Soil invertebrate response to petroleum contaminants in subantarctic soils, and implications for remediation efforts.

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Declaration

This thesis has not been submitted in any form for a higher degree to any other university or institution.

Many people contributed to the work presented in this thesis, and this is acknowledged and specified at the beginning of each chapter.

The work carried out for this thesis did not require the approval of the Macquarie University Ethics Review Committee. However, all necessary collection and biosecurity permits for work on Macquarie Island were obtained through the Tasmanian Department of Primary Industries, Parks, Water and Environment. These are detailed at the beginning of each chapter where relevant.

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Thesis Summary

Subantarctic islands hold scientific and economic importance, but power to support operations in this region is predominantly produced by diesel-fuelled generators, and several leaks and spills have created areas of contaminated soil. This thesis aims to assess the toxicity of highly weathered petroleum contaminants to soil biota on Macquarie Island, in the context of its unique subantarctic climate and biogeography.

In the first ever review of petroleum contaminants in subantarctic soils, their effects on endemic biota were found to be extremely variable. Limitations and opportunities for contaminant management were also identified for the sensitive subantarctic environment, and the benefits of management techniques with minimal collateral disturbance were brought into relief.

In a field-based experiment, several biotic, environmental and chemical factors were investigated to determine the most important drivers of soil invertebrate assemblages on subantarctic Macquarie Island. Overall, environmental factors that could be linked to physical soil disturbance held greater influence over soil invertebrate assemblages than did petroleum contaminants.

In the first of two laboratory-based toxicity tests, well-aged diesel was not found to affect survival at the highest test concentration in either *Microscolex macquariensis* (an earthworm endemic to Macquarie Island) or *Eisenia fetida* (a common model test species of earthworm). Sub-lethal endpoints were more sensitive, though in some cases the effect was hormetic. These results suggest that the addition of diesel to a soil may increase its carbon content, thereby stimulating microbial activity and increasing the amount of food available to the worms. Finally, to simplify chemical analyses, a typical weathered diesel profile was synthesised using six hydrocarbon types. The mixture toxicity adhered more closely to a concentration addition joint action scenario than independent action. When tested individually, alkyl napthalenes and cycloalkanes were the most toxic, whereas *n*-alkanes and branched alkanes were less toxic.

Together, these findings will guide the focus of remediation efforts, with implications for how contaminant management techniques are selected for environmentally sensitive sites such as subantarctic islands. In particular, the end goals of contaminant management must be well-defined, and the broader impacts of any works should be taken into consideration.

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Abbreviations

AAD	Australian Antarctic Division
AADC	Australian Antarctic Data Centre
C	carbon
DTA	direct toxicity assessment
EC_x	x% effective concentration
HTGEU	high temperature gradient extraction unit
IC_x	x% inhibitory concentration
ISO	International Organization for Standardization
LOI	loss on ignition
LC_x	x% lethal concentration
MDR	model deviation ratio
NOEC	no observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbon
PRB	permeable reactive barrier
rpm	revolutions per minute
SAB	special Antarctic blend diesel
SE	standard error
sp.	species (singular)
spp.	species (plural)
TOC	total organic carbon
TPH	total petroleum hydrocarbon
UCM	unresolved complex mixture
VOC	volatile organic compound

Chapter 1:

Introduction

1.1. Background

An increase of human activities in the high latitudes has resulted in a higher incidence of petroleum spills in these areas (Yang *et al.*, 2009). The subantarctic region – that within a few degrees of latitude either side of the Antarctic Convergence (Figure 1.1) – holds research and economic importance to biologists, meteorologists, climatologists, atmospheric scientists, geologists, seismologists, fisheries operators and increasingly, to tourism operators. However, sustaining a human presence in such remote and challenging conditions comes with many logistical hurdles, including the supply of power. This is primarily produced by diesel generators, but the large volumes of fuel required to sustain operations in the region must be transported by ship, and a number of contamination events have occurred as a result of the transfer and storage of these fuel supplies.

Soil health is paramount to the health of above-ground ecosystems, with bacteria, fungi, macroinvertebrates and plants being vital to decomposition and nutrient cycling processes (Doran and Zeiss, 2000). The contamination of soils by petroleum hydrocarbons is known to perturb the health and balance of soil biota, being mutagenic in isopods and springtails (van Gestel, 2012), limiting reproduction in annelid worms (Mooney *et al.*, 2013), favouring some microbial groups to the detriment of natural communities (van Dorst *et al.*, 2014), and inhibiting seed germination and growth in plants (Adam and Duncan, 2002; Macoustra *et al.*, 2015). Such contamination is of particular concern in the subantarctic, because the ecosystems of cold regions are thought to be more sensitive to disturbance than those of warmer regions (Snape *et al.*, 2008; Yang *et al.*, 2009; Greenslade and Convey, 2012). Indeed, of the flora, fauna and ecosystems investigated from the subantarctic region, most have been shown to be highly vulnerable to petroleum contamination (e.g. Coulon & Delille 2006; Macoustra et al. 2015; Mooney 2013; Mooney et al. 2013; van Dorst et al. 2014). For this reason, it is desirable to rehabilitate petroleum-contaminated soils. If left untreated it is estimated that contaminants would persist for centuries (Rayner *et al.*, 2007).

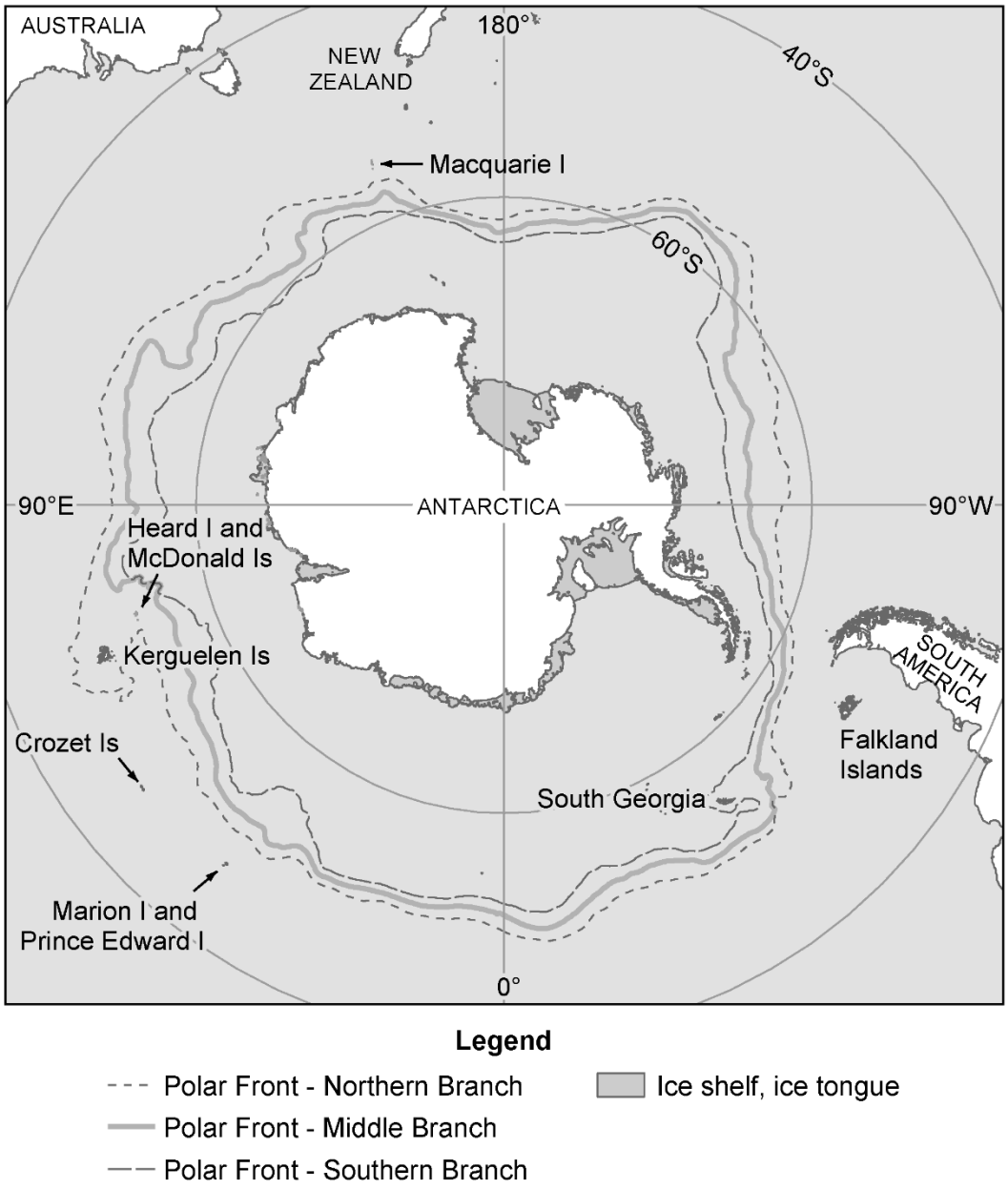

Figure 1.1: Map of subantarctic islands (Credit: David Smith, Australian Antarctic Data Centre)

Petroleum oils and fuels contain different blends of hydrocarbons to confer different properties to the product, such as combustibility, viscosity, lubrication or suitability for use in particular climates (Hutcheson *et al.*, 1996; Filler, Snape and Barnes, 2008). Different hydrocarbon groups vary in their toxic effects on soil biota, so the environmental impacts of petroleum spills vary according to the contaminant substance (Hutcheson *et al.*, 1996). After a contamination event, the lighter components of a petroleum oil mixture will be lost from a soil relatively quickly as they volatilise (Atlas, 1981). Simpler molecules – such as *n*-alkanes and branched alkanes – will also be

catabolised by soil bacteria and fungi, a process known as biodegradation (Atlas, 1981). This progression is highly contingent on climate, soil properties and other environmental conditions, an effect known as weathering. After extended weathering, what remains is a residue of heavier hydrocarbons that are difficult to identify, resistant to further biodegradation, and which may be sorbed to organic material and fine particles in the soil.

In general, soil remediation techniques tend to focus on increasing either or both volatilisation and biodegradation. This is particularly necessary for the subantarctic scenario, where the unique environmental conditions inhibit the extent to which these factors may progress naturally (Snape *et al.*, 2008; Yang *et al.*, 2009). For example, low temperatures reduce the vapour pressure of petroleum hydrocarbons in soil, as well as retarding the microbial action that would break down those molecules (e.g. Coulon et al. 2005). Similarly, the constant precipitation typical of these regions may cause some soil types to become water-logged, thereby causing anaerobic conditions that may also limit microbial action (Rayner *et al.*, 2007). Other, sandier soil types are relatively low in nutrients, potentially causing microbial action to be rate-limited, but the naturally high salinity of these coastal soils (Mallis, 1988) may render the addition of further nitrogen salts inappropriate. Indeed, nutrient addition has been shown to increase toxicity to microbes on both Macquarie and Kerguelen Islands (Walworth et al. 2007; Delille, Coulon et al. 2007).

Contaminated soils at less remote sites would ordinarily be removed for treatment *ex situ*, but transporting thousands of tonnes of contaminated material by ship to a treatment facility would be impracticable. For the same reasons, it would also be impracticable to construct a treatment facility on site (e.g. Troxler et al. 2010). Furthermore, subantarctic soils are valuable and support a range of endemic biota (Bergstrom and Chown, 1999). For these reasons, *ex situ* treatment methods are generally thought to be infeasible. However, complications are also associated with many on-site techniques such as biopiles and landfarming, which require large areas of space, and disturb otherwise healthy areas. These techniques have been used with some success in the Arctic and on Antarctica (Aislabie, Saul and Foght, 2006; McWatters *et al.*, 2016), but soil biodiversity values in these high polar areas are far lower than that of subantarctic islands, where soils are rarely frozen (Filler, Snape and Barnes, 2008). The options remaining for *in situ* remediation techniques – those that leave the soil structure largely intact – are also limited. Microbioventilation and air sparging have been used on

Macquarie Island with a degree of success, but the efficacy of this method has been limited, and some disturbance to the soil profile is inherent in its use (Rayner *et al.*, 2007). More recently, permeable reactive barriers have been effective at preventing contaminant plumes from spreading with the movement of groundwater (Freidman et al. 2017).

For these reasons, remediation works in the subantarctic are particularly resource intensive, and a degree of environmental disturbance is inherent in the application of most techniques. The development of robust remediation targets, then, is imperative to effective rehabilitation, and raises the question of how much remediation is enough remediation; petroleum contaminants will continue to exert a deleterious effect on soil biota if concentrations are too high, but if targets are too conservative then resource use will continue unnecessarily, and disruption to soil ecosystems will be prolonged. The endpoint for soil rehabilitation works is generally marked by having reduced contaminant concentrations to a level considered acceptable for a given purpose. That is to say, returning a contaminated soil to its former level of health requires a far more extensive and delicate rehabilitation effort than if it were to be used as construction fill (NEPC 1999), as was the case for remediated soils at Casey Station on Antarctica (McWatters *et al.*, 2016). However, no target thresholds specific to subantarctic islands currently exist for any jurisdiction. Such targets are derived primarily from laboratory-based, single-species toxicity tests carried out in accordance with international guidelines (e.g. ISO 2008; OECD 2004); but field-based experiments are also of value, particularly when assessing the success of long-term remediation works.

A substantial body of research has emerged in recent years, aiming to develop toxicity thresholds for petroleum-contaminated soils – primarily for Macquarie Island, but also for the Crozet and Kerguelen archipelagos (e.g. Bramley-Alves et al. 2014; Coulon and Delille 2006; Delille, Coulon et al. 2007; Macoustra et al. 2015; Mooney et al. 2013; Wasley et al. 2015). Most of this work has been conducted using fresh diesel as the test contaminant, or fuels aged for up to four weeks. Research conducted into the toxicity of fresh fuel is vital to understanding the effects of new contamination events, but the propensity for weathering to alter the composition and toxicity of petroleum mixtures over time (Atlas, 1981; Alexander, 2000) means that results of these studies may be of limited relevance to the development of suitable remediation targets for decades-old contaminants in soils.

1.2. Thesis aims and structure

The aim of this thesis is to assess the toxicity of weathered petroleum contaminants to soil biota on the subantarctic Macquarie Island. This has been achieved through four objectives:

1. To collate existing knowledge relevant to petroleum contaminants in subantarctic soils more broadly;
2. To identify the relative influence of various environmental properties on soil invertebrate assemblages, with a view to informing contaminant management approaches;
3. To determine the toxicity to soil biota of weathered diesel; and
4. To identify the relative toxicities to soil biota of the different hydrocarbon groups that comprise weathered fuels.

Each chapter has been prepared as a stand-alone manuscript suitable for publication, so some repetition is inherent between chapters.

Chapter 2 addresses the first objective, and presents the first ever review of knowledge of petroleum contamination of soils on subantarctic islands. The matter is one of international significance, and there is evidence that findings may be transferrable between management jurisdictions. In the last decade, the body of literature on the topic has become substantial, and this chapter outlines the challenges associated with working in this unique biogeographic scenario, takes stock of our current knowledge, and identifies gaps remaining in our understanding of how to rehabilitate subantarctic soils contaminated by petroleum products.

Chapters 3, 4 and 5 present experimental findings addressing some of these gaps using three broad categories of direct toxicity assessment (Warne, 2003): field-based testing, site-specific laboratory-based testing, and standardised testing. Chapter 3 addresses objective two by developing a novel approach to assess the drivers of soil invertebrate assemblages on subantarctic Macquarie Island. By comparing the influence of petroleum contaminant concentration, vegetation and soil properties, the findings of this study highlight the importance of thinking beyond contaminant concentration alone when rehabilitating disturbed soils.

Chapters 4 and 5 examine the toxicity of aged diesel to earthworms, such as that which remains decades after a fuel spill. Objective three is addressed in chapter 4. Here,

internationally recognised guidelines developed for use with the common model species *Eisenia fetida* using a standardised soil mixture (Organisation for Economic Co-operation and Development, 2004; International Organization for Standardization, 2008) were adapted for use with natural soils from Macquarie Island, and the endemic earthworm *Microscolex macquariensis*. This experiment also investigated the potential for *E. fetida* to be used as a surrogate for *M. macquariensis* in toxicity testing, as the latter is known to be difficult to obtain in sufficient numbers for ecotoxicological studies (Mooney *et al.*, 2013).

The experiment described in chapter 5 addresses objective four, adhering more strictly to toxicity testing guidelines with the use of a standard soil mixture and a stronger focus on *E. fetida.* By developing a mixture of six synthetic hydrocarbons to resemble the primary components of contaminant plumes on Macquarie Island, chemical analyses are simplified. Furthermore, by subsequently testing the constituent parts of this mixture individually, it may be possible to identify the most pertinent hydrocarbon groups for remediation engineers to focus on. This is the first time such a technique has been used for the toxicological testing of petroleum contaminants in soil macroinvertebrates. Finally, the over-arching themes, limitations and implications of these four chapters are discussed in chapter 6. Additional research is required to develop remediation targets specific to the subantarctic scenario. However, it is intended that the findings of this thesis contribute to the identification of contaminant management approaches that are ecologically appropriate for the region.

Chapter 2:
Ecosystem effects and the management of petroleum-contaminated soils on subantarctic islands

Statement of contribution

Contributor	Concept development and research	Revisions and editing
Ingrid Errington	90	60
Tim Spedding	5	10
Dan Wilkins	5	10
Grant Hose		10
Cath King		10

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Abstract

Human activity in the Polar Regions has resulted in petroleum contamination of soils. In this context, subantarctic islands are a unique management challenge for climatic, biological and logistical reasons. In this review we identify the main abiotic factors affecting petroleum-contaminated soils in the subantarctic environment, the primary effects of such contamination on biota, and lessons learned with regards to remediation techniques in this region. The sensitivity of biota to contamination depends on organism life stage, soil properties, and the degree of contaminant weathering. Initial studies using species endemic to subantarctic islands suggest that for fresh diesel fuel, sensitivities may range between 103 and 20,000 mg total petroleum hydrocarbons (TPH) kg^{-1} soil. Diesel that has undergone a short period of weathering is generally more toxic, with sensitivities ranging between 52 and 13,000 mg TPH kg^{-1} soil for an earthworm and a grass respectively (based on EC_{20} and IC_{50} values). A sufficient body of data from which to develop remediation targets for existing spills in the region does not yet exist for the region, but there has been a recent increase in research attention to address this data gap. A range of remediation methods have also now been trialled, and techniques such as in-ground aeration and nutrient addition have achieved some success. Passive management techniques such as permeable reactive barriers and phytoremediation are in preliminary stages of investigation for the region and show promise, not least because they cause less collateral disturbance than other methods.

Keywords

Ecotoxicology; Macquarie Island; Crozet; Kerguelen; Marion; collembola; cold climate.

2.1. Introduction

An increase of human activities in the high latitudes has resulted in an increased incidence of petroleum spills in these areas (Yang *et al.*, 2009). The subantarctic region – that within a few degrees of latitude either side of the Antarctic Convergence – presents some unique management considerations in the event of a spill, primarily stemming from the fact land is mainly confined to small islands isolated by vast expanses of ocean (Figure 1.1). This affects biogeography, climate and the logistics of human activities. The region has attracted relatively little research, despite its significance to biology, meteorology, geology, climatology, seismology, atmospheric science, fisheries and increasingly, to tourism. In light of this burgeoning importance, research stations are maintained on many of the islands. However, sustaining a human presence in this remote and challenging environment comes with many logistical challenges, including the supply of power, typically produced by diesel generators. Large quantities of fuel are transported by ship to sustain stations and research activities in the region. Consequently, a number of contamination events have occurred as a result of the transfer and storage of fuel supplies. While policies, attitudes and practice have improved in recent years, the risk of new fuel spills will persist whilst bulk quantities of fuel are being transported and stored. Although some success has been achieved with wind power on the Falkland Islands (Otley *et al.*, 2008), and solar and wind power are used in field huts on Macquarie Island, to date renewable sources of energy have been used only in small scale applications (Scherell, 2001). The status quo may be changing, as the British Antarctic Survey recommissioned a hydro-electric plant on South Georgia in 2007 to reduce the diesel requirements of the research station and museum, the latter of which is a major tourist attraction (Gilkes Ltd., 2007). In 2011, Belgium also demonstrated a zero-emission Antarctic base that may serve as a model for other nations that run bases in the subantarctic (International Polar Foundation, 2011).

Petroleum contamination in soil is known to be mutagenic in isopods and springtails (van Gestel, 2012), to limit reproduction in annelid worms (Mooney *et al.*, 2013), and to inhibit seed germination and shoot growth in plants (Adam and Duncan, 2002; Macoustra *et al.*, 2015). Cold region ecosystems are thought to be more sensitive to damage by spills and disturbance than those of warmer climes (Snape *et al.*, 2008; Yang *et al.*, 2009; Greenslade and Convey, 2012), and much of the studied flora and fauna of subantarctic islands have been shown to be vulnerable to petroleum contamination (e.g. Macoustra et al. 2015; Mooney 2013; van Dorst et al. 2014). Furthermore, these islands are

used heavily by marine birds and mammals for breeding (Selkirk, Seppelt and Selkirk, 1990), which may be exposed to petroleum products via dermal contact with contaminated soils. As such, it is desirable that contaminated soils be remediated effectively and efficiently, using techniques that minimise further ecological damage by physical disturbance. However, the biotic and abiotic conditions in the subantarctic are such that petroleum contamination may have a half-life in the range of tens to hundreds of years, so may persist for centuries if not remediated (Rayner *et al.*, 2007).

In addition to the unique logistical and biological concerns, subantarctic islands have a distinct climate (Table 2.1), which is quite different to that of equivalent more land-dominated latitudes in the northern hemisphere. This distinct climate affects the chemistry and behaviour of petroleum contamination in soils. For example, air temperatures are some of the most consistent year-round anywhere on Earth because of the mediating effect of the surrounding ocean on the climate. Winters are mild relative to similar northern latitudes, but the warmth experienced over summer at equivalent northern latitudes is also absent, so the seasonal boost to microbial activity on subantarctic islands is much reduced (Table 2.1). Mean annual rainfall varies greatly between subantarctic islands, but in all cases is very regular, with ≥1 mm of precipitation occurring on up to 274 days of the year (Table 2.1). This persistent rainfall, combined with abundant vegetation and low rates of biological decay, can give rise to organic, waterlogged soils that are low in oxygen, which in turn can alter the microbial community and potentially limit the rate of biodegradation (Rayner *et al.*, 2007). Finally, strong winds and large waves are able to pass across the Southern Ocean largely unimpeded by land, and the average wind speed on subantarctic islands is upwards of 30 km hr^{-1} year round (Table 2.1). The combination of rough seas and high winds creates substantial sea spray, causing high soil salinity near the coast where most human settlements, and thus contamination, is likely to occur (Mallis, 1988).

Because of the logistical challenges, and unique biological and climatic traits of subantarctic islands, much of the knowledge pertaining to the remediation of petroleum contaminated soils in northern high latitudes and other parts of the world may be of limited applicability to the subantarctic (Snape *et al.*, 2003; Walworth *et al.*, 2007). As such, it is imperative that we better understand these unique interactions for successful management of contaminated soils in this region.

The aim of this review is to provide an overview of the current knowledge of petroleum contamination in terrestrial environments on subantarctic islands, and the primary methods used for contaminated site remediation. Until recently there has not been a sufficient volume of work to warrant a specific review of the unique subantarctic case, but this situation has changed. Many early studies focussed on the Crozet and Kerguelen Archipelagos following a 20,000 L diesel spill on Île de la Possession (e.g. Coulon & Delille 2006), with an increasing volume of work investigating Macquarie and other islands in the last decade. Related topics have been reviewed previously, such as human impacts on the subantarctic region more generally (Bergstrom and Selkirk, 2007), conservation management (De Villiers *et al.*, 2005), oil pollution in Antarctica (Raymond *et al.*, 2016), petroleum remediation in cold climates (Snape *et al.*, 2008; Yang *et al.*, 2009) and remediation in polar regions (Aislabie, Saul and Foght, 2006; Camenzuli *et al.*, 2014). Where appropriate, studies from other regions and laboratory-based experiments have been included here for both depth and contrast. A smaller quantity of literature exists on petroleum contamination in marine and intertidal systems in the subantarctic region (e.g. Marcos et al. 2009; Pelletier et al. 2004), but its review is beyond the scope of this paper.

2.2. Petroleum contamination on subantarctic islands

2.2.1. Contamination events

Most subantarctic settlements and research stations are powered by diesel generators, so diesel fuel is therefore the most widely used and studied contaminant fuel in the subantarctic (Cooper and Condy, 1988; Delille and Pelletier, 2002; Rayner *et al.*, 2007). The most common sources of petroleum contamination are leaks that may occur when pumping fuel from a ship to a land-based storage facility, the subsequent failure of fuel storage or pipe infrastructure on land, and human error in fuel storage, transfer and handling processes (Table 2.2). It is, however, worth noting that incidents are not always reported and rarely publicised, particularly in historical cases. For this reason, it may be more relevant to consider factors such as the nature and extent of human activities as indicators of likely contamination (outlined in Table 2.2), as well as a case-by-case consideration regarding the maintenance of infrastructure and the presence and maintenance of bunding. No specific studies of soil contamination by fuels are known to exist for South Georgia Islands, South Sandwich Islands, Prince Edward Islands, or the Falkland Islands. For some of these locations, however, contamination is likely and signs

Table 2.1: Key climate characteristics of a selection of subantarctic islands.

		January (summer) temperature (°C)	July (winter) temperature (°C)	Mean annual precipitation (mm)	Mean days precipitation ≥ 1 mm	Mean wind speed (km hr^{-1})	Reference
Macquarie Island	54°30′S 158°57′E	5.3–8.8	1.6–4.9	981	218.3	35	Australian Bureau of Meteorology, 2016
Heard and McDonald islands	53°06′S 73°31′E	3.7–5.2	-0.8–0.3	1300–1900	274 (approx.)	30	Australian Antarctic Division, 2005
Kerguelen Archipelago	49°21′S 70°13′E	4.1–11.2	-0.8–4.8	850	246	35	Institut Polaire Français, 2016; National Oceanic and Atmospheric Administration, 2016
Possession Island	46°25′S 51°59′E	4.5–10.4	1.4–6.0	1783	173	Unknown	Météoclimat, 2016
Marion Island	46°46′S 37°51′E	4.8–10.6	1.7–6.6	2399	247	Unknown	National Oceanic and Atmospheric Administration, 2016
South Georgia Island	54°17′S 36°30′W	1–8	-5–1	1455	154	Unknown	Cappelen and Jensen, 2001

Table 2.2: Reported petroleum contamination events on subantarctic islands.

Location	Years active	Population	Known contamination	Reference
Base Alfred Faure, Crozet Archipelago (France)	1961 – present	25–50	1997: A broken pipe on Île de la Possession leaked 20,000 L of diesel fuel, resulting in a 1350 m^2 area of oiled soil.	Delille and Pelletier, 2002; IPF, 2017
Kerguelen Archipelago (France)	1949 – present	50–100	1980: A Russian tanker ran aground at Grande Terre, spilling 850 m^3 of an unspecified type of petroleum.	Cripps and Priddle, 1991; IPF, 2017
Macquarie Island (Australia)	1948 – present	15–40	2002: A spill of SAB diesel resulted in 180 metric tons of highly-contaminated soil near the main power house, with hotspots up to approximately 25,000 mg TPH kg^{-1} when measured in 2003 (Australian Transport Safety Bureau, 1988; Deprez, Arens and Locher, 1994; Rayner *et al.*, 2007).	AAD, 2012; ATSB, 1988; Deprez et al., 1994; Rayner et al., 2007
			1975: An unknown volume or presumably SAB was lost when the settling tank over-flowed. Only known from a mention in the station log book.	
			1988: A spill event of unknown volume near the 'fuel farm' fuel storage area. Anecdotal evidence only. Test results were consistent with SAB.	
			1987: The running aground of the MV *Nella Dan*, spilling 270,000 L of marine diesel fuel and 6000 L of lubricating oil in the near-shore area. Reports vary as to how much pollution was observed on the beach.	

			Pre 1975: Mixed contamination due to the practice of burning off waste oils in a pit near the old power house.	Deprez et al., 1994
Heard Island (Australia)	1947 – 1955	Approx. 10	Contamination is poorly known, but an estimated 1000-3000 m^2 of soil is contaminated at concentrations of up to 5500 mg TPH kg^{-1}. Failure of abandoned fuel drums are the main source, in addition to other unknown sources.	AAD, 2014; Stark et al., 2003
Prince Edward Islands (South Africa)	1948 – 1988	12–80	Little detail is available, but 'small spills [of diesel] have occasionally occurred. The deliberate dumping of an unknown quantity of diesel at the Marion Island station killed hundreds of rockhopper penguins in 1980.	Cooper and Condy, 1988; Venter, 2009
Falkland Islands	Inhabited since 1764	2800	Soil contamination is not mentioned in the most recent Falkland Islands State of The Environment Report, and little other information is available. However, domestic and municipal diesel- and kerosene-fuelled generators are common. The Islands have the highest population and longest history of human inhabitation of any subantarctic island. Taken in combination with its military history, as well as the offshore drilling and fisheries in its waters, it seems very probable that some terrestrial contamination would exist.	Otley et al., 2008

South Georgia Islands (Britain/ Norway)	Since the late 18th century	Various	Two research stations are operated by Britain. King Edward Point Research Station (population: 12-22) was intermittently operational from 1969-1982, and re-established permanently in 2001. Bird Island Research Station (population: 4-10) was intermittently operational from 1957 – 1982, and has been permanently operational since then. Five abandoned Norwegian whaling stations all have derelict fuel tanks. Leaks from these tanks were noted from 1972, and in 1979 benthic sediments near stations were found to be very highly contaminated, at over 50,000 mg kg^{-1} TPH. A clean-up operation was first carried out in 1990/91 at the largest station, Grytviken (operational: 1904– 1964). The tanks at Grytviken were not finally emptied until a second operation in 2003, so leaks may have continued for over 30 years since first noted. The nature and extent of the 'clean-up' works is unclear, and has not yet been investigated for other whaling stations.	Bourne, 1985; Cripps and Priddle, 1991; Pasteur and Walton, 2006; Purcell Miller Tritton LLP, 2011
South Sandwich Islands (Britain)	1976 – 1983	Unknown	No petroleum soil contamination is known. Two small settlements were in operation during Argentine-British tensions from approximately 1976 – 1983. No whaling stations are known to have existed here.	Headland, 2009

of contamination have been incidentally noted by unrelated studies (Table 2.2). Many of these islands have a history of human habitation from a time before a general consciousness existed around avoiding soil pollution, in most cases as far back as the sealing and whaling era of the late 18th century (Delille, Coulon and Pelletier, 2004; Selkirk, 2007). Hence despite the lack of available data, it is a reasonable assumption that petroleum contamination occurs on some or all of the islands that have had a history of human habitation.

2.3. Abiotic processes affecting petroleum contamination

2.3.1. Temperature

Temperatures on subantarctic islands are cool year-round with the mediating effect of the ocean preventing substantial warming over summer, while also engendering relatively mild winters (Table 2.1). Petroleum contamination is generally more persistent in cold soils, a function of both decreased volatility and decreased microbial activity with decreasing temperature.

Petroleum-based fuels and oils contain a mixture of volatile organic compounds (VOCs) and heavier compounds in ratios that confer different properties to the blend, such as lubrication or the ability to work in cold conditions. However, at low temperatures even lighter fractions may persist in the soil long after a spill because their volatility is reduced, while heavier fractions are less easily metabolised by soil microbiota under such conditions (McIntyre *et al.*, 2007). Lower molecular weight aromatic compounds are known to be more acutely toxic to biota, whereas heavier compounds such as polycyclic aromatic hydrocarbons (PAHs) can be more chronically toxic (Yang *et al.*, 2009). There is a general positive association between temperature, petroleum volatility and biodegradation, but the interaction between these properties in soil is complex. In an Arctic landfarming study, Paudyn et al. (2008) found that the effect of temperature on net TPH loss was contingent on aeration, though variability was high: with no aeration, TPH loss decreased with temperature, but this relationship reversed with increasing degrees of aeration. Furthermore, at low temperatures some VOCs may not have sufficient energy to evaporate, causing them to remain in the soil for longer, thereby extending the period of acute toxicity and delaying the beginning of microbial degradation of heavier compounds (Atlas and Bartha, 1972). Mesocosms containing natural soil from Kerguelen Island that were incubated at 10°C and 20°C underwent a greater reduction of total petroleum hydrocarbons (TPH) for both diesel and crude oil

than did mesocosms kept at 4°C (Coulon *et al.*, 2005). However, Microtox® assays indicated that toxicity was lowest in the 4°C treatment (Coulon *et al.*, 2005). Furthermore, contamination at the soil surface reduces albedo (that is, reduces reflectance and increases absorption of the Sun's radiation), which in the Antarctic context has been shown to increase the soil temperature by up to 10°C compared with uncontaminated surrounding soil (Balks *et al.*, 2002). This warming could potentially boost microbial activity, and may also be sufficient to prevent soils from freezing, which is known to halt or substantially delay biodegradation (Eriksson, Ka and Mohn, 2001). Finally, a diurnal freeze-thaw cycle between -5°C and 7°C (as is common in subpolar regions) has been shown to increase both the rate and extent of biodegradation when compared to microcosms maintained at a constant temperature of 7°C (Eriksson, Ka and Mohn, 2001). These complex interactions between temperature and biodegradation may help explain why in an *in situ* study on Kerguelen Island, mesocosms covered in black plastic showed little improvement in biodegradation rates, despite being 2°C warmer than uncovered treatments (Delille, Coulon and Pelletier, 2004).

2.3.2. Soil properties and hydrology

Soil types in the subantarctic can vary greatly, being of organic (Otley *et al.*, 2008), volcanic (Stark *et al.*, 2003), young oceanic crust (Selkirk, Seppelt and Selkirk, 1990) or older continental crust in origin (Bergstrom and Chown, 1999). Accordingly, soil types may have very different properties. For example, the Macquarie Island isthmus features coarse sandy, low-organic soils of approximately 3% TOC adjacent to highly organic, peaty soils of 48% TOC (Mooney *et al.*, 2013). In addition, soils may be quite thin, such as Heard Island, where soils may be less than 0.5 m deep before reaching bedrock (Stark *et al.*, 2003), or may be up to 6 m deep in the case of some rapidly accumulated coastal bogs on Macquarie Island (Comfort, 2014).

Following a 20,000 L leak of diesel fuel in 1997 on Île de la Possession (Delille and Pelletier, 2002) it was found that the properties of the soil substrate, soil moisture in particular, influenced natural attenuation rates of fuel contaminants (Coulon and Delille, 2006). The climate of subantarctic islands tends to be of constant mist and drizzle (Table 2.1), so soils often remain waterlogged with low oxygen availability for extended periods (Bergstrom and Chown, 1999; Rayner *et al.*, 2007). Oxygen is required for the catabolism of most hydrocarbon substrates under aerobic conditions, so it follows that biodegradation may be strongly rate-limited when soil oxygen is low (Leahy and Colwell, 1990). Indeed, saturated, hypoxic soils were considered to be the primary

cause of the very slow natural biodegradation rates observed at one contaminated site on Macquarie Island (Rayner *et al.*, 2007). Furthermore, the microbial community itself may shift as anaerobic taxa become favoured, however anaerobic biodegradation rates tend to be far lower than aerobic rates because of fewer possible degradation pathways (Leahy and Colwell, 1990). Aerobic microbial taxa are by far the most commonly investigated taxa for bioremediation applications (Foght, 2008), but this focus has limited relevance to anaerobic subantarctic soils, unless artificial oxygenation is possible.

Hydrophobic contaminants strongly sorb to organic matter in soils (Karickhoff, Brown and Scott, 1979). For this reason, in highly organic soils, spilled fuel may be retained in the upper surface layers (Adam and Duncan, 2002). In low-organic, coarse sandy soils, fuel will migrate downwards in the soil profile. On the Macquarie Island isthmus, this downwards migration, combined with warmer temperatures and higher rates of volatilisation, has given rise to low TPH concentrations at the soil surface (Deprez, Arens and Locher, 1994; van Dorst *et al.*, 2014; Wasley, Mooney and King, 2015). However, at depths from 0.2 to 2 m, these same studies have reported fuel concentrations exceeding 20,000 mg TPH kg^{-1} dry soil. The migration potential of a fuel is also dependent on its constituent parts; for example, BTEX compounds have low soil organic carbon coefficients, so are relatively mobile in soils compared to other contaminants (Piwoni and Keeley, 1990). Water tables are generally shallow (occurring at the surface in the case of mires, or 2-4 m below the soil surface), so contaminant plumes on Macquarie and Possession islands have migrated with ground water, eventually reaching the marine environment (Deprez, Arens and Locher, 1994; Delille and Pelletier, 2002). Hydrocarbon sheen has been observed in this near-shore environment under some circumstances (Rayner *et al.*, 2007). These hydrogeological movements are dependent on complex subsurface topography, and in one case on Macquarie Island the plume of a new spill was calculated to have spread at least 13 m from its source in one year following the spill incident (Rayner *et al.*, 2007). As such, it can be difficult to predict the extent of a contaminant plume (Deprez, Arens and Locher, 1994) and plan remediation works without detailed subsurface investigation. Such investigation may cause substantial physical disturbance to the soil profile.

2.4. Toxicity and effects of petroleum contamination on soil biota

Subantarctic islands typically exhibit relatively low terrestrial biodiversity because, until recent human visitation, species dispersal was dependent on propagules crossing vast distances of ocean carried by birds, wind and ocean currents (Frenot *et al.*, 2005; Chown, Greenslade and Marshall, 2006). This typically low diversity within the microbial community is important because, with fewer species, functional redundancy is likely to be low, thereby rendering subantarctic ecosystems particularly susceptible to disturbances (Terauds, Chown and Bergstrom, 2011), such as petroleum spills and subsequent remediation works.

2.4.1. Microbes

Microbes are so well suited to the task of breaking down petroleum hydrocarbons that remediation science tends to focus on promoting this activity, sometimes in combination with volatilisation and containment. This microbial process is known as biodegradation, and has been observed to result in bacterial abundances at least four orders of magnitude greater than those of uncontaminated soils on Crozet Island (Delille and Pelletier, 2002). Most relevant to the subantarctic context are psychrophiles, microbes that function best below 15°C and that are greatly impaired above 20°C (Gunn Rike, Schiewer and Filler, 2008). These bacteria and fungi catabolise the petroleum molecules into other organic compounds such as alcohols, acids and fatty acids, which may in turn be degraded further (Atlas, 1981). In general, the susceptibility of petroleum hydrocarbon species to microbial catabolism decreases from *n*-alkanes to branched alkanes, branched alkenes, monoaromatics, other cyclic alkanes, PAHs and heavy asphaltenes (Yang *et al.*, 2009), indicating the susceptibility of end-groups to enzymatic oxidisation (Abbasian *et al.*, 2015). However, knowledge of the paths by which particular compounds are broken down is patchy because, in addition to the sheer number of possible hydrocarbons, the degradation pathway for each compound depends on a range of interacting abiotic factors such as soil temperature, oxygen availability, soil chemistry and the specific chemistry of the contaminant (Atlas, 1981; Leahy and Colwell, 1990). Microbes with the potential to metabolise petroleum hydrocarbons are specialised but appear ubiquitous, and occur naturally on Crozet Island (Delille and Pelletier, 2002) and Macquarie Island (Ferrari, Zhang and van Dorst, 2011; van Dorst *et al.*, 2014). For example, though sensitive to the toxic effects of petroleum contaminants, some fungal

groups endemic to Macquarie Island (Ferrari, Zhang and van Dorst, 2011) and the Antarctic peninsula (Hughes, Bridge and Clark, 2007) have been identified as strong candidates for bioremediation. Similarly, in mesocosm experiments on Kerguelen Island, alkanes were found to degrade faster than aromatic compounds (Delille, Coulon and Pelletier, 2004; Coulon *et al.*, 2005), and this trend was enhanced by the application of fertiliser (Delille, Coulon and Pelletier, 2007). Degradation of both alkanes and PAHs in both diesel and crude oil mesocosms was near complete after four years from the start of the experiment, yet significant toxicity was still indicated by a Microtox® test (Delille, Coulon and Pelletier, 2007). This could have been because the least toxic compounds were metabolised first, while the most toxic compounds persisted in the soil, or could have been due to the toxicity of the metabolic by-products. This sensitivity of microbial communities to even low concentrations of residual fuel compounds has also been noted in a mesocosm study on Macquarie Island (Crane, 2016). Soil spiked with a simplified range of hydrocarbons selected to mimic those found in nearby contaminant plumes differed in the structure and function of microbial assemblages relative to solvent controls, even at the lowest nominal concentration of 50 mg kg^{-1} (Crane 2016; Table 2.3). This experiment did not include a non-solvent control (i.e. mixing only), however, which would have been a valuable inclusion to determine the impact of the hexane solvent.

The majority of studies on the response of microbes to petroleum contamination have focused on their utility in the remediation process (e.g. Atlas 1981; Ferrari et al. 2011; Foght, 2008; Leahy & Colwell 1990). Relatively few studies, however, have considered these organisms an important component of the soil ecosystem and themselves susceptible to the toxicity of hydrocarbons (Schafer, Snape and Siciliano, 2007; van Dorst *et al.*, 2014; Crane, 2016). As soil health underpins the health of the broader ecosystem through nutrient cycling, this under-representation of microbial taxa in toxicity testing may be a substantial gap in our understanding of the true environmental effect of petroleum spills. Traditional toxicity testing focused on individual species, but recent advances in genetic approaches allow potentially thousands of microbial species from dozens of phyla, representing a whole, naturally occurring assemblage, to be tested simultaneously. With this in mind, the soil microbial community of Macquarie Island is now known to be highly sensitive to special Antarctic blend (SAB) fuel (Table 2.3; Crane 2016; Ferrari et al. 2011; van Dorst et al. 2014). Petroleum contaminants compromise bacterial species richness, evenness and diversity, leaving only a few species dominating

Table 2.3: Threshold total petroleum hydrocarbon concentrations for soil biota on Macquarie Island. Where multiple sub-lethal indicators were tested (for example, in earthworms, avoidance, cocoon production and juvenile production), only the most sensitive indicator has been presented here.

Taxa	Test compound	TOC of test soil (%)	Threshold concentration (mg TPH kg^{-1})	Test description	Reference
Microbes	Fresh SAB	4.46	IC_{20}: 190	Nitrification	Schafer et al., 2007
Microbes (1700 species)	Fresh SAB	5 – 36	EC_{20}: 155	Nitrification	van Dorst et al., 2014
Microbes	A mixture of hydrocarbons resembling that found in a weathered SAB spill, delivered in a hexane solvent.	Not given	NOEC < 50 (nominal concentration)	Bacterial laccase and nitrification gene response	Crane, 2016
***Microscolex macquariensis* (earthworm)**	Fresh SAB	48	LC_{50}: 2322 EC_{20}: 127	Chronic survival Juveniles hatched	Mooney et al., 2013
	Fresh SAB	3	LC_{50}: 103	Acute survival	
	SAB, aged 4 weeks at 8°C	48	LC_{50}: 1364 EC_{20}: 1089	Chronic survival Cocoon production	
	SAB, aged 4 weeks at 8°C	3	EC_{20}: 52	Juveniles hatched	
Springtails (community)	Fresh SAB	5 – 40	EC_{10}: 700 – 1000	Spiked turf	Mooney, 2013
Mites (community)	Fresh SAB	5 – 40	EC_{10}: 1700	Spiked turf	Mooney, 2013

Plants (various)	SAB, aged 14 days at 15°C	2.3	IC_{50}: 2040 – 12,200 IC_{50}: 80 – 1,050 IC_{50}: 780 – 13,000	Germination Root growth Shoot growth	Macoustra et al., 2015
		6.5	IC_{50}: >1600 IC_{50}: 80 – 830 IC_{50}: 88 – 1600	Germination Root growth Shoot growth	
***Poa foliosa* (grass)**	Fresh SAB	13.6*	Not determined; leaf condition was reduced above 20,000 mg kg^{-1}	Root and shoot biomass and condition	Bramley-Alves et al., 2014

TOC: total organic carbon; SAB: special Antarctic blend diesel; TPH: total petroleum hydrocarbons; ICx: the concentration causing x% inhibition of an endpoint; ECx: the concentration that causes an effect in x% of a test population; LCx: the concentration that causes x% mortality in a test population; NOEC: the concentration at which there is no observed effect. *Test medium was a commercial compost, not natural Macquarie Island soil.

the resultant assemblage (van Dorst *et al.*, 2014; Crane, 2016). Similarly, very low SAB concentrations increase fungal diversity, and concentrations above 250 mg kg^{-1} were found to substantially reduce diversity (Ferrari, Zhang and van Dorst, 2011).

2.4.2. Macroinvertebrates

Knowledge on the response of soil invertebrate assemblages to existing petroleum contamination on subantarctic islands is limited. Only one study has investigated patterns in the field, the primary findings of which indicate that vegetation coverage was a better predictor of springtail assemblages than contamination, possibly because by the time of sampling, surface contamination was minimal compared to the underlying plume (Wasley, Mooney and King, 2015). Mooney (2013) spiked turf cores with fresh SAB to examine its toxicity to springtails and mites (Table 2.3), though this work is of most relevance to understanding the initial effects of new spills more so than the development of remediation targets. Mite assemblages were more resilient to fresh SAB contamination than springtails, and toxicity to both groups was generally found to be ameliorated by raising soil organic content (Mooney, 2013).

Single species toxicity assays have only been carried out for subantarctic islands using one earthworm species, *Microscolex macquariensis* (Mooney *et al.*, 2013), which is endemic to Macquarie Island (Greenslade & van Klinken 2006). These experiments focused on SAB (either fresh or aged *in vitro* for four weeks), using two naturally occurring soils (a sandy, low-carbon soil and a peaty, highly organic soil). Consistent with other studies (e.g. Macoustra et al. 2015), high soil carbon content was found to greatly ameliorate the toxic effects of the petroleum contamination. Aging (volatilising) fuel also commonly reduces its toxicity (Atlas, 1981), a trend evident even with the relatively short (four week) period of aging used by Mooney et al. (2013). However, toxicity was greater for aged fuel than for fresh fuel in highly organic soils (Mooney *et al.*, 2013). This trend was not anticipated, but may have been an artefact of the experimental process: concentrations in the fresh fuel treatments would have decreased substantially in the early stages of the experiment because of volatilisation, whereas the aged treatments had already gone through this process so gross concentration would have remained higher for the duration of the test.

Further studies into the effects of weathered diesel fuel on soil macroinvertebrates are needed and much of the foundational work required to do so already exists. The invertebrates of Macquarie Island are well described (Greenslade and van Klinken,

2006), and some less comprehensive work on soil invertebrates has been carried out for South Georgia Island (Greenslade and Convey, 2012), Marion Island (Gabriel *et al.*, 2001), and Heard Island (Chown, Greenslade and Marshall, 2006). Nothing is known to have been published regarding soil invertebrates on French subantarctic islands, however soil invertebrate diversity is typically low in the region, with many phyla occurring on multiple islands (Bergstrom and Chown, 1999; Chown, Greenslade and Marshall, 2006; Greenslade and Convey, 2012). Thus, any studies carried out for one particular island will likely contribute to our general understanding of soil community dynamics for subantarctic islands (Gabriel *et al.*, 2001; Terauds, Chown and Bergstrom, 2011; Greenslade and Convey, 2012). Furthermore, the springtail species recommended for single-species toxicity testing on Macquarie Island (e.g. *Parisotoma insularis, Tullbergia* sp., *Cryptopygus caecus*, *C. antarcticus* and *Folsomotoma punctata*) (Mooney 2013; Wasley et al. 2015) are likely to be of relevance to other subantarctic islands. Finally, vegetation coverage as a whole has been identified as one of the primary influences on collembolan community dynamics, more so than the degree of petroleum contamination (Wasley, Mooney and King, 2015). In this manner, remediation and monitoring processes *per se* may cause substantial physical disturbance to the soil structure, and necessitate the removal of vegetation in order to install equipment, thereby affecting soil invertebrate assemblages secondarily.

2.4.3. Plants

Plants may be impacted by petroleum contamination in soils, but may also affect contamination through their potential for phytoremediation; the latter is discussed below (see Section 2.5.6). Germination was found to be inhibited in SAB contaminated soils for seven native Macquarie Island plants, and a further three species failing to sprout at all (Macoustra *et al.*, 2015). The effect on germination was species dependent, dose dependent, and varied with soil organic content (Table 2.3). It is thought that a hydrophobic film of hydrocarbons forms around the seed, acting as a physical barrier to the water and oxygen required for germination (Adam and Duncan, 2002). Root and shoot length were more sensitive to contamination than germination rate, which may be because young plants lack the protection of a seed coat but are not yet protected by a thicker epidermal layer, as in adult plants (Bramley-Alves *et al.*, 2014; Macoustra *et al.*, 2015). Similar to other taxonomic groups, such as earthworms (Mooney, 2013; Mooney *et al.*, 2013), the sensitivity of plants to contamination tends to be reduced in soils with higher organic content. This was found to generally hold true in terms of germination

and root length, but not for shoot length, for which toxicity was found to increase with organic content (Macoustra *et al.*, 2015). To explain this anomaly, it was suggested that the increased nutrient levels associated with increased organic matter enhanced root uptake, and with that, any sorbed petroleum hydrocarbons. These differences in life stage sensitivities are of particular importance on subantarctic islands when considered in tandem with the persistent nature of petroleum contamination and the short growing season of plants in these regions.

2.5. Remediation methods

A substantial body of research exists on remediation techniques for petroleum contamination in lower latitudes (Khan, Husain and Hejazi, 2004). At higher latitudes towards the poles, *in situ* methods are often limited by abiotic factors (Section 2.3), whereas offsite 'dig-and-haul' methods are logistically and environmentally impracticable (Snape *et al.*, 2008; Camenzuli *et al.*, 2014) and financially prohibitive (Rayner *et al.*, 2007). A number of methods to promote the natural biodegradation and attenuation of petroleum in soil have been investigated on subantarctic islands.

2.5.1. Aeration

Aeration through microbioventing was trialled *in situ* in water-logged soils on Macquarie Island, enhancing oxygen respiration rates (and with it, biodegradation) from negligible levels to 3-25 mg kg^{-1} TPH per day (Rayner *et al.*, 2007). However, the trial assumed that the enhanced respiration measured by the buried oxygen sensor array would be uniform throughout the subsurface, and that biodegradation would continue at rapid rates until the soil was remediated. These assumptions turned out to be overly simplistic, based upon unpublished results from a larger, multi-year microbioventing remediation project on Macquarie Island (Dan Wilkins, pers. comm.). It was concluded that heterogenous sub-surface conditions, including buried organic and beach cobble horizons, limited the distribution of oxygen and nutrients. This microbioventing method involved shallow trenches (< 30 cm deep) and drilled injection rods, which needed to be removed and periodically re-installed in new locations. Although substantially less disruptive than excavation, this disturbance to the soil profile and vegetation coverage may be counter-productive potential phytoremediation processes (Section 2.5.6; Chapter 3). Furthermore, in this project, an air compressor (electricity supplied by a diesel generator) was run constantly to pump air through the subsurface injection rods, which has its own environmental costs. Single-well aeration was trialled

on Macquarie Island, but was deemed largely unsuccessful owing to the shallow water table in the location trialled, and consequent formation of subsurface air channelling (Rayner *et al.*, 2007). A passive bioventing system (e.g. Auer et al. 1996) was also trialled at Macquarie Island using one-way valves to take advantage of barometric pumping, but due to operational constraints the system was installed in an area of coarse sandy soils that were not oxygen limited, resulting in inconclusive data (AAD, unpublished data). A second unpowered aeration technique was also trialled on Macquarie Island, whereby the high winds typical of the latitude (Table 2.1) were intercepted by large plastic funnels to ventilate subsurface contaminated soils. However, large quantities of sand were also mobilised by the wind, and even with funnels placed 1 m above the ground, the infrastructure was quickly clogged with sand (AAD, unpublished data).

2.5.2. Biopiles

On the Antarctic continent, large-scale biopiles have been used with success, reducing SAB concentrations in 370 tonnes of soil by a factor of four over a five-year period (McWatters *et al.*, 2016). In the subantarctic, biopiles have shown to be effective on a much smaller pilot scale of 4 kg on Kerguelen Island (Delille, Pelletier and Coulon, 2007). Tilling, warming, venting and the application of fish compost significantly increased the abundance of hydrocarbon degrading bacteria and reduced TPH by 93-99.8% over only one year, though notable residual toxicity remained in biopiles treated with fish compost (Delille, Duval and Pelletier, 2008). However, a substantial contamination event is likely to create many hundreds or thousands of tonnes of contaminated soil requiring treatment (Delille and Pelletier, 2002; Rayner *et al.*, 2007; McWatters *et al.*, 2016). At such scales, heating and venting become challenging and costly (McWatters *et al.*, 2016), and the efficacy of the techniques developed on Kerguelen Island is yet to be determined at a scale many orders of magnitude greater than the pilot. In addition, implications of disturbance of the soil profile on such a large scale of operation should be considered. Similarly, the biosecurity concerns associated with the application of natural fertilisers such as fish compost (or the logistics associated with sterilisation) may be prohibitive to this technique being applied outside of a laboratory.

2.5.3. Containerisation (Biocells or Bioreactors)

Half- and quarter-volume steel shipping containers have been used by the Australian Antarctic Division to temporarily store contaminated soil for treatment at Macquarie

Island, and at Antarctic stations. By installing a false floor and piping in these units, water and air may be recirculated, thereby allowing for the remediation of approximately four cubic metres of soil at a time (Pal and Heath, 1998). These types of systems are known in the literature as bioreactors (e.g. Mudliar et al. 2010) or biocells (e.g. Pal & Heath 1998). Extensive site disturbance is required to excavate the contaminated soil, yet containerisation provides a controlled environment in which to enhance biodegradation rates and thus facilitate rapid on-site reuse. No published data currently exists regarding the efficacy of containerisation in subpolar regions, but the technique provides an intermediate solution between larger scale biopiling and in-ground remediation techniques.

2.5.4. Nutrient amendment

Soil nitrogen deficiency has been identified as one of the primary limitations for microbial activity in cold climate soils, and as such, nitrogen is often added to soils to promote biodegradation (Walworth 2007). However, though a reduction in TPH is generally observed with nutrient addition, toxicity may be increased, perhaps even as a direct result of the fertiliser. Inorganic nitrogen tends to be added in the form of ammonium and/or nitrate salts, which at high concentrations increases soil salinity, that may then inhibit microbial activity (Walworth *et al.*, 2007). For Macquarie Island soil tested in a laboratory, inhibition of microbial activity occurred following applications of ammonium chloride at concentrations above 625 mg kg^{-1} of dry soil (Walworth *et al.*, 2007). The same study emphasised that calculating nitrogen concentration as a function of soil moisture is more appropriate than for dry soil, as osmotic stress is more important than concentration, but the latter is presented here for the sake of comparability with other studies: the values presented by Walworth et al. (2007) are lower than those reported by previous studies on Antarctic soils by at least 900 mg kg^{-1} (Ferguson *et al.*, 2003), and on Arctic soils by up to 5000 mg kg^{-1} (Mohn and Stewart, 2000). This difference was attributed to the already high salinity of Macquarie Island coastal soils from sea spray created by the rough surf and high winds typical of the latitude (Table 2.1; Walworth et al. 2007). As such, recommendations for nitrogen application in other high-latitude regions may be inappropriate for use on subantarctic islands dominated by highly active marine processes. Furthermore, the application of a commercial fertiliser to mesocosms on Kerguelen Island had a limited but positive effect on biodegradation rates in unvegetated soils for both diesel and crude oil but was ineffective in already nutrient-rich vegetated soils (Delille, Coulon and Pelletier, 2004,

2007; Delille, Duval and Pelletier, 2008). Also, while fertiliser addition boosted degradation rates for both diesel and crude oil, overall toxicity was actually increased in Microtox® assays when compared to unfertilised treatments, though without knowing the form in which the nitrogen is present it is difficult to determine how it might be contributing to toxicity (Coulon *et al.*, 2004).

2.5.5. Permeable reactive barriers

Permeable reactive barriers (PRBs) are a containment technique for contaminated sites, 'remediating' water that passes through the barrier. PRB's have been used successfully at Casey station in Antarctica since 2005 (Mumford et al. 2013; 2014). Groundwater is funnelled using impermeable wings through a filtration cage, whereupon hydrocarbons are adsorbed before being biologically degraded. This *in situ* approach is low maintenance and once installed requires few resources. Though the hydrology will be altered and some soil structure lost locally during installation of the wings, this disturbance is once-off and relatively minor compared to full site excavation. PRBs also allow for other techniques such as phytoremediation to be carried out concurrently. A trial unit was installed on Macquarie Island in December 2014 and preliminary results are positive, with TPH in groundwater leaving the PRB at or below reporting limits (Freidman, Gras, *et al.*, 2017). The technique can also be used to intercept excess nutrients in cases where they were applied to the contaminant source zone to accelerate biodegradation. While PRBs are a recognised containment technique to minimise environmental impacts, it is important to recognise that they do not remediate the contaminated source soils.

2.5.6. Phytoremediation

Phytoremediation by native plant species shows promise as a method for hydrocarbon remediation in the subantarctic environment, because the method preserves the soil profile and promotes restoration of the original edaphic systems. However, thus far the technique has not been actively used to treat any spills on subantarctic islands. The tussock grass *Poa foliosa* has been identified as being extremely tolerant of SAB contamination, with little toxicity observed at the highest treatment concentration of 40,000 mg kg^{-1} (Bramley-Alves *et al.*, 2014). Furthermore, soil in planted treatments originally contaminated at 10,000 mg kg^{-1} SAB was remediated to have undetectable TPH concentrations within eight months. The breakdown of SAB by *P. foliosa* was correlated with microbial populations (Bramley-Alves, 2009). As such, it is thought that

phytoremediation by *P. foliosa* proceeds via a phytodegradation pathway, possibly by the exudation of enzymes to encourage microbial activity in the root zone (Bramley-Alves, 2009). This result was obtained using a commercial potting mix (13% TOC) rather than natural soils from Macquarie Island, but does highlight the potential for an endemic species to be used in phytoremediation processes. Large, tussock-forming *Poa* species are found on most subantarctic islands, such as *P. cookii* on Kerguelen, Crozet, Heard, McDonald and Prince Edward Islands (Selkirk, 2007), and *P. flabellata* on the Falkland Islands and South Georgia Island (Otley *et al.*, 2008). There may be some logistical challenges associated with enabling *Poa* roots to reach deep strata of contamination, but the genus may be an ideal candidate for phytoremediation of the shallow soils that also harbour the edaphic communities in the region and, as such, warrants further attention.

There is some evidence that unvegetated sites – be they natural or denuded by disturbance – experience lower albedo and thus higher temperatures, thereby stimulating microbial biodegradation (Braddock, Lindstrom and Prince, 2003). However, vegetation coverage has been found to have an overall positive effect on biodegradation and toxicity reduction outcomes. The 1997 leak of diesel on Possession Island surfaced partially in a dry, unvegetated area, and partially in a wet, vegetated area (Coulon and Delille, 2006). The plant species occurring in the vegetated site were not identified, but TPH was reduced by approximately 10% more in the vegetated site than the unvegetated site, which, though marginal, may indicate value in the further investigation of the role natural subantarctic plant systems could play in bioremediating contaminated soils. Fertiliser did improve biodegradation in the dry area but not in the vegetated area, indicating that the process was nutrient limited in the former (Coulon and Delille, 2006). A subsequent study established that vegetated soil on nearby Kerguelen Island had nitrogen and carbon levels almost four times that of adjacent unvegetated soil (Coulon *et al.*, 2004), and here again, fertiliser had a negligible effect on biodegradation in the vegetated treatments (Delille, Coulon and Pelletier, 2004).

The establishment of vegetation cover to provide phytoremediation is perhaps the least disruptive of the available remediation approaches. The rate and extent of remediation provided by this approach is similar to other *in situ* remediation efforts such as nutrient amendment, but without the inherent risks (such as increasing soil salinity and toxicity) and additional resource requirements (Walworth *et al.*, 2007). Furthermore, intact vegetation stabilises the surface layers of soil, preventing the remobilisation of

contaminated topsoil in the high-wind environments typical of subantarctic islands. This being the case, it is recognised that access and maintenance requirements for infrastructure may render revegetation of some contaminated sites impracticable.

2.5.7. Vermi-remediation

Some success has been reported in other regions with the use of earthworms to increase soil aeration and mixing and thereby biodegradation, a process known as vermi-remediation (e.g. Contreras-Ramos et al. 2006; Schaefer & Juliane, 2007; Hickman & Reid, 2008). As yet this ecosystem service has not been investigated for the subantarctic context, but may warrant attention in light of the necessity to limit the environmental impacts of remediation works in this region. Though the subantarctic islands represent the southernmost distribution of earthworms, candidate endemic species do occur such as *Microscolex macquariensis* on Macquarie Island, and *Microscolex kerguelarum* in the Kerguelen archipelago and on Marion Island. Indeed, when placed in natural Macquarie Island soils spiked with SAB fuel aged for 23 weeks, *M. macquariensis* survival was unaffected and mass increased at the highest concentration tested (approximately 550 mg kg^{-1}; Chapter 3).

2.6. Remediation guidelines for petroleum-contaminated soils on subantarctic islands

Petroleum contamination is often measured as TPH, expressed most commonly as a mass of contaminant per unit of dry soil (e.g. mg kg^{-1}), or less commonly as a percentage of soil dry weight. The usefulness of TPH as a measure of toxicity is recognised as limited (Hutcheson *et al.*, 1996), as it does not take into account the difference in toxicities between different chemical constituents within the fuel or the stage of degradation. However, it persists in the literature as few suitable, inexpensive alternatives have been identified, and it allows for comparability between different studies, sites and species, and for setting overall guideline targets to be set for remediation. There is a paucity of literature dedicated to developing remediation targets for use in the subantarctic context, but it has been suggested that targets developed for temperate zones are too low when applied to the subantarctic scenario (Schafer 2007). In light of this, there is a need to develop more comprehensive targets to advise the progress and environmentally acceptable completion point of remediation works.

The toxicity of petroleum contaminants to biota is dependent on a wide range of factors, such as the original contaminant, the degree of weathering that the contaminant has been subject to, the bioavailability of the contaminant, and other biotic and abiotic factors. Sensitivities vary greatly between taxa, and for this reason it is recommended for the development of environmental quality guidelines that toxicity assays be carried out for at least eight species from at least four taxa for a given scenario (van Gestel, 2012). Though a substantial body of work exists regarding contamination on the Crozet and Kerguelen archipelagos, all work thus far that has focused on petroleum toxicity to biota has been developed for Macquarie Island. Of taxa studied to date, microbes have been the most sensitive group, followed by macroinvertebrates and plants (Table 2.3). However, most studies to date have focused on fresh SAB diesel ($C_{9\text{-}18}$) as the contaminant, and only limited work has been done to test the toxicity of weathered petroleum spills that are upwards of 20 years old (Table 2.2). As such, there is not yet a sufficient body of research to develop targets for remediation works in progress at contaminated subantarctic sites, as toxicity is known to vary greatly with the degree of weathering of the contaminant. Existing remediation guidelines for the C_{10}-C_{25} fraction in other cold-climate areas vary greatly between jurisdictions, ranging from 100 mg kg^{-1} in Norway, to 12,500 mg kg^{-1} in Alaska (Snape *et al.*, 2008).

2.7. Conclusions

Existing soil remediation targets developed for petroleum contamination at lower latitudes may be inappropriate for use on subantarctic islands. This is primarily because the unique biological and geophysical environments of these islands give rise to high conservation values, but also limits natural biodegradation processes. These factors also complicate the logistics of carrying out remediation works. An increasing body of work over the past few decades can now inform our response to such contamination in the subantarctic context. Remediation techniques such as aeration and nutrient amendment have been trialled with some success, while preliminary studies into more passive techniques such as containment by permeable reactive barriers and phytoremediation show strong promise, not least because they are less resource intensive and potentially less environmentally disruptive than other methods. As yet it is not possible to derive a comprehensive remediation target for the existing decades-old diesel spills that occur on subantarctic islands. Further work targeting the toxic effects of highly weathered, aged fuels in soils would allow the development of such targets. This would require the

inclusion of more specific indicators of toxicity than the commonly used measure of TPH.

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Chapter 3:
The influence of vegetation and soil properties on invertebrate communities in a diesel-contaminated soil

Statement of contribution

Contributor	Field work	Species ID	Geochemical and soil analyses	Statistical analysis and interpretation	Revisions and editing
Ingrid Errington		100	50	70	60
Grant Hose	50		10	30	20
Alex Michie	50		10		
Cath King					10
Sarah Houlahan			30		5
Simon George					5

Soil and invertebrate samples were collected under permits ES12226 and FA13033 from the Tasmanian Department of Primary Industries, Parks, Water and Environment.

Abstract

Soil health is important for the functioning of all terrestrial ecosystems, but may be impacted by contamination. Soil contamination may in turn necessitate rehabilitation and remediation works, but many of the techniques currently used cause physical disturbance to the soil structure, which may in itself affect soil assemblages. An understanding of the relative influence of these two types of disturbance on soil biota is needed to inform in situ remediation activities. Subantarctic Macquarie Island provides an ideal location to study these interactions because soil biodiversity is naturally low and a number of diesel spills have undergone active in situ remediation in recent years. In this study, soil cores were collected in triplicate from 21 locations. Springtails were extracted and identified to genus/species level. Total petroleum hydrocarbon (TPH) concentrations were measured at the surface (0-32,000 mg kg^{-1}) and at 0.5 m depth (0-740 mg kg^{-1}) at each site, as was vegetation coverage and a range of soil properties. The relationships between these data were examined using distance-based linear models.

Together, all environmental variables (vegetation and soil properties) explained a total of 76% of the variation in springtail assemblages. Soil properties alone accounted for 52% of the variation in springtail assemblages, of which bulk density was most important followed by soil conductivity and pH. Vegetation cover by the four plant taxa accounted for 34% of variation observed, with *Leptinella plumosa* and *Poa foliosa* having the greatest influence. Surface and underlying TPH concentration did not have a significant effect on springtail assemblages. Overall, factors that can be linked to physical soil disturbance had greater influence over springtail assemblages than did soil contamination. This finding may influence the selection of the most appropriate contaminant management approach for environmentally sensitive sites.

Keywords

Contamination; biogeography; community; subantarctic; invasive

3.1. Introduction

Soil health underpins the function of above-ground ecosystems, with soil biota being critical for nutrient cycling and decomposition (Doran and Zeiss, 2000). The ability of a soil ecosystem to maintain its structure and function is a consequence of biotic, abiotic and anthropogenic factors, such as soil chemical and physical properties (Doran and Zeiss, 2000). Changes to these factors may alter soil communities, and, in turn, overall soil health. However, the complex interactions between the physical and chemical properties of soil, and the biota living in it, are often difficult to replicate under laboratory conditions (Alexander, 2000). For this reason, field-based studies are of greater relevance for understanding how soil biota respond to disturbance than laboratory-based studies. However, the variety both within and between soil systems limits our observational ability, with a lack of suitable taxonomic keys and knowledge restricting many field-based studies to genus-, family- or even order-level identification of the taxa present (e.g Blakely et al. 2002; Kimberling et al. 2001; Leinaas et al. 2015). Furthermore, the long-term history of disturbance for a site is often difficult to determine, making it problematic to draw conclusions about interactions between soil biota and soil properties.

Disturbances to soil may be biological (e.g. the removal of plant material), physical (e.g. the loss of soil structure), or chemical (e.g. the introduction of a contaminant). In the case of chemical disturbance, the number of incidents of contamination involving petroleum-based products has increased in recent years as products are transported longer distances, a trend that is particularly true for cold regions (Yang *et al.*, 2009). Petroleum contamination is known to alter not only soil chemistry, but soil properties such as oxygen availability and soil water penetrability (Gillet and Ponge, 2005). Following a petroleum contamination event, the most volatile fractions will quickly evaporate, leaving behind more persistent heavier molecular weight compounds such as polycyclic aromatic hydrocarbons (PAHs), which are often resistant to biodegradation (Atlas, 1981). The complex and chromatographically poorly-resolved compounds in a biodegraded petroleum spill are known as the unresolved complex mixture (UCM) (e.g. Reddy et al. 2002). Some of these hydrocarbons are directly toxic to some taxa, but for other taxa they may act as a food source (Blakely, Neher and Spongberg, 2002; van Dorst *et al.*, 2014). In both scenarios, however, petroleum contamination may cause major changes in soil communities.

Subantarctic Macquarie Island (54°30'S, 158°57'E) provides an ideal environment to isolate and examine potential drivers of soil invertebrate diversity. Its extreme remoteness and young age have given rise to naturally low biodiversity in the terrestrial environment, making it relatively simple to identify soil invertebrates to the species level, while also limiting the effects of differing vegetation or soil types (Selkirk, Seppelt and Selkirk, 1990; Terauds, Chown and Bergstrom, 2011; Greenslade and Convey, 2012; Wasley, Mooney and King, 2015). Cold region ecosystems may be more sensitive to disturbance than those of warmer climates (Snape *et al.*, 2008; Yang *et al.*, 2009; Greenslade and Convey, 2012), perhaps because of limited species redundancy (Freckman and Virginia, 1997), so subantarctic assemblages may be more sensitive to the effects of contaminants than those elsewhere. Furthermore, potential sources of disturbance are limited and site histories are relatively well known. A continuous human presence has been maintained on Macquarie Island since 1948, but impacts in this time have generally been strongly controlled and well-recorded (Chapter 2). Of concern on Macquarie Island, however, are a number of diesel spills that have occurred since the 1970s – the last major incident occurred in 2001 (Chapter 2). Soil temperatures are relatively low year round, though rarely freezing, with no true summer season to boost natural biodegradation (Australian Bureau of Meteorology 2016; see Chapter 2 for further detail). Similarly, precipitation is persistent throughout the year: rain falls on approximately two out of three days (Australian Bureau of Meteorology, 2016). This constant precipitation can cause some soil types to become water-logged and low in oxygen, again limiting microbial activity and natural biodegradation rates (Rayner *et al.*, 2007).

In this unique biogeographical environment, petroleum contamination is predicted to remain for centuries if no action is taken (Rayner *et al.*, 2007), but there are challenges associated with this. The extreme remoteness of Macquarie Island renders *ex situ* soil remediation impracticable for logistical reasons. *In situ* remediation efforts have been underway since 2003, primarily in the form of air sparging, microbioventilation and nutrient amendment (Rayner *et al.*, 2007; Walworth *et al.*, 2007). However, these processes themselves may disrupt soil ecosystems. Microbioventilation and monitoring infrastructure must be regularly dug up to be moved, causing a subsequent loss of soil structure and the possible liberation of contaminants from deeper, more highly-contaminated soil strata (Rayner *et al.*, 2007). Similarly, the addition of nitrogen salts to boost microbial activity has been shown to be inappropriate for use on the coastal soils

of Macquarie Island, because these soils are already highly saline and any further increases may reduce microbial activity rather than enhance it (Walworth *et al.*, 2007). Initial field observations of soil invertebrate communities on Macquarie Island suggested that petroleum contamination may have a minimal effect on soil invertebrate species assemblages, but sites that are known to have a history of contamination still tend to exhibit lower invertebrate abundance and diversity (Wasley, Mooney and King, 2015). A general model of soil biota response to disturbance is impossible to define, but changes in the community structure of soil biota – including arthropods, worms, bacteria and plants – have been linked to tilling (Moradi *et al.*, 2013), the liberation of contaminants by breaking up soil interstitial spaces (Jiang *et al.*, 2016), the loss or simplification of vegetation cover (Kimberling, Karr and Fore, 2001; Callaham *et al.*, 2006) and the loss of soil structure (Kimberling, Karr and Fore, 2001). These forms of disturbance are all likely to occur as a matter of course with some soil remediation techniques. Understanding which factors most strongly influence the dynamics of soil invertebrate ecology is important because such knowledge has implications for how best to restore or improve soil health in this unique biological and environmental scenario.

The taxonomy of the arthropods of the subantarctic islands is now well described, a process made simpler by the relatively low biodiversity of these isolated islands compared to that of mainland areas (Chown and Convey, 2016). Springtails (subclass: Collembola) were selected as the focal group of this study because the taxonomy of the springtails on Macquarie Island is better known than for other groups such as mites (Greenslade and van Klinken, 2006), and there is evidence that the presence of non-native springtails may be correlated with disturbed or contaminated sites (Greenslade and Majer, 1993; Leinaas *et al.*, 2015). Furthermore, the species richness of springtails is well known to be negatively affected by disturbance (Ponge *et al.*, 2003), while the group are also known to be primary colonisers following a soil contamination event (Greenslade and Majer, 1993). A number of introduced springtail species have become naturalised on Macquarie Island (Greenslade and Convey, 2012), and these may serve as a further line of evidence when considering the effects of soil contamination on springtail assemblages.

The aims of this study were to identify the factors that most strongly influence springtail diversity in the vicinity of the Macquarie Island research station. To this end, we asked:

- What are the relative effects of petroleum concentration, physical soil properties and vegetation cover on springtail assemblages?
- In what ways do species traits (e.g. invasivity and life history) reflect differences in species assemblages?
- What implications do these findings have for the management of contaminated sites?

3.2. Methods

3.2.1. Sample collection

Soil samples were collected on Macquarie Island in the summer of 2012-2013, from 21 locations within the vicinity of the research station (Appendix 1). Locations were selected to include the areas surrounding the fuel farm and powerhouse, which are known to be contaminated by petroleum residues (Wasley, Mooney and King, 2015), as well as from an area thought to be relatively undisturbed, at the south-westernmost extent of the Isthmus. Sampling locations were limited to sandy soils, so as to control for the influence of soil type on springtail assemblages and soil properties.

At each location, a 2 x 2 m square quadrat was established. Within this quadrat, three pairs of soil cores were collected from the soil surface from three randomly chosen places. Soil cores were taken using a segment of PVC pipe (70 mm Ø x 70 mm) in a hand corer. From these paired cores, one core was used for invertebrate extraction and the other for the analysis of soil properties. Percentage vegetation coverage was recorded for each plant species present in a square 0.5 x 0.5 m surrounding each core. A further single soil sample was collected from a depth of 0.5 m at each location and analysed for TPH, with a corresponding core for invertebrate extraction.

3.2.2. Extraction and identification of springtails

Soil cores for invertebrate extraction were inverted over 2 mm mesh and placed in a high temperature gradient extraction unit (HTGEU) for 96 hours, following Gabriel et al. (2001). The HTGEU was held at 25°C for 48 hours before being increased to 30°C for the remaining 48 hours. Cold water (approximately 10°C) flowed through the base of the HTGEU. Springtails were initially preserved in propylene glycol which was then drained off through 63 µm mesh and invertebrates were transferred to 100% ethanol for long term preservation. All springtails were identified to species level, with the exception of the genus *Megalothorax,* using the key developed by Greenslade and van Klinken (2006),

and from this it was noted whether each species was native or introduced to Macquarie Island (*sensu* Greenslade and Convey 2012).

3.2.3. Soil properties

Approximately 10 g of soil from each core collected for soil property analyses was placed in a plastic jar with 50 mL of MilliQ filtered water making a 1:5 soil:water suspension (Rayment and Higginson, 1992). The samples were placed on an orbital shaker at 15 rpm for 60 minutes (Rayment and Higginson, 1992) before pH and conductivity were measured using hand-held meters and probes (Hanna Instruments, Rhode Island, USA).

Moisture content from each soil core was determined by weighing the soil before and after drying at 70°C to constant mass. Bulk density was calculated by dividing dry soil mass by corer volume. The dry soil was then homogenised, and particle size was determined by placing a 55 g sub-sample in a mechanical sieve shaker for five minutes. This yielded three fractions: silt (<63 µm), sand (<2 mm) and gravel (>2 mm). Total organic content (TOC) was determined by loss on ignition. The mass of a sub-sample (10 ± 0.5 g) of dry, homogenised soil was recorded to four significant figures, then placed in a muffle furnace at 550°C for 24 hours. The samples were transferred to an oven at 70°C to avoid reabsorption of atmospheric moisture, and removed from the oven one at a time to record their post-ignition mass, from which the percentage organic mass could be calculated.

3.2.4. Petroleum hydrocarbon analysis

Soil for TPH analysis was collected directly into a clean glass jar. Each sample was sealed with a Teflon-lined lid, refrigerated at 4°C and transferred to the Organic Geochemistry laboratory at Macquarie University (Australia) for analysis. Each sample was homogenised, and a sub-sample of wet soil (15 ± 0.1 g) was removed and placed in beaker with 30 mL of a 9:1 mix of dichloromethane:methanol. The samples were solvent extracted using an ultrasonicator bath (Ultrasonics Australia, Sydney) for 25 minutes. The samples were allowed to settle before decanting the extract solution. This sonication process was repeated a further two times. The three extracts were pooled and reduced to 0.5 mL using a nitrogen blow-down system. An injection standard containing cyclooctane and 1,4-dichlorobenzene was added to all extracts. These standards were chosen due to their differences in retention time, and because they are not typically found in petroleum products or indigenously in the environment. The extracts were then analysed using gas chromatography-mass spectrometry (GC-MS) on an Agilent

7890A GC coupled to a Leco Pegasus time-of-flight-MS and an Agilent 7683 ALS auto-sampler (Santa Clara, CA, USA). The extracts were injected (1 µL) into a split/splitless injector operating at 310°C in splitless mode onto a J&W DB5MS column (60 m × 0.25 mm i.d., 0.25 mm film thickness) coated with modified 5% phenyl 95% methyl silicone, with helium as the carrier gas (1.5 mL/min, constant flow). The temperature programme was 40°C (held for two minutes), increasing to 310°C at a rate of 4°C/min (held for 45 minutes). The detector voltage of the MS was between 1600 V and 1850 V.

The concentration of all compounds within the C_{9-18} range was measured by GC-MS, as this is the typical molecular weight range of fresh special Antarctic blend (SAB) diesel (Brown *et al.*, 2016), and also because, at masses above C_{18}, many samples contained an odd-over-even *n*-alkane pattern characteristic of plant lipids that were naturally present in the samples. The abundance of each hydrocarbon was calculated relative to the injection standards. The sediment remaining after sonication was dried to constant mass at 70°C and weighed, and any mass lost by extraction was considered to be negligible. The TPH of each sample was calculated in milligrams of hydrocarbons per kilogram of dry sediment (mg kg^{-1}).

3.2.5. Data analyses

Before statistical analysis, all soil property parameters were averaged and springtail species counts were pooled for each of the 21 sampling locations, in order to reduce heterogeneity in the analysis. For multivariate analyses, surface and underlying TPH concentrations were log transformed to improve linearity. Some springtail species occurred in large numbers, while others were very uncommon and zero counts for some species were often recorded. A dummy variable of 1 was therefore added to the abundance of each taxa for each sample to eliminate zero counts, and the data was square-root transformed to reduce the dominance of highly abundant species in the analysis. All environmental variables (TPH, soil properties and vegetation cover) were normalised and a resemblance matrix was calculated using Euclidean distance. The resemblance matrix for springtail species counts was calculated using Bray-Curtis similarity. A stepwise distance-based linear model (DISTLM) was constructed using R^2 as the selection criterion (Clarke and Gorley, 2006).

Pearson's correlations were used to identify relationships between vegetation cover and soil properties such as bulk density and moisture content, as well as between surface and underlying TPH concentrations. Further correlations were conducted to identify

relationships between individual species and surface TPH concentration, as well as the four factors found to be important in the sequential analysis of the DISTLM (i.e. bulk density, conductivity, pH and coverage by two plant species).

The significance level (α) for all analyses was 0.05. Multivariate analyses were carried out using PRIMER with PERMANOVA (Clarke and Gorley, 2006). Univariate analyses were carried out in Minitab 17.0 (Minitab Inc., 2010).

3.3. Results

3.3.1. Soil properties and contamination

Soils contained a minimum of 96.8% sand, with most of the remainder being gravel and only a small fraction being silt. Soils were slightly acid with an average pH of 6.12. Soil pH was moderately negatively correlated with conductivity ($r = -0.259$; $p = 0.040$). Vegetation cover was strongly negatively correlated with bulk density ($r = -0.799$, $p < 0.001$) and soil moisture ($r = -0.765$; $p < 0.001$).

Total petroleum hydrocarbon concentrations at the surface were highly variable, both between and within sampling locations (Appendix 2). Surface TPH concentrations ranged from below detection limits to approximately 32,000 mg kg^{-1}, with an average of 1402 mg kg^{-1}. TPH concentrations at a depth of 0.5 m ranged from below detection limits to approximately 740 mg kg^{-1}, with an average of 111 mg kg^{-1}. TPH concentrations in the surface soil generally did not correlate with or reflect the TPH concentrations found at 0.5 m depth ($r = 0.087$; $p = 0.497$).

3.3.2. Springtail assemblages

Thirteen springtail taxa were identified in the cores, four of which were introduced to Macquarie Island (Table 3.1; Figure 3.1). Springtail abundances were highly variable, ranging from zero to 2573 individuals per core, with an average of 275 individuals. Three colony-forming species, *Parisotoma insularis*, *Folsomotoma punctata* and the introduced *Ceratophysella denticulata*, were identified, and these taxa dominated the assemblages wherever they were found (Figure 3.1). Of the 21 soil cores collected from a depth of 0.5 m, 14 contained no springtails, and of the remaining seven, six cores contained five or fewer individuals, and one core contained 17 individuals. In light of these extremely low

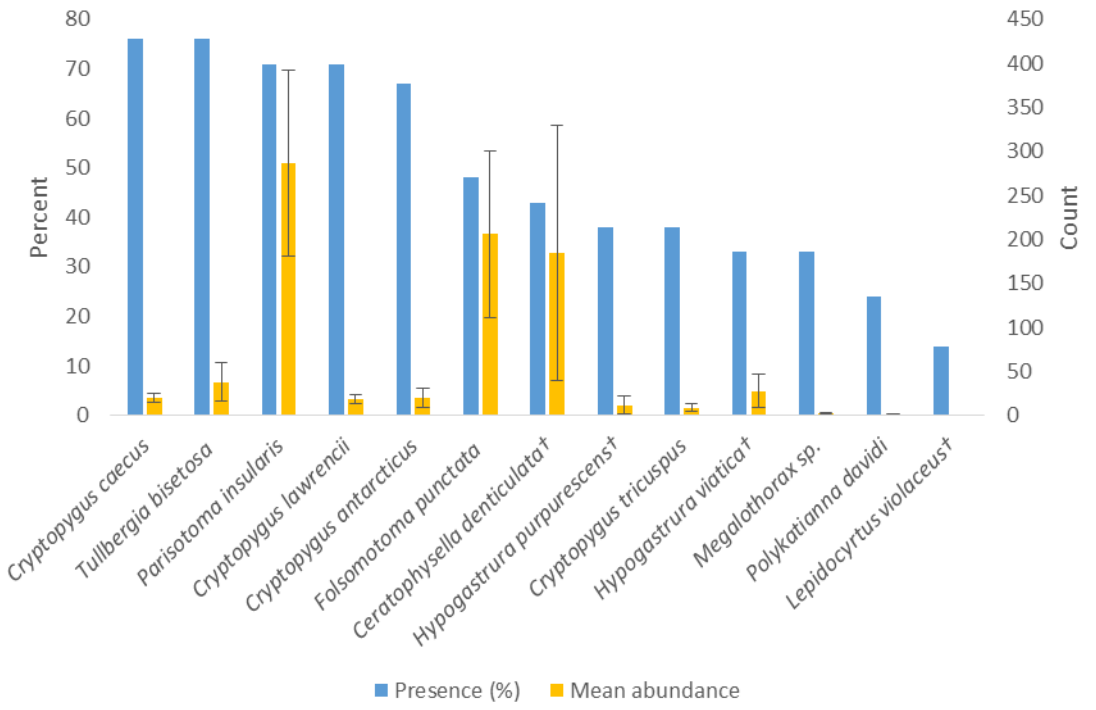

Figure 3.1: Springtail presence and mean abundance (± SD) in soil cores collected at surface level on Macquarie Island. † denotes introduced species.

frequencies and abundances, and the possibility that those individuals that were collected may have fallen into the collection pit from the surface, no further analyses were conducted for springtails collected from below the surface.

The full stepwise DISTLM explained a total of 75.6% of the variation observed in springtail assemblages. When tested individually, bulk density, pH, percentage silt and soil moisture each had a significant effect on springtail assemblages, as did percentage cover by the tussock grass *Poa foliosa* and the herbaceous ground-cover *Leptinella plumosa* (Table 3.2). Springtail assemblages were not significantly affected by the TPH concentration of either surface or underlying soils (Table 3.2). In the sequential test of all variables, bulk density was most strongly related to springtail assemblages, and was fitted first (R^2 = 0.241; Table 3.2). After bulk density, soil conductivity (R^2= 0.111), pH (R^2 = 0.076) and percentage cover of *L. plumosa* (R^2 = 0.069) together also explained a significant proportion of the variation in springtail assemblages (Table 3.2). The forced addition of further variables, including TPH concentrations, did not result in a significant increase in the variation explained by the DISTLM. When fitted together, all eight soil property variables accounted for 52.2% of the variation observed in springtail assemblages (an average of 6.5% per property; Table 3.2). Similarly, vegetation cover

Table 3.1: Springtail abundances and their correlation with environmental factors. Data are based on soil cores taken at 21 locations near the Macquarie Island research station, and are presented in order of percentage species presence.

Species	**Pearson's corelations: r (p)**					
	Surface TPH	**Underlying TPH**	**Vegetation cover**	**Bulk density**	**pH**	**EC**
Cryptopygus caecus	-0.20 (0.44)	-0.19 (0.13)	**0.63** (0.01)	**-0.30** (0.02)	0.13 (0.32)	-0.08 (0.56)
Tullbergia bisetosa	0.33 (0.14)	0.06 (0.63)	-0.11 (0.64)	-0.01 (0.97)	0.23 (0.07)	-0.22 (0.09)
Parisotoma insularis	-0.15 (0.38)	-0.10 (0.43)	**0.47** (0.00)	**-0.55** (0.00)	0.14 (0.28)	0.19 (0.13)
Cryptopygus lawrencii	-0.09 (0.51)	-0.09 (0.47)	0.07 (0.06)	**-0.29** (0.02)	0.17 (0.19)	-0.09 (0.48)
Cryptopygus antarcticus	-0.18 (0.70)	-0.07 (0.60)	**0.57** (0.04)	-0.07 (0.58)	0.11 (0.39)	0.012 (0.89)
Folsomotoma punctata	-0.26 (0.26)	-0.17 (0.19)	**0.46** (0.04)	-0.18 (0.17)	0.10 (0.46)	0.06 (0.66)
Ceratophysella denticulata†	0.09 (0.72)	**-0.45** (0.00)	**0.45** (0.04)	**-0.35** (0.01)	0.02 (0.88)	**0.25** (0.05)
Hypogastrura purpurescens†	0.09 (0.69)	**-0.31** (0.01)	**0.44** (0.05)	**-0.31** (0.02)	0.04 (0.74)	0.24 (0.06)
Cryptopygus tricuspus	-0.01 (0.97)	-0.03 (0.84)	0.33 (0.14)	**-0.29** (0.02)	0.15 (0.24)	0.03 (0.83)
Hypogastrura viatica†	-0.09 (0.69)	-0.23 (0.07)	**0.51** (0.02)	-0.24 (0.06)	0.01 (0.94)	0.15 (0.24)
Megalothorax sp.	-0.08 (0.73)	0.07 (0.61)	0.23 (0.31)	**-0.32** (0.01)	**0.28** (0.01)	0.02 (0.86)
Polykatianna davidi	-0.35 (0.12)	0.05 (0.66)	0.25 (0.28)	-0.24 (0.06)	0.24 (0.06)	-0.04 (0.78)
Lepidocyrtus violaceus†	-0.29 (0.20)	-0.09 (0.48)	0.36 (0.11)	**-0.32** (0.01)	0.18 (0.15)	0.14 (0.27)

† denotes introduced species. Pearson's correlation values in bold are significant ($p < 0.05$). Vegetation cover represents all plant species combined. EC is electrical conductivity

Table 3.2: Relationship between springtail assemblages and soil properties (buk density; EC; pH; particle sizes; moisture and TOC), vegetation properties (percentage cover of four plant species) and TPH contamination (at surface and at a depth of 0.5 m). Marginal tests indicate the relationship between environmental variables and assemblages individually. Sequential tests indicate the relationship between environmental variables and assemblages determined by Distance-based linear models (DistLM). Partial R^2 values indicate the relationship of a variable once those listed above it have already been fitted to the stepwise model.

Variable	**Marginal test**		**Sequential test**		
	Individual r^2	***p***	**Partial r^2**	**Cumulative r^2**	***p***
Bulk density	0.241	**0.001**	0.241	0.241	**0.001**
EC	0.055	0.342	0.111	0.352	**0.005**
pH	0.131	**0.023**	0.076	0.429	**0.020**
% *Leptinella plumosa*	0.145	**0.008**	0.069	0.498	**0.025**
% *Poa foliosa*	0.145	**0.008**			
% silt	0.117	**0.035**			
% moisture	0.116	**0.032**			
% *Calitriche sp.*	0.082	0.114			
TOC	0.080	0.086			
Surface TPH	0.045	0.468			
Underlying TPH	0.043	0.484			
% *Poa annua*	0.042	0.573			
% sand	0.038	0.576			
% gravel	0.033	0.680			

EC: electrical conductivity; TPH: total petroleum hydrocarbons; TOC: total organic carbon. Bold indicates $p < 0.05$.

The abundance of many springtail species, including the three most common introduced species (*C. denticulata*, *H. purpurescens* and *H. viatica*), was strongly correlated with percentage vegetation cover (Table 3.1). Of the six species that were not correlated with vegetation cover – *T. bisetosa, C. lawrencii, C. tricuspus, Megalothorax* sp., *P. davidi* and *L. violaceus* – the latter four were relatively uncommon and found only in small numbers, so these poor correlations may reflect the small sample size. Bulk density was negatively correlated with eight of 13 species, including three of the four introduced species (Table 3.1). The relationship was most pronounced for the most abundant species, *P. insularis* (r = -0.55). Though conductivity and pH were both an important influence on springtail assemblages in the DISTLM, they were each only moderately correlated with the abundance of a single taxon (Table 3.2). Conductivity was correlated with the abundance of the most common introduced species, *C. denticulata* (r = 0.25), whereas pH was correlated with the relatively uncommon *Megalothorax sp.* (r = 0.28). No species' abundance was significantly correlated with surface TPH concentration, and only two species' abundance was moderately, negatively correlated with underlying TPH (both introduced hypogastrurids; Table 3.1).

3.4. Discussion

3.4.1. Drivers of springtail assemblages

Petroleum contamination, whether in surface soils or in an underlying plume, accounted for very little of the variability observed in springtail assemblages. Similarly, surface contaminants did not affect the abundance of individual species. Underlying TPH concentration was moderately, negatively correlated with the abundance of only two out of 13 species, both of which were introduced hypogastrurids: the highly abundant *C. denticulata* and the less abundant *H. purpurescens*.

When combined, soil properties had the greatest influence on springtail assemblages, followed by coverage by two plant species, *L. plumosa* and *P. foliosa*. On an average per-factor basis this trend was reversed, with each species of plant having a relatively greater influence on springtail assemblages than each individual soil property. This latter trend has been identified previously following the cessation of mining operations, whereby the complexity of the vegetation community was found to be the main driver of springtail assemblages (Greenslade and Majer, 1993). Similarly, the recovery rate of springtail species richness and abundance following fire disturbance has been shown to be contingent on vegetation type (Janion-Scheepers *et al.*, 2016). The most important

factors influencing springtail assemblages were soil bulk density, coverage by *L. plumosa* and *P. foliosa,* and soil pH (each explaining 24.1, 14.5, 14.5 and 13.1% respectively). Plant diversity was low in this study, with only four taxa identified, two of which were relatively uncommon (*Calitriche sp.* and the introduced *Poa annua)*. This was likely because sample sites were selected to represent only one soil type, in order to limit any confounding effect due to variation in soil properties.

Bulk density was strongly associated with vegetation coverage, which may be a result of plant roots aerating the soil, or decayed vegetation increasing the proportion of light organic material. As such, the strong influence of bulk density on springtail assemblages may lend further weight to the importance of vegetation to those assemblages. Similarly, soil pH was positively correlated with electrical conductivity, an indicator of soil salinity. Soil acidity is known to be increased by the addition of nitrogen-salt based fertilisers (Vitousek *et al.*, 1997), which have been used on Macquarie Island to enhance bioremediation by preventing nutrient limitation of the microbial biodegradation processes (Schafer, Snape and Siciliano, 2007). Indeed, soil cores with notably low pH values (< 5.75) were collected mainly from locations with a history of intensive remediation works (e.g. the region surrounding the main power house). However, while both pH and conductivity were important factors in the multivariate model of effects on springtail assemblages as a whole, neither were strongly correlated with the abundance of individual springtail taxa.

Vegetation coverage was an important influence on springtail assemblages, and on the abundance of most individual taxa. Plants have previously been shown to affect how subantarctic soils respond to petroleum hydrocarbon contamination (Chapter 2). For example, the plume of diesel caused by a leaking pipe on Possession Island surfaced partially in an unvegetated area, and partially in a vegetated area (Coulon and Delille, 2006). The soil TPH concentration was reduced by at least 10% more in the vegetated site than the unvegetated site within a year of the leak.

The link between vegetation and soil invertebrate assemblages may be particularly important in light of the close link between vegetation and bulk density, the most important influence on springtail assemblages determined by this study. It stands to reason that vegetation coverage would be affected by the physical disturbance of surface soils, and indeed, the loss of a soil's structure would also affect bulk density. While the types of physical soil disturbance and their potential effects are too complex for a

generalised model to be developed, evidence indicates that physical disturbance and changes to plant coverage are an important influence on below-ground ecosystems. For example, in the low-Arctic, plant removal and soil tilling alone caused changes to bacterial and fungal communities that remained evident even six years following disturbance (Mikola, Sørensen and Kytöviita, 2014). Furthermore, physical disturbance to soils and changes to vegetation have been shown to impact the ecology of arthropods, worms, bacteria and plants (Kimberling, Karr and Fore, 2001; Callaham *et al.*, 2006; Moradi *et al.*, 2013; Jiang *et al.*, 2016).

3.4.2. Invertebrate species, traits and life-histories

Other studies on Macquarie Island have reported more diverse springtail species lists (e.g. Mooney 2013; Terauds et al. 2011; Wasley et al. 2015), but it should be noted that those studies covered a number of soil types, whereas this study deliberately collected samples only from one soil type to control for the ameliorating effect of soil TOC on petroleum toxicity (e.g. Macoustra et al. 2015; Mooney et al. 2013). As such, the lower species richness recorded in this study was to be expected.

Mites were also observed in samples collected from the soil surface, occurring in similar numbers to springtails (in line with the findings of Mooney, 2013), along with a number of less abundant taxa such as worms, book lice, beetle larvae and dipterans. Two thirds of the cores collected from a depth of 0.5 m contained zero springtails and the remaining third contained very few individuals. Similarly low numbers of mites were also observed in the samples from depth, and no other taxa were noted (Errington, unpublished data). As such, it was felt that the few springtails and mites present had likely fallen into the collection pit during the digging process. Several previous experiments based on Macquarie Island have used surface-dwelling soil biota to investigate their response to petroleum contaminants (Mooney, 2013; Macoustra *et al.*, 2015; Wasley, Mooney and King, 2015). However, surface soils are known to often contain low TPH concentrations, even with highly contaminated underlying soils (Wasley et al. 2015). Petroleum contaminants tend to percolate downwards through the soil profile over time, to a depth below that of soil invertebrate habitat (Deprez, Arens and Locher, 1994). Furthermore, subsurface movements are known to be highly dynamic; the plume of a spill on Macquarie Island spread at least 13 m from its source in its first year (Rayner *et al.*, 2007). As such, an area with a high underlying TPH concentration may never have been contaminated at the surface, so surface biota may only be exposed to volatile compounds. For these reasons, it may be that springtails and

other surface-dwelling soil biota are simply not a relevant receptor when considering the toxicity and rehabilitation requirements of decades-old fuel spills.

Phenotypic plasticity has been shown to be important for drought tolerance in subantarctic springtails, a trend that likely favours introduced species over native ones (Chown *et al.*, 2007). Further investigation of such life-history traits with regards to soil contamination and physical disturbance may elucidate the causes of the relationships observed here. For example, in subarctic springtails, life-history traits have been linked to fire disturbance response (Malmström, 2012). Taxa living on the soil surface were more resilient than those found in deeper strata; sexually reproducing species were more resilient than parthenogenic species; and species with morphometry suggesting an ability for fast dispersal were more resilient than those without (Janion-Scheepers *et al.*, 2016).

Four introduced species of springtails were identified in this study. The three hypogastrurids (*C. denticulata*, *Hypogastrura viatica* and *H. purpurescens*) were common and abundant, particularly *C. denticulata* (Figure 3.1). The tendency for these species to be present in large numbers (as opposed to lone individuals) may reflect their use of pheromones to facilitate large aggregations (Mertens and Bourgoignie, 1977). The fourth introduced species, *Lepidocyrtus violaceus*, was the least common species identified in this study, found at only three of the 21 sampling locations (Figure 3.1). These three locations, however, were in areas relatively close to human infrastructure, and with known underlying petroleum contamination (Wasley, Mooney and King, 2015).

Previous studies have shown introduced springtail species to be associated with, and abundances perhaps even facilitated by, degraded or disturbed sites (e.g. Fountain and Hopkin 2004; Gabriel et al. 2001; Greenslade and Convey 2012; Leinaas et al. 2015). It is therefore expected that the presence of non-native species may be an indicator of disturbance. Indeed, in the case of Macquarie Island and other subantarctic islands (Kerguelen, Marion and South Georgia), introduced springtails have consistently been associated with human structures (Gabriel *et al.*, 2001; Greenslade and Convey, 2012). In this study, however, springtail abundance was not correlated with surface TPH concentration for any of the four introduced species, and only moderately correlated with TPH in the underlying soil (0.5 m depth) for two species (Table 3.1). These relationships were negative, so underlying contamination did not favour these two species. Indeed, the highest numbers of introduced springtails were found furthest from

the research station at the south-westernmost extent of the Isthmus, with no known history of petroleum contamination or disruption to the soil profile. In addition to their colonial nature, introduced hypogastrurids have been noted to negatively impact the abundance and diversity of other species (Terauds, Chown and Bergstrom, 2011; Leinaas *et al.*, 2015), thereby further altering the community dynamics. Finally, introduced springtails – including other hypogastrurid species – have been reported to prefer moist conditions with high organic content (Gabriel *et al.*, 2001; Leinaas *et al.*, 2015), such as those provided by locations with higher vegetation cover. In this light, the more disturbed locations sampled in this study with reduced vegetation cover may simply not provide suitable habitat for the particular introduced species present on Macquarie Island.

Introduced plant species have also been noted to influence introduced springtail assemblages, particularly with regards to their commensal relationship to each other. For instance, Leinaas et al. (2015) noted a cascading effect of disturbance on springtail community dynamics, whereby disturbance (grazing) promoted the dominance of an introduced species of plant, which in turn facilitated the dominance of an introduced hypogastrurid over native springtail taxa. However, in contrast to this finding, the only introduced plant species recorded in this study (the short grass, *Poa annua*) was not a significant influence on springtail assemblages (Table 3.2).

3.4.3. Ecotoxicity, contaminant management and risk assessment

It is clear from previous studies that the native fauna and flora of Macquarie Island vary greatly in their sensitivity to petroleum contaminants in soil (Chapter 2). For SAB that has undergone a short period of aging, the range of sensitivity lies between 52 and 13,000 mg kg^{-1} TPH for a native earthworm and a grass, based on EC_{20} and IC_{50} values respectively (Bramley-Alves et al. 2014; Mooney et al. 2013). In light of this, the lack of an effect on springtail assemblages caused by soil TPH concentrations identified by this study was unexpected. However, a more recent field-based study indicated relatively little effect of soil TPH concentrations on soil invertebrates (Wasley, Mooney and King, 2015), similar to the findings presented here. Furthermore, the soil TPH concentrations detected in this study were generally quite low compared to previous field surveys of soils on Macquarie Island (Rayner *et al.*, 2007; Wasley, Mooney and King, 2015), which may reflect natural biodegradation over time as well as the success of contaminant management works that have been underway since 2003. Some TPH concentrations measured in this study were still very high (> 30,000 mg kg^{-1}) though, well above the

threshold concentrations identified for other taxa by previous laboratory-based studies (Chapter 2). Indeed, remediation guidelines for the C_{10}-C_{25} fraction in other cold-climate jurisdictions range from 100 mg kg^{-1} in Norway, to 12,500 mg kg^{-1} in Alaska (Snape *et al.*, 2008). This may reflect the limitations of aging contaminants under non-field conditions, or the possibility that traditional testing methods are prone to over-estimating toxicity (Alexander, 2000).

Alternatively, the findings of this study may add to a mounting body of work indicating that TPH concentration alone is not a strong indicator of toxicity to soil biota (e.g. Al-Mutairi et al. 2008; Coulon et al. 2005; Jiang et al. 2016), and as such may be an inadequate metric for assessing real-world rehabilitation requirements for a number of related reasons. First, TPH provides no indication of which hydrocarbons are present, nor how toxic or how bioavailable they are. This is pertinent to this study because some sampling locations with no known history of petroleum contamination returned relatively high surface TPH concentrations, but low concentrations at a depth of 0.5 m. Surface soils on Macquarie Island have generally been shown to be clean (< 200 mg kg^{-1} TPH), even above a highly contaminated plume (> 5000 mg kg^{-1} TPH), because highly dynamic subsurface movements carry the contaminant plume into new areas (Rayner *et al.*, 2007; Wasley, Mooney and King, 2015). As such, some of the high TPH concentrations reported in this study may not have actually been from a petroleum source. Indeed, a number of these locations are used by moulting elephant seals and crèching penguin colonies (pers. obs.), whose faeces have been reported to introduce high quantities of sterols and especially phytol to the soil surface, the latter close to the range of SAB (C_9-C_{18}; Huang et al., 2011). Other possible naturally occurring contributors to TPH include low molecular *n*-alkanes, *n*-alkenes and alkanals, which are known to occur in Macquarie Island soil (Huang et al. 2011), and which may be derived from algae, bacteria or plant material. Similarly, TPH provides no indication of how degraded (and thus resilient to further biodegradation) the contaminant is. Furthermore, microbial catabolism of hydrocarbons via oxidation may produce toxic intermediaries (e.g. acids or alcohols). Some of these intermediaries could be a component of TPH if the compounds are GC-resolvable, but highly functionalised and polar compounds may not be detected by GC-MS, and thus would not be included in TPH.

This study has shown that subantarctic soil invertebrate communities may be sensitive to physical disturbances of the soil, particularly with respect to how such disturbance might affect vegetation and bulk density. This finding may have implications for

contamination management, as it indicates that the physical disturbance to soils inherent in some *in situ* remediation techniques (e.g. biopiles, landfarming or ventilation) should be taken into account in decision-making processes. Soil invertebrate communities were also found to be sensitive to pH and conductivity, so remediation methods that involve the addition of nutrients to soils in the form of nitrogen salts may also be counter-productive to restoring the health of soil invertebrate communities (Walworth *et al.*, 2007). The potential for the toxic effects of a petroleum spill to be exacerbated by remediation works has rarely been considered in previous research. For environmentally sensitive, remote sites such as Macquarie Island, suitable remediation options available to land managers are restricted: the *ex situ* treatment of soil is unfeasible for logistical and environmental reasons, while many *in situ* techniques may also impact soil communities. However, less intensive contamination management techniques do exist (Chapter 2). Due to the strong association between invertebrate communities and plant cover, our findings suggest that methods that use or maintain plant cover are likely to expedite the recovery of the soil ecosystem. For example, the subantarctic tussock grass *Poa foliosa* has been demonstrated to show strong potential for phytoremediation (Bramley-Alves *et al.*, 2014), while revegetating an area following the completion of remediation works may facilitate the re-establishment of soil invertebrate communities.

3.5. Conclusion

The most important factors influencing springtail assemblages in this study were soil properties such as bulk density, pH and conductivity, as well as coverage by some plant species. Importantly, petroleum contamination, whether at the soil surface or in an underlying plume within the soil profile, did not significantly affect springtail assemblages. These results may serve to inform environmental decision making generally: soil invertebrate communities are closely linked to vegetation coverage and soil properties, and activities that alter these factors will likely impact soil biota more broadly. The benefits of carrying out remediation works of any sort must be balanced against the impacts due to physical disturbance inherent in those works.

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Chapter 4:

A comparative study on the toxicity of weathered diesel to a subantarctic earthworm, *Microscolex macquariensis,* and a model earthworm species, *Eisenia fetida*

Statement of contribution

Contributor	Experiment design and execution	Field work	Geochemical analyses	Statistical analysis and interpretation	Revisions and editing
Ingrid Errington	100	100	50	70	60
Grant Hose				30	30
Cath King					10
Sarah Houlahan			50		

Soil and *Microscolex macquariensis* were collected under permits ES15206 and FA13981 from the Tasmanian Department of Primary Industries, Parks, Water and Environment.

Abstract

The use of model test organisms simplifies many types of biological testing, but the application of these tests to real-world ecotoxicological scenarios may be limited. This may be of particular relevance to the development of rehabilitation targets for diesel-contaminated soils on subantarctic Macquarie Island, because the unique biogeographical context there may render guidelines developed for other locations unsuitable. In this study, the toxic response of two species of earthworm was compared in terms of survival, cocoon production, mass change and avoidance behaviour. *Microscolex macquariensis* is endemic to Macquarie Island and is a candidate species for ecotoxicological testing, but cultures are difficult to establish. *Eisenia fetida* is a commonly used model test organism that is easily cultured, and which may be a suitable substitute for *M. macquariensis*. To investigate this possibility, diesel was added to natural soils from Macquarie Island and aged for 23 weeks, before being mixed with clean soil to create a range of test concentrations.

Survival was not significantly affected within the six-week exposure period in either species at 19 mg total petroleum hydrocarbons (TPH) kg^{-1} dry soil, the highest concentration tested. Cocoon production in *M. macquariensis* was more than twice as sensitive as in *E. fetida* (EC_{10} of 3.12 mg kg^{-1} and 8.11 mg kg^{-1} respectively), though the response of *E. fetida* was hormetic in nature. The mass of both species increased in all treatments, again indicating a possible hormetic effect. Finally, in the avoidance test, both species showed a preference for contaminated soils over control soils. Combined, these findings indicate that the addition of diesel to the soil may increase soil carbon, thereby stimulating microbial activity and increasing the amount of food available to the worms. The responses of the two species were not sufficiently similar that *E. fetida* could be used as a surrogate for *M. macquariensis* in future testing.

4.1. Introduction

The use of model test organisms simplifies comparisons of results between studies, allowing trends to be more easily recognised and theories to be developed, while simplifying and standardising methods for the culturing of sufficient individuals for testing (Ankeny and Leonelli, 2011). These benefits remain true for ecotoxicological investigations, but the real-world application of results obtained using model species may be limited because different species will be affected by a toxicant in different ways, and because the natural distribution range of model test species is limited. Such differences may be of particular importance when developing rehabilitation targets for contaminated land, and for this reason it is vital that ecotoxicological tests be conducted for a range of locally-occurring taxa under local environmental conditions (van Gestel, 2012).

Contamination of soils by petroleum is known to occur on a number of subantarctic islands, where the unique cold, wet climate greatly reduces rates of natural biodegradation (Section 2.3). These islands that occur within a few degrees of the subantarctic convergence (Figure 1.1) are of high conservation value because they are the only land on which many marine mammals and birds can breed in the Southern Ocean (Selkirk, Seppelt and Selkirk, 1990). For this reason, there is a strong imperative to rehabilitate contaminated land in these regions, but the body of research to inform such works is currently limited. Like many of these islands, Macquarie Island has experienced a number of petroleum spills since the 1970s– predominantly of the light special Antarctic blend (SAB) diesel (Table 2.2). Rehabilitation and remediation efforts on Macquarie Island began in 2003 (Rayner *et al.*, 2007), but the ecotoxicological thresholds of native soil biota living in soil contaminated by highly weathered diesel fuel are largely unknown. This is, in part, because the island's remoteness limits access to native species for ecotoxicological testing. Such work is highly resource intensive, particularly because of the time-consuming nature of collecting sufficient numbers of native animals to conduct toxicity assays (Mooney, 2013). As such, there is an incentive in this circumstance to use model species in place of native species, if possible.

Common toxicity test guidelines for terrestrial earthworms, such as those developed by the International Organization for Standardization and the Organisation for Economic Co-operation and Development (e.g. ISO 2008; OECD 2004), have primarily focussed on European species such as *Eisenia* spp., *Enchytraeus* spp. and *Lumbricus* spp.. However, it

is unclear whether it would be appropriate to extrapolate the results of studies on these model species to inform remediation targets for subantarctic islands. The earthworm *Microscolex macquariensis* (Beddard 1896) is endemic to Macquarie Island, and was the subject of the first toxicity tests of petroleum products on multi-cellular terrestrial species for any subantarctic island (Mooney, 2013; Mooney *et al.*, 2013). A representative of the *Microscolex* genus is native to most subantarctic islands (Dyne and Jamieson, 2004), so further study within the genus may be of use in the consideration of similar contaminated sites elsewhere in the region. Ecotoxicological studies conducted to date, however, focus on fresh SAB fuel, or spiked soils aged for only 2-4 weeks, which may not reflect the composition of fuel residues after decades of slow weathering (Mooney *et al.*, 2013; Macoustra *et al.*, 2015). Furthermore, these studies have examined relatively high total petroleum hydrocarbon (TPH) concentrations in the soil that would reflect those of a recent spill where little volatilisation or biodegradation has occurred. There is, however, recent evidence that TPH concentrations at the soil surface (where most soil invertebrates live) are relatively low on Macquarie Island (Wasley, Mooney and King, 2015). This may reflect the success of decades of remediation works, or perhaps natural processes such as the percolation of petroleum contaminants to deeper strata, the highly active movement of groundwater near sea level on Macquarie Island (Rayner *et al.*, 2007), and the lability of surface layers in high-wind conditions (Table 2.1). For these reasons, it is important to identify toxicity thresholds for native species, in weathered contaminants reflective of those occurring in contaminated field conditions and at low concentrations. This study aims to identify toxicity threshold concentrations in terms of mortality, reproduction and behaviour, for the earthworm *M. macquariensis* living in soil historically contaminated by diesel fuel. This study also aims to determine whether the commonly used model species, *Eisenia fetida* (Savigny 1826), might be an appropriate substitute species for future tests.

4.2. Methods

4.2.1. Study animals and husbandry

Microscolex macquariensis (Order: Haplotaxida; Family: Lumbricidae) is predominately an endogeic species, living in the upper few centimetres of the soil profile (Greenslade and van Klinken, 2006) and within decaying plant material near the surface (pers. obs.). The European earthworm *E. fetida* (Order: Haplotaxida; Family: Lumbricidae) is generally considered an epigeic species, predominately found at the surface litter layer

of the soil (Schaefer and Juliane 2007), but is commonly found at depths of up to 10 cm deep. As such, the two have overlapping habitats and likely similar ecological functions. *M. macquariensis* individuals were collected from uncontaminated soils on the southwest extent of the isthmus on Macquarie Island in March 2016. The collected worms were returned to Macquarie University and cultured in clean soil sourced from Macquarie Island. The *E. fetida* culture (supplied by Vermifert, Yass, Australia) was maintained in organic compost. Both species were fed hydrated oats *ad libitum* (OECD, 2004), and kept in a dark room at 11 ± 1°C.

4.2.2. Preparation of soil

Soil was collected from the southwest extent of the isthmus on Macquarie Island, away from the station and from any known anthropogenic contamination or disturbance in April 2015, before being transported to Macquarie University (Sydney, Australia) and stored at 3°C for seven months. The soil was homogenised, and nine subsamples were taken for soil property analyses. Approximately 10 g of soil from each sample was placed in a plastic jar with 50 mL of MilliQ filtered water making a 1:5 soil:water suspension (Rayment and Higginson, 1992). The samples were placed on an orbital shaker at 15 rpm for 60 minutes (Rayment and Higginson, 1992) before pH and conductivity were measured using hand-held meters and probes (Hanna Instruments, Rhode Island, USA). A further 65 g of soil was dried at 70°C to constant mass, and particle size was determined by placing approximately 55 g of soil in a mechanical sieve shaker for five minutes. This yielded three fractions: silt (< 63 µm), sand (< 2 mm) and gravel (> 2 mm). Total organic content (TOC) was determined by loss on ignition using the remainder of the dry soil (10 ± 0.5 g) by combustion at 550°C for 24 hours.

MilliQ water was added to the clean stock soil to amend the soil moisture to 15%. This soil was then mechanically mixed for nine minutes in 3 kg batches with a sufficient volume of SAB to achieve an initial concentration of 50,000 mg SAB kg^{-1} dry soil (Breville, Melbourne, Australia). This spiked soil was stored under a fume hood at a depth of 10 cm in plastic trays lined with aluminium foil. The fume hood was at ambient laboratory temperature (approximately 22 ± 2°C). This temperature was warmer than outside ambient temperatures on Macquarie Island (generally in the range of 2-9°C, though seasonably variable), thereby enhancing the rate of volatilisation and degradation. The soil was turned manually on a weekly basis, whereupon MilliQ water was also added to maintain moisture levels at 15%. This regime was maintained for 160 days before further preparation for each experiment.

4.2.3. Chronic toxicity and reproductive test

Chronic toxicity assays were based on the OECD guideline for the testing of chemicals 222 (OECD 2004). The aged, contaminated soil (Section 4.2.2) was mechanically mixed with clean soil from the same field source to prepare a gradient of test concentrations, using eight clean:contaminated ratios (100:0, 75:25, 50:50, 25:75, 13:87, 6:94, 3:97 and 0:100). This method of soil preparation was selected in preference to initially adding SAB in different volumes to a number of stock soils (c.f. Macoustra et al. 2015; Mooney et al. 2013) because there is evidence that the initial contaminant concentration affects the rate of biodegradation and the hydrocarbon species present, which subsequently affects toxicity (Mooney *et al.*, 2013). Three replicate jars (75 mm Ø x 85 mm) were prepared for each concentration treatment, for each of the two species. When small quantities of soil were mixed in pilot studies, this created an uneven distribution of contaminant within the soil matrix. For this reason each treatment concentration was mixed as a single batch. In this manner, a total of 1845 g (dry weight equivalent) of soil was prepared for each treatment concentration. Three 15 g (dry weight equivalent) samples from each treatment were collected from random locations within the mixing bowl and frozen for later hydrocarbon analysis. The remaining soil was divided between six jars (300 g of soil per jar, dry weight equivalent).

For each jar, six sexually mature worms with a well-developed clitellum were randomly selected from the culture, cleaned, blotted dry, weighed, and then placed on the surface of the soil (50 g soil per worm, equivalent to that recommended by OECD guidelines (2004)). This process was repeated for *E. fetida* and *M. macquariensis*, for a total of 144 worms per species. An additional three jars were prepared with uncontaminated soil without any worms, in order to estimate how many worms or cocoons might naturally occur in the soil. The jars were then covered with aluminium foil secured by a rubber band and returned to the dark room maintained at 11 ± 1°C. Worms were fed hydrated oats *ad libitum*. The foil was removed from the jars once a week for six weeks to remove and replace uneaten oats, to allow for aeration, and to maintain soil moisture at 15%.

After six weeks, surviving worms from each jar were counted, rinsed with water, blotted dry and re-weighed. The soil was returned to the jar and incubated for a further six weeks to allow juveniles to hatch from any cocoons. It was assumed that any further biodegradation throughout the testing period would not be significant, so no final sample for geochemical analysis was taken at this stage as this would likely remove some juveniles and cocoons. Jars were then placed in a warm water bath (40 ± 2°C) to

encourage any hatched juvenile worms to rise to the surface. Juvenile worms were counted until no more appeared at the surface for 30 minutes. The soil was then wet-sieved through 0.5 and 1 mm gauge sieves to assist in finding cocoons. Any cocoons were placed in a beaker of water, with those that floated being recorded as hatched, and those that sank as unhatched (OECD 2004). The soil-only control (with no worms added) contained an average of 1.5 hatched and 5.5 unhatched *M. macquariensis* cocoons per jar, and these values were subtracted from cocoon counts for each replicate before analysis. As expected, no *E. fetida* cocoons were found in the soil-only control. Cocoons of *M. macquariensis* were approximately 1 mm in diameter and dark brown in colour, whereas those of *E. fetida* were approximately 3 mm in diameter and lighter in colour.

The endpoints measured in this test were:

- Adult survival after six weeks.
- Adult mass change.
- Number of juveniles and cocoons (hatched or unhatched) after 12 weeks.

4.2.4. Avoidance test

This test was derived from the International Standard 17512-1:2008 (ISO, 2008). As for the chronic test (Section 4.2.3), aged contaminated soil (Section 4.2.2) was mechanically mixed with clean field-collected soil to prepare a gradient of eight clean:contaminated ratios (100:0, 75:25, 50:50, 25:75, 13:87, 6:94, 3:97 and 0:100). In this way, a total of 1545 g (dry weight equivalent) of soil was prepared for each treatment. Three 15 g samples from each treatment were collected from random locations within the mixing bowl and frozen for later hydrocarbon analysis. Using a plastic divider in the middle of a plastic container (120 x 170 x 35 mm), uncontaminated control soil was placed in one half and contaminated test soil placed in the other (250 g on each side). Three replicate containers were prepared for each treatment, for both species.

The plastic divider was removed, and five sexually mature worms of one species were placed on the centre line with some part of their body touching both soil types (120 worms per species). Plastic lids were secured to each container before being returned to the dark temperature-controlled room. All containers were aligned along the same axis of the room, but rotated randomly to control for any directional effects within the room.

After four days the test and control soils were separated by re-inserting the divider, and the number of worms on both soil types was recorded. Worms cut in half by the divider

were recorded as 0.5 on each side (this occurred in four of the 24 *M. macquariensis* containers, and six of the 24 *E. fetida* containers). Worms found on the lid of the container were considered to be displaying avoidant behaviour, and as such their number was combined with those found in the uncontaminated soil. It was assumed that no significant change in contaminant concentration occurred during the short duration of this experiment, so the concentrations used for analysis were those taken immediately before the start of the experiment.

4.2.5. Geochemical analysis of soil

Each 15 g soil sample was placed in a beaker with 30 mL of a 9:1 mix of dichloromethane: methanol. The samples were solvent extracted using an ultrasonicator bath (Ultrasonics Australia, Sydney) for 25 minutes. The samples were allowed to settle before decanting the extract solution, and this sonication process was repeated a further two times. The three extracts were pooled and reduced to 0.5 mL using a nitrogen blow-down system. The remaining sediment was dried to constant mass at 70°C and removed from the oven one at a time for weighing, in order to calculate mg of contamination in terms of the mass of dry soil.

Elemental sulphur was removed from the soil extracts with activated copper. The extracts were further reduced to 0.2 mL, then passed through a heat-activated silica column to remove polar compounds. The column bed was set using 3.5 mL of 4:1 hexane:dichloromethane, and the extracts were passed through the column using a further 3 mL of the hexane:dichloromethane mixture. The resulting extract was reduced to 0.5 mL volume, and an injection standard containing cyclooctane, 1,4-dichrolobenzene, p-terphenyl-d14 and 1-bromoicosane or tetracosane was added to all extracts. These standards were chosen due to their differences in retention time, and because they are not typically found in petroleum products or indigenously in the environment.

The extracts were then analysed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890A GC coupled to a Leco Pegasus time-of-flight-MS and an Agilent 7683 ALS auto-sampler (Santa Clara, CA, USA). The extracts were injected (1 µL) into a split/splitless injector operating at 310°C in splitless mode onto a J&W DB5MS column (60 m × 0.25 mm i.d., 0.25 mm film thickness) coated with modified 5% phenyl 95% methyl silicone, with helium as the carrier gas (1.5 mL/min, constant flow). The temperature programme was 40°C (held for two minutes), increasing to 310°C at a rate of

4°C/min (held for 45 minutes). The detector voltage of the MS was between 1600 V and 1850 V.

The concentration of all compounds within the C_{9-18} range was measured, reflecting that of fresh SAB diesel (Brown *et al.*, 2016), calculated relative to the injection standards. The dry sediment mass was used to calculate TPH concentrations in milligrams of contaminant per kilogram of dry soil (mg kg^{-1}), with any mass lost in the extraction process considered to be negligible.

4.2.6. Statistical analyses

Measured TPH concentrations were compared to nominal dilution ratios using a Pearson's correlation analysis to verify the accuracy of measurements (Minitab Inc., 2010). Subsequent analyses were conducted using measured concentration values. All further analyses were carried out using the DRC package version 3.0-1 (Ritz and Streibig, 2005) in R version 3.3.1 (R Core Team, 2016). Assumptions of normality and homogeneity of variance were examined using q-q plots and plots of residuals, respectively. Concentration response curves were then estimated by fitting a four parameter, nonlinear regression model. A series of log-logistic, log-normal and Weibull curves were fitted to the data for three chronic responses for each species: survival, mass change and cocoon production. The Akaikes information criterion of each fitted curve were compared to determine the best fitting model for each response type. The parameters of the model were estimated with maximum likelihood, using starter values determined by the program's self-starter tool. A similar process was used to analyse avoidance behaviour, using the proportion of worms found in the contaminated side of the soil as the response variable and dose as the explanatory variable (Ritz and Streibig, 2005). In these cases, a three parameter log-logistic curve was not a significant improvement on a two parameter curve when compared with an ANOVA test, so analyses were based on the simpler two parameter curve. Effective concentration values (EC10 and EC50) were extrapolated from the fitted curve, and, where possible, compared between species using a confidence interval ratio test ($\alpha = 0.05$; Wheeler et al. 2006).

4.3. Results

4.3.1. Soil physico-chemical properties

Soils were slightly acidic and predominantly sandy, containing only small fractions of silt or gravel (Table 4.1). Organic content was low at approximately 3% (Table 4.1).

Though the measured TPH concentrations were very low for the highest spiked concentration (mean ± SD: 19.2 ± 1.5 and 42.5 ± 6.8 mg kg^{-1} for the chronic and avoidance assays respectively; n = 3), the measured values were still significantly correlated with those that would be expected from the nominal dilution ratios for the chronic (Pearson's r = 0.06, p < 0.001) and avoidance (r = 0.85, p < 0.001) tests. This represents a loss of three orders of magnitude from the original addition of 50,000 mg SAB kg^{-1} dry soil. The mean pristane:C_{17} ratio of the stock spiked soil was 2.8 ± 0.1, and 0.9 ± 0.0 for the phytane:C_{18} ratio, indicating a high degree of biodegradation.

4.3.2. Toxicity responses

4.3.2.1. Control mortality

There was no mortality for the model species *E. fetida* in the control treatment. Control mortality for the endemic Macquarie Island species, *M. macquariensis* was, on average, 22%, ranging from zero to two worms out of six dying before the end of the six-week exposure period. It is not possible to know at what point during the exposure period these individuals died, as worms were concealed within the soil. Given the 100% survival observed in one control replicate as well as in some contaminated treatments, it was not deemed appropriate to correct for control mortality as per Abbott (1925). Pre-exposure handling may have been the cause of this mortality, rather than the test conditions *per se*. As such, further analyses were continued, despite control mortality being higher than the guideline for test validity of less than 10% (OECD 2004).

4.3.2.2. Chronic toxicity and reproduction

Survival of either species was not significantly affected at any TPH concentration tested (Table 4.2). Despite this, however, mortality in *E. fetida* did increase at the highest TPH concentration tested (19 mg kg^{-1}), suggesting that this concentration was approaching the threshold of survival for this species. A yellow exudate of coelomic fluid was observed on the epidermis of some *E. fetida* individuals in treatments with a TPH concentrations higher than 8 mg kg^{-1}.

Table 4.1: Properties of test soil.

Property	Mean (SE)
pH	6.39 (0.14)
Conductivity (µS)	171.89 (19.21)
Total organic carbon (%)	3.08 (0.29)
Particle size (%)	
Silt (<63 µm)	0.22 (0.05)
Sand (63 µm - 2 mm)	97.84 (0.32)
Gravel (> 2mm)	1.94 (0.33)

Table 4.2: Effective concentration toxicity thresholds (mg kg^{-1}) of aged diesel for two species of earthworm, *Eisenia fetida*, a commonly used model species, and *Microscolex macquariensis*, endemic to subantarctic Macquarie Island.

Species	ECx	Survival	Total Cocoons	Mass change	Avoidance
Eisenia fetida	EC10	> 19*	8.11 (7.37 – 8.84) p <0.001	10.75 (0.88 – 20.61) p = 0.99	ND
	EC50	> 19*	8.31 (7.98 – 8.63) p <0.001	18.78 (0.00 – 92.12) p = 0.90	ND
Microscolex macquariensis	EC10	> 19*	3.12 (2.28 – 3.95) p <0.001	0.01 (0.00 – 0.24) p = 0.99	1.09 (0.00 - 3.44)
	EC50	> 19*	4.62 (3.03 – 6.21) p <0.001	0.14 (0.00 – 2.35) p = 0.90	116.87* (0.00 - 426.98)

EC10 and EC50 values indicate the TPH concentration in soil that caused an effect in 10 and 50% of the test animals, respectively. Values in parentheses indicate 95% confidence limits; lower limits were rounded up to zero if negative. * indicates EC estimates that exceed the highest test concentration. ECx values were compared between species using a confidence interval ratio test, and the significance of this comparison is provided as a p value. ND indicates that an ECx value could not be determined.

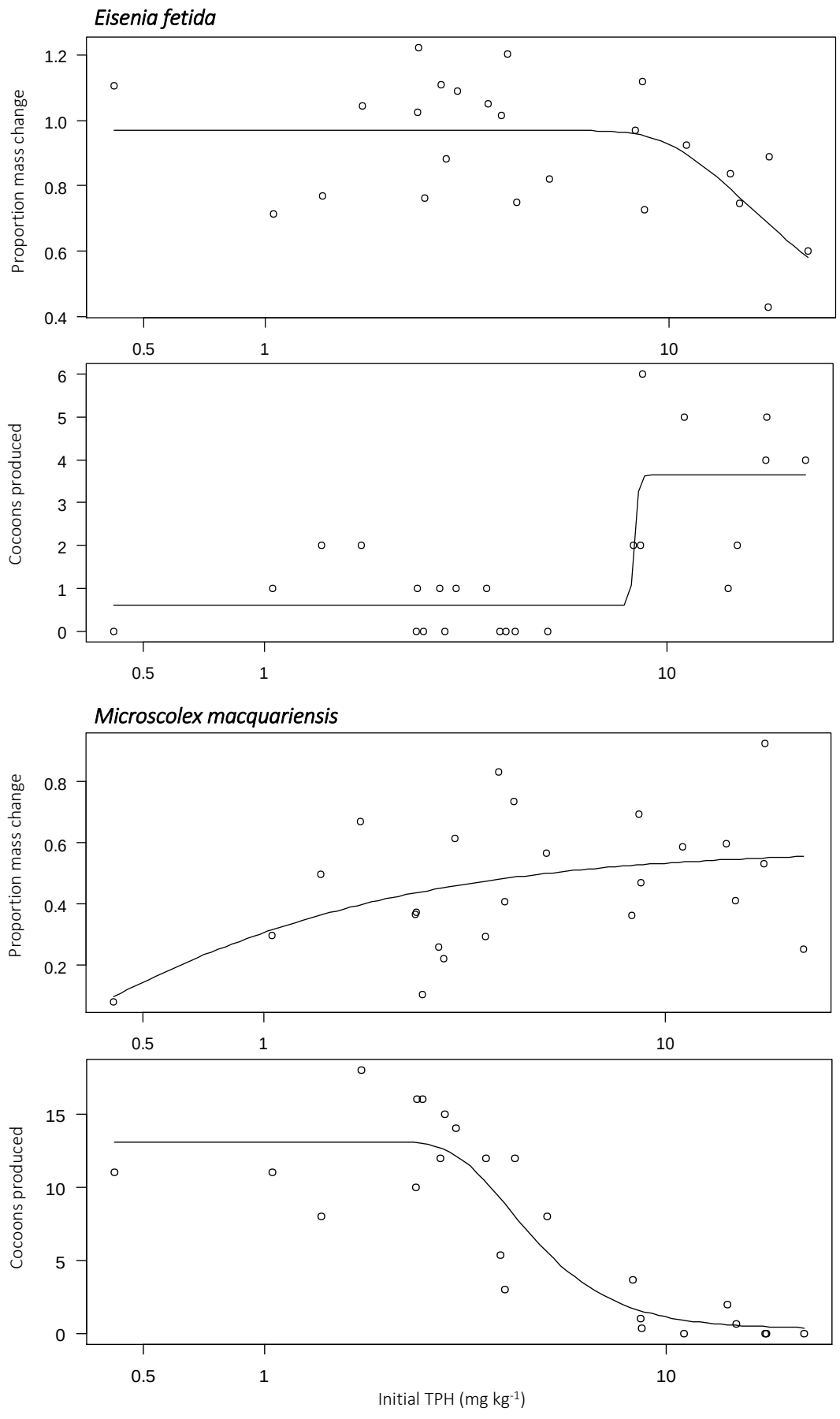


Figure 4.1: Dose-response curves for sub-lethal endpoints, for two species of earthworm.

For sub-lethal effects and end points, *M. macquariensis* was generally more sensitive to TPH than was *E. fetida*. The EC10 and EC50 values for cocoon production were significantly different between the two species (Table 4.2). Cocoon production in *M. macquariensis* declined with increased TPH concentration, and zero cocoons were produced in the highest treatment (Figure 4.1). In contrast, for *E. fetida*, TPH exposure appeared to stimulate a potentially hormetic response: cocoon production increased approximately five-fold, but with no subsequent reduction in cocoon numbers within the concentration range tested (Figure 4.1). The percentage of hatched cocoons and number of juveniles in each treatment were highly variable and showed no correlation with TPH concentration across both species.

Body mass of both species increased throughout the six-week exposure period in all treatments and in controls (Figure 4.1), suggesting that test animals, though sexually mature, were still relatively young. This increase in size, however, was more pronounced in contaminated treatments, again indicating a stimulatory or possible hormetic effect (Figure 4.1). The relative increase in mass was greater in *M. macquariensis* than in *E. fetida*. For *M. macquariensis,* individuals in treatments of approximately 5 mg kg^{-1} were on average 45% larger than those in the control soil, whereas for *E. fetida,* mass increase peaked at 20% larger at approximately 3 mg kg^{-1} (Figure 4.1). However, mass gain was highly variable in both species, so the difference in ECx values between species was not significant (Table 4.2).

4.3.2.3. Avoidance behaviour

A greater proportion of worms were found in the contaminated half of the container in the avoidance test for both *E. fetida* and *M. macquariensis* for most treatment concentrations, indicating a habitat preference for the contaminated soil (Figure 4.3). EC10 values determined for *M. macquariensis* were very low, while EC50 values exceeded the highest concentration tested. This disparity may be indicative of the need for a wider range of test concentrations, or may reflect the high variability recorded in these assays (Table 4.2). No meaningful concentration response curve could be fitted for avoidance behaviour in *E. fetida*.

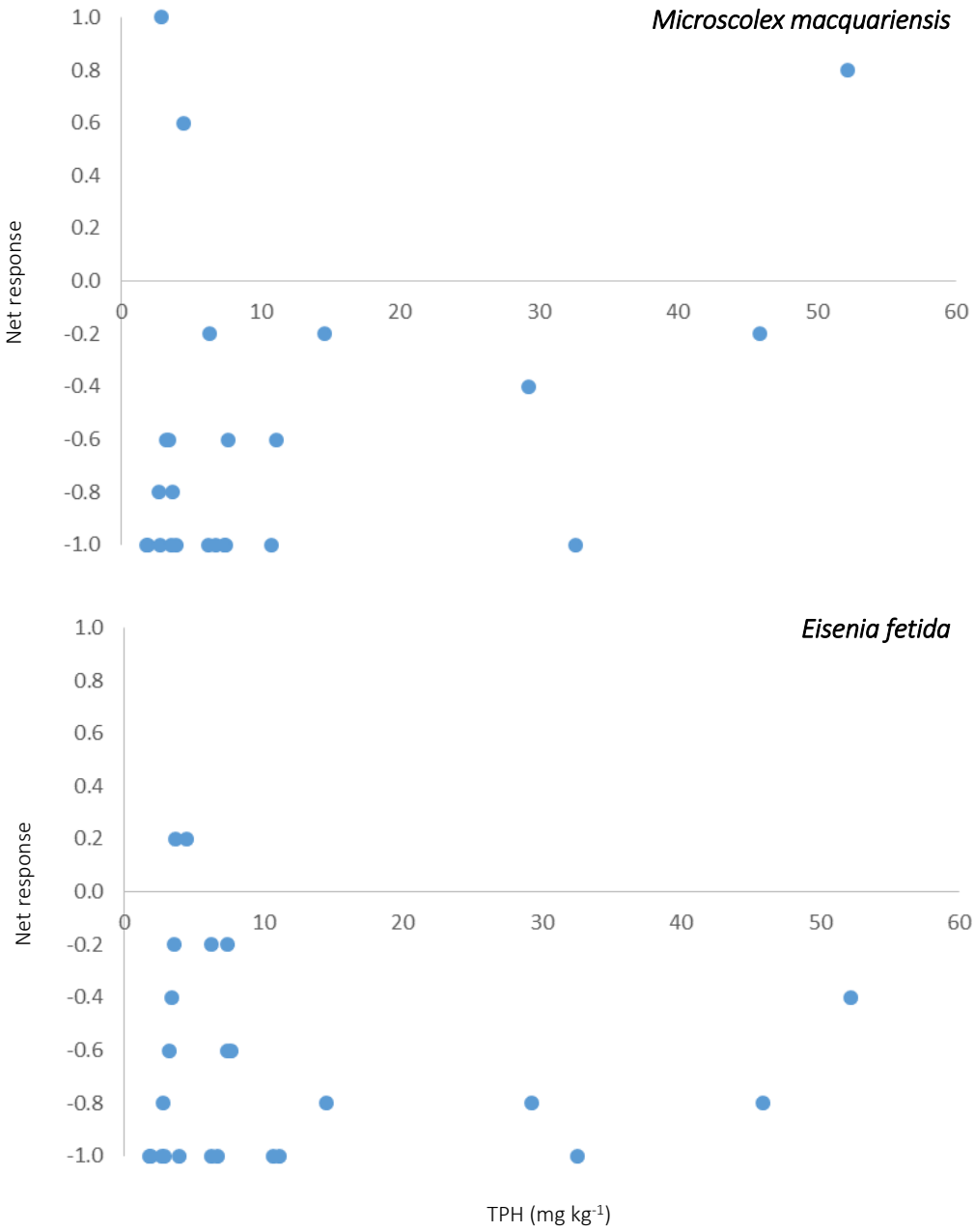


Figure 4.2: Net response avoidance behaviour in two species of earthworm.

NR = (A-T)/n × 100, where NR = net response, A = number of worms found avoiding the test soil, T = the number of worms found in the test soil, and n = the total number of worms. A positive NR value indicates avoidant earthworm behaviour, whereas a negative value indicates earthworm preference for the test soil.

4.4. Discussion

4.4.1. Responses to fuel contamination

Soil TPH concentrations were lower than anticipated after 23 weeks of aging, with only 0.05% remaining from an initial nominal concentration of 50,000 mg kg^{-1}. Few previous studies have aged soils freshly amended with diesel under laboratory conditions, and of those that have, most opted for an aging period of less than one month (e.g. Macoustra et al. 2015; Mooney 2013; Zytner et al. 2006). Consequently, there are no similar studies with which to compare these results. However, given the strong correlation between the measured concentrations and those expected from the nominal dilutions, the test soils can be considered well mixed, so discrepancies are unlikely to be caused by a heterogeneous distribution of contaminants. Ultrasonication with DCM and methanol in a 9:1 ratio is an established method for hydrocarbon extraction (Hoshino *et al.*, 2015), so while it is possible that other solvent mixtures may have been more appropriate to this particular circumstance (e.g. Berset et al. 1999; Guerin 1999), there is no reason to doubt that these measured values are a reasonable representation of TPH concentration. Furthermore, the resulting TPH concentrations here closely reflect those occurring in surface soils in known contaminated areas on Macquarie Island (Wasley, Mooney and King, 2015).

The survival of both species was not affected at the low concentrations tested. This result was not unexpected in light of a related study, which identified an LC50 value of approximately 1364 mg kg^{-1} for *M. macquariensis* in SAB-contaminated soils aged for only four weeks (Mooney *et al.*, 2013), compared to the 23 weeks of aging in the present study. In that study, SAB toxicity increased with the degree of aging for some scenarios. However, fuel toxicity is generally considered to decrease with weathering, either because the most acutely toxic compounds volatilise soon after a contamination event (Atlas, 1981), or because the compounds that are not biodegraded are inherently not bioavailable (Alexander, 2000). For this reason, the findings of Mooney et al. may have been an artefact of the experiment design whereby a rapid decrease of TPH concentration in freshly contaminated soils counteracts the higher toxicity of more reactive hydrocarbon species (Mooney *et al.*, 2013). Also, Mooney et al. (2013) used a soil with a higher organic content (48%, compared to 3% in the present study). Such high organic matter content is known to greatly ameliorate the toxic effects of petroleum contaminants (Mooney *et al.*, 2013). As such, it is possible that the toxicity thresholds

determined by Mooney et al. (2013) may be an underestimate of those needed for more highly weathered diesel in carbon-poor soils.

The mass of *M. macquariensis* was positively correlated with TPH concentration, whereby worms increased in mass by approximately 45% at 5 mg kg^{-1} relative to controls. This increase in mass was maintained even at the highest concentrations tested. *E. fetida* mass was up to 20% greater in treatments than controls, but this effect was quite clearly hormetic, with average worm mass being greatest at a TPH concentration of approximately 3.1 mg kg^{-1}, and decreasing below that of control treatments at approximately 8.5 mg kg^{-1}.

In *E. fetida*, increasing TPH concentrations were also associated with a five-fold increase in cocoon production at the highest TPH concentration tested relative to the controls. No such trend was observed for cocoon production in *M. macquariensis*, which followed a negative sigmoidal exposure response, with no stimulatory effect detected at the concentrations tested. These results indicate that cocoon production in *M. macquariensis* is more sensitive to highly weathered SAB contamination than it is to SAB-contaminated soils that have only been aged for four weeks, which had an EC_{20} value of 1089 mg kg^{-1} (Mooney *et al.*, 2013). This EC_{20} value is two orders of magnitude greater than an EC_{20} calculated for comparability for this study (3.5 mg kg^{-1}) following an aging period of 23 weeks.

The exudation of coelomic fluid on the epidermis of *E. fetida* is a known indication of stress in the species (Zirbes *et al.*, 2012), and was observed on some individuals at concentrations above 8 mg kg^{-1}. However, such a response has only previously been noted in response to pathogens or predators (but see Bundy et al. 2001), and no reports are known of such a response in toxicity assessments. It is unclear whether this exudate reflected a toxic response to petroleum contaminants *per se* in this study. The response was, however, consistent with other sub-lethal endpoints, so the observation here may emphasise the utility of coelomic fluid as a stress indicator in *E. fetida*. No coelomic fluid exudate was observed on the epidermis of *M. macquariensis* individuals.

Both *M. macquariensis* and *E. fetida* moved towards the contaminated soil at the low TPH concentrations tested in this study. Taken together with the negligible impact of SAB on survival, as well as its stimulatory effect on cocoon production and mass, these results may indicate a response to a changed habitat more so than an over-compensatory physiological response, as is the primary accepted mode of hormesis

(Calabrese, 2005). Specifically, earthworms may be responding to the increase in soil carbon with the introduction of petroleum hydrocarbons, particularly in the case of naturally low-carbon soils, such as that tested here. As more carbon is available for metabolic assimilation by earthworms as they ingest soil, worm mass would intuitively increase, leading to a likely increase in fecundity (Hartenstein, Neuhauser and Kaplan, 1979). The addition of weathered petroleum to soil may increase worm mass by one of two paths. First, petroleum hydrocarbons introduced to the soil may act as a direct food source for the earthworms, though there is limited evidence available regarding their ability to metabolise such compounds (Alexander, 2000). More likely, though, is a secondary response to the stimulation of soil bacterial or fungal biomass, which is known to be a primary food source for earthworms. It is well established that some microbial groups are able to metabolise petroleum hydrocarbons, and that these communities are stimulated at low to medium TPH concentrations (van Dorst *et al.*, 2014). As such, microbial biomass is more likely than 'raw' petroleum hydrocarbons to be the food source responsible for the increase in earthworm mass evident in this study.

4.4.2. Implications for contaminant management

The findings of this study indicate a sub-lethal response in *M. macquariensis* at soil TPH concentrations far below that of current guidelines for a range of comparable jurisdictions. While caution must be taken when extrapolating the findings of laboratory-based toxicological studies to real-world scenarios, particularly when incorporating the uncertainty of a weathered contaminant (Alexander, 2000), the existing maximum concentration for petroleum hydrocarbons in the C_{10}-C_{25} fraction for other cold-climate jurisdictions range from 100 mg kg^{-1} in Norway, to 12,500 mg kg^{-1} in Alaska (Snape *et al.*, 2008), over two orders of magnitude greater than the low effect concentrations (EC_{10} values) determined here.

In this study, however, some of the low EC_{10} values reflect hormesis at TPH concentrations far lower than those commonly tested. Hormesis is a controversial issue within the toxicology field, and the relevance of the phenomenon to real-world ecosystems has been a topic of intense discussion in the past decade (Chapman, 2002; Calabrese, 2005; Kefford *et al.*, 2008). This is particularly the case when considering how to incorporate hormetic effects within the ecological risk assessment process. Indeed, perhaps because of the controversy, no field-based assessments of hormesis in contaminated lands are known to exist (see also: Chapman 2002). The potential impact, however, of a key soil architect being substantially larger in size or producing many

more offspring warrants further investigation. Moreover, the burrowing of earthworms through the soil is known to increase the biodegradation rate of petroleum hydrocarbons, sometimes termed vermiremediation (Hickman and Reid, 2008). This is thought to occur because earthworm locomotion increases soil aeration, while the ingestion of soil exposes contaminants to microbes in the earthworm gut and liberates contaminants trapped in interstitial soil gaps (Schaefer and Juliane, 2007; Hickman and Reid, 2008). As such, a hormetic increase in the size and abundance of earthworms may actually be beneficial in terms of promoting bioremediation. This may be particularly the case in light of the avoidance test results, where *M. macquariensis* was attracted to soil contaminated with weathered SAB. Unfortunately, a concurrent treatment containing contaminated soils but no worms was beyond the scope of this study, but such an addition in future experiments could determine whether members of the *Microscolex* genus may be candidates for targeted vermiremediation of contaminated soils on subantarctic islands.

4.5. Conclusion

The commonly used model species of earthworm, *E. fetida,* differed in its response to weathered SAB contamination compared with *M. macquariensis,* which is endemic to subantarctic Macquarie Island. For this reason *E. fetida* should not be considered an acceptable substitute for *M. macquariensis* in toxicity testing assays. Furthermore, the low TPH concentrations tested in this study reflect those reported in surface soils of areas known to be contaminated by highly weathered petroleum on Macquarie Island. No mortality was observed in either species at the concentrations tested, but even extremely low concentrations elicited a substantial response with regards to body condition and fecundity. In some cases this response was hormetic in nature, and these findings contribute to the ongoing ecotoxicological debate regarding how stimulatory responses should be incorporated into the ecological risk assessment process.

Chapter 5:
The toxicity of petroleum hydrocarbon mixtures to the earthworms *Microscolex macquariensis* and *Eisenia fetida*

Statement of contribution

Contributor	Experiment design and execution	Field work	Geochemical analyses	Statistical analysis and interpretation	Proof reading and editing
Ingrid Errington	80	100	50	70	60
Grant Hose	10			30	30
Cath King					10
Sarah Houlahan			50		
Simon George	10				

Soil and *Microscolex macquariensis* were collected under permits ES15206 and FA13981 from the Tasmanian Department of Primary Industries, Parks, Water and Environment.

Abstract

Petroleum hydrocarbons are common contaminants in soils, nearly always occurring in complex mixtures such as diesel. The weathering of these mixtures may change their composition substantially, but it is difficult to discriminate which compounds remain in the residue. As such, the replication of field conditions for ecotoxicological studies is difficult, and a synthetic weathered diesel mixture for use in toxicity testing is a valuable alternative. In the present study, a mixture of six compounds (docosane, pristane, benz[a]anthracene, decalin, adamantane and diisopropyl naphthalene) representing different hydrocarbon types (*n*-alkanes, branched alkanes, polycyclic aromatic hydrocarbons, cycloalkanes and alkyl naphthalenes) was prepared to resemble the weathered diesel occurring in contaminated soils on subantarctic Macquarie Island. The toxic effects of this mixture were investigated using two species of earthworm: *Microscolex macquariensis*, endemic to Macquarie Island, and *Eisenia fetida*, a common model test species. The relative toxicity of each constituent hydrocarbon was also determined for *E. fetida*.

In general, *E. fetida* was more sensitive to the synthetic weathered diesel mixture than *M. macquariensis*. In the latter, survival, cocoon production, and mass were not affected by exposure to any test soils up to the maximum concentration tested (20,000 mg hydrocarbons kg^{-1} dry soil). For *E. fetida*, cocoon production was the most sensitive endpoint (EC_{10}: 393 mg kg^{-1}), followed by mass change (EC_{10}: 439 mg kg^{-1}). Unexpectedly, both species showed a preference for the contaminated soil over clean control soil, an effect that may be related to the hormetic response caused by some of the hydrocarbon compounds when tested individually. The joint mode of toxicity for the mixture did not strongly adhere to either a concentration addition or independent action model. The mixture tended towards antagonism for the mortality endpoint, but it was generally synergistic for sub-lethal effects. This variability may also be attributable to hormetic effects caused by some compounds.

5.1. Introduction

Petroleum hydrocarbons are common soil contaminants globally (Cermak *et al.*, 2010). These contaminants nearly always occur in a complex mixture of different hydrocarbon types, with changes in the nature and composition of that mixture over time depending on environmental factors. Lighter hydrocarbon compounds are lost to volatilisation, and simple hydrocarbons are broken down by soil microbes in a process known as biodegradation (Yang *et al.*, 2009). What remains is a residue of the hydrocarbon groups that have low volatility and high resistance to further biodegradation.

It is not simple to discriminate the constituent parts of this residue, and for this reason it is commonly referred to as the unresolved complex mixture (UCM; Brassington et al. 2007). Furthermore, a degree of uncertainty exists in understanding how toxic the UCM is to soil biota. Some advocate that these larger, more hydrophobic molecules are less bioavailable, and therefore pose little toxic risk (Alexander, 2000). Conversely, there are numerous examples whereby a reduction in TPH following remediation works of petroleum contaminated soils coincided with an increase in toxicity of the soil to microbes (Coulon *et al.*, 2005; Jiang *et al.*, 2016), plants (Al-Mutairi, Bufarsan and Al-Rukaibi, 2008; Jiang *et al.*, 2016) and macroinvertebrates (Mooney *et al.*, 2013). This may indicate that the UCM persists for so long in soil precisely because it is so toxic, and consequently unable to be catabolised by hydrocarbon-consuming bacteria and fungi. It is thought this increase in toxicity is caused by the production of intermediary metabolites during the biodegradation process, which may increase the mobility of the parent compound, thereby increasing its bioavailability (Ahn *et al.*, 2006; Zytner, Salb and Stiver, 2006).

Ecotoxicological testing for real-world petroleum contaminated soils is inherently complex. Field-based experiments are of greater similarity to what would occur in the natural environment than laboratory-based tests, but field experiments are often impracticable, and toxicant effects may be difficult to separate from environmental effects (Alexander, 2000). For this reason, ecotoxicological studies tend to be dominated by laboratory-based experiments. The difficulty associated with replicating field conditions *in vitro* means that these tests usually focus on a single contaminant and a single species. It is perhaps unsurprising, then, that results obtained under laboratory test conditions are often different to results obtained when the method is applied in the field (Zytner *et al.*, 2001). In light of these difficulties, as well as the uncertainty regarding UCM components and toxicity, there is value in developing a synthetic

weathered petroleum mixture to reflect existing field contamination for use in toxicity testing. Such a mixture, containing a number of known compounds that represent different hydrocarbon types, would simplify chemical analyses (Walker and Colwell, 1974; Zytner, Salb and Stiver, 2006). Furthermore, the subsequent investigation of each constituent compound as an individual toxicant may assist in identifying the most toxic portions of the UCM, and therefore the most relevant hydrocarbon groups on which to focus remediation efforts.

Many studies have tested mixtures of petroleum hydrocarbons, but the selection of the hydrocarbon compounds and their relative concentrations is often arbitrarily justified according to toxic units (e.g. Barata et al. 2005), or simply divided equally in the mixture (e.g. Walker and Colwell, 1974). Only one other study is known to have tested a consortium of hydrocarbon compounds developed to reflect that of a weathered petroleum spill, in that case investigating its effects on microbial respiration (Zytner et al. 2006). As yet, however, a model petroleum mixture has not been used to test the toxicity of weathered soil contaminants on macroinvertebrates. Petroleum hydrocarbons are generally accepted to have a narcotic mode of action (León Paumen *et al.*, 2009), and to have an additive effect in mixtures (e.g. Barata et al. 2005). If the molecular site of action of different contaminants is the same, then joint action when mixed is likely to be a function of simple concentration addition (Warne, 2003; Cedergreen *et al.*, 2008). However, some exceptions to narcosis in invertebrates do exist for hydrocarbon compounds (e.g. Sverdrup et al. 2002). If the molecular sites of action are different, then independent joint action may be a more appropriate model for the toxicity of a mixture (Cedergreen *et al.*, 2008; Phyu *et al.*, 2011).

The subantarctic Macquarie Island provides an ideal scenario in which to investigate the toxicity of weathered petroleum products on soil biota. A number of contamination events have occurred since the 1970s, with the most recent in 2002 resulting in 180 metric tons of soil that is highly-contaminated by special Antarctic blend (SAB; a light diesel fuel), with hotspots up to approximately 25,000 mg TPH kg^{-1} (Rayner *et al.*, 2007). However, the cold temperatures and water-logged soils typical of subantarctic islands greatly retard natural microbial biodegradation rates (see Chapter 2; Rayner et al. 2007), and *ex situ* remediation techniques are not possible because the biodiversity values and extreme remoteness render such methods both environmentally undesirable and logistically unfeasible (Chapter 2). An increasing body of work focused on Macquarie Island soil contamination and toxicity has been developed in recent years (Chapter 2),

and the endemic earthworm *Microscolex macquariensis* has emerged as a suitable species for ecotoxicological testing (Mooney *et al.*, 2013). Toxicity tests are well-defined for earthworms and have been applied broadly throughout the world (van Gestel, 2012), but were developed for the commonly used model test species, *Eisenia fetida* (Organisation for Economic Co-operation and Development, 2004; International Organization for Standardization, 2008). The use of model species can be valuable for several reasons, such as ease of culturing and comparability of results from different studies. However, it is uncertain how relevant toxicity thresholds obtained for a model species might be for an organism endemic to a unique environment such as that of Macquarie Island. Unfortunately, the field collection of a sufficient number of *M. macquariensis* individuals required for a full ecotoxicological assay is prohibited by logistical and time constraints (Mooney *et al.*, 2013), and cultures with this species are difficult to maintain in a laboratory (pers. obs.). As such, a case may be made for the substitution of the model species *E. fetida* in place of *M. macquariensis*, if petroleum contaminants can be demonstrated to illicit a similar response from the two species.

The aim of this study was to use an artificial mixture of hydrocarbon compounds to investigate the toxicity of highly weathered diesel fuels to soil invertebrates. Toxicity to two species of earthworm was compared: *M. macquariensis*, which is endemic to subantarctic Macquarie Island, and *E. fetida*, which is a commonly used model species for toxicity testing. The individual toxicity of each of the six constituent hydrocarbon compounds from the mixture was also tested for *E. fetida*, and this was used to determine the type of joint action for such a mixture. Aggregation behaviour in earthworms as an indicator of toxicity was also investigated as a potential new sensitive sub-lethal endpoint.

5.2. Methods

5.2.1. Study animals and husbandry

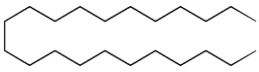
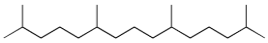
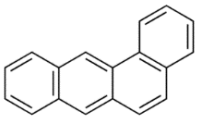
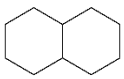
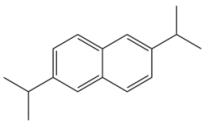
Microscolex macquariensis were collected from uncontaminated soils at the southwestern-most extent of the isthmus on Macquarie Island in March 2016. The collected worms were returned to Macquarie University and cultured in clean soil collected from Macquarie Island. *Eisenia fetida* were supplied by Vermifert (Yass, Australia) and were cultured in organic compost. Both species were fed hydrated oats *ad libitum* (OECD 2004), and soil moisture content was maintained by the addition of

MilliQ water. All cultures were kept in the dark, in a temperature-controlled room maintained at 11 ± 1°C.

5.2.2. Selection of hydrocarbon compounds for model weathered diesel mixture

The mixture components and their ratios were selected based on previous studies, which examined the chemistry of soils at contaminated sites at Macquarie Island (S. George, unpublished data). From this, six commercially available hydrocarbons (supplied by Sigma Aldrich) were used to create a model artificial weathered diesel mixture, representative of the primary hydrocarbon types identified from the contaminated soils at Macquarie Island (Table 5.1). The test concentrations for this mixture and for each individual compound were based on pilot studies (I. Errington, unpublished data).

Table 5.1: Composition of model weathered diesel mixture.

Compound type	**Representative compound**	**Structure**	**Mix proportion (mass %)**
***n*-alkanes**	Docosane		55.510
Branched alkanes	Pristane		44.408
Polycyclic aromatic hydrocarbons (PAHs)	Benz[a]anthracene		0.061
Cycloalkanes	Decalin		0.007
	Adamantane		0.007
Alkyl naphthalenes	Diisopropyl-naphthalene (DIPN; mixed isomers)		0.008

5.2.3. Preparation of test soils

The test concentrations of the mixture, and of each individual compound, were chosen to reflect the range of measured field concentrations on Macquarie Island and to allow for the assessment of sub-lethal effects (Tables 5.2, 5.3 and 5.4; Wasley et al. 2015). A solution was prepared for both the model weathered diesel mixture and for the individual hydrocarbon compounds, using *n*-hexane as a solvent. Each treatment received the same volume of solution (containing the required volume of appropriate toxicant stock solution and an additional volume of hexane).

The test soil matrix for this study was adapted from OECD guidelines (OECD 2004), combining quartz sand (washed and dried; Seymour's Building Supplies, Sydney), clay (Boral, Sydney) and certified organic compost (sieved to 4 mm and dried; Australian Native Landscapes, Sydney, Australia) in a ratio of 80:17:3 respectively. Each treatment concentration was mechanically mixed (Planetary Mixer; Breville, Melbourne, Australia) as a single batch before being divided between experimental containers. Replicate batches of soil were not prepared individually because pilot studies showed that mixing was uneven when small volumes of soil were prepared. For each treatment concentration of each compound, the hexane solution was added to the sand with sufficient milliQ water for a final soil moisture (by mass) of 15%. The sand and contaminant solutions were mixed for three minutes so that the test compound was uniformly distributed throughout the sand without binding to any clay or organic material. The clay and compost were then added to the mixer and combined for a further three minutes. Two 7 g soil samples were collected randomly from the mixing bowl for chemical analysis.

5.2.4. Chronic toxicity testing

Chronic toxicity assays were adapted from existing guidelines (OECD, 2004). Two replicates were prepared for each test compound, for each concentration (target concentrations for the mixture and for each compound are given in Tables 5.2 and 5.3). To this end, 300 g dry weight equivalent of the prepared soil was placed loosely into a glass jar (75 mm Ø x 85 mm), before being left open for 2-3 hours in a fume cupboard to allow the hexane solvent to evaporate. A solvent control using the maximum volume of hexane received by any treatment was also prepared to account for any solvent effects.

Sexually mature worms, each with a well-developed clitellum, were used for testing. For each replicate jar, six worms were randomly selected from culture, rinsed with water, blotted dry, weighed as a group (from which mean worm mass for that group was

calculated), and then placed on the surface of the soil. For the weathered diesel mixture, this process was repeated for both *E. fetida* and *M. macquariensis.* For the individual hydrocarbon assays, only *E. fetida* was used, because numbers of field-collected *M. macquariensis* were limited. An additional treatment was prepared without any worms, using non-spiked soil to estimate how many worms or cocoons might naturally occur in the soil. The jars were covered with aluminium foil secured by a rubber band and maintained at 11 ± 1°C. The foil was initially perforated with a pin, as per the methods of Roeloffs et al. (2016) with the far smaller worm, *Enchytraeus cripticus*, but this was observed to allow *E. fetida* to push through the perforations and escape the test jar. Perforated foil was therefore replaced by unperforated foil in week three of the test period. This initial problem increased variability in mortality data for some treatments, and may have caused an overestimation of mortality in extreme cases because it was not possible to distinguish a missing worm from a deceased one due to their rapid decay. The foil was removed from the jars once a week for the initial six weeks of the experiment to remove and replace uneaten hydrated oats, to allow for aeration, and to replenish lost soil moisture.

After six weeks, surviving worms from each jar were counted, washed, blotted dry and re-weighed in a group. Worms from treatments that had received the mixture of hydrocarbons were then placed, with no individuals touching, in a food-grade plastic container (120 x 170 x 35 mm). The movement of the worms was recorded using a video camera for five minutes.

Worm aggregation behaviour in the mixture assays was recorded in terms of the time taken for three or more worms to locate each other and remain together for the remainder of the video period. Any worms that escaped the container in this period were placed back near the centre of the container, again not touching any other individuals. Treatments in which worms had not formed an aggregation within the five-minute video period were recorded as having taken 300 seconds for analysis purposes.

Once the worms were removed, the soil was returned to the jar and incubated for a further six weeks to allow juveniles to hatch from any cocoons that had been laid. After six weeks, 7 g of soil was collected from the centre of each jar for chemical analysis. The percentage of each constituent hydrocarbon in the synthetic weathered diesel mixture remaining in the soil after the 12-week experiment period was calculated for the highest treatment concentration tested (20,000 mg kg^{-1}). This was used to determine the relative

impact of the two earthworm species on degradation of the mixture, and to compare the degradation of each constituent hydrocarbon.

Jars were then placed in a warm water bath (40 ± 2°C) to encourage any hatched juvenile worms to rise to the surface. Juvenile worms were counted until no more appeared at the surface for 30 minutes. The soil was then wet-sieved through 0.5 and 1 mm gauge sieves to assist in finding cocoons. Any cocoons were placed in a beaker of water, with those that floated being recorded as hatched, and those that sank as unhatched (OECD 2004).

The endpoints measured from this test were:

- Adult survival after six weeks.
- Adult mass change after six weeks.
- Number of juveniles and cocoons (hatched or unhatched) after 12 weeks.
- Adult aggregation behaviour after six weeks, recorded in terms of time taken for three or more earthworms to find each other and remain together.

5.2.5. Avoidance testing

This test was based on existing international standard guidelines (ISO 2008). Using a plastic divider in the middle of a plastic container (120 x 170 x 35 mm), uncontaminated control soil was placed in one half and contaminated test soil placed in the other (250 g dry weight equivalent on each side). Two replicate containers were prepared for each concentration treatment for both the mixture and for each individual compound (Tables 5.3 and 5.4).

At the start of the test the plastic divider was removed, and five sexually mature worms were placed on the centre line, ensuring that some part of their body touched both soil types (five worms per species per compound; 50 g of dry weight equivalent of each soil type available to each worm). Plastic lids were secured to each container before being returned to the temperature-controlled room. All containers were aligned along the same axis of the room, but the test side of each container for each concentration was alternated along that axis, to control for any directional effects within the room.

After 96 hours the test and control soils in each container were separated by re-inserting the divider, and the number of worms found in both soil types was recorded. Worms cut in half by the divider were recorded as 0.5 on each side (this occurred in only five of the 14 model weathered diesel mixture containers, and three of the 74 individual compound

containers). Worms found on the lid of the container were considered to be displaying avoidance behaviour, and as such their count was included with those found in the uncontaminated soil. It was assumed that no significant change in contaminant concentration occurred during the short duration of this experiment, so the samples taken to determine exposure concentrations were collected immediately before the start of the experiment (Tables 5.3 and 5.4).

5.2.6. Analysis of hydrocarbon concentrations

The two 7 g soil samples taken from each concentration prior to starting each assay were pooled prior to extraction, giving a total of 14 g of moist soil. Similarly, the 7 g samples taken from each replicate jar at the completion of the chronic test were also pooled before extraction.

Each 14 g sample was placed in a beaker with 30 mL of a 9:1 mix of dichloromethane: methanol. The samples were solvent extracted using an ultrasonicator bath (Ultrasonics Australia, Sydney) for 25 minutes. The samples were allowed to settle before decanting the extract solution. This sonication process was repeated twice more. The three extracts were pooled and reduced to 0.5 mL using a nitrogen blow-down system. A substantial quantity of fine clay was suspended in the extraction solution in each round of sonication, and it was therefore unavoidable that some of this was lost from the original sample in the decanting process.

Elemental sulphur was removed from the soil extracts with activated copper. The extracts were further reduced to 0.2 mL, and then passed through a heat-activated silica column to remove polar compounds. The column bed was set using 3.5 mL of 4:1 hexane:dichloromethane, prior to passing the extracts through the column using a further 3 mL of the hexane:dichloromethane mixture. The resulting extract was reduced to 0.5 mL volume, and an injection standard of anthracene was added to all extracts. Anthracene was chosen because it does not co-elute with the target hydrocarbons, nor is it typically found indigenously in the environment. The extracts were then analysed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890A GC coupled to a Leco Pegasus time-of-flight-MS and an Agilent 7683 ALS auto-sampler (Santa Clara, CA, USA). The extracts were injected (1 µL) into a split/splitless injector operating at 310°C in splitless mode onto a J&W DB5MS column (60 m × 0.25 mm i.d., 0.25 mm film thickness) coated with modified 5% phenyl 95% methyl silicone, with helium as the carrier gas (1.5 mL/min, constant flow). The temperature programme was

40°C (held for 2 minutes), increasing to 310°C at a rate of 4°C/min (held for 45 minutes). The detector voltage of the MS was between 1600 V and 1850 V. In light of the fine clay particles lost in the decanting process, as well as the uniformity of soil constituent parts and the uniformity of soil moisture in this study, the extracted 14.0 g soil sample was adjusted to take into account a 15% moisture content. This value was then used to calculate the concentration of target hydrocarbons in mg of contaminant per kg of dry soil (mg kg^{-1}).

5.2.7. Statistical analyses

Measured exposure concentrations for each contaminant were compared with the nominal target concentration using Pearson's correlations ($\alpha = 0.05$), in order to verify the efficacy of the preparation and chemical analysis processes (Minitab Inc., 2010).

Percent mortality in the controls and solvent controls were calculated to determine test acceptability (< 10%; OECD, 2004). In the case of uncertainty, a general linear model was used to compare mortality between the two controls. Dose-response modelling was conducted using the DRC package version 3.0-1 (Ritz and Streibig, 2005) in R (version 3.3.1; R Core Team 2016). Assumptions of normality and homogeneity of variance were examined using q-q plots and plots of residuals, respectively.

For the chronic toxicity tests, dose-response curves were estimated by fitting a four parameter, nonlinear regression model. A series of log-logistic, log-normal and Weibull curves were fitted to the data for each analysis, and the Akaikes information criterion of each fitted curve was used to determine the best fitting model for each response type. The parameters of the model were estimated with maximum likelihood, using starter values determined by the program's self-starter tool. Lethal and effective concentrations (LC_{10}, LC_{50}, EC_{10} and EC_{50}) were extrapolated from the fitted curve. Where possible, LCx and ECx values were compared for each endpoint between compounds, using a confidence interval ratio test ($\alpha = 0.05$; Wheeler et al. 2006).

The dose-response curves for the avoidance behaviour test were calculated using the proportion of worms found in the contaminated side of the soil as the response variable and dose as the explanatory variable (Ritz and Streibig, 2005). Effective concentration values (EC_{10} and EC_{50}) were derived from the best fitting log-logistic response curves.

5.2.8. Expected mixture toxicity and type of joint action

The expected mixture effect on survival, mass change and cocoon production was calculated for *E. fetida* using the results of each constituent compound when tested

individually. The concentration addition (CA) mode of joint action was calculated using Equation 1 (Phyu *et al.*, 2011):

Equation 1:

$$ECx_{mix} = \left(\sum_{i-1}^{n} \frac{P_a}{ECx_a} \right)^{-1}$$

where ECx_{mix} is the effective concentration of a mixture causing an effect of x%, P_a is the proportion of compound a in the mixture, and ECx_a is the effective concentration of component a that would cause x% effect when tested individually. Where ECx_a values were unable to be determined accurately (i.e. values were greater than the highest test concentration), the highest test concentration was used as a conservative estimate. For this reason, confidence intervals could not be meaningfully calculated.

The expected mixture effect under an independent action (IA) model was calculated for the highest mixture test concentration of 20,000 mg kg^{-1} using Equation 2 (Phyu *et al.*, 2011):

Equation 2:

$$BR_{mix} = 1 - [(1 - BR_a)(1 - BR_b) \cdots (BR_n)]$$

where BR_{mix} is the predicted biological response to the mixture, and BR_a, BR_b and BR_n the biological responses to mixture components a, b and n, respectively. The net biological response for each endpoint was calculated by subtracting the modelled mixture *BR* for each compound from the modelled *BR* of control soil. Where sigmoidal dose-response models could not be fitted for a particular compound, a linear function was fitted in Excel 2013 (Microsoft Corporation, 2012), from which a *BR* was estimated. For this reason, confidence intervals could not be meaningfully calculated.

For a quantitative indication of the type of joint action for each endpoint, the model deviation ratio (MDR) was calculated using the following ratio (Belden, Gilliom and Lydy, 2007):

Equation 3:

$$MDR = \frac{Expected}{Observed}$$

An MDR of 1 would indicate that the two values are identical, and so adhere to that particular model. Taking the high variance in this dataset and the inability to calculate confidence intervals, it was required that the expected and observed toxicities differed by more than 50%, to allow for a more conservative buffer for biologically insignificant deviations. This is a more conservative buffer than previous studies that have used a value of only 30% (Phyu *et al.*, 2011). Thus, an MDR value less than 0.5 would indicate that the toxicity of each individual compound is, overall, ameliorated by the presence of the other compounds, so the type of joint action is antagonistic. Conversely, an MDR value greater than 1.5 indicates that when combined, the toxicity of each compound is, overall, enhanced by the presence of the others, and so the type of joint action is synergistic.

5.3. Results

5.3.1. Hydrocarbon concentrations

In the case of both the synthetic weathered diesel mixture and the individual hydrocarbons, measured contaminant concentrations were up to four orders of magnitude lower than the target concentration (Tables 5.2, 5.3 and 5.4). Indeed, decalin was below the detection limit in all but the two highest test concentrations (Table 5.2). Measured concentrations were also highly variable and correlated poorly with their target concentration, with the *M. macquariensis* mixture ($r = 0.46$, $p = 0.18$) and adamantane ($r = 0.23$, $p = 0.32$) being of particular concern. Adamantane, benz[a]anthracene and docosane exist as solids under normal laboratory conditions, so they may have formed a patchy distribution as the hexane solvent evaporated. Due to these inconsistencies in measured hydrocarbon concentrations, dose response curves were modelled using nominal contaminant concentrations.

In the hydrocarbon mixture, the total hydrocarbon concentration in treatments containing *E. fetida* declined to only 0.4% of the original measured concentration for the highest treatment concentration of 20000 mg kg-1 (Figure 5.1). By comparison, the decline in total concentration of the target hydrocarbons in treatments containing *M. macquariensis* was less extensive, though still substantial at 22.8% of the original concentration remaining (Figure 5.1). In the *E. fetida* treatments, the final concentration of each constituent compound from the mixture was near or below the detection limit, making the comparison of the relative loss of each compound infeasible. For treatments containing *M. macquariensis*, decalin and DIPN concentrations declined the most:

decalin concentrations were below detection limits at the end of the 12-week experiment period, and only 2.5% of DIPN remained. Adamantane and docosane were more persistent: 85.6 and 47.5% remained in the test soil after 12 weeks, respectively.

Table 5.2: Target and measured test concentrations for chronic toxicity tests of single hydrocarbon compounds in test soils.

Target concentration (mg kg^{-1})	**Measured concentration (mg kg^{-1})**					
	Docosane	**Pristane**	**Benz[a] anthracene**	**Decalin**	**Adamantane**	**DIPN**
0	BDL	BDL	BDL	BDL	BDL	BDL
25	NT	NT	3.5	BDL	BDL	0.3
50	NT	NT	17.6	BDL	BDL	5.1
100	NT	NT	26.3	BDL	BDL	8.6
250	0.1	1.8	32.3	BDL	BDL	23.2
500	0.1	6.1	62.9	BDL	BDL	NT
1000	0.2	62.7	NT	BDL	3.4	NT
2500	2.3	35.2	NT	0.4	255.8	NT
5000	21.9	28.6	NT	0.5	77.5	NT
10,000	94.9	28.8	NT	NT	22.0	NT
20,000	NT	114.9	NT	NT	NT	NT

Measured concentrations given here are the geometric mean of the concentration at day zero and at day 42. DIPN indicates mixed isomers of diisoproyl naphthalene. BDL indicates that a concentration was below detection limits. NT indicates that the concentration was not selected for testing based on pilot studies.

Table 5.3: Measured concentrations of a hydrocarbon mixture developed to model that found in weathered diesel contamination, to two species of earthworm.

Target (mg kg^{-1})	**Measured concentration (mg kg^{-1})**		
	Avoidance test (*Eisenia fetida*)	**Chronic test**	
		Microscolex macquariensis	***Eisenia fetida***
0	BDL	BDL	BDL
500	42.2	4.3	1.7
1000	82.4	TM	8.2
2500	590.8	TM	TM
5000	806.6	464.7	37.7
10,000	431.9	851.4	52.5
20,000	659.7	308.2	38.9

Measured exposure concentrations for the chronic test are the geometric mean of the initial concentration and the concentration at day 42. BDL indicates that a concentration was below detection limits. TM indicates total mortality at that concentration, so a geometric mean concentration could not be calculated for these treatments.

Table 5.4: Target and measured test concentrations of single hydrocarbon compounds, for avoidance toxicity testing with the earthworms *Microscolex macquariensis* and *Eisenia fetida.*

Target (mg kg^{-1})	**Measured concentration (mg kg^{-1})**					
	Docosane	**Pristane**	**Benz[a] anthracene**	**Decalin**	**Adamantane**	**DIPN**
0	BDL	BDL	BDL	BDL	BDL	BDL
25	NT	NT	2.48	NT	NT	0.02
50	NT	NT	2.41	BDL	NT	5.13
100	NA	NA	7.39	BDL	NA	5.93
250	2.53	1.43	30.90	BDL	0.02	NT
500	4.08	9.02	55.96	BDL	0.00	NT
1,000	0.65	83.43	NT	BDL	3.80	NT
2,500	20.76	51.90	NT	0.24	77.34	NT
5,000	9.52	25.92	NT	NT	15.43	NT
10,000	262.14	28.54	NT	NT	27.22	NT

Measured concentrations given here are the initial concentration at the start of the test. DIPN is diisopropyl naphthalene. BDL indicates that a concentration was below detection limits. NT indicates that the concentration was not tested for that compound. NA indicates that the chemical analysis for this concentration was not successful, so is not available.

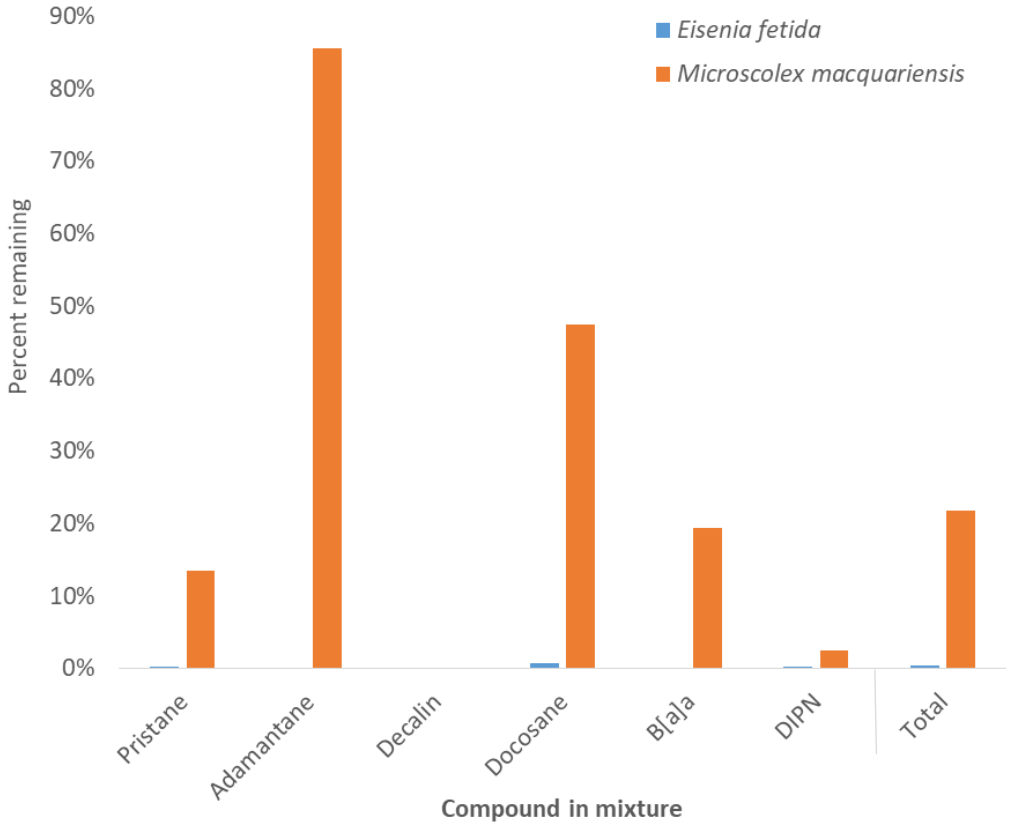


Figure 5.1: Percentage of hydrocarbons remaining in a synthetic weathered diesel mixture after the 12-week experiment period, for two species of earthworm. The nominal initial hydrocarbon concentration was 20,000 mg kg^{-1} dry soil.

5.3.2. Solvent effects

Mortality of *E. fetida* in the solvent control was, on average, 16.7% (± 8.6 SE). This is above the validity threshold criteria of 10% (OECD 2004). However, though mortality in the hexane controls was higher than that of the control treatments (8.3 ± 4.5% SE), this difference was not significant between the two groups (GLM with Dunnett's comparison: T=0.93, p = 0.373), so further analyses were continued. Mortality of *M. macquariensis* in the solvent control was more substantial at 75 ± 35.4%, compared to 0% for the control. However, mortality was otherwise generally low in treatment soils for *M. macquariensis* (Table 5.3). Indeed, mortality was nil in some treatments, which also received a large volume of hexane. It is possible that this species is simply highly sensitive to pre-experiment handling, so analyses were continued.

5.3.3. Toxicity of mixture to *Eisenia fetida* and *Microscolex macquariensis*

5.3.3.1. Observed toxicity

As noted in Section 5.3.1, dose response curves were modelled using nominal (rather than measured) concentrations. In general, *E. fetida* was more sensitive to the synthetic weathered diesel mixture than was *M. macquariensis*, with no toxic effect observed at the maximum concentration tested (20,000 mg kg^{-1}) for any endpoint except avoidance behaviour. As such, the toxicological response and relative sensitivity of the two species could not be statistically compared.

For *E. fetida*, total cocoon production was the most sensitive endpoint measured, and had a much narrower 95% confidence interval than mass change (Table 5.5; Figure 5.2). No cocoons were found in the procedural controls containing no worms.

The net avoidance response was highly variable for both species, ranging from a strong avoidance of the contaminated soil to a strong preference for it. Indeed, unexpectedly, both species showed a preference for the contaminated soil over clean control soil (Figure 5.2), and all worms were found on the contaminated side of the container at the highest concentration tested (20,000 mg kg^{-1}). For aggregation behaviour (measured as time taken for three or more worms to form a group), the EC_{10} and EC_{50} estimates were well above the maximum concentration tested (Table 5.5).

5.3.3.2. Predicted toxicity of synthetic weathered diesel mixture to *Eisenia fetida*

The toxic effects caused by the hydrocarbon mixture (Section 5.3.3.1) were generally not well predicted by either the CA or IA models using the dose response curves calculated for the individual compounds (Table 5.6). In the case of survival, both models indicated that the toxicity of the individual compounds (Section 5.3.4) was ameliorated when mixed, though this effect was far stronger under the IA scenario than under the CA scenario. For the endpoint of mass change, a CA model was appropriate, whereas under the IA model the toxicity of each compound was enhanced when mixed. Finally, the predicted effect of the mixture on cocoon production varied greatly with the model type: under a CA scenario, the joint action of individual compounds was five-fold more toxic than measured values, but under an IA scenario were four times less toxic. While neither model was consistently a strong predictor of joint action type, deviations from predicted toxicities tended to be less extreme using the CA model than the IA model.

Table 5.5: Toxicity thresholds of a hydrocarbon mixture developed to model that found in weathered diesel contaminated soils at subantarctic Macquarie Island, to two species of earthworm. All concentrations are in mg of the contaminant mixture per kg dry soil.

Species	EC/LCx	Survival	Total Cocoons	Mass change	Avoidance	Aggregation
Eisenia fetida	10	NC	392 (306 – 477)	439 (35 – 844)	17 (-72 – 105)	>20,000*
	50	NC	598 (513 – 683)	708 (277 – 1140)	2784 (-648 – 6217)	>20,000*
Microscolex macquariensis	10	>20 000*	NC	>20 000*	43 (-80 – 166)	>20,000*
	50	>20 000*	NC	>20 000*	875 (-29 – 1779)	>20,000*

EC_{10} and EC_{50} values indicate the total concentration of a synthetic aged diesel solution in soil (mg kg^{-1}) that caused an effect in 10 and 50% of the test animals, respectively. Values in parentheses indicate 95% confidence limits. * indicates estimates that exceeded the highest test concentration so should be interpreted with caution. NC indicates a curve that could not be calculated.

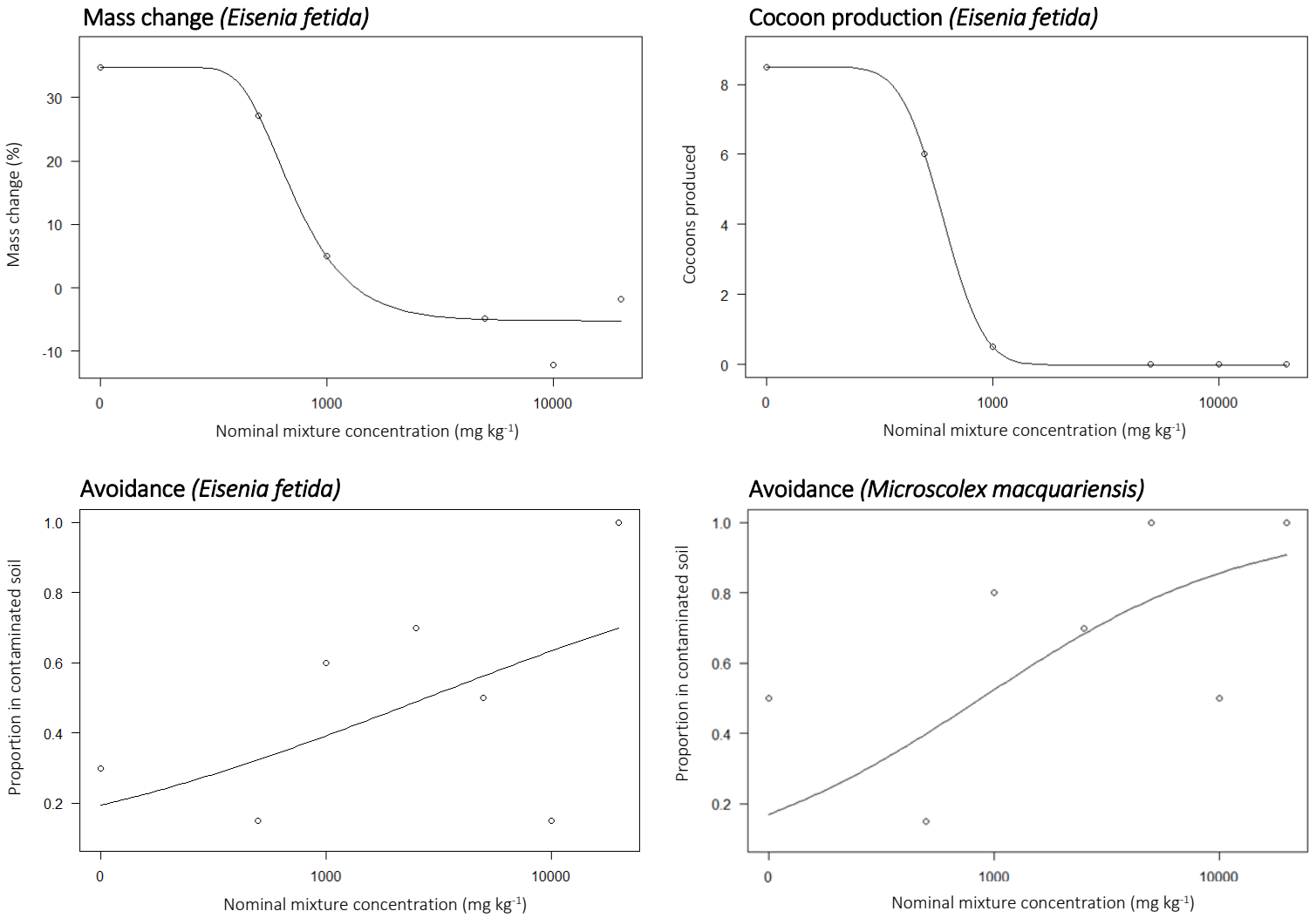


Figure 5.2: Dose-response curves for three different endpoints, for two species of earthworm. The nominal initial hydrocarbon concentration was 20,000 mg kg^{-1} dry soil.

Table 5.6: Summary of the toxicity of a mixture of hydrocarbons selected to resemble highly weathered diesel in soil at subantarctic Macquarie Island, indicating the observed effect, and that expected under either two theoretical scenarios: concentration addition (CA) and independent action (IA).

Endpoint	Joint action model	Expected effect	Observed effect	MDR	Joint action type
Survival	CA (LC_{10})	9272	>20,000	0.46	Antagonistic
	IA (BR%)	5.4	58.3	0.09	Antagonistic
Mass change	CA (EC_{10})	599	439 (35 – 844)	1.36	CA
	IA (BR%)	70.4	36.5	1.93	Synergistic
Cocoon production	CA (EC_{10})	1979	392 (306 – 477)	5.05	Synergistic
	IA (BR%)	2.0	8.5	0.24	Antagonistic

Values in parentheses indicate 95 % confidence limits, where possible. The effect under the CA scenario is an LC/EC_{10} value (mg kg^{-1}), whereas under the IA scenario it is in terms of the percent biological response (BR%). The model divergence ratio (MDR) is the ratio of the predicted mixture response and the observed response. An MDR of 1 indicates that a mixture adheres to that particular model. An MDR less than 1 indicates antagonism among mixture compounds, whereas an MDR greater than 1 indicates synergism; a difference of at least 50% is required here to account for biologically insignificant divergences from the model.

5.3.4. Toxicity of individual compounds to *Eisenia fetida*

In the single compound exposures, mortality was only recorded for DIPN, benz[a]anthracene and decalin. DIPN was significantly more lethal than benz[a]anthracene, but not more so than decalin because of the high variance in the response of earthworms in decalin (Table 5.7). ECx estimates for pristane and docosane were greater than the maximum concentrations tested, indicating relatively low toxicity. While a sigmoidal dose-response curve for adamantane could not be determined, there was little mortality recorded for this contaminant, though the variance did appear to increase with higher concentrations.

Cocoon production was highly variable, including in control treatments where numbers ranged from zero to nine cocoons produced within the six-week test period. Adamantane was more toxic than decalin at the EC_{10} level, but the two were not significantly different at the EC_{50} level (Table 5.7). No cocoons were produced by worms

in soil containing DIPN at any concentration. For adamantane, concentrations above than 250 mg kg^{-1} prevented cocoon production, while for pristane and decalin this threshold was 1000 mg kg^{-1}. Cocoon production in docosane and benz[a]anthracene treatments remained highly variable up to the maximum concentration tested (10,000 and 500 mg kg^{-1} respectively). No cocoons were found in the soil-only controls (i.e. without worms).

Worms in the control soil increased in mass by an average of 46% during the six-week exposure period, suggesting that while the worms were sexually mature (i.e. had a well-developed clitellum) they had not yet reached an adult size. Docosane had no effect on mass, pristane caused a 16% reduction in mass at concentrations above 5000 mg kg^{-1}, and adamantane caused a 20% reduction at concentrations above 1000 mg kg^{-1}. DIPN caused a strong reduction in mass gain up to 50 mg kg^{-1} (some worms did not grow at all during the exposure period), and 100% mortality at concentrations above this. Benz[a]anthracene caused a highly variable decrease in mass compared with worms in control soils: exposed worms did not increase in size at 100 mg kg^{-1}, but increased in size by approximately 25% at the highest concentration of 500 mg kg^{-1}. Interestingly, decalin, though it caused complete mortality at concentrations of 2000 mg kg^{-1} and above, also caused a hormetic effect on mass, whereby *E. fetida* increased by up to 69% in size after six weeks at 100 mg kg^{-1} (i.e. approximately 50% larger than worms in the control), reducing to a 25% mass increase in the 1000 mg kg^{-1} treatments (i.e. 46% smaller than controls). For the compounds that allowed for ECx values to be calculated, in general, adamantane had the greatest effect on mass change, followed by decalin and pristane (Table 5.7).

In most instances, *E. fetida* avoided contaminated test soils, responding particularly strongly for docosane, DIPN and adamantane. For some compounds, however, worms preferred the contaminated soils over the clean soils, even at high concentrations. This trend was particularly notable for soils contaminated by pristane and decalin, and to a lesser extent by benz[a]anthracene.

Table 5.7: Toxicity of hydrocarbon compounds tested individually to the earthworm *Eisenia fetida.*

Compound	EC/LCx	Survival	Total Cocoons	Mass change	Avoidance
Pristane	10	>20,000*	NC	276 [a] (142 - 411)	1596 [a] (217 – 29,76)
	50	>20,000*	NC	716 [a] (450 - 981)	9308 [a] (2029 – 16,586)
Docosane	10	>10,000*	>10,000*	NC	NC
	50	>10,000*	>10,000*	NC	NC
Adamantane	10	NC	75 (-187 – 336)	88 [b] (5 – 377)	NC
	50	NC	100 [a] (41 – 158)	482 [b] (309 – 656)	NC
Diisopropyl naphthalene	10	34[a] (-208 – 275)	NC	NC	15 [a] (-33 – 63)
	50	41[a] (-109 – 190)	NC	NC	19 [a] (-12 – 50)
Decalin	10	1506[ab] (-1788 – 4800)	199 (-574 – 972)	226 [c] (-16 – 469)	209 [a] (-35 – 454)
	50	1746 [ab] (-1860 – 5346)	704 [a] (-1043 – 2451)	403 [c] (-191 – 997)	2304* [a] (-397 – 5004)
Benz[a] anthracene	10	22[b] (-130 – 173)	>500*	>500*	NC
	50	73[b] (-172 – 317)	>500*	>500*	43 [a] (-1137 – 1223)

EC10 and EC50 values indicate the compound concentration in soil (mg kg^{-1}) that caused an effect in 10 and 50% of the test animals, respectively. Values in parentheses indicate 95% confidence limits. * indicates estimates that exceeded the highest test concentration so should be interpreted with caution. NC indicates a curve that could not be calculated. Superscript letters denote ECx values that are not significantly different between compounds for that endpoint (i.e. column in table), at that percentage effect.

5.4. Discussion

5.4.1. Relative hydrocarbon degradation in a synthetic weathered diesel mixture

The relative degradation of different hydrocarbon compounds in the synthetic weathered diesel hydrocarbon mixture was highly variable in treatments containing *M. macquariensis*. Decalin (a cycloalkane) and DIPN (an alkyl naphthalene) were below or close to detection limits, whereas docosane (an *n*-alkane) and adamantane (a cycloalkane) were more persistent: 85.6 and 47.5% still remained in the test soil after 12 weeks, respectively. Cycloalkanes are known to be resistant to microbial biodegradation (Atlas, 1981), so the comprehensive loss of decalin is likely due to its relatively high vapour pressure, rather than biodegradation. Generally, the most susceptible hydrocarbon type to degradation processes are *n*-alkanes (Atlas, 1981), so the persistence of docosane was not expected. However, under normal laboratory conditions both adamantane and docosane exist as solids, so these results may reflect a non-uniform distribution throughout the soil matrix, rather than resistance to biodegradation.

Hydrocarbon compounds in soil can degrade differently when added individually or within a mixture of other hydrocarbons, possibly because some compounds are preferentially degraded by soil microbes, thereby delaying the onset of degradation for other compounds (Zytner, Salb and Stiver, 2006). For example, over 30 days the degradation of naphthalene was only 17% complete when present on its own, but enhanced to 45% when present in 'synthetic diesel fuel' mixture (Zytner, Salb and Stiver, 2006). Contrary to this, the degradation of phenanthrene was inhibited by the presence of other hydrocarbon compounds, where it was reduced from 26% to 9% (Zytner, Salb and Stiver, 2006). Unfortunately such a comparison is not possible in this study for *E. fetida*, because the concentrations of any remaining individual mixture components were too low for further analyses.

5.4.2. Mixture toxicity

The mixture tested was not highly toxic to either *M. macquariensis* or *E. fetida*. While the mixture contained six different compounds, docosane and pristane comprised 99.9% of the mix, a true reflection of field ratios of hydrocarbon types. Individually, however, these two compounds were not highly toxic (Section 5.4.3). In the case of docosane, no effect was detected for any endpoint. Pristane did have a notable negative effect on mass change, but was actually preferred over control soil in the avoidance test. As such, the

low mixture toxicity is consistent with the results from the tests of individual hydrocarbon compounds.

Despite the hydrocarbon mixture being more degraded in treatments that contained *E. fetida* than *M. macquariensis, E. fetida* was more sensitive in terms of mass change and cocoon production. This result was not expected in light of findings from Chapter 3, in which the two species were exposed to whole aged diesel, and wherein EC_{10} and EC_{50} values for mass change were an order of magnitude greater for *E. fetida* than for *M. macquariensis,* and double for cocoon production. Earthworms are known to enhance the degradation of hydrocarbons (Section 5.4.6), an effect that is thought to be a function of improved soil aeration, as well as the mixing of microbes and hydrocarbons in the earthworm gut (Hickman and Reid, 2008). In general, direct metabolism of petroleum hydrocarbons by annelid worms is not thought to occur (Hickman and Reid, 2008; Roelofs *et al.*, 2016), so it is unlikely that an increased gut uptake caused the toxic response. As such, the toxic effect observed here is more likely due to a passive diffusion process (Shi, Xu and Hu, 2014).

5.4.3. Toxicity of individual hydrocarbons

Only DIPN, benz[a]anthracene and decalin caused a measurable effect on survival, with sub-lethal effects such as cocoon production and mass change being more sensitive endpoints. When tested individually, DIPN was the most toxic compound from the mixture, followed by benz[a]anthracene, adamantane and decalin. Indeed, no cocoons were produced in soils containing DIPN, even at the lowest concentration of 25 mg kg^{-1}. *E. fetida* was not highly sensitive to the two primary components in the mixture: docosane did not affect any endpoint at the maximum concentration tested (10,000 mg kg^{-1}), and pristane only affected mass change at relatively high concentrations (Table 5.7). Decalin caused a hormetic effect on mass; individuals in the 100 mg kg^{-1} treatment were approximately 50% larger than control animals after six weeks, but 46% smaller than control animals in the 1000 mg kg^{-1} treatments, followed by 100% mortality at concentrations of 2000 mg kg^{-1} and above. This range is consistent with that described by Calabrese (2005), whereby the stimulatory phase of a hormetic response is typically within 30-60% that of controls. Hormetic responses in sub-lethal endpoints have been noted previously for petroleum hydrocarbons. For example, a slight increase in reproductive endpoints was reported for the annelid worm *Enchytreus albidus* in response to exposure to phenanthrene (Amorim *et al.*, 2011).

5.4.4. Joint action types

The mixture tested in this study did not adhere strongly to either a CA or IA mode of joint action, and effects varied between and within endpoints. However, deviations from the expected effects under a CA scenario tended to be less extreme than under an IA scenario. The dose-response curves calculated for the individual compounds (used here to predict joint toxicity) contained a high degree of variance (Table 5.6). Combined with the inability to derive meaningful confidence intervals for predicted toxicities, the lack of consensus in favour of one particular model is, perhaps, unsurprising. Both joint action models for survival determined the compounds to be antagonistic when in mixture, but sub-lethal endpoints (mass change and cocoon production) tended towards synergism. This may be linked to the hormetic effects discussed in Section 5.4.3. The overall improvement in earthworm health and function facilitated by the addition of soil carbon may be sufficient to overcome the antagonistic joint action of the mixture for sub-lethal endpoints.

Hydrocarbons, including those tested here, are generally thought to share a narcotic mode of action (León Paumen *et al.*, 2009), which would suggest additive toxicity is likely when mixed (Barata *et al.*, 2005). For example, León Paumen et al. (2009) tested six polycyclic aromatic compounds on two soil invertebrates (the springtail *Folsomia candida* and the annelid worm *Enchytraeus cripticus*), which caused a narcotic effect in 71% of cases. However, some exceptions do exist. Anthracene caused a more specific (though unknown) mode of action than narcosis in the springtail *Folsomia fimetaria* (Sverdrup, Torben and Krogh, 2002). Furthermore, phenanthrene caused very different transcriptional responses in *F. candida* and *E. crypticus*, resulting in a higher toxicity to the former than to the latter (Roelofs *et al.*, 2016). The funnel hypothesis states that mixture toxicities tend towards additivity with an increase in the number of constituent compounds, with a threshold being apparent at approximately 10 compounds (Warne and Hawker, 1995). In this study, although the mixture contained six compounds, it was dominated (99.9%) by only two of those compounds, perhaps behaving more as a binary mixture than a six-part one. As such, the mixture tested here may not be sufficiently complex for the funnel hypothesis to inform our understanding of its mode of joint action.

5.4.5. Avoidance and aggregation behavioural responses

Both species appeared to prefer the contaminated soil over the control soil in the mixture assays, as well as in some individual compounds, particularly decalin and

pristane. There are two possible explanations for this behaviour. First, pristane comprised 44.4% of the mixture, so it may be posited that the preference for high concentrations of pristane seen in the individual compound tests also drove the preference for high concentrations of the mixture. Indeed, pristane caused no mortality at the highest concentration tested (20,000 mg kg^{-1}), and had relatively high EC_{10} and EC_{50} values for mass change and avoidance. In this case, the increase of total soil carbon caused by spiking may have promoted microbial biomass, and so increased food available to the worms. Indeed, the synthetic weathered diesel used by Zytner et al. (2006) caused microbial counts to increase by an order of magnitude at the test concentration of 5000 mg kg^{-1}. Second, in the case of more toxic compounds, earthworms might be so impaired by the contamination that they are not able to move away from it. At the conclusion of the avoidance assays, worms in some treatments appeared in poor condition, were generally unresponsive and some had segments beginning to decay.

Aggregation behaviour was not significantly related to the concentration of the model weathered diesel mixture in either *E. fetida* or *M. macquariensis,* and does not warrant further investigation as an indicator of sub-lethal toxic effects in earthworms. Interestingly, though the behaviour was observed in this study for *E. fetida*, and is known to be a common response in the species to other stressors such as pathogens or predators (Zirbes *et al.*, 2012), the behaviour was far less common in *M. macquariensis*.

5.4.6. Vermi-remediation potential

The efficacy of *E. fetida* for enhancing the degradation of persistent polycyclic aromatic hydrocarbons (PAHs) has been noted previously, with the extent of biodegradation for anthracene and benzo[a]pyrene more than doubled by its presence within 11 weeks, and phenanthrene completely removed (Contreras-Ramos, Alveraz-Bernal and Dendooven, 2006). The potential for vermi-remediation to be utilised in the management of contaminated soils on Macquarie Island was not initially an aim of this study. For this reason, no contaminated treatments without earthworms were included in the experiment design, so it is not possible to directly compare the rate of loss with and without worms. However, the 77.2% reduction in total mixture concentration in the presence of *M. macquariensis* in this study is substantial, though not nearly as large as that in the presence of *E. fetida* (99.6%; Figure 5.1). For this reason, further investigation into the use of *M. macquariensis* in the contamination management strategy for Macquarie Island may be warranted. Indeed, a member of the *Microscolex* genus is

found on all subantarctic islands, many of which also have areas contaminated by petroleum products (Chapter 2), so such a study may be more broadly relevant to the entire subantarctic region.

5.5. Conclusion

In this study, a synthetic mixture of various hydrocarbon types was developed to resemble the consortium of hydrocarbons that occur in soils contaminated by highly weathered diesel at subantarctic Macquarie Island. Of the compounds tested, alkyl naphthalenes and cycloalkanes were the most toxic to the commonly used model earthworm species *E. fetida*. *Microscolex macquariensis* – an earthworm endemic to Macquarie Island – was shown in this case to be less sensitive to the mixture of hydrocarbons than *E. fetida*, a result that is not consistent with other studies. The joint mode of toxicity for this mixture did not strongly adhere to either a CA or IA model, and while the mixture tended towards antagonism for the mortality endpoint, it was generally synergistic for sub-lethal effects. This variability may be linked to the hormetic effects observed for some compounds. These findings may be useful to guide remediation efforts and priorities, as well as future ecotoxicological testing.

Chapter 6:
Discussion and conclusions

6.1. Introduction

The aim of this thesis was to investigate the toxicity of weathered petroleum contaminants to soil biota on subantarctic Macquarie Island, and this was achieved through four different avenues:

1. Chapter 2 presents the first ever review of petroleum hydrocarbon contaminants on subantarctic islands, collating almost three decades of research conducted by research groups from several countries, and identifying points of intersection where results from a study may be of relevance to other islands.
2. Chapters 3 to 5 explore the toxicity of diesel contamination to the biota of Macquarie Island, using each of the three broad categories of direct toxicity assessment (DTA) described by Warne (2003): field-based testing, standardised testing, and intermediary site-specific laboratory-based testing.
    a. In Chapter 3, the effect of existing diesel contaminated soils on springtail assemblages at Macquarie Island was investigated using a field-based observational experiment;
    b. In Chapter 4, internationally standardised toxicity test procedures were adapted to be more specific to the Macquarie Island scenario, utilising natural rather than artificial soils, and attempting to replicate a highly weathered fuel spill using real diesel;
    c. In Chapter 5, those standardised testing procedures were adhered to more strictly, in order to determine the effect of a synthetic hydrocarbon mixture and its constituent parts on a common model species of earthworm, as well as an earthworm endemic to Macquarie Island. Chapter 5 was the first ever study to use a model contaminant mixture representative of highly weathered fuel to examine toxicity to a soil macroinvertebrate.

Through the investigations in this thesis, a comprehensive picture of the toxic effects of highly weathered petroleum contamination to soil biota on Macquarie Island has begun

to emerge. The results of each specific chapter have been discussed therein, and this chapter seeks to consider all the findings together, identifying overarching themes and study limitations, as well as implications for both land managers and future research in this area.

6.2. Key findings and themes

6.2.1. The importance of vegetation

In Chapter 2, vegetation emerged as an important factor influencing the toxicity of petroleum contaminants in soil. This may be because vegetation is associated with a higher soil TOC, which has been shown to reduce the bioavailability of petroleum compounds (e.g. Macoustra et al. 2015; Mooney et al. 2013), but might also reflect the contribution of some plants to bioremediation *per se*, a process termed phytoremediation (Bramley-Alves *et al.*, 2014). Chapter 3 further highlighted the importance of vegetation with regards to its influence on soil invertebrate communities. On a per-factor basis, vegetation coverage was the strongest influence on springtail assemblages, exceeding the effect of soil properties or soil TPH concentration. The implications of this theme are explored further in Section 6.4.3 of this chapter.

6.2.2. Sensitivity of earthworms

Chapters 4 and 5 compared the responses of two species of earthworms to petroleum contaminants in soil. *Microscolex macquariensis* is endemic to Macquarie Island and has been used previously for ecotoxicological testing (Mooney *et al.*, 2013). However, it can be difficult to collect a sufficient number of *M. macquariensis* individuals for toxicity testing from the field (Mooney *et al.*, 2013), and the species is not easily kept in culture (pers. obs.). *Eisenia fetida*, however, is native to Europe, though naturalised on all continents except Antarctica, and is used worldwide as a model test species. In contrast to *M. macquariensis*, *E. fetida* is easy to obtain and maintain in culture, so there is incentive to use *E. fetida* as a surrogate species in place of *M. macquariensis* in toxicity testing. However, the two species were found to differ quite substantially and inconsistently in their responses to petroleum hydrocarbons (Chapters 4 and 5). For example, *M. macquariensis* was many times more sensitive to aged SAB than *E. fetida,* depending on the endpoint used. In the case of the synthetic weathered diesel mixture, however, *M. macquariensis* showed very little toxicological response, as no effect was demonstrable for any chronic endpoint at the highest concentration tested (20 000 mg kg^{-1}). This is in stark contrast to *E. fetida*, for which EC_{10} values were only 393 mg kg^{-1} and

439 mg kg^{-1} for cocoon production and mass change, respectively. As such, *E. fetida* is not an appropriate surrogate for *M. macquariensis* in terms of the toxic effects of weathered diesel.

The potential for the use of earthworms – including *E. fetida* – to enhance the biodegradation rate of petroleum contaminated sites has been noted previously (Contreras-Ramos, Alveraz-Bernal and Dendooven, 2006; Schaefer and Juliane, 2007). This was further indicated by the experiment described in Chapter 5, whereby TPH concentrations in replicates containing *E. fetida* were only 0.04% of an original starting concentration of 20,000 mg kg^{-1} after a six-week exposure period. The reduction in TPH concentration in replicates containing *M. macquariensis* was less extensive (22.8% remaining), perhaps reflecting the smaller size of *M. macquariensis* and correspondingly lower soil turnover rates compared to *E. fetida*. This does, however, still represent a substantial reduction in TPH for such a short period, perhaps indicating the potential of *M. macquariensis* as a native vermi-remediation candidate. The possibility for vermi-remediation on Macquarie Island was beyond the scope of this thesis, but further research is warranted in order to verify that the presence of *M. macquariensis* does enhance degradation rates relative to treatments containing no worms, and that the observed concentration reduction is not solely attributable to volatilisation or microbial action.

A number of assays from Chapters 4 and 5 revealed a degree of hormesis (i.e. an initially stimulatory effect on an endpoint at low concentrations of a toxicant, with an inhibitory response at higher concentrations). For the aged SAB assays (Chapter 4) and for some hydrocarbon compounds when tested individually (Chapter 5), worms increased in size or produced more cocoons in response to low contaminant concentrations, and inhibition only occurred at higher concentrations (in some cases, however, an inhibitory phase was not observed at the highest concentration tested, so further testing would be required to confirm the assumption that the response is truly hormetic and not simply stimulatory). The extent of the stimulatory responses reported here was in line with the range generally observed in studies where hormesis is reported, being 30-60% greater than controls for a particular endpoint (Calabrese, 2005). Hormetic trends are often difficult to detect, because few studies include a sufficient number of low-range test concentrations. While not unprecedented (e.g. Amorim et al., 2011; Laughlin et al., 1981), a hormetic response to petroleum hydrocarbons has not been reported frequently for macroinvertebrates.

Furthermore, for some toxicants, worms also preferred the contaminated soil over the control soil in the avoidance tests (primarily aged SAB, decalin and pristane). This was most likely because, by amending soil with petroleum hydrocarbons, the TOC of the soil increased, thereby prompting an increase in microbial biomass (Zytner, Salb and Stiver, 2006), subsequently increasing the food available to worms. The manner in which hormetic effects might manifest in real-world contexts is poorly understood, nor is it clear how land managers should incorporate such effects into the ecological risk assessment process (Chapman, 2002; Kefford *et al.*, 2008). It has, however, been posited that:

> *In ecological applications an anthropogenic increase of a population is, or at least should be, of equal concern to a decrease in a population effect.*
>
> Kefford et al. 2008

6.3. Limitations

6.3.1. The wrong canaries

Chapter 3 investigated the effect of petroleum contaminants on soil invertebrates, and found no significant relationship between springtail assemblages and TPH concentrations in surface soils, or with TPH concentrations in underlying soils. Indeed, the sub-lethal effects caused by low contaminant concentrations (e.g. reduced reproductive indicators and body condition) as identified in Chapters 4 and 5, are likely to be stronger under field conditions. Here, inter-species competition and shifts in available niches may cause even minor variations in competitive advantage to significantly influence community assemblages (van Straalen, 1998). Even so, no response to soil contamination was identified in springtail assemblages. This being the case, it was noted here and elsewhere (Deprez et al. 1994; Wasley et al. 2015) that soils on Macquarie Island tend to be more heavily contaminated in lower strata than at the surface. It was also noted here that very few soil invertebrates were found in cores collected at a depth of 0.5 m. If the soil invertebrates of Macquarie Island are not commonly found in the most contaminated strata, and those at the surface are not affected by underlying contamination, then they may not be the most appropriate receptor with which to investigate the toxic effects caused by decades-old petroleum contaminants. The implications of this are discussed further in Section 6.4.2.

6.3.2. Geochemical analyses

For Chapters 3, 4 and 5, the target hydrocarbons were extracted from the soil by placing a sample in a beaker containing 30 mL of a 9:1 mix of dichloromethane:methanol, and placing this in an ultra-sonicator for an initial 25 minutes. The extract solution was decanted, and the sonication process was repeated twice more. The three extracts were pooled, reduced, and an injection standard was added before analysis using GC-MS.

In the field-collected soil samples from Chapter 3, with some exceptions, TPH concentrations were lower than anticipated based on the results of a similar study (Wasley, Mooney and King, 2015). This may be due to the success of a long-term remediation program in the intervening period between sample collections in these two studies. In Chapter 4, where SAB diesel was added at a concentration of 50,000 mg kg^{-1} before being aged for 23 weeks, the 40 mg kg^{-1} measured at the conclusion of the aging period was far below the concentration that was anticipated. However, such an extensive period of aging had not previously been carried out, so there was little precedent upon which to estimate a final concentration. Finally, in Chapter 5, decalin was near undetectable at the start of the experiment, while all other compounds were measured to be two to three orders of magnitude lower than the target concentration. It was partly for this reason that nominal concentration values were used for most analyses in Chapter 5. This may have been caused by the high volatility of some compounds (such as decalin), or the non-homogeneous mixing of some compounds that exist as solids under ambient laboratory conditions.

When each of these cases is considered alone, the low hydrocarbon concentrations measured for each experiment may be accounted for. Taken together, however, these results suggest that the extraction method used for the geochemical analyses here may not have been ideal for this scenario. Other studies have successfully used a shorter sonication process with the same solvent mixture (e.g. Hoshino et al. 2015), but alternative solvent mixtures are also often used, such as varying ratios of DCM, hexane, acetone and toluene (e.g. Berset et al. 1999; Guerin 1999). These alternative solvent mixtures may be better suited to the soil matrices demonstrable and target contaminants tested here, but may also enhance the extraction of non-target compounds that damage analysis equipment (e.g. sulphuric or polar compounds). The addition of a standard prior to extraction is highly sample-, time- and matrix-dependant, so would poorly reflect the extent of binding and adsorption undergone by hydrocarbons added to soils months or years prior. This makes the determination of

percent recovery inaccurate and prone to errors. The broad trends identified in this thesis remain valid relative to each other, but the precise threshold concentrations should be validated before using these results to inform contaminant management practices at Macquarie Island.

6.3.3. A lack of clarity in the discipline

An unanticipated theme identified throughout this thesis was a lack of clarity and conflicting findings in the literature on a number of topics. For example, at low temperatures there is evidence that the toxicity of petroleum hydrocarbons might increase because acutely toxic compounds are not able to volatilise and so remain in the soil (Atlas and Bartha, 1972), or might decrease because toxic compounds become less bioavailable (Walker and Colwell, 1974). Similarly, weathering is generally understood to decrease the toxicity of diesel because acutely toxic compounds tend to volatilise soon after a contamination event (Atlas, 1981), or because the compounds that are not biodegraded are inherently not bioavailable (Alexander, 2000). Furthermore, a reduction in TPH concentration is often the primary indicator of rehabilitation success (e.g. Snape et al. 2008) but there is substantial evidence to suggest that a reduction in TPH is not always strongly linked to a reduction in toxicity, in some cases increasing with biodegradation or weathering (e.g. Al-Mutairi et al. 2008; Coulon et al. 2005; Jiang et al. 2016; Mooney et al. 2013).

The field of petroleum contamination is not new, nor is it experiencing a period of rapid development; a 1981 review by Atlas still cited regularly almost four decades later. As such, this variability between studies and findings is not due to limited research. Instead, the conflicting results are likely because of the high degree of condition-specificity of findings. For example, with all other factors held constant, an increase in soil TOC may cause an apparently highly toxic compound to seem relatively benign (Macoustra et al. 2015; Mooney et al. 2013). Similarly, an increase in temperature from 4°C to 10°C caused a significant increase in TPH loss in a subantarctic mesocosm experiment, but a further increase from 10°C to 20°C made little difference, perhaps because the naturally-occurring microbial community was poorly adapted to warmer temperatures (Delille, Pelletier and Coulon, 2007). Furthermore, the limitations involved when replicating field conditions in a laboratory and the subsequent extrapolation of those laboratory findings to real-world scenarios (Alexander, 2000) means that comparability between studies is inherently difficult. Findings are likely to be highly site specific, while cultured test animals and standardised testing methods

may be inadequate proxies for real-world populations (Martin *et al.*, 2014). This is a limitation of the work presented in this thesis – while the findings presented here add to our overall understanding of cold-climate petroleum ecotoxicology, their relevance and application to contaminant management scenarios must still be carefully considered.

6.4. Implications of findings

6.4.1. Contaminant management techniques

In light of the findings of Chapters 2 and 3, common contaminant management techniques may not be appropriate for the subantarctic context. For example:

- Aeration requires the disturbance of the soil profile to install and maintain infrastructure (Rayner *et al.*, 2007).
- Biopiles have achieved substantial success on the Antarctic continent, even in the face of more adverse environmental conditions than those of the subantarctic (McWatters *et al.*, 2016). However, subantarctic soils contain higher biodiversity and a relatively rich and varied flora and fauna compared to Antarctic soils, which may be harmed by the biopile process.
- Nutrient addition using nitrogen salts has been shown to increase salinity in the already highly saline soils of coastal Macquarie Island, thereby potentially negatively impacting microbial communities (Walworth *et al.*, 2007). The addition of fertilisers was found to enhance the rate of TPH reduction on Kerguelen Island, but increased toxicity to microbes (Delille, Coulon and Pelletier, 2007).
- Nutrient amendment using a natural fish emulsion yielded a degree of success in a mesocosm study on Kerguelen Island (Delille, Duval and Pelletier, 2008), but the biosecurity considerations associated with the broad-scale application of foreign biological matter to a highly environmentally sensitive site may be substantial.

6.4.2. The goals of soil contaminant management works

Springtail assemblages were not significantly affected by highly weathered petroleum contaminants in soils on Macquarie Island (Section 6.3.1; Chapter 3), but vegetation and soil properties were identified to be strong influences (Chapters 2 and 3). This gives rise to a new question for both researchers and land managers: is the physical disturbance to soils caused by many remediation techniques more deleterious to soil invertebrate

communities than the petroleum contamination such works aim to reduce? Certainly, in the low-arctic, changes to bacterial and fungal communities caused by plant removal and soil tilling alone remained evident even six years following the disturbance (Mikola, Sørensen and Kytöviita, 2014). If physical disturbance is indeed more disruptive than petroleum contamination, a second, more philosophical question arises: what level of priority do we confer upon soil invertebrates when considering soil health and site rehabilitation? If, however, soil invertebrates are not deemed to be high-priority receptors in the risk assessment process, alternative taxa must be considered. For animals at Macquarie Island, this is predominantly seabirds and seals. Such taxa are only likely to come into dermal contact with localised hydrocarbon sheens, but dermal uptake in mammals and birds has been little studied (Ball and Truskewycz, 2013). A number of plant species are also likely to be affected by petroleum contamination (Macoustra *et al.*, 2015), but these, similar to invertebrates, would also be negatively impacted by physical soil disturbance.

The extreme toxicity of fresh diesel fuel to a range of Macquarie Island taxa is well established (Mooney et al. 2013; Mooney 2013; van Dorst et al. 2014), so a new contamination event would necessitate immediate action. However, the most appropriate response to decades-old contamination is less obvious. Soil ecosystems are most certainly damaged by a petroleum contamination event (e.g. Macoustra et al. 2015; Mooney 2013; van Dorst et al. 2014), but if, after a point, that damage cannot be undone without perpetuating disturbance in other ways, a rehabilitation goal beyond simply reducing TPH concentrations should be considered. Indeed, a similar question regarding diesel contamination on Possession Island in the Crozet archipelago has been posed previously, asking:

> *At what point is intervention no longer useful?*
>
> Coulon and Delille 2006

Such questions are well beyond the scope of this thesis, but their answers are vital to defining the goals of soil contaminant management efforts, particularly for sensitive subantarctic environments.

6.4.3. Alternative rehabilitation and remediation techniques

The ability to monitor the progress of remediation works will continue to rely on physical access to the most contaminated strata (i.e. the digging of holes and inherent

physical disturbance to a localised area). However, alternative contaminant management techniques exist that cause less physical or chemical disturbance to soils than the techniques outlined in the previous section. For example, permeable reactive barriers (PRBs) have achieved high rates of success in both the Antarctic and subantarctic (Freidman, Gras, *et al.*, 2017; Freidman, Terry, *et al.*, 2017). This infrastructure necessitates an initial disturbance to the soil profile for installation, but subsequent effects are minimal. PRBs do not actively remediate contaminated soils, but capture contaminants in groundwater, preventing a plume from spreading, and enabling the periodic removal of contaminants trapped in the spent absorption materials.

As discussed in Section 6.2.1, vegetation is an important influence on soil invertebrate communities and biodegradation. At least one plant from Macquarie Island has been identified as a strong candidate for phytoremediation: the native grass *Poa foliosa* showed little toxic stress at concentrations far higher than those recently measured in the most contaminated areas of the island (Bramley-Alves *et al.*, 2014; Wasley, Mooney and King, 2015). Similarly, the earthworm *M. macquariensis*, which is endemic to Macquarie Island, may show potential as a candidate for vermi-remediation (Section 6.2.2), a technique which may complement phytoremediation, both of which may be used in tandem with PRBs. Large, tussock-forming members of the *Poa* genus are found on all subantarctic islands, as are members of the *Microscolex* genus, so such applications may be more broadly relevant beyond the focal location of this thesis, Macquarie Island.

6.5. Conclusions

This thesis aimed to assess the toxicity of weathered petroleum contaminants to soil biota on the subantarctic Macquarie Island. This was achieved through a literature review of existing knowledge, as well as a combination of field-based testing, standardised laboratory experiments, and a site-specific laboratory-based experiment.

There is potential for the findings presented here to be applied to subantarctic islands other than Macquarie Island because environmental conditions among these islands are similar and many of the species present are the same or are closely related. However, the context-specificity of this discipline was also highlighted, both in the findings presented here and within the broader literature. Even broader trends vary greatly according to taxa, soil properties and the precise toxicant being tested. Petroleum contaminants in soils have long been demonstrated to negatively affect biota, and this thesis helps to

better define the long-term effects of highly weathered diesel fuel on subantarctic soil ecosystems. The results presented here will be used to inform target remediation concentrations for existing contamination on Macquarie Island. However, these results also highlight the importance of thinking beyond contaminant concentration alone when rehabilitating and remediating disturbed soils. If the ability for ecosystems to begin recovering from disturbance is also taken into consideration, then the specific contaminant management techniques employed may be of similar importance to the target contaminant concentration *per se*.

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Appendix 1:

Sample location and total petroleum hydrocarbon (TPH) data from Chapter 3

Site	Longitude	Latitude	Location description	Replicate	TPH (C_9-C_{18}; mg/kg)
1	158.9364452	- 54.50039367	Central isthmus, near Fuel Farm	Surface 1	22
				Surface 2	24
				Surface 3	missing
				Depth	166
2	158.9362449	-54.5005504	Central isthmus, near Fuel Farm	Surface 1	12
				Surface 2	14
				Surface 3	12
				Depth	163
3	158.9362689	-54.50062909	Central isthmus, near Fuel Farm	Surface 1	11
				Surface 2	19
				Surface 3	8
				Depth	19
4	158.9362958	-54.50024309	Central isthmus, near Fuel Farm	Surface 1	18
				Surface 2	missing
				Surface 3	17
				Depth	52
5	158.9362009	-54.50030817	Central isthmus, near Fuel Farm	Surface 1	missing
				Surface 2	missing
				Surface 3	14
				Depth	56
6	158.9361516	-54.5003489	Central isthmus, near Fuel Farm	Surface 1	29
				Surface 2	18
				Surface 3	missing
				Depth	37
7	158.9390446	-54.49883394	North isthmus, near main station	Surface 1	82
				Surface 2	missing
				Surface 3	missing
				Depth	325
8	158.939109	-54.4988754	North isthmus, near main station	Surface 1	5
				Surface 2	missing
				Surface 3	55
				Depth	15
9	158.938994	-54.49888055	North isthmus, near main station	Surface 1	16
				Surface 2	21
				Surface 3	21
				Depth	52

10	158.9390588	-54.4987396	North isthmus, near main station	Surface 1	11
				Surface 2	18
				Surface 3	49
				Depth	missing
11	158.9390483	-54.49878326	North isthmus, near main station	Surface 1	24
				Surface 2	12
				Surface 3	2
				Depth	20
12	158.9391318	-54.49878721	North isthmus, near main station	Surface 1	missing
				Surface 2	12
				Surface 3	103
				Depth	12
13	158.9387401	-54.49895243	North isthmus, near main station	Surface 1	10
				Surface 2	
				Surface 3	26
				Depth	12
14	158.9388872	-54.49896808	North isthmus, near main station	Surface 1	55
				Surface 2	missing
				Surface 3	243
				Depth	missing
15	158.9389502	-54.49905054	North isthmus, near main station	Surface 1	20
				Surface 2	17
				Surface 3	32
				Depth	14
16	158.9390729	-54.4991173	North isthmus, near main station	Surface 1	8
				Surface 2	16
				Surface 3	8
				Depth	748
17	158.9391365	-54.49915327	North isthmus, near main station	Surface 1	100
				Surface 2	8
				Surface 3	9
				Depth	2
18	158.9391794	-54.49919753	North isthmus, near main station	Surface 1	22
				Surface 2	20
				Surface 3	10
				Depth	12
19	158.9320178	-54.50171586	Southwest isthmus	Surface 1	17
				Surface 2	5
				Surface 3	15
				Depth	23

20	158.9321215	-54.50168873	Southwest isthmus	Surface 1	19
				Surface 2	missing
				Surface 3	15
				Depth	Missing
21	158.9321243	-54.50178258	Southwest isthmus	Surface 1	20
				Surface 2	22
				Surface 3	13
				Depth	7

Appendix 2:

Presentation slides

This presentation was awarded the inaugural prize for *Best Science Communication* at the Society for Environmental Toxicology and Chemistry (SETAC) conference in October 2016.

These slides were developed prior to a complete dataset having been collected, so the analyses presented here differ to those in the main thesis body.

A comparison of field- and lab-based risk assessment methods for determining the toxicity of weathered diesel to subantarctic soil invertebrates

Ingrid Errington[1]

Ass. Prof. Grant Hose [1]

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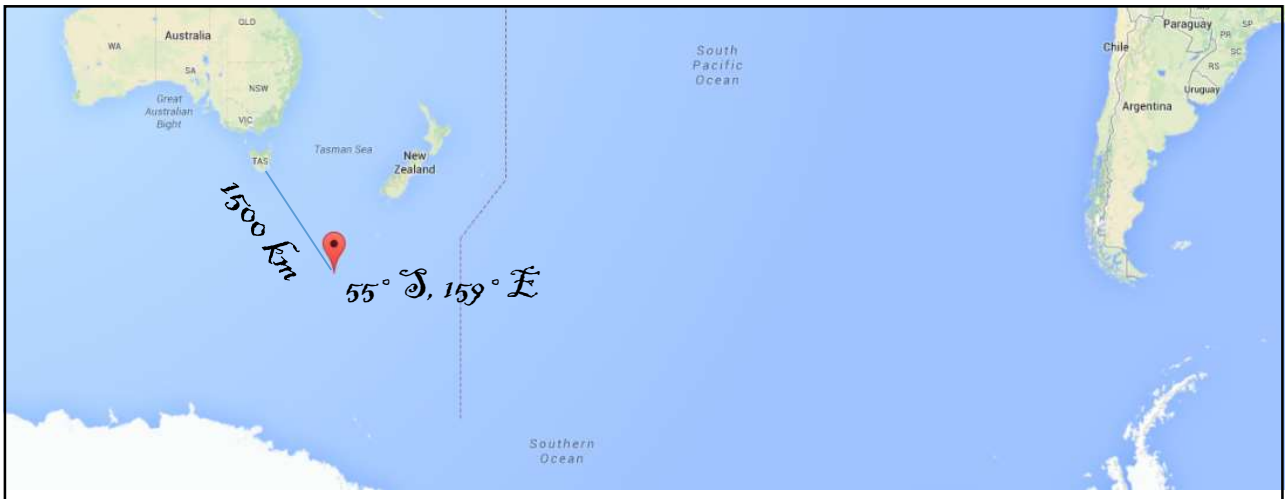
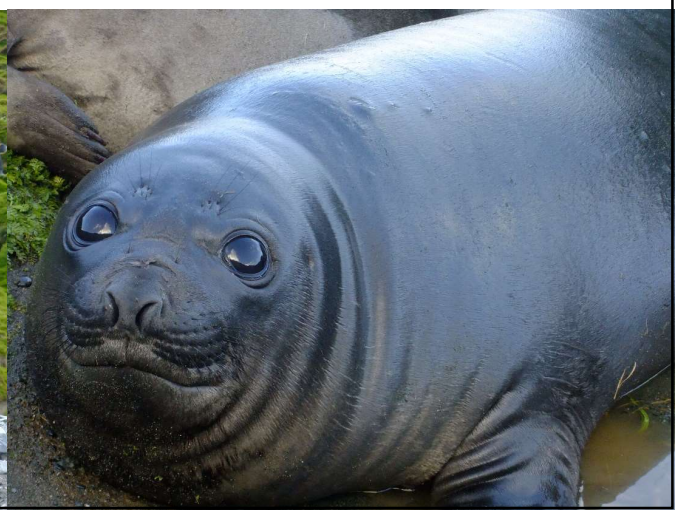
MACQUARIE University

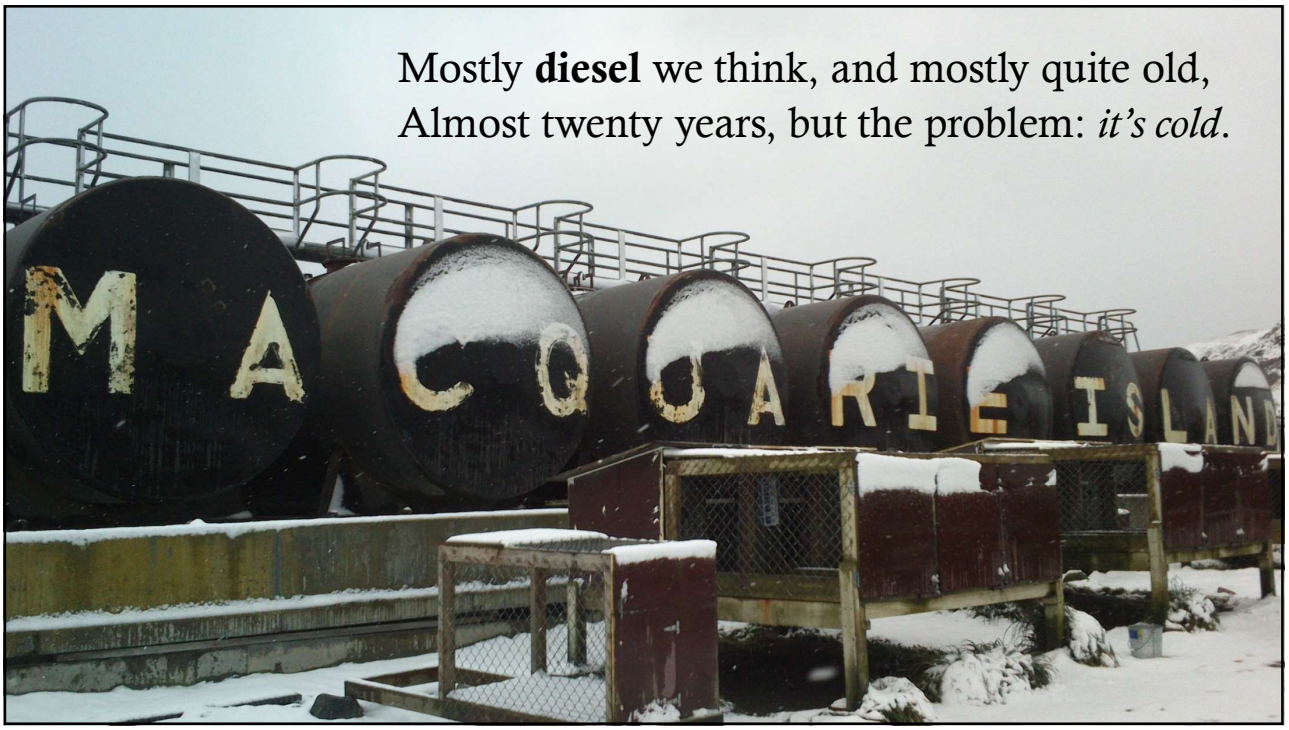
Australian Government
Department of the Environment
Australian Antarctic Division



A dot of an island, its name is Macquarie,
Can be found way down south, let me tell you a story.

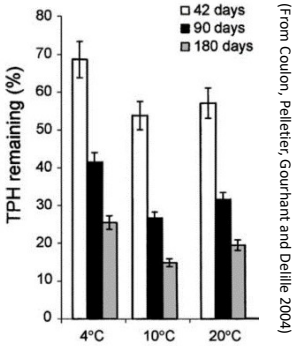
A World Heritage Area, with seals, birds and krill,
But all is not well – you see there have been **some spills**…





Mostly **diesel** we think, and mostly quite old,
Almost twenty years, but the problem: *it's cold*.

In warmer climes, it's fair to assume,
That the hydrocarbons would soon be consumed.



Remediation here's slow, so unless we assist,
It's likely the fuel will, for **centuries**, persist.

(Rayner et al. 2007)

evaporate,

are known to

blend

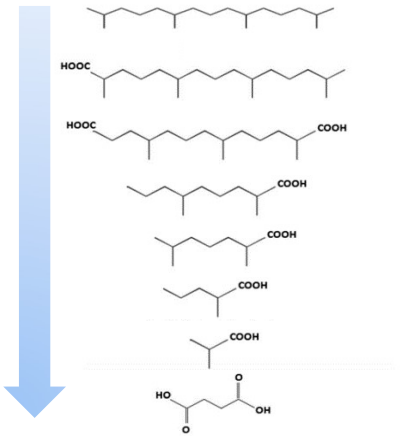
Light parts of a fuel

While simpler molecules

break down

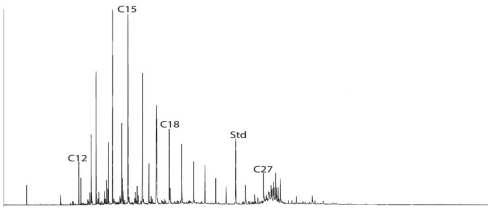
into lower

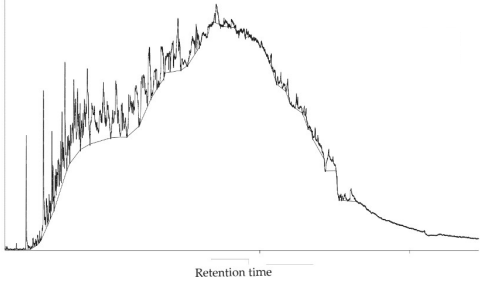
weights.



(Adapted from Abbasian, Lockington, Mallavarapu and Naidu, 2015)

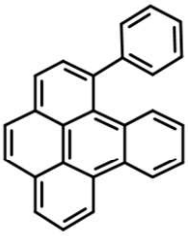
But then we are left with a mess in the soil,
An Unresolved Complex Mix of the remnants of oil.

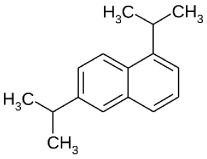


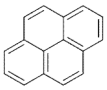


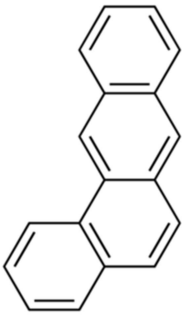
Unpublished data

UCM:
Unresolved
Complex
Mixture









It's unclear what's in the UCM,
But polycyclic aromatic hydrocarbons
Are toxic to life; retard seed germination,
So doing something about it is our obligation.

And we are doing something, but it's very expensive,

It's a long way away,

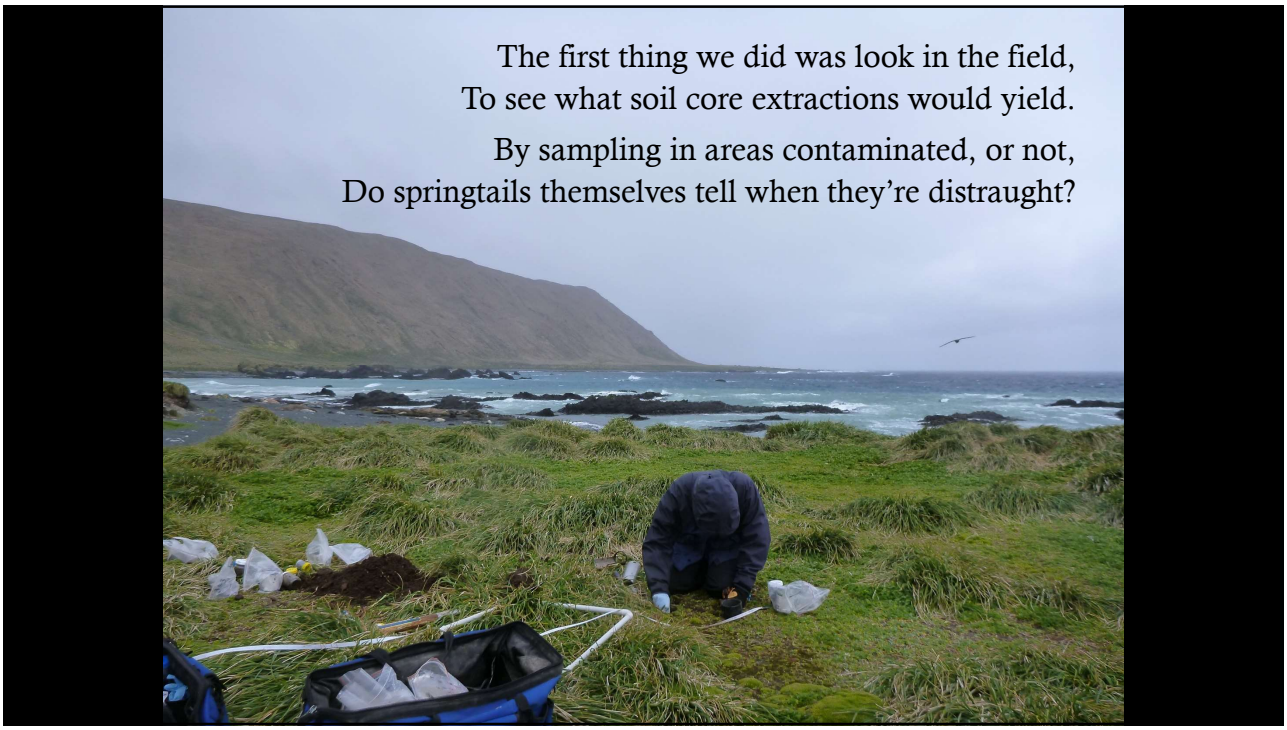
and environmentally intensive.

Existing targets are vague and broad.
For a subantarctic island their use may be flawed,

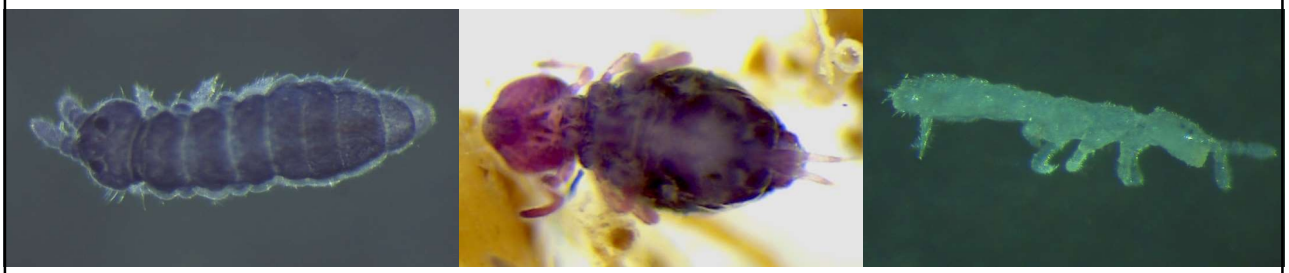
Tasmanian guidelines:
- 65 mg/kg for C6 to C9 compounds
- 1000 mg/kg for C10 to C36 compounds

They were developed for the mainland,
Where conditions are milder and resources at hand.

This was my aim, then: **to find a concentration,**
Where soil bugs are healthy in fuel contamination.



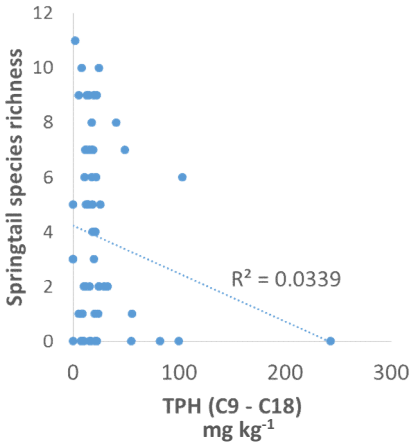
A high temperature gradient extracted the beasties,
And 28 000 were identified to species.

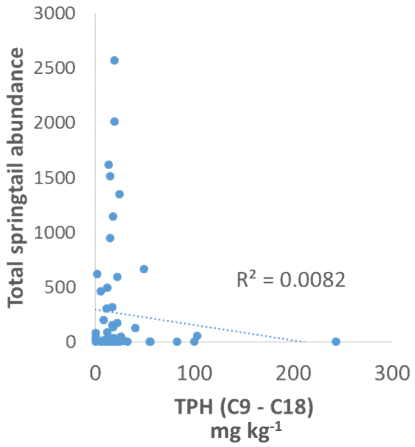


Chemistry was analysed with GC-MS,
Which is a difficult machine, but no, I digress,
TPH and degradation were thus quantified,
It's a work in progress, but for today, it's a guide.

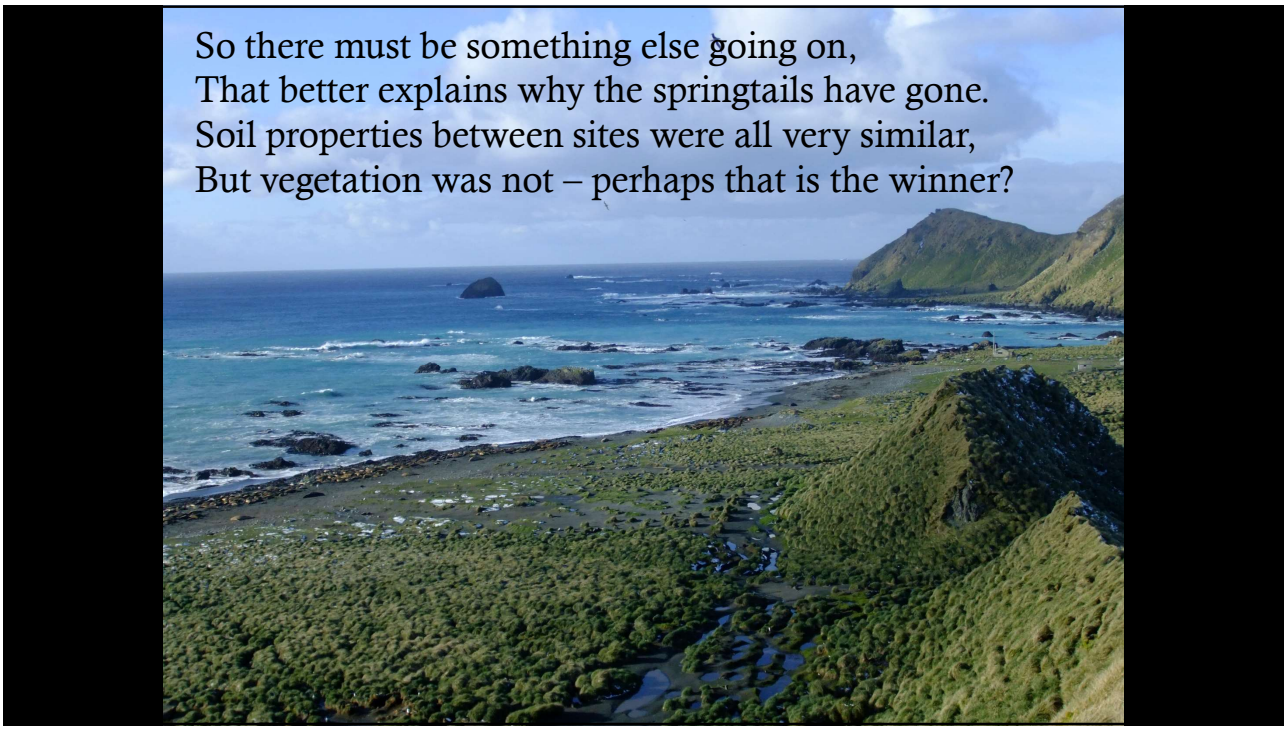
TPH: Total Petroleum Hydrocarbons

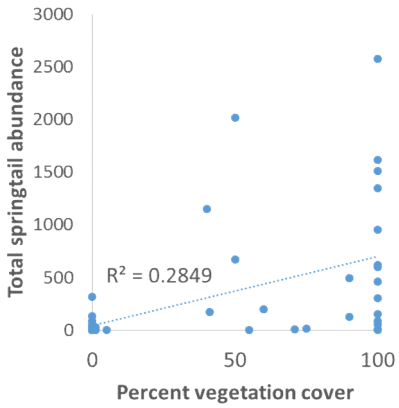
So here we are, but the R squared is **poor**,
In terms of richness, abundance and more.
While fresh diesel spills affect surface biota (Mooney 2013),
Old spills don't do much – hardly one iota.

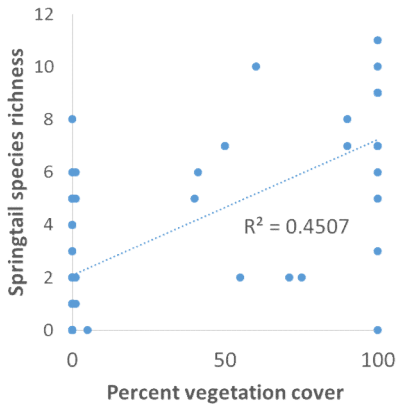




It became clear why though, through doing this chore,
Fuel percolates down a few feet or more.
The springtails however, live in the root zone,
About five centimetres – very few live below.







Yes, a bit better, but the implication's debated…
See, plant coverage and disturbance can be easily related.

And disturbance can be caused by remediation,
Through digging and turning and fertilisation!

and other things too!

And so we come to the next part of my tale,
This time a lab-based bioassay,
Using two species of earthworm, one model, one endemic,

Model species:
Eisenia fetida

Endemic species:
Microscolex macquariensis

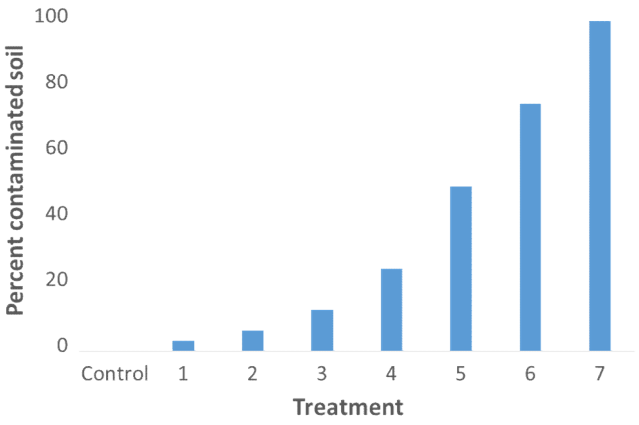
The results of which are hopefully less polemic.

Clean Macquarie Island soil, I did amend,
With copious Special Antarctic Blend.

This SAB diesel is fairly light,
Which means much will evaporate, even overnight.

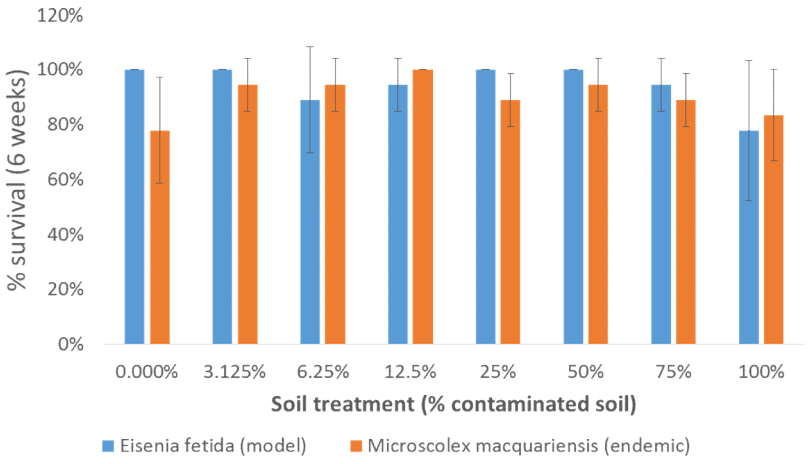
To mimic the UCM of a decades-old spill,
Within a PhD timeframe makes a student feel ill.
So I did my best, I gave it twenty-three weeks,
Far longer than most tox-test studies like this.

It sat under a fume hood, for near half a year,
Turned weekly, with moisture maintained, to be clear.
And then I prepared a gradient of pollution,
By adding clean soil in geometric dilution.

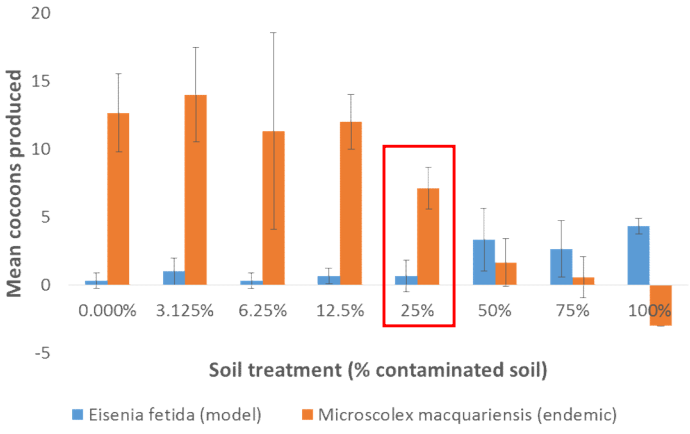


The worms were weighed! The experiment started!
Six weeks later I counted those that departed.
In another six weeks I counted cocoon production,
My results were in: survival, mass, reproduction.

Survival for both species was generally high,
Higher concentrations are needed if retried.

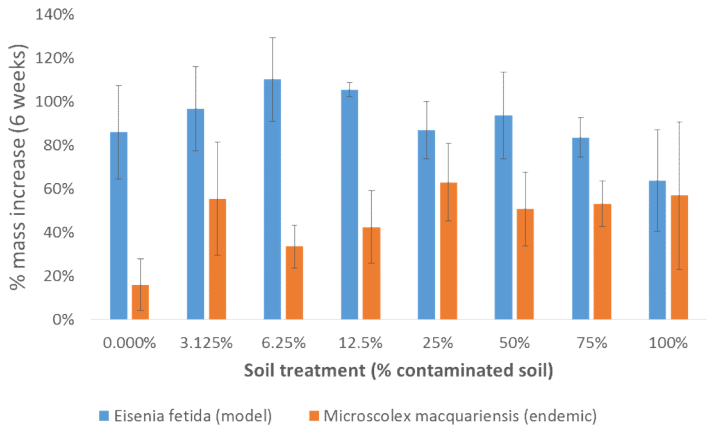


Reproduction tells more; it's not so consistent.
For a start, *Eisenia* seems far more resistant!
In *Microscolex*, the endemic species, however,
Cocoon production heads south here at this measure.

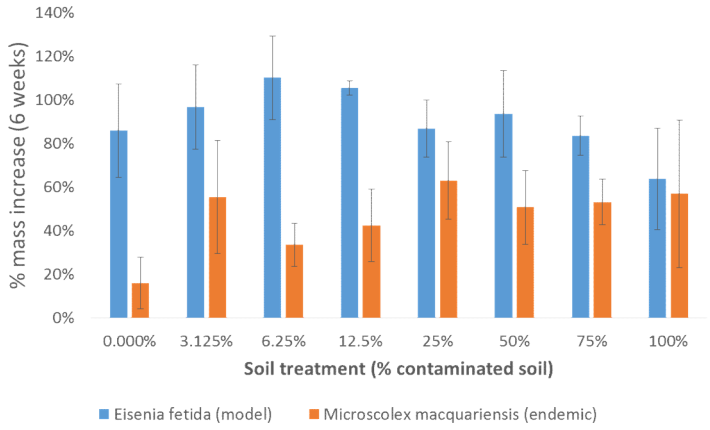


A cautionary tale, then, for model species use:
A strong reliance could cause one to deduce,
That a system's very resilient to stuff,
While the local species may be far less tough.

And finally **mass change** – are worms smaller or bigger?
Does this metric help find a value that triggers?
Higher concentrations would still further advise,
But both species definitely increased in size.



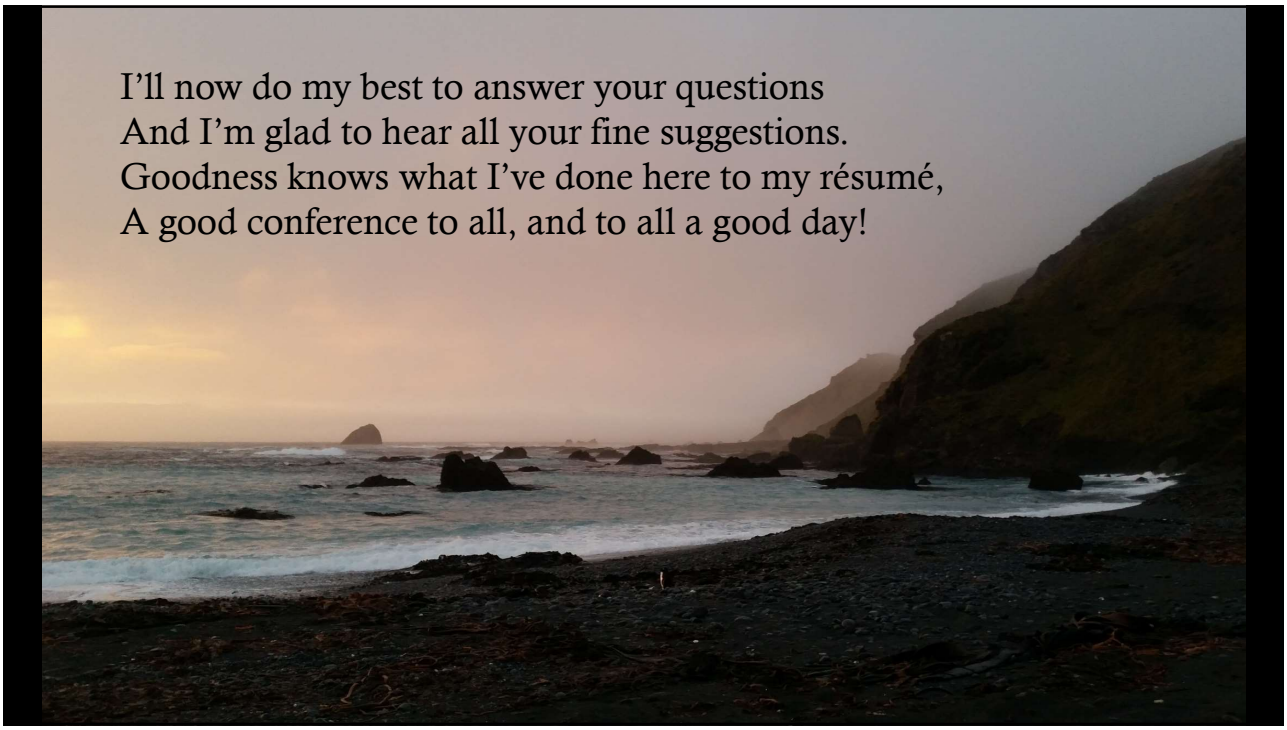
This is known as a hormetic trend,
Where low concentrations actually transcend,
The toxic effects caused by a compound,
Then at high concentrations, the toxicity's re-found.



How could a positive effect come to be,
From something thought to be so nasty?
Well, microbes can eat those fuel molecules,
And worms can then eat the fat animalcules.

So for all this work, what's the magic number,
That makes soil critters sleep the eternal slumber?
Or when some other effect can be seen,
To know when to consider our dirty soil, clean?

We've seen some effect, but not yet enough,
Any plan we came up with would be very rough
Taking a risk assessment approach,
Might also help find better receptors to broach.


I'll now do my best to answer your questions
And I'm glad to hear all your fine suggestions.
Goodness knows what I've done here to my résumé,
A good conference to all, and to all a good day!