants exposed to Special Antarctic Blend fuel. Fuel concentration measured as total petroleum hydrocarbon (TPH, mg/kg). *Where standard deviation was not calculated by ToxCalc. ^Concentrations lower than lowest test concentration.

Endpoint	Species	Organic carbon soil type	IC_{50} (mg TPH/kg)	TOC normalised IC_{50} (mg TPH/kg)
Germination (%)	*Deschampsia chapmanii*	High	>10,400	>1,600
		Low	4,800±800	2,090±350
	Luzula crinita	High	>10,400	>1,600
		Low	4,700±700	2,040±300
	Colobanthus muscoides	High	>10,400	>1,600
		Low	5,400±1,400	2,350±610
	Epilobium pendunculare	High	>10,400	>1,600
		Low*	28,000	12,200
Root length (mm)	*Deschampsia chapmanii*	High	2,300±960	350±150
		Low	>2,400	>1,050
	Luzula crinita	High	4,100±970	630±160
		Low^	190±10	80±4
	Colobanthus muscoides	High	>5,400	>830
		Low	>2,400	>1,040
	Epilobium pendunculare	High	190±40	80±20
		Low	>2,400	>1,040
Shoot length (mm)	*Deschampsia chapmanii*	High	1,230±130	190±20
		Low^	2,300±780	1,000±340
	Luzula crinita	High	570±140	88±20
		Low	1,800±1,400	780±600
	Colobanthus muscoides	High	7900±50	1,600±10
		Low	>13,300	>5,800
	Epilobium pendunculare	High*	9,060±60	1,390±10
		Low	>30,000	>13,000

organic carbon soils. A dose-dependent increase in microbial respiration (IC_{20} of 350 mg fuel/kg) has been reported in fuel contaminated soils up to 50,000 mg SAB/kg in the presence of sufficient nitrogen [14], which illustrates that the additional nutrients from the organic matter (particularly TKN) in the high OC soil may be important in facilitating the microbial activity and degradation of the fuel.

During hydrocarbon exposures, acute toxicity is likely caused by lighter, more volatile hydrocarbons with a lower equivalent carbon number; heavy fractions with a high equivalent carbon number are more likely to cause chronic toxic effects [34-37]. Toxicity of petroleum hydrocarbons is strongly correlated with hydrocarbon fractions that have lower boiling points and octanol-water coefficients [$\log(K_{ow})$], especially those within the nC10–nC19 range [12, 37-38]. Differences in toxicity between hydrocarbon fractions has been addressed by Environment Canada, and with some exceptions, hydrocarbons have been split into four specific fractions (F1-F4) as the basis in their tiered risk assessment framework (F1 nC6–nC10; F2 nC11–nC16; F3 nC17–nC34; F4; ≥nC35) [39]. In terms of their toxicity, the fractions may be ranked in the following order F1>F2>F3>F4 which is consistent with the hypothesis that toxicity decreases with increasing equivalent carbon number. Due to the rapid volatilisation of nC6–nC10 hydrocarbons (F1) [35], hydrocarbons with an equivalent carbon number between nC11–nC16 (F2) represent the fraction with the greatest toxicological effect [12]. This is significant in the context of SAB fuel as it is primarily composed of nC9–nC14 hydrocarbons, a range that encompasses some of the most toxic components [2].

The effect of SAB on seed germination and seedling growth in subantarctic plants. Plant responses to contaminated soils are influenced by both the physiological characteristics of the plant species and the physical and chemical parameters of the soil [4]. Thus assessing the toxicity of fuel-contaminated soils to plants requires multiple plant species, different biological endpoints and soils that reflect site conditions and the range of soil properties at that site.

In this study, each test species differed in its response to SAB-contaminated soils across three endpoints (germination, root and shoot growth; Figure 2). Initial tests with freshly harvested seeds revealed that not all species were able to germinate under controlled laboratory conditions. This may be due to unsuitable conditions for plant germination in the laboratory setting, or alternatively to seed maturity or dormancy mechanisms. Dormancy is an innate "whole-seed" characteristic that defines the environmental conditions that must be satisfied to initiate germination. Dormancy will prevent germination until specific

environmental conditions trigger testa rupture, and these conditions are typically species-specific [40-41]. In seed viability bioassays, seeds of seven of the twelve plant species were able to germinate on high organic control soils, whereas only four species germinated on the low OC soil. The effect of OC content on the species germination success suggests that seed dormancy was not the only mechanism influencing germination.

When soils were spiked with SAB, germination was completely inhibited in three species, all of which were dicot herbs (*M. fontana*, *C. corymbosa* and *Colobanthus* sp.). This suggests that these species are particularly vulnerable to SAB-contaminated soils, and risk being lost from the impacted region, potentially decreasing species diversity at contaminated sites. However, *M. fontana* (a perennial, mat-forming, herb) was found growing at the fuel-spill sites during seed collection (Table 2). This suggests that either *M. fontana* seed production is more tolerant to soil-SAB concentrations than germination, that the SAB-concentration in soils at the seed collection site was lower than bioassay concentrations, or/and that other factors are influencing the field-based germination.

The fact that the three species with 100% germination inhibition were dicot herbs is likely to be coincidental, as two of the species that did germinate were monocot and dicot herbs. Robson et al. [42] also noted high variability in test species tolerance to hydrocarbon-contaminated soil, with drastic dose-dependent reductions in biomass and relative growth rate (RGR) in some species, while others were not impacted. This variation was related to the RGR, with plants with low RGR showing the least impact from fuel contamination [42], which is consistent with our findings, where higher tolerance to fuel contaminated soil occurred in slower growing species. Interestingly species with low RGR are also more successful on nutrient-deficient soils [43].

The seed coat also plays a major role in species-specific variability in germination success. The coat protects the seed prior to germination by preventing mechanical or chemical damage to the embryo. To assess the protective nature of the seed coat from fuel contamination, Amakiri and Onofeghara [44] soaked seeds of *Capsicum frutescens*in crude oil from between 1 h to > 32 weeks prior to planting. Whilst *C. frutescens* maintained 100% germination regardless of the period of pre-exposure of seeds to oil, the time to achieve germination increased significantly in seeds pre-exposed for longer periods, delaying seed emergence [44]. This implies that germination was physically inhibited by the oil coating on the seeds, preventing imbibition and likely increasing microbial respiration around the seeds, thereby depleting available oxygen. This physical inhibition can be overcome in time as the hydrocarbons degrade, enabling water and oxygen to penetrate the seed. Thus, if inhibition of

germination is overcome as a result of a non-renewed coating of oil, it is possible that the three herbs that germinated on control soils but not on SAB-contaminated soils in the current study, may have germinated had the test duration been increased beyond 28 d. The four species that did successfully germinate on both the low and high OC soils (*C. muscoides*, *D. chapmanii*, *E. pendunculare*, and *L. crinita*) had comparable sensitivities to SAB contaminated soils, with similar 28-d $IC5_{50}$ values based on germination inhibition (except *E. pendunculare* on sandy soil, Figure 2).

Germination was more tolerant than root growth on SAB-contaminated soils for all of the native subantarctic species, and was generally more tolerant when the soils contained higher OC. Once germination had occurred, the level of soil OC had no significant effect on SAB toxicity for root growth ($p > 0.05$), however, higher OC enhanced the SAB toxicity on shoots, with a greater inhibition of the shoot growth. Thus, the added organic matter reduced the toxicity of SAB contaminated soils in germination tests, but increased toxicity in terms of above ground shoot growth. This may be related to nutrient cycling within contaminated soils, as microbial-driven nitrification was inhibited within SAB-contaminated fuels in subantarctic regions (IC_{20} at 660 mg SAB/kg soil over 21 days [14]), most likely due to the enzymatic inhibition of ammonia monooxygenase (AMO), however, denitrification process were very tolerant.

The greater sensitivity of root growth than germination to SAB contaminated soils (Figure 2) is most likely because the roots are in direct contact with the soil. Roots are highly permeable tissues of the early seedlings that are no longer protected by the hard, impermeable seed coat. Because early roots have yet to develop protective layers, such as a thickened epidermis and cuticle, the lipid bilayer may be more vulnerable to the presence of hydrocarbons. The lipid bilayer structure of the plasma membrane is, in essence, a colloidal micelle and as such, will follow the same principles of other colloidal micelles [45]. This includes the solubilisation when a foreign molecule is incorporated into the colloid. As hydrocarbons are readily solubilised, they are able to move into the plasma membrane and displace fatty acids, thereby reducing the integrity of the membrane. This in turn causes the cell to become increasingly permeable, eventually leading to collapse of the cell [45]. In addition, the high tolerance of the shoots with the dicots *C. muscoides* and *E. pendunculare* to SAB contaminated soils may be related to the epigeal germination. Monocots undergo hypogeal germination in which the cotyledons remain below ground where they are more exposed to the surrounding soil contamination (such as *D. chapmanii* and *L. crinita*), whereas

dicots perform epigeal germination pushing the cotyledons above ground upon emergence from the seed, away from the contaminated soils.

In this study, three biological endpoints were used to evaluate the effect of SAB contaminated soils on germination, and shoot and root growth on subantarctic plant species. Given the mode of action of hydrocarbon toxicity discussed above and in past research [12, 38, 46-48], root growth was expected to be the most sensitive endpoint as roots are in direct contact with contaminated soil. In contrast, inhibition of shoot growth is thought to arise, not only from direct contact with hydrocarbons, but also as a result of systemic shock of translocation of hydrocarbons to stems [46]. Unexpectedly shoot growth was less adversely affected on the low than on the high OC soil. This could be due to the plant favouring shoot growth over root growth given the less hospitable nature of the low organic carbon soil for root growth (which has higher wet bulk density and lower water holding capacity and porosity, Table 1).

Using the IC_{50} data, the sensitivity of each endpoint was ranked using "1" as most sensitive to "3" as least sensitive (Table 5). The ranking is presented for both the high and low OC soils following 28-d exposure to concentration series of SAB-contaminated soils. Overall the least sensitive endpoint was germination, and the most sensitive endpoint was root growth, but this varied between root and shoot growth depending on the species and the soil type tested (Figure 2, Table 5). Other studies have also shown that germination success is a less sensitive endpoint than root and shoot growth, resulting in an underestimation of the toxicity of fuel-contaminated soils [36, 40]. Interestingly for *D. chapmanii* and *E. pendunculare*, the order of sensitivity of endpoints was conserved between soil types, but this order differed between soil types for *L. crinita* and *C. muscoides* (Table 4). These species sensitivities to SAB-contaminated soils are far greater than that of the tolerant subantarctic grass *P. foliosa* following an 8 month exposure (no impact on biomass production, plant morphology and photosynthetic efficiency), which demonstrated ideal characteristics of a plant with phytoremediation properties [22] rather than a toxicity test species.

This study contributes to the growing evidence of species-specific responses of plants to petroleum hydrocarbon contamination, as well as differences in sensitivity of test end points. This study therefore highlights the need for toxicity tests to include multiple species from different phylogenetic groups, a range of end points, and multiple soil types that are representative of the impacted environment, in order to have a comprehensive and accurate site-specific risk-assessment.

Table 5. Comparison of endpoint sensitivity (based on IC_{50} values) ranked for each subantarctic plant species from the most sensitive (1) to the least sensitive (3). The ranking is presented for both the high and low organic carbon soils following 28-d exposure to SAB-contaminated soils. Where the same number appears, there was no significant difference in the sensitivity of endpoints.

Species	High Organic Carbon Soil		Low Organic Carbon Soil	
Deschampsia chapmanii	Shoot	1	Shoot	1
	Root	1	Root	1
	Germination	2	Germination	2
Luzula crinita	Shoot	1	Root	1
	Root	2	Shoot	2
	Germination	3	Germination	3
Colobanthus muscoides	Root	1	Root	1
	Shoot	2	Germination	2
	Germination	2	Shoot	3
Epilobium pendunculare	Root	1	Root	1
	Shoot	2	Germination	2
	Germination	2	Shoot	2

Conclusions

In this study, site-specific remediation targets for subantarctic plants on fuel contaminated soils utilised multiple species and early life cycle endpoints (germination, root length and shoot length) to fully assess toxicity endpoints. Variable germination rates between species under control conditions highlights that not all native subantarctic plants were suitable for use in laboratory toxicity testing or for year round testing. Of the species that were suitable for laboratory bioassays, germination, root and shoot growth varied with exposure to fuel contaminated soil, with root and shoot growth exhibiting greater sensitivity than germination. The TPH concentrations of contaminated soils required to induce growth inhibition was very high, and only likely to be experienced directly at a spill site, but due to the climate of subantarctic regions, the high concentrations may persist over time, so these high concentrations are environmentally relevant. Future research is required to optimise test procedures for the species identified as suitable test species in this study, primarily to identify the factors around the seed viability, and the effect of seed dormancy on subantarctic seed germination during laboratory bioassays.

In order to derive remediation targets and environmental quality guidelines, Species Sensitivity Distribution (SSD) models require toxicity data from a minimum of eight species from at least four taxonomic groups. Prior to this study there was no toxicity data available for sensitive early life stages of native subantarctic plants exposed to TPH. Therefore the data obtained here makes a significant contribution to the SSD model currently being developed to

guide remediation activities at fuel contaminated sites at Macquarie Island and in subantarctic regions more generally.

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