Fungal diversity in Sub-Antarctic Macquarie Island and the effect of hydrocarbon contamination on this fungal diversity

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Publication Date:
2012

DOI:
https://doi.org/10.26190/unsworks/15665

Fungal diversity in Sub-Antarctic Macquarie Island and the effect of hydrocarbon contamination on this fungal diversity

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A thesis submitted in fulfillment of the requirements for the degree of

Master of Philosophy in Science

Supervisor: Dr. Belinda Ferrari

Co Supervisor: Dr. Brendan Burns

PLEASE TYPE

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: Zhang

First name: Chengdong Other name/s:

Abbreviation for degree as given in the University calendar: Master of Philosophy in Science

School: School of Biotechnology and Biomolecular Sciences Faculty: Faculty of Science

Title: Fungal diversity in Sub-Antarctic Macquarie Island and the effect of hydrocarbon contamination on this fungal diversity

Abstract 350 words maximum: (PLEASE TYPE)

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Acknowledgments

First I would like to thank Australian Antarctic Division for funding and supporting this research. I very much appreciated my supervisor Belinda Ferrari for sharing her research experience and improving my writing skills. I am particularly grateful to Josie van Dorst for soil samples collection and statistical analysis assistance and Tristrom Winsley for his sharing of valuable experience. Finally, I feel truly grateful my parents for economic support to my tuition fees and living expense, my wife Jia Sun for her supporting on my life.

List of abbreviations

AAD: Australian Antarctic Division

ANARE: Australian National Antarctic Research Expeditions

ANOVA: Analysis of Variance

BLAST: Basic Local Alignment Search Tool

BSA: Bovine Serum Albumin

BTEX: Benzene, Toluene, Ethylbenzene and Xylene

CFU: Colony Forming Units

COI: Mitochondrial Cytochrome C Oxidase I

CRBA: Cooky Rose Bengal Agar

CRBB: Cooky Rose Bengal Broth

CTAB: Hexadecyltrimethylammonium bromide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic acid

EF1α: Elongation Factor-1 Alpha

EFM: Epi-Fluorescence Microscopy

ETS: External Transcribed Spacer

FFM: Fuel Farm

IGS: Intergenic spacers

ITS: Internal Transcribed Spacer

LSU: Large Subunit

NCBI: National Centre for Biotechnology Information

MDS: Non-Metric Multidimensional Scaling

MPH: Main Power House

OTUs: Operational Taxonomic Units

PAH: Polyaromatic Hydrocarbon

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PEG: Polyethylene Glycol

PRIMER: Plymouth Routines In Multivariate Ecological Research

PVPP: Polyvinylpolypyrrolidone

RPB: RNA Polymerase II Subunit

rDNA: Ribosomal Deoxyribonucleic Acid

RFLP: Restriction Fragment Length Polymorphism

rRNA: Ribosomal Ribonucleic Acid

sp.: Species

SAB: Special Antarctic Blend Diesel Fuel

SEM: Scanning Electron Microscope

SNA: Special Nutrient Agar

SNB: Special Nutrient Broth

SSMS: Soil Substrate Membrane System

SSU: Small Subunit

TAE: Tris-acetate-EDTA

TEM: Transmission Electron Microscope

TPH: Total Petroleum Hydrocarbon

Tris: Tris(hydroxymethyl)aminomethane

w/v: weight/volume

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Abstract

Fungi form the largest group of eukaryotic organisms and are widely distributed on Earth. Estimates suggest that at least 1.5 million species exist in nature, yet only 5% have been recovered into pure culture. The fungal diversity of Sub-Antarctic Macquarie Island soil is largely unknown. In this study, a low nutrient fungal culturing approach was developed and used alongside a traditional high nutrient approach to recover Macquarie Island fungi from pristine and a series of Special Antarctic Blend (SAB) diesel fuel spiked soil samples. The low nutrient culturing approach recovered a significantly different ($P<0.01$) fungal population compared with the high nutrient media approach. In total, 91 yeast and filamentous fungi species were recovered from the soil samples, including 63 yet unidentified species.

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1 Introduction

1.1 *Fungi*

Fungi, also known as *Mycota*, are the largest group of eukaryotic organisms forming a five Kingdom system (Whittaker, 1959). Based on recent molecular biological analysis, fungi are considered to be derived from the same unicellular ancestors as animals (*Metazoa*), whereas they have been suggested to have evolved independently exhibiting an equal position to animals and plants (*Viridiplantae*) (Figure 1.1) (Shalchian-Tabrizi *et al.*, 2008; Gherbawy and Voigt, 2010). According to conservative estimates, at least 1.5 million fungal species actually exist in nature with no more than 5% of them recovered into pure culture (Hawksworth, 2001). Most recently, the predicted number of species has increased to 5.1 million (Blackwell, 2011). Hence, there are a large number of fungal species still waiting to be discovered, described and exploited.

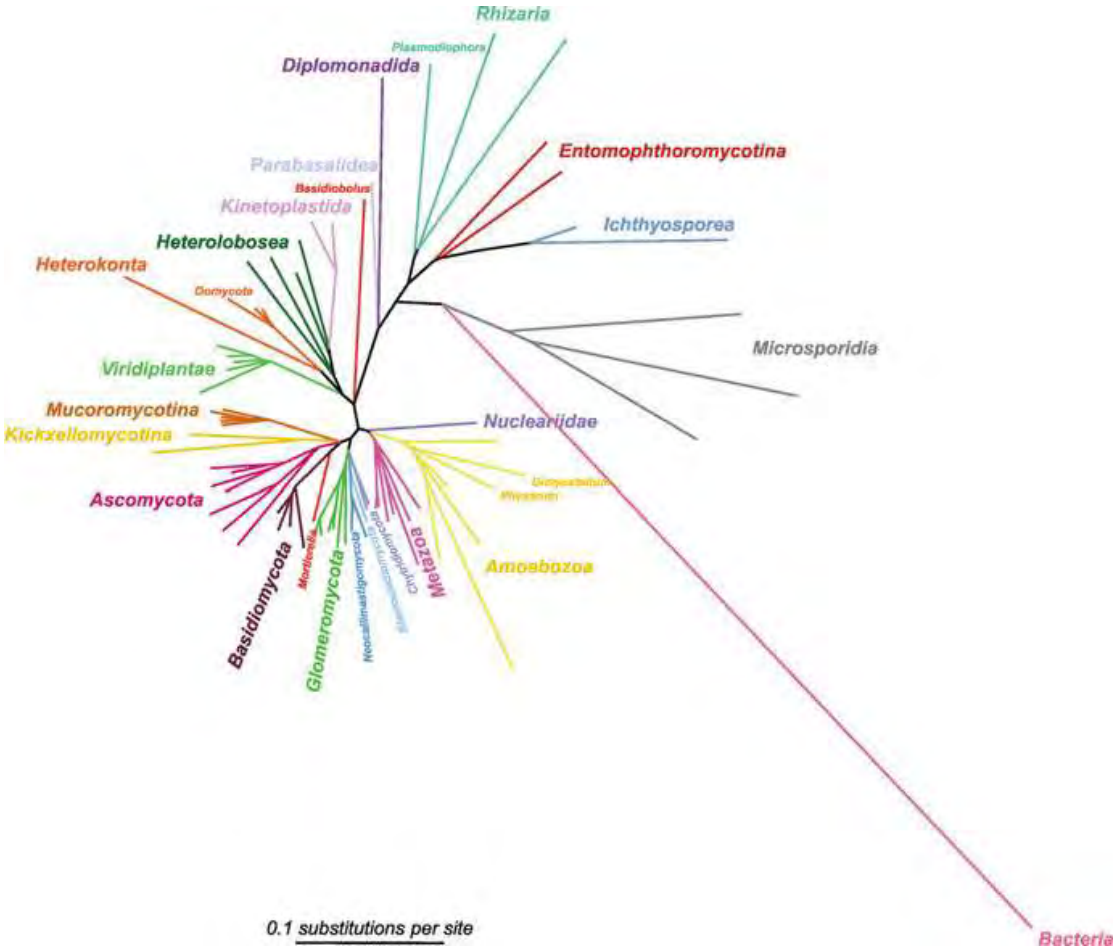


Figure 1.1 Measured evolutionary position of fungi and other eukaryotic groups based on amino acid sequences comparison of EF1α, actin, β-tubulin and their homologous present in outgroup (Gherbawy and Voigt, 2010).

1.1.1 Fungal Diversity

Fungi are widely distributed on Earth (Figure 1.2), from the mountains (Broady and Weinstein, 1998) and rivers (Wuczkowski *et al.*, 2003) to fields (Laughlin and Stevens, 2002; Hunt *et al.*, 2004)) and forests (Landeweert *et al.*, 2003), and from oceans (Singh *et al.,* 2010) to the poles (Connel *et al.*, 2006; Arenz and Blanchette, 2011). Fungi have been isolated from extreme environments, including those high in salinity (Mohamed and Martiny, 2010), hydrocarbons (April *et al.*, 2000; Prenafeta-Boldu *et al.*, 2001), anoxic zones (Jebaraj *et al.*, 2010), hydrothermal areas (Calvez *et al.*, 2009) and radionuclide contaminated areas (Dadachova *et al.*, 2007). Fungi have also been well described as human (Vicente *et al.*, 2008; Sudhadham *et al.*, 2010) and plant pathogens (Sundelin *et al*., 2009).

Until 2008, there were 99,108 accepted fungal species that have been described (Kirk *et al.*, 2008). This number has risen significantly over last three years with approximately 1,200 novel fungal species discovered per annum (Blackwell, 2011). Based on Hibbett *et al.* (2007), the latest classification of the Kingdom *Fungi* describes one sub-kingdom, seven phyla, ten sub-phyla, 35 classes, 12 sub-classes, 129 orders and 536 families (Figure 1.3) (Cannon and Kirk, 2007). The traditional phylum *Zygomycota* is no longer accepted in the classification system and their members are now distributed within other phyla. The order *Blastocladiales* of phylum *Chytridiomycota* has been upgraded to phylum *Blastocladiomycota*, and phylum *Microsporidia* is now considered a member of *Fungi* (Hibbett *et al.,* 2007).

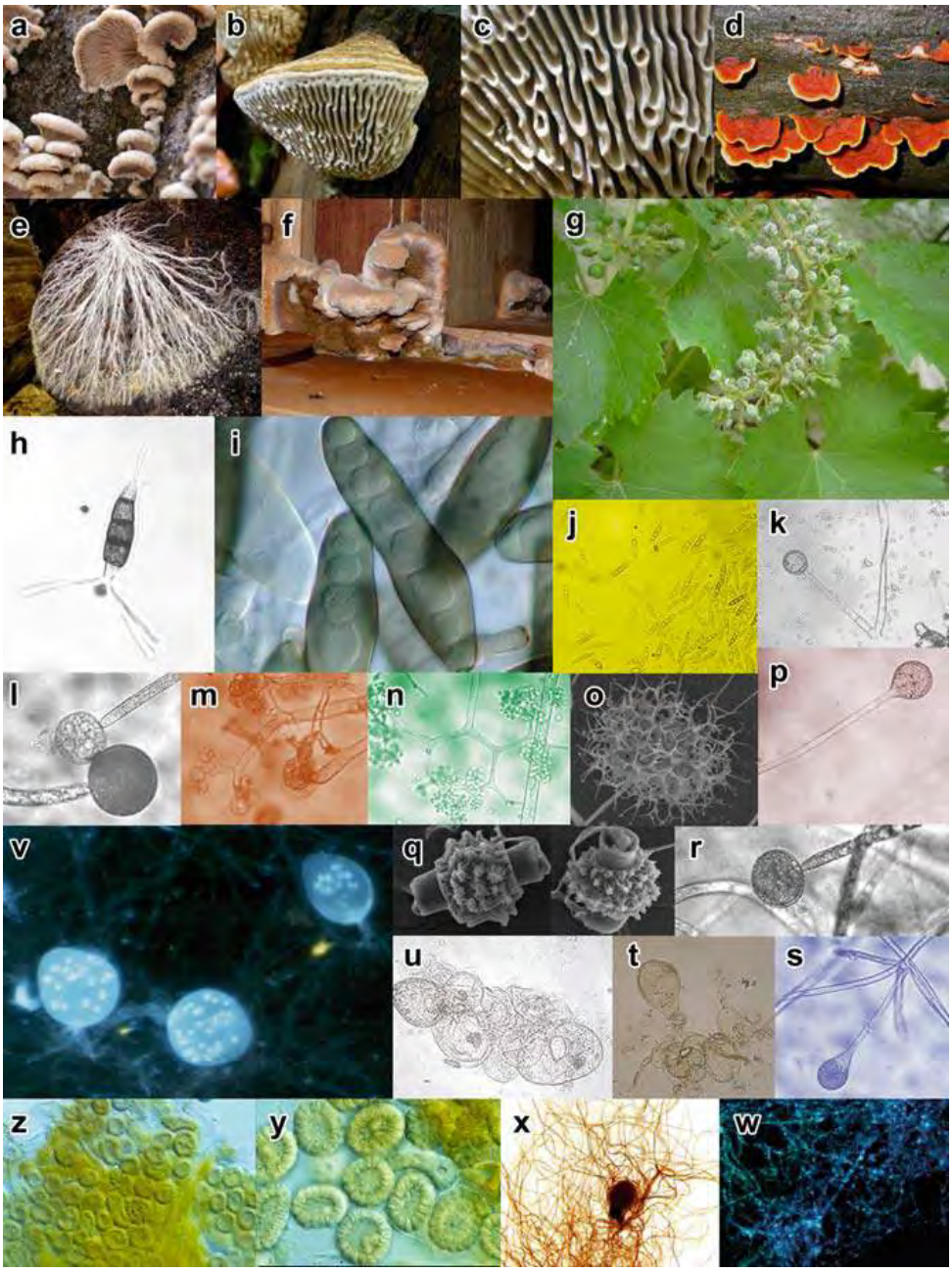


Figure 1.2 The fungal diversity on Earth. (a) *Schizophyllum sp.*, (b) and (c) *Daedalea sp.*, (d) *Trametes sp.*, (e) *Antrodia sp.*, (f) *Serpula sp.*, (g) *Plasmopara sp.*, (h) *Pestalotiopsis sp.*, (i) *Bipolaris sp.*, (j) *Fusarium sp.*, (k) and (r) *Mucor sp.*, (l) *Helicostylum sp.*, (m) *Thamnidium sp.*, (n) and (o) *Dichotomocladium sp.*, (p) and (s) *Absidia sp.*, (q) *Lentamyces sp.*, (t) and (u) *Caecomyces sp.*, (v) and (x) *Neocallimastix sp.*, (w) *Anaeromyces sp.*, (y) *Sorosphaera sp.* (Gherbawy and Voigt, 2010).

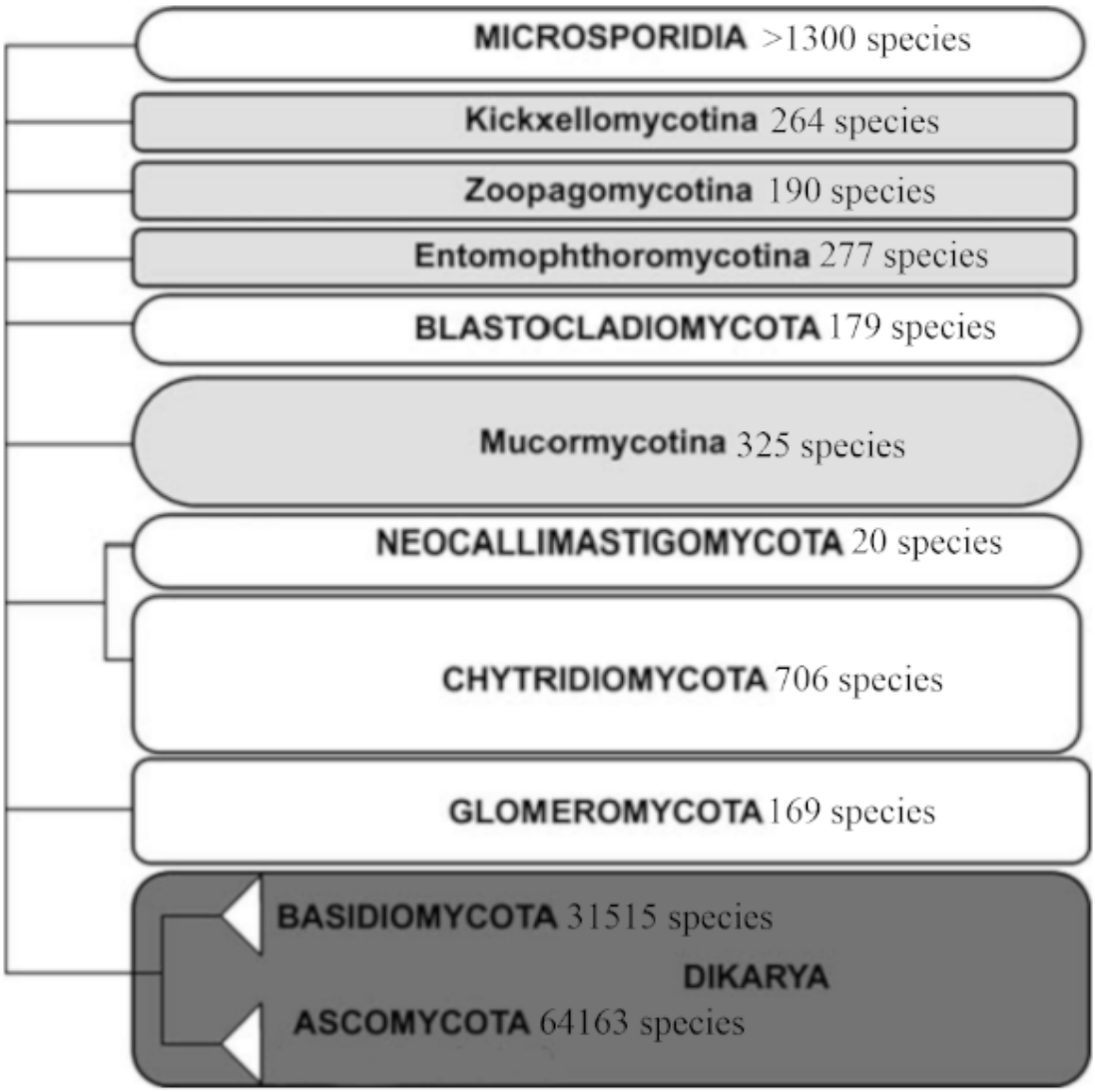


Figure 1.3 Phylogenetic tree of *Fungi* at the phylum and sub-phylum *incertae sedis* level (Hibbett *et al.*, 2007). The branch lengths are not proportional to genetic distances. Species number for each taxa are adopted from *Ainsworth & Bisby's Dictionary of the fungi 10th ed.* (Kirk *et al.*, 2008).

1.1.2 Antarctic Fungal Diversity

Mycologists have investigated Antarctic fungal diversity since the mid-nineteenth century (Hughes and Bridge, 2010) with the majority carried out on different locations including maritime, continental and Sub-Antarctic regions. These investigations were previously based on cultivation and morphological identifications only (Broady and Weinstein, 1998; Frate and Caretta, 1990; Kerry, 1990; Vishniac, 1996; McRae and Seppelt, 1999). However, most recently they have also been based on molecular identification and phylogenetic analysis (Arenz *et al*., 2006; Blanchette *et al.*, 2004; Bridge & Newsham, 2009; Connell *et al*., 2006; Duncan *et al*, 2008; Sung *et al.*, 2008; Arenz *et al*., 2010; Arenz and Blanchette, 2011).

Antarctica is considered the last pristine site on Earth and provides unique opportunities for fungal biodiversity and ecology investigations (Malosso *et al.*, 2006). Compared with the large number of described fungi species in temperate regions, the understanding of fungal diversity in the Antarctic and sub-Antarctic regions is poorly understood. So far, approximately 1,000 accepted fungal species have been reported from Antarctic regions (Bridge and Newsham, 2009). Most of the reported Antarctic fungi species are cosmopolitan, while a small proportion is proposed to be indigenous (Ruisi *et al.*, 2007). In addition, only a few species are considered psychrophilic, the remainder are considered to be cold tolerant mesophilic strains (Hughes and Bridge, 2010).

The list of Antarctic fungi is diverse, most of the frequently isolated species belong to genus *Acremonium*, *Aspergillus*, *Cadophora, Chalara*, *Chrysosporium*, *Cladosporium*, *Cunninghamella*, *Cryptococcus*, *Debaryomyces, Dotatomyces*, *Fusarium*, *Geomyces*, *Mortierella*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phialophora*, *Phoma*, *Pseudeurotium*, *Scolecobasidium*, *Scytalidium*, *Thelebolus*, *Thermomyces*, *Tolypocladium*, *Torulaspora, Trichophyton* and *Verticillium*. (Frate and Caretta, 1990; Vishniac, 1996; Arenz *et al.*, 2006; Connell *et al.*, 2006; Kostadinova *et al.*, 2009; Arenz *et al.*, 2010; Arenz and Blanchette, 2011). Lichens are the predominant flora in the terrestrial ecosystem of Antarctica (Lee *et al.*, 2008) and over 1,500 lichenicolous fungi have been identified around the world (Kirk *et al.*, 2008). However, only limited Antarctic lichenicolous fungal species, such as *Arthonia parmeliae*, *Carbonea antarctica*, *Dactylospora dobrowolskii*, *Phacopsis usneae*, *Sphaerellothecium buelliae*, and *Weddelomyces gasparriniae* have been described (Olech and Alstrup, 1996; Hawksworth and Iturriaga 2006). *Deschampsia antarctica* Desv. and *Colobanthus quitensis* (Kunth) Bartl. are the only two angiosperms present in Antarctica, recent investigations on endophytic fungi associated with these Antarctic plants showed that a large portion of the isolated fungi were unidentified species and therefore distinct fungal communities exist when compared with temperate regions (Rosa *et al.*, 2009; Rosa *et al.*, 2010). Interestingly, several fossil fungi have also been discovered from Antarctic Triassic silicified peat deposits, such as *Mycocarpon asterinum* (Taylor and White, 1989).

Due to the extreme climatic conditions, Antarctic regions provide the opportunity for cold adapted fungal species discovery (Kostadinova *et al.*, 2009). The enzymatic systems of these fungi may provide great potential for biotechnological exploitation and application (Cavicchioli et al., 2011). *Antarctomyces psychrotrophicus*, an indigenous psychrophilic species can produce an extracellular antifreeze protein with maximum thermal hysteresis activity under alkaline conditions, which differ from other reported antifreeze proteins (Stchigel *et al.*, 2001; Xiao *et al.*, 2010). Recent investigations on the antioxidant response of fungal strains isolated from Antarctica revealed great antioxidant enzyme abilities compared with mesophilic strains and included *Alternaria sp.*, *Geomyces sp.*, *Lecanicillim sp.*, *Cladosporium sp.*, *Monodictys sp.*, *Penicillium sp.*, *Epicoccum sp.* and *Aspergillus sp.* (Gocheva *et al.*, 2009; Tosi *et al.*, 2010).

In 2001, Aislabie *et al.* isolated high numbers of *Phialophora sp.* from oil contaminated soils collected in three different Antarctic locations, whereas in the control soils *Chrysosporium sp.* and *Geotrichum sp.* were dominant, suggesting that *Phialophora sp.* was a potential hydrocarbon degrader. Hughes *et al.* (2007) showed that many Antarctic species may be tolerant to a range of aliphatic and aromatic hydrocarbons and included *Mollisia sp.*, *Penicillium sp.*, *Mortierella sp.*, *Trichoderma sp.* and *Phoma sp.*. The Ross Island Region, a major Antarctic location has been researched for fungal wood destroying or cellulose degradation activity. From this site, Arenz *et al.* (2006) identified 71 fungal isolates, Duncan *et al.* (2008) recovered 29

fungal species and Blanchette *et al.* (2010) isolated 69 fungal cultures. In these studies the *Cadophora sp.*, *Cladosporium sp.* and *Geomyces sp.* were the most frequently isolated fungal species (Blanchette *et al.* 2004; Farrell *et al.*, 2011).

1.1.3 Sub-Antarctic Macquarie Island Fungal Diversity

To date, knowledge on Sub-Antarctic Macquarie Island fungal diversity is severely limited with only one relatively recent report. In 1990, Kerry isolated a group of leaf and litter fungi including *Acremonium zonatum*, *Ascochyta stilbocarpae*, *Aureobasidium pulllans*, *Camarosporium metableticum*, *Geomyces pannorum*, *Motierella gamsii*, *Penicilium brevi-compactum*, *Penicillium cyclopium*, *Phoma exioua*, *Phoma sp.*, *Rhodesiopsis gelatinosa* and *Stagonospora ischmaemi* (Kerry, 1990). In this report, the Macquarie Island fungi were considered to be cold tolerant mesophilic strains, they exhibited slow growth at 4°C and an optimum growth rates occurred at 15°C.

1.2 Fungal Identification Techniques

Correct and rapid identification of fungi is not only practical for fungal biodiversity investigations but are also meaningful for clinical, agriculture and biotechnology applications (Guarro *et al.*, 1999). Classical fungal identification methods are complicated and severely limited with extensive professional training required. Many species simply classified based on morphology, results in inconsistencies with true phylogenetic relationships. Past fungal taxonomic systems include De Bary (1884),

Gaumann (1962), Martin (1950), Whittaker (1969) and the Ainsworth system (1973) (Kirk *et al.*, 2008). Currently, the two most influential fungal taxonomy systems in use are *The Mycota VII* (Mclaughlin *et al.*, 2001a, 2001b) and *Ainsworth & Bisby's Dictionary of the fungi 10th ed.* (Kirk *et al.*, 2008). After entering the era of molecular biology, many novel disciplines and technologies have been introduced to update fungal classification and identification systems which not only support the development of rapid and unambiguous detection and identification of fungi, but also establishes phylogenetic relationships coherently (Gherbawy and Voigt, 2010).

1.2.1 Molecular Biology Characterisation

With the development of the Polymerase Chain Reaction (PCR) technique in the last century (Guarro *et al.*, 1999), molecular identification was introduced into modern mycology and has subsequently increased fungal biodiversity and taxonomy investigations as it is rapid, highly specific and reliable (Chase and Fay, 2009; Begerow *et al.*, 2010). Molecular identification is primarily based on the amplification of a fungal target gene or DNA fingerprinting. Currently, accepted molecular targets include the internal transcribed spacer (ITS), ribosomal small subunit (SSU), ribosomal large subunit (LSU), α- and β-tubulin, actin, RNA polymerase II subunit (RPB), elongation factor-1 alpha (EF1α) and mitochondrial cytochrome C oxidase I (COI) genes (Min and Hickey, 2007; Chase and Fay, 2009; Seifert, 2009; Begerow *et al.*, 2010). Recently, SSU, LSU, ITS, EF1α and RPB genes were used for phylogenetic analysis to reconstruct the early evolution of *Fungi* (James *et al.*, 2006).

Additionally, the latest higher-level phylogenetic re-classification of *Fungi* was also based on these molecular targets (Hibbett *et al.*, 2007).

1.2.1.1 Ribosomal DNA Genes

The most widely used molecular targets for fungal classification and identification are the ribosomal DNA (rDNA) genes (Guarro *et al.*, 1999). The rDNA gene sequences are highly conserved containing over 100 copies per fungal genome and include both conserved and highly variable domains (Wu *et al.*, 2003). Therefore, they have been used to determine the genetic relationship among fungi (Guarro *et al.*, 1999). In Eukaryotes, ribosomal RNAs (rRNA) are encoded by 18S, 5.8S and 28S rDNA genes. The rDNA genes are repetitive transcriptional units and are separated by intergenic spacers (IGS). Each unit contains two external transcribed spacers (5' and 3' ETS), 18S rDNA, internal transcribed spacer 1 (ITS1), 5.8S rDNA, internal transcribed spacer 2 (ITS2) and 28S rDNA (Figure 1.4) (Gherbawy and Voigt, 2010). The overall length of each unit is between approximately 7.7 and 24 kb (Guarro *et al.*, 1999).

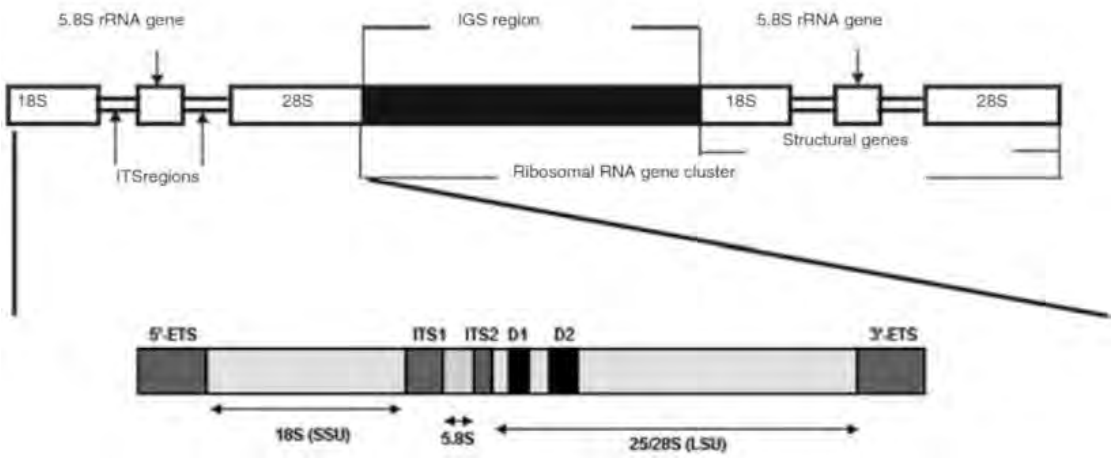


Figure 1.4 Schematic of rDNA gene clusters in the fungal genome, modified from *Molecular Identification of Fungi* (Gherbawy and Voigt, 2010). The upper figure represents the locations of repetitive rDNA gene units and spacer regions (ITS and IGS). The lower figure represents the locations of 5' ETS, 18S rDNA gene (or SSU), ITS1, 5.8S rDNA gene, ITS2, 28S rDNA gene (or LSU) with D1-D2 variable regions and 3' ETS in each repetitive rDNA gene unit.

The 18S rDNA gene contains conserved and variable domains (Guarro *et al.*, 1999). Amplifying and sequencing the variable domains using specific primers have been used to identify a wide range of fungal groups (Smit *et al.*, 1999; Wu *et al.*, 2003). However, they are not always suitable for fungal species or strain identification as too conserved (Hunt *et al.*, 2004). In some cases, partial or complete fungal 18S DNA sequences have been verified against traditional morphological classification systems (Haase *et al.*, 1995; Okada *et al.*, 1997; de Hoog and van den Ende, 1998; Abdullahi *et al.*, 2005).

The 5.8S rDNA nucleotide sequences are short and highly conserved, and are rarely used for fungal molecular identification or phylogenetic analysis (Guarro *et al.*, 1999; Nilsson *et al.*, 2008). However, combining 5.8S rDNA with ITS1 and ITS2 regions provides the most popular method for fungal identification and phylogenetic investigations, and ITS sequences are the most abundant gene sequences available in public databases (Table 1.1) (Begerow *et al.*, 2010). The ITS1, 5.8S rDNA and ITS2 are collectively called the ITS. ITS1 and ITS2 are moderately conserved domains. This conservative property is relatively consistent within species but obvious differences occur between species (Uijthof, 1996). Additionally, the sizes of ITS region are distinct among various eukaryotic species (Korabecna, 2007). This feature has led to the ITS regions being a suitable target for the molecular identification of fungal species. Occasionally, approximately 3% ITS intraspecies variability within *Dikarya* and higher in other basal fungal lineages may lead to two different species being mistakenly identified (Nilsson *et al.*, 2008).

The 28S rDNA contains greater variation within domains than the 18S rDNA, and may be useful for fungal phylogenetic studies. The D1-D2 variable domain of the 28S rDNA gene is often used for yeast classification and identification (Guarro *et al.*, 1999; Mueller *et al.*, 2004). This target is also used for *Basidiomycetes* and filamentous *Ascomycetes* identification (Mueller *et al.*, 2004). Studying 28S rDNA variable domains are likely to be a reliable method for fungal classification and identification. However, only a small proportion of fungal 28S rDNA gene sequences are available

in public databases (Table 1.1).

Table 1.1 The number of partial fungal identification DNA sequences in GenBank Database (Begerow *et al.*, 2010).

Target Gene	**Number of Sequences**
ITS	147,042
SSU	136,588
LSU	115,228
β-tubulin	14,921
Actin	4,124
RPB2	8,316
EF1α	7,235
COI	581

1.2.1.2 DNA Restriction Fragment Length Polymorphism (RFLP)

In recent years, DNA fingerprinting technologies have emerged (Gherbawy and Voigt, 2010). These methods have been widely used for fungal classification, particularly targeting chromosomal DNA genes. RFLP is the use of restriction enzymes to recognise and digest specific nucleotides sequences or restriction sites located on DNA molecular, therefore generating different sized restriction fragments after gel electrophoresis. Variable fragment sizes are used to distinguish between different DNA samples (Gherbawy and Voigt, 2010). The RFLP technique is based on the variation of DNA sequences, such as point mutations, translocation, inversion, deletion and transposition in the evolutionary process. It has become an effective fungal classification research method at the intraspecies, interspecies and the population level (Guarro *et al.*, 1999; Gherbawy and Voigt, 2010).

RFLP on ITS PCR amplicons is widely used as they highlight differences in nucleotide sequence as well as the variability in the length of ITS regions (Garro *et al.*, 1999). Farmer and Sylvia (1998) studied 64 ectomycorrhizal fungal ITS RFLP maps with 7 restriction enzymes, the result showed that the RFLP maps corresponded to their morphological species. Fischer and Wagner (1999) analysed the ITS RFLP patterns with 3 restriction enzymes for 52 species of lignicolous *Basidiomycetes*, and suggested that RFLP analysis as a tool for fungal identification. Recently, PCR-RFLP analysis was applied for the identification of plant pathogens (Matsushita and Suzuki, 2005). PCR-RFLP analysis has been widely applied as a rapid human fungal pathogens identification tool for clinical purposes, such as the identification of *Candida* species (Morace *et al.*, 1997), *Trichophyton* species (Mochizuki *et al.*, 2003) and members of Order *Mucorales* (Machouart *et al.*, 2006).

1.2.2 Morphological Characterisation

Fungal morphological identification was developed on the basis of morphological features since ancient times for macrofungi and the eighteenth century for microfungi (Carlile *et al.*, 2001). The observation of morphological characteristics following cultivation on appropriate culture medium is still a commonly used fungal identification method (Guarro *et al.*, 1999).

The major characteristic of *Ascomycetes* is that they produce an ascus during the teleomorph (sexual phase). If a fungal strain produces ascospores in its ascus,

regardless of any other characteristics, it is considered an *Ascomycetes*. Within the *Ascomycetes*, the morphological identification of teleomorphs is based on characteristics of the ascus, ascocarp or ascospore (Figure 1.5 (A)-(H)) (Carlile *et al.*, 2001). Morphological classification of the anamorph (asexual phase or imperfect fungi) is based on conidia during development. Conidia are developed from conidiogenous cells on conidiophores with *Blastic* and *Thallic* conidiogenesis (Figure 1.5 (I)-(P)). *Blastic* conidiogenesis are hypha or conidiophores that bud from the location of growth (Kirk *et al.*, 2008). *Thallic* conidiogenesis are conidia formed by fragmentation of hypha (Mueller *et al.*, 2004).

Figure 1.5 Morphological features of *Ascomycetes*. Photos were taken from *Fungal Families of the World* (Cannon and Kirk, 2007). (A) Ascocarp of *Cucurbitaria laburni* (B) Ascocarp of *Stigmatula astragali* (C) Asci and ascospores of *Hysterographium fraxini* (D) Asci and ascospores of *Fremitomyces punctatus* (E) Asci and ascospores of *Eurotium chevalieri* (F) Asci and ascospores of *Chaetothyrium javanicum* (G) Asci and ascospores of *Podostroma alutaceum* (H) Ascospores of *Xenonectriella ornamentata* (I) *Blastic* conidiogenesis of *Amorphotheca resinae* (J) *Blastic* conidiogenesis of *Clonostachys solani* (K) *Blastic* conidiogenesis of *Botrytis cinerea* (L) *Blastic* conidiogenesis of *Sporothrix sp.* (M) *Thallic* conidiogenesis of *Geotrichum candidum* (N) *Thallic* conidiogenesis of *Ceratocystis fimbriata* (O) Conidiogenous cells, micro- and macro-conidia of *Cylindrocarpon didymum* (P) Conidia of *Helicoön pluriseptatum.*

Particular characteristics of *Basidiomycetes* that differ from other fungi is they produce basidiospores from basidium during sexual reproduction. Additionally, clamp connections (Figure 1.6 (A)) and ballistospores (discharge spore into the air from sterigma) are the other two specific characteristics examined at the microscopic level. Within *Basidiomycetes*, the morphological identification of teleomorphs is mainly based on the characteristics of basidium (Figure 1.6 (B)-(E)) (Carlile *et al.*, 2001). Similar to *Ascomycetes*, anamorph identifications are also based on *Blastic* and *Thallic* conidiogenesis features (Figure 1.6 (F)) (Guarro *et al.*, 1999).

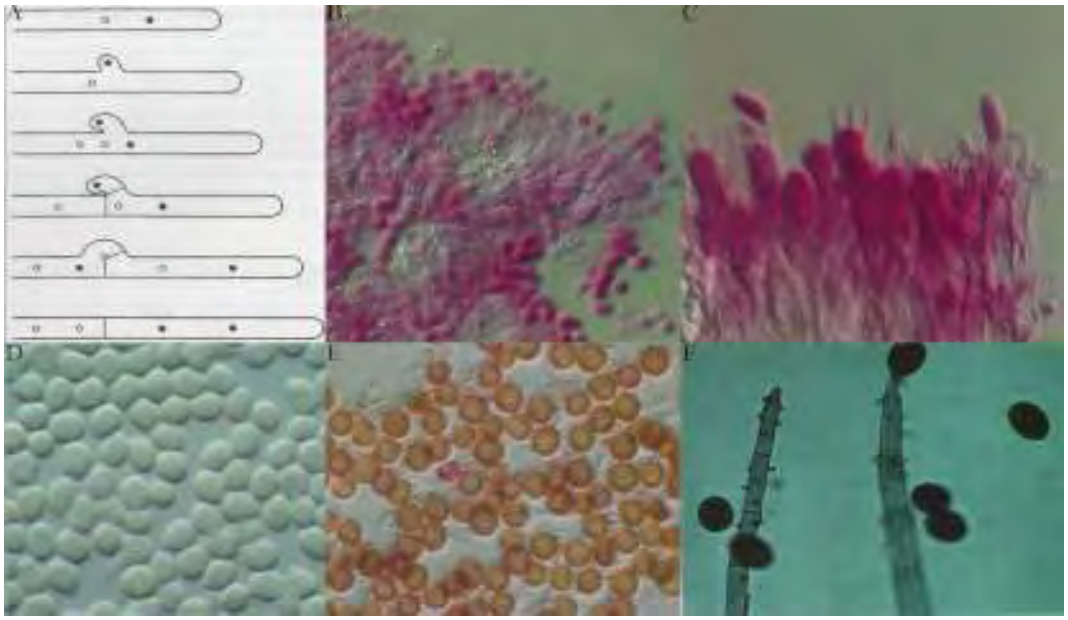


Figure 1.6 Morphological features of *Basidiomycetes*. (A) Image taken from *The Fungi (2nd ed.)* (Carlile et al., 2001) and remaining images from *Fungal Families of the World* (Cannon and Kirk, 2007). (A) Steps in the formation of clamp connection (B) Basidia and basidiospores of *Lycoperdon molle* (C) Badisia and basidiospores of *Septobasidium bogoriense* (D) Basidiospores of *Phakopsora vitis* (E) Basidiospores of *Scleroderma citrinum* (F) *Blastic* conidiogenesis of *Botryobasidium conspersum* (anamorph: *Acladium conspersum*).

1.3 Limitations in Fungal Cultivation Studies

Recovery of fungi from environmental samples into pure culture is a pre-requisite for specimen collection, morphological description and species identification. However, fungal isolation has been severely limited (Bridge and Spooner, 2001; Anderson and Cairney, 2004). Compared to the estimated 1.5 million fungal species, less than 5% have been recovered (Hawksworth, 2001). For example, Calvez *et al*. (2009) recovered 21 fungal strains from an environmental sample. By comparison, in the same samples 7,425 fungal small subunit (SSU) rRNA gene sequences were obtained. While Jebaraj *et al*. (2010) obtained 268 distinct fungal RFLP patterns from sediment samples collected in Arabian Sea, yet only 26 fungal cultures were recovered.

1.3.1 Reasons for Low Species Recovery

In order to recover a broad range of fungi from environmental samples, serial dilutions are always recommended (Arenz and Blanchette, 2011). However, the recovery rate is not high. A crucial point is the selection of culture medium. The ideal culture medium to recover a diverse fungal spectrum should be non-selective or selective media with an equitable growth rate for all species should be used (Mueller *et al*., 2004).

In fact, most of frequently used artificial fungal culture media, such as potato dextrose (PDA) and malt extract agar (MEA) are highly selective (Mueller *et al*., 2004). These media may create conditions that favor species capable of rapid germination and

growth. Thus, rapidly growing species outcompete slower growing species. In order to suppress the growth rate of fast growing species, fungal-toxic agents are recommended to be added to culture media at sub-lethal doses, such as rose bengal, cyclosporin A, cycloheximide or dichloran (Mueller *et al*., 2004). Ottow and Glathe (1968) compared the fungal colony numbers growing on rose bengal malt extract agar versus malt extract agar, the results showed that more fungal isolates grew on rose bengal malt extract agar. In addition, many fungal species are not adapted to growth in nutrient rich culture media or require special growth requirements (Mueller *et al*., 2004; O'Brien *et al.*, 2005).

1.3.2 Novel Cultivation Methods for Bacteria

Similar to fungi, traditional bacterial cultivation has been severely limited with less than 1% of species recovered (Kaeberlein *et al.*, 2002; Ferrari *et al.*, 2005). Thus, novel bacterial cultivation methods were developed over the last 2 decades. These methods were based on simulating natural environments, limiting nutrients and extending incubation times and have enabled the recovery of previously "uncultivable" bacteria from a range of environments (Kaeberlein *et al.*, 2002; Leadbetter, 2003; Green and Keller, 2006). For example, Janssen *et al*. (2002) isolated several novel species of the Division *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* using 1/100 diluted medium from pasture soils and extended incubation periods. Ferrari *et al*. (2008) developed a powerful soil substrate membrane system (SSMS) to simulate natural environment for soil bacterial

cultivation. This technique has been successfully applied to the recovery of mercury resistant (Rasmussen *et al.*, 2008) and fastidious soil bacteria (Ferrari and Gillings, 2009), including members of the bacterial Candidate Division TM7 (Ferrari *et al.*, 2005).

Similar reports are limited for describing greater fungal diversity. In the only report published, Collado *et al.* (2007) developed a high-throughput dilution-to-extinction culturing technique to recover fungi from plant litter. The result showed a significant increase in species richness compared to a traditional nutrient rich culturing method. In this study, total 88 fungal species were recovered from five litter samples, whereas only 32 species were recovered by both methods. 73 species recovered using dilution-to-extinction approach was significantly higher than 47 species isolated using traditional isolation approach. In addition, 34 singletons were isolated using dilution culturing which was significantly higher than 14 singletons isolated using the traditional method, and they mainly belonged to unidentified fungus.

1.4 Petroleum Contamination in Polar Regions

In last two decades, human activities increasing in the Arctic and the Antarctica, such as tourism, shipping and scientific expedition have resulted in a serious degree of petroleum contamination to Polar Regions (Hughes and Stallwood, 2005). Due to human habitation, Arctic regions have suffered from frequent petroleum contamination events, particularly large-scale crude oil spills (Poland *et al.*, 2003). In

March 1989, the *Exxon Valdez* oil tanker striking on a reef in Alaska led to the release of approximately 11 million gallons of crude oil, which polluted 1,300 miles of coastline and therefore, the local ecosystem was almost devastated (Peterson *et al.*, 2003). In 1994, one thousand tons of crude oil spilled from pipelines in the Usinsk region of Russia, this disastrous spill was eight times greater than the *Exxon Valdez* event (Poland *et al.*, 2003). Petroleum contamination in Antarctic regions is less common than Arctic regions, with several rare large-scale oil spills reported (Hughes and Stallwood, 2005). The largest spill was caused by a resupply/tourist ship "*Bahia Paraiso*" and resulted in 680,000 liters of diesel fuel released into Arthur Harbor in 1989 (Karl, 1992). Furthermore, medium size accidental oil spills have also been reported. For example, over 20,000 liters of diesel fuel were spilled in Sub-Antarctic Crozet Island in 1997 (Delille and Pelletier, 2002; Coulon and Delille, 2006) and between 1000 - 10,000 liters of diesel fuel were spilled in Sub-Antarctic Macquarie Island in the last two decades (Rayner *et al.*, 2007).

1.5 Fungal Degradation of Hydrocarbons

Fungi are considered as natural decomposers that are capable of biodegradation of a wide range of organic compounds including hydrocarbons. Generally, hydrocarbons include aliphatic, monoaromatic and polyaromatic compounds that are the components of petroleum (Hughes and Bridge, 2010). Fungi utilise aliphatic hydrocarbons as a sole carbon source for growth and have been well described (Markovetz *et al.*,1968; April *et al.*, 1998; April *et al.*, 2000; Yamada-Onodera *et al.*,

2002; Husaini *et al.*, 2008). Biodegradation of a wide range of aromatic hydrocarbons by fungi have also been reported (Prenafeta-Boldu *et al.*, 2006), thus fungi may contribute significantly to bioremediation of petroleum hydrocarbon contaminated soil.

1.5.1 Aliphatic Hydrocarbons

Up to 90% of the volume of petroleum is C_{14}-C_{20} alkanes (Stroud *et al.*, 2007). In 1968, Markovetz *et al.* evaluated a group of filamentous fungal species including *Cunninghamella sp.*, *Alternaria sp.*, *Cephalosporium sp.*, *Penicillium sp.*, *Fusarium sp.*, *Spicaria sp.* and *Aspergillus sp.* for the ability to assimilate a series of alkanes and alkenes containing different chain lengths. In 2000, April *et al.* reported the filamentous fungi *Phialophora sp.*, *Aspergillus sp.*, *Oidiodendron sp.*, *Pseudallescheria sp.*, *Neosartorya sp.*, *Penicillium sp.* and *Rhizopus sp.* isolated from northern and western Canada to be able to degrade n-C_{12}-n-C_{26} aliphatic fractions of crude oil. Recently, similar fungal strains were isolated from used motor oil contaminated sites in Malaysia (Husaini et al., 2008). In addition, *Penicillium simplicissimun*, a polyethylene-degrading fungus was also observed to utilise alkanes up to 50 carbons in length (Yamada-Onodera, 2002).

1.5.2 Monoaromatic Hydrocarbons

Monoaromatic hydrocarbons are collectively known as BTEX (benzene, toluene, ethylbenzene and xylene isomers). They are present in petroleum and solvents applied

in the chemical industry (Prenafeta-Boldu *et al*., 2001). Many fungal species have been reported to be BTEX degrading strains (Middelhoven, 1993; Cox *et al.*, 1993; Cox *et al.*, 1996; Prenafeta-Boldu *et al.,* 2001; Prenafeta-Boldu *et al.*, 2006; De Hoog et al., 2006). *Exophiala sp.* have been widely isolated and reported as a BTEX degrading fungi; *Exophiala jeanselmei* can grow on styrene and styrene-related compounds (Cox *et al.*, 1993, 1996) and is able to assimilate a range of monoaromatic compounds (Middelhoven, 1993); *Exophiala xenobiotica* has been frequently isolated from sites rich in monoaromatic hydrocarbons (De Hoog et al., 2006); While Prenafeta-Boldu *et al.* (2001) reported that *Exophiala sp.*, *Cladophialophora sp.*, *Leptodontium sp.*, *Pseudeurotim sp.* and *Cladosporium sp.* may utilise toluene as sole carbon and energy source.

The biodegradation of monoaromatic hydrocarbons by fungi is associated with oxidation by lignin degradation related enzymes, cytochrome P450 monooxygenases (*Cox et al.*, 1996; Prenafeta-Boldu *et al.*, 2006) and methylation (Hughes and Bridge, 2010). The metabolic pathways for degradation of monoaromatic hydrocarbons are not well understood. Nevertheless, the metabolic pathways of styrene and toluene by fungi has been summarised in Figure 1.7.

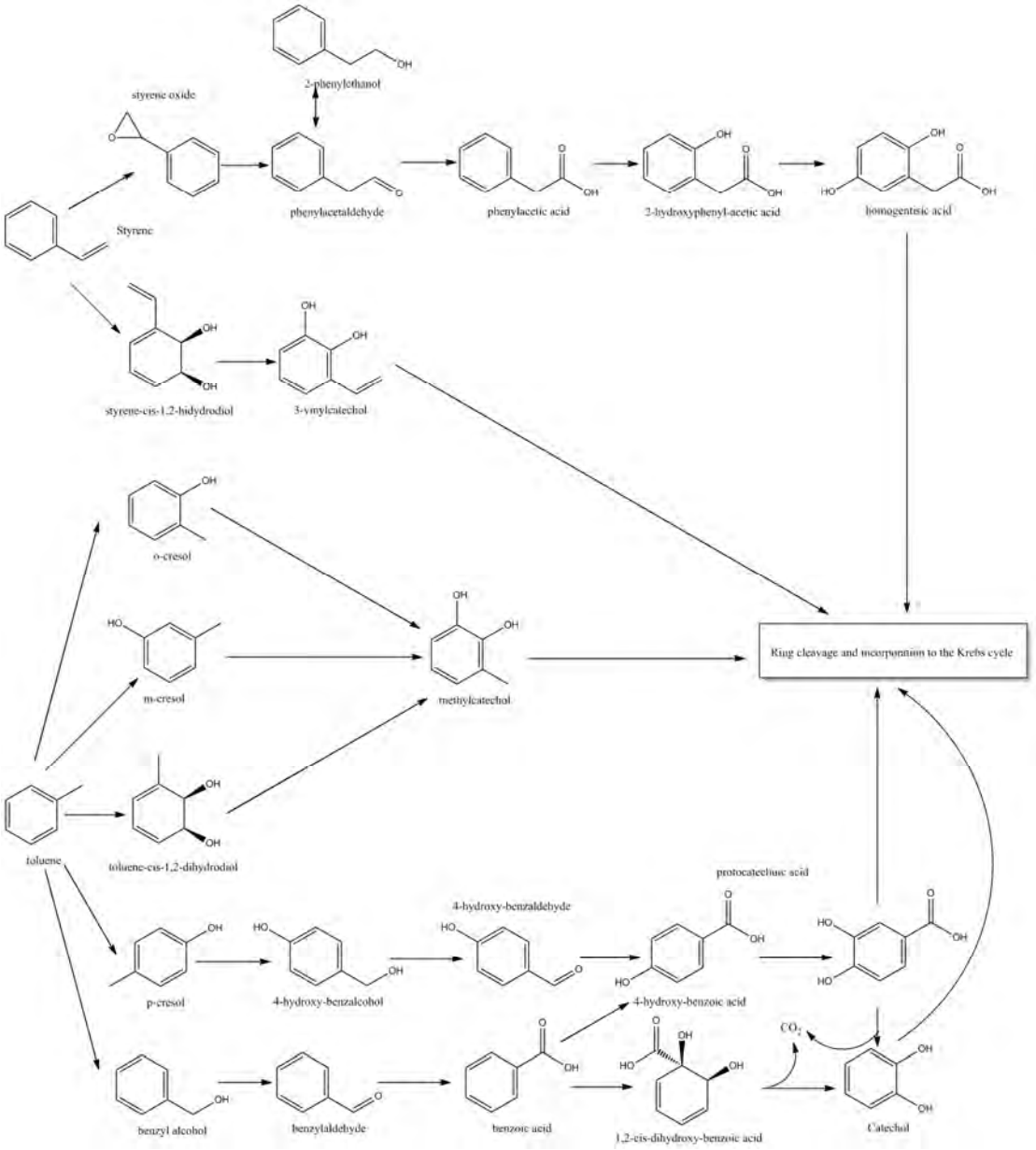


Figure 1.7 Metabolic pathways of styrene and toluene biodegradation by fungi. Adopted from (Prenafeta-Boldu *et al.*, 2006).

1.5.3 Polyaromatic Hydrocarbons

Polyaromatic hydrocarbons (PAHs) are present in crude oil and coal tar. Only a limited number of fungal species have been reported as PAHs degrading fungi. PAHs are toxic to most eukaryotes, including fungi (Hughes and Bridge, 2010). The

degradation of PAHs by lignin degrading fungi is referred to as the non-specific radical oxidation by white rot fungi (Aust, 1995; Haritash and Kaushik, 2009; Syed *et al.*, 2010). The pathway of degradation is not well understood. The oxidation reactions are catalysed by a range of extracellular oxidative enzymes, such as catalases, polyphenol oxidases and lignin peroxidases secreted by white rot fungi (Hughes and Bridge, 2010). Some white rot fungal species have been reported as PAHs degrading fungi, including *Phanerochaete chrysosporium,* which is an efficient PAHs degrading fungus and thus has been applied in bioreactor systems for PAHs degradation (Liao *et al*., 1997 & Tekere *et al*., 2007). While *Pleurotus ostreatus* may biodegrade a range of PAHs with high efficiency (Marquez-Rocha *et al.*, 2000).

The degradation pathway of PAHs by non-lignin degrading fungi is catalysed by cytochrome P450 monooxygenase targeting aromatic ring oxidation followed by hydroxylase participation (Hughes and Bridge, 2010). Rare fungi have been reported as PAHs non-lignin degrading fungi. Sarraswathy and Hallbery (2002) isolated a *Trichoderma harzianum* strain and four *Penicillium sp.* strains from a former gasworks site. These strains were able to degrade pyrene, which is a four ring PAH. *Rhodotorula glutinis* has been reported as a PAH degrading yeast which utilised phenanthrene, a three ring PAH as sole carbon and energy source (Romero *et al.*, 1998).

1.6 Sub-Antarctic Macquarie Island and Petroleum Contamination

Macquarie Island (54°37'53"S, 158°52'15"E) is a sub-Antarctic island located 1500 km south of Tasmania. The annual temperature range is from 3°C in June and July to 7°C in January with precipitation frequently. It serves as a critical habitat to over one million seabirds and many other mammals. Since 1948, a permanent Australian National Antarctic Research Expeditions (ANARE) research station has operated at the northern isthmus of the island (Figure 1.8 (A) and (B)). In order to satisfy the research stations energy needs, Special Antarctic Blend (SAB) diesel fuel has been transported, stored and used on the island. Poor protocols and accidental fuel spills have resulted in petroleum hydrocarbon contamination occurring at the New Main Power House (MPH) with approximately 180 metric tons of soils highly contaminated by SAB diesel fuel in 2002, approximate 100 metric tons of soils were moderately contaminated at Old MPH before 1994 and approximate 600 metric tons of soils were low to moderately contaminated at the Fuel Farm (FFM) before 1994 (Figure 1.8 (C)) (Rayner *et al.*, 2007).

Natural biodegradation rates are slow and limited by oxygen and nutrient availability, soil moisture and the low temperatures on the island. Therefore, a micro-bioventing system aimed at increasing the oxygen and nutrient concentrations has been employed on the New MPH, Old MPH and FFM contaminated sites to increase the biomass of hydrocarbon degrading microorganisms. However, the biodegradation activity is

thought to be limited by indigenous psychrotolerant bacteria *in situ* (Rayner *et al.*, 2007).

The bacterial diversity present in Macquarie Island soil and the effect of SAB diesel fuel contamination on this diversity was recently investigated by combining a novel cultivation approach, the SSMS and traditional DNA sequencing strategies (van Dorst, 2009). Sixteen bacterial isolates were recovered with two potentially novel genera and two potentially novel families isolated. An ecotoxicology (EC25) assessment of SAB diesel fuel was determined and revealed that the aerobic fraction of bacteria was highly susceptible to 354.81 mg fuel/kg soil. Whereas, there was no obvious effect of SAB diesel fuel contamination on the anaerobic fraction.

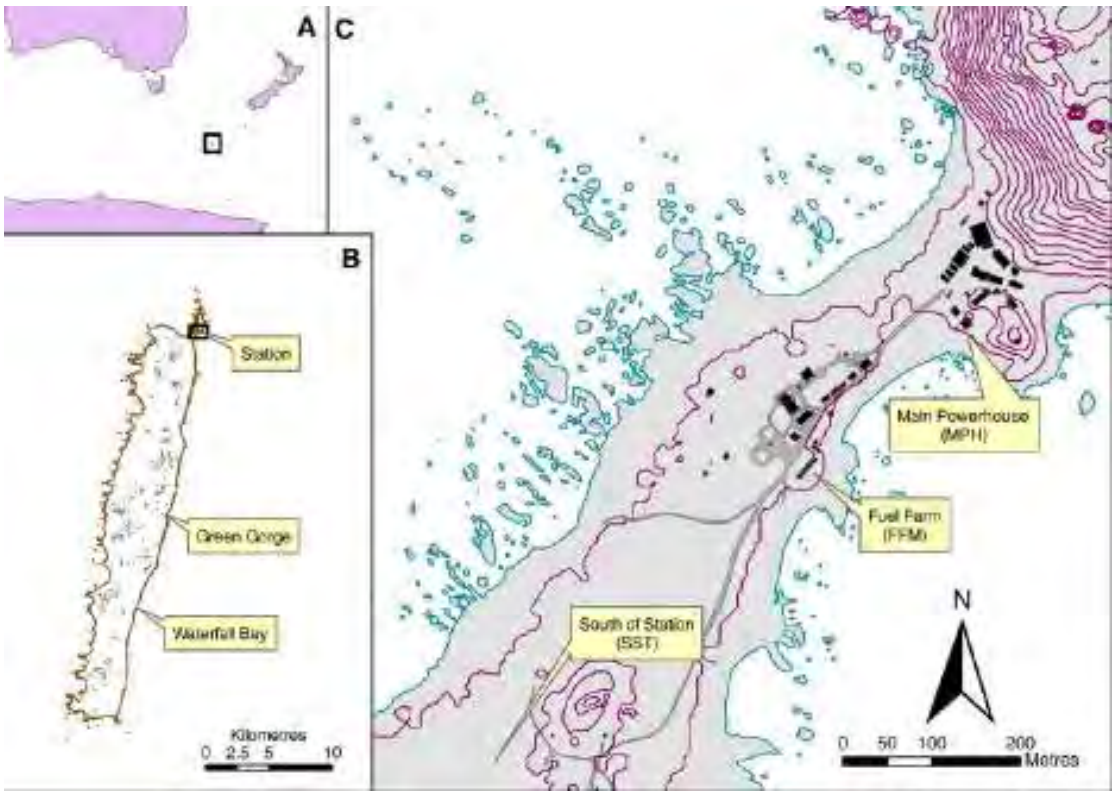


Figure 1.8 (A) Location of Sub-Antarctic Macquarie Island (B) Location of research station (C) Location of the Main Power House and Fuel Farm (Rayner *et al.*, 2007).

1.7 Project Aims

An understanding of fungal diversity on Sub-Antarctic Macquarie Island is limited and the effect of SAB diesel fuel on fungal diversity on the island is unknown. A traditional nutrient rich and a low nutrient media approach will be used for the cultivation of the fungal community from both pristine and SAB diesel fuel spiked soil samples from Sub-Antarctic Macquarie Island. The principal aim will to develop and apply a diluted media approach to recover diverse bacteria from soil, as such an approach has not yet been adopted for fungi. A comparison of the isolates recovered by both culture methods will enable the most suitable approach to be adopted when determining the effect of SAB diesel fuel on fungal diversity from a range of soil plots, as well as to see if this type of method is suitable for fungi.

The second aim of my thesis is to determine the effect of SAB diesel fuel contamination on fungal diversity present in a range of Macquarie Island soil plots by spiking SAB diesel fuel to soil samples to final concentrations of 0, 50, 100, 250, 500, 1,000, 10,000 and 20,000 mg/kg, respectively. Additionally, isolating fungal species from soils spiked with high concentrations of SAB diesel fuel will result in a library of isolates for further characterisation for petroleum hydrocarbon degradation capability which could be applied in the future bioremediation strategies.

Chapter 2 Materials and Methods

2.1 Field Collections

Uncontaminated soil samples were collected near the Australian Antarctic Research station at Sub-Antarctic Macquarie Island (54°37'53"S, 158°52'15"E) during February in 2009 (Figure 2.1). The collection site was chosen due to the similar soil physical properties to the research stations SAB diesel fuel contaminated Main Power House (MPH). At the control collection site, the soil plots were selected ranging in organic carbon from 0% to 50% for sample excavation (Figure 2.2). Approximately 1 kg pristine soil was excavated using a spade aseptically from the top 25 cm of each 1 m x 1 m plot. An additional soil core for chemical analysis was collected as close to the initial shallow pit as possible.

The soil carbon gradient and soil humidity analysis were performed at the research station on the Island. The soil plots were named P1 to P10, which indicated the increasing carbon gradient from the lowest P1 to the highest P10. Four of the soil plots were used for fungal diversity investigation in this study. For each plot, Josie van Dorst divided the bulk soil into eight 125 g soil samples and then spiked with SAB diesel fuel to final concentrations of 0, 50, 100, 250, 500, 1,000, 10,000 and 20,000 mg/kg, respectively. The spiked soil samples were homogenised through mixing and were incubated for 10 days at 10°C. Prior to fungal diversity analysis, the final total petroleum hydrocarbon (TPH) concentration in the soils were measured using gas chromatography by the Australian Antarctic Division (AAD) (Table 2.1).

Due to the differences between the nominal and final measured SAB concentration in the soils was observed, the concentrations of SAB in soil samples were grouped as low (actual 0 to 249 mg/kg), medium (actual 250 to 5,000 mg/kg) and high (actual over 5,001 mg/kg) rather than does not exist SAB concentrations for partial data analysis in this study. Nominal 0 to 250 mg/kg for P2, nominal 0 to 500 mg/kg for P4, nominal 0 to 100 mg/kg for P6 and P9 were grouped as low; nominal 500 to 1,000 mg/kg for P2, nominal 1,000 mg/kg for P4, nominal 250 to 1,000 mg/kg for P6 and P9 were grouped as medium; nominal 10,000 to 20,000 mg/kg for P2, P4, P6 and P9 were grouped as high.

Table 2.1 Final TPH concentrations in SAB diesel fuel spiked soil samples used in this study.

Nominal SAB diesel fuel Concentration (mg/kg)	**Final TPH Concentration (mg/kg)**			
	P2	**P4**	**P6**	**P9**
0	<50	<50	81	116
50	65	<50	88	74
100	65	<50	115	94
250	169	84	388	569
500	344	247	800	1,210
1000	757	628	3,401	1,976
10000	12,763	9,744	25,289	20,331
20000	15,680	15,030	18,740	32,254

Figure 2.1 Macquarie Islands Australian Antarctic Research Station (Taken by Josie van Dorst). The location of Main Power House and soil sample collected site as labeled were similar in terrain.

Figure 2.2 Locations of the ten soil plots taken at the sample collection site. For this study, P2, P4, P6 and P9 were selected for analysis of fungal diversity and were based on selection of a range of samples over a varying carbon gradient.

2.2 Culturing Sub-Antarctic Macquarie Island Fungi

2.2.1 Preparation Soil Suspensions

P2, P4, P6 and P9 were selected for fungal diversity analysis. In order to estimate the dilution factors, each soil suspension was prepared a 10^{-1} concentration by suspending 1 g of soil in 10 ml of sterile distilled water in a 15 ml sterile plastic tube. The suspensions were vortexed for 5 min and then allowed to settle for a further 5 min. Subsequently, serial dilutions were made with sterile distilled water between 10^{-2} and 10^{-5}.

2.2.2 Fungi Cultivation

Standard 1X and low nutrient 0.1X Cooke Rose Bengal Agar (CRBA) plates (150 x 15 mm Petri Dish, Sarstedt, Adelaide Australia) were used as the fungi isolation media. Initially, a 20X stock of Cooke Rose Bengal Broth (CRBB) was prepared by adding 100 g glucose (Ajax Finechem, Taren Point, Australia), 50 g soytone (Bacto Laboratories, Liverpool, Australia), 10 g KH_2PO_4 (Ajax Finechem), 5 g $MgSO_4 \cdot 7H_2O$ (Ajax Finechem) and 350 mg Rose Bengal (Sigma-Aldrich, Castle Hill, Australia) and sterile distilled water to a final volume of 1 L (Altas, 2004). Then, the 20X CRBB stock was diluted to 1X CRBA by mixing 50 ml stock with 950 ml sterile distilled water and 20 g agar (Oxoid, Adelaide, Australia). 0.1X CRBA was prepared by mixing 5 ml stock with 995 ml sterile distilled water and 20 g agar.

Potato dextrose agar (PDA) was prepared by adding 39 g PDA (Sigma-Aldrich) with

1 L sterile distilled water and was used for sub-culturing to obtain pure fungal cultures. In order to inhibit bacterial growth on media, streptomycin sulfate (Sigma-Aldrich) was added at a final concentration of 50 mg/L at 46°C after autoclaving media.

Fungi were isolated following spread plating of 1 ml of each of the 10^{-3}, 10^{-4} and 10^{-5} soil dilutions onto CRBA plates in triplicate. Based on Kerry (1990) the optimum growth temperatures for the majority Sub-Antarctic Macquarie Island's fungi is 15°C. Thus, the incubation temperature was set at 15°C and the incubation time was three weeks. Following primary incubations, depending on the fungal macroscopic morphology, a small piece of each selected strain was placed onto fresh PDA for a second incubation step. In each case, hyphal tips, colonies or spores were picked and placed onto fresh media using an inoculating needle and the process was repeated until pure cultures obtained. All of the incubations were carried out in freeze-thaw cycle incubator (Sanyo, Lane Cove West, Australia) at 15°C in the dark.

A small number of fungal isolates isolated from CRBA were not able to grow on PDA. In these cases, a special nutrient agar (SNA) was used. The SNA was developed from CRBA and consisted of 10 g glucose (Ajax Finechem), 5 g soytone (Bacto Laboratories), 1 g KH_2PO_4 (Ajax Finechem), 0.5 g $MgSO_4 \cdot 7H_2O$ (Ajax Finechem) and 20 g agar into 1 L sterile distilled water.

2.3 Fungal Morphological Characterisation

For microscopic characterisation of fungal isolates, two types of microscope slides were prepared. For a traditional slide preparation, one drop of sterile distilled water was placed onto a clean microscope glass slide (Livingstone, Rosebery, Australia). Fungal hypha were picked using an inoculating needle and were placed onto the drop of water, followed by a coverslip (LabServ, Scoresby, Australia) and sealed using nail polish.

The second type of microscope slide was a modified slide culture used for characterising microscopic morphological features (Harris, 1986). Firstly, tweezers, surgical blades (Swann-Morton, Sheffield, United Kingdom) and clean glass slides (Livingstone) were sterilised by immersing in 75% ethanol and flammed. Secondly, a $1\,cm^3$ agar plug was cut from the fungal colony edge on the agar plate. The agar plug was placed upside down onto a glass slide. Then, the microscope slide was placed in a sterile petri dish or a 50 ml sterile plastic tube, and incubated at 15°C for 7 days. Finally, the agar plug was removed and one drop of sterile distilled water was added followed by a coverslip and the slide was sealed with nail polish. All of the microscope slide preparations were performed in a PCII Biosafety hood to prevent cross contamination. The microscope slides were analysed using a BX61 Epi-Fluorescence Microscope equipped with a DP71 digital camera (Olympus, Mt Waverley, Australia) for image analysis.

2.4 Fungal Genomic DNA Extraction

There were three alternative methods used for fungal genomic DNA extractions. For the first round, *prep*GEM™ (ZyGEM, Hamilton, New Zealand) was used as described by Ferrari *et al* (2007). If no DNA or no PCR products (Section 2.5) were produced then a second round of DNA extracts were prepared using a modified bead-beating method. Finally, the CTAB method was performed for those fungi that were still not producing PCR amplicons.

2.4.1 Genomic DNA Extraction Using The *prep*GEM™ Method

The *prep*GEM™ tissue kit previously used for parasite DNA extractions was modified for fungi here (Ferrari *et al.,* 2007). For each sample, a portion of a colony was scraped from the agar plates using an inoculating loop and transferred into a PCR tube containing 89 µl ultra pure water, 10 µl of 10 x buffer and 1 µl *prep*GEM™ enzyme. PCR tubes were then incubated at 75°C for 15 min followed by 95°C for 5 min using a MyCycler Thermocycler (Bio-Rad, Gladesville, Australia). The DNA extracts were then transferred into fresh 1.5 ml tubes and centrifuged at 14,000 rpm for 1 min. Finally, the DNA lysates were removed from the precipitated residue and placed into a 500 µl tube and stored at -20°C until required.

2.4.2 Genomic DNA Extraction Using The Modified Glass Bead-Beating Method

Based on the principle of FastPrep® instruments from MP Biomedicals, a simplified

FastPrep® bead-beating method was developed and applied here. A 5 mm^3 agar plugs containing a fungal colony were cut from agar plates using sterile surgical blades. Each piece of agar was then placed into a screw cap 2 ml microfuge tube (Sarstedt) containing 0.5 g of 0.1 mm and 0.5 mm glass beads (Mo Bio, Carlsbad, USA) and 1 ml ultra pure water. Tubes were homogenized using the FastPrep® 120 instrument (MP Biomedicals, Seven Hills, Australia) for 40 sec at a speed setting of 6.0. Finally, the DNA extracts were incubated at 95°C for 5 min and centrifuged at 14,000 rpm for 1 min. The DNA lysates were removed and placed in a 500 µl tube for storage at -20°C until required.

2.4.3 Genomic DNA Extraction Using A Modified CTAB Method

Based on the standard CTAB DNA extraction method described by Ralph and Bellamy (1964), a modified CTAB genomic DNA extraction method was adopted. A 5 mm^3 agar plug containing a fungal colony from the agar plate was cut using sterile surgical blades and was placed into a screw cap 2 ml microfuge tube containing 0.5 g of 0.1 mm and 0.5 mm glass beads (Mo Bio), 20 mg polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich) and 500 µl hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich) pre-heated to 55°C and vortexed briefly. To prepare 100 ml CTAB extraction buffer, 2 g CTAB, 28 ml of 5M Sodium chloride (Ajax Finechem), 4 ml of 5 M EDTA (Applichem, Taren Point, Australia), 10 ml of 1M Tris (Bio-Rad) and 58 ml sterile distilled water were placed into a 250 ml Schott bottle and mixed briefly. Then a portion (500 µl) of a 25:24:1 ratio of phenol: chloroform: isoamylalcohol

mixture (Sigma-Aldrich) was added into the microfuge tube. The sample was homogenised using the FastPrep® 120 instrument for 30 sec at a speed setting of 5.0. The sample was incubated for 1 hour at 70°C and centrifuged at 14,000 rpm for 10 min. After that, the supernatant was removed into a fresh 1.5 ml microcentrifuge tube and an equal volume of a 24:1 ratio of chloroform: isoamylalcohol was added. The sample was vortexed briefly and centrifuged again at 14,000 rpm for 10 min. The supernatant was then transferred into a fresh 1.5 ml tube and 2 volumes of 20% w/v polyethylene glycol (PEG) precipitation buffer (BDH Laboratory Supplies, Poole, England) was added and the sample was incubated for a further two hours at RT. Following this incubation step the sample was centrifuged at 14,000 rpm for 10 min, the supernatant was removed and the pellet was air dried. Finally, the pellet was resuspended in 30 µl ultra pure water and the DNA lysate was stored in a 500 µl tube at -20°C until required.

2.5 PCR Amplification of Fungal Genes

The fungal internal transcribed spacer (ITS) gene sequences were amplified using previously published ITS specific primer sets (Table 2.2). In addition, fungal 18S rDNA gene sequences and fungal 28S rDNA gene sequences were also amplified using published protocols (Table 2.2). All PCR primer sets were obtained from Integrated DNA Technologies (McLeans Ridges, Australia) and PCR's were carried out using a MyCycler Thermocycler.

Table 2.2 Primer sets used for fungal gene PCR amplification and sequencing

Primer set	Sequence	Target	References
NL1	5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'	fungal 28S rDNA	Wuczkowski *et al.* (2003)
NL4	5'-GGT CCG TGT TTC AAG ACG G-3'	fungal 28S rDNA	Wuczkowski *et al.* (2003)
ITS1	5'-TCC GTA GGT GAA CCT GCG G-3'	fungal ITS	White *et al.* (1990)
ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'	fungal ITS	White *et al.* (1990)
NSI1	5'-GAT TGA ATG GCT TAG TGA GG-3'	fungal ITS	Martin and Rygiewicz (2005)
NLB4	5'-GGA TTC TCA CCC TCT ATG AC-3'	fungal ITS	Martin and Rygiewicz (2005)
EF3	5'-TCC TCT AAA TGA CCA AGT TTG-3'	fungal 18S rDNA	Smit *et al.* (1999)
EF4	5'-GGA AGG GRT GTA TTT ATT AG-3'	fungal 18S rDNA	Smit *et al.* (1999)
fung5	5'-GTA AAA GTC CTG GTT CCC C-3'	fungal 18S rDNA	Smit *et al.* (1999)

2.5.1 Fungal ITS Gene PCR Amplification

The fungal ITS gene sequences were amplified using the primer set ITS1/ITS4 (White *et al.,* 1990) and yielded 500 bp-700 bp size amplicons. The PCR was performed in 50 µl reaction mix containing 27.75 µl of ultra pure water, 10 µl of 5X PCR buffer, 1 µl of 10 mM dNTP, 1µl of 10 µM for forward and reverse primers, 4 µl of 25 mM $MgCl_2$, 0.25 µl (5 u/µl) GoTaq Flexi DNA polymerase (Promega, Annandale, Australia) and 5 µl of DNA template. The PCR program consisted of initial denaturation step at 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 2 min followed by a final extension step at 72°C for 5 min.

The NSI1/NLB4 primer set (Martin and Rygiewicz, 2005) was also used which targets the ITS region and was used if the ITS1/ITS4 primer set did not produce a PCR amplicon and yielded approximately 500bp-700bp size products. The PCR mix for the NSI1/NLB4 primer set was identical to the ITS1/ITS4 primer set above. The PCR program consisted of an initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 60°C for 40 sec, 72°C for 40 second followed by a final extension at 72°C for 5 min.

2.5.2 Fungal 18S rDNA Gene PCR Amplification

Fungal 18S rDNA gene sequences were amplified using the primer set EF4/EF3 (Smit *et al.,* 1999) and yielded approximately 1.5 kb size products. Each 50 µl reaction mix consisted of 27.75 µl of ultra pure water, 10 µl of 5X PCR buffer, 1 µl of 10 mM dNTP, 1 µl of 10 µM primer EF4, 1 µl of 10 µM primer EF3, 4 µl of 25 mM $MgCl_2$, 0.25 µl (5 u/µl) GoTaq Flexi DNA polymerase and 5 µl of DNA template. The PCR program consisted of an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 3 min followed by a final extension at 72°C for 10 min.

2.5.3 Fungal 28S rDNA Gene PCR Amplification

The fungal 28S rDNA gene D1-D2 region was amplified using the primer set NL1/NL4 (Wuczkowski *et al.,* 2003) and yielded approximately 800-1000 bp size products. Each 50 µl reaction mix consisted 27.75 µl of ultra pure water, 10 µl of 5X

PCR buffer, 1 µl of 10 mM dNTP, 1 µl of 10 µM primer EF4, 1 µl of 10 µM primer EF3, 4 µl of 25 mM $MgCl_2$, 0.25 µl (5 u/µl) GoTaq Flexi DNA polymerase and 5 µl of DNA template. The PCR program consisted of initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 2 min followed by a final extension at 72°C for 10 min.

2.6 Agarose Gel Electrophoresis

PCR products and a 100 bp DNA ladder (Promega) were visualised using gel electrophoresis on a 2% agarose gel (w/v). The agarose gel was made up 40 mM TAE (Tris-Acetate-ethylenediaminetetraacetic acid) buffer (Applichem) with the addition of SYBR safe DNA gel stain (Invitrogen, Mulgrave, Australia) at concentration of 0.01%. The gel was visualised using a Syngene GelDoc System (Syngene, Frederick, USA) under blue light transilluminator emission.

2.7 Restriction Fragment Length Polymorphism (RFLP) Analysis

PCR products were digested with the restriction enzymes *Hae* III and *Rsa* I (Promega), respectively as described by Imad *et al.* (2010) and Korabecna (2007). Briefly, 15 µl PCR product, 2.63 µl ultra pure water, 2 µl 10X buffer C, 0.2 µl 100X BSA (Promega) and 0.17 µl each restriction enzyme were placed in 96 well PCR plates (Bio-Rad), sealed with Microseal Foil (Bio-Rad) and incubated overnight at 37°C. The digested products were visualised on a 2% agarose gel with SYBR safe DNA gel stain (Section

2.6). Based on the DNA fingerprinting for both restriction enzymes, each different ribotype was selected for DNA sequencing (Section 2.8).

2.8 DNA Sequencing

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Donacaster, Australia) according to the manufacturer's instructions. The NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA) was used for determination of DNA concentrations after purification. Primers ITS1 and ITS4 were used for the fungal ITS region DNA sequencing; primers EF4, EF3 and fung5 were used for the fungal 18S rDNA region sequencing; primers NL1 and NL4 were used for the fungal 28S rDNA region sequencing.

DNA sequencing was performed in a 20 µl reaction mix containing 1 µl DNA template (20-50 ng/µl), 16.44 µl ultra pure water, 0.06 µl primer, 1 µl Big Dye Terminator (Applied Biosystems, Mulgrave, Australia) and 1.5 µl 5X Big Dye Buffer. The sequencing reactions were carried out using the MyCycler Thermocycler and consisted of 25 cycles of 10 sec at 96°C, 5 seconds at 50°C and 4 min at 60°C.

The sequencing PCR products were transferred to a clean 1.5 ml tube with the addition of 5 µl 125 mM EDTA and 60 µl 100% ethanol and vortexed briefly. Samples were precipitated overnight at RT. Subsequently, samples were centrifuged for 20 min at 14,000 rpm and supernatants discarded. Samples were washed with 250 µl 70%

ethanol and centrifuged for a further 10 min at 14,000 rpm. Finally, supernatants were discarded and samples were dried using a heat block at 90°C for 1 min. The sequencing analysis was carried out using a ABI 3730 sequence scanner (Applied Biosystems) in the Ramaciotti Centre Sequencing Facility (School of Biotechnology and Biomolecular Sciences, UNSW, Sydney).

2.9 Long-Term Isolate Preservation

A 15% glycerol solution was prepared for long-term preservation of fungal isolates at -80°C. After preparation, the glycerol solution was sterilised three times by autoclaving for 30 min at 15 psi 121°C. For spore producing isolates or fungal mycelia grow on agar surface cultures, 2 ml of the preserving fluid was placed on the surface of agar plates and fungi were gently scraped off with a pipette. Then use pipette to transfer 1 ml of the spore or mycelial suspension into two 2 ml screw cap polypropylene vials (Corning Incorporated, New York, USA). For non sporulating isolates or fungal mycelia that grew deep within the agar cultures, several 5 mm^3 agar plugs were cut from agar plates using a sterilised surgical blade and were placed into a screw cap polypropylene vial containing 0.5 - 1.0 ml of the 15% glycerol solution. The vials were frozen at -20°C overnight, then transferred to an ultra low temperature (-80°C) freezer for long-term preservation. All of the isolates were checked for viability after 7 days of storage.

2.10 Identification and Phylogenetic Analysis

DNA sequences (Section 2.8) were analysed against the GenBank database within National Centre for Biotechnology Information (NCBI) using blastn. Then morphological information was combined with ITS sequence matches obtained from GenBank for isolate identification. Fungal ITS sequences for all isolates were submitted and deposited into GenBank database under accession numbers JN104511-JN104578 (Table 3.2). For phylogenetic analysis, alignment for fungal isolate ITS sequences were carried out using ClustalX 2.1 and the best-fit models of nucleotide substitution was statistically selected by jModelTest. Finally, the phylogenetic tree was constructed using MEGA 5.05 by the Maximum Likelihood statistical method.

2.11 Statistical Analysis

In order to determine if the low and high nutrient approach recovered different fungal communities for some diesel fuel concentrations but not others, one way analysis of similarity (ANOSIM) test was used to test each fuel concentration individually. Furthermore, two way ANOSIM test was conducted to determine if the recovered fungal communities was significantly different between two nutrient approach for overall diesel fuel concentrations as well. The ANOSIM test was carried out using software PRIMER 6 (Plymouth Routines In Multivariate Ecological Research) (Primer-E Ltd, Ivybridge, United Kingdom). The PRIMER 6 is widely conducted for multivariate statistical analysis in ecological and environmental science, particularly

for the analysis of species distribution and abundance (Clarke and Gorely, 2006). After raw data from each SAB diesel fuel concentration and culture medium was collected, the data were compiled into a data matrix within PRIMER 6. Then the fungal assemblage data was pre-treated to balance the relative contribution of dominant species by square-root transformation. After creating a resemblance matrix the similarity between samples was calculated on the basis of the Bray-Curtis coefficient (Clarke and Green, 1988). The similarity results representing the biological relationship were then examined using hierarchical clustering with group-average linkage and non-metric multi-dimensional scaling (NMDS) ordination plots. The distances between pairs of samples on the NMDS ordination plot reflect the relative similarity of species composition. Clusters (at various levels of similarity) superimposed on the ordination plot, ascertained the level of agreement between the hierarchical clustering with group-average linkage and NMDS methods (Clarke and Warwick, 2001). Global *R* values that resulted from these analyses provided an absolute measure of how separated the groups were, on a scale of 0 for groups that are indistinguishable, to a value of 1 that the samples are more similar within same group than other groups. To determine the significance of the variables between samples, fuel concentration and media dilution were tested against 999 permutations.

A two way analysis of variance (ANOVA) statistical method was used to determine if the recovered fungal diversity from 4 investigated soil plots was affected by organic carbon concentrations or by different SAB diesel fuel concentrations. Here, the 3 null

hypothesis were 1) "there was no difference among the levels of organic carbon concentrations in pristine soil plots", 2) "there was no difference among the levels of different SAB diesel fuel concentrations in spiked soil plots" and 3) "there was no difference between organic carbon concentrations in pristine soil plots with different SAB diesel fuel concentrations". The P value was set up at 0.05, and the null hypothesis will be rejected if $P<0.05$. Furthermore, a one way ANOVA statistical method was used to determine if the recovered total colony forming units were affected by different SAB diesel fuel concentrations. The null hypothesis was "there is no difference among the levels of different SAB diesel fuel concentrations". The P value was set up at 0.001, and the null hypothesis will be rejected if $P<0.001$. Both ANOVA statistical methods were carried out using SigmaPlot 12 (Systat Software Inc., Chicago, USA).

In order to compare the diversity of fungi recovered from the investigated soils with different SAB treatments, Shannon's diversity index and Simpson diversity index were used to determine whether there was any correlation between the recovered fungal communities and different TPH values in the soils. After raw data of fungal species from each soil sample was collected, the data was processed as a data matrix within PRIMER 6, the diversity index was calculated. The greater the Shannon's diversity index, the greater the diversity. By comparison, the lower the value for the Simpson diversity index the higher the diversity.

The Shannon's diversity index is defined as

$$H' = -\sum_{i=1}^{S} \pi_i \log(\pi_i)$$

where π_i is the proportion of individuals belonging to the *i*th species in the data of interest, and the log base is defined as 2 in here.

Additionally, the Simpson diversity index is defined as

$$\lambda = \sum_{i=1}^{R} p_i^2$$

Where p_i is the proportional abundances.

Chapter 3 Fungal Cultivation Using Both A High and A Low Nutrient Media Approach

3.1 Experimental design

In this study, the experiment was designed to recover Macquarie Island fungal species from pristine and SAB spiked to nominal final concentrations of 0, 50, 100, 250, 500, 1,000, 10,000 and 20,000 mg/kg soil P4 using standard 1X and low nutrient 0.1X CRBA media. Spread plating of each of the 10^{-3}, 10^{-4} and 10^{-5} soil dilutions onto CRBA plates in triplicate. When fungal colonies formed on the plates, each morphological distinct isolate was sub-cultured on PDA until pure cultures were obtained and followed molecular identification. Finally, all the recovered fungal OTUs were adopted phylogenetic analysis for tree construction, ANOSIM test to determine if there was a significantly different between two approaches and one way ANOVA test to determine if the recovered total OTUs were effected by the different SAB diesel fuel concentrations.

3.2 Background and Aims

Since 1948, a permanent Australian Antarctic Research station has operated at the northern isthmus of Macquarie Island, which has resulted in soil contamination caused largely by SAB diesel fuel spills (Schafer *et al.*, 2007). Compared with macrobiota, the biodiversity of microbiota and the effect of SAB diesel fuel on microbial diversity on Macquarie Island is still largely unknown, especially mycota.

Novel cultivation methods including diluted culture media have been widely adopted to recover previously undescribed bacteria but similar approaches have not yet been reported for fungal cultivation. The aim of this chapter was to apply a low nutrient media approach as well as a high nutrient media approach to recover greater fungal diversity from Macquarie Island soils. Furthermore, in order to investigate the impact of petroleum contamination on fungal biodiversity, soil samples were spiked with SAB diesel fuel to nominal final concentrations of 0, 50, 100, 250, 500, 1,000, 10,000 and 20,000 mg/kg, respectively and fungal species from each soil were isolated.

3.3 Fungal Cultivation From Soil

After approximately 21 days of incubation at 15°C, fungal colony formation was observed on the majority of high and low nutrient CRBA plates (Figure 3.1). In total, 9,699 fungal isolates were recovered from the range of soil samples investigated. Based on the results of a combination of distinct morphological characteristics from (1) culture colour: whether a particular culture colour present or not or with variable colour, such as white, black, grey, pink, green and yellow in these isolates; (2) culture reverse colour: whether a particular culture reverse colour present or not or with variable colour, such as white, black, pink and yellow in these isolates; (3) smell: whether a particular odor was secreted or not, such as garlic, aromatic, strong, unpleasant and sweet in these isolates; (4) culture position: the culture at least partly superficial or only immersed. Finally, 156 fungal isolates were selected for sub-cultivation on PDA until pure cultures were obtained for molecular identification.

A comparison of fungal colonies formed on high or low nutrient media showed a species shift between low SAB (nominal 0, 50, 100, 250 and 500 mg/kg), medium SAB (nominal 1,000 mg/kg) and high concentrations of SAB diesel fuel (nominal 10,000 and 20,000 mg/kg) contaminated soil (Figure 3.1). In a longitudinal comparison of fungal colonies formed for spiked soil samples from the same plot, a visible distinction between high and low nutrient media in terms of recovered species diversity was observed. This difference was observed for all concentrations except the high SAB contaminated soil samples (Figure 3.1).

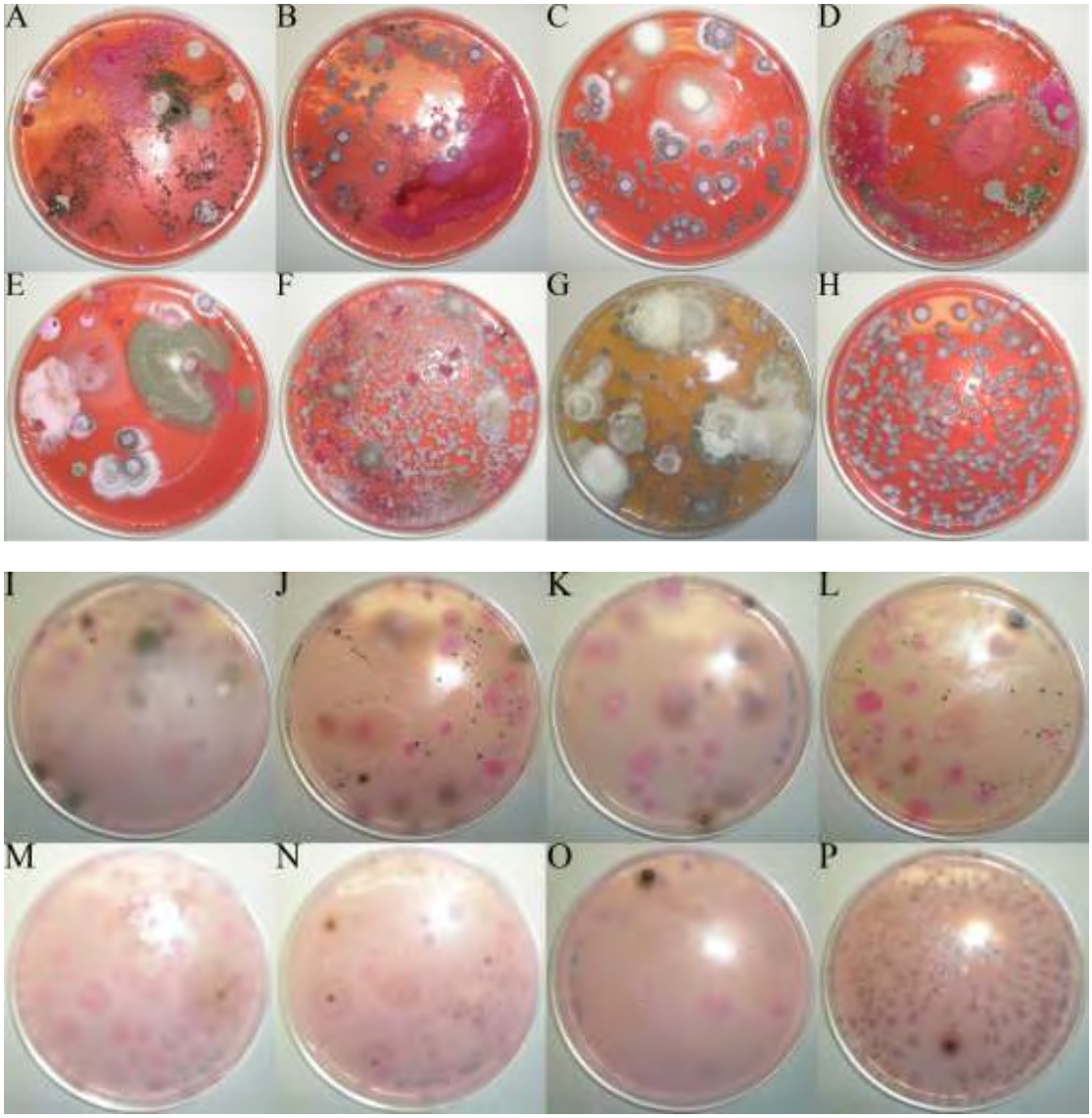


Figure 3.1 Growth of fungal colonies after cultivation on high nutrient media (A-H) versus low nutrient media (I-P). From left to right: soil samples were spiked with SAB diesel fuel to nominal final concentrations of 0, 50, 100, 250, 500, 1,000, 10,000 and 20,000 mg/kg, respectively. Comparing the fungal colonies formed on both culture media, a visible shift in fungal diversity was observed between low SAB (nominal 0, 50, 100, 250 and 500 mg/kg), medium SAB (nominal 1,000 mg/kg) and high concentrations (nominal 10,000 and 20,000 mg/kg) of SAB diesel fuel. The pristine soil (A and I) revealed the greatest fungal diversity. As the SAB diesel fuel concentration approached nominal 50 (B and J), 100 (C and K), 250 (D and L) and 500 mg/kg (E and M), the soil samples showed less fungal diversity and distinct fungal populations were formed among them. When SAB diesel fuel concentration exceeded nominal 1,000 mg/kg, only limited fungal species were recovered and they were the dominant species present in the soil samples. The most abundant fungal species isolated from nominal 1,000 (F and N) and 20,000 mg/kg (H and P) SAB diesel fuel was *Pseudeurotium bakeri*, and *Arthroderma sp.* 1 was the dominant species recovered from nominal 10,000 mg/kg (G and O) SAB diesel fuel. The fungal shift was observed clearly on high nutrient media (A-H) images, but images were blurred on low nutrient media (I-P) due to the weak colour background of the media.

3.4 DNA Extraction and PCR Amplification

DNA extraction of the 156 fungal isolates was most successful using the *prep*GEM Tissue Kit with 129 ITS gene PCR amplification products obtained when amplified with primer set ITS1/ITS4 (Figure 3.2). For the remaining 27 isolates, a modified bead-beating method was used for DNA extraction. As a result, ITS gene PCR amplification products were obtained from a further 5 strains (Figure 3.3), but the remaining 22 strains did not produce a PCR product. Finally, the CTAB method was used for DNA extraction of the remaining 22 isolates. However, the ITS gene PCR amplification still failed to produce a PCR product (data not shown) with primer sets ITS1/ITS4 and NSI1/NLB4. In summary, 134 ITS gene PCR amplification products were produced with primer set ITS1/ITS4 yielding products ranging between 500bp-700bp for distinct isolates (Figure 3.2 and 3.3). Additionally, a unique 1kb product was obtained from isolate CZ24 (Section 3.9).

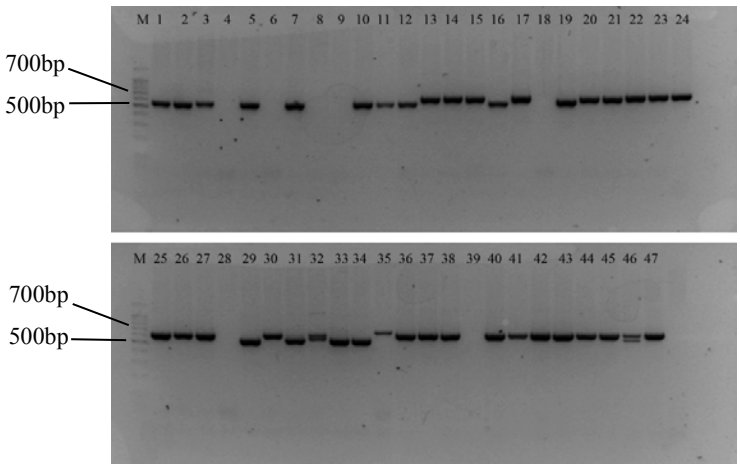


Figure 3.2 Forty seven fungal ITS rDNA PCR amplification products were obtained using primer set ITS1/ITS4. The DNA was extracted using *prep*GEM Tissue Kit. M: 100 bp molecular weight marker; Lane 1-47: fungal ITS rDNA PCR amplification products. The size of these products ranged from 500-700 bp for distinct isolates.

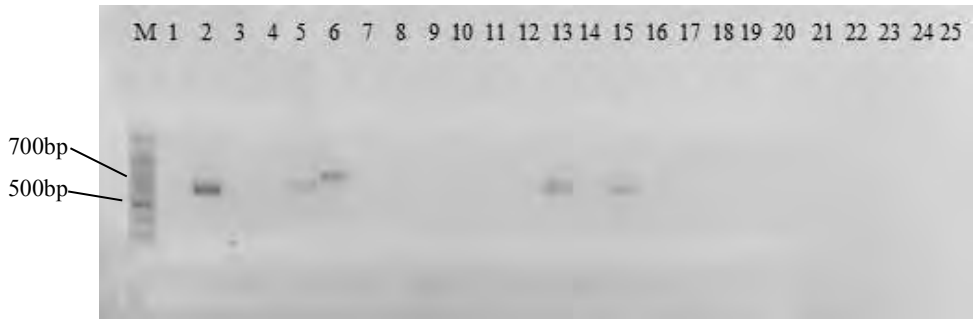


Figure 3.3 Twenty five PCR products from remaining 27 fungal ITS rDNA PCR amplification products with primer set ITS1/ITS4. The DNA was extracted using a modified bead-beating method for the 27 fungal strains. M: 100 bp molecular weight marker; Lane 1-25: fungal ITS rDNA PCR amplification products. The size of these products ranged from 500-700 bp for distinct isolates.

3.5 RFLP Analysis of Isolates

In total 134 ITS gene PCR amplification products obtained with primer set ITS1/ITS4 were digested by restriction enzymes (Figure 3.4). Following comparative analysis based on the *Hae* III and *Rsa* I RFLP patterns, 69 different ribotypes were selected for DNA sequencing (Section 2.8) and taxonomic designation using BLASTn (Section 2.10).

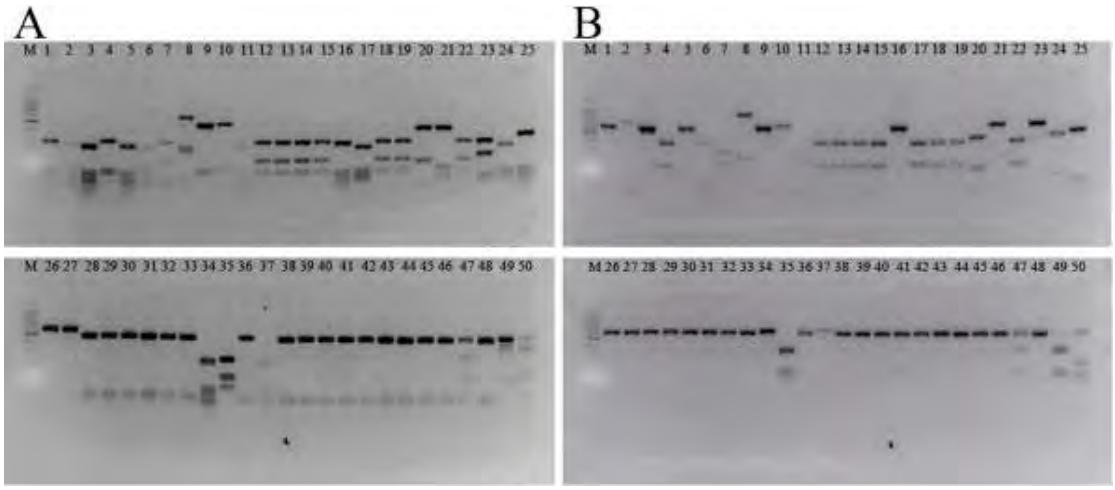


Figure 3.4 Fifty fungal ITS rDNA PCR-RFLP products on 2% agarose gels. (A) *Hae* III RFLP pattern. (B) *Rsa* I RFLP pattern. M: 100 bp molecular weight marker; Lane 1-50: fungal ITS rDNA PCR-RFLP products.

3.6 Identification of Fungal Isolates

Finally, there were 91 fungal species recovered from 9,699 isolates obtained from one plot of 8 spiked soil samples, including 69 species sequenced (Section 3.4) and 22 species were not sent for sequencing (Section 3.3). Of the 69 different species sequenced, and then combined with macroscopic and microscopic morphological information, 28 isolates were identified to the species and 21 to the genus level (Table 3.1). Due to the low match similarity or unique morphological characteristics, another 20 isolates were not able to be identified to the genus level and were termed unidentified fungus 1 to 18, 41 and 42. These isolates were identified to phylum level based on microscopic morphological information. 52 species (57% of total species) were identified as phylum *Ascomycota*; 15 species (16% of total species) were identified as phylum *Basidiomycota*; one species (1% of total species) was identified as *Mucoromycotina*, a sub-phylum *incertae sedis*. Of the remaining 23 unidentified fungal species (25% of total species), 22 species (24% of total species) did not yield ITS gene PCR amplification products using primer sets ITS1/ITS4 and NSI1/NLB4 (Section 3.3) and were termed unidentified fungus 19 to 40 (Table 3.1), one species (1% of total species) could not be classified into any fungal phylum due to its unusual ITS gene sequence and morphological characteristics (Section 3.9).

The distribution and abundance data (Table 3.1) showed that only 11 (12% of total species) fungal species were recovered using both the high and low nutrient media. *Antarctomyces psychrotrophicus* (4.97%), *Arthroderma sp.* 1 (3.97%), *Geomyces*

pannorum (1.84%) and *Pseudeurotium bakeri* (86.75%) were the most dominant species among the pristine and SAB diesel fuel contaminated soil samples. Furthermore, *Arthroderma sp.* 1 (CZ02), *Exophiala sp.* (CZ06), *Leptodontidium sp.*1 (CZ25) and *Hypocrea sp.* (CZ90) represented potentially novel species based on low ITS sequence identity similarity combined with distinct morphological characteristics to the its closest cultured organism. *Arthroderma sp.* 1 and *Exophiala sp.* were prevalent from pristine to high SAB diesel contaminated soil samples. In particular, *Pseudeurotium bakeri* (CZ03) was isolated from all of the SAB diesel contaminated soil samples except pristine soil sample and was the most abundant fungal species present in the range of soil samples examined. This species was highly abundant by 100-fold as the SAB diesel concentration reached nominal 1,000 and 20,000 mg/kg of soil compared with lower levels of contamination.

In order to investigate the species distribution over the range of SAB diesel fuel spiked soil samples, these fungal isolates were classified to eight class, one sub-phylum *incertae sedis* and four unclassified groups (Figure 3.5). The effect of increasing concentration of SAB diesel fuel on diversity at the this level showed that fungal diversity increased at low concentrations and decreased at higher concentrations of SAB diesel fuel. Furthermore, with increasing concentrations of SAB diesel fuel, the population shifted towards an overall increase in unidentified fungal species. This effect was observed on both high and low nutrient media. At nominal 0-50 mg/kg SAB diesel fuel, fungal isolates recovered were diverse from

class *Agaricomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Microbotryomycetes*, *Saccharomycetes*, *Sordariomycetes*, *Tremellomycetes* and *incertae sedis* sub-phylum *Mucoromycotina* to small proportion of unidentified fungal species. Then a shift to class *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Sordariomycetes* and small proportion of unidentified species occurred at nominal 100 mg/kg SAB diesel fuel with less diversity. At nominal 250-500 mg/kg, a second shift was observed with increasing diversity to class *Agaricomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Saccharomycetes*, *Sordariomycetes*, *Tremellomycetes* and high proportion of unidentified species. At high concentrations exceeding nominal 1,000 mg/kg, the diversity became limited to the class *Eurotiomycetes*, *Leotiomycetes* as well as several unidentified species from the dilute media only (Table 3.1 and Figure 3.5).

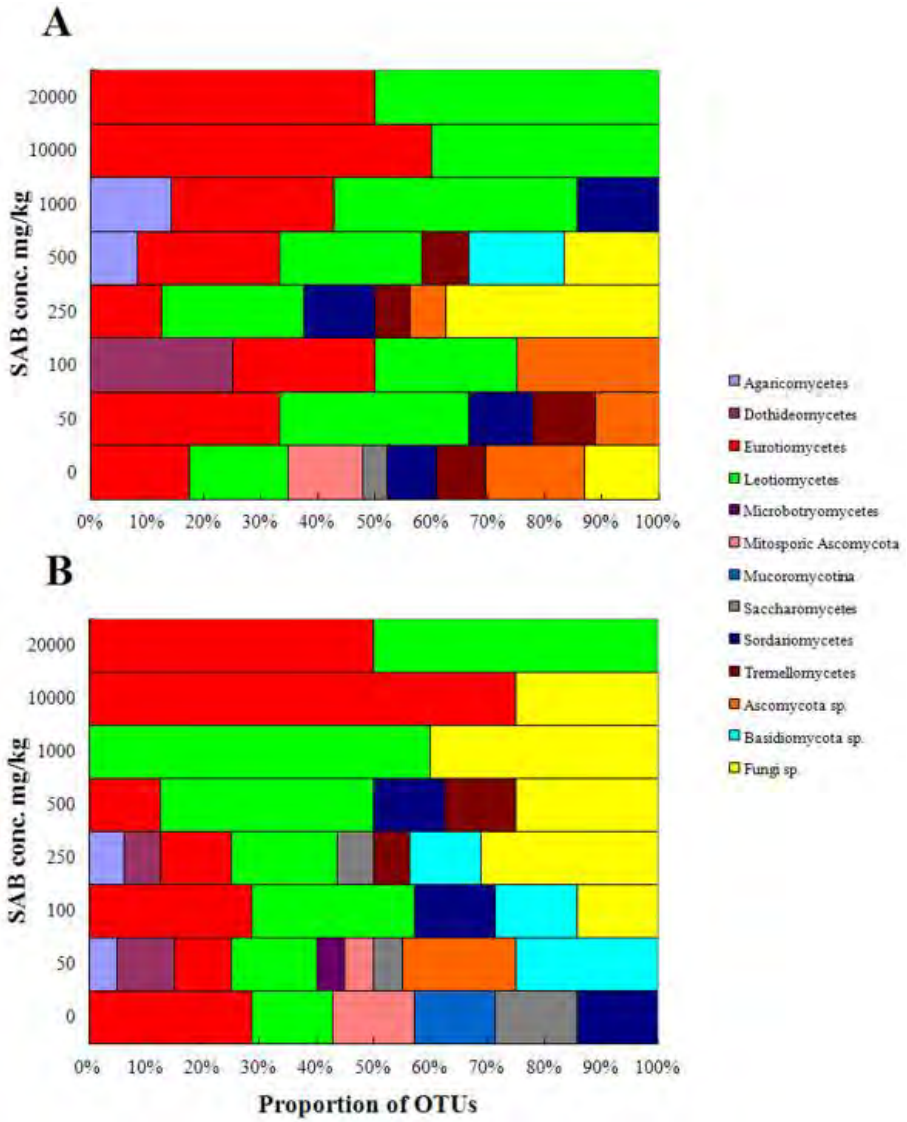


Figure 3.5 The distribution of fungal isolates at class level over a range of SAB diesel fuel spiked soil samples. (A) The OTU distribution in high nutrient media (B) The OTU distribution in dilute nutrient media. With increasing SAB diesel fuel concentrations in soil samples the fungi isolates exhibited more diversity at low concentrations and was limited at higher concentrations.

Table 3.1 List of recovered fungal OTUs from entire 1X and 0.1X nutrient culture media, distribution, molecular and morphologic identification, ITS sequence matches, isolation abundance and frequency, GenBank accession number for fungal isolates recovered from pristine and SAB contaminated Sub-Antarctic Macquarie Island soil samples.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
Ascomycota									
CZ01	0;50;250;500;1000	*Antarctomyces psychrotrophicus*	*Ascomycota sp.* HM589217.1 100%	*Antarctomyces psychrotrophicus* FJ911878.1 99%	266	216	482	4.97	JN104511
CZ02	0;50;100;250;500;10000	*Arthroderma sp.1*	*Arthroderma melis* AJ877216.1, 91%	*Arthroderma melis* AJ877216.1 91%	91	294	385	3.97	JN104512
CZ29	100	*Arthroderma sp.2*	*Arthroderma quadrifidum* EU181451.1 99%	*Arthroderma quadrifidum* EU181451.1 99%		1	1	0.01	JN104536
CZ34	10000	*Aspergillus fumigatus*	*Aspergillus fumigatus* HQ285578.1 100%	*Aspergillus fumigatus* HQ285578.1 100%		1	1	0.01	JN104538
CZ44	20000	*Aspergillus tubingensis*	*Aspergillus tubingensis* HQ728255.1 100%	*Aspergillus tubingensis* HQ728255.1 100%		1	1	0.01	JN104547
CZ42	50;100	*Cladosporium cladosporioides*	*Cladosporium sp.* HQ829411.1 100%	*Cladosporium cladosporioides* HQ671181.1 100%	1	6	7	0.07	JN104545
CZ81	500	*Coniochaeta ligniaria*	*Coniochaeta sp.* HQ657319.1 99%	*Coniochaeta ligniaria* AY198390.1 99%		1	1	0.01	JN104572
CZ21	0;50;250	*Debaryomyces subglobosus*	*Debaryomyces subglobosus* FN675240.1 99%	*Debaryomyces subglobosus* FN675240.1 99%	1		1	0.01	JN104528

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ64	250	*Doratomyces nanus*	*Doratomyces nanus* FJ914706.1 99%	*Doratomyces nanus* FJ914706.1 99%	1		1	0.01	JN104558
CZ40	1000	*Doratomyces stemonitis*	*Doratomyces sp.* FJ914686.1 99%	*Doratomyces stemonitis* FJ914658.1 99%	3		3	0.03	JN104543
CZ06	0;50;250;500;10000;20000	*Exophiala sp.*	*Exophiala sp.* AB488490.1 100%	*Exophiala salmonis* GU586858.1 98%	6	5	11	0.11	JN104516
CZ05	0;50;250;500;1000	*Geomyces pannorum*	*Onygenales sp.* GU212399.1 100%	*Geomyces pannorum* DQ189224.1 99%	130	48	178	1.84	JN104515
CZ20	0	*Geomyces sp.1*	*Geomyces pannorum* DQ779788.1 94%	*Geomyces pannorum* DQ779788.1 94%	1		1	0.01	JN104527
CZ55	250	*Geomyces sp.2*	*Geomyces pannorum* AJ608968.1 97%	*Geomyces pannorum* AJ608968.1 97%	1		1	0.01	JN104553
CZ56	250	*Geomyces sp.3*	*Chrysosporium pseudomerdarium* EU823311.1 96%	*Chrysosporium pseudomerdarium* EU823311.1 96%	1		1	0.01	JN104554
CZ38	10000	*Geomyces sp.4*	*Geomyces pannorum* DQ779788.1 94%	*Geomyces pannorum* DQ779788.1 94%	1		1	0.01	JN104541
CZ78	0;50;250	*Gloeotinia temulenta*	*Verticillium sp.* FJ948142.1 100%	*Gloeotinia temulenta* EU287812.1 100%	2	3	5	0.05	JN104570
CZ90	50	*Hypocrea sp.*	*Hypocrea koningii* FJ025200.1 99%	*Hypocrea koningii* FJ025200.1 99%	6	6	12	0.12	JN104578
CZ08	0	*Leptodontidium orchidicola*	*Leptodontidium orchidicola* GU586841.1 99%	*Leptodontidium orchidicola* GU586841.1 99%		7	7	0.07	JN104517

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ25	0;50	*Leptodontidium sp.1*	*Leptodontidium orchidicola* GU586841.1 97%	*Leptodontidium orchidicola* GU586841.1 97%	2	1	3	0.03	JN104532
CZ19	0	*Leptodontidium sp.2*	*Leptodontidium orchidicola* GU586841.1 98%	*Leptodontidium orchidicola* GU586841.1 98%	1		1	0.01	JN104526
CZ30	100	*Mollisia dextrinospora*	*Mollisia dextrinospora* AY259134.1 100%	*Mollisia dextrinospora* AY259134.1 100%		5	5	0.05	JN104537
CZ22	0	*Neonectria radicicola*	*Neonectria radicicola* GU934548.1 99%	*Neonectria radicicola* GU934548.1 99%	1		1	0.01	JN104529
CZ87	500	*Oidiodendron sp.*	*Oidiodendron griseum* AF307765.1 99%	*Oidiodendron griseum* AF307765.1 99%	1		1	0.01	JN104576
CZ26	0;1000	*Oidiodendron truncatum*	*Oidiodendron truncatum* AF062809.1 100%	*Oidiodendron truncatum* AF062809.1 100%	2		2	0.02	JN104533
CZ77	50	*Paecilomyces sp.1*	*Paecilomyces sp.* DQ187954.1 100%	*Penicillium daleae* DQ132832.1 99%		1	1	0.01	JN104569
CZ14	0	*Paecilomyces sp.2*	*Paecilomyces carneus* HQ660442.1 94%	*Paecilomyces carneus* HQ660442.1 94%	1		1	0.01	JN104521
CZ23	0	*Paecilomyces sp.3*	*Paecilomyces carneus* HQ660442.1 94%	*Paecilomyces carneus* HQ660442.1 94%	1		1	0.01	JN104530
CZ91	50	*Penicillium biourgeianum*	*Penicillium biourgeianum* HM469395.1 100%	*Penicillium biourgeianum* HM469395.1 100%	2		2	0.02	JN104579
CZ37	10000	*Penicillium coprophilum*	*Penicillium coprophilum* FJ613111.1 100%	*Penicillium coprophilum* FJ613111.1 100%	1		1	0.01	JN104540

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ36	10000	*Penicillium sp.1*	*Penicillium sp.* FJ379809.1 99%	*Penicillium griseolum* EF422848.1 95%	1		1	0.01	JN104539
CZ39	1000	*Penicillium sp.2*	*Penicillium sp.* FJ491803.1 99%	*Penicillium cecidicola* DQ123648.1 96%	1		1	0.01	JN104542
CZ51	250	*Penicillium spinulosum*	*Penicillium spinulosum* HM469405.1 99%	*Penicillium spinulosum* HM469405.1 99%		1	1	0.01	JN104551
CZ28	100	*Podospora tetraspora*	*Podospora tetraspora* GQ922573.1 99%	*Podospora tetraspora* GQ922573.1 99%		2	2	0.02	JN104535
CZ48	250	*Preussia funiculata*	*Preussia funiculata* AY943059.1 100%	*Preussia funiculata* AY943059.1 100%		1	1	0.01	JN104550
CZ71	50	*Preussia sp.*	*Preussia flanaganii* AY943061.1 99%	*Preussia flanaganii* AY943061.1 99%		2	2	0.02	JN104563
CZ03	50;100;250;500;1000;10000; 20000	*Pseudeurotium bakeri*	*Pseudeurotium bakeri* GU934582.1 99%	*Pseudeurotium bakeri* GU934582.1 99%	4613	3801	8414	86.75	JN104513
CZ60	250	*Tolypocladium sp.*	*Beauveria geodes* U19037.1 98%	*Beauveria geodes* U19037.1 98%	1		1	0.01	JN104556
CZ16	0	*Torula caligans*	*Torula caligans* FJ478093.1 100%	*Torula caligans* FJ478093.1 100%	1		1	0.01	JN104523
CZ13	0	*Trichophyton sp.*	*Trichophyton eboreum* AJ876907.1 92%	*Trichophyton eboreum* AJ876907.1 92%	2		2	0.02	JN104520
CZ17	0	Unidentified fungus 02	Fungal sp. HM439547.1 99%	*Tricladium angulatum* AY204608.1 92%	1		1	0.01	JN104524

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ18	0	Unidentified fungus 03	*Cystodendron sp.* DQ914672.1 100%	*Hymenoscyphus monotropae* AF169309.1 91%	1		1	0.01	JN104525
CZ15	0	Unidentified fungus 05	*Epacris microphylla* root associated fungus AY268185.1 98%	*Aureobasidium pullulans* HQ637297.1 96%	1		1	0.01	JN104522
CZ76	50	Unidentified fungus 07	Fungal endophyte FN392308.1 98%	*Thozetella havanensis* EF029184.1 87%		1	1	0.01	JN104568
CZ74	50	Unidentified fungus 08	*Trimmatostroma sp.* EU707580.1 99%	*Penidiella rigidophora* EU019276.1 95%		1	1	0.01	JN104566
CZ72	50	Unidentified fungus 10	*Chrysosporium europae* AJ007843.1 92%	*Chrysosporium europae* AJ007843.1 92%		2	2	0.02	JN104564
CZ73	50	Unidentified fungus 11	*Ascomycete sp*. AY303610.1 97%	*Myrmecridium schulzeri* EU041772.1 97%		1	1	0.01	JN104565
CZ89	50	Unidentified fungus 14	Fungal sp. FJ235965.1 97%	*Clathrosphaerina zalewskii* EF029222.1 90%	1		1	0.01	JN104577
CZ11	0	Unidentified fungus 16	*Chrysosporium vespertilium* AJ007846.1 91%	*Chrysosporium vespertilium* AJ007846.1 91%	1		1	0.01	JN104519
CZ58	250	Unidentified fungus 18	*Kernia sp.* FJ946487.1 95%	*Kernia pachypleura* DQ318208.1 90%	1		1	0.01	JN104555
CZ43	50	Unidentified fungus 41	*Ascomycota sp.* GU985215.1 100%	*Mollisia cinerea* AY259135.1 93%	1		1	0.01	JN104546
CZ09	0	*Volutella ciliata*	*Volutella ciliata* GU586855.1 98%	*Volutella ciliata* GU586855.1 98%	6	10	16	0.16	JN104518

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
Mucoromycotina									
CZ04	0	*Mortierella sp.*	*Zygomycete sp.* AJ608979.1 94%	*Mortierella minutissima* EU484265.1 95%		5	5	0.05	JN104514
Basidiomycota									
CZ45	250	*Ceriporia lacerata*	*Ceriporia lacerata* HQ331078.1 98%	*Ceriporia lacerata* HQ331078.1 98%		1	1	0.01	JN104548
CZ63	0;50;250;500	*Cryptococcus gastricus*	*Cryptococcus gastricus* EU266562.1 99%	*Cryptococcus gastricus* EU266562.1 99%	49	15	64	0.66	JN104557
CZ27	0	*Cryptococcus terricola*	*Cryptococcus terricola* FN298664.1 100%	*Cryptococcus terricola* FN298664.1 100%	5		5	0.05	JN104534
CZ86	500	*Lenzites elegans*	*Lenzites elegans* HQ331076.1 99%	*Lenzites elegans* HQ331076.1 99%	1		1	0.01	JN104575
CZ67	50	*Peniophora sp.*	*Peniophora sp.* HM595565.1 98%	*Peniophora laxitexta* FJ882040.1 97%		1	1	0.01	JN104559
CZ68	50	*Sporobolomyces ruberrimus*	*Sporobolomyces ruberrimus var. Albus* AF444581.1 99%	*Sporobolomyces ruberrimus var. Albus* AF444581.1 99%		1	1	0.01	JN104560
CZ41	1000	*Thanatephorus cucumeris*	*Thanatephorus cucumeris* FR670341.1 99%	*Thanatephorus cucumeris* FR670341.1 99%	1		1	0.01	JN104544
CZ75	50	Unidentified fungus 01	*Skeletocutis diluta* JF692197.1 88%	*Skeletocutis diluta* JF692197.1 88%		2	2	0.02	JN104567
CZ70	50	Unidentified fungus 04	*Sporobolomyces inositophilus* AF444559.1 90%	*Sporobolomyces inositophilus* AF444559.1 90%		1	1	0.01	JN104562

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ83	500	Unidentified fungus 06	*Phlebia livida* AB084618.1 95%	*Phlebia livida* AB084618.1 95%	2		2	0.02	JN104573
CZ85	500	Unidentified fungus 09	Fungal sp. FJ609284.1 99%	*Sistotrema brinkmannii* DQ093737.1 94%	3		3	0.03	JN104574
CZ79	50	Unidentified fungus 12	*Polypore sp.* AJ537411.1 93%	*Wrightoporia bracei* JF692199.1 93%		1	1	0.01	JN104571
CZ69	50	Unidentified fungus 13	*Phanerochaetaceae sp.* HM595569.1 88%	*Phanerochaete chrysosporium* AB361644.1 88%		1	1	0.01	JN104561
CZ47	50;100;250	Unidentified fungus 15	*Phlebiopsis gigantea* FJ791151.1 99%	*Phlebiopsis gigantea* FJ791151.1 99%		5	5	0.05	JN104549
CZ52	250	Unidentified fungus 17	*Sistotrema brinkmannii* HM535376.1 93%	*Sistotrema brinkmannii* HM535376.1 93%		2	2	0.02	JN104552
Unidentified									
CZ12	0	Unidentified fungus 19	Data not available	Data not available	1		1	0.01	
CZ61	250	Unidentified fungus 20	Data not available	Data not available	2		2	0.02	
CZ65	250	Unidentified fungus 21	Data not available	Data not available	1		1	0.01	
CZ66	250	Unidentified fungus 22	Data not available	Data not available	1		1	0.01	
CZ57	250	Unidentified fungus 23	Data not available	Data not available	1		1	0.01	
CZ62	250	Unidentified fungus 24	Data not available	Data not available	2		2	0.02	
CZ59	250	Unidentified fungus 25	Data not available	Data not available	1		1	0.01	
CZ84	500	Unidentified fungus 26	Data not available	Data not available	1		1	0.01	
CZ88	500	Unidentified fungus 27	Data not available	Data not available	1		1	0.01	

Table 3.1 Continued.

Isolate	SAB concentration in soil (mg/kg)	Identification	Closest match (accession number, % identity)	Closest cultured organism (accession number, % identity)	1X	0.1X	Total	%	Accession number
CZ07	0	Unidentified fungus 28	Data not available	Data not available		6	6	0.06	
CZ10	0	Unidentified fungus 29	Data not available	Data not available		1	1	0.01	
CZ31	100	Unidentified fungus 30	Data not available	Data not available		1	1	0.01	
CZ49	250	Unidentified fungus 31	Data not available	Data not available		1	1	0.01	
CZ50	250	Unidentified fungus 32	Data not available	Data not available		1	1	0.01	
CZ53	250	Unidentified fungus 33	Data not available	Data not available		1	1	0.01	
CZ54	250	Unidentified fungus 34	Data not available	Data not available		1	1	0.01	
CZ46	250	Unidentified fungus 35	Data not available	Data not available		1	1	0.01	
CZ82	500	Unidentified fungus 36	Data not available	Data not available		1	1	0.01	
CZ80	500	Unidentified fungus 37	Data not available	Data not available		1	1	0.01	
CZ32	1000	Unidentified fungus 38	Data not available	Data not available		1	1	0.01	
CZ33	1000	Unidentified fungus 39	Data not available	Data not available		1	1	0.01	
CZ35	10000	Unidentified fungus 40	Data not available	Data not available		1	1	0.01	
CZ24	10000	Unidentified fungus 42	*Acanthamoeba polyphaga* AF526432.1 89%	*Acanthamoeba polyphaga* AF526432.1 89%		1	1	0.01	JN104531
Total					5227	4472	9699	100.00	

3.7 Phylogenetic Diversity of Recovered Fungal Species

Based on the ITS gene sequence information, phylogenetic trees were constructed for the fungal phylum *Ascomycota* (Figure 3.6) and phylum *Basidiomycota* (Figure 3.7), respectively. Strain CZ04 was identified as *Mortierella sp.*, which was a unique isolate belonging to the genus of *incertae sedis* sub-phylum *Mucoromycotina*. The resulting phylogenetic tree was constructed together with *Basidiomycota*. Strain CZ24 was an unusual species based on ITS gene sequence information and morphological characteristics, so that it was not classified into any fungal phylum in this study. Phylogenetic analysis and other characteristic results about strain CZ24 are represented in Section 3.9.

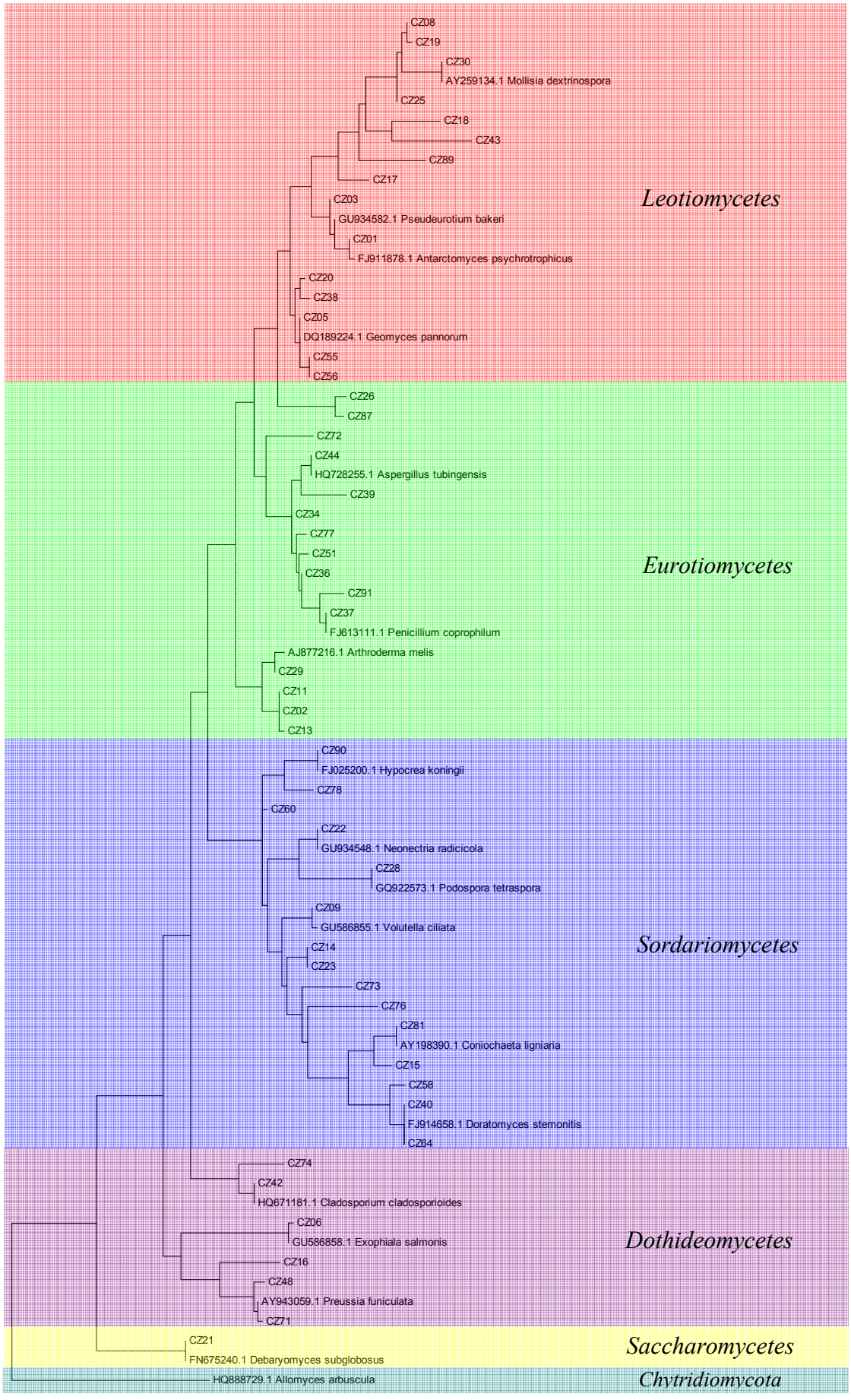

CZ08
CZ19
CZ30
AY259134.1 Mollisia dextrinospora
CZ25
CZ18
CZ43
CZ89
CZ17
CZ03
GU934582.1 Pseudeurotium bakeri
CZ01
FJ911878.1 Antarctomyces psychrotrophicus
CZ20
CZ38
CZ05
DQ189224.1 Geomyces pannorum
CZ55
CZ56
Leotiomycetes
CZ26
CZ87
CZ72
CZ44
HQ728255.1 Aspergillus tubingensis
CZ39
CZ34
CZ77
CZ51
CZ36
CZ91
CZ37
FJ613111.1 Penicillium coprophilum
Eurotiomycetes
AJ877216.1 Arthroderma melis
CZ29
CZ11
CZ02
CZ13
CZ90
FJ025200.1 Hypocrea koningii
CZ78
CZ60
CZ22
GU934548.1 Neonectria radicicola
CZ28
GQ922573.1 Podospora tetraspora
CZ09
GU586855.1 Volutella ciliata
CZ14
CZ23
CZ73
CZ76
CZ81
AY198390.1 Coniochaeta ligniaria
CZ15
CZ58
CZ40
FJ914658.1 Doratomyces stemonitis
CZ64
Sordariomycetes
CZ74
CZ42
HQ671181.1 Cladosporium cladosporioides
CZ06
GU586858.1 Exophiala salmonis
CZ16
CZ48
AY943059.1 Preussia funiculata
CZ71
Dothideomycetes
CZ21
FN675240.1 Debaryomyces subglobosus
Saccharomycetes
HQ888729.1 Allomyces arbuscula
Chytridiomycota
0.05

Figure 3.6 Phylogenetic tree of *Ascomycota* based on ITS gene sequence comparisons of 52 *Ascomycota* isolates, as well as closest related cultured isolates and an outgroup (*Allomyces arbuscula*) from fungal phylum *Chytridiomycota*. The tree was constructed using MEGA 5.05 by Maximum Likelihood statistical method with General Time Reversible model for setting up. These *Ascomycota* isolates were classified into five class groups (*Leotiomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Dothideomycetes* and *Saccharomycetes*) based on the latest classification of *Fungi* (Hibbett *et al.*, 2007).

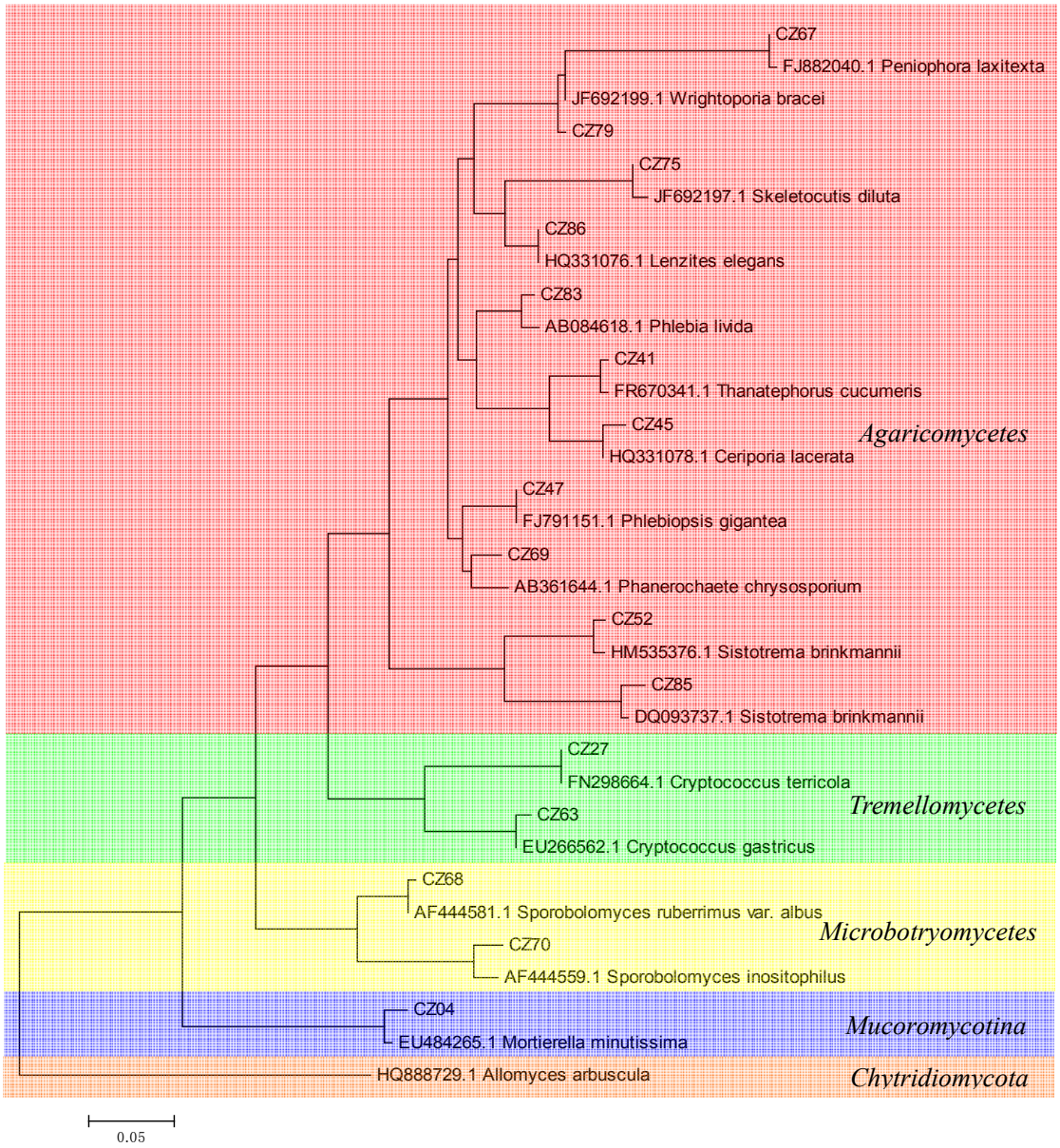


Figure 3.7 Phylogenetic tree of *Basidiomycota* based on ITS gene sequence comparisons of 15 *Basidiomycota* isolates, one *Mucoromycotina* isolate (CZ04), their closest related cultured isolates and an outgroup (*Allomyces arbuscula*) from fungal phylum *Chytridiomycota*. The tree was constructed using MEGA 5.05 by Maximum Likelihood statistical method with General Time Reversible model for setting up. These *Basidiomycota* isolates were classified into three class groups (*Agaricomycetes*, *Tremellomycetes* and *Microbotryomycetes*) based on latest classification of *Fungi* (Hibbett *et al.*, 2007).

3.8 Comparing the Recovered Species Richness Between High Nutrient and Low Nutrient Media

Of the 91 fungal species recovered, the high nutrient culture approach recovered 52 species (57% of total species) from among 5,227 isolates and the low nutrient approach recovered 50 species (55% of total species) from among 4,472 isolates. Both culture media recovered high proportion of singletons, which in total represented 67% of total species richness. Additionally, a high number of unidentified species were recovered from both culture media, 34 species (37% of total species) in high nutrient and 33 species (36% of total species) in low nutrient media (Table 3.2).

Table 3.2 Fungal communities recovered from plot 4 soil samples using both the high and low nutrient culture media approaches.

	High nutrient media	Low nutrient media	Combined media
Total isolates	5,227	4,472	9,699
Species richness	52	50	91
Species/soil sample	9.6±6.7	8.9±6.2	9.3±6.2
Singletons	32	29	61
Unidentified species	34	33	63

The one way ANOSIM test on each nominal SAB diesel fuel concentration individually showed significant difference between high and low nutrient media approaches when the nominal SAB diesel fuel concentration below 1,000 mg/kg (including 1,000 mg/kg), as the global R value from the lowest 0.222 to the highest 1 and P value of 0.1 (Table 3.3). Additionally, due to the global R value -0.111 and P

value of 0.8 for 10,000 mg/kg and global R value -0.074 and P value of 0.6 for 20,000 mg/kg, there was no significant difference between high and low nutrient media approaches from these two nominal SAB diesel fuel concentrations (Table 3.3).

Table 3.3 The ANOSIM test results of each fuel concentration individually, the results presented the global R value as well as P value.

Nominal SAB diesel fuel Concentration (mg/kg)	Global R value	P value
0	0.778	0.1
50	1	0.1
100	0.852	0.1
250	0.667	0.1
500	0.704	0.1
1000	0.222	0.1
10000	-0.111	0.8
20000	-0.074	0.6

The two way ANOSIM statistical analysis on the recovered community distribution using NMDS plot showed a significant difference between high and low nutrient media approaches due to a global R value of 0.542 and P value of 0.001 (Figure 3.8). Here, the selection of media contributed a major role in altering the recovered species distribution. The SAB diesel fuel concentration within the spiked soil samples used for cultivation resulted in an even greater effect on recovered community composition as revealed by a statistically significant global R value of 0.916 and P value of 0.001. Furthermore, a greater number of novel species were recovered from the low nutrient media, including two unidentified species from the 10,000 mg/kg SAB spiked soil samples (Table 3.1).

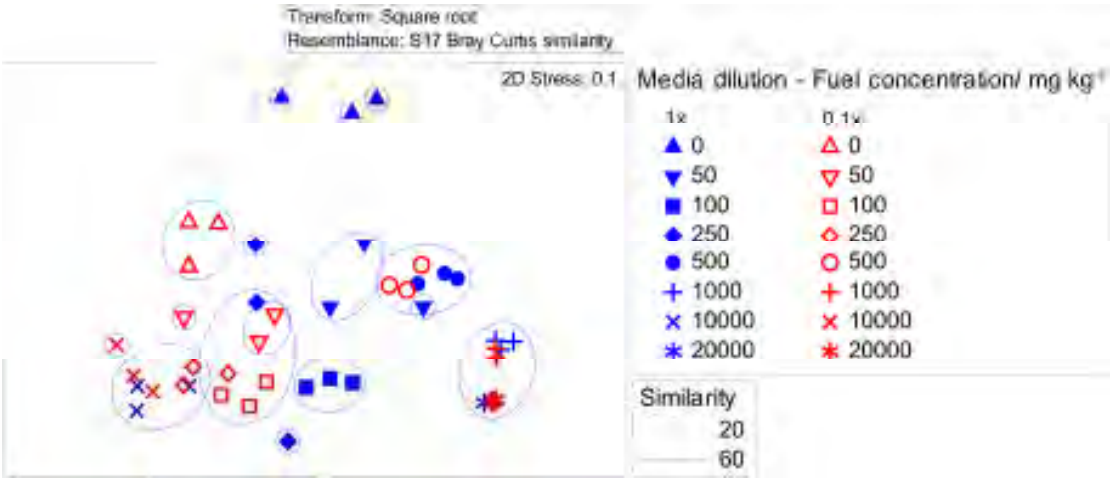


Figure 3.8 Non-metric multi-dimensional scaling (NMDS) configuration plot comparing the species composition of P4 fungal communities recovered from high and low nutrient media for each SAB diesel fuel spiked soil sample. The NMDS plot of similarities was calculated on the basis Bray-Curtis coefficient using square root transformed fungal diversity data. The NMDS plot was shown superimposed clusters within dashed lines for 60% similarity boundaries (green line) and continuous ellipses for 20% similarity boundaries (blue line). The results confirmed that the two different media approaches did a significant alter the recovered species distribution due to a global R value of 0.542 and P value of 0.001. The SAB diesel fuel concentration within the spiked soil samples resulted in an even greater effect on recovered species distribution as revealed by a global R value of 0.916 and P value of 0.001.

3.9 Characterisation of Strain CZ24

Strain CZ24 was a singleton isolated from the low nutrient media from soil spiked with SAB diesel fuel at 10,000 mg/kg. This strain was not able to grow on PDA and sub-culturing was subsequently carried out on special nutrient agar (SNA) (Section

2.2.2). After seven days cultivation on SNA, colony size reached 0.1 mm - 0.5 mm diameter and was a grey-white colour. After 14 days cultivation, no further growth was observed. Microscopic analysis of morphological characteristics showed *mycelium* were present consisting of rare branched, aseptate, smooth, 1 μm diameter hyphae and grey-white colour colony; no *conidia* and *conidiophores* were observed (Figure 3.9).

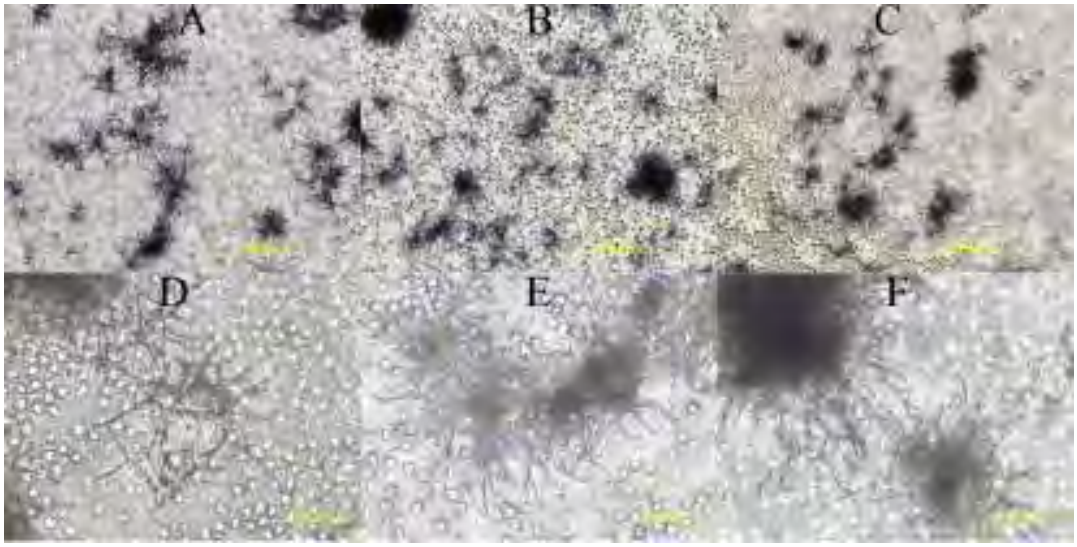


Figure 3.9 Morphological characteristics of strain CZ24 using the Olympus BX61 Epi-Fluorescence Microscope equipped with a DP71 digital camera. (A), (B) and (C) colonies on SNA under 10X magnification; (D), (E) and (F) colonies on SNA under 50X magnification. Scale bars: A-C = 500 μm, D-F = 100 μm. Colonies showed 0.1 mm - 0.5 mm in diameter with 1 μm diameter hyphae. Furthermore, there were some unknown crystal-like particles only formed around colonies.

PCR amplification targeting the 18S rDNA gene using primer set EF4/EF3, the ITS gene with primer set ITS1/ITS4 and the 28S rDNA gene with primer set NL1/NL4,

was carried out. The PCR amplification products were successfully obtained with approximate 1.4 kb length for 18S rDNA, 1 kb length for ITS gene and 700 bp length for 28S rDNA gene. After DNA sequencing and taxonomic designation using BLASTn, 18S rDNA shows a 96% similarity to *Pithoascus langeronii* which is a fungal species belonging to phylum *Ascomycota*. However, ITS sequence data revealed 89% identity to *Acanthamoeba polyphaga* and 97% identity to *Acanthamoeba castellanii* for 28S rDNA, both organisms are members of genus *Acanthamoeba* belonging to kingdom *Amoebozoa* (Table 3.3).

Interestingly, CZ24 was not only shown to be tolerant to SAB diesel fuel at 10,000 mg/kg, but antibacterial activity was observed with positive results from antimicrobial bioassays observed for range of gram positive and negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus subtilus*, *Micrococcus luteus* and *Staphylococcus aureus*.

Table 3.4 Phylogenetic analysis of strain CZ24 target 18S rDNA, ITS and 28S rDNA genes, closest matches and evolutionary position of closest match organism.

Gene	**Closest match (accession number, % identity)**	**Evolutionary position of closest match organism**
18S rDNA	*Pithoascus langeronii* GQ280421.1 96%	*Fungi*
ITS	*Acanthamoeba polyphaga* AF526432.1 89%	*Amoebozoa*
28S rDNA	*Acanthamoeba castellanii* GU001160.1 97%	*Amoebozoa*

3.10 Conclusion

Antarctica fungi has been isolated from many locations, from 10 species recovered on Windmill Islands (McRae and Seppelt, 1999) to 19 taxa identified at King George Island with the Shannon diversity index from the lowest 0 to the highest 2.02 (Rosa *et al.*, 2010). In this study, 91 OTUs represented the lowest 0.010 to the highest 2.546 of Shannon diversity index (Table 4.2), which was higher than King George Island, and this index value was close to the diversity recovered in temperate zone (Hunt *et al.*, 2004). Therefore, Sub-Antarctic Macquarie Island showed great soil fungal diversity with 91 filamentous fungi species recovered from one soil plot. Here, the low to high nutrient media approach gave similar species richness results, but the low nutrient media approach recovered a significantly different fungal community composition compared with high nutrient media from a range of SAB diesel spiked soil, and therefore enhanced the fungal diversity recovered ($P<0.01$). Of the 63 (69% of total species) unidentified species, the majority were potentially indigenous novel species on Macquarie Island due to the low ITS match similarity or unique morphological characteristics. Further morphological description is required for species identification.

In addition, a large number of fungal species were recovered from medium to high levels of SAB diesel spiked soils. Several were similar to known hydrocarbon degrading fungi including *Arthroderma sp.*, *Aspergillus sp.*, *Cryptococcus sp.*, *Exophiala sp.*, *Geomyces sp.*, *Penicillium sp.* and *Pseudeurotium sp.*. However,

further characterisation on the potential for hydrocarbon degradation is required to determine if they are not only tolerant but also capable of assimilating petroleum hydrocarbons.

Chapter 4 Effect of Hydrocarbon Contamination on Fungal Diversity in Macquarie Island soil

4.1 Experimental design

In this study, the experiment was designed to investigate the impact of SAB diesel fuel contamination on fungal species recovered from 4 soil plots (P2, P4, P6 and P9) of various carbon content (Table 4.1). The soil was divided into eight samples and spiked with SAB diesel fuel to final concentrations of 0, 50, 100, 250, 500, 1000, 10,000 and 20,000 mg/kg, respectively. Here, due to potential operational errors, such as asymmetrical homogenising and physical evaporation, differences between the nominal and final measured SAB diesel fuel concentration in the soils was observed (Table 2.1). In this case, the concentrations of SAB in soil samples were grouped as low (actual 0 to 200 mg/kg), medium (actual 201 to 5,000 mg/kg) and high (actual over 5,001 mg/kg) rather than does not exist SAB concentrations.

In this chapter, the fungi were isolated with spread plating of 10^{-3} soil dilutions only onto low nutrient 0.1X CRBA media in triplicate. When fungal colonies formed on the plates, each morphological distinct isolate as determined by colony characteristics such as colour and shape was sub-cultured on PDA until pure cultures were obtained. Finally, a two way ANOVA test was used to determine if the recovered fungal communities from 4 investigated soil plots was affected by organic carbon concentrations or by different SAB diesel fuel as the level of significance was 0.05; Shannon's diversity index and Simpson diversity index were calculated to determine

whether there was any correlation of fungal groups recovered from the investigated soils with different SAB concentrations.

4.2 Background and Aims

In Chapter 3, the effect of hydrocarbon contamination on Sub-Antarctic Macquarie Island soil fungal communities revealed a dramatic shift occurred from low to high concentrations of SAB diesel fuel. Within the 91 fungal species recovered, a couple of dominant species: *Antarctomyces psychrotrophicus, Arthroderma sp.*, *Geomyces pannorum* and *Pseudeurotium bakeri* contributed to species richness in medium and highly contaminated soils. In particular, *Pseudeurotium bakeri* was isolated from all of the SAB diesel fuel spiked soil samples and was the most abundant fungal species represented. These dominant fungal species may play a role in hydrocarbon degradation and thus may potentially aid in bioremediation efforts. However, only one soil plot was examined.

The aim of this chapter was to determine if the dominant fungal species recovered from P4 were also found in a range of soil plots sampled from Macquarie Island. In addition, the effect of hydrocarbon contamination on fungal communities in a range of soil plots using the low nutrient media was investigated to find out what the effect of SAB diesel fuel on fungal communities was and finally to determine what concentration of SAB diesel fuel resulted in a major change on fungal diversity and abundance.

4.3 Fungal Cultivation From Soils

In this chapter, the optimum 10^{-3} soil dilution was used for fungal isolations from SAB diesel fuel spiked soils from 4 plots (P2, P4, P6 and P9) of varying carbon content (Table 4.1). After 21 days of incubation at 15°C, fungal colonies were present from all soil samples. Based on macroscopic morphological characteristics each unique isolate was sub-cultured on PDA until pure cultures were obtained. The molecular identification was not performed for unknown isolates due to time constraints therefore unknown isolates were designated as OTUs only.

Table 4.1 The carbon content of the four soil plots examined in this chapter. The carbon content was expressed as a percentage of total weight. (Carbon content was analysed by the AAD).

Plot number	Carbon Content (% weight)
P2	1.097
P4	3.146
P6	12
P9	26.99

A small portion of OTUs could be identified based on morphological characteristics as they were previously identified from P4 in Chapter 3. Overall, there were 14 OTUs recovered from P2, 27 OTUs recovered from P6, 19 OTUs recovered from P9 and 25 from P4. A two-way ANOVA test was used here to determine the fungal diversity affecting factor with the hypothesis that 1) "there was no difference among the levels of organic carbon concentrations in the pristine soil plots", 2) "there was no difference among the levels of different SAB diesel fuel concentrations in spiked soil plots" and

3) "there was no difference between organic carbon concentrations in pristine soil plots with different SAB diesel fuel concentrations". As a result, the carbon content found in soil plots did not contribute to the diversity of recovered fungal species ($P>0.05$). However, there was a statistically significant difference ($P<0.05$) among the diversity recovered from soils spiked with increasing concentrations of SAB diesel fuel. Therefore, SAB diesel fuel contributed to fungal species diversity among the four spiked soil plots examined.

4.4 Recovered Fungal Species Abundance

With increasing concentrations of SAB diesel fuel, a significant difference in total CFU/g was observed in all soil plots ($P<0.001$) (Figure 4.1). Thus, SAB diesel fuel was a crucial factor contributing to the total CFUs recovered among the four soil plots analysed. This difference in total CFUs was due to the presence of several dominant species: *Pseudeurotium bakeri* (OTU 14) was the most abundant species present in all soil plots and was found to be widely distributed in soils spiked with low to high concentrations of SAB diesel fuel (Figure 4.2 A, B, C and D). This particular OTU (*Pseudeurotium bakeri*) increased in abundance by 100-fold as the SAB diesel fuel concentration approached medium and high in P4, medium and high in P6, and low in P9 (Figure 4.1). Isolate OTU 9 was abundant at medium and high concentrations of SAB diesel fuel but was only isolated from P2 (Figure 4.2 A); *Arthroderma sp.* 1 (OTU 13) contributed to the dominant fungal colony recovered in soil samples spiked with low to high concentrations of SAB diesel fuel in P4 and low to high in P9

(Figure 4.2 B and 4.2 D); *Antarctomyces psychrotrophicus* (OTU 15) contributed to the dominant fungal colony recovered at medium SAB concentrations of diesel fuel in P4 (Figure 4.2 B).

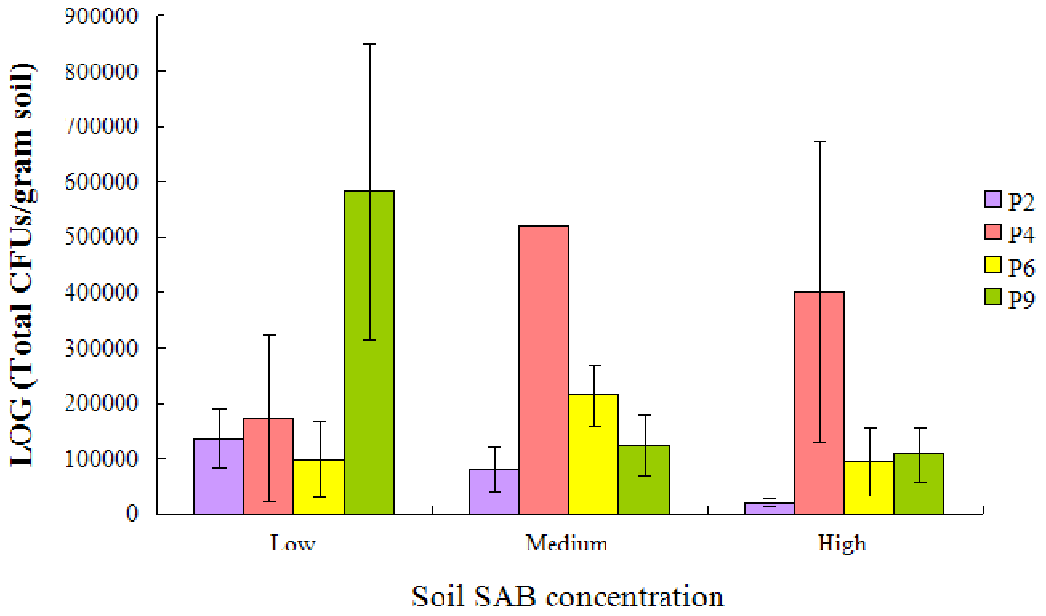
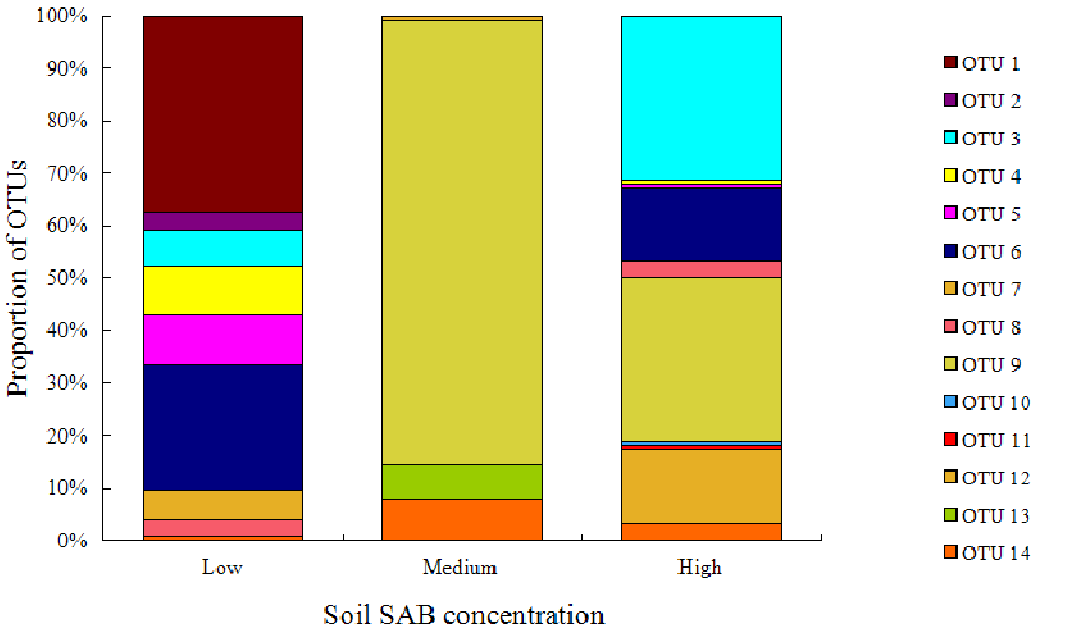
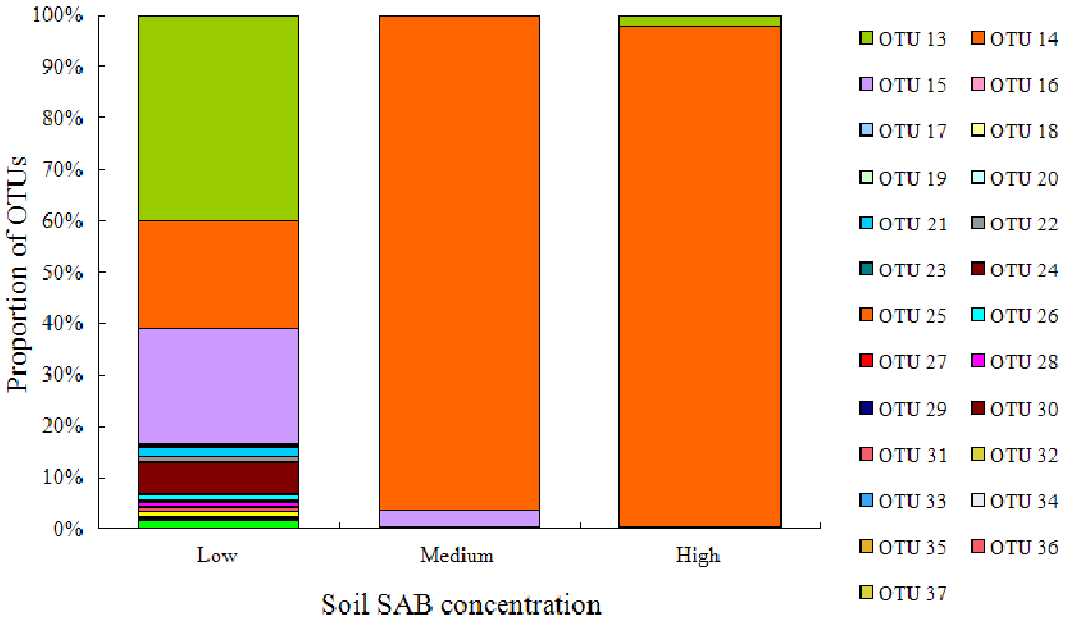


Figure 4.1 The average abundance of fungal isolates recovered from four soil plots spiked with a range of SAB diesel fuel. The spiked soil samples were grouped into low, medium and high levels of SAB.

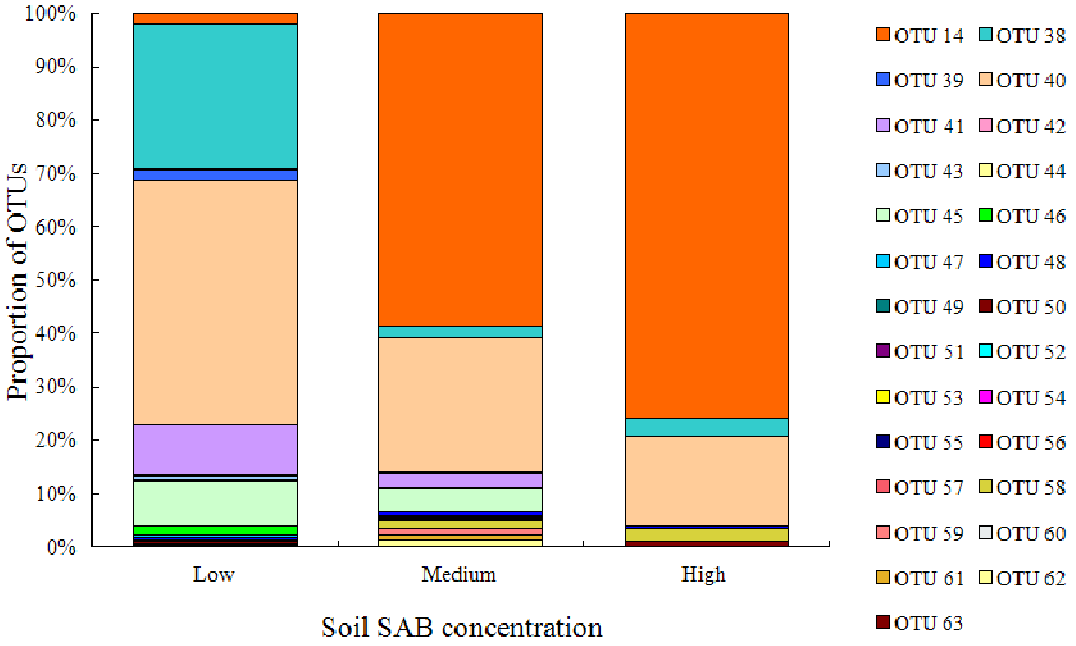
A


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Proportion of OTUs
Low
Medium
High
Soil SAB concentration
OTU 1
OTU 2
OTU 3
OTU 4
OTU 5
OTU 6
OTU 7
OTU 8
OTU 9
OTU 10
OTU 11
OTU 12
OTU 13
OTU 14

B


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Proportion of OTUs
Low
Medium
High
Soil SAB concentration
OTU 13
OTU 14
OTU 15
OTU 16
OTU 17
OTU 18
OTU 19
OTU 20
OTU 21
OTU 22
OTU 23
OTU 24
OTU 25
OTU 26
OTU 27
OTU 28
OTU 29
OTU 30
OTU 31
OTU 32
OTU 33
OTU 34
OTU 35
OTU 36
OTU 37

C


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Proportion of OTUs
Low
Medium
High
Soil SAB concentration
OTU 14
OTU 38
OTU 39
OTU 40
OTU 41
OTU 42
OTU 43
OTU 44
OTU 45
OTU 46
OTU 47
OTU 48
OTU 49
OTU 50
OTU 51
OTU 52
OTU 53
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OTU 55
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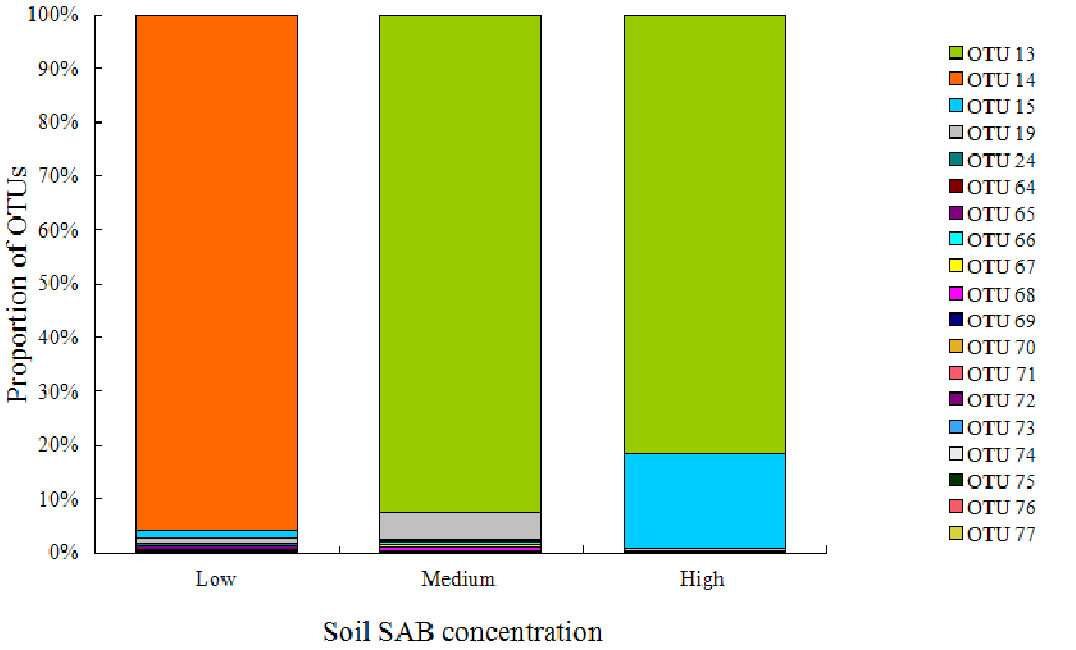
OTU 57
OTU 58
OTU 59
OTU 60
OTU 61
OTU 62
OTU 63

D


100%
90%
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10%
0%
Proportion of OTUs
Low
Medium
High
Soil SAB concentration
OTU 13
OTU 14
OTU 15
OTU 19
OTU 24
OTU 64
OTU 65
OTU 66
OTU 67
OTU 68
OTU 69
OTU 70
OTU 71
OTU 72
OTU 73
OTU 74
OTU 75
OTU 76
OTU 77

Figure 4.2 The distribution and abundance of fungal isolates recovered from soil plots over range of SAB diesel fuel spiked soil samples. (A) Fungal isolates recovered from P2. (B) Fungal isolates recovered from P4. (C) Fungal isolates recovered from P6. (D) Fungal isolates recovered from P9. With increasing SAB diesel fuel concentrations, consistent trends were observed with a major species shift at low, medium and high. When SAB diesel fuel concentration exceeded high, all the soil plots showed a decline in diversity and only a limited number of species were isolated.

4.5 Recovered Fungal Species Distribution

A comparison on the Shannon's diversity index and Simpson diversity index for the recovered fungal species from the four examined soil plots (Table 4.2 and Table 4.3) showed that the fungal population was more diverse in non spiked and nominal 50 mg/kg SAB soils. With increasing SAB diesel fuel concentrations, the diversity index declined to relatively low levels, and the most notable change in diversity index consistently occurred when concentrations of SAB diesel fuel was high. This trend was also observed in the species distribution figures (Figure 4.2 A, B, C and D), all of the soil plots exhibited a major species shift when SAB diesel fuel concentrations increased from low to medium. These mid-level concentrations resulted in a visible distinction in fungal species diversity.

Table 4.2 The Shannon diversity index (H') of the recovered fungal species from P2, P4, P6 and P9 against nominal SAB diesel fuel concentration.

Plot number	Diversity index (H') in nominal SAB concentration (mg/kg)							
	0	50	100	250	500	1000	10000	20000
P2	2.102	1.218	1.442	1.488	0.365	0.873	0.745	0.000
P4	2.546	1.242	1.293	0.943	1.579	0.233	0.443	0.010
P6	0.669	2.271	1.829	1.793	0.288	2.619	0.873	1.531
P9	2.532	2.397	0.022	0.088	1.280	1.856	0.036	0.124

Table 4.3 The Simpson diversity index (λ) of the recovered fungal species from P2, P4, P6 and P9 against nominal SAB diesel fuel concentration.

Plot number	Diversity index (λ) in nominal SAB concentration (mg/kg)							
	0	50	100	250	500	1000	10000	20000
P2	0.267	0.575	0.402	0.470	0.894	0.701	0.755	1.000
P4	0.205	0.592	0.502	0.708	0.416	0.932	0.865	0.998
P6	0.773	0.314	0.417	0.448	0.920	0.182	0.668	0.357
P9	0.215	0.221	0.996	0.981	0.472	0.417	0.993	0.967

In P2, P4 and P9, a gradual decline in species richness was observed with increasing concentrations of SAB diesel fuel from low to medium (Figure 4.3). However, P6 showed higher diversity than the other soil plots combined with increased diversity at low to medium concentrations SAB diesel fuel. Finally, when the SAB diesel fuel concentration reached high, all soil plots revealed a decline in diversity and only a limited number of species were isolated.

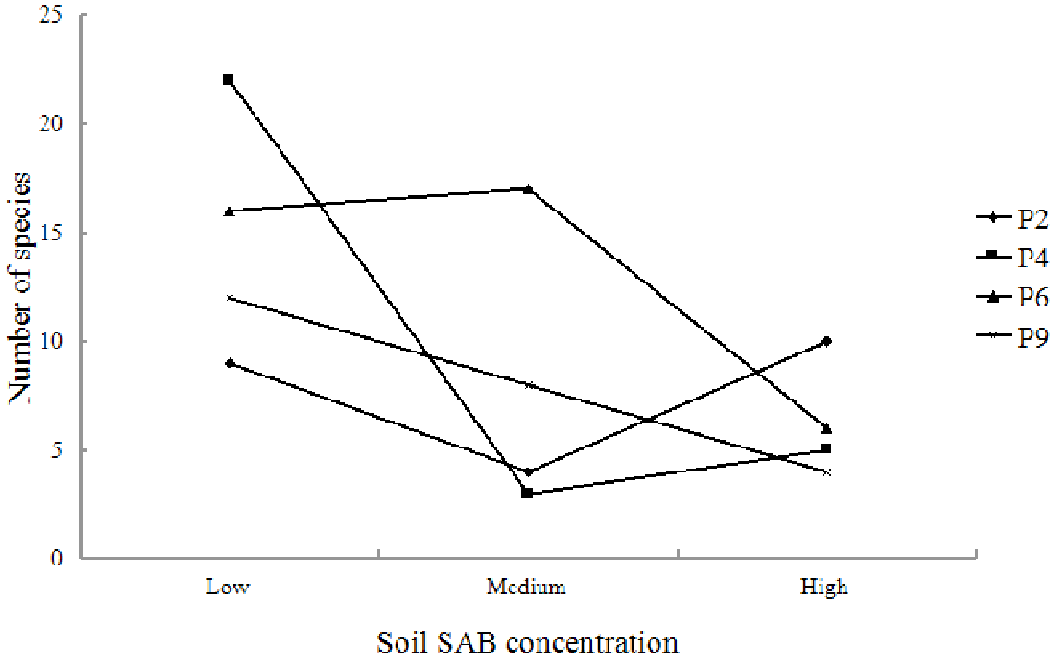

Figure 4.3 The effect of increasing concentrations of SAB diesel fuel on the number of fungal species recovered from soil plots spiked with SAB diesel fuel. The species number was an combined number formed from the number of colonies obtained for triplicates from each soil sample.

In P2, the number of OTUs ranged between 3-6 at nominal 0-10,000 mg/kg (actual 0-12,763 mg/kg) SAB diesel fuel and declined to one at nominal 20,000 mg/kg (actual 15,680 mg/kg) SAB diesel fuel. OTU 2 and 5 were only isolated from P2 pristine soil samples and were not found in SAB diesel fuel spiked soil samples. OTU 1 was a dominant species present in nominal 0-250 mg/kg (actual 0-169 mg/kg) SAB diesel fuel but disappeared when SAB diesel fuel exceeded nominal 500 mg/kg (actual 344 mg/kg). OTU 3 and 4 were dominant species in P2 pristine soil samples, and were rarely isolated from P2 low concentration of SAB diesel fuel. OTU 6, 7 and

8 contributed to a major species shift in diversity at nominal 50 mg/kg (actual 65 mg/kg) SAB diesel fuel and were not isolated when SAB diesel fuel concentration exceeded nominal 500 mg/kg (actual 344 mg/kg). The second species shift was due to the dominant isolate OTU 9 which appeared at nominal 500 mg/kg (actual 344 mg/kg) SAB diesel fuel. At nominal 20,000 mg/kg (actual 15,680 mg/kg) SAB diesel fuel, only one species was isolated (OTU 12). Furthermore, although *Arthroderma sp.* 1 (OTU 13) and *Pseudeurotium bakeri* (OTU 14) were not dominant species in P2, they were previously isolated from SAB diesel fuel spiked soil samples in P4 in Chapter 3 (Table 3.1) and were also found in P6 and P9 (Figure 4.2 C and D).

In P4, all of the isolates were designated in Chapter 3 (Table 3.1). The effect of SAB diesel fuel on fungal species diversity showed that fungal diversity decreased gradually as SAB diesel fuel increased, with 8 species at nominal 0-50 mg/kg (actual less than 50 mg/kg) and only two at nominal 20,000 mg/kg (actual 15,030 mg/kg) soil. However, at nominal 500 mg/kg (actual 247 mg/kg) SAB diesel fuel there was an exception with 7 species recovered (Figure 4.2). At nominal 0 mg/kg (actual less than 50 mg/kg) SAB diesel fuel, the fungal isolates showed high diversity. Then a shift occurred at nominal 50 mg/kg (actual less than 50 mg/kg) SAB diesel fuel followed by another shift at nominal 500 mg/kg (actual 247 mg/kg) SAB diesel fuel to five different isolates recovered. When the SAB diesel fuel concentration exceeded nominal 1,000 mg/kg (actual 628 mg/kg), the fungal diversity became limited to three species and a third species shift occurred at nominal 10,000 mg/kg (actual 9,744

mg/kg) SAB diesel fuel (Figure 4.2).

In P6, the effect of increasing concentration of SAB diesel fuel on fungal species diversity showed that fungal diversity increased at low concentrations and decreased at high concentrations of SAB diesel fuel, this trend was not observed for the other plots (Figure 4.2). The number of fungal OTUs decreased 4- fold from 12 at nominal 250 mg/kg (actual 388 mg/kg) SAB to 3 at nominal 500 mg/kg (actual 800 mg/kg) SAB, and subsequently increased 3-fold at nominal 1,000 mg/kg (actual 3,401 mg/kg) SAB diesel fuel (Figure 4.3). In un-spiked soil samples, the fungal isolates showed less diversity and a species shift was observed at nominal 50 mg/kg (actual 88 mg/kg) SAB diesel fuel. At nominal 500 mg/kg (actual 800 mg/kg), the fungal species became less diverse and *Pseudeurotium bakeri* (OUT14) increased in abundance by 100-fold compared with low levels of contamination. A third shift occurred at nominal 1,000 mg/kg (actual 3,401 mg/kg) SAB with a large proportion of previously recovered OTUs from low concentrations of SAB diesel fuel not isolated and a further five novel isolates (OTU 58 ~ OTU 62) contributed to species richness. When SAB diesel fuel concentration exceeded nominal 10,000 mg/kg (actual 25,289 mg/kg) with diversity limited to *Pseudeurotium bakeri* (OTU 14) which increased in abundance by 100-fold compared with low levels of SAB diesel fuel spiked samples (Figure 4.2 C).

In P9, fungal species diversity decreased with low concentrations, increased at medium concentrations and decreased at high concentrations of SAB diesel fuel

(Figure 4.2). At nominal 0-50 mg/kg (actual 116-74 mg/kg), fungal isolates showed greatest diversity. Isolates OTU 72 ~ OTU 77 and *Antarctomyces psychrotrophicus* (OTU 15) were recovered from these two soil samples only. A first shift occurred at nominal 100 mg/kg (actual 94 mg/kg) SAB diesel fuel where the abundance of *Pseudeurotium bakeri* (OTU 14) increased 500-fold. A second shift was observed as SAB diesel fuel concentration approached nominal 250 mg/kg (actual 569 mg/kg) with *Arthroderma sp.* 1 (OTU 13) emerging as the dominant species. A third shift was observed at nominal 20,000 mg/kg (actual 32,254 mg/kg), with less diversity present and *Pseudeurotium bakeri* (OTU14) was again the dominant species (Figure 4.2).

4.6 Conclusion

In this chapter, the low nutrient culture approach was used to recover fungal communities from a further three soil plots. For each plot, SAB diesel fuel was shown to be significant factor effecting fungal species diversity ($P<0.05$) and abundance ($P<0.001$).

At SAB diesel fuel concentrations of nominal 50 mg/kg, all of the examined soil plots revealed a major species shift in diversity, while a limited number of fungal species were recovered when SAB diesel fuel concentration exceeded nominal 10,000 mg/kg (Figure 4.3). Therefore, the fungal diversity was affected at low and high concentrations of SAB diesel fuel. By comparison, the total CFUs were not affected by the high concentrations of SAB diesel fuel but it is important to understand that

this was due to a few dominant species thriving in SAB contaminated soil samples at concentrations exceeding nominal 10,000 mg/kg (Figure 4.1). This Macquarie Island's fungal diversity and abundance data can be used to further develop ecotoxicological assessments and therefore assist in future remediation attempts on the island.

Although the majority of fungal isolates recovered in P2, P6 and P9 were not identified, a small portion were identified that were also prevalent in P4 (Figure 3.1 and Table 3.1). *Arthroderma sp.* 1 and *Pseudeurotium bakeri* were the most abundant and widely distributed species among spiked soil samples from each plot and were found to contribute to the dominant species richness in soils contaminated with high concentrations of SAB diesel fuel. These isolates are now major candidates for characterisation for their potential role in petroleum hydrocarbon degradation in the future.

Chapter 5 Discussion

In the last decade, novel bacterial cultivation methods based on simulating natural environments, the use of limited nutrients and/or extended incubation times have been developed (Kaeberlein *et al.*, 2002; Ferrari *et al.*, 2008). The application of these novel approaches has resulted in the recovery of slow growing and previously undescribed bacterial species from various environmental samples (Janssen *et al.*, 2002; Ferrari *et al.*, 2005; Rasmussen *et al.*, 2008). Here, a low nutrient fungal cultivation approach was developed and applied for the isolation of Sub-Antarctic Macquarie Island soil fungi alongside a traditional standard nutrient approach. Combined, these two cultivation approaches resulted in the recovery of 91 yeast and filamentous fungal species from one soil plot. The novel low nutrient approach recovered a significantly different ($P<0.01$) fungal population compared with traditional approach and therefore played a critical role in enhancing recovered fungal diversity. In particular, the two media types recovered different fungal communities when SAB concentrations at low and medium level but not for high level. It means that the different fuel concentrations have has different effects on the fungal communities. It also means that when assessing the effect of fuel contamination on the soil fungal communities, the choice of cultivation method is important. The diversity of fungal species recovered from Macquarie Island soil here highlights the fact that novel bacterial cultivation approaches should be widely adopted for fungi, including cultivation using various types of culture media and simulating natural environments.

In Antarctic regions, fungi have been isolated from various substrata, such as soil, water, animal and vegetation, and in each case specific endemic communities have been discovered (Ruisi *et al.*, 2007). This includes the keratinophilic fungal species *Chrysosporium sp.* and *Geomyces sp.* isolated from Antarctic bird feathers (Frate and Caretta, 1990), while novel lichenicolous fungi have also been isolated from Antarctic and sub-Antarctic regions (Hawksworth and Iturriaga, 2006; Sung *et al.*, 2008). Endophytic fungi associated with Antarctic plants have been found to exhibit significantly distinct fungal communities compared with temperate regions and most of the reported endophytic fungal isolates were previously undescribed fungi (Rosa *et al.*, 2009; Rosa *et al.*, 2010). However, little is known about Macquarie Island soil micro-fungi (Kerry, 1990), lichenicolous fungi (Hawksworth and Iturriaga, 2006), plant associated fungi (Laursen *et al.*, 1997), higher fungal species (Laursen *et al.*, 2002) or animal associated fungi. Due to the unique climate and geographical location, Macquarie Island hosts specific endemic flora and fauna, including at least 41 vascular plant species (Copson, 1984), 141 lichen species (Kantvilas and Seppelt, 1992) and thousands of animals (Erskine *et al.*, 1998). Therefore, in order to extend the spectrum of fungal diversity obtained from Macquarie Island, future investigations should not only focus on soil micro-fungi species diversity, but also on specimen collection and investigations from lichens, plants and animals.

Antarctic fungi have been investigated from many locations and have shown great diversity (Bridge and Newsham, 2009). For example, 10 fungal species were

recovered from Windmill Islands (McRae and Seppelt, 1999); 14 fungal isolates have been identified from Taylor Valley (Connell *et al.*, 2006); 19 fungal taxa have been identified at King George Island (Rosa *et al.*, 2010). The Ross Sea Region has been a frequently investigated site and exhibits high diversity (Farrell *et al.*, 2011). In 2006, Arenz *et al.* recovered 71 fungal taxa from soils and historic wood collected from Ross Island and Blanchette *et al.* (2010) isolated 69 fungal cultures from Shackleton's Historic Hut on Cape Royds. In this study, the four investigated soil plot with different nominal concentrations of SAB contamination have been represented the high value of Shannon diversity index from the lowest 0 to the highest 2.619 (Table 4.2), and most of the index values were higher than the fungal diversity index from the lowest 0 to the highest 2.02 at King George Island (Rosa *et al.*, 2010). These values were similar to the level of fungal Shannon diversity index in temperate zone which were based on the cultivation, such as the fungal diversity of grassland soils in Monmouthshire, England (Hunt *et al.*, 2004) and marsh sediment in Rhode Island, USA (Mohamed and Martiny, 2011). Compared these index values with the recent soil fungal diversity investigated by pyrosequencing, the results showed approximate half values with the high throughput method (Lim *et al.*, 2010). Therefore, it was not unexpected that high fungal diversity was recovered from one soil site in this study.

Fungal isolates recovered from soil samples based on cultivation methods only do not reveal the complete soil fungal community profile (Smit *et al.*, 1999). Therefore, a culture-independent method based on total soil DNA extraction and construction of

target gene clone libraries would complement our fungal diversity data (O'Brien *et al.*, 2005). Furthermore, the application of high-throughput sequencing targeting the ITS or 18S rDNA genes would produce a more comprehensive profile of fungal diversity. Recently Roche 454 GS FLX pyrosequencing has been reported to obtain 1 million sequence reads of 500 million base pairs of sequence information in a single run at relatively low cost (Voelkerding *et al.*, 2009). This strategy has been adopted for environmental fungal diversity investigations. In 2009, Buee *et al.* obtained 166,350 ITS sequences from six different forest soil samples which corresponded to approximately 1,000 OTUs. The investigations of plant associated fungal species richness and diversity on *Quercus sp.* using Roche 454 GS FLX pyrosequencing revealed extremely diverse communities (Jumpponen and Jones, 2009) and the communities were seasonal dynamics (Jumpponen *et al.*, 2010). This technology has not yet been adopted for Antarctic fungal diversity investigations.

Of the 91 fungal species recovered from one soil plot, high proportion of isolates (69% of total species) could not be identified to species level using standard molecular techniques targeting the ITS region (Table 3.1). Of these, 41 unidentified isolates exhibited low ITS sequence matches and distinct morphological characteristics to the closest cultured organisms, while the remaining 22 isolates yielded no amplification products. While estimates suggest that only 5% of the estimated 1.5 million estimated fungal species have been recovered into pure culture (Hawksworth, 2001), the available fungal ITS data is limited with only 1% of ITS

sequences currently deposited in public sequence databases (Nilsson *et al.*, 2010). Furthermore, like bacterial phylogenetics, most widely used fungal primer sets were designed using a limited diversity of sequences over twenty years ago (White *et al.*, 1990). Thus, it is not surprising that a high proportion of environmental fungi can not be successfully identified using these ITS primer sets (Martin and Rygiewicz, 2005; Begerow *et al.*, 2010). Alternative targets such as 18S rDNA, 28S rDNA genes and Mitochondrial cytochrome C oxidase I gene (COI) offer an alternative target but even greater limitations on available sequence data in public databases restricts this application (Guarro *et al.*, 1999; Crous *et al.*, 2008). Therefore, there is a requirement to design updated fungal specific ITS primer sets that target a greater range of fungal species.

The recovered fungal species from Sub-Antarctic Macquarie Island confirmed the presence of both indigenous and cosmopolitan fungal species found in the Antarctic continent (Ruisi *et al.*, 2007). These include the predominant *Ascomycota* phylum (57%), a small proportion of *Basidiomycota* phylum (16%) and *Mucoromycotina* Subphylum *incertae sedis* (1%). *Antarctomyces psychrotrophicus* has been repeatedly reported as an Antarctic indigenous fungal species isolated from various locations of Antarctica (Arenz *et al.*, 2006; Arenz *et al.*, 2010; Stchigel *et al.*, 2001). This study is the first report of the species being isolated from a Sub-Antarctic region. In addition, unidentified species isolated here are potentially indigenous fungal species and require further characterisation. By comparison, identified species, such as

Cladosporium cladosporioides, *Geomyces pannorum*, *Cryptococcus sp.* and *Penicillium sp.* are proposed to be cosmopolitan species that have also been isolated from Antarctica soils (Frate and Caretta, 1990; Vishniac, 1996; Arenz *et al.*, 2006; Connell *et al.*, 2006; Kostadinova *et al.*, 2009; Arenz *et al.*, 2010; Arenz and Blanchette, 2011) and temperate soils such as river Danube of Vienna in Austria and Manchester in United Kingdom (Wuczkowski *et al.*, 2003; Cosgrove *et al.*, 2007).

Chemical contaminants are known to effect terrestrial ecosystems due to the alteration of natural soil chemistry (Saterbak *et al.*, 1999; Fernandez et al., 2005; Oliveira and Pamulha, 2006). Petroleum hydrocarbon contamination of soil results in serious ecotoxicological effects on the wildlife in polar regions (Peterson *et al.*, 2003; Poland *et al.*, 2003), as well as the microbial ecology (Karl, 1992; Aislabie *et al.*, 2001; Hughes and Stallwood, 2006). In temperate regions, earthworm lethality assays, seed germination and plant growth assays are often used as indicators for ecotoxicological assessments of petroleum hydrocarbon contamination (Salanitro *et al.*, 1997; Saterbak *et al.*, 1999, Dorn and Salanitro, 2000). Soil microorganism respiration tests have also been developed recently and have been recommended as indicators for soil ecotoxicological assessments (Fernandez *et al.*, 2005; Plaza *et al.*, 2010). In polar regions, only limited soil toxicity information on petroleum hydrocarbon contamination has been reported which suggests that current temperate zone clean-up standard are too liberal for Sub-Antarctic island soil (Schafer *et al.* (2007). On Macquarie Island, the ecotoxicological effects of SAB diesel fuel has been determined

on soil microorganisms by targeting respiration rates including nitrification, denitrification, carbohydrate utilization and total soil respiration. Such assessments have been used to model EC20's (concentration that results in a 20% change from the control response) and subsequent EC20 estimates have varied between 16 to 950 mg/kg soil (Schafer *et al.* (2007). More recently, van Dorst (pers. comm., 2011) estimated an EC25 of 354.81 mg fuel/kg soil by targeting Macquarie Island's aerobic fraction of the bacterial community following the application of novel culturing method and there was no obvious effect of SAB diesel fuel contamination on the anaerobic fraction. In the future, the data obtained in this study should also be used to determine what concentration of SAB diesel fuel affected 25% of the natural fungal population (EC25). Together a combination of bacterial, fungal and worm EC25's can be used for the development of a comprehensive ecotoxicological assessment of Macquarie Island. This information can then be incorporated into the development of site-specific management guidelines and will be important in determining when bioremediation has been successfully completed on Macquarie Island.

The presence of potential hydrocarbon degrading bacterial communities on Macquarie Island has been investigated previously (van Dorst, 2009; Powell *et al.*, 2010). In 2009, van Dorst reported a shift toward hydrocarbon degrading bacterial species when SAB diesel fuel concentration exceeded 10,000 mg/kg in spiked Macquarie Island soils. For the bacterial population, the total CFU's decreased when SAB diesel fuel concentrations increased in soil samples. By comparison in this study, the fungal total

CFU's increased but consisted of reduced diversity at high concentrations SAB diesel fuel. An increase in fungal total CFUs has also been reported by Obire and Anyanwu (2009) and Nkwelang *et al.* (2008) and suggests that petroleum hydrocarbon contamination has an adverse impact on soil fungal diversity, enhancing the population toward hydrocarbons degraders (Obire and Anyanwu, 2009).

In this study, the preliminary investigation of the effect of SAB diesel fuel contamination on fungal diversity on Macquarie Island soils showed a statistically significant affect ($P<0.05$) on recovered fungal diversity combined with a significant affect ($P<0.001$) in colony abundance with increasing SAB diesel fuel concentrations. Therefore, SAB diesel fuel was a crucial factor affecting the natural diversity of soil fungi. This finding agrees with one previous report that showed a significant difference in total fungal CFU's and diversity ($P<0.05$) from soil spiked with 0%, 0.5%, 1%, 3% and 5% crude oil in a tropical region. In this case, total fungal CFUs increased and fungal diversity declined as crude oil concentrations increased (Obire and Anyanwu, 2009).

Of the total fungal isolates recovered from one soil plot, a couple of the identified species were also abundant in SAB diesel fuel contaminated soil samples across all 4 soil plots (Table 3.1). Of these *Exophiala sp.*, *Geomyces pannorum, Cladosporium cladosporioides* and *Penicillium sp.* have been reported to be tolerant to and potentially degraders of petroleum hydrocarbons (April *et al.*, 2000; Prenafeta-Boldu

et al., 2001; Hughes *et al.*, 2007). *Antarctomyces psychrotrophicus* is an indigenous Antarctic fungal species that has been isolated from the soils of King George, South Shetland (Stchigel *et al.*, 2001), and Ross Island (Arenz et al., 2006; Arenz *et al.*, 2010) as well as marine macroalgae associated fungi from King George Island (Loque *et al.*, 2010). This is the first known report of *Antarctomyces psychrotrophicus* isolated from petroleum hydrocarbon contaminated soils in an Antarctic region.

Arthroderma sp. 1 and *Pseudeurotium bakeri* were also highly prevalent and were the dominant species in all SAB diesel fuel contaminated soil samples. *Arthroderma sp.* is genus of keratinolytic fungi which has been reported to be involved in the degradation of petroleum hydrocarbons in soil (Ulfig *et al.*, 2003; Przystas *et al.*, 2007). *Pseudeurotium sp.* has been isolated from Ross Sea Region and McMurdo Dry Valleys of Antarctica (Arenz *et al.*, 2006; Arenz and Blanchette, 2011). One of the species members, *Pseudeurotium zonatum* has been shown to be capable of growth on volatile aromatic hydrocarbons as the sole carbon source (Prenafeta-Boldu *et al.*, 2001). In this study, *Pseudeurotium bakeri* increased in abundance by 100-fold as SAB diesel fuel concentration approached 1,000 and 20,000 mg/kg in plot 4, and increased 500-fold at 100 mg/kg concentration of SAB diesel fuel in plot 9. Therefore, the isolates of *Pseudeurotium bakeri*, as well as the novel species of *Arthroderma* sp recovered from Macquarie Island are two potential candidates for investigation of a potential role in petroleum hydrocarbon degradation.

The interesting strain CZ24 recovered from 10,000 mg/kg concentration of SAB diesel fuel spiked soil was also shown to tolerate high concentrations of petroleum hydrocarbons here. Phylogenetic results showed that the 18S rDNA sequence of CZ24 showed 96% similarity to *Pithoascus langeronii* which is a fungal species belonging to *Ascomycota* phylum, it also exhibited 94% similarity to a large number of other *Ascomycota sp.* However, typical teleomorph or anamorph morphological characteristics of *Ascomycota* were not observed for CZ24. Other morphological characteristics, such as *mycelium* could not assist in the identification and classification of this species into any fungal phylum. The ITS sequence of CZ24 revealed 89% similarity to *Acanthamoeba polyphaga* and 97% similarity to *Acanthamoeba castellanii* for the D1/D2 region of 28S rDNA. The relatively conserved 18S rDNA region data suggests CZ24 belongs to the Fungi Kingdom, but highly variable ITS and D1/D2 regions of 28S rDNA suggests the evolutionary position of CZ24 is close to *Acanthamoeba*, a genus belonging to Kingdom *Amoebozoa*. Molecular phylogeny analysis provided conflicting data for the classification of CZ24. CZ24 has now been observed to exhibit antibacterial activity with positive results for antimicrobial bioassays for a range of gram positive and negative bacterial species (data not shown). Therefore, sequencing the genome of CZ24 and analysis of its cell wall constituents is required for further phylogenetic analysis. In addition, the production of extracts for future analysis of CZ24 potential for production of antibacterial compounds is essential for determining any value towards anti-bacterial, cytotoxic, or chemical effects.

In this study, a high number of fungal species were isolated from Macquarie Island. These fungal isolates included several novel species and previously described hydrocarbon degrading fungal species. The isolates recovered from soils spiked with high levels of SAB diesel fuel require further characterisation to determine if they in fact play a role in hydrocarbon degradation and whether potential cold-adapted enzymes and their processes could be exploited in future bioremediation efforts. The fungal diversity data obtained here can be now used for the development of a comprehensive ecotoxicological assessment of Macquarie Island and to aid in establishing when site-specific contamination clean up attempts have been successful. This data is vital for the development of guidelines for management of diesel fuel contaminations in the future.

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