Who ate my phenanthrene?

An investigation of hydrocarbonoclastic organisms in pristine and contaminated soils

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Abstract

The microbial community dynamics of two soil types (sand and clay) were assessed using culture-independent techniques to investigate the difference in microbial dynamics of soils of different type and contamination histories. This was then used to determine if targets for remediation strategies can be predicted based on baseline community structure alone.

Two novel nucleic acid extraction techniques were developed for clayey and sandy soils and the profiling technique of Polymerase Chain Reaction (PCR) Temperature Gradient Gel Electrophoresis (TGGE) was optimised. A comparison between the laboratory standard profiling method, denaturing gradient gel electrophoresis (DGGE), and TGGE determined that although there was a difference in banding pattern of communities, there was no statistical difference in the results of the diversity, thus, strengthening the argument for use of TGGE over DGGE, especially due to the greatly reduced experimental run times and requirement of smaller sample volumes.

Baseline fungal communities of the two soil types were investigated using TGGE profiling of the internal transcribe regions (ITS) regions of the rRNA gene. In sandy soils, the most contaminated test pit had the highest fungal diversity. The fungal profiles were dominated by species from the class Eurotiomycetes and included the well-known hydrocarbon degrading species of *Aspergillus* and *Eurotiales*. Dominance of these species did not change depending on contamination level, suggesting a level of adaptability to multiple carbon sources. There appeared to be no correlation between fungal species diversity and contaminant level in the clayey soil type. Similar dominant fungal species were identified in the clay communities to those found in the sandy soils, all of which were part of the phylum *Ascomycota*. The clayey soils had a higher species diversity and range-weighted richness compared to sandy soils, which may be due to the *pore connectivity theory*. As a result of low water connectivity in soils the formation of diverse communities is promoted through creation of microhabitats.

The prediction of contaminant mineralisation was undertaken on the sandy soils using PCR amplification of 16S rRNA, ITS region profiling and 'prediction primers' (PAH-RHD$_\alpha$ GN-F and GN-R for Gram-negative bacteria and PAH-RHD$_\alpha$ GP-F and GP-R for Gram-positive bacteria). Stable isotope probing (SIP) was used to track the active degraders of ^{13}C-labelled phenanthrene. Baseline profiling indicated that there was very little difference in fungal diversity but a significant difference in bacterial diversity dependent on contamination history. The pristine soil had the highest fungal diversity at baseline, although the contaminated soil had the highest bacterial diversity. Identification of the dominant fungal and bacterial species highlighted the presence of organisms capable of degradation of various petroleum-based compounds and other anthropogenic compounds regardless of contamination history. Community response after the simulated contamination event (^{14}C-phenanthrene) showed that the microbial community in the deep pristine and shallow contaminated soils were the most able to adapt to the presence of phenanthrene. The similarity in the microbial community structure of the well adapted soils demonstrated that a highly adaptable fungal community in these soils enabled a rapid response to the introduction of a contaminant. Ten fungal and 15 bacterial species were identified as active degraders of phenanthrene. The fungal degraders were dominated by the phylum *Basidiomycota* and *Ascomyota* including the genus *Crypotococcus* and *Tremellales*. Bacterial degraders include the genus *Alcanivorax*, *Marinobacter* and *Enterococcus*.

There was little synergy between dominant baseline microbes, predicted degraders and those that were determined to be actually degrading the contaminant. It can be concluded from this work that a prediction of the bioremediation potential of a soil cannot be made based solely on baseline microbial diversity. Furthermore, the assessment of baseline communities tends to grossly underestimate the ability of a microbial community (by around 80–90%) or potentially identify inaccurate remediation targets. This work has demonstrated that there are many complex interactions that occur once a soil is exposed to a contaminant and that a simplistic investigation of the microbial

community is not sufficient to determine how a soil will respond to a contamination event.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Abbreviations

µg	Microgram
μg^{-1}	Per microgram
µl	Microlitre
μl^{-1}	Per microlitre
µm	Micrometer
%	Percent
±	Positive and negative standard deviation from the mean
≥	Greater than or equal to
°	Degree
°C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
A	Adenine
APS	Ammonium persulphate
AUD	Australian Dollar
B	C,G,T
bp	Base pairs
BSA	Bovine serum albumin
BTEX	Aromatic compounds: benzene, toluene, ethylbenzene and xylene
C	Cytosine
CaCl	Calcium chloride
CO_2	Carbon dioxide
CsCl	Cesium chloride
CTAB	Cetyl trimethylammonium bromide
d	Day
D	A,G,T
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
Fo	Functional organisation
g	Gram
G	Guanine
h	Hour
H	Hydrogen
H	A,C,T
H’	Shannon Weaver diversity Index
HCl	Hydrogen chloride
HMW-PAH	High molecular weight PAH

ITS	Internal transcribed spacer
J	Shannon Weaver Equitability Index
K	G,T
Kb	Kilobases
Kg	Kilogram
KH_2PO_4	Potassium di-hydrogen orthophosphate
L	Litre
l^{-1}	Per litre
Lcc	Laccase
LMW	Low molecular weight
LMW-PAH	Low molecular weight PAH
LnP	Lignin peroxidase
LOR	Limit of reporting
M	Meter
M	Molar
M	A,C
MBG	Molecular biology grade
MC	Moisture content
MgSO4	Magnesium sulphate
min	Minute/minutes
min^{-1}	Per minute
Ml	Millilitre
mM	Millimolar
MnP	Manganese-dependent peroxidase
MPN	Most probable number
msa	Multiple sequence alignment
N	Nitrogen
N	A,C,G,T
NB	Note
$Na_2HPO_4.7H_2O$	Di-sodium hydrogen orthophosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium di-hydrogen orthophosphate
NaOH	Sodium hydroxide
NEPM	National Environment Protection Measure
Ng	Nanogram
NH_4Cl	Ammonium chloride
O	Oxygen
OTU	Operational Taxonomic Unit
P	Phosphorus
P:C	Phenol : chloroform : isoamyl alcohol
PAH	Polycyclic aromatic hydrocarbon
PAH-RHD_α	PAH ring-hydroxylating dioxygenase alpha subunit
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PL	Parento-Lorenz evenness curve
qPCR	Quantitative PCR / real time PCR

R	A,G
rDNA	Ribosomal DNA
RHD	Ring-hydroxylating dioxygenase
RHD_{α}	Ring-hydroxylating dioxygenase α subunit
RNA	Ribonucleic acid
Rr	Range-weighted richness
rRNA	Ribosomal RNA
S	Second
S	G,C
s^{-1}	Per second
SDS	Sodium dodecyl sulfate
SIP	Stable isotope probing
sp.	Species
SPB	Sodium phosphate buffer
T	Thymine
TAE	Tris-acetic acid EDTA
TBE	Tris-boric acid EDTA
TE buffer	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
TGGE	Temperature gradient gel electrophoresis
TPH	Total petroleum hydrocarbon
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TTGE	Temporal temperature gradient electrophoresis
UK	United Kingdom
US	United States of America
V	Volts
V	A,C,G
W	A,T
WHC	Water holding capacity
X	Unknown amino acid
Y	C,T

1 Introduction

1.1 Crude Oil Pollution and Composition

Oil pollution through accidental release on land and in marine areas can cause highly adverse effects to humans, plant and animal life and the environment. Oil spills are mainly as a result of human activity (exploration and transport of oil, refining and storage, road run off, burning of fuels) but naturally occurring oil seeps can also be sources of crude oil in the environment (Chaîneau *et al.*, 2003; Khan *et al.*, 2004). Some of the most well-known oil spills have occurred in the marine environment but have also affected the land in the process. One of these spills was the *Exxon Valdez* disaster where a reported 37,000 tonnes of crude oil from the stricken ship washed ashore at Prince William Sound, Alaska in 1989 (The International Tanker Owners Pollution Federation Limited, 2014). This disaster was also the first high profile field demonstration of bioremediation (Fahy, 2003) and is discussed further in Section 1.2.3. A more recent spill was the *BP Deepwater Horizon* accident in 2010 where an estimated 492,000 tonnes of oil was release into the ocean after an explosion on an oil rig operating in the Gulf of Mexico (U.S. Coast Guard; U.S. Geological Survey, 2010).

Crude oil is a complex mixture of hydrocarbons, many of which are known to be carcinogenic, teratogenic, and mutagenic (Dou *et al.*, 2008; Liao *et al.*, 2014). It also contains small quantities of oxygen-, sulphur- and nitrogen-containing compounds and trace amounts of organometallic compounds (Gutnick and Rosenberg, 1977). Components of crude oil can be grouped into four classes according to their differing solubility in organic solvents and water. The classes are the saturates or aliphatics (n- and branched alkanes and cycloparaffins), the aromatics (mono-, di-, and polycyclic aromatic compounds; containing one or more benzene rings), the resins (aggregates with various building blocks such as pyridines, quinolines, sulfoxides and amides) and the least soluble of all fractions, the asphaltenes (aggregates of molecules with condensed aromatic and naphthenic rings connected by paraffin chains) (Sugiura *et al.*, 1997; Philp, 2005). Weathered oil, which is generally found as a contaminant in the

environment, is dominated by complex mixtures of the aliphatic and polycyclic aromatic hydrocarbons (PAHs) and thus are a major environmental concern (Frenzel *et al.*, 2009). Figure 1.1 shows examples of the chemical structures of crude oil fractions.

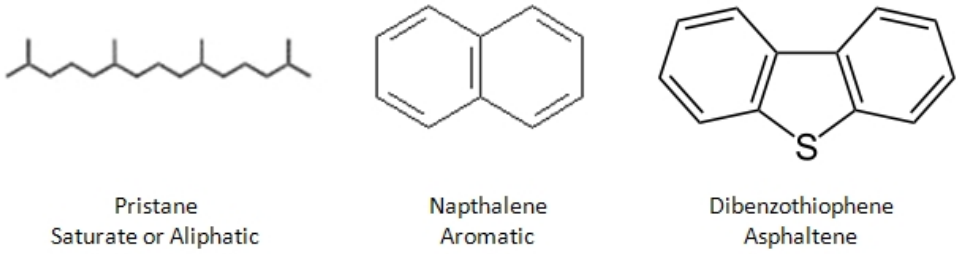

Figure 1.1 Representative chemical structures of aliphatic, aromatic and asphaltene fractions of crude oil. Adapted from Philp *et al*. (2005).

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic fused-ring aromatic compounds consisting of hydrocarbon molecules of two or more fused benzene or aromatic rings produced naturally and from anthropogenic sources (Cerniglia, 1992; Muckian *et al.*, 2007). PAHs exist as a complex mixture in many different petroleum based products such as tar and creosote and as such are widespread pollutants in the environment (Singleton *et al.*, 2006). Common areas of pollution are soils and waters surrounding gas plants, oil refineries, air bases, petrol stations and chemical manufacturing facilities (Juhasz *et al.*, 2005; Seo *et al.*, 2009). Of most concern are the higher molecular weight PAHs (HMW-PAHs) as they present a significant threat to human health due to their mutagenic and carcinogenic properties and thus 16 PAH compounds are recognised as priority pollutants by the United States Environmental Protection Agency (US EPA) and the European Union (EU) (Figure 1.2) (Juhasz *et al.*, 2005; Muckian *et al.*, 2007; Muckian *et al.*, 2009). PAHs are persistent pollutants in the environment due to their hydrophobicity, low water solubility and strong tendency to absorb to the soil matrix (Cerniglia, 1992; Chen and Aiken, 1999). All of these factors contribute to low PAH bioavailability and thus low biodegradation rate (see Section 1.3.2 for further details) (Wang *et al.*, 2009). The PAH that is the main focus of this project is phenanthrene, as it is one of the most abundant PAHs in the environment and has been used as a model compound to study both

bacterial and fungal biodegradation (Dai *et al.*, 2009; Kanaly and Harayama, 2010).

Phenanthrene is a three-ring PAH which is acutely toxic due to its ability to form an epoxide (Juhasz *et al.*, 2005). Phenanthrene is found in high concentrations in PAH-contaminated sediments, surface soils and waste sites (Moody *et al.*, 2001; Regonne *et al.*, 2013).

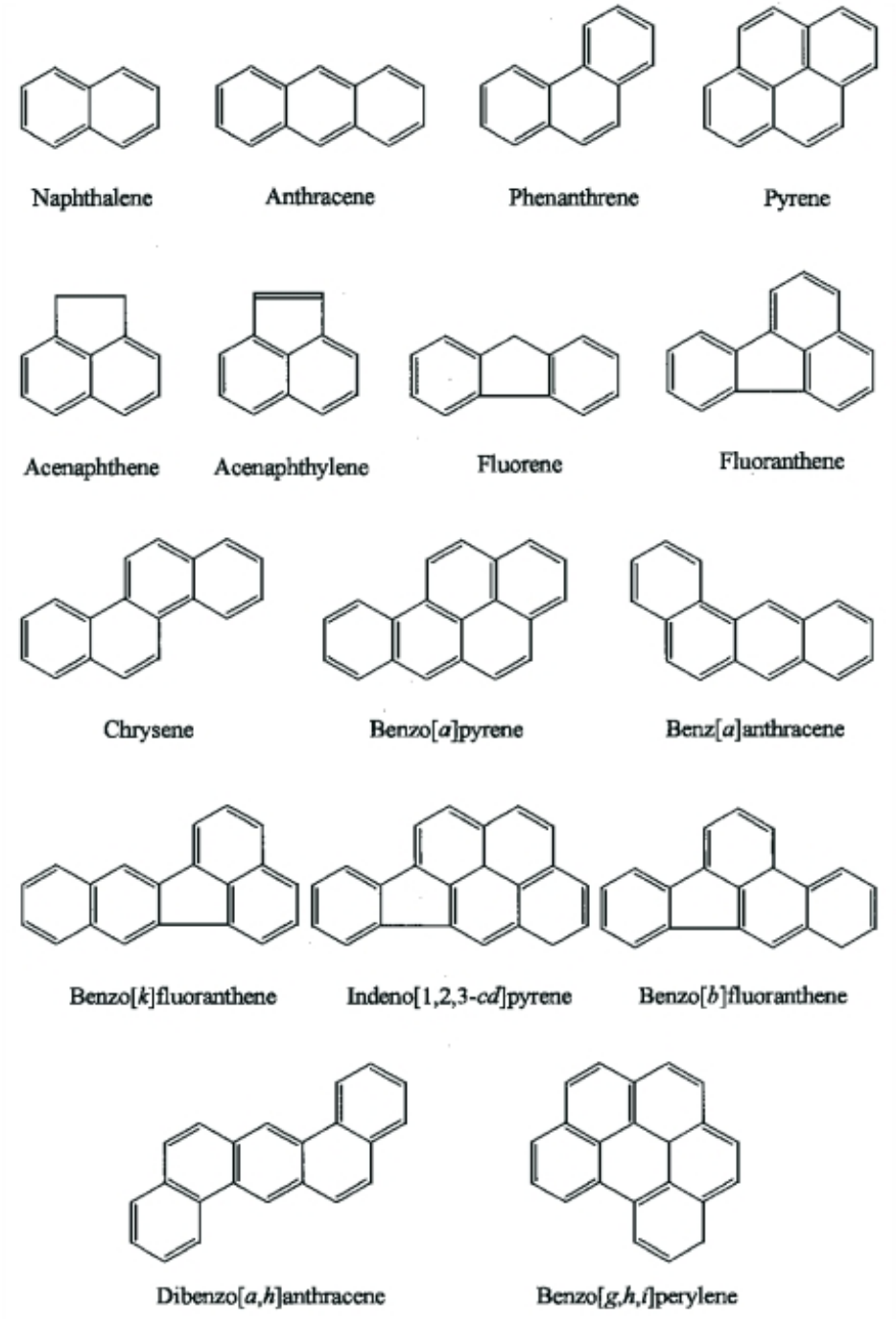

Figure 1.2 Chemical structures of the 16 Priority PAH Compounds identified by the US EPA. Taken from Habe & Omori (2003).

1.2 Remediation

As a result of the toxicity associated with oil pollution, there is a need to detoxify or remediate contaminated environments. Remediation methods are generally divided into two categories: *in-situ* remediation methods and *ex*-situ remediation methods (Reddy, 2008).

Traditionally treatment of polluted soils has involved physical methods where polluted soils were either excavated and disposed to landfill or isolated *in situ* by the use of various barriers to prevent movement of pollutants off-site or contact between humans and the pollutants.

The most common treatment is disposal to landfill; in South Australia an estimated 87,000 tonnes per annum of polluted soil is disposed of to landfill sites (SKM, 2013). This is becoming a non-viable option due to gradual changes in disposal regulations, which have resulted in increased fees and liabilities for landfill disposal. In some countries, the *in-situ* containment of contaminants is considered as waste disposal and therefore, subject to the same stringent regulations, permitting processes and liabilities (Doak, 2004; Scullion, 2006). Thus these practices are becoming less prevalent, increasing the demand to develop alternate, more sustainable techniques.

Remediation treatment approaches may be classified as physical, chemical and biological, all with varying degrees of success, mainly due to a dependence on the specific pollutant involved. In many cases a combination of all three approaches are used to provide the most effective treatment (Alexander, 1994; Scullion, 2006).

1.2.1 Physical Remediation Techniques

As mentioned before, physical remediation techniques are becoming less widely used, but when coupled with other types of remediation strategies they can result in the successful removal of pollutants (Scullion, 2006). Several of the physical treatment methods are listed below; not all treatments are dealt with in detail as this is beyond the scope of this project. Physical treatments include;

Vitrification or Thermal treatment: This involves applying high temperature to the contaminated substrate (i.e. soil) which results in the destruction of organic pollutants or creates a ceramic-like material which traps inorganic pollutants (Khan *et al.*, 2004). The end products are usually disposed of in landfill (Norris *et al.*, 1999; Pope *et al.*, 2000).

Vapour extraction/air sparging: This promotes the volatilisation of pollutants (i.e. BTEX) in the unsaturated (subsurface portion of the soil where macropores/ cavities contain both water and air) and saturated zones (subsurface portion where macropores are completely filled with water) (Gomez-Lahoz *et al.*, 1995). Air sparging also increases the dissolved oxygen available within the soil, which in turn stimulates aerobic biodegradation (Park *et al.*, 2005).

Soil washing/pump and treat: This involves extraction and treatment of polluted groundwater which is then recirculated to gradually desorb more pollutants from the soil matrix. This is an effective method for treating hydrophilic pollutants (i.e. aniline and phenols) as the water is able to remove hydrophilic pollutants from the soil matrix (Tiller, 1996; Mulligan *et al.*, 2001; Nathanail and Bardos, 2004). This method has limited effectiveness for pollutants with low aqueous solubility and a strong affinity for particular soil fraction (i.e. PAHs) (Scullion, 2006; Li *et al.*, 2014)

The physical treatments above can enhance the action of biological treatment by increasing the pollutants bioavailability. Physical remediation processes are most effective in coarse-textured soils and pollutants that are more soluble or volatile (Scullion, 2006).

1.2.2 Chemical Remediation Techniques

Chemical treatments are more widely applied to polluted groundwater, although some have been applied to soil. These treatments involve the application of chemicals to a medium to either destroy or convert pollutants into less toxic forms, to extract them or to immobilise them (Scullion, 2006). Chemical techniques are highly effective for dechlorination of polychlorinated biphenyls (PCBs) and halogenated alkanes, although their effectiveness is highly dependent

on soil organic matter level and pH (Wood, 2001). Chemical remediation techniques include;

Soil flushing: This involves the 'flooding' of contaminated soils with extraction solutions (i.e. acids, solvents, surfactants) that mobilise contaminants to an area where the contaminated water is extracted and collected for treatment thereby removing the contaminant mass from the soil (Khan *et al.*, 2004). This process is effective in removal of inorganic metals, volatile organic carbons, petroleum hydrocarbons and pesticide contaminants (Juhasz *et al.*, 2003; Khan *et al.*, 2004).

Solidification and stabilisation: These processes reduce the mobility of hazardous contaminants through physical and chemical means. Stabilisation generally involves the conversion of the contaminant into a less soluble, immobile, and less toxic form (Khan *et al.*, 2004). Solidification reduces contaminant mobility through encapsulation in a monolithic solid with high structural integrity (Suthersan, 1997). Solidification and stabilisation are most effective with heavy metal and other inorganic contaminants, although they have been shown to be effective in the treatment of low level organic contaminated soils (Khan *et al.*, 2004; Leonard and Stegemann, 2010; Kogbara *et al.*, 2012).

Chemical Oxidation: This involves the introduction of chemicals (i.e. hydrogen peroxide or Fenton's reagent, permanganates and ozone) which form hydroxyl radicals that act as strong oxidants to destroy organic contaminants (Kong *et al.*, 1998; Gan *et al.*, 2009). Chemical oxidants have been shown to be effective on a broad suite of contaminants ranging from inorganic compounds (such as cyanides) to chlorinated aliphatic compounds and complex aromatic compounds (Venkatadri and Peters, 1993).

1.2.3 Biological Remediation Techniques (Bioremediation)

The majority of techniques used in physical and chemical remediation techniques require high energy input and are considered to be non-cost effective, non-environmentally friendly or non-sustainable. Comparisons of remediation costs have shown that a physical treatment method such as on-site incineration can be between $227-$909 per cubic metre, whereas

bioremediation can be between $3–$341 per cubic metre (Philp, 2005) making bioremediation a more popular and affordable option.

Bioremediation is the use of living microbes to degrade environmental pollution or the application of a biological treatment to clean up hazardous chemicals through natural biological systems (Ball, 2006). During various bioremediation processes, organic molecules undergo transformations involving enzymes resulting in the complete conversion of an organic molecule to inorganic products (Alexander, 1994; Scullion, 2006). There are many organic contaminants that are amenable to bioremediation (Figure 1.3); however the effectiveness of bioremediation is dependent on the contaminant, its bioavailability and the environments microbial capacity (Juhasz and Naidu, 2000; Adetutu *et al.*, 2012). The major advantage of bioremediation is that it can be conducted *in situ*, which removes the cost and liability of transport and minimises site disruption. It also eliminates the need to find an area where the removed soil can be treated (Scullion, 2006).

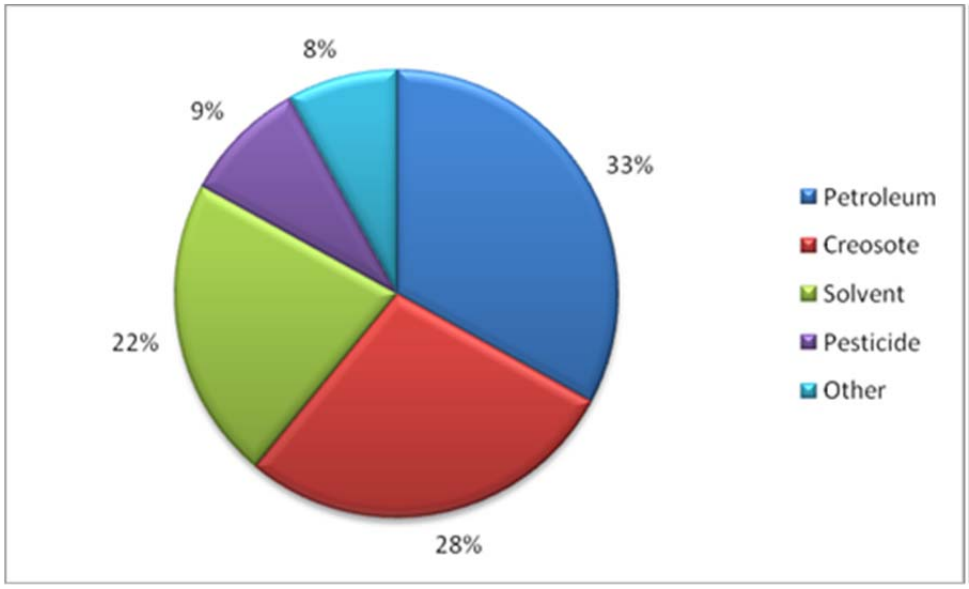

Figure 1.3 The major types of waste chemicals amenable to bioremediation. Adapted from Ball *et al*. (2006)

The specific bioremediation processes that are used depends on the contaminant type and characteristics of the environment studied. For example, hydrocarbon degrading organisms are present in most soils; they may be as low as 0.1% of soil microbiota in pristine ecosystems, whereas they can dominate oil

contaminated soil (Greenwood *et al.*, 2009). Bioremediation strategies can involve any of the following techniques, whether it is *in situ*, on-site, or in a bioreactor.

Natural Attenuation: is generally a 'hands-off' process, which allows the endogenous microbes to degrade the pollutant without any addition of exogenous macronutrients or microbes (Alexander, 1994).

Biostimulation: accelerates the rate of bioremediation by promotion of the growth conditions of the endogenous microbes by addition of exogenous macronutrients which are often limited in contaminated environments, namely nitrogen and phosphorus (Atlas, 1981; Scullion, 2006; Muckian *et al.*, 2009). Biostimulation often results in a more rapid onset of degradation, although some studies have found that degradation rates converge with time, with no marked improvement in overall treatment compared with natural attenuation (Margesin *et al.*, 2003; Sarkar *et al.*, 2005).

Bioaugmentation: is used if there is a lack of adapted microorganisms for pollutant degradation (i.e. hydrocarbon) or insufficient microbial capacity for degradation. The endogenous community is augmented by seeding hydrocarbon-degrading (hydrocarbonoclastic) microbes into the environment (often as well as nutrient addition), so that biodegradation is created and stimulated (Waly *et al.*, 1997; Scullion, 2006). However, the survival of the exogenous inoculums is often a limitation to this process (Wang *et al.*, 2009). The introduced microbes may not be adapted to thrive in the specific conditions by either incompatible conditions or competition from the endogenous community, thus resulting in slow or no bioremediation (Singer *et al.*, 2005; Silva *et al.*, 2009; Wang *et al.*, 2009). This can sometimes be overcome by isolation and culture of endogenous microbes with the capacity to degrade the contaminant with subsequent re-introduction at increased concentrations (El Fantroussi and Agathos, 2005).

Bioremediation ideally results in pollutants being permanently eliminated by conversion to harmless substances such as carbon dioxide, water and ethane

(Ball, 2006), which makes bioremediation environmentally safe and therefore is generally well accepted by the public (Swannell, 2003). These advantages all contribute towards bioremediation being a lower cost and low energy method for degrading organic contaminants in soil, groundwater and shorelines (Swannell, 2003).

1.3 PAH degradation

PAHs once exposed to the environment can be degraded via biotic and abiotic mechanisms, with the chief process for natural elimination of PAHs from contaminated environment being microbial degradation (Cerniglia, 1984). A wide variety of organisms are known to metabolize PAHs. Contaminated environments typically contain a wide variety of bacteria, fungi and algae capable of PAH degradation which all have different metabolic pathways and substrate ranges (Grossman *et al.*, 2000; Toledo *et al.*, 2006; Silva *et al.*, 2009; Regonne *et al.*, 2013)

1.3.1 Abiotic PAH degradation

There are several processes that can occur to reduce the concentration of PAHs in the environment that do not involve microbial degradation.

Transfer processes cause the relocation of PAHs without altering their structure via volatilisation, absorption, leaching or erosion (Kleineidam *et al.*, 2002). The tendency for loss of PAHs through these methods decreases as the molecular weight of the compound increases (Ahangar, 2010).

Chemical degradation alters the structure of the compounds to generally less toxic compounds, through naturally occurring chemical processes such as oxidation-reduction or photochemical exposure (Wick *et al.*, 2011).

Sequestration processes relocate PAHs into long-term storage without altering the structure, via adsorption or diffusion (Wick *et al.*, 2011). The effectiveness of these processes is highly dependent on the pore size distribution in soils (i.e. percentage and type of clay) and the amount and nature of organic matter present (Semple *et al.*, 2003; Ahangar, 2010). Sequestration can govern how

effective biotic PAH degradation is due to the bioavailability of PAHs to organisms capable of degradation (Kleineidam *et al.*, 2002).

1.3.2 Biotic PAH degradation

A wide variety of bacteria and fungi have been observed to be capable of PAH degradation (Table 1.1), using varying metabolic pathways and substrate ranges under both aerobic and anaerobic conditions. Historically the majority of degradation studies have been in aerobic environments as these reactions are the more favoured and often more rapid (Grossman *et al.*, 2000; Toledo *et al.*, 2006; Silva *et al.*, 2009). However, anaerobic conditions are often promoted when the degree of contamination is very high thereby limiting oxygen flow due to soil pore saturation or clogging (Gan *et al.*, 2009).

The rate of PAH degradation is indirectly proportional to the number of aromatic/ benzene rings present in the molecule (Cerniglia, 1992); thus LMW-PAHs are more readily biodegradable than higher weight compounds. For bacterial degradation, this is usually due their inability to incorporate the HMW-PAH into the cell due to its large size (Canet *et al.*, 2001). The degradation rate of HMW-PAHs is also controlled by desorption kinetics, which over time reduce due to hydrophobic PAHs being sequestered into the soil matrix (Scullion, 2006; Muckian *et al.*, 2009). Other factors that affect PAH biodegradation rates are temperature, pH, soil type, aeration, nutrients, depth, diffusion, microbial adaptations or capacity, bioavailability, previous chemical exposure, water availability, sediment toxicity, physico-chemical properties of the PAH, concentration of the PAH and seasonal factors (Cerniglia, 1992; Muckian *et al.*, 2009; Wick *et al.*, 2011). Biodegradation of PAHs is highly regio- and stereo-selective and the specific pathway involved is highly dependent on the molecular weight of the PAH and the type of microbes involved (Gibson and Subramanian, 1984; Cerniglia, 1992). The degradation pathway for aromatic compounds also depends on the whether fungi or bacteria are degrading the compound (Figure 1.4).

Table 1.1 Example of microbial genera associated with PAH degradation. Adapted from Cerniglia (1992), Juhasz & Naidu (2000) and Seo *et al.* (2009).

Compound degraded	Bacterial Genera	Fungal Genera
Naphthalene	*Acinetobacter, Alcaligenes, Marinobacter, Brevundimonas, Burkholderia, Cycloclasticus, Pseudomonas, Rhodococcus, Sphingomonas*	*Aspergillus, Candida, Cunninghamella, Gilbertella, Linderina, Panaeolus, Penicillium, Rhizophlyctis, Thannidium, Zygorhynchus*
Anthracene	*Alcaligenes, Beijernickia, Comamonas, Cycloclasticus, Janibacter, Mycobacterium, Rhodococcus, Sphingomonas*	*Bjerkandera, Cunninghamella, Cladosporium, Daedaela, Penicullium, Phanerochaete, Ramaria, Rhizopus, Trametes*
Phenanthrene	*Acidovorax, Acinetobacter, Arthrobacter, Burkholderia, Cycloclasticus, Flavobacterium, Micrococcus, Mycobacterium, Nocardioides, Pseudomonas, Streptomyces, Staphylococcus, Sphingomonas,*	*Aspergillus, Bjerkandera, Cunninghamella, Curvularia, Penicillium, Phanerochaete, Pleurotus, Syncephalastrum, Trametes*
Fluoranthene	*Acidovorax, Arthrobacter, Janibacter, Mycobacterium, Pseudomonas, Sphingomonas, Stenotrophomonas, Terrabacter*	*Aspergillus, Bjerkandera, Cryptococcus, Cunninghamella, Flamulina, Laetiporus, Penicillium, Pleurotus,*
Pyrene	*Acidovorax, Bacillus, Burkholderia, Mycobacterium, Pseudomonas, Rhodococcus, Xanthamonas*	*Agrocybe, Cunninghamella, Kuehneromyces, Penicillium, Phanerochaete, Syncephalastrum, Trammetes,*

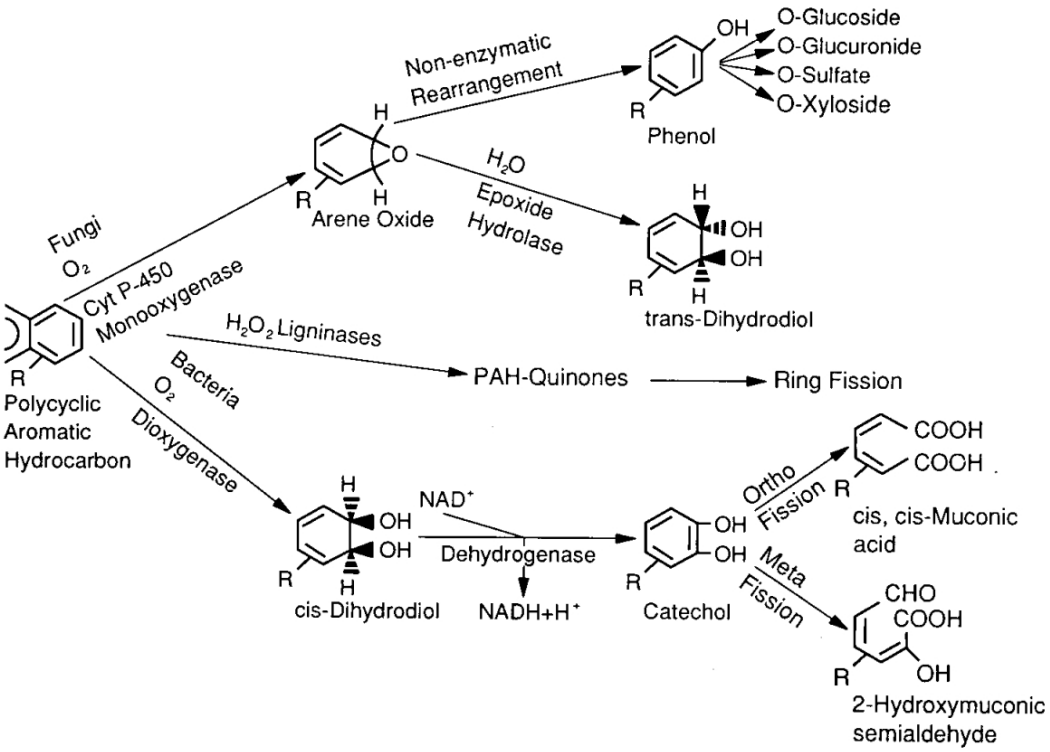

Figure 1.4 Pathways for the microbial degradation of polycyclic aromatic hydrocarbons. Taken from Cerniglia (1992).

1.3.2.1 Bacterial PAH degradation

Initial bacterial degradation involves the incorporation of molecular oxygen into the aromatic nucleus/ring. This reaction is catalysed by multicomponent dioxygenase enzymes (also known as ring-hydroxylating dioxygenase or RHD) to form *cis*-dihydrodiol (Albaiges *et al.*, 1983; Cerniglia, 1992; Saito *et al.*, 1999; Juhasz and Naidu, 2000; Singleton *et al.*, 2009). This initial ring oxidation is usually the rate limiting step of biodegradation of PAHs (Cerniglia, 1992). The enzyme *cis*-dihydriol dehydrogenase then re-aromatises the aromatic nucleus of the *cis*-dihydrodiol to form dihydroxylated intermediates (Cerniglia, 1984); further oxidisation of the intermediates leads to the formation of catechol (Gibson and Subramanian, 1984). The next step in bacterial metabolism is confirmation-dependent aromatic ring fission. If the hydroxyl groups of the dihydroxlated intermediate are in the *ortho*-position (the groups are in positions 1 and 2 of the aromatic ring) then oxygenolytic cleavage occurs between the two hydroxyl groups by intradiol (*ortho*) cleaving dioxygenase resulting in the formation of *cis,cis*-muconic acid (Juhasz and Naidu, 2000). If the hydroxyl

groups are in the *meta*-position (groups are on positions 1 and 3) the cleavage occurs adjacent to the hydroxyl groups catalysed by the enzyme extradiol (*meta*) cleaving dioxygenase forming 2-hydroxymuconic semi-aldehyde (see Figure 1.4) (Cerniglia, 1992).

This entire process is referred to as the upper catabolic pathway of PAH degradation (Cerniglia, 1992; Habe and Omori, 2003). Once the first aromatic ring of the PAH molecule is degraded, the second ring is attacked in the same manner and so on (Atlas and Bartha, 1981). Degradation via the upper degradation pathway (ring cleavage) results in the production of succinic, fumaric, pyruvic and acetic acids and aldehydes and the by-products of this reaction are carbon dioxide and water (Juhasz and Naidu, 2000; Kasai *et al.*, 2006). The cleavage products are utilised by microbes for the synthesis of cellular constituents and energy (Wilson and Jones, 1993; Habe and Omori, 2003).

Biodegradation of phenanthrene can occur through multiple pathways depending on which organism is attacking the compound. In general, phenanthrene is degraded to 1-hydroxy-2-naphthoate (compound I) through 5 intermediate compounds (compounds II to VI) (Figure 1.5).

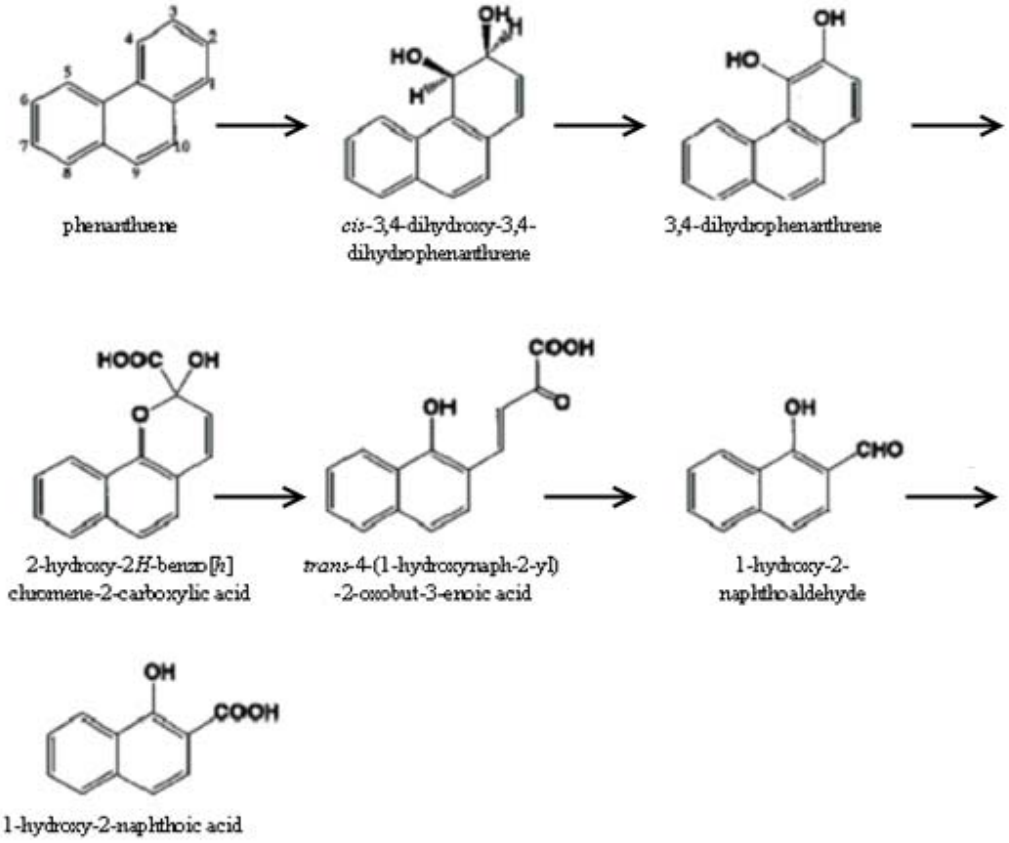

Figure 1.5 Initial bacterial degradation pathway of Phenanthrene. Adapted from Habe & Omori, (2003).

Further metabolism of 1-hydroxy-2-naphthoate involves either hydroxylation to form 1,2-dihydroxynaphthalene (compound A-II) which enters the naphthalene degradation pathway resulting in salicyclic acid (A-VI), or that aromatic ring is cleaved to form *trans*-2'-carboxy-benzalpyruvic acid (compound B-II) which undergoes further enzymatic degradation to from tricarboxylic cycle intermediates via metabolites phthalate (compound B-IV) and protocatechuic acid (compound B-V) (Habe and Omori, 2003) (Figure 1.6).

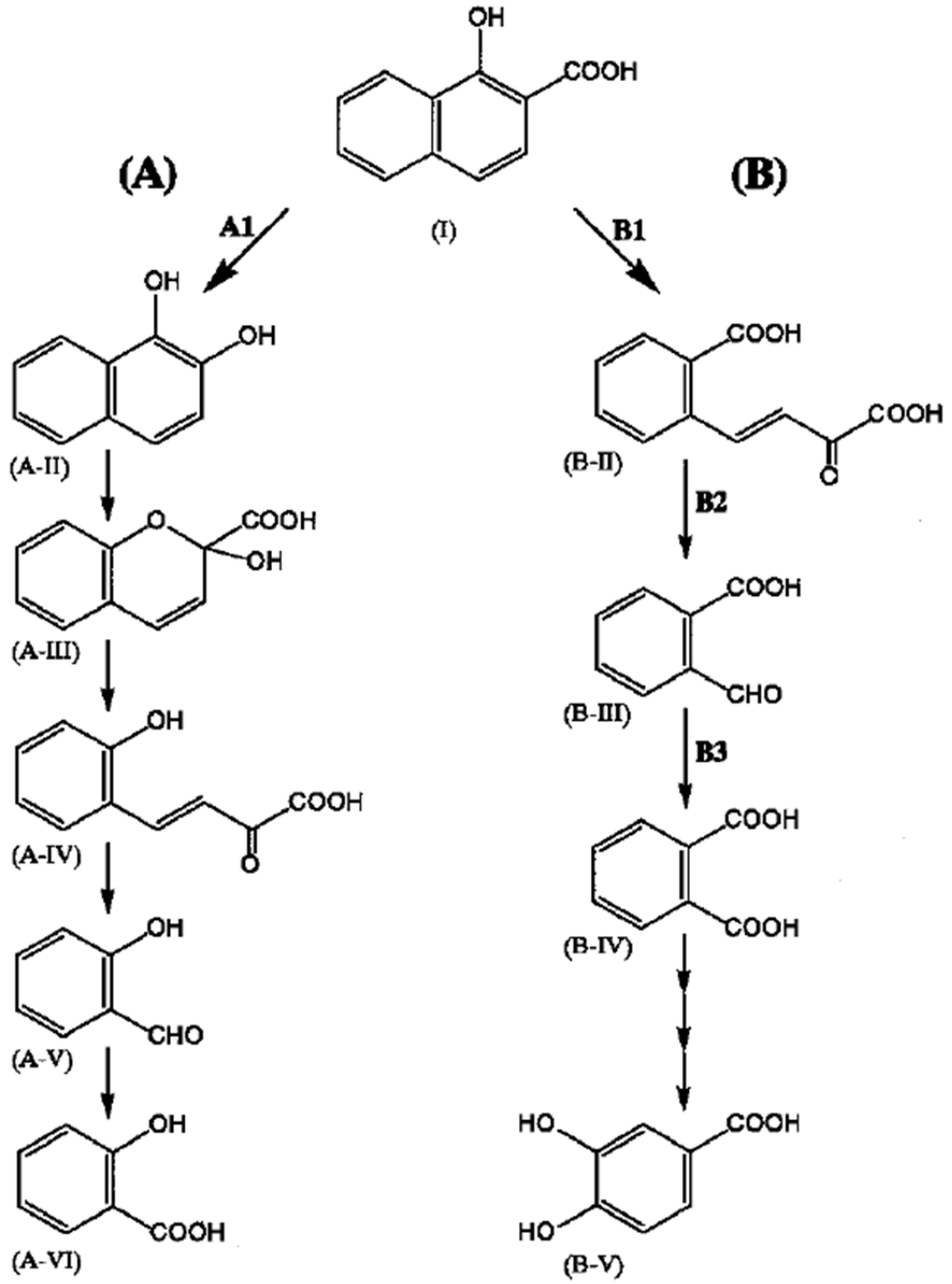

Figure 1.6 Catabolic Pathways of 1-Hydroxy-2-naphthoic acid in the Phenanthrene Degradation Pathway. Taken from Habe & Omori, (2003)

The catabolic enzymes involved in the degradation of various PAHs have been well studied and as mentioned previously, the first hydroxylation step is performed mainly by aerobic bacteria that contain the PAH-ring hydroxylating dioxygenase (PAH-RHD) system (Cébron *et al.*, 2009). Homologous PAH-RHD

enzymes are encoded by specific genes present in both Gram-positive (GP) and Gram-negative (GN) bacterial species, with the arrangement of these genes varying with the type of bacteria (Habe and Omori, 2003; Zhou *et al.*, 2006; Cébron *et al.*, 2009).

1.3.2.2 Fungal PAH degradation

Similarly to bacteria, the initial step of fungal PAH metabolism involves the introduction of atmospheric oxygen to the aromatic nucleus. Non-ligninolytic fungi tend to utilize cytochrome P-450 monooxygenase enzymes to incorporate oxygen, resulting in arene oxide intermediates (Figure 1.4). These intermediates can either undergo further metabolism by epoxide hydrolase to form *trans*-dihydrodiols, or undergo non-enzymatic rearrangement to form phenol which is then conjugated with sulphate, glucuronic acid, or glucose (Cerniglia, 1992). Ligninolytic fungi produce lignin peroxidases and manganese-dependent peroxidase that degrade lignin-related compounds and catalyse the oxidation of PAHs to quinines (Cerniglia, 1993; Dai *et al.*, 2009). The metabolites from fungal metabolism are generally less mutagenic than the parent compound but are not fully degraded; at this point bacteria continue the metabolism (Scullion, 2006).

Literature reviews suggest that fungal extracellular enzymes initiate the degradation of HMW-PAHs, removing the need to incorporate the pollutant into the cell, producing smaller metabolites which are then further metabolised by bacteria (Smit *et al.*, 1999; Canet *et al.*, 2001; Anderson and Cairney, 2004; Scullion, 2006; Silva *et al.*, 2009; Balaji *et al.*, 2014). The extracellular enzymes also catalyse the decomposition of plant residues, releasing nutrients into the soil that help sustain and stimulate microbial growth (Smit *et al.*, 1999). Decomposition also breaks down organic matter that pollutants have sorbed to, thus releasing the pollutants for microbial degradation (Wang *et al.*, 2009). Moreover, fungal hyphae have the ability to penetrate contaminated soil to reach pollutants, giving fungi a significant advantage over bacteria (Smit *et al.*, 1999; Pointing, 2001; Wang *et al.*, 2009). Even though most research points to the fact that fungi initiate metabolism of HMW-PAHs, it has been shown that Gram-positive bacteria dominate communities in older PAH-polluted sites

(Uyttebroek *et al.*, 2006; Cébron *et al.*, 2008). It has also been shown that Gram-positive bacteria are able to increase PAH bioavailability in aged contaminated soils due to biosurfactant and biofilm formation, which together enable these bacteria to undertake the majority of the initial PAH degradation (Bastiaens *et al.*, 2000; Johnsen and Karlson, 2004; Leys *et al.*, 2005). This all provides strength to the argument that when devising remediation strategies, especially those of older PAH-polluted sites, both fungal and bacterial community dynamics should be investigated and promoted.

1.4 Microbial Ecology: Methods for investigation / characterisation

It is well known that microbes with hydrocarbonoclastic abilities are ubiquitous within the soil environment; generally HMW-PAHs are degraded by fungi, while lower molecular mass compounds are predominately degraded by bacteria (Scullion, 2006), making the total microbial community of interest for study in terms of the bioremediation of PAHs. It is well recognised that less than 1% of the microbial diversity of soil can be cultured (Amann *et al.*, 1995; Torsvik and Øvreås, 2002); furthermore, the use of viable cell counting methods, such as plate counting techniques, is inappropriate for spore-producing organisms such as fungi and *Actinomycetes* (Smit *et al.*, 1999; Gallagher *et al.*, 2005; Muckian *et al.*, 2009). Culture-dependent techniques are also laborious, time consuming and most importantly, selective and biased for growth of specific microorganisms (Zengler *et al.*, 2002). The introduction of molecular microbial ecology, applied molecular methods such as polymerase chain reaction (PCR) based community profiling has, to some extent overcome these limitations (Rogers and McClure, 2003). Many of these techniques exploit the 16S rRNA gene in prokaryotes and the 18S rRNA gene in eukaryotes, which encode for the small subunit of the ribosome that is critical to the function of all organisms. Thus, these genes are very highly conserved between species (Thies, 2006). Detection, identification and assessment of the diversity of contaminant degrading microbial populations can be made by detection of these organism-specific/gene-specific gene

sequences which can also be quantified using real time PCR (qPCR) (Rogers and McClure, 2003).

Utilisation of molecular ecology has resulted in a tremendous increase in the knowledge of microbial community dynamics and the existence of formerly unknown microbes and thus culture-independent descriptions of microbial communities now dominate the literature in all areas of microbial ecology (Van Hamme *et al.*, 2003; Muckian *et al.*, 2009). Advances in a procedure called stable isotope probing (SIP) has even further improved the profiling and elucidation of the active portion of the soil microbial community (Manefield *et al.*, 2002a; Gallagher *et al.*, 2005; Manefield *et al.*, 2007; Neufeld *et al.*, 2007c; Huang *et al.*, 2009; Chen and Murrell, 2014) and will be a focus of this project, discussed further in Section 1.4.2.

Comparison of soil microbial communities and monitoring of response changes can also be achieved through analysis of phenotypical and physiochemical (functional) profiles. A common phenotypical analysis is that of phospholipid fatty acids (PFLAs). Phospholipids are essential membrane components of all living cells and have great structural diversity, coupled with high biological specificity (Zelles 1999). PFLA analysis exploits the fact that certain groups of microorganisms have different “signature” fatty acids (Tunlid & White, 1992). The PFLAs patterns for communities are determined through PFLA extraction which are then identified and quantified by chromatographic retention time and mass spectral comparison (Grayston *et al.*, 2004). It has been shown that rapid changes in microbial community structure can be detected by changes in PLFA patterns (Garland 1997; Zelles 1999). PLFAs can also be utilised as a biomarker for SIP (see Section 1.4.2). The functional diversity of communities can also be assessed through their patterns of substrates utilisation. The process involved incubation of soil suspensions in microtitre plates (commercially available as Biolog™) containing sole carbon sources and a salt which changes colour as the substrate is metabolised (Degens & Harris, 1997; Kirk *et al*., 2004). The communities’ response can then be assessed for the overall rate of colour development, richness and evenness of response (or diversity) and the pattern,

or relative rate of utilisation to enable a community level physiological profile (Garland, 1997).

Another considerable advance in the investigation of soil microbial communities, but outside the scope of this project, has come with the advent of next-generation sequencing platforms and associated bioinformatics tools which have enabled the use of high-throughput sequencing for rapid, cultivation-independent and relatively low-cost investigations of the metagenome (the study of the collective microbial genomes) of a community (Torsvik and Øvreås, 2002; Simon and Daniel, 2009; Xu *et al.*, 2014). Metagenomics has allowed the assessment and exploitation of the taxonomic and metabolic diversity of varying microbial communities on an ecosystem level (Simon and Daniel, 2009). One of the largest metagenomics dataset to date, comprised of 7.7 million sequences and 6.3 billion base pairs, was generated during the *Global Ocean Sampling* expedition (Simon and Daniel, 2009).

The development of metagenomics has also permitted the identification of the most frequently represented functional genes and metabolic pathways that are relevant in a given ecosystem and has allowed for comparison of systems (comparative metagenomics) (Tringe *et al.*, 2005; Simon and Daniel, 2009).

The improvement in next generation sequencing has also seen a boom in other 'omics' technologies including metatranscriptomics, metaproteomics and metabolomics. The formation of metagenomic complementary DNA (cDNA) libraries from messenger RNA (metatranscriptomics) has allowed identification of the expressed biological signatures in complex ecosystems (Simon and Daniel, 2011), although this is still rare due to the difficulties in processing of environmental RNA samples (Zhou and Thompson, 2002). Metaproteomics aims at assessing the immediate catalytic potential of a microbial community (Simon and Daniel, 2011) although this technique is challenged by uneven species distribution, broad ranging protein expression levels within microorganisms and the large genetic heterogeneity within microbial communities (Mocali and Benedetti, 2010; Schneider and Riedel, 2010). Metabolomics is the application of techniques to analyse the interactions of organisms with their environment, such

as identifying the stress from abiotic (such as xenobiotic exposure or temperature) and biotic stressors (such as competition) (Lankadurai *et al.*, 2013). Increasingly, researchers are finding that a combination of all 'omics' technologies is necessary to gain a comprehensive understanding of the complex microbial communities (Simon and Daniel, 2009; Bell *et al.*, 2014).

1.4.1 Phylogenetic investigation using Polymerase Chain Reaction

1.4.1.1 Investigation of Bacterial Diversity

Bacteria with the ability to degrade contaminants such as hydrocarbons and PAHs are ubiquitous in the environment (Atlas, 1981), thus understanding the diversity of the bacterial community is imperative. The ability to profile members of the bacterial community is accomplished through the targeting of the ribosomal DNA. In prokaryotes, the ribosome is comprised of three molecules of varying sizes; 5S, 16S and 23S. The 16S rRNA gene encodes for the small subunit of the ribosome, which is critical to the function of all organisms and therefore is highly conserved between species allowing the use of universal probes or primers to target these conserved regions (Thies, 2006). These conserved regions are flanked by semi-conserved and hypervariable regions, as demonstrated in the secondary structure of *Escherichia coli* 16S rRNA (Figure 1.7). There are nine hypervariable regions (V1 to V9) within the 16S rRNA are common targets for phylogenetic investigation (Chakravorty *et al.*, 2007). A commonly used and successful universal primer pair is the 'Muyzer' primers, developed by Muyzer and colleagues (Muyzer *et al.*, 1993) which target the V3 variable region. These primers have been used universally in bacterial diversity studies since their development and, because of this, they have been adopted for use in this study.

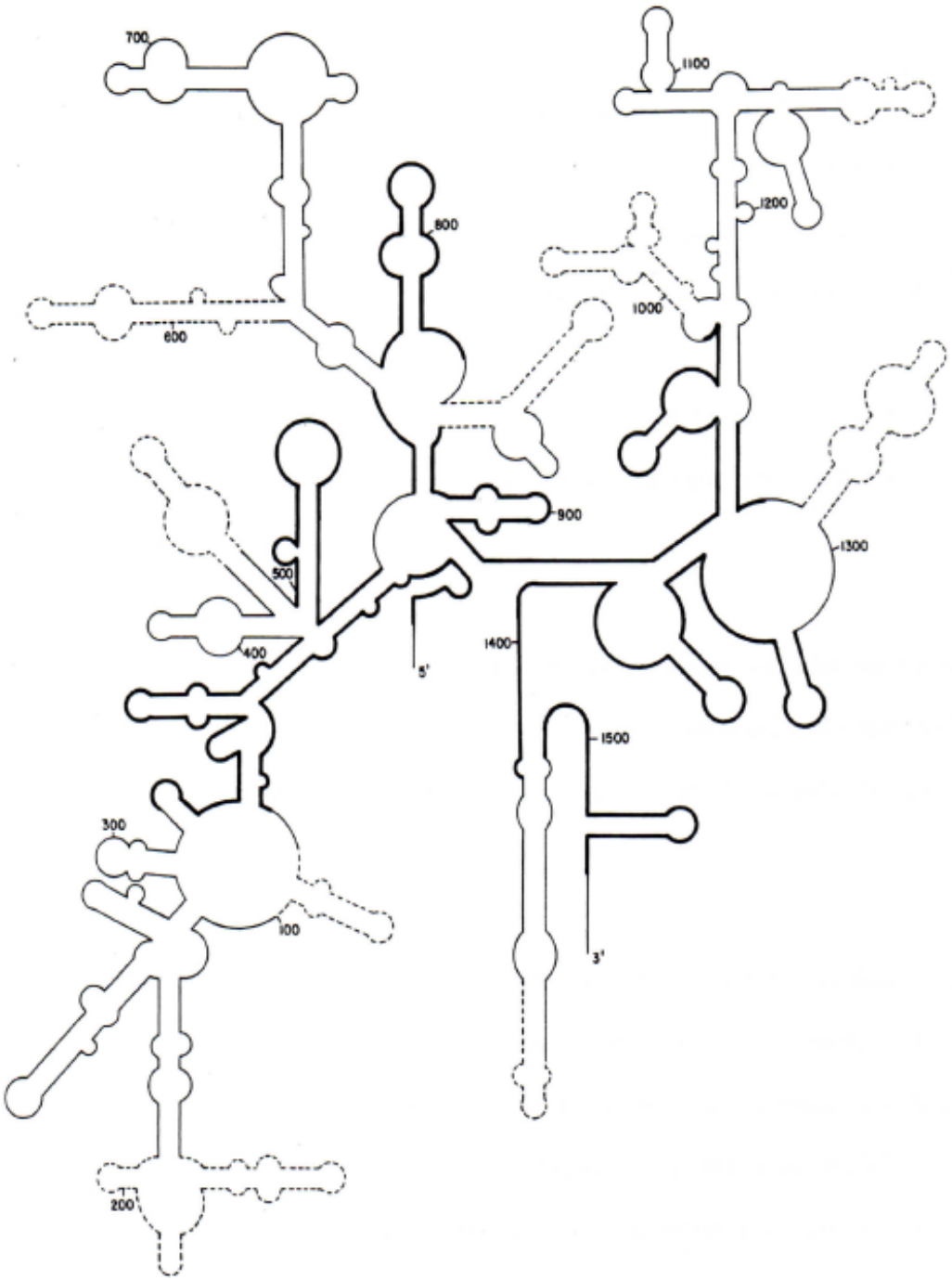

Figure 1.7 Schematic map of the secondary structure of *Escherichia coli* 16S rRNA. The nearly universal regions are shown in bold lines, the semi-conserved regions in normal lines and the hyper-variable regions in dashed lines. (Gutell *et al.,* 1985*,* cited in Fahy, 2003).

1.4.1.2 Investigation of Fungal Diversity

Understanding of the diversity and activity of the soil fungal community remains poor relative to that of soil bacterial communities and it is not uncommon for articles that purport to review aspects of 'soil microbial ecology' to consider only

bacteria (Anderson and Cairney, 2004). Yet it is known that fungi can play an important role in the degradation of PAHs, especially those of high molecular weight (Wang *et al.*, 2009). As discussed previously (Section 1.3.2), fungi possess many attributes over bacteria that make them important targets for remediation strategies and thus it is important to investigate their community during contamination events.

Similar to bacterial diversity investigations, the ribosomal DNA genes are the targets for fungal community investigations. Fungal rDNA genes are a multiple-copy gene family comprised of highly similar sequences (typically 8-12kb each) arranged as tandem repeats; each repeat is comprised of the coding regions for the primary rDNAs and one or more non-coding spacer regions (intergenic spacer regions) (Anderson and Parkin, 2007). The most common targets for microbial ecology studies within these gene clusters are the genes encoding for 18S rRNA and 25/28S rRNA and the internal transcribed spacer (ITS1 and ITS 2) region that incorporates the 5.8S rRNA gene (Figure 1.8). The variant evolution rate of the nucleotide sequences of the rDNA genes provides a means for analysing phylogenetic relationships over a broad range of taxonomic levels (White *et al.*, 1990; Anderson *et al.*, 2003a). The slowest evolving sequence is the nuclear small-subunit rDNA (18S rDNA) resulting in a lack of variation between closely related species, thus providing a useful target for studying distantly related organisms whereas the mitochondrial rDNA genes evolve more rapidly and are useful for investigations at the ordinal or family level (Smit *et al.*, 1999; Anderson and Cairney, 2004). A further limitation of using the 18S rDNA gene can occur when using certain PCR primers which detect other 18S rDNA sequences especially in environmental soil samples, thus interfering with the community profile produced with molecular ecology techniques (Smit *et al.*, 1999). The ITS region, located between the 18S rDNA and 28S rDNA genes and incorporating the 5.8S rDNA gene have a far faster rate of evolution and provides a greater sequence variation between closely related species (White *et al.*, 1990; Anderson and Cairney, 2004). Thus the ITS region is a popular choice for species level identification of fungal taxa in environmental DNA pools and

therefore will be the main target for the fungal community profiling of this project.

When selecting primers for fungal diversity investigation primers are required that will cover a broad range of taxa without losing specificity or introducing amplification bias towards a particular taxonomic group (Smit *et al.*, 1999; Anderson *et al.*, 2003b). To do this nested PCR is often employed, where the first round select for a broad range of taxa by targeting the 18S rDNA sequence, which can also detect plant or eukaryotic DNA in certain circumstances (White *et al.*, 1990; Gardes and Bruns, 1993). This is circumvented by the second round of PCR by selecting for a primer that targets the ITS region (Anderson and Cairney, 2004). Primer pairs that target 18S rDNA, such as EF4/EF3 and EF4/fung 5 (Smit *et al.*, 1999) have been shown to produce a bias towards amplification of *Basidiomycota* and *Zygomycota*. However Anderson and colleagues (2003b) reported that by using the same primer pairs in conjunction with further sets of ITS target primers (EF4 (Smit *et al.*, 1999)/ITS4 (White *et al.*, 1990) and ITS1F (Gardes and Bruns, 1993)/ITS2 (White *et al.*, 1990)) resulted in a relatively equal proportion of sequences representing the four main fungal phyla (i.e. *Chytridiomycota*, *Zygomycota*, *Ascomycota* & *Basidiomycota*); see Figure 1.8 for primer binding sites on the rDNA gene.

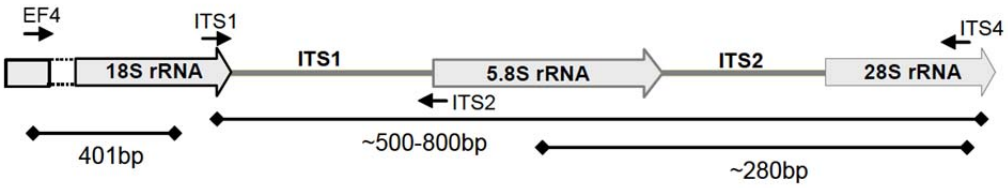

Figure 1.8 Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations. Adapted from White *et al*. (1990) and Embong *et al.* (2008)

Using the ITS region also has possible limitations which need to be factored in when interpreting results. The ITS region can persist in the DNA pool of a sample even after the contributing species have become metabolically inactive or functionally less important (Ostle *et al.*, 2003). Targeting the rRNA molecules extracted from environmental samples can overcome this limitation (Anderson and Parkin, 2007). Some reports however suggest that RNA analysis of fungal communities is difficult since the main precursor rRNA molecule (containing 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 25/28S rRNA) is transcribed by RNA polymerase I as a single molecule which then undergoes post-transcription processing in which the ITS regions are cleaved (Hibbett, 1992; Anderson and Parkin, 2007; Manter and Vivanco, 2007). Yet it has recently been shown that it is possible to capture the ITS region prior to this cleavage event since metabolically active fungi are constantly transcribing precursor rRNA molecules and 80% of all RNA transcribed in growing cells at any point in time is precursor rRNA (Paule and Lofquist, 1996; Anderson and Parkin, 2007). The ITS region will be the target for all fungal community diversity experiments conducted during this project.

1.4.1.3 PAH degradation functional gene in bacteria

A key target for analysis of functional genes involved in PAH degradation are the genes that code for the unique ring-hydroxylating dioxygenase (RHD) or initial dioxygenase (Margesin *et al.*, 2003; Hickey *et al.*, 2012). This exploits the fact that no matter what organism is degrading phenanthrene the first step is always incorporation of molecular oxygen into the aromatic nucleus (Cerniglia, 1992), see Section 1.3 for detail about PAH degradation pathways. Investigation of the functional genes that encode PAH-RHD provides a valuable tool to assess the dominant organisms in the environment as well as the relationship between specific microbial populations and the performance of the degradation processes or potential degradation capacity (Watanabe and Hamamura, 2003; Zhou *et al.*, 2006; Singleton *et al.*, 2009; Ding *et al.*, 2010).

The RHD is a multicomponent enzyme containing four subunits (Figure 1.9); α subunit (large), β subunit (small), ferredoxine, and ferredoxin reductase, which occur in different orientations dependent on the organism (Cerniglia, 1992; Habe

and Omori, 2003; Singleton *et al.*, 2009). The gene encoding the RHD α subunit (generally termed *phn*Ac) has two highly conserved regions thought to be critical in substrate recognition, which makes them good targets for PCR (Habe and Omori; Bordenave *et al.*, 2008; Kumar and Khanna, 2009). The conserved regions within the α subunit are the Rieske centre, incorporating a conserved protein sequence (–Cys-X-X-His-X-) of the [2Fe-2S] binding site and the ring hydroxylating ring domain, primarily a conserved Asn-Trp-Lys motif preceding the iron binding site (Habe and Omori 2003; Bordenave *et al.*, 2008; Cébron *et al.*, 2008; Ding *et al.*, 2010).

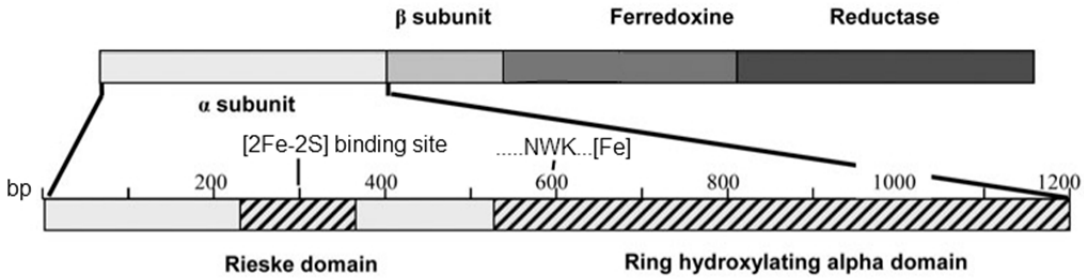

Figure 1.9 Organisation of the PAH-RHD operon, using *Pseudomonas putida* NCBI 9816-4 as an example organism. Adapted from Zhou *et al*. (2006) and Bordenave *et al*. (2008).

Early investigations into the initial ring-hydroxylating dioxygenases (RHD_α) genes in bacteria identified two main phylogenetic group clustering, the *nid*A-like and *nah*Ac-like dioxygenase genes, Figure 1.10 shows the phylogenetic neighbour-joining tree of RHD_α genes (Habe and Omori, 2003; Cébron *et al.*, 2008). Each group encompasses dioxygenase components derived from Gram-negative and Gram-positive bacteria, respectively (Bordenave *et al.*, 2008). Several studies (Saito *et al.*, 1999; Moser and Stahl, 2001; Gomes *et al.*, 2007; Bordenave *et al.*, 2008; Cébron *et al.*, 2008; Singleton *et al.*, 2009) have developed degenerate primers that target these groups, most requiring separate primer sets for each bacterial group or nested PCR to detect both which is not suitable for procedures such as real-time PCR. Zhou and colleagues (2006) devised primers (Nid-for/rev & Nah-for/rev) that were able to detect a broad range of bacteria, but it was later reported that these primer sets were unable to detect organisms from key genera such as *Acidovorax*, *Sphingomonas* and *Nocardioides* (Cébron *et al.*,

2008; Singleton *et al.*, 2009). Recently, Ding *et al*. (2010) developed degenerate primers that targeted the PAH-RHD$_\alpha$ subunit of both Gram-positive and Gram-negative species in one PCR reaction (PAH-RHD$_\alpha$-396F & PAH-RHD$_\alpha$-696R); the primers were tested *in silico* against 281 putative PAH-RHD$_\alpha$ genes and on total community DNA extracted from contaminated soil samples. The efficiency of this primer set was increased by the inclusion of a 5 cycle pre-amplification step for both normal and qPCR. The PAH-RHD$_\alpha$ primer sets developed by Ding *et al*. (2010) will be used for all PAH specific bacterial community experiments conducted during this project.

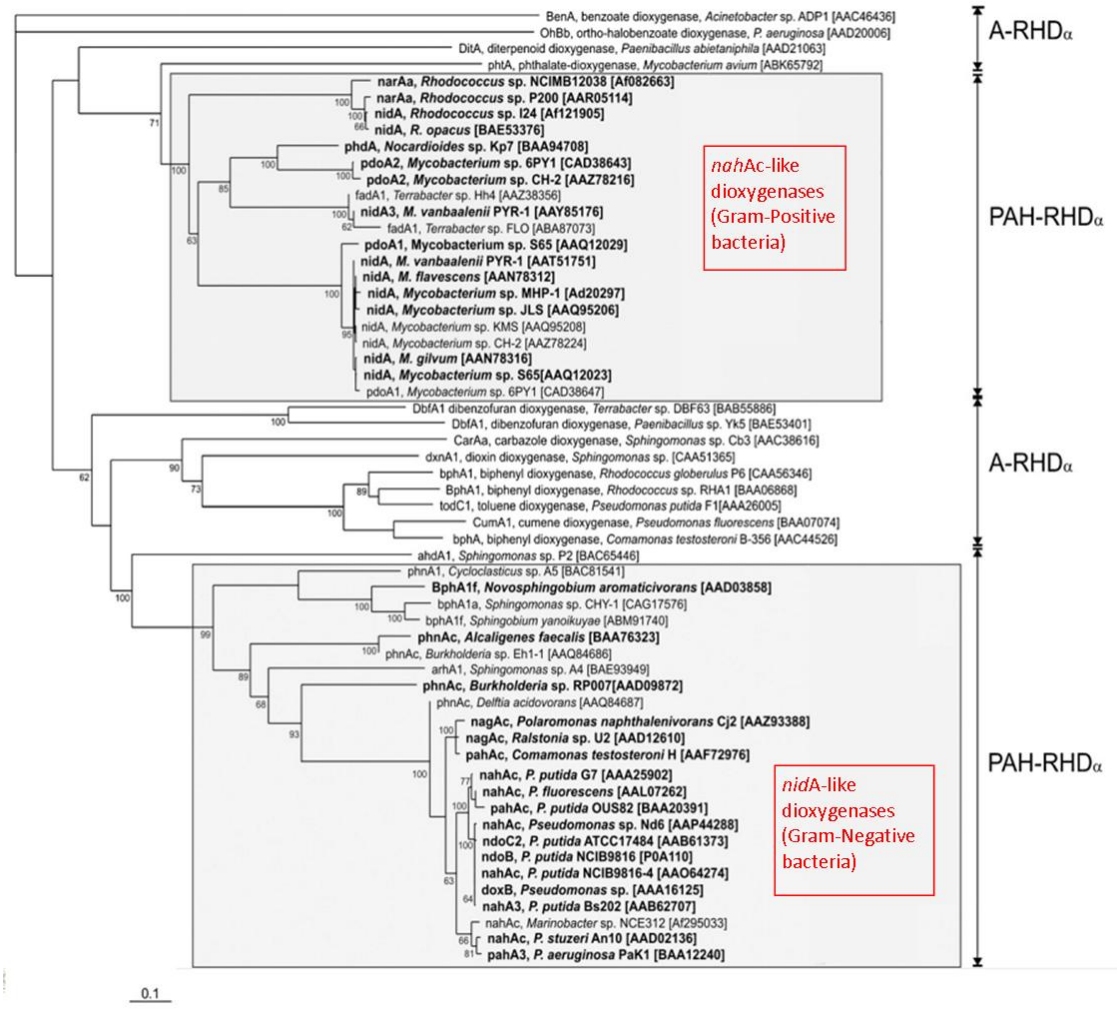

Figure 1.10 Phylogenetic neighbour-joining tree of ring-hydroxylating dioxygenase alpha subunit amino acid sequences. Taken from Cebron *et al*. (2008)

1.4.1.4 PAH degradation genes in fungi

As discussed in Section 1.3, fungal degradation of PAHs occurs through the production of extracellular enzymes. Common enzymes that are associated with fungal degradation include lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (*lcc*) (Cerniglia, 1993; Cerniglia, 1997; Han *et al.*, 2004; Wang *et al.*, 2009), making the genes that encode these enzyme key targets for analysis of a soil microbial community's functional capacity for PAH degradation. Pointing *et al.* (2005) successfully utilised three primer pairs that targeted the catalytic and conserved domains for each gene and these primers will be utilised in this project.

1.4.2 Stable Isotope Probing (SIP)

Stable Isotope Probing (SIP) offers a great potential for wide application in microbial ecology, by offering a cultivation independent means of investigating the effect of changes in environmental conditions on the microbiota (Radajewski *et al.*, 2000). Traditional isolation methods enable identification of organisms capable of degrading pollutants, yet little information is gained on the overall relevance of the cultivated species to the degradation of pollutants in the systems from which they were isolated, especially in complex systems such as soil (Aitken *et al.*, 2004). SIP is based on the premise that physiologically active organisms will incorporate carbon and nitrogen from stable isotopically labelled substrates into its biomarkers when the labelled substrate is supplied as the sole energy source (Neufeld *et al.*, 2007c). These 'heavy' (labelled) biomarkers can then be separated from 'light' (unlabelled) biomarkers by high speed centrifugation (Figure 1.11). This basic principle is not new; Meselson and Stahl (1958) showed that DNA labelled with ^{15}N could be separated via density-gradient centrifugation from DNA containing ^{14}N. However the use of SIP to label biomarkers, such as polar lipid derived fatty acids, DNA and RNA, enabling the identification of the metabolically active microorganisms has only recently been introduced.

Boschker *et al*. (1998) were the first to publish a stable isotope probing experiment, which identified organisms able to oxidise methane in fresh water sediment using ^{13}C-labelled methane. The target or labelled biomarker, polar lipid derived fatty acids (PLFAs) was extracted, separated and analysed via isotope ratio mass spectrometry. Specific phylogenetic groups produced unique PFLA profiles which were used to reveal the dominant organisms within the sediment. A major limitation of the PFLA-based SIP (PFLA-SIP) technique is that the level of resolution is relatively low, because identification relies on comparing environmental signatures to those of cultured relatives (Manefield *et al.*, 2004).

The recent combination of SIP with molecular biology methods, such as PCR-based analyses of 16S rDNA genes, has shifted the biomarker interest to DNA and RNA, resulting in high resolution solutions to phylogenetic investigations (Manefield *et al.*, 2004; Dumont and Murrell, 2005). Radajewski and colleagues (2000) were the first to prove that ^{13}C-labelled DNA could be isolated from a mixed microbial community. They demonstrated that the 'heavy' DNA, isolated from an oak forest soil pulsed with labelled methanol ($^{13}CH_3OH$), was separated from natural (unlabelled) DNA due to a difference in buoyant density by density centrifugation in a caesium chloride gradient. The heavy DNA was then used to construct a clone library which was subsequently identified via sequencing. Successful application of SIP requires a high degree of isotopic enrichment to enable adequate separation due to buoyant density (Manefield *et al.*, 2004). For DNA-SIP this generally results in increased pulse length to ensure enough time for populations to undergo at least two cell divisions (Lueders *et al.*, 2004b). This can lead to a process called cross-feeding which can confound results. Another limitation of DNA-SIP is that the species identified under this process do not directly represent the metabolically active members of the community, which can also contribute to slow or minimal incorporation of the labelled substrate (Manefield *et al.*, 2002b). In contrast the synthesis of RNA can be rapid; Whitby *et al*. (2005) demonstrated that over an 8 hour period labelled carbon accumulated in RNA almost 10-fold more rapidly than DNA and labelling can

occur without the need for DNA synthesis and replication, hence RNA-SIP is more indicative of active populations (Gallagher *et al.*, 2005; Madsen, 2006). This is why in recent years a shift towards RNA-SIP has occurred. Manefield *et al.* (2002a) were the ground breakers in using RNA as a targeted biomarker. They successfully identified bacteria that dominated the degradation of phenol in an industrial wastewater treatment plant. The methodology was analogous to DNA-SIP in that 'heavy' RNA was separated via density centrifugation on caesium trifluoroacetate gradients; reverse transcription PCR and sequencing was then undertaken to provide phylogenetic information (Manefield *et al.*, 2004). After biomarkers have been subjected to SIP, they are suitable for many downstream applications such as PCR, community profiling (denaturing/temperature gradient gel electrophoresis), phylogeny, microarrays, cloning and metagenomics.

Both DNA- and RNA-SIP have been applied to investigate a myriad of compounds in various environments, such as benzene in contaminated groundwater (Kasai *et al.*, 2006), benzoate in coastal sediments (Gallagher *et al.*, 2005), phenol from an aerobic industrial bioreactor (Manefield *et al.*, 2002a) and naphthalene from PAH-contaminated soil (Manefield *et al.*, 2007). The majority of publications have used carbon ($^{13}C/^{12}C$), oxygen ($^{18}O/^{16}O$), and nitrogen ($^{29}N/^{28}N$) as probes since they can leave signals in organic material (plant or animal) and inorganic constituents (carbon dioxide, water, soils, sediments, fossils and rocks) (Hopkins *et al.*, 1998; Neufeld *et al.*, 2007a).

As mentioned before, there is a trade-off between the length of time and concentration of substrate pulse necessary to achieve sufficient ^{13}C-labeling of biomarkers without confounding results due to cross-feeding of the label. For this reason Cebron *et al*. (2007) recommend selecting DNA as the biomarker since recovery of pure RNA from soil is difficult; yet there has been a recent shift towards RNA-SIP (Kasai *et al.*, 2006; Lueders *et al.*, 2006; Madsen, 2006; Whiteley *et al.*, 2006; Manefield *et al.*, 2007; Qiu *et al.*, 2008) due to its' rapid turnover rate within the cell. Therefore pulse times and substrate concentrations representative of environmental concentrations are an important consideration when designing SIP experiments (DeRito *et al.*, 2005). Another important step

during SIP is to limit the effect cross-feeding has on results. It is also important to fractionate gradients after centrifugation and analyse each fraction as opposed to the common practice of using a needle to remove 'heavy' bands when gradients are stained with ethidium bromide (see Figure 1.11) (Manefield *et al.*, 2007).

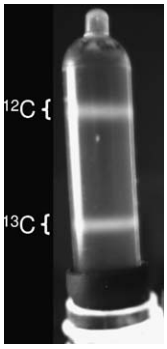

Figure 1.11 Example of the caesium chloride gradient formed after high speed centrifugation. Stained with ethidium bromide, showing ^{12}C unlabelled DNA and ^{13}C-labelled DNA forming two distinct bands. Taken from Neufeld *et al.* (2007a).

Cross-feeding can cause issues when trying to identify organisms that are responsible for the primary metabolism of compounds; however it can also gain insight into population interactions with fungi and predatory food webs in ecosystems by tracing the flow of the isotopic label through trophic levels (Lueders *et al.*, 2004b) since heterotrophs or predators grown in the presence of the labelled substrate (i.e. ^{13}C-labelled CO_2) will have biomass derived from mixtures of labelled and unlabelled carbon (DeRito *et al.*, 2005; Cébron *et al.*, 2007). However, detection of the label becomes more difficult with each step through the food web, but tracing trophic levels can be enhanced by coupling SIP with stable isotope mass spectrometry (DeRito *et al.*, 2005).

Thus, the use of RNA- and DNA-SIP solely or in combination provides a robust culture-independent method to elucidate the identity of microorganisms (in a short time frame) and link this to their function in degradation of a contaminant, which is a limitation of other popular molecular techniques such as fluorescent *in-situ* hybridisation (Gray and Head, 2001; Lueders *et al.*, 2004a; Lueders *et al.*, 2004b Dumont, 2005 #56; Chen and Murrell, 2014).

Recently, research has begun to link the process of SIP with next generation sequencing and metagenomics, enabling an effective alternative to large-scale whole-community metagenomic studies by specifically targeting the organisms or biochemical transformations of interest, thereby reducing the sequencing effort and time-consuming bioinformatic analyses of large datasets (Uhlik *et al.*, 2013; Mazard and Schafer, 2014). However, at the commencement of this project such techniques were novel and therefore cost prohibitive; furthermore the low-throughput of SIP and requirement for isolation of sufficient DNA for metagenomic library construction and shot-gun sequencing are major limitations with current techniques (Uhlik *et al.*, 2013; Chen and Murrell, 2014; Mazard and Schafer, 2014).

1.4.3 DGGE and TGGE

A popular method for separation and identification of species detected from environmental samples is denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). These methods allow the separation of same sized DNA fragments based on sequence. DNA fragments are separated by electrophoresis in polyacrylamide gels containing a gradient of denaturing substances. In DGGE a chemical gradient is created through the use of urea and formamide, whereas TGGE creates a temperature gradient. The use of sequence separation was adapted to microbial ecology by Muyzer *et al.* (1993) using the V3 variable region of the 16S rRNA with a GC clamp to prevent total denaturation. Since then, countless studies have utilised DGGE and to a lesser extent TGGE, to profile various communities including the Archaea and Eukaryotes as well as role-specific communities such as sulphur-reducers or nitrogen fixing species.

A significant benefit of DGGE and TGGE is that the gels can be scanned to analyse the pattern of bands for further comparative analysis (Ogino *et al.*, 2001). Furthermore, electrophoresed fragments can be directly excised from the gel, amplified and sequenced thereby by-passing cloning, making identification much quicker.

1.5 Project Aims

A considerable amount of past literature has dealt with how culturable bacteria respond to anthropogenic agents (Cerniglia, 1992; Margesin and Schinner, 1999; Juck *et al.*, 2000; Margesin *et al.*, 2003; Sarkar *et al.*, 2005), yet little is known about the long term effects these compounds have on soil microbial communities, nor how these communities are influenced by different soil types or histories and type of pollution (Muckian *et al.*, 2007; Ding *et al.*, 2010). Similarly, PAH degradation has been the subject of many studies in recent decades, although the majority of the work has been culture-dependent, which struggles to elucidate the complex interactions of PAH degrading microorganisms in soil. This can be attributed in part to the fact that these environments typically contain a variety of different PAH degrading organisms with different metabolic pathways and substrate ranges. Investigation of multiple organisms is imperative to help drive remediation practices (Grossman *et al.*, 2000; Toledo *et al.*, 2006; Silva *et al.*, 2009; Uhlik *et al.*, 2013). Therefore, investigation into the long term effects of anthropogenic agents, such as the model 3 ring polycyclic aromatic hydrocarbon phenanthrene, using powerful non-culture dependent techniques (i.e. SIP and TGGE) will provide insight into the community dynamics and help guide development of better remediation strategies (Bell *et al.*, 2013).

It has been suggested that microbial community structure in polluted environments are influenced by the complexity of chemical mixtures present, time or exposure and it is thought this generally leads to a reduction in microbial diversity (MacNaughton *et al.*, 1999; Bordenave *et al.*, 2008). Many bioremediation strategies rely on stimulation of endogenous microbial communities for pollutant reduction, working on the premise that endogenous microbes that have become adapted through a history of exposure to hydrocarbons would be better able to survive and respond to further contamination events compared to microbes with no such pre-exposure or adaption (Cerniglia, 1992; Peters *et al.*, 2005; Muckian *et al.*, 2007; Lamberts *et al.*, 2008; Liang *et al.*, 2012). This forms the key aim of this project, which is to

investigate the effects of a contamination event on soils with different contamination histories. Molecular microbiology techniques, including PCR and TGGE will be employed in this project to test the hypothesis *that pristine environments will be able to better adapt to contamination events due to an increased microbial diversity*. This investigation can therefore be broken into several smaller aims as follows:

- Development of robust and reliable molecular extraction and comparison techniques (TGGE vs DGGE) that can be applied to different soil types (Chapter 3);
- Determination of community dynamics in soil of differing types and contamination histories which may result in differing contamination responses (Chapter 4) to gain a baseline understanding of communities;
- Identification of 'key player' microbes involved in the degradation of a model PAH (Chapter 5); and
- Using the information gained in the above steps to determine if a prediction can be made about a soils' ability to respond to a contamination event based on its baseline microbial community to test the main project hypothesis (Chapter 5).

2 Materials & Methods

2.1 Soil Sampling, Storage and Analysis

2.1.1 Soil Sampling and Storage

All soil samples were collected from an oil refinery on the coast of South Australia, by Coffey International in October 2007 and June 2008 (see Figure 4.1 and Figure 4.2 for locations of the test pits). Samples were obtained using a backhoe and were collected using sterile equipment and gloves, in accordance with the Australian Standard for best practice sampling of potentially contaminated soils (Commonwealth of Australia, 2005).

Each soil sample (approximately 200 g) was transferred to a sterile collection bag, sealed air-tight and stored on ice at the site. Once transferred to the laboratory, samples were stored at -20°C. Smaller sub-samples (5 g) were collected and stored at -80°C.

2.1.2 pH Determination

The pH of each soil sample was determined by the using 1:5 soil to water ratio, according to the Soil Survey Standard Test Method (Department of Sustainable Natural Resources, 2008a). Five grams of soil was placed into a beaker and air dried overnight. Twenty-five millilitres of deionised water was then added and mixed well. The pH of each sample was then measured using a pH/Ion 510 meter (Eutech Instruments Inc.).

2.1.3 Soil Moisture Content (%)

The moisture content (MC) of each soil sample was determined by the loss in mass of samples after drying in an oven (the gravimetric water content), according to the Soil Survey Standard Test Method (Department of Sustainable Natural Resources, 2008b). Three grams of soil was added to clean pre-weighed (to 2 decimal places) crucibles and placed in a 60°C oven until a constant weight had been achieved, in approximately 72 hours. Constant weight was achieved when the difference in weight of a sample, brought to room temperature in a

desiccator, after two successive periods did not exceed 0.1% of its original sample weight. Duplicates of each sampled were analysed.

The MC of the soil could then be determined as a percentage of the total dry soil weight using the following formula:

$$MC\ (\%) = \frac{W_2\ -\ W_3}{W_3\ -\ W_1}$$

where W_1 is the weight of crucible (g),

W_2 is the weight of moist soil + crucible (g)

W_3 is the weight of dry soil + crucible (g)

2.1.4 Water Holding Capacity

The water holding capacity of a soil was determined using a volumetric method in triplicate, according to the Soil Survey Standard Test Method (Department of Sustainable Natural Resources, 2008b). Fifty grams of moist soil was placed into funnels with filter paper (Whatman #1) and clipped tubing attached to the end of the funnel with a volumetric flask placed underneath the funnel. A control was run with no soil in order to test the volume of water retained by the filter paper. Fifty millilitres of deionised water (dH_2O) was poured into the funnel and allowed to saturate the soil for 30 mins, after which the clip was removed from the tubing and the water was allowed to collect in the bottom flask for a further 30 mins. The final volume of water was measure and the water holding capacity was calculated using the following formulas:

$$A\ = 50 - \left(V_{filter\ paper}\ +\ V_{water\ eluted}\right)$$

where $V_{\text{filter paper}}$ is volume of water retained by filter paper (blank tests),

$V_{\text{water eluted}}$ is volume of water eluted from funnel.

$$WHC\ (ml.100g^{-1}\ fresh\ soil) = 2A + MC\ (\%)$$

where MC is the moisture content of the soil tested.

(Department of Sustainable Natural Resources, 2008b)

2.1.5 Soil Classification and Contaminant Indicators of Samples

All soil samples were classified according to the Australian Standard and the Unified Soil Classification System (U.S. Army Corps of Engineers and U.S. Bureau of Reclamation, 1952; Commonwealth of Australia, 1993). The standard recommends describing the soil as "XY", where the prefix "X" is the major soil ground and the suffix "Y" is the descriptor. The major soil groups, descriptors and symbols are summarised in Table 2.1.

Table 2.1 Unified Soil Classification System

Major Soil Group	Descriptor
Gravel (G) Sand (S)	Well graded (W) Poorly graded (P) Silty (M) Clayey (C)
Silt (M) Clay (C) Organic (O)	Low plasticity (L) Medium plasticity (I) High plasticity (H)

Potential visual and olfactory indicators of petroleum hydrocarbon contamination was also noted for test soils, the presence of an indicator was based on odour or appearance as recommended in the National Environment Protection Measure (NEPM) guidelines (National Environmental Protection Council, 1999).

2.2 Culture of Microorganisms

2.2.1 Media

2.2.1.1 Minimal (M9) Media

M9, a mineral medium (Sambrook and Russell, 2001), was prepared for enrichment cultures with 1 x M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% carbon source in sterile dH_2O. M9 Salts stock comprised of 64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl in 1000 ml dH_2O. The standard medium was

supplemented with filter sterilised benzene (80 $mg.l^{-1}$) or phenanthrene (0.75 $mg.l^{-1}$) as the sole carbon source. Agar was added at 15 $g.l^{-1}$ for plates.

2.2.1.2 Tryptone Soya Broth (TSB)

According to manufacturer's instructions, 30 g of TSB was added to deionised water to a final volume of 1000 ml. The mixture was aliquoted and autoclaved.

2.2.1.3 Luria Broth (LB)

Ten grams of tryptone, 5 g NaCl and 5 g yeast extract was added to deionised water to a final volume of 1000 ml. The pH was adjusted to 7.0 using NaOH or HCl (1.0 M, as appropriate) before the final volume was reached. The mixture was aliquoted and autoclaved

2.2.1.4 Bushnell Hass (BH) Broth

Bushnell Hass Broth (3.27 g) was added to deionised water to a final volume of 1000 ml. The mixture was then boiled to dissolve the medium completely. The mixture was aliquoted and autoclaved

2.2.2 Microcosm Standardisation

Replicate microcosms/cultures were supplemented with the same concentration of phenanthrene using a mastermix. Twenty millilitres of mastermix solution was prepared in 20 ml serum bottles, thus leaving a minimum headspace. BH media and ^{12}C-phenanthrene (dissolved in hexane) (total volume 20 ml) were added to bottles to obtain a concentration of 200 $mg.l^{-1}$. The bottles were sealed with PTFE-lined butyl septa. The mastermix solutions were allowed to equilibrate overnight at 25°C, and shaken intermittently to aid dissolution.

Spiking of microcosm/cultures was carried out by adding 1 ml per 10 ml of media to already sealed sterile serum bottles, to obtain a final phenanthrene concentration of 20 $mg.l^{-1}$.

2.2.3 PAH Positive Bacterial Strains

Known PAH degrading strains were obtained from research partners at the University of South Australia, and maintained at -80°C in 1 ml 20% glycerol in nutrient broth. Strains were streaked onto nutrient agar plates, incubated at

25°C and monitored for single colony growth. After incubation, single colonies were transferred into LB medium and incubated overnight. The cultures were then centrifuged for 10 min at 4°C at 12,000 x *g* (Heraeus™ Centrifuge) and the supernatant removed. The pelleted cells were washed with 1 ml of sodium phosphate buffer (SPB; 93.2 ml 1.0 M Na_2HPO_4; 6.8 ml 1.0 M NaH_2PO_4; pH 8.0) and resuspended in BH broth.

Five hundred microlitres of the resuspend cells was added to prepared BH media supplemented with 20 $mg.l^{-1}$ of phenanthrene (utilising a phenanthrene mastermix-see Section 2.1.4) and incubated at 25°C overnight. Aliquots of the overnight culture were then subjected to nucleic acid extraction, refer to Section 2.6.1.

2.3 Most Probable Number (MPN)

Microbial enumeration of culturable bacteria present in soil samples was determined using the Most Probable Number (MPN) method. Initially, 1.0 g of soil was added to 9 ml of TSB media and mixed. One millilitre of the suspension was added to 9 ml of media, the process was repeated until a 1 million fold dilution was achieved. The dilutions were incubated for 24-48 hours at 21°C and samples were scored positive or negative for growth.

2.4 ^{14}C Mineralisation Microcosm Experiments

^{14}C-phenanthrene mineralisation experiments were prepared to examine the capacity of soil microorganisms to mineralise PAHs (i.e. converted to $^{14}CO_2$). Experiments were prepared in duplicate 30 ml sealable vessels with a GC vial attached to the inner wall, with a final volume of 5 g of soil (Adetutu *et al.*, 2012). Initially, 1.5 g of soil was added to the mesocosm together with 1 ml phenanthrene/methanol mixture (0.75 $mg.\ ml^{-1}$). The methanol was then left to evaporate off and the remaining quantity of soil was mixed in. One millilitre of 30 mM sodium azide was added to control mesocosms to inhibit microbial growth.

Soil water holding capacity (WHC) was then adjusted to 40-60% water holding capacity (see Section 2.1.4) using 800–1000 µl of minimal salts media (no carbon

source) to achieve a C:N:P molar ratio of 100:10:1 (Dandie *et al.*, 2010). One millilitre of 0.1 M NaOH was added to the GC vial and approximately 1 µC of ^{14}C-phenanthrene added to the vessel and the lid sealed. Vessels were incubated in the dark at 25°C for 25 days and destructive sampling of the NaOH trap was undertaken on days 1, 2, 3, 4, 7, 9, 11, 16, 18, 23 and 25. At the various time points, all of the NaOH was removed and distributed into 0.5 ml aliquots and mixed with scintillation fluid (performed in duplicate) and placed in a scintillation counter (Beckman LS3801) used to determine the ^{14}C counts in disintegrations per minute (dpm). After each sampling event, fresh NaOH was added and the samples were weighed to determine if moisture had been lost, if so sterile water was added to maintain the 40-60% WHC.

The percentage of phenanthrene mineralised (or CO_2 evolved) was then calculated by determining the cumulative scintillation count across all time points, then dividing this by the initial ^{14}C-phenanthrene concentration, thus reporting the cumulative evolution of $^{14}CO_2$ (% evolved). Standard error was also calculated for all samples.

2.5 ^{13}C and ^{12}C Phenanthrene Incubation

Soils that were to be utilised in SIP experiments were first incubated with ^{13}C- or ^{12}C-phenanthrene to enable detection of phenanthrene degrading organisms. Experiments were prepared in 125 ml sealable vessels with a final volume of 6 g of soil and 20 µg.g^{-1} of phenanthrene.

Initially, 1.0 g of soil was added to the mesocosm with 0.5 ml of the ^{12}C-phenanthrene/methanol mastermix (0.24 mg.ml^{-1}) or 1.2 ml ^{13}C-phenanthrene (0.1 mg.ml^{-1} 99% ^{13}C in nonane, Novachem). The methanol and nonane were then left to evaporate off, after which the remaining quantity of soil was mixed in.

Soil water holding capacity was then adjusted to 60% using 890–1160 µl of minimal media (no carbon source) to achieve a C:N:P molar ratio of 100:10:1. Vessels were incubated at 25°C in the dark for 5 days. After incubation,

destructive sampling of the mesocosm was undertaken and samples stored at -80°C for further processing (refer to Section 2.6.3).

The length of incubation time was determined from the results of the ^{13}C mineralisation study (Chapter 5.3.3). After 4 days, all soils had a minimum phenanthrene mineralisation of 10%, which was considered an appropriate length of time to run the SIP incubation enabling sufficient incorporation of the label (^{13}C) into the nucleic acids of organisms metabolising phenanthrene but not enough time to confound results due to cross feeding (Lueders *et al.*, 2004a; Manefield *et al.*, 2004; Manefield *et al.*, 2007).

The variability between duplicate community profiles was shown to be minimal during the baseline investigation of soil samples, therefore experiments were not run in duplicate due to ^{13}C-labelled phenanthrene being cost prohibitive.

2.6 Molecular Analysis

2.6.1 Nucleic Acid Extraction

2.6.1.1 Sandy Soils

One gram of glass beads (<106 µm, 150-212 µm and 212-300 µm diameter) were added to a 2.5 ml microtube and sterilised. Under sterile conditions, 0.6 g soil and 800 µl of 100 mM sodium phosphate buffer (SPB; 93.2 ml 1.0 M Na_2HPO_4; 6.8 ml 1.0 M NaH_2PO_4; pH 8.0) together with 260 µl sodium dodecyl sulphate (SDS; 10% (w/v) SDS; 0.5 M Tris-HCl pH 8.0; 0.1 M NaCl) were added, vortexed briefly and then mixed using a Mini-Beadbeater (BIOSPEC Products, Oklahoma, USA) for 2 cycles of 30 s at around 4 $m.s^{-1}$, with samples placed on ice for 2 mins between cycles (method adapted and optimised from Henckel *et al*. (1999) and Lueders *et al*. (2004b), see Chapter 3.1). The mixture was centrifuged at 4°C for 3 min at 12,000 x *g*, and the supernatant removed to a sterile 1.5 ml microtube. One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 4°C for 3 min at 12,000 x *g*. The supernatant was collected and one volume of chloroform: isoamyl alcohol (24:1) added. The mixture was again centrifuged at 4°C for 3 min at 12,000 x *g*. The supernatant was carefully removed and nucleic acids precipitated from solution using 2 volumes of

polyethylene glycol (PEG) solution (30% (w/v) PEG6000; 1.6 M NaCl) and incubated at room temperature for 2 hours. The samples were then centrifuged at 4°C for 30 min at 12,000 x *g*. The pellet was washed with 1 volume of 70% molecular biology grade (MBG) ethanol, centrifuged at 4°C for 10 min at 18,000 x *g*, air dried and resuspended in 60 µl sterile MBG water.

2.6.1.2 Clayey Soils

One gram of glass beads (<106 µm, 150-212 µm and 212-300 µm diameter) were added to a 2.5 ml microtube and sterilised. Under sterile conditions, 0.5 g soil with 500 µl of cetyl trimethylammonium bromide (CTAB) extraction buffer (10% w/v CTAB; 0.7 M NaCl) and 0.5 ml phenol: chloroform: isoamylalcohol (25:24:1) were added to the tube. The sample was then mixed with a Mini-Beadbeater (BIOSPEC Products, Oklahoma, USA) for 2 cycles of 20 s at around 4 $m.s^{-1}$, with samples placed on ice for 1 min between cycles (adapted from Mahmood *et al*., 2005). The mixture was centrifuged at 4°C for 5 min at 16,000 x *g*, and the supernatant removed to a clean 1.5 ml microtube. DNA was precipitated from solution by addition of two volumes of PEG solution (30% (w/v) PEG6000; 1.6 M NaCl) and centrifuged at 4°C for 10 min at 18,000 x *g*. The pellet was washed with 1 volume of 70% MBG ethanol, centrifuged at $4^{\circ}C$ for 10 min at 18,000 x *g*, air dried and resuspended in 50 µl sterile TE buffer (10 mM Tris-Cl, 0.5 M EDTA pH 8.0).

2.6.1.3 Bacterial Culture

One millilitre of overnight culture was centrifuged (5 min; 4°C; 14,000 x *g*) to pellet cells (Sambrook and Russell, 2001). The supernatant was discarded and 0.5 ml of 0.1 M SPB (89.6 ml 1.0 M Na_2HPO_4; 10.4 ml 1.0 M NaH_2PO_4; pH 7.8) was used to resuspend the pellet. The mixture was transferred to 2 ml tubes containing 0.5 g glass beads (<106 µm, 150-212 µm and 212-300 µm diameter) and a further 0.5 ml of SPB (pH 7.8). This was then mixed in a Mini-Beadbeater (BIOSPEC Products, Oklahoma, USA) for 2 cycles of 20 s at around 4 $m.s^{-1}$, the samples were placed on ice for 2 mins between cycles. The mixture was then centrifuged at $4^{\circ}C$ for 5 min at 16,000 x *g*; the supernatant was then removed to a clean microtube. One volume of phenol: chloroform: isoamyl alcohol (25:24:1)

was added and centrifuged at 4°C for 5 min at 16,000 x *g*. The supernatant was carefully removed and added to a fresh microtube containing one volume of chloroform: isoamyl alcohol (24:1) and centrifuged (4°C; 10 min; 16,000 x *g*). The supernatant was carefully removed and nucleic acids precipitated from solution using 1:10 volume 3 M sodium acetate and 1 vol 99% MBG ethanol and incubated at -20°C for 2 hours. The samples were then centrifuged (4°C; 10 min; 16,000 x *g*) and the pellet washed with 1 vol 70% MBG ethanol, air dried and resuspended in 50 µl MBG water.

2.6.1.4 Fungal Mycelium

Under sterile conditions, 0.5 g of glass beads (<106 µm, 150-212 µm and 212-300 µm diameter) and 0.5 ml SPB was placed into a sterile microtube (Sambrook and Russell, 2001). A sterile loop was used to collect mycelium from a pure plate isolate and added to the tube. Five hundred microlitres of CTAB extraction buffer (10% w/v CTAB; 0.7 M NaCl) and 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and then mixed with a Mini-Beadbeater (BIOSPEC Products, Oklahoma, USA) for 2 cycles of 20 s at around 4 $m.s^{-1}$. The mixture was centrifuged at 4°C for 5 min at 16,000 x *g* and the supernatant removed to a clean microtube. One volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 4°C for 5 min at 16,000 x *g*. The supernatant was carefully removed and nucleic acids precipitated from solution using 2 volumes of PEG solution (30% (w/v) PEG6000; 1.6 M NaCl) and incubated at room temperature for 2 hours. The samples were then centrifuged at 4°C for 30 min at 12,000 x *g*. The pellet was washed with 1 volume of 70% MBG ethanol, centrifuged at 4°C for 10 min at 18,000 x *g*, air dried and resuspended in 50 µl MBG water.

2.6.2 DNase treatment of RNA and cDNA synthesis

Extracted nucleic acid was treated with RNase free DNase after RNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Ten micrograms of RNA was resuspended in 1 X DNase I reaction Buffer (New England Biolabs) to a final volume of 100 µl. Two units of DNase I (2 $U.\mu l^{-1}$) was added and thoroughly mixed before incubation at 37°C for

10 mins. The DNAse was then inactivated by the addition of 1 µl 0.5 M EDTA and incubation at 75°C for 10 mins.

Single strand cDNA was then produced through reverse transcriptase PCR using an iScript™ cDNA synthesis kit (BioRad). One microgram of RNA template was added to 4 µl 5 X iScript reaction mix containing oligo(dT) and random hexamer primers, 1 µl iScript reverse transcriptase and MBG water to a final volume of 20 µl. The mixture was mixed thoroughly and incubated using the following protocol: 5 mins at 25°C; 30 mins at 42°C; 5 mins at 85°C. Synthesised cDNA was then stored at–20°C.

2.6.3 Stable Isotope Probing (SIP)

2.6.3.1 DNA SIP

Equilibrium (isopynic) density gradient centrifugation and gradient fractionation were conducted in caesium chloride (CsCl) gradients. Gradients were loaded with 500 ng of DNA extracted from ^{13}C- and ^{12}C-phenanthrene incubations (Section 2.5). Gradient buffer (GB) (100 mM Tris-HCl; 100 mM KCl; 1 mM EDTA) to a total volume of 1.2 ml was then mixed with DNA. The volume of CsCl stock required to achieve the gradient buoyant density of 1.725 g.ml^{-1} was determined using the following formula;

$$V_{CsCl\ Stock} = \frac{V_{DNA/GB\ Solution}}{(d_{CsCl\ stock} - d_{desired}) \times 1.52}$$

where v is volume,

d is density,

$d_{desired}$ is the desired gradient density of 1.725 g.ml^{-1}

(Neufeld *et al.*, 2007b)

The appropriate volume of CsCl stock solution was mixed with the DNA/GB solution and carefully added to polyallomer Optiseal centrifuge tubes (13 x 51 mm), sealed, and spun in a NVT 90 rotor in an Optima L-100 XP ultracentrifuge (Beckman Coulter, USA) at 44,100 rpm and 20°C for 36–40 h. Gradients were fractionated from below by displacement with MBG water by using a syringe pump at a flow rate of 3.3 µl.s^{-1}. The buoyant density of gradient fractions was

determined by weighing known volumes on a four-figure milligram balance. Fractions with density of around 1.75 $g.ml^{-1}$ are indicative of fractions containing ^{13}C-labelled DNA (Neufeld *et al.*, 2007b) and were targeted during amplification and fingerprinting.

DNA was isolated from target gradient fractions by PEG precipitation with 20 µg of glycogen then ethanol washed and resuspended in 30 µl of TE buffer. Gradient fractions were checked for the presence of DNA by standard agarose gel electrophoresis with 1 X SYBR® Gold (Invitrogen, OR) staining.

2.6.3.2 RNA SIP

Equilibrium (isopynic) density gradient centrifugation and gradient fractionation were conducted in caesium trifluoroacetate (CsTFA) gradients consisting of 4.080 ml of a 1.99 $g.ml^{-1}$ CsTFA solution (GE Healthcare, UK), 153 µl deionised formamide in a final volume of 5.1 ml of MBG H_2O. Gradients were loaded with 500 ng of total RNA extracted from ^{13}C- and ^{12}C-phenanthrene incubations (Section 2.5) in polyallomer Optiseal centrifuge tubes (13 x 51 mm), sealed, and spun in a NVT 90 rotor in an Optima L-100 XP ultracentrifuge (Beckman Coulter, USA) at 64,000 rpm and 20°C for 36 h (Whiteley *et al.*, 2007). Gradients were fractionated from below by displacement with water by using a syringe pump at a flow rate of 3.3 $\mu l.s^{-1}$. The buoyant density of gradient fractions was determined by weighing known volumes on a four-figure milligram balance. Fractions with density of around 1.795 $g.ml^{-1}$ are indicative of fractions containing ^{13}C-labelled RNA (Kasai *et al.*, 2006; Whiteley *et al.*, 2007) and were targeted during amplification and fingerprinting.

RNA was isolated from target gradient fractions by precipitation with isopropanol. Gradient fractions were checked for the presence of RNA by standard agarose gel electrophoresis, stained with 1 X SYBR® Gold (Invitrogen, OR).

2.6.4 Polymerase Chain Reaction (PCR)

2.6.4.1 Bacterial 16S rRNA

Optimised touchdown PCR conditions were used to amplify the partial sequence of the 16S rRNA gene. PCR was performed using the primer set 341F (5'-CCT ACG GGA GGC AGC AG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3'), a 40-base pair (bp) GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GG-3') was attached to the 5' end of the 341F primer for amplification of products subjected to TGGE or DGGE (Muyzer *et al.*, 1993; Adetutu, 2005). Standard touchdown cycling conditions were: 5 mins at 95°C, (30 s at 95°C; 30 s at 65°C; 60 s at 72°C) x 10 with annealing temperature decreasing by 1°C per cycle, (30 s at 95°C; 30 s at 55°C; 60 s at 72°C) x 25; 10 mins at 72°C. Each 25 µl reaction contained 5 µl template DNA (diluted as required), 1 unit KAPA2G Robust DNA Polymerase (KAPA BioSystems), 1x KAPA2G Buffer B with 1.5 mM $MgCl_2$, 0.5 mM additional $MgCl_2$, 0.2 mM dNTP , 0.4 µM of each primer.

2.6.4.2 Fungal ITS

Optimised nested PCR conditions were used to amplify the ITS regions of the 5.8S rRNA gene. The first round of nested PCR was performed using the primer set EF4 (5'- GGA AGG GRT GTA TTT ATT AG-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') (White *et al.*, 1990; Smit *et al.*, 1999). The product was then used as template for the next round of PCR using the primer set ITS1F (5'- CTT GGT CAT TTA GAG GA AGT AA-3') and ITS2 (5'- GCT GCG TTC TTC ATC GAT GC-3') (White *et al.*, 1990; Gardes and Bruns, 1993). A 40 bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GG-3') was attached to the 5' end of the ITS1F primer for amplification of products subjected to TGGE or DGGE (Gardes and Bruns, 1993; Muyzer *et al.*, 1993). Standard cycling conditions for round one were: 5 mins at 94°C, (30 s at 94°C; 30 s at 55°C; 30 s at 72°C) x 35; 5 mins at 72°C. Conditions for round two were: 5 mins at 95°C, (30 s at 95°C; 30 s at 58°C; 30 s at 72°C) x 35; 5 mins at 72°C. Each 25 µl reaction contained 5 µl template DNA (diluted as required), 1 unit KAPA2G Robust DNA Polymerase (KAPA BioSystems), 1x KAPA2G Buffer B with 1.5 mM $MgCl_2$, 1 x KAPA2G Enhancer, 0.5 mM additional $MgCl_2$, 0.2 mM dNTP , 0.4 µM of each primer.

2.6.4.3 Ring Hydroxylating Dioxygenase (RHD) Primers

Ding *et al.* (2010) Primers

The following PCR conditions were used to amplify the partial sequence of the PAH-RHD$_\alpha$ gene. PCR was performed using the primer set 396F (5'- ATT GCG CTT AYC AYG GBT GG-3') and 696R (5'-ATT ACC GCG GCT GCT GG-3'). Standard cycling conditions included a pre-amplification step, as per Ding *et al*. (2010). Conditions were : 5 mins at 94°C, (1 min at 94°C; 2 mins at 46°C; 1 min at 72°C) x 5, (1 min at 95°C; 1 min at 58.5°C; 1 min at 72°C) x 30, 10 min at 72°C. Each 20 µl reaction contained 2 µl template DNA (diluted as required), 0.4 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 1 x Phusion HF Buffer(containing 1.5 mM $MgCl_2$), 0.2 mM dNTP, 0.3 µM forward primer and 0.6 µM reverse primer.

Cébron *et al.* (2008) Primers

The following optimised nested PCR conditions were used to amplify the partial sequence of the PAH-RHD$_\alpha$ gene. PCR was performed using the following primer sets and annealing temperatures as listed in Table 2.2.

Table 2.2 Primer sequences and associated annealing temperatures

Primer Pair	Forward Primer		Reverse Primer		Annealing Temp (°C)
	Name	Sequence (5'-3')	Name	Sequence (5'-3')	
PAH-RHD$_\alpha$ GP	GP-F	CGG CGC CGA CAA YTT YGT NGG	GP-R	GGG GAA CAC GGT GCC RTG DAT RAA	54.0
PAH-RHD$_\alpha$ GN	GN-F	GAG ATG CAT ACC ACG TKG GTT GGA	GN-R	AGC TGT TGT TCG GGA AGA YWG TGC MGT T	57.0

A 40 bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GG-3') was attached to the 5' end of the GP-F and GN-F primer for amplification of products subjected to TGGE.

The first round cycling conditions were : 1 min at 98°C, (10 s at 98°C; 30 s at 54°C or 57°C; 10 s at 72°C) x 35, 5 mins at 72°C. Each 20 µl reaction contained 2

µl template DNA (diluted as required), 0.4 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 1 x Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 3% DMSO, 0.2 mM dNTP , 0.3 µM of each primer.

The second round cycling conditions included touchdown annealing temperature steps, which were: 1 min at 98°C, (10 s at 98°C; 30 s at 64–54°C or 67–57°C; 10 s at 72°C) x 10, (10 s at 98°C; 30 s at 54°C or 57°C; 10 s at 72°C) x 35, 5 mins at 72°C. Each 20 µl reaction contained 2 µl template DNA (diluted as required), 0.4 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 1 x Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 3% DMSO, 0.5 mM additional $MgCl_2$, 0.2 mM dNTP and 0.3 µM of each primer.

Designed PAH-RHD$_\alpha$ Primers

The following PCR conditions were used to amplify the partial sequence of the PAH-RHD$_\alpha$ gene. PCR was performed using the following primer sets and annealing temperatures as listed in Table 2.3.

Table 2.3 Designed primer pairs, sequences and annealing temperature

Primer Pair	Forward Primer		Reverse Primer		Annealing Temp (°C)
	Name	Sequence (5'-3')	Name	Sequence (5'-3')	
GP-1	GP-524F	TTY GKM WSS BTS GAY CCR MAK G	GP-659R	CSR MRS BKR WYT TCC AGT TBS C	56.5
GP-2	GP-524F	TTY GKM WSS BTS GAY CCR MAK G	GP-662R	TBT CSR MRS BKR WYT TCC AGT T	54.0
GN-1	GN-5625F	TGC ARY TAY CAC GGS TGG	GN-5913R	TCM RYV GGH RYY TTC CAG TT	55.0
GN-2	GN-5625F	TGC ARY TAY CAC GGS TGG	GN-5913X-R	AAR TTT TCM RYV GGH RYY TTC CAG TT	52.0
GN-3	GN-5679X-F	CCR TTY GAR AAR GRD YKK TAY SRY G	GN-5952R	TGM GTC CAM CCV AYG TGR TA	55.0

Cycling conditions were: 30 s at 98°C, (10 s at 98°C; 30 s at various temperatures; 10 s at 72°C) x 35, 5 mins at 72°C. Each 20 µl reaction contained 2 µl template DNA (diluted as required), 0.4 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 1x Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTP and 0.3 µM of each primer.

Fungal PAH degradation primers

The following PCR conditions were used to amplify the lignin peroxidase (LnP), manganese-dependent peroxidase (MnP) and laccase (*Lcc*) enzymes that are known to be involved in fungal degradation of PAHs. PCR was performed using the following primer sets and conditions, listed below (Table 2.4).

Table 2.4 Fungal PAH degrading enzyme primers (Pointing *et al.*, 2005)

Primer Pair	Forward Primer		Reverse Primer		Reference
	Name	Sequence (5'-3')	Name	Sequence (5'-3')	
LnP	LnP-F	SCB AAC ATY GGY CTY GAC GA	LnP-R	TCS ABG AAG AAC TGS GWG TC	(Reddy and D'Souza, 1998)
MnP	MnP-F	GMR ATG GCC TTC RRT TCY T	MnP-R	TTA KGC AGG RCC RTY GAA CT	(Bogan *et al.*, 1996)
Lcc	Lcc-F	CAY TGG CAY GGN TTY TTY CA	Lcc-R	RTG RCT RTG RTA CCA RAA NGT	(Bogan *et al.*, 1996)

Cycling conditions were: (3 min at 94°C, 1 min at 50°C, 1 min at 72°C) x 1, (1 min at 94°C; 1 min at 50°C; 1 min at 72°C) x 45, 10 mins at 72°C. Each 20 µl reaction contained 2 µl template DNA, 0.4 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific), 1x Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTP and 0.3 µM of each primer.

2.6.5 Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were analysed using the DCode Universal Mutation Detection System (Bio-Rad Inc., USA) using 8% polyacrylamide gel in 1 X TAE. A 40 to 60% urea-formamide denaturant gradient was created using a Mini-Peristaltic pump

(CBS Scientific Co., USA) (Roling *et al.*, 2002). The 40% gradient was formed using 6.9 ml 70% denaturant (6% acrylamide, 29.4 $g.l^{-1}$ urea, 30% formamide) and 5.1 ml 0% denaturant (6% acrylamide). The 60% gradient was formed using 10.3 ml 70% denaturant (12% acrylamide, 29.4 $g.l^{-1}$ urea, 30% formamide) and 1.7 ml 0% denaturant (6% acrylamide). Before gradient solutions were loaded into the pump, 130 µl of 10% ammonium persulphate (APS) and 13 µl of tetramethylethylenediamine (TEMED) were added to each. This was to aid in the gel setting. A 32 well comb was used to create the stacking gel, using 6 ml of 0% denaturant with 60 µl APS and 6 µl TEMED. Between 12 to 19 µl of PCR product (based on the intensity of the PCR product when electrophoresed) was mixed with 2 µl of 6 X Blue/Orange loading dye (Promega) then loaded into the stacking gel. Electrophoresis was performed for 16 h at a constant voltage of 60 V at 60°C.

2.6.6 Temperature Gradient Gel Electrophoresis (TGGE)

The PCR products were analysed using the TGGE MAXI System (Whatman Biometra®, Germany) using 8% polyacrylamide gel (7 M urea) in 1 X TBE. The gel was cast with one backing plate with polybond film and a slotted front plate to create wells. Before casting the gel, 72 µl 10% APS and 99 µl TEMED was added to 45 ml of polyacrylamide to commence polymerisation. Gels were left to polymerise for a minimum of three hours. Between 5 to 10 µl of PCR product (based on the intensity of the PCR product when electrophoresed) was mixed with 1 µl of 6 X Blue/Orange loading dye (Promega) then loaded into the stacking gel. Electrophoresis was performed for 8 h at a constant voltage of 220 V with a temperature gradient of $55.0^{\circ C}$ to $68.0^{\circ C}$ for 16S rRNA products, $50.0^{\circ C}$ to 58.0°C for ITS products, and $51.0^{\circ C}$ to $60.0^{\circ C}$ for RHD_{α} products.

2.6.7 Gradient Gel Electrophoresis Visualisation

2.6.7.1 Silver Staining

Gels to be used for community analysis were stained using a silver staining method (McCraig *et al.*, 2001) as follows: the gel was placed in Fixing Solution I (10% ethanol; 0.5% glacial acetic acid) for 2 hours and then incubated with

shaking in freshly prepared staining solution (0.1% [w/v] silver nitrate) for 20 min. The gel was then developed in fresh solution (0.01% [w/v] sodium borohydride; 0.4% formaldehyde; 0.4% [w/v] sodium hydroxide) until bands appeared, generally after 20 mins. The developing process was fixed used Fixing Solution II (0.75% [w/v] sodium carbonate) for 10 mins and preserved for 20 mins in ethanol-glycerol preservative (25% ethanol; 10% glycerol). The stained gel was scanned with an Epson Perfection V700 Photo scanner and saved as a .tif file for analysis.

2.6.7.2 SYBR ®Gold Staining

Gels to be used for further molecular analysis were stained using a 1 X SYBR Gold solution in 10 X TBE buffer for 15 mins, then visualised using a VersaDoc™ (BioRad).

2.6.8 Agarose Gel Photography

Genomic DNA was visualised in 0.9% (w/v) agarose gels and 2% (w/v) agarose gels were used for PCR products. Gels were either stained using a 0.5 $\mu g.ml^{-1}$ ethidium bromide bath, a 1 X SYBR® Gold bath (Invitrogen, OR), or pre-cast with SYBR® Safe (Invitrogen, OR) according to manufacturer's instructions.

2.6.9 Band Excision

2.6.9.1 Agarose gels

Agarose gels were visualised using a Safe Imager™ 2.0 Blue Light Transilluminator (Invitrogen). Representative bands of interest were excised from the gel using a sterile scalpel and placed into labelled sterile 1.5 ml microtubes. DNA was then eluted from the gel using QIAquick gel extraction kit (Qiagen), as per the manufacturer's instructions.

2.6.9.2 Polyacrylamide gels

For improved visualisation polyacrylamide gel were placed on a standard light box, bands were then excised and incubated in approximately 2 volumes of filter sterilised elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (v/v) SDS) for 4 h at 37°C (McKew *et al.*, 2007). DNA was

precipitated out of the supernatant with 2 volumes of filter sterilised 99% ethanol, incubated for 2 h at -20°C then centrifuged at 4°C for 10 mins at 13,000 rpm. The pellet was washed with 1 volume filter sterilised 70% ethanol, air dried and resuspended in 20 µl sterile dH_2O.

2.6.10 Gradient Gel analysis

TotalLab analysis package, (Version TL120; Nonlinear Dynamics, USA) was used to calculate the intensity and mobility of the bands within the DGGE/TGGE gels. For calculation of band intensity volumes, minimum peak threshold of the software was set to 21 using the Rolling Ball method in order to reduce background noise. Band intensity values were averaged for replicates of treatments and banding patterns converged. Two bands were thought to be related if they migrated the same distance down the gel (Nakatsu *et al.*, 2000) and therefore each band was considered to be an operational taxonomic unit (OTU) or species (Dilly *et al.*, 2004).

Banding patterns were analysed using TotalLab to generate similarity profiles using the unweighted pair group method with mathematical averages (UPGMA).

The banding patterns were also analysed using the Shannon Weaver diversity Index (*H'*) and Equitability Index (*J*). The Shannon Weaver diversity Index is a general diversity value which increases as the number of species (OTUs) increase and the Equitability Index is a measure of the relative abundance of the different species (OTUs) in the sample (Dilly *et al.*, 2004). The closer *J* is to 1.0 the more even the community with fewer dominant species. The Shannon Weaver diversity, Equitability Indices and standard errors were calculated using the following formulas in Microsoft® Office Excel 2003;

$$H' = -Pi \times \ln Pi$$

where *Pi* is the proportion of the community that is made of species *i* (intensity of band I / total intensity of all bands in lane)

ln is the natural logarithm.

(Ogino *et al.*, 2001)

$$J = {H'}/{\ln ni}$$

where *ni* is the total number of species (bands) in a lane.

(Adetutu, 2005)

Pareto-Lorenz (PL) evenness curves were used to estimate functional organization (Fo) within the microbial community, by scoring communities at the interception of the *y*-axis projection with the vertical 20% *x*-axis (Wittebolle *et al.*, 2008). This method assumes that the distribution of species within a microbial community relates to its capacity to optimize and conserve functionality (Marzorati *et al.*, 2008). Therefore the lower the PL score the lower the functional organization within a community as very few or no species are dominant. The more a PL curve deviates from the perfect 45^0 line the less evenness can be observed in the structure of the community meaning a smaller number of different species are present in dominant numbers. Therefore a Fo less than 30 is considered to have low functional organisation and therefore high evenness with no species present in high concentrations. Fo between 30–70 represents a medium organisation with the most fitting species present in high numbers with the majority present in decreasing amounts. A Fo score above 70 is considered high and represents a specialised community in which a small amount of species are dominant.

Range weighted richness (Rr) was used to calculate the carrying capacity of the microbial community according to the following equation:

$$Rr = N^2 \times Tg$$

where N is the total number of bands,

Tg is the percentage of temperature gradient of the sample analysed

(Marzorati *et al.*, 2008).

The higher a carrying capacity of an environment is (i.e. the number of individuals that the environment can support) the higher is the probability it can host a high number of bands, where communities with an Rr below 10 are

adverse or restricted to colonisation environments (i.e. contaminated soil) and a community with a Rr greater than 30 are typically habitable environments with a high microbial diversity.

Statistical significance was tested using the Student's t-test, in accordance with standard protocol (Adetutu, 2005).

2.6.11 Sequencing

Bands were excised according to the method in Chapter 2.6.9. Re-amplification was performed by PCR as described in Chapter 2.6.4. Products were gel purified and prepared for sequencing as per Australian Genome Research Facility (Qld, Australia) purified DNA sample protocol. Duplicates of each sample were sent (one with the forward primer and one with the reverse).

Chromatograms of the sequences received from AGRF were checked and edited for quality using Sequencher™ (Sequencer Version 5.0, Gene Codes Corporation, USA) and sample replicates were aligned and consensus sequences created. Homology sequences were compared with the Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information (NCBI) using a BLAST algorithm (http://www.ncbi.nlm.gov.library.vu.edu.au/BLAST) for comparison of a nucleotide query sequence against a nucleotide sequence database (blastn) to determine putative identities of the bands. Species were matched with the highest identity scoring sequence entry.

3 Method Development

3.1 Extraction Methods from specific soil types

The key basis of this project was to use molecular microbial ecology to investigate microbial community dynamics, through the application of stable isotope probing (SIP). While there are several commercial kits available for extraction of nucleic acids (both RNA and DNA) from various materials (e.g. soil, water, oil), many of these kits extract nucleic acid of high purity but low concentration, thereby limiting further downstream applications (Whiteley *et al.*, 2006; Feinstein *et al.*, 2009). As SIP requires separation of DNA and RNA before any amplification occurs (i.e. prior to PCR), both a high purity and high concentration extract from relatively small quantities of soil is required. Therefore the first aim of this chapter was to develop a robust and reliable nucleic acid extraction method that could be routinely applied to different soil types (clayey and sandy soils). An effective and efficient extraction method also needs to manage or negate the co-extraction of soil humic substances that can inhibit the activity of enzymes (Tebbe and Vahjen, 1993). Furthermore, DNA can be tightly bound to clay soil particles making extraction difficult (Andersen *et al.*, 1998; Braid *et al.*, 2003); thus two different extraction techniques were required, one to extract DNA from sandy soils and a second to extract DNA from clayey soils with high humic substances.

3.1.1 Extraction from Sandy Soils

Initial experimentation into method development was undertaken using a sandy soil (TP9, a hydrocarbon contaminated soil) with soil taken from two depths, 0-1 m and 1-2 m. The experiment involved comparison of two methods, a phenol: chloroform: isoamyl alcohol (P:C) extraction method, and a commercially available kit FastDNA® Spin Kit for Soil (MP Biomedical). The P:C method represents the standard laboratory protocol and involved mechanical lysis in the presence of P:C for 2 x 30 s and a phenol: chloroform and isopropanol DNA precipitation step. The Spin Kit was used according to manufacturer's instructions, which again involved mechanical lysis with various buffers and then

column purification and elution of extracted DNA. Figure 3.1 shows an agarose gel of the extracts using the two methods as visual determination of DNA concentration of was the only available method of quantification available at the time of experimentation. The figure shows that genomic DNA was extracted using both methods; however the DNA extracted using the P:C method showed smearing, possibly due to shearing of DNA which could affect its quality. When bacterial 16S rRNA PCR of these extracts was attempted using the protocol described in Chapter 2.5.7.1, no product was amplified for the kit extracts despite the original DNA band appearing clear (Figure 3.1). In contrast some faint products of the correct size (246 bp) were visible in P:C extracts (Figure 3.2). Given this, it was decided to pursue optimisation of the P:C method.

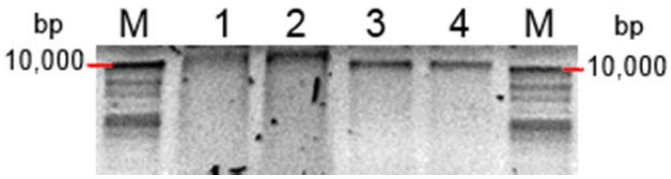

Figure 3.1 Comparison of initial extraction methods from sandy soils. 0.9% agarose gel. M: Marker, Promega 1 kb DNA Ladder; Lane 1: TP9 0-1m P:C extract; Lane 2: TP9 1-2m P:C extract; Lane 3: TP9 0-1m FastDNA® extract; Lane 4: TP9 1-2m FastDNA® extract.

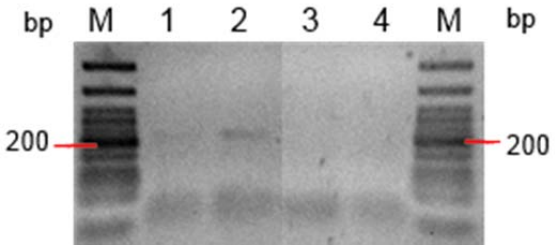

Figure 3.2 Bacterial PCR (341F & 518R) of initial extraction methods from sandy soils. 2.0% agarose gel of 16S rRNA PCR product. M: Marker, New England Low Molecular Weight Ladder; Lane 1: TP9 0–1 m P:C extract; Lane 2: TP9 1-2m P:C extract; Lane 3: TP9 0-1m FastDNA® extract; Lane 4: TP9 1-2m FastDNA® extract.

The next step in method development was to trial methods adapted from Henckel *et al*. (1999) and Lueders *et al*. (2004b). These methods were similar to the P:C method initially trialled, but varied in mechanical lysis time and buffers used. The method developed by Henckel *et al*. (1999) utilised mechanical lysis (1 x 45 s) in the presence of phosphate buffer and phenol: chloroform followed by isopropanol precipitation of DNA, whereas, the method of Lueders *et al*. (2004b)

was less abrasive, as soil was beaten (1 x 40 s) with sodium phosphate buffer (SPB) and sodium dodecyl sulphate (SDS) and the phenol: chloroform purification step was completed after mechanic lysis. DNA was then precipitated using polyethylene glycol (PEG). The resultant DNA extracts from both methods are shown in Figure 3.3.

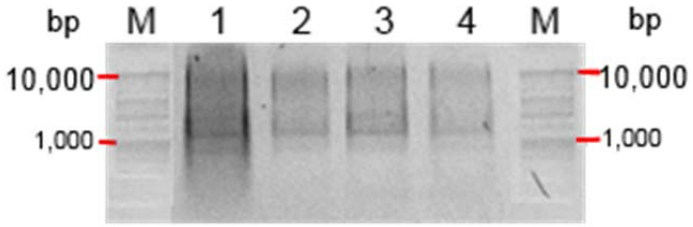

Figure 3.3 Comparison of adapted Henckel and Lueders extractions methods from sandy soils. 0.9% agarose gel. M: Marker, Promega 1 kb DNA Ladder; Lane 1: TP9 0-1 m Henckel extract; Lane 2: TP9 1-2 m Henckel extract; Lane 3: TP9 0-1 m Lueders extract; Lane 4: TP9 1-2 m Lueders extract.

Both methods extracted DNA although the Lueders *et al*. (2004b) method appeared to have co-extracted DNA and RNA, which was promising as downstream applications of the extract would require both DNA and RNA and if the one protocol could extract both nucleic acids an reduction in processing time would be advantageous.

Further optimisation of beating times and the addition of a cooling step between beating to reduce degradation of the nucleic acid of the Lueders method was undertaken to ensure that this extraction method produced good quality DNA and RNA with a satisfactory yield. The optimised method effectively co-extracted DNA and RNA from only freshly incubated soil, as shown in Figure 3.4. In contrast this methodology (lanes 1 and 2) did not extract RNA from stored soils, possibly due to degradation of RNA under storage conditions.

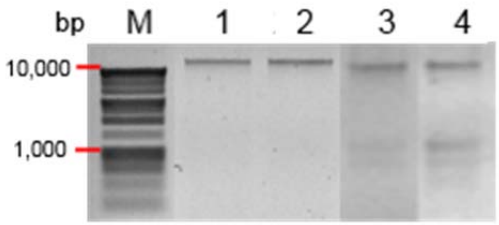

Figure 3.4 Extracts from sandy soils using optimised adapted Lueders method. 0.9% agarose gel. M: Marker, Promega 1 kb DNA Ladder; Lane 1: TP9 at 0-1 m; Lane 2: TP9 1-2 m; Lane 3: incubated TP9 at 0-1 m; Lane 4: incubated TP9 1-2 m.

3.1.2 Extraction from Clayey Soils

Given that clayey soils have a tendency to strongly bind DNA it was expected that the optimised method for sandy soils would not be sufficient to extract nucleic acids from these types of soil. Initially, nucleic acid extraction of the clayey soil type (FTF5–hydrocarbon contaminated soil) using the sandy soil method (Chapter 2.6.1) demonstrated that this was in fact the case, as no visible DNA or RNA was extracted (visualisation through gel electrophoresis in 0.9% agarose) and 16S rRNA PCR products could not be obtained (data not shown).

Several commercially available kits were used in the laboratory for extraction of nucleic acids from soils so the next step was to trial two of these kits, the PowerSoil® DNA Isolation Kit (Mo-Bio) and FastDNA® Spin Kit for Soil (MP Biomedical). Both kits used mechanical lysis in the presence of buffers and subsequent purification through membrane binding and centrifugation. The PowerSoil® kit claims to be better suited for soil samples with high humic acid content, such as clays, due to a patented inhibitor removal technology as part of the extraction process (Mo Bio Laboratories, 2013). Extractions were completed as per the manufacturer's instruction for both kits and again did not produce visible DNA extracts.

Given that several attempts to extract DNA from the clayey soil type had been unsuccessful it was decided to assess the number of viable microbial cells present in the soil. Most probable number (MPN) testing was carried out as described in Section 2.3 to determine if the number of culturable organisms was the limiting factor for extraction. For comparison the two different soil types, sandy and clayey, were tested as well as soil with different contamination

exposure histories, i.e. pristine and previously contaminated. A killed control was run in parallel with test samples, with no growth observed during testing. Results of the MPN test demonstrated that there was between 10^3 and 10^6 organisms per gram of soil in the clayey soil type (Figure 3.5). Interestingly the number of organisms in the sandy soil was generally lower than this, ranging between 10^2–10^3 organisms per gram of soil. The results of the MPN testing indicated that a smaller number of organisms were present in the sandy soils and therefore nucleic acid extraction from clayey soils should be more achievable than from sandy soils. It was also observed that an increased number of organisms were present in the shallow (0-1m) clay sample compared to all other samples tested.

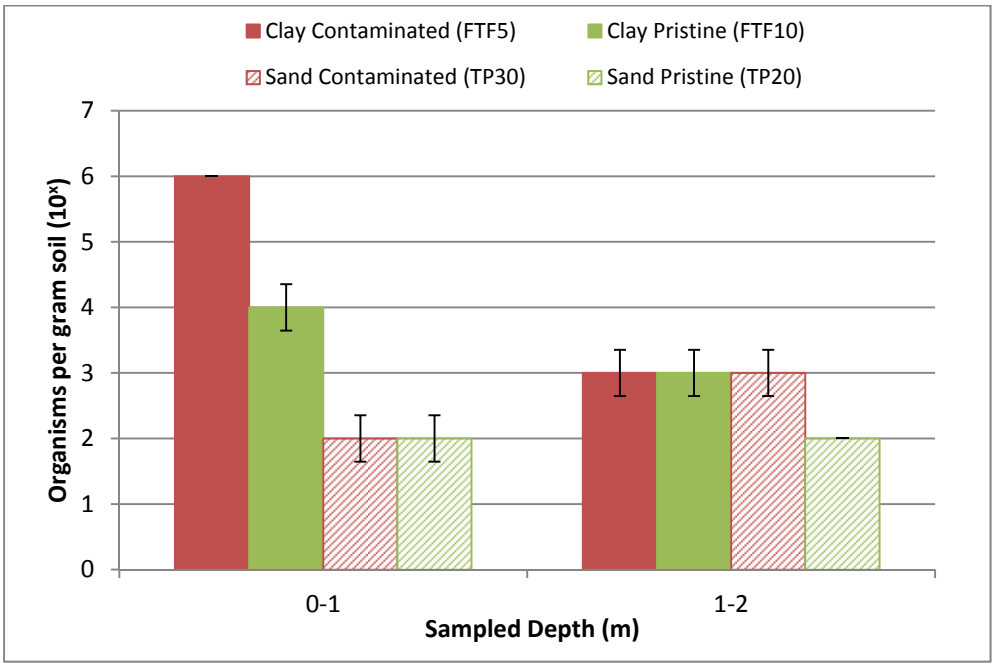

Figure 3.5 MPN testing of different soil types with varying contamination histories and depths. n=2

Given that the presence of organisms should not be a limiting factor, it was thought that the extractions from the commercial kits had been successful but had extracted only a small concentration of DNA. To confirm this PCR amplification of 16S rRNA was attempted from kit extracts. Figure 3.5 demonstrates that amplification of 16S rRNA was possible and that DNA had in fact been extracted from clayey soils using the commercial kits, although at very low concentrations that could not be visualised on agarose gels. Low DNA

concentration of extracts was confirmed using a NanoDrop 1000 spectrophotometer (Thermo Scientific) with concentrations ranging from 2–10 $ng.\mu l^{-1}$. These concentrations were too low to be able to use for further downstream applications, i.e. SIP, without PCR amplification.

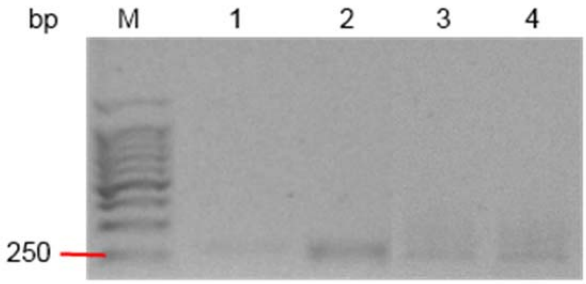

Figure 3.6 16S rRNA PCR products from DNA extracts from clayey soils using commercially available kits. 2.0% agarose gel of 16S rRNA PCR product. M: Marker, Promega 1kb Ladder; Lane 1: FTF5 0-1 m PowerSoil® extract; Lane 2: FTF5 0-1 m FastDNA® extract; Lane 3: FTF5 1-2 m PowerSoil® extract.

As commercial kits were not producing concentrated DNA extracts the next step was to go back to first principles and attempt a phenol: chloroform extraction. Mahmood *et al*. (2005) had developed a method for DNA extraction from similar soil types that were to be used for SIP so this method was trialled. The method utilised mechanical lysis in the presence of phenol: chloroform: isoamyl alcohol and a CTAB extraction buffer for 1 x 30s and a PEG and ethanol DNA precipitation step. Various beating times (1 x 30s, 1 x 15s and 2 x 20s) were trialled with the addition of a cooling step between beating in the hope of preventing degradation of DNA during extraction. Figure 3.7 and Figure 3.8 show that the beating time of 2 x 20s was the only method to successfully extracted visible DNA; furthermore this method produced the strongest 16S rRNA product.

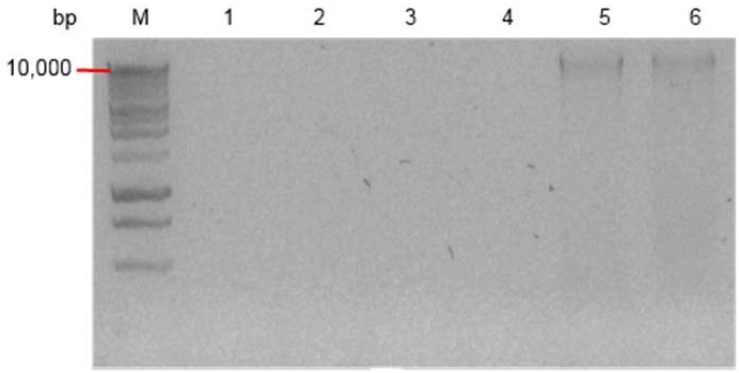

Figure 3.7 DNA extracts using an adapted Mahmood *et al*. (2005) first principles method. 0.9% agarose gel**.** M: Marker, Promega 1 kb DNA Ladder; Lane 1: FTF5 0-1 m, beat 1 x 30s; Lane 1: FTF5 1-2 m, beat 1 x 30s; Lane 3: FTF5 0-1 m, beat 2 x 15 s; Lane 4: FTF5 1-2 m, beat 2 x 15 s; Lane 5: FTF5 0-1 m, beat 2 x 20 s; Lane 6: FTF5 0-1 m, beat 2 x 20 s.

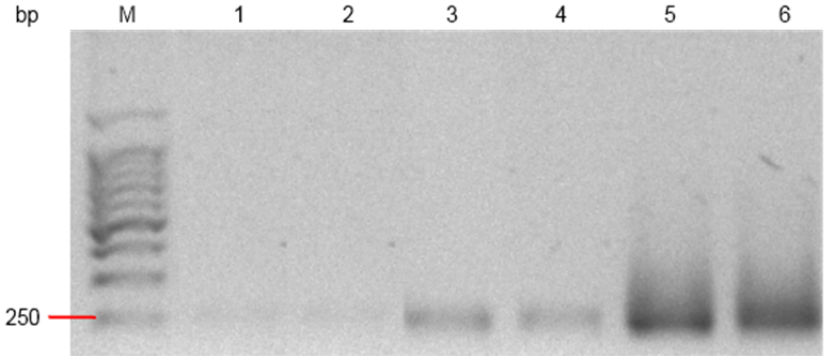

Figure 3.8 16S rRNA products from adapted Mahmood *et al*. (2005) DNA extracts. 2.0% agarose gel of 16S rRNA PCR product. M: Marker, Promega 1kb Ladder; Lane 1: FTF5 0-1 m, beat 1 x 30 s; Lane 2: FTF5 1-2 m, beat 1 x 30 s; Lane 3: FTF5 0-1 m, beat 2 x 15 s; Lane 4: FTF5 1-2 m, beat 2 x 15 s; Lane 5: FTF5 0-1 m, beat 2 x 20 s; Lane 6: FTF5 0-1 m, beat 2 x 20 s.

Although this adapted method was able to extract DNA it was still at concentrations too low to be useful for SIP applications. Given the considerable time already invested in the project to get to this point, and the time that would be required to further optimise this protocol, it was decided that the clayey soil type would be utilised for investigation of diversity of the endogenous microbial communities within different soil types (Chapter 4). Only the sandy soil would be used in investigating whether a prediction about a communities' response to a contamination event could be made using SIP (Chapter 5).

3.2 Ring-Hydroxylating Dioxygenase (RHD) Primer Design

One of the key aims of this project was to determine if by investigating the structure of the endogenous microbial community we could determine how the community would adapt to a contamination event. Various methods were to be

utilised as part of this project including TGGE and real-time PCR (qPCR) to test the hypothesis that if there is a high number of organisms containing "degradation" genes then the community should be able to adapt well to a contamination event.

The model contaminant utilised for this project was phenanthrene, a three-ring polycyclic aromatic hydrocarbon, and it is well known that the key genes in the initial bacterial aerobic degradation of phenanthrene are those that code for a unique multicomponent ring-hydroxylating dioxygenase (RHD). This dioxygenase is involved in the incorporation of oxygen into the aromatic nucleus regardless of the organism mineralising the compound (Cerniglia, 1992; Margesin *et al.*, 2003) making this gene a good target for community profiling.

Several studies have developed primers that target conserved regions within the RHD, one of which is an iron binding site within the Rieske domain of the alpha subunit of the RHD enzyme (RHD_{α}). Phylogenetic investigations as part of previous research identified two main phylogenetic group clusters associated with degradation, the *nid*A-like (Gram-negative bacteria) and *nah*Ac-like dioxygenase genes (Gram-positive bacteria) (Habe and Omori, 2003; Cébron *et al.*, 2008). Although many researchers have developed primers to target the two groups there has been limited success in primers that target both groups with one round of amplification. Two publications (Cébron *et al.*, 2008; Ding *et al.*, 2010) developed primers that were successfully used for qPCR and these primers were the first target of this project. Cebron *et al*. (2008) primers (PAH-RHD_{α} GN-F & GN-R and PAH-RHD_{α} GP-F & GP-R) targeted the Gram-negative and Gram-positive bacteria separately whereas Ding *et al*. (2010) developed a set of degenerate primers that targeted both Gram-positive and Gram-negative species (PAH-RHD_{α}-396F & PAH-RHD_{α}-696R).

As the one set of primers developed by Ding *et al*. (2010) amplified both phylogenetic clusters these were the first to be attempted as part of this project. The primers were tested according to the conditions described by Ding *et al*. (2010) using DNA template from known PAH degraders and environmental soil extracts from a pristine soil (TP20) and hydrocarbon contaminated soil (TP30).

The known PAH degrading bacteria template DNA was extracted from *Mycobacterium gilvum* ATCC 700033 (Gram-positive) and *Pseudomonas aeruginosa* PA01 (Gram-negative). The bacteria were grown from stocks, supplied by research partners from the University of South Australia in liquid cultures with phenanthrene as the sole carbon source. Environmental template DNA was extracted from soil samples incubated as part of the experiments described in Chapter 5, again in the presence of phenanthrene as the sole carbon source.

The PCR conditions recommended by Ding *et al*. (2010) included an initial pre-amplification step with annealing temperature of 46°C for 2 min. Results from various attempts to optimise cycling conditions discovered that they were very unspecific and generated multiple bands, with the correct sized bands (320 bp) being very faint in both pure culture extracts and environmental samples (Figure 3.9).

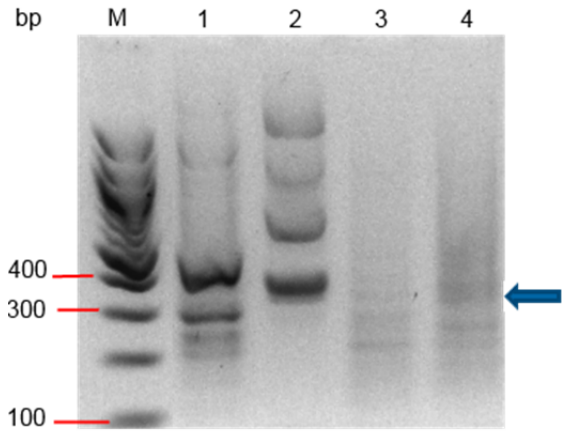

Figure 3.9 PCR amplification from various DNA extracts using Ding *et al*. (2010) primers. 2.0% agarose gel. M: Marker, Promega 100bp Ladder, ➡ target band size (320bp); Lane 1: Gram-negative bacteria *Pseudomonas aeruginosa*; Lane 2: Gram-positive bacteria *Mycobacterium gilvum*; Lane 3: pristine sandy test soil (TP20 0-1m); Lane 4: contaminated sandy test soil (TP30 0-1m).

Further attempts were made to increase the specificity of these primers, which involved the removal of the pre-amplification step and determination of the best annealing temperature using gradient PCR, but the primers still produced multiple bands and the target band was not the dominant band (Figure 3.10).

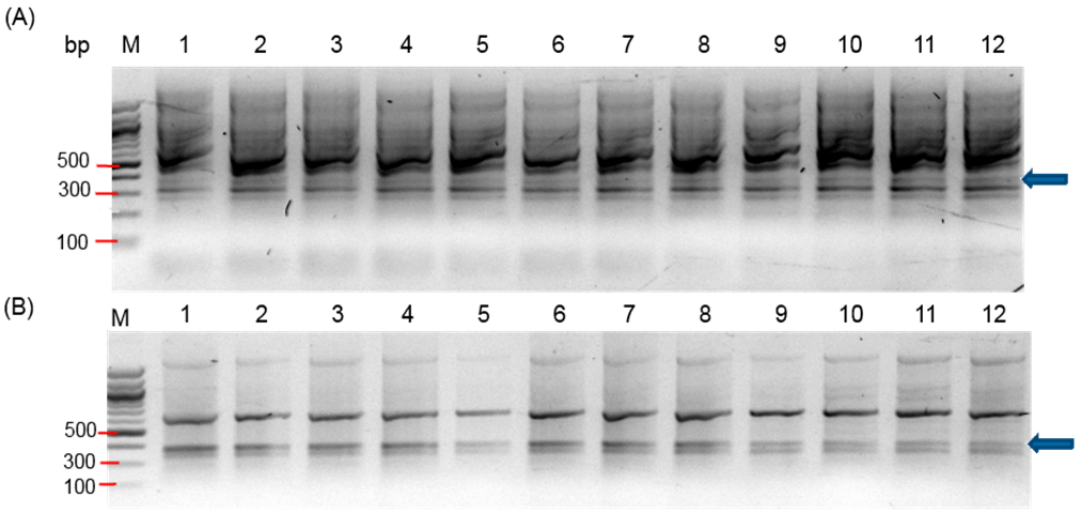

Figure 3.10 PCR amplification of Gram-positive and Gram-negative bacteria using Ding *et al*. (2010) primers with various annealing temperatures. 2.0% agarose gel. M: Marker, Promega 100bp Ladder, ➡ target band size (320 bp). (A) Gram-positive bacterium *M. bacterium*, (B) Gram-negative bacterium *P. aeruginosa*, Annealing temperatures : Lane 1:55°C, Lane 2: 56°C, Lane 3: 57°C, Lane 4: 58°C, Lane 5: 59°C, Lane 6: 60°C, Lane 7: 61°C, Lane 8: 62°C, Lane 9: 62°C, Lane 10: 63°C, Lane 11: 64°C, Lane 12: 65°C.

The two primer sets developed by Cébron *et al*. (2008) were the next group to be tested in this project. The primers were tested using the conditions stated in the publication using DNA template from a known PAH degrader and an environmental soil extract (TP30 0-1 m), as described for the testing of the primers developed by Ding *et al*. (2010). Again, optimisation was unsuccessful as amplification was unspecific (Figure 3.11). Interestingly for both sets of primers the most dominant band of the appropriate size was from the template of the opposite cluster, i.e. the target band size of 292 bp using the Gram-positive primers GP-F and GP-R was the brightest in lanes 3 and 6 of Figure 3.11A which were amplified from DNA from the Gram-negative bacterium *P. aeruginosa*.

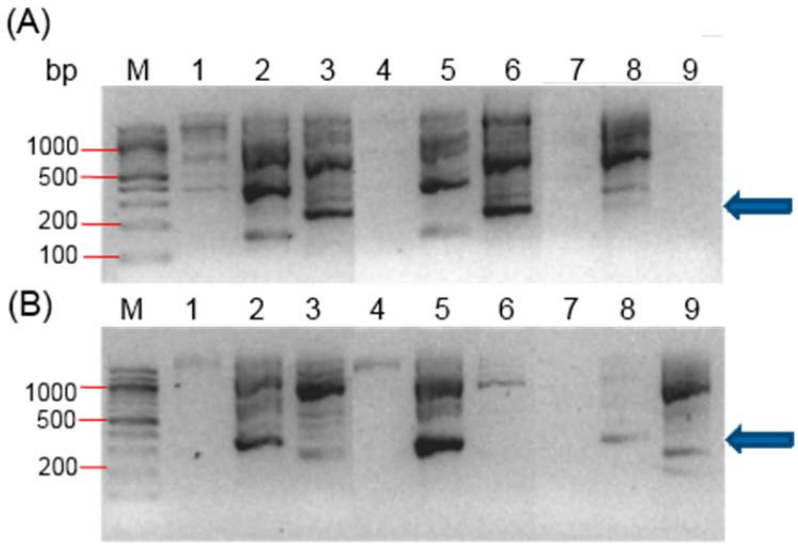

Figure 3.11 PCR amplification of Gram-positive and Gram-negative bacteria using Cébron *et al*. (2008) primers. 2.0% agarose gel. M: Marker, Promega 100bp Ladder, ➡. target band size. (A) Gram-positive primer pair, target size 292 bp, (B) Gram-negative primer pair, target size 306 bp, Lanes 1–3: normal PCR conditions, Lane 1: Environmental soil DNA template (TP30 0-1m), Lane 2: Gram-negative template DNA, Lane 3: Gram-positive template DNA, Lanes 4–6: PCR with addition of BSA, Lane 4: Environmental soil DNA template (TP30 0-1m), Lane 5: Gram-negative template DNA, Lane 6: Gram-positive template DNA. Lanes 7–9: PCR with addition of DMSO, Lane 7: Environmental soil DNA template (TP30 0-1m), Lane 8: Gram-negative template DNA, Lane 9: Gram-positive template DNA.

Given the lack of success with the above primers, the next stage was to design primers that could be used in qPCR to again target the conserved iron binding site of the RHD_{α} for Gram-negative and Gram-positive bacteria that were more specific than the other primers trialled to this point.

Primers for PCR amplification of the RHD_{α} genes from the two cluster groups were designed based on all available Gram-positive and Gram-negative RHD_{α} gene sequences deposited in GenBank (as of 24 June 2011). The criteria for primer design were as follows:

I. target two areas of conservation less than 300 bp apart

II. product size of around 200-300 bp

III. similar melting points close to 60°C

A total of 20 and 35 sequences were aligned for the Gram-positive and Gram-negative clusters, respectively, using BioEdit Sequence Alignment Editor. The resultant multiple sequence alignments (msa) are provided in Appendix A.I. The RHD_{α} gene commenced at approximately 539 bp in the Gram-positive msa and

approximately 5650 bp for the Gram-negative msa. Two potentially primer pairs were designed for Gram-positive RHD_{α} and three for the Gram-negative cluster based on product size, number of self-dimers, GC content and melting points, which was analysed using PrimerAnalyser (Kalendar *et al.*, 2011). The proposed primer sequences were tested *in-silico* using BLAST-n to test specificity. A summary of the selected primer pairs is provided in Table 3.1.

Table 3.1 Summary of RHD_{α} primer pairs

Primer Pair	Primer	Sequence (5'-3')	Length (bp)	Tm (°C)	GC content (%)	Self-dimers	Product Size (bp)
Gram Positive							
GP-1	GP-524F	TTY GKM WSS BTS GAY CCR MAK G	22	59.1	55	0	156
	GP-659R	CSR MRS BKR WYT TCC AGT TBS C	22	58.8	56	2	
GP-2	GP-524F	TTY GKM WSS BTS GAY CCR MAK G	22	59.1	55	0	160
	GP-662R	TBT CSR MRS BKR WYT TCC AGT T	22	57.1	47	3	
Gram Negative							
GN-1	GN-5625F	TGC ARY TAY CAC GGS TGG	18	56.1	58	0	306
	GN-5913R	TCM RYV GGH RYY TTC CAG TT	20	56.0	50	2	
GN-2	GN-5625F	TGC ARY TAY CAC GGS TGG	18	56.1	58	0	312
	GN-5913X-R	AAR TTT TCM RYV GGH RYY TTC CAG TT	26	58.1	40	2	
GN-3	GN-5679X-F	CCR TTY GAR AAR GRD YKK TAY SRY G	25	57.7	47	1	292
	GN-5952R	TGM GTC CAM CCV AYG TGR TA	20	54.6	53	0	

Primers were tested again using DNA extracted from known PAH degrading bacteria and an environmental contaminated soil sample (TP30). Various numbers of amplifications were attempted using the five sets of primers, including the use of a high fidelity hot start DNA polymerase (Phusion Hot Start II, Thermo Scientific), with little to no success. Similarly to the primers that were already trialled, all primer sets either resulted in multiband profiles of non-specific product that were generally much larger than the target size (ranging from 156–312 bp) or no product was detected at all (Figure 3.12).

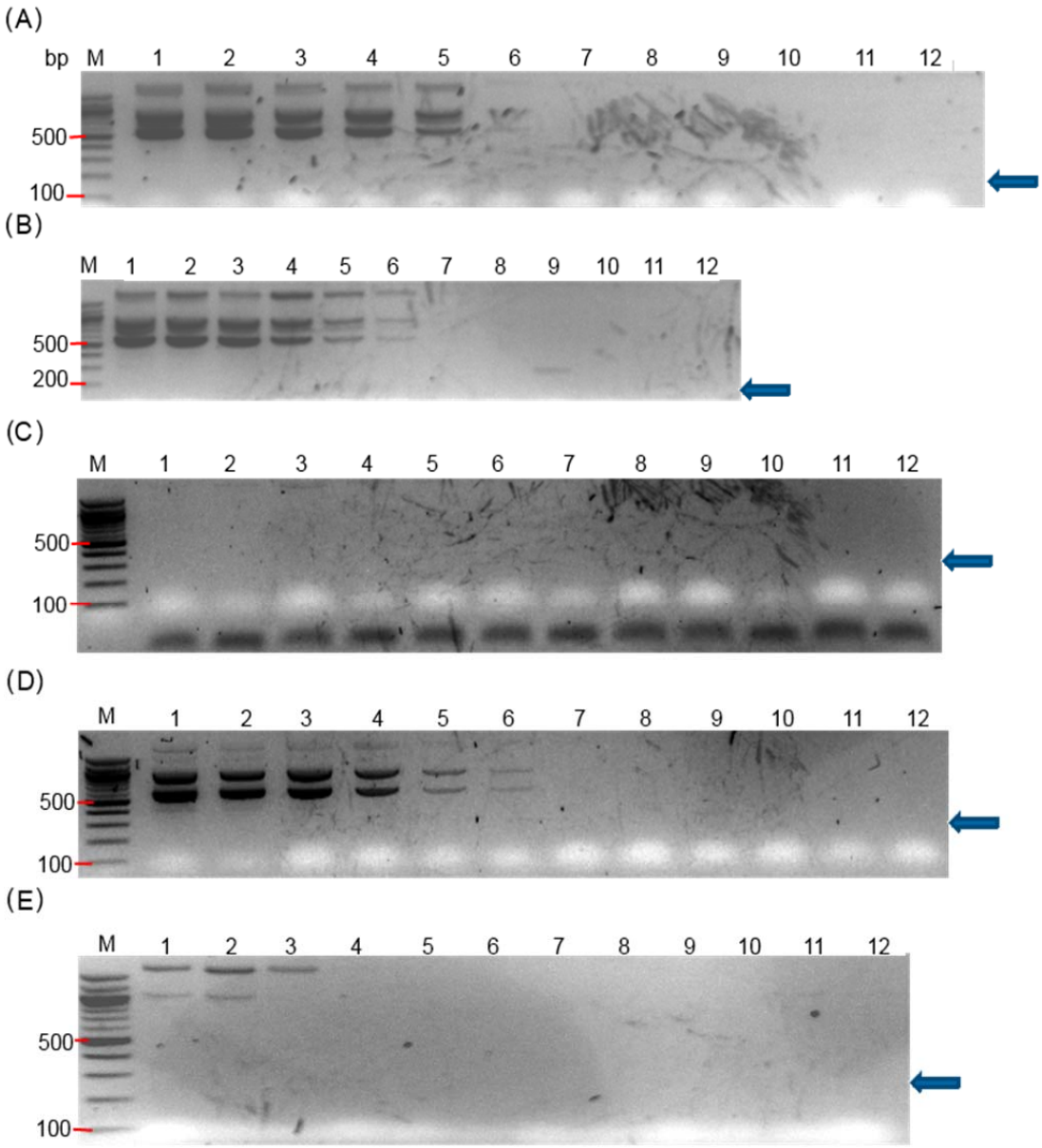

Figure 3.12 PCR amplification of designed primers for Gram-positive and Gram-negative RHD$_{\alpha}$ gene in bacteria. 2.0% agarose gel. M: Marker, Promega 100bp Ladder, ⇨ target band size. (A) GP-1 primer pair target size 160 bp, (B) GP-2 primer pair target size 160 bp, (C) GN-1 primer pair, target size 306 bp, (D) GN-2 primer pair, target size 312 bp, (E) GN-3 primer pair, target size 292 bp. Positive control for Gram-positive primers *M. gilvum;* Positive Control for Gram-negative primers *P. aeruginosa.* Lanes 1–8: gradient PCR of 52°C–64°C with positive control DNA template; Lane 9: Environmental DNA template (TP30 0-1m); Lane 10: opposite positive control DNA template; Lane 11: *Escherichia coli* template DNA; Lane 12: negative control.

Given the lack of success with designed primers amplifying target products in one round of amplification, the plan to use qPCR to quantify the number of phenanthrene degrading species present in the community was not continued. This phase of the project was however not completely discarded as there was the potential that nested PCR, like that required for the amplification of the fungal ITS region, could be used to obtain bands of the correct size. It was also thought that by using nested PCR to add a GC clamp to the 5' end of the forward primer the products could then be profiled using TGGE.

A 40 bp GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GG-3') was added to the forward primers of all pairs that had already been tested. However, only one of each group of the designed primers was selected for this work (GP-1 and GN-3). The first round of PCR was completed with the normal forward and reverse primers, as described in Chapter 2.5.7.3. The first round PCR products were then used as the template for a nested PCR to introduce the GC-clamp. PCR conditions were the same as in the first round except for the addition of DMSO (3%) and a touchdown cycle from 68–58°C during the annealing step to help reduce unspecific primer binding. Figure 3.13 provides the results from both rounds of the nested PCR. The only primer that produced bands of the targeted size was the Gram-negative pair of Cébron *et al*. (2008) (346 bp) but again the darkest bands were present in Gram-positive samples, i.e. the opposite sample of the primer targets (Figure 3.13).

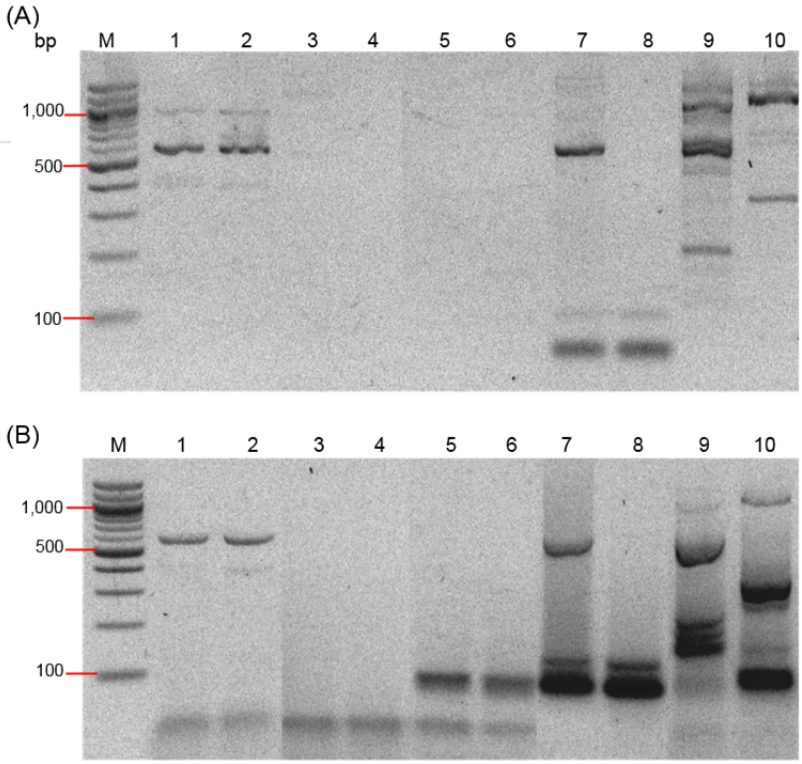

Figure 3.13 Nested PCR amplification of designed primers, Ding *et al*. (2010) and Cébron *et al*. (2008) primers with GC-clamps. 2.0% agarose gel. M: Marker, Promega 100bp Ladder. (A) Round one; (B) Round two. Lane 1: designed Gram-negative primers with Gram-positive template; Lane 2: designed Gram-negative primers with Gram-negative template, target size R1: 292 bp, R2: 332 bp; Lane 3: designed Gram-positive primers with Gram-positive template, target size R1: 156 bp, R2: 196 bp; Lane 4: designed Gram-positive primers with Gram-negative template; Lane 5: Ding primers with Gram-positive template, target size R1: 320 bp, R2: 360 bp; Lane 6: Ding primers with Gram-negative template, target size R1: 320 bp, R2: 360 bp; Lane 7: Cébron Gram-positive primers with Gram-positive template, target size R1: 292 bp, R2: 332 bp; Lane 8: Cébron Gram-positive primers with Gram-negative template; Lane 9: Cébron Gram-negative primers with Gram-positive template; Lane 10: Cébron Gram-negative primers with Gram-negative template, target size R1: 306 bp, R2: 346 bp.

The primers of Cébron *et al.* (2008) were selected for further investigation as they seemed the most promising. Optimisation of these primers was completed using both pure culture extracts and environmental soil samples, resulting in the first round of the nested PCR being completed as above. This was then run on an agarose gel and the products of the correct size (306 bp for Gram-negative and 292 bp for Gram-positive) excised and eluted using a QIAquick gel extraction kit (Qiagen). The eluted template was then subjected to another round of PCR with the GC-clamped forward primer, normal reverse primer and increased $MgCl_2$ (0.5 mM) concentration. Figure 3.14 displays both rounds of the optimised nested PCR from environmental soil extracts. This method was applied to both DNA and

cDNA and resultant PCR products were then subjected to TGGE as part of further investigations within Chapter 5.

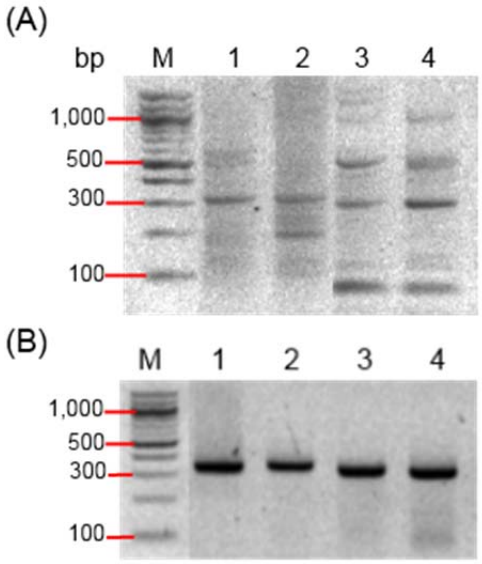

Figure 3.14 Optimised Nested PCR amplification of GC-clamped Cébron *et al.* (2008) primers from environmental soil DNA extract. 2.0% agarose gel. M: Marker, Promega 100bp Ladder. (A) Round one, target size GN-306 bp, GP-346 bp; (B) Round two, target size GN-292 bp, GP-332 bp. Lane 1: Gram-negative primers, TP30 0-1m template; Lane 2: Gram-negative primers, TP30 1-2m template; Lane 3: Gram-positive primers, TP30 0-1m template; Lane 4: Gram-positive primers, TP30 1-2m template.

3.3 Comparison of TGGE and DGGE

One of the main culture-independent applications employed throughout this project was fingerprinting of the microbial communities using gradient gel electrophoresis. Most commonly, denaturing gradient gel electrophoresis (DGGE) is used, which utilises a chemical gradient to denature DNA molecules based on sequence and resultant melting behaviours. However, one of the main drawbacks of DGGE is the lack of reproducibility of chemical gradients across multiple gels (Rosenbaum and Riesner, 1987; Viglasky, 2013). An alternative to chemical gradients was first described in the late 1980's which utilised a temperature gradient (TGGE) to denature molecules (Rosenbaum and Riesner, 1987). This was a major advance in reproducibility as the temperature could be set at the same graduation of multiple gels enabling a large increase in the number of communities that could be assessed as there was no longer a limitation of only being able to analyse one gel. Aside from the increase in

reproducibility and reliability of gradients, TGGE offers a decreased set up time, along with a reduction in consumable cost and a considerable reduction in sample volume required (Viglasky, 2013).

The use of DGGE for community profiling was standard practice within the laboratory but little work had been undertaken into TGGE. Given the potential for TGGE to be a valuable tool in increasing the number of samples analysed while reducing processing time, development of a reliable method for running TGGE for bacterial and fungal communities was required. TGGE requires around half the sample volume of DGGE and therefore there may be the potential for TGGE to underestimate the community diversity by not detecting low density members of the community. Therefore an investigation into whether there was a difference in the diversity of communities when analysed by the two different fingerprinting tools was completed.

Optimisation of TGGE run times and gradients were completed for each PCR target. TGGE gels were cast and electrophoresis set up according to the manufacture's instruction, see Chapter 2.6.9. The run time of TGGE was attempted to be shortened, however times less than 8 hours did not provide sufficient separation of bands to allow for useful interpretation, regardless of the temperature gradient or product size. Gradients were optimised using a trial and error process so that the following optimised gradients were determined for each PCR product (Table 3.2).

Table 3.2 Optimised TGGE gradients for specific PCR products used in this project

Primer Pairs		Target	Optimised Gradient
341F-GC	518R	Bacterial 16S rRNA	52.5–65.0°C
ITS1F-GC	ITS2	Fungal ITS of 18S rRNA	50.0–58.0°C
GN-F-GC	GN-R	Bacteria Gram-negative RHD_{α}	51.0–60.0°C
GP-F-GC	GP-R	Bacteria Gram-negative RHD_{α}	51.0–60.0°C

The next phase was to compare the diversity results from identical samples run on TGGE and DGGE to determine if there was a difference in community diversity based on the analysis tool used. Soil samples used for this experiment were from a hydrocarbon contaminated (TP30) and pristine (TP4) sandy soil across varying sample depths. DNA was extracted and amplified using 18S rRNA ITS primers as per the optimised methods described in Chapter 2.6. Products, in duplicate were loaded on a 40–60% gradient DGGE gel and a 50.0–58.0°C gradient TGGE. Gels were silver stained, scanned and analysed using TotalLab analysis package (Version TL120; Nonlinear Dynamics, USA) and are shown in Figure 3.15. The community diversity and equitability indices were calculated and presented in Figure 3.16.

The banding patterns between the two gels types appeared to vary, however the diversity indices do not seem to be affected. The diversity indices calculated from the TGGE gel were slightly higher than those from DGGE gels for the pristine soil with no trend observed for the contaminated soil. There was no trend showing that equitability indices were dependent on the analysis method either. Statistical analysis of the indices demonstrated that there was no significant difference between the diversity and equitability of a community when determined by TGGE or DGGE (Student *t*-test, $p>0.05$).

Given the above, it was determined that although TGGE uses greatly reduced sample volumes this does not bias the community profile and is therefore a reliable method for community profiling which has many advantages over DGGE, including reproducibility and processing time.

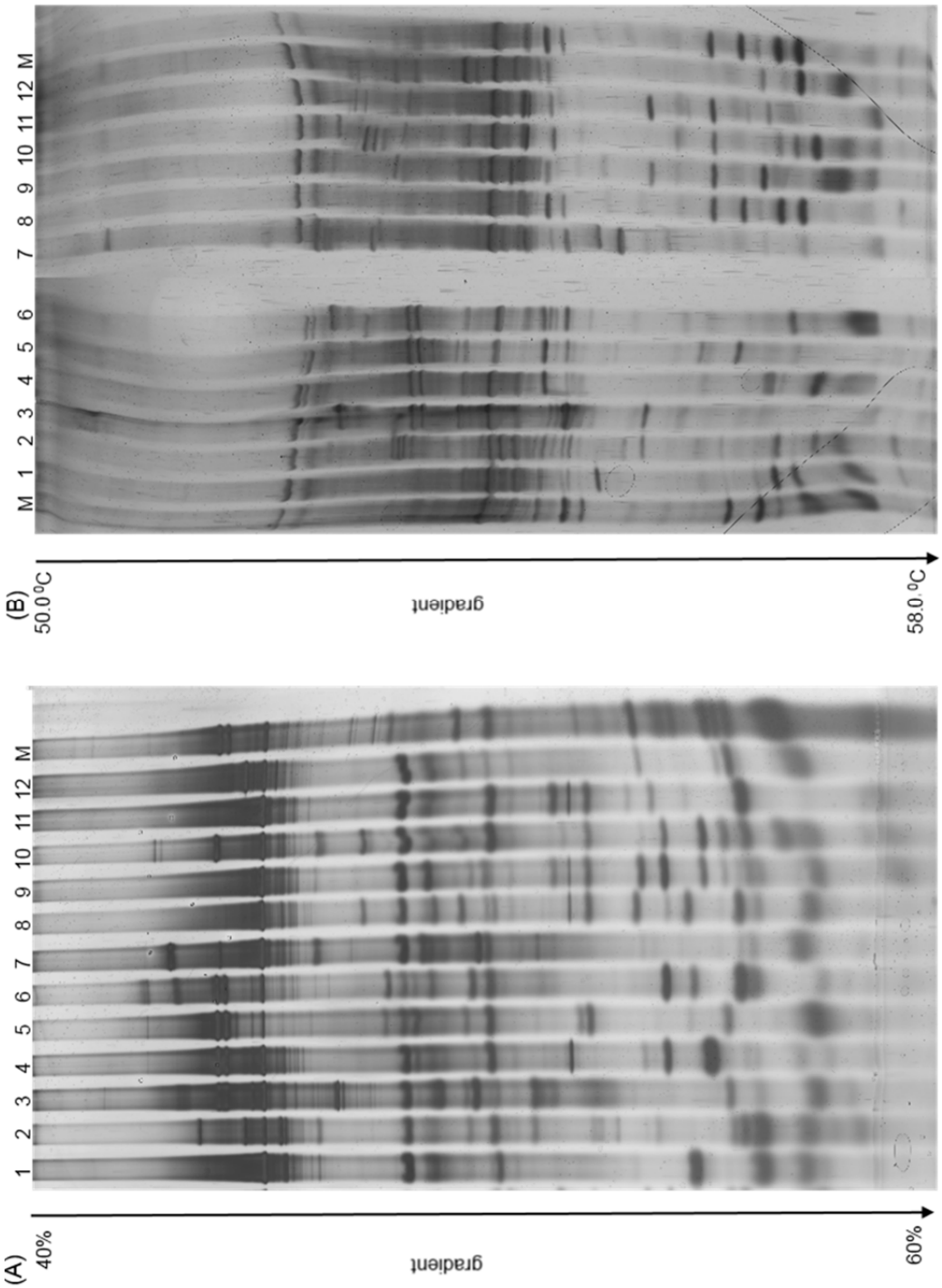

Figure 3.15 Comparison of DGGE and TGEE of same fungal ITS products. M: Marker Lane, (A) DGGE gradient 40 – 60 %, (B) TGGE gradient 50.0 – 58.0ºC. Lanes 1 – 2: TP4 1.0m; Lanes 3 – 4: TP4 1.5m; Lanes 5 – 6: TP4 1.5m; Lanes 7 – 8: TP30 1.0m; Lanes 9 – 10: TP30 1.5m; Lanes 11 – 12: TP30 1.5m.

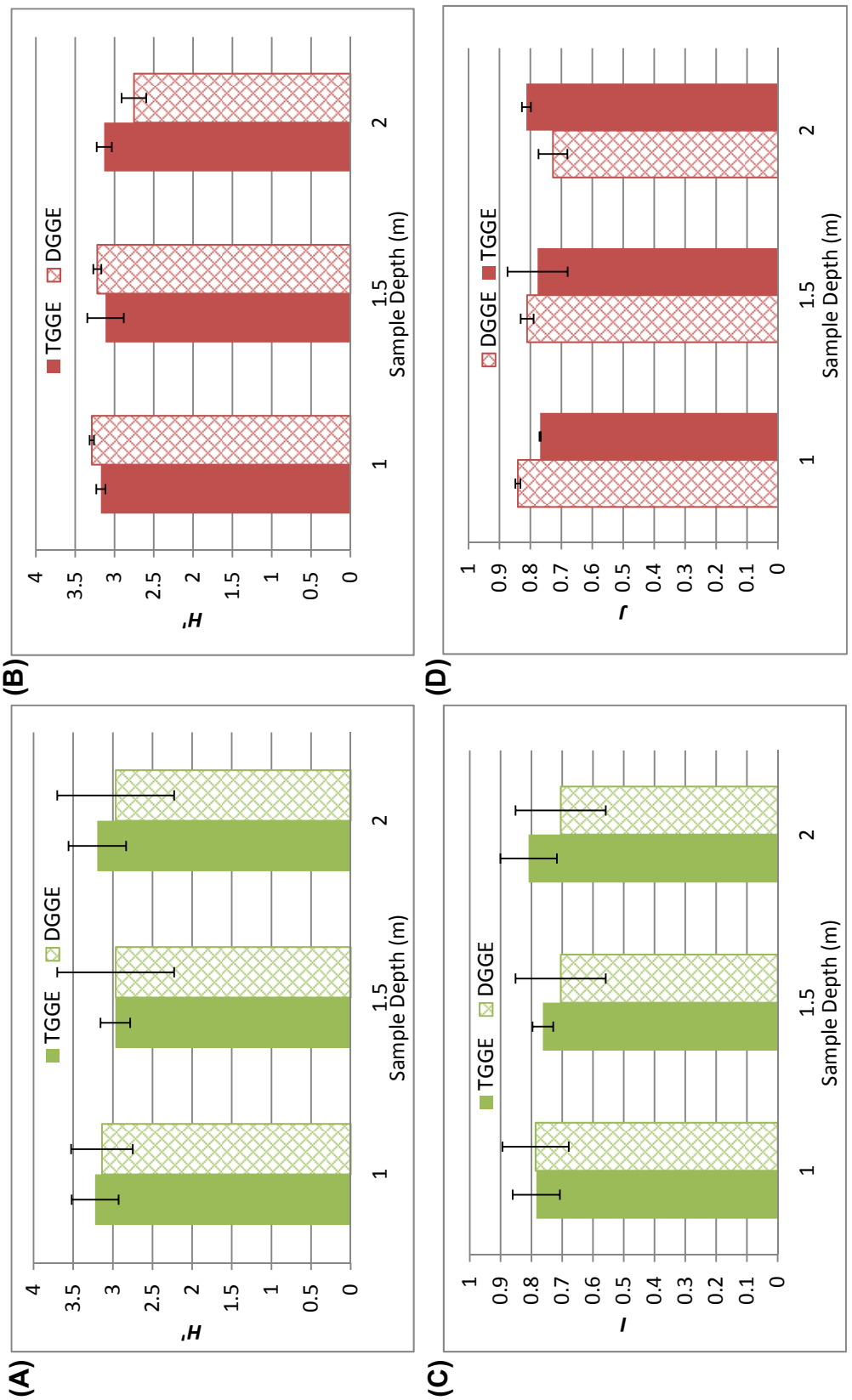

Figure 3.16 Comparison of Shannon-Weaver diversity (*H'*) and Equitability Indices (J) for pristine (TP4: A and C) and contaminated (TP30: B and D) soils. n= 2

3.4 Conclusion

The key basis of this project was to use molecular microbial ecology to investigate microbial community dynamics, through the application of stable isotope probing (SIP). SIP requires that the nucleic extraction method used produces extracts of high quality and concentration. Two soil types, clayey and sandy, were the focus of this project and given that clayey soils bind DNA tightly to particles two different extraction techniques were required, one to extract DNA from sandy soils and a second to extract DNA from clayey soils with humic substances.

During extraction method development, an interesting side note was discovered. The number of culturable bacteria was generally higher in the clayey soils (10^3 and 10^6 organisms per gram of soil) tested using MPN method compared to the sandy soils (10^2–10^3 organisms per gram of soil). Furthermore, the shallow clay sample had an increased number of organisms compared to all other samples tested.

Several extraction methods were trialled from both clayey and sandy soils, including commercially available kits. It was discovered that although the commercial kits extracted DNA of high quality from both soil types the concentration was insufficient to be utilised for downstream SIP applications that cannot rely on PCR amplification to increase sample concentration. A method adapted from Lueders *et al*. (2004b) was optimised such that both DNA and RNA were co-extracted from sandy soils.

Development of an extraction method from clayey soils was achieved; however concentrations were not sufficient for SIP applications and therefore it was decided that the sandy soil type would be utilised in further experiments involving SIP (Chapter 5). The clayey soils and associated extraction method would be utilised in experiments looking at any difference in community dynamics based on soil type and contamination levels (Chapter 4).

Development of PCR that could target the alpha subunit of the Ring-Hydroxylating Dioxygenase enzyme required testing of several different primers

and an attempt was made to design specific primers. All attempts resulted in non-specific binding of primers and non-dominant bands of interest. It was hoped that these primers could be used in qPCR but the lack of specify led to curtailment of this approach. However, through the use of nested PCR a GC-clamp was successfully joined to the 5'-end of the forward primers designed by Cébron *et al*. (2008) enabling the products to be profiled using gradient gel electrophoresis (TGGE and DGGE).

Optimisation and comparison of the widely used DGGE with the less used TGGE revealed that although the banding patterns of the two profiling techniques appeared different there was no statistical difference in the diversity and equitability of a community when determined by TGGE or DGGE (Student t-test, $p>0.05$). Therefore it was confirmed that TGGE would be a valued profiling tool for this project.

The methods developed and optimised in this chapter are to be employed in the remaining chapters of this project, Chapter 4 will utilises the DNA extraction protocols and TGGE to investigate the difference, if any, in community profiles of different soil types with differing contamination levels, and Chapter 5 uses all methods developed to determine if a prediction can be made about how a sandy soil will adapt to multiple contamination events.

4. Fungal Community Structure of Different Soil Types Contaminated with Petroleum Hydrocarbons

4.1 Introduction

Crude oil is a complex mixture of total petroleum hydrocarbons (TPH), including aliphatics, aromatics, resins and asphaltenes (Sugiura *et al.*, 1997; Philp, 2005). A key contaminant of concern within crude oil mixtures are polycyclic aromatic hydrocarbons (PAHs) due to their hydrophobicity, low water solubility, strong tendency to absorb to the soil matrix and their significant threat to human health due to mutagenic and carcinogenic properties (Juhasz *et al.*, 2005; Muckian *et al.*, 2007; Frenzel *et al.*, 2009; Muckian *et al.*, 2009). PAHs are therefore key targets for remediation strategies.

Microorganisms with the ability to degrade anthropogenic contaminants are ubiquitous within the natural soil environment (Scullion, 2006). A broad range of bacteria, fungi and algae are capable of TPH and PAH degradation. An increasing number of remediation technologies are exploiting the natural microbial activity within the environment to degrade these contaminants. Therefore developing a better understanding of the structures of these communities and how the indigenous organisms may differ based on contaminant levels is an important step for design of more efficient and effective remediation strategies of contaminated soils.

Literature suggests that an important initial step in the degradation of high molecular weight hydrocarbons such as PAHs relies on the action of fungal extracellular enzymes such as oxidoreductases, laccases and lignin peroxidases (Cerniglia, 1993; Anderson and Cairney, 2004; Scullion, 2006; Balaji *et al.*, 2014). These enzymes can break the pollutant down to smaller metabolites, thus removing the limiting step of pollutant incorporation into the cell, as is the case for bacterial degradation (Scullion, 2006). The smaller compounds can then undergo further metabolism by other fungi and/or bacteria (Smit *et al.*, 1999; Canet *et al.*, 2001; Silva *et al.*, 2009).

Most research activities have been focused on bacteria to the detriment of fungi, despite their key roles in hydrocarbon and PAH degradation. Therefore an investigation of the fungal community was the focus of this chapter in an attempt to bridge the knowledge gap of fungal community structure and activity in contaminated soils. Within this chapter, molecular microbial ecology methods were used to investigate the taxonomic diversity, functional diversity and dynamics of indigenous surface and subsurface fungal communities of two different soil types (clayey and sandy) with varying levels of contamination.

4.2 Methods

4.2.1 Site and sampling location

Soil samples used in this project were obtained from a former oil refinery on the coast of South Australia. Samples were collected from two main areas within the site, one area was a sandy beach surrounding a wharf historically used to load large oil tankers (samples labelled TP and collected in October 2007). The second sampling area was located in a former tank farm (samples labelled FTF and collected in June 2008) where spillages of crude oil were known to have occurred (see Figure 4.1 and Figure 4.2 for sample locations). Six test pits locations, 3 pits at each sampling location, were excavated to nominal depths of 2.0 and 3.0 m below ground level using a backhoe. Soil samples (1 kg) were collected from the bucket and stored in appropriate containers and temperature (- 80°C).

Figure 4.1: **Locations of Test Pits for Sandy Soils**

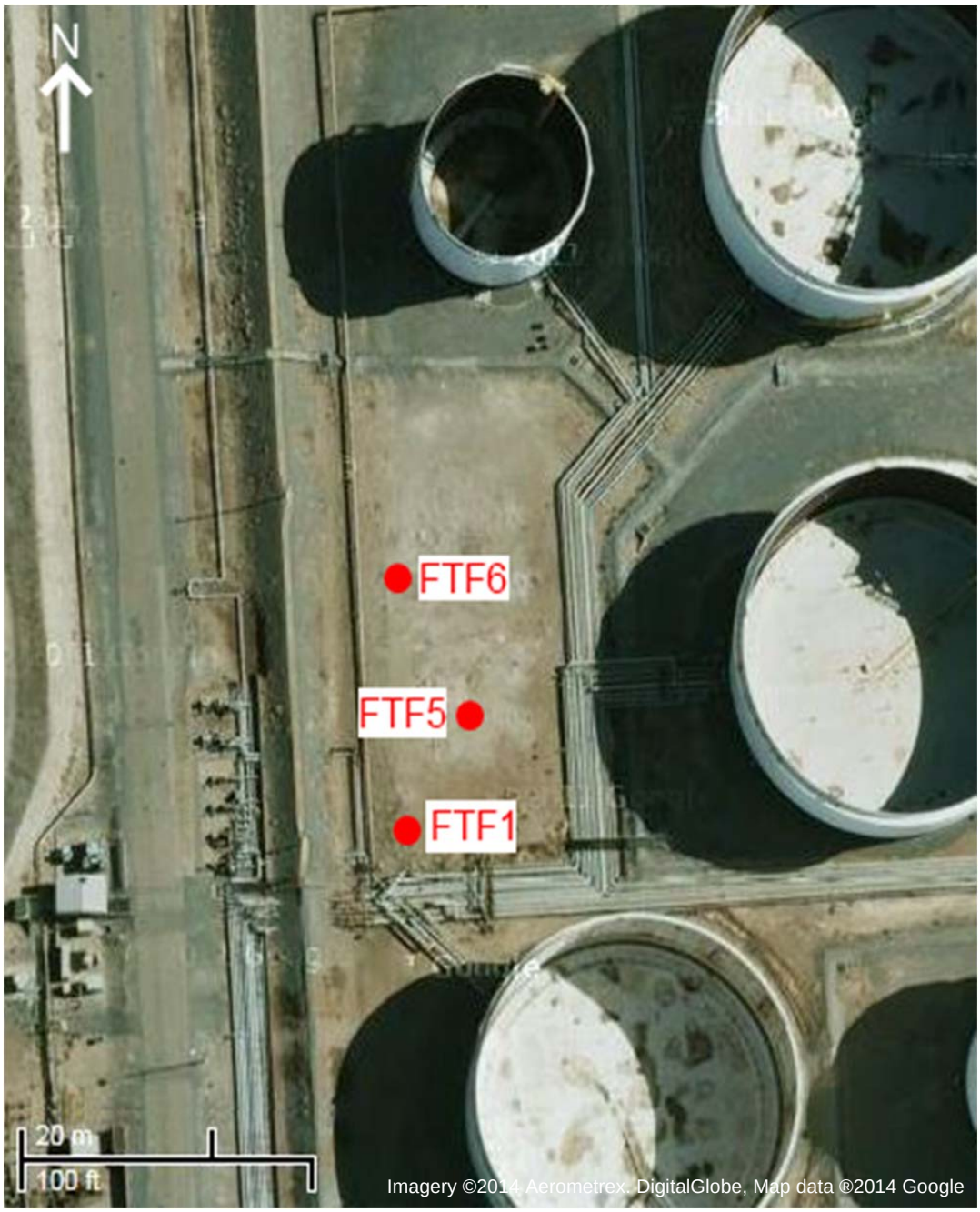

Figure 4.2: **Locations of Test Pits for Clayey Soils**

4.2.2 Physio-chemical analysis of soils

Sub-samples of soils from varying depths within each test pit location were stored in laboratory supplied containers and transported to a National Association of Testing Authorities (NATA) accredited laboratory for chemical analysis. Soil samples were tested for the presence of heavy metals, total petroleum hydrocarbons (TPH), benzene, toluene, ethyl benzene, xylene (BTEX),

halogenated aliphatic and aromatic compounds, phenolic compounds and PAHs using methods governed by Australian Standards and the National Environment Protection Measure (NEPM) guidelines (National Environmental Protection Council, 1999). The following depths were analysed for each test pit: TP4 (1.5 m, 2.0 m); TP9 (1.0 m, 1.5 m, 2.0 m); TP30 (1.0 m, 1.5 m, 2.0 m); FTF1 (0.5 m, 1.0 m, 2.0 m); FTF5 (0.5 m, 1.5 m, 3.0 m), FTF6 (0.0 m, 0.5 m, 2.5 m). It should be noted that as this testing was completed as part of a different project not every depth that was used in community profiling was analysed for contaminant concentration.

The results of the laboratory testing were used to score (low or no, medium and high) the relative contamination level of the soil compared to the other soil samples of similar type.

Soils were also tested for pH, moisture content and classified according to the USCS system and potential hydrocarbon contamination indicators noted (Chapter 2.1).

4.2.3 Fungal Community Profiling

Soil samples from three depths (1.0 m, 1.5 m and 2.0 m) for sandy soils and seven depths (0.0 m, 0.5 m, 1.0 m, 1.5 m, 2.0 m, 2.5 m and 3.0 m) for clayey soils at each location were selected for fungal community profiling. The variability in sample retrieval and depth across the locations was outside the control of the researcher as the soils were retrieved as part of a contaminated site investigation. The location of the sandy soils had no known history of underground disturbance therefore the excavation to 2.0 m was considered sufficient for site investigation purposes, soil samples from this location were not collected between surface and one metre due to site limitations. The clayey soils were sampled from within a former tank farm which contained underground product lines; therefore the extent of investigation was increased to 3.0 m.

Samples were stored on ice and DNA was extracted using mechanical lysis and precipitation based on soil type (Chapter 2.6.1).

Nested PCR amplifications targeting the fungal 5.8S rRNA ITS region were carried out using primer pairs EF4 with ITS4 and ITS1F with ITS2. A 40 bp GC clamp was included on the ITS1F primer to allow for separation using TGGE. Template DNA was used at a dilution of 1:10 for round one of the nested PCR (refer to Chapter 2.6.4 for specific cycling conditions). Template was diluted to reduce the effect of PCR inhibitors co-extracted during DNA extraction.

Five to ten microlitres of PCR products were loaded in duplicate, volumes loaded were dependent on band intensity in an agarose gel, and electrophoresed for 8 h using a temperature gradient of 50.0°C to 58.0°C. The resultant gels were silver stained and scanned for diversity analysis (Chapter 2.6.7.1 and 2.6.8). Shannon Weaver diversity and Equitability Indices were calculated as well as Pareto-Lorenz evenness curves and a UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced using the TotalLab Software package (Chapter 2.6.10). Representative bands of interested were excised from a re-run TGGE gel stained using SYBR Gold and sent for sequencing analysis.

Amplification of key fungal PAH degrading enzymes (lignin peroxidase, manganese-dependent peroxidase and laccase) was attempted using primer pairs LiP (Reddy and D'Souza, 1998), MnP (Bogan *et al.*, 1996) and *lcc* (Bogan *et al.*, 1996) but were unsuccessful due to unspecific primer binding resulting in multi-banded profiles with bands of the correct size not being the most dominant.

4.3 Results

4.3.1 Physio-chemical analysis of soils

Results of pH and moisture content testing of the soils is provided in Figure 4.3. These results were used to assist in the classification of the soil based on the dominant soil type, during which the appearance and indication of potential contamination were also noted (See Table 4.1 for classification of the test soils).

The pH was similar in both sandy and clayey soils and was slightly basic (ranging from 8.41 to 8.84 in sandy soils and 8.0 to 8.8 in clayey soils); there appeared to

be no depth dependent trend. The moisture content was much higher in clayey soils; values ranged between 13.9 to 22.5%, compared to sandy soils (values between 3.1 to 10.2%). This is consistent with the different characteristics and properties of the two soil types (Heath, 2004). The moisture content appears to increase with depth in sandy soils whereas that of clayey soils was generally decreasing, with the exception of FTF5 which increased in moisture content at 2.0 m and then decreased.

Table 4.1 Classification of test soils

Sample	Major Soil Group	Description	Potential contamination indicators	Contami-nation Level
TP4	Sand	Orange medium grained, well graded sand. Increasing clay with depth, around 2.0 m	None observed	Low
TP9	Sand	Yellow/brown medium grained, well graded sand	Increasing hydrocarbon odour, slight black staining with depth (≥ 1.5 m)	Medium
TP30	Sand	Yellow/brown medium grained, well graded sand	Dark black staining present ≥ 1.5 m. Strong hydrocarbon odour	High
FTF1	Clay	Brown/black silty clay, fine grained, plasticity increasing with depth	None observed	Low
FTF5	Clay	Brown/black clay, fine grained, medium plasticity	Slight hydrocarbon odour	Medium
FTF6	Clay	Red/brown clay, fine grained, low–medium plasticity	Strong hydrocarbon odour	High

NB: Contamination level was determined using chemical analysis results and scored relative to other samples of the same soil type (see Table 4.2).

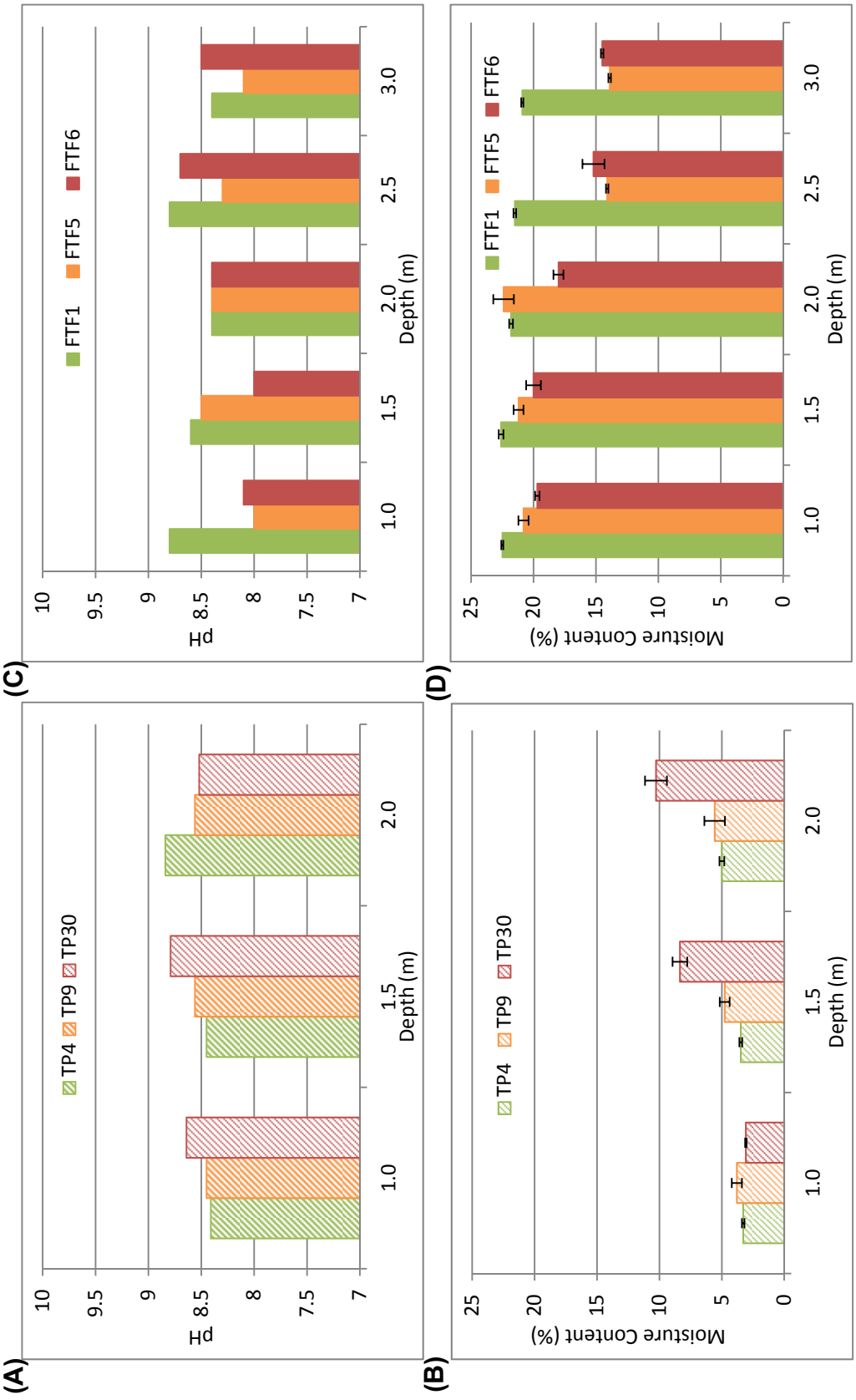

Figure 4.3 Comparison of pH and moisture content for sandy and clayey soils across varying sample depths. n=3 (A) pH of sandy soils, (B) moisture content of sandy soils, (C) pH of clayey soils, (D) moisture content of clayey soils

Results of the laboratory chemical testing are provided in Appendix A.2 with results compared against current legislative contamination guideline levels (the NEPM health and ecological values for industrial sites) (National Environmental Protection Council, 1999). A summary of the key contaminant concentrations (TPH, BTEX and PAHs) within the test soils is provided Table 4.2. All other analytes (metals, phenols and other hydrocarbons) were generally below the limit of laboratory reporting (LOR) or NEPM guidelines.

The sandy soils were generally less contaminated compared to the clayey soils with lighter end TPH fractions (C_6–C_{28}) detected in TP9 and TP30 in depths less than 1.5 m, as well as total PAH detected in the 1.5 m TP30 sample. TPH, BTEX and Total PAH were detected in FTF5 and FTF6 clayey soil samples, with the highest concentrations being in FTF6 at 2.5 m. Samples from TP30, FTF5 and FTF6 had concentrations of TPH, BTEX and Total PAH above the NEPM Health Investigation Guideline levels for industrial soils indicating that samples have concentrations of petroleum based hydrocarbons at levels that could present a health risk. Based on the analytical chemical results the soils were classified into relative contamination levels (low, medium or high); refer to Table 4.1.

Table 4.2 Summary of key contaminant concentrations (mg.kg^{-1}) in test soils

Sample	Depth (m)	TPH					B	T	E	X	Total PAH
		C_6-C_9	C_{10}-C_{14}	C_{15}-C_{28}	C_{29}-C_{36}	C_{10}-C_{36}					
Sandy Soils											
TP4	1.5	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	2.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
TP9	1.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	-
	1.5	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	2.0	**44**	**180**	**640**	<LOR	**870**	<LOR	<LOR	<LOR	<LOR	<LOR
TP30	1.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	1.5	**34**	**500**	**1230**	<LOR	**1780**	<LOR	<LOR	<LOR	<LOR	**1.2**
	2.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
Clayey Soils											
FTF1	0.5	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	1.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	2.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	-
FTF5	0.5	**65**	**50**	<LOR	<LOR	**150**	<LOR	**3.2**	**0.6**	**4**	<LOR
	1.5	**1420**	**2790**	<LOR	<LOR	**2890**	<LOR	**39.6**	**34.7**	**589**	-
	3.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	**0.6**	**8.4**
FTF6	0.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	0.5	**4580**	**4260**	<LOR	<LOR	**4360**	**43.2**	**734**	**168**	**1295**	**25.2**
	2.5	<LOR	**440**	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	**0.7**	<LOR

NB: LOR limit of reporting;-not analysed; shaded boxes are values above the NEPM HIL guidelines.

4.3.2 Fungal Community Profiles

4.3.2.1 Sandy Soils

The TGGE gel of all three sandy soils across varying depths is provided in Figure 4.4 and the resultant UPGMA and diversity are provided in Figure 4.5 and Figure 4.6, respectively. The fungal profiles shows there were several bands that were present in all contaminant levels and sample depths. The majority of the bands present across all samples were the most dominant bands in the profile. There appeared to be no large shift in community profile across sample depth or contaminant level. There was good correlation in banding patterns between duplicates for all 1.0 m samples; there was some variability in duplicate banding for other samples although most duplicates were grouped within the same clade.

The Shannon Weaver diversity (H') and Equitability Indices (J) were similar across all contamination levels, with the highest values being in the 1.5 m samples, with the exception of equitability in TP4 at 2.0 m and diversity of TP9 also at 2.0 m (Figure 4.6A&B). The more contaminated test pit (TP30) had a statistically significantly higher diversity at 1.0 m compared to the other test pits depths (TP30 *H'* value 3.22±0.06, Student *t*-test $p>0.05$). There was no statistical difference between the equitability and diversity of TP4 and TP9 (Student *t*-test $p<0.05$). This was mirrored in the UPGMA analysis (Figure 4.5) where TP4 and TP9 samples tended to cluster together. The dendrogram also demonstrates that the two clusters formed were more related to sample depth than contamination level. The deeper samples from TP4 (1.5 m) and TP9 (1.5-2.0 m) and all samples from TP30 formed one cluster and the remaining samples formed the second.

Functional organisation of the different samples was interpreted using PL evenness curves (Figure 4.6C-D). The Fo for sandy soils ranged from 60% to 76%, with the highest Fo in all test pits being the shallower sample (1.0 m). This suggests that all soils have medium to high functional organisation, which slightly increases with depth. Therefore in the deeper sandy samples there are several species that are dominant and present in high numbers in the

community; however other species are still present in numbers to retain functional diversity, given that the higher the Fo and the further the PL curve deviates from the perfect evenness line, the more specialised the community is (Marzorati *et al.*, 2008). The numbers of non-dominant species decreases with sample depth such that the deeper samples tended to have the lowest Fo. This is supported by the low Equitability Index values for the shallower samples.

The highest contamination level soil (TP30) has the most even community (i.e. lowest average Fo of 66%); the Fo increased as the contamination level decreased, such that TP4 had the highest average Fo (72%). This suggests that the higher the contamination level the more even the community was.

The range-weighted richness (Rr) of each sample was calculated and averaged to determine the habitability of the community (Figure 4.6F). The Rr of the most contaminated test pit, TP30, was the highest across all depths and ranged from $13.8(\pm1.3)x10^2$ to $17.7(\pm3.0)x\ 10^2$. This indicates that the community is medium, meaning that this soil has the ability to sustain a medium range of different fungal species. All other soils had a low Rr suggesting that these environments prevented colonisation and restricted fungal diversity.

After analysis of the dynamics of the test pits, five representative bands of interest were excised from the TGGE and sequenced to determine the identity of the dominant organisms present in the samples (bands in red boxes in Figure 4.4). Sequences were matched with the highest identity score in NCBI (Table 4.3). Final sequences of excised bands can be found in Appendix A.3.

Table 4.3 Summary of sequence identifications of bands excised from TGGE gel

Band	Accession Number	Closest Match	Identity (%)	Taxonomic Phylum/ Class
1	JN659472.1	*Eurotiales sp.* AP-2012 strain ThNM026	92	*Ascomycota/ Eurotiomycetes*
2	KC812355.1	*Aspergillus sp.* 2 GP-2013 strain HC1	94	*Ascomycota/ Eurotiomycetes*
3	EU167606.1	*Mycosphaerella pyri* strain CBS 100.86	93	*Ascomycota/ Dothideomycetes*
4	FR799279.1	*Plastismatia glauca*	89	*Ascomycota/ Lecanoromycetes*
5	HE864321.1	*Aspergillus fumigatus*	99	*Ascomycota/ Eurotiomycetes*

The fungal species that were present across all test pits were identified as *Eurotiales sp., Mycosphaerella pyri* and *Aspergillus fumigatus* (bands 1, 3 & 5). It should be noted that identity matches below 99% are considered ambiguous and therefore the identities may represent a related species to those matched. Furthermore, the species sequence may in fact be representative of novel or previously uncharacterised organisms. The *Eurotiales*-like organism was identified as the species with the highest abundance, where abundance was measured as the number of times a particular band appeared in all 18 lanes of Figure 4.4. Band 2, identified as an *Aspergillus*-like *sp.* was found in the deeper TP4 sample and all TP9 samples. Band 4 was present in only the deeper samples of TP30 and was related to *Plastismatia glauca*. The majority of the organisms identified were from the class Eurotiomycetes.

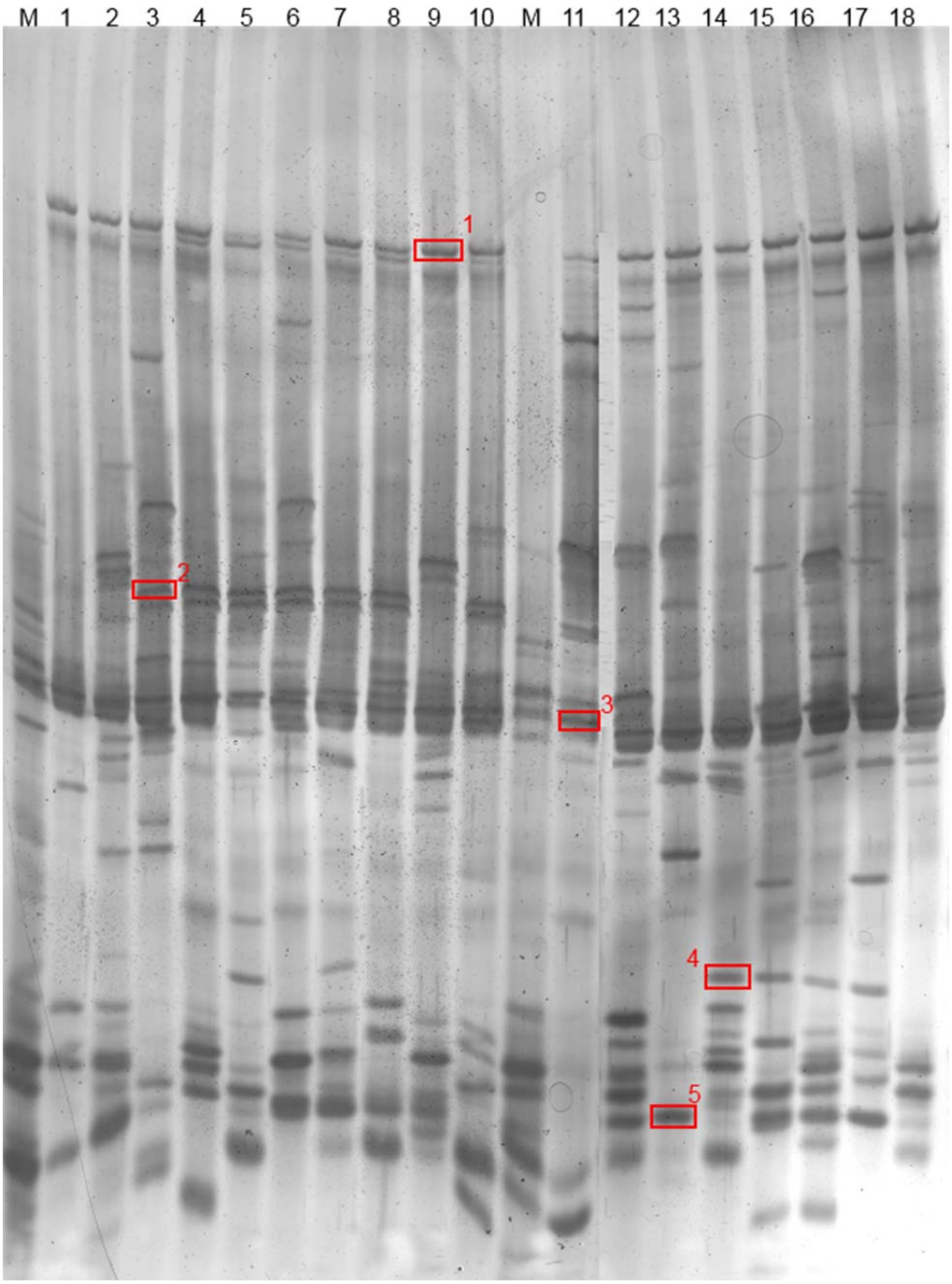

Figure 4.4 TGGE (50.0–58.0°C) of fungal community from sandy soil type. M: Marker. Lanes 1-2: TP4 1.0 m; Lanes 3-4: TP4 1.5 m; Lanes 5-6: TP4 2.0 m; Lanes 7-8: TP9 1.0 m; Lanes 9-10: TP9 1.5 m; Lanes 11-12: TP9 2.0 m; Lanes 13-14: TP30 1.0 m; Lanes 15-16: TP30 1.5 m; Lanes 17-18: TP30 2.0 m. Bands in red boxes were excised for sequencing.

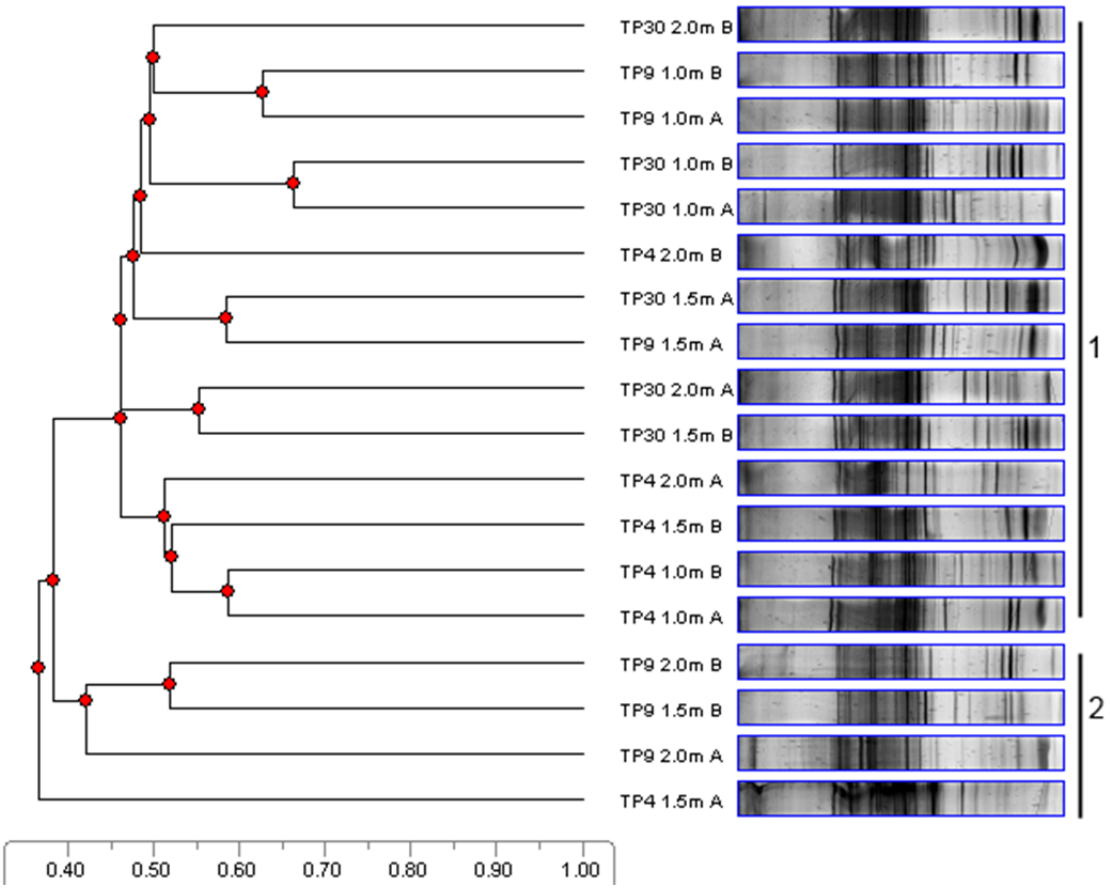

Figure 4.5 UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of rRNA ITS amplification from sandy soils. n=2 The scale bar represents similarity as a proportion of 1 while numbers 1 and 2 represent different fungal community clusters.

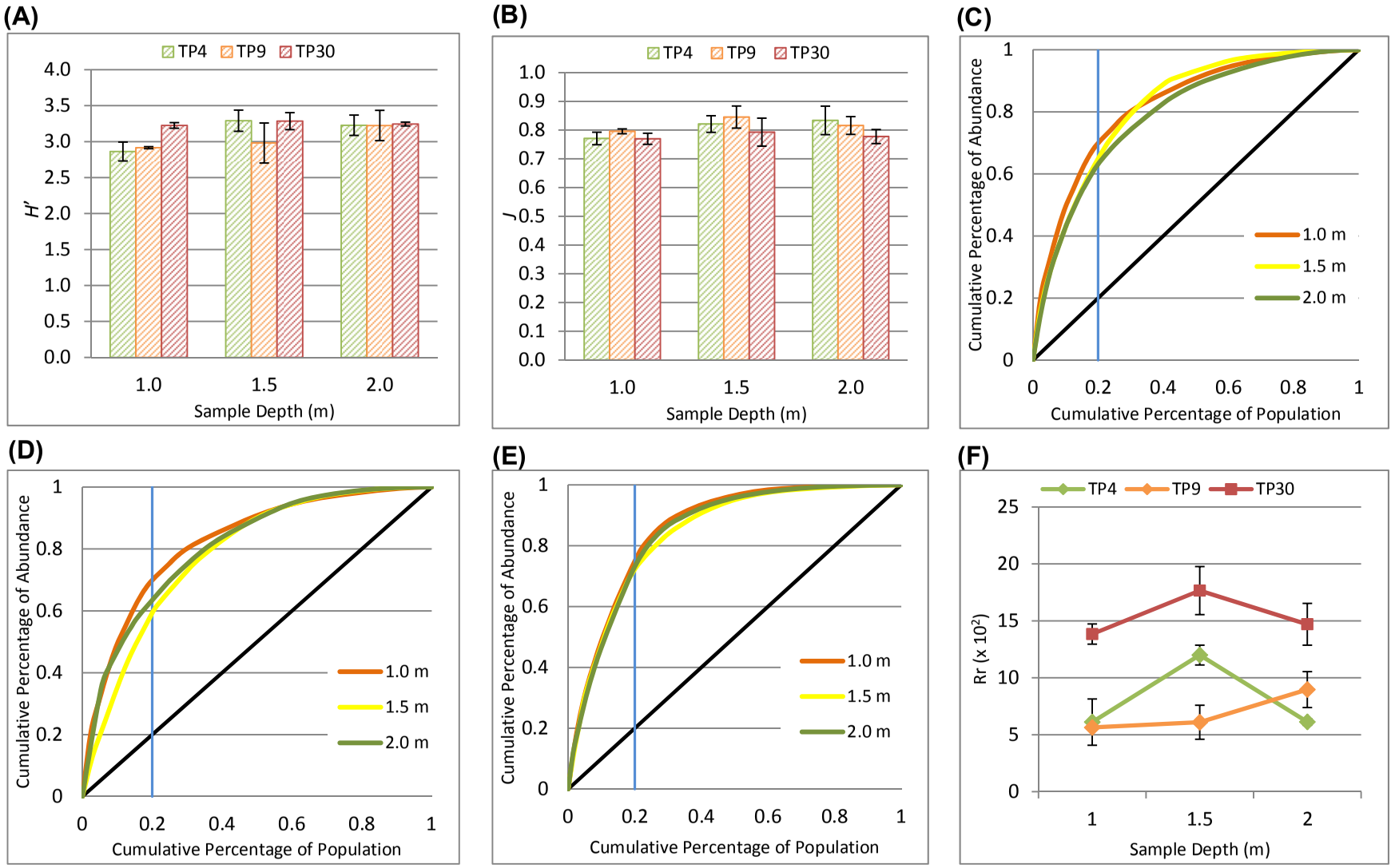

Figure 4.6 Shannon Weaver Indices and Functional Organisation curves for sandy soils. n=2 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve for TP4, (D) Fo for TP9, (E) Fo curve for TP30; (F) Range-weighted richness (Rr) for sandy soils. The black 45° line in C-E indicates the perfect evenness line of a population.

4.3.2.2 Clayey Soils

The TGGE gel of all three clayey soils across varying depths is provided in Figure 4.7 and the resultant UPGMA and dynamics are provided in Figure 4.8 and Figure 4.9, respectively. The fungal profiles show there were a few dominant species that were present in all contaminant levels and sample depths. The banding patterns of FTF1 and FTF5 appeared similar whereas FTF6 had a different pattern. There was good correlation in banding patterns between duplicates, with the exception of FTF6 2.5 m samples.

The UPGMA analysis (Figure 4.8) did not reveal any distinct clustering of samples dependent on contamination level or diversity, as the seven clusters formed contained various sample depths for all three test pits (or contamination levels).

Generally the shallow samples were the most diverse and even (*H'* and *J* values ranged from 3.62 (±0.14) and 0.79 (±0.02) at FTF5 to 3.78 (±0.18) and 0.86 (±0.01) in FTF6) and these values decreased with depth; however there was a slight increase in diversity at around 1.5–2.0 m for all test pits (Figure 4.9A-B). The diversity and equitability of FTF5 greatly decreased at 1.5 m in comparison to the other test pits (*H'*=2.17±0.81; *J*=0.46±0.18). This sample depth corresponds to the highest contamination concentration at this location. A comparison of the diversity and equitability of the three test pits demonstrated that there was no statistically significant difference between FTF1 and FTF5 but there was a difference between FTF6 at the other test pits (Student *t*-test $p>0.05$).

The functional organisation of the different samples was interpreted using PL evenness curves (Figure 4.9C-D). The Fo for clayey soils ranged from 62% to 87%, with the lowest Fo in all test pits being the shallowest sample (0.0 m). The majority of the communities had a Fo above 70% meaning that the clayey soils had a high functional organisation, which generally increased with depth, meaning that the shallower samples have fewer species that were dominant with other members of the community present in high enough numbers to retain functional diversity. The functional diversity tended to decrease with

depth such that the 2.5 or 3.0 m samples had the highest Fo, with the exception of FTF6 where the least even community was in the 1.5 m sample. This was supported by the fact that the Shannon Weaver diversity and Equitability Indices were highest in the shallower samples.

The range-weighted richness (Rr) of each sample was calculated and averaged to determine the habitability of the community (Figure 4.9F). The Rr of all clayey soils was medium to high, ranging from $10.9(\pm1.04)x10^2$ (FTF1 1.0 m) to $44.5(\pm1.2)x10^2$ (FTF1 2.5 m). The Rr of the low and medium contamination level soils tended to increase with depth to 2.5 m with a decrease in the deepest sample, with the medium contaminant soil (TP9) tending to have the highest Rr across all sample depths. The Rr of the highest contamination level (TP30) soil remained relatively stable across all depths except for a large spike in the 2.0 m sample. The majority of the clayey samples had a medium Rr and only a few deeper samples for all contamination levels had a high Rr. This suggests that the clayey soils have the ability to sustain a medium range of fungal species, with the ability to sustain species diversity increasing with depth regardless of contaminant level.

After analysis of the diversity of the test pits, five bands of interest were excised and sequenced to determine the identity of the dominant organisms present in the samples (bands in red boxes in Figure 4.7). Sequences were matched with the highest identity score in NCBI (Table 4.4). Final sequences of excised bands can be found in Appendix A.3.

Table 4.4 Summary of sequence identifications of bands excised from TGGE gel

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Taxonomic Phylum/ Class
1	JX945654.1	Uncultured *Aspergillus sp.*	93	*Ascomycota/ Eurotiomycetes*
2	KC237297.1	*Pezizomycotina sp.* DMRF-8	99	*Ascomycota/ unclassified*
3	EU167606.1	*Mycosphaerella pyri* strain CBS 100.86	96	*Ascomycota/ Dothideomycetes*
4	FR799279.1	*Plastismatia glauca*	79	*Ascomycota/ Lecanoromycetes*
5	HE864321.1	*Aspergillus fumigatus*	95	*Ascomycota/ Eurotiomycetes*

Band 1, similar to an *Aspergillus sp.* was present in FTF1 and FTF5 at all depths and was one of the most dominant bands across these samples. Band 2 represented a species that was present across all test pits and was identified as *Pezizomycotina sp.* This species was one of the most dominant bands across all depths in FTF1 and the surface samples of FTF5; however it was not dominant in the other communities. Bands 3 and 5 were present in FTF5 and FTF6 only and were similar to *M. pyri* and *A. fumigatus*, respectively. The organisms similar to *P. glauca* (Band 4) were present in one depth in both FTF1 (2.5 m) and FTF5 (0.0 m). All species identified were from the phylum *Ascomycota*.

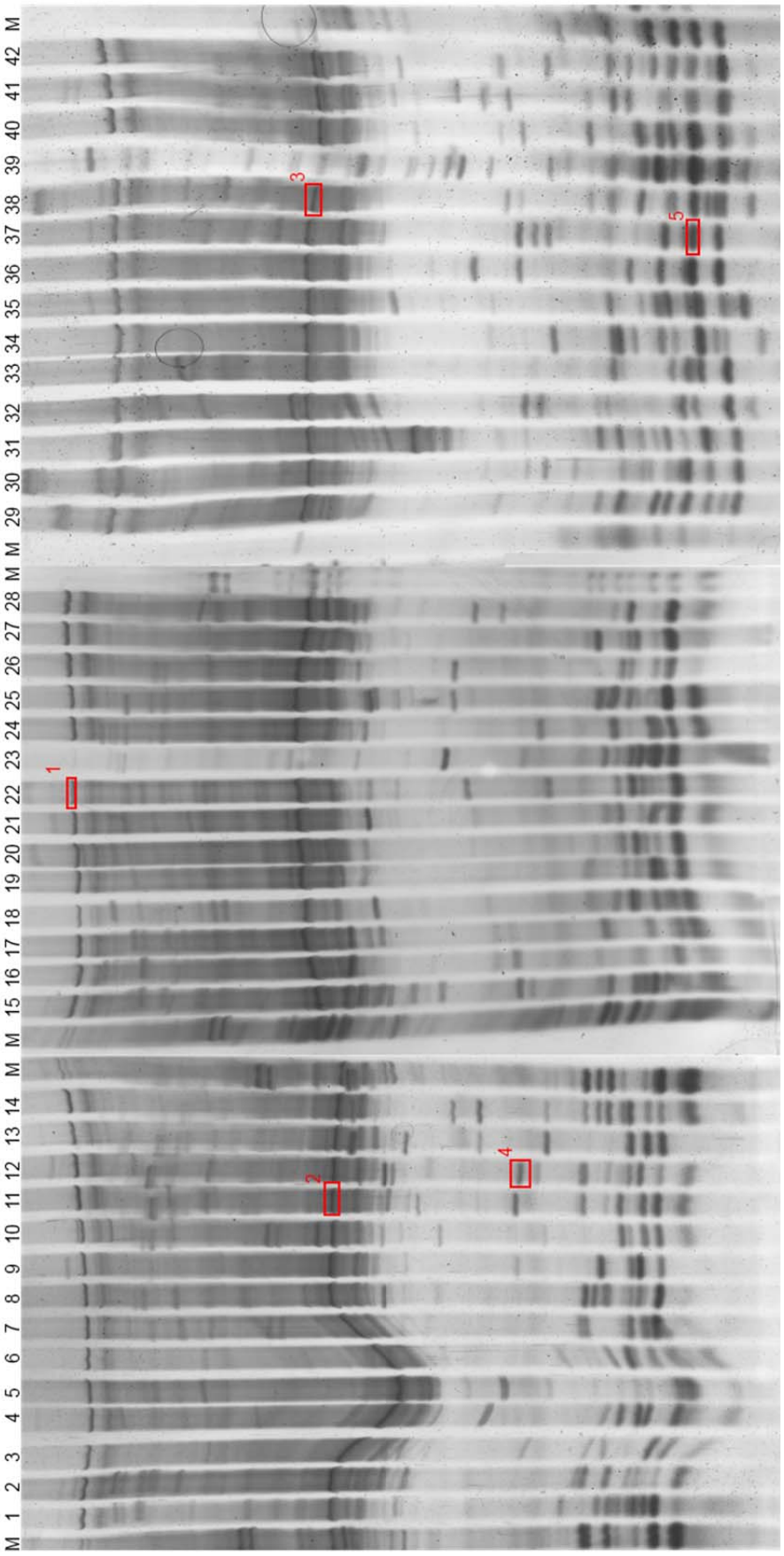

Figure 4.7 TGGE (50.0–58.0°C) of fungal community from clayey soil type. M: Marker. Lanes 1-2: FTF1 0.0 m; Lanes 3-4: FTF1 0.5 m; Lanes 5-6: FTF1 1.0 m; Lanes 7-8: FTF1 1.5 m; Lanes 9-10: FTF1 2.0 m; Lanes 11-12: FTF1 2.5 m; Lanes 13-14: FTF1 3.0 m; Lanes 15-16: FTF5 0.0 m; Lanes 17-18: FTF5 0.5 m; Lanes 19-20: FTF5 1.0 m; Lanes 21-22: FTF5 1.5 m; Lanes 23-24: FTF5 2.0 m; Lanes 25-26: FTF5 2.5 m; Lanes 27-28: FTF5 3.0 m; Lanes 29-30: FTF6 0.0 m; Lanes 31-32: FTF6 0.5 m; Lanes 33-34: FTF6 1.0 m; Lanes 35-36: FTF6 1.5 m; Lanes 37-38: FTF6 2.0 m; Lanes 39-40: FTF6 2.5 m; Lanes 41-42: FTF6 3.0 m. Bands in red boxes were excised for sequencing.

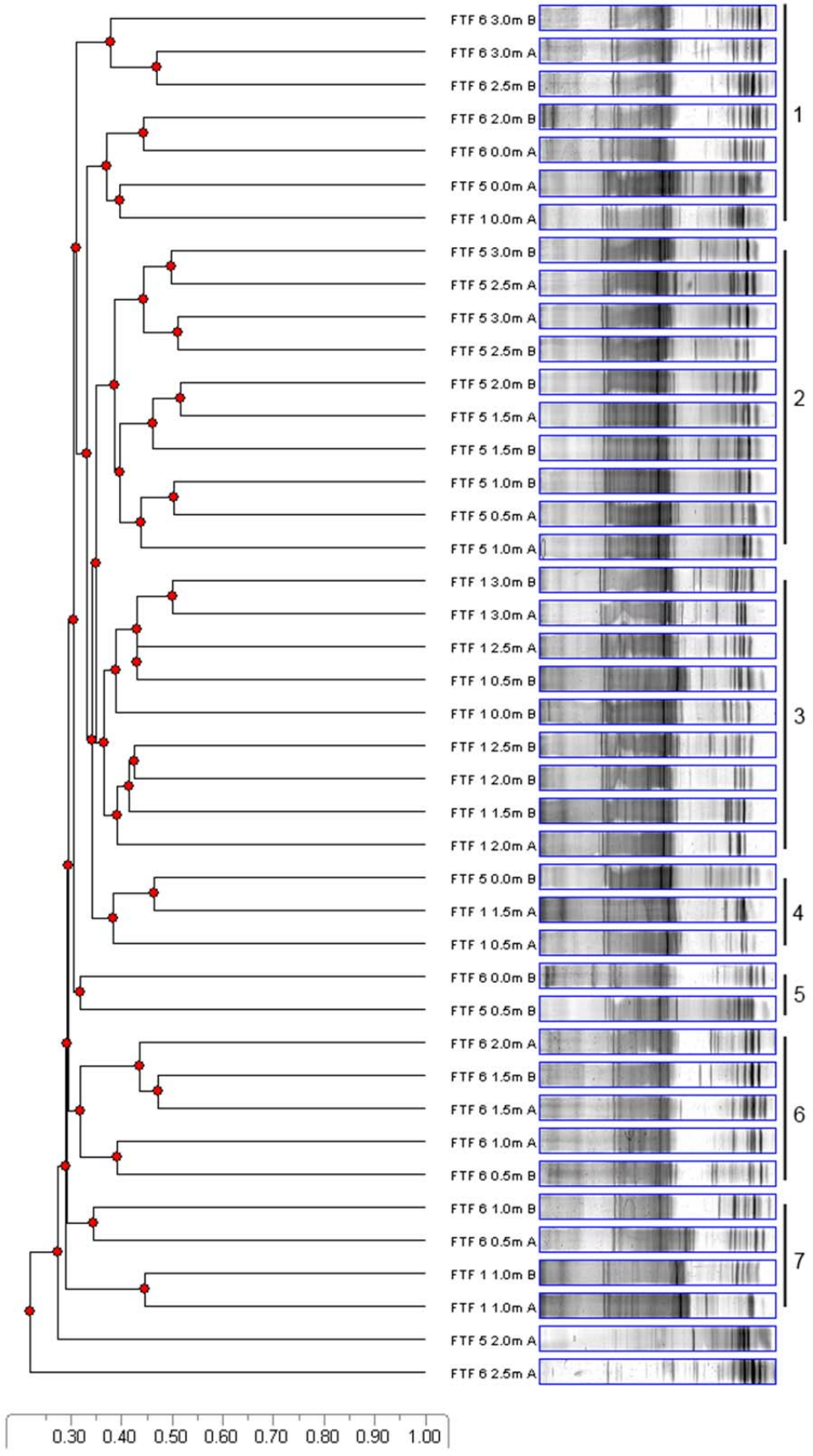

Figure 4.8 UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of rRNA ITS amplification from sandy soils. n=2 The scale bar represents similarity as a proportion of 1 while numbers 1 to 7 represent different fungal community clusters.

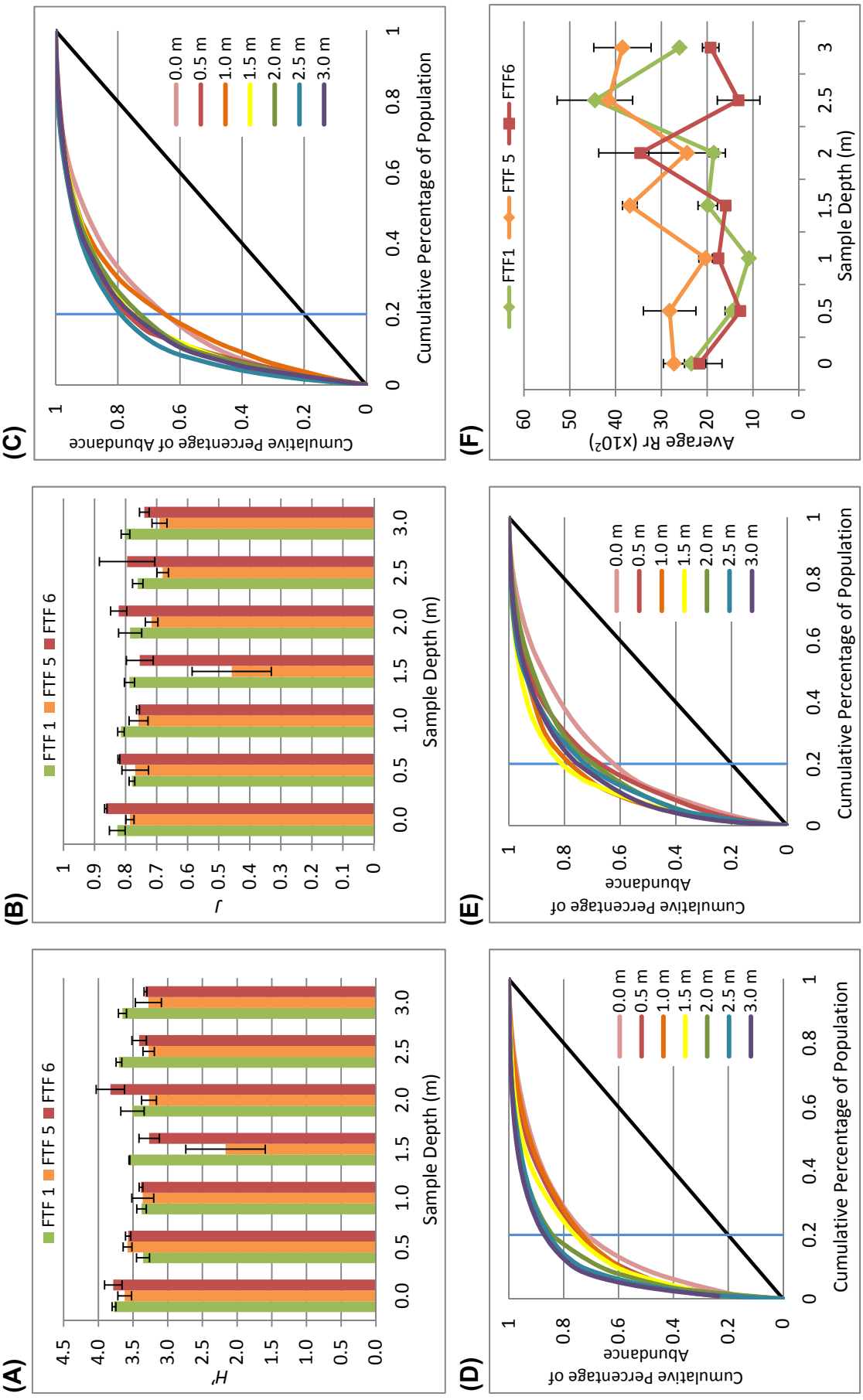

Figure 4.9 Shannon Weaver Indices and Functional Organisation curves for clayey soils. n=2 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve for FTF 1, (D) Fo for FTF 5, (E) Fo curve for FTF 6; (F) Average Range-weighted richness (Rr) for clayey soils. The black 45° line in C-E indicates the perfect evenness line of a population.

4.3.2.3 Fungal Community Dynamics Comparison

For ease of comparison of fungal community dynamics in the different soil types, the Shannon-Weaver indices, functional organisation and range-weighted richness calculations were averaged for each soil (for similar depths, i.e. 1.0 m, 1.5 m and 2.0 m) and are presented in Figure 4.10.

Figure 4.10A demonstrates that on average the clayey soil type had a higher diversity and functional organisation (*H'*=3.30; Fo=76%) compared to the sandy soils (*H'*=3.14; Fo=69%), whereas the equitability of the different communities were similar, although the clayey soils was slightly lower (*J*=0.80 for sand compared to *J*=0.74 for clay). This suggests that the clayey soil communities have more species present but the communities are organised such that there are only a few key species are dominant.

Figure 4.10B–D presents the difference in diversity indices based on soil type and contamination level. Generally the soils with the lowest and highest contamination levels had the highest average diversity, equitability and functional organisation, with the exception of the equitability and functional organisation of sandy soils. The most even community in the sandy soil was that of the medium contamination level (*J*=0.85) and the functional organisation within the sandy soil decreased with contamination level (Fo=72% low; Fo=66% high).

The average range-weighted richness (Rr) of the tests soils was calculated and is presented in Figure 4.10E. This demonstrates that the clayey soils ($16.5\pm2.3\times10^2$–$23.2\pm5.9\times10^2$) had around double the carrying capacity of the sandy soils ($8.1\pm2.8\times10^2$–$15.4\pm1.6\times10^2$), meaning that the clayey type soils were a more habitable environment and could support more diversity. The most habitable environments were the medium contaminant level clayey soil (FTF5; $Rr=23.2\pm5.9\times10^2$) and the high contamination level sandy soil (TP30; $Rr=15.4\pm1.6\times10^2$). The carrying capacity of sandy soils tended to increase with contamination level whereas the Rr peaked in the medium contaminant level and then remained relatively constant in clayey soils.

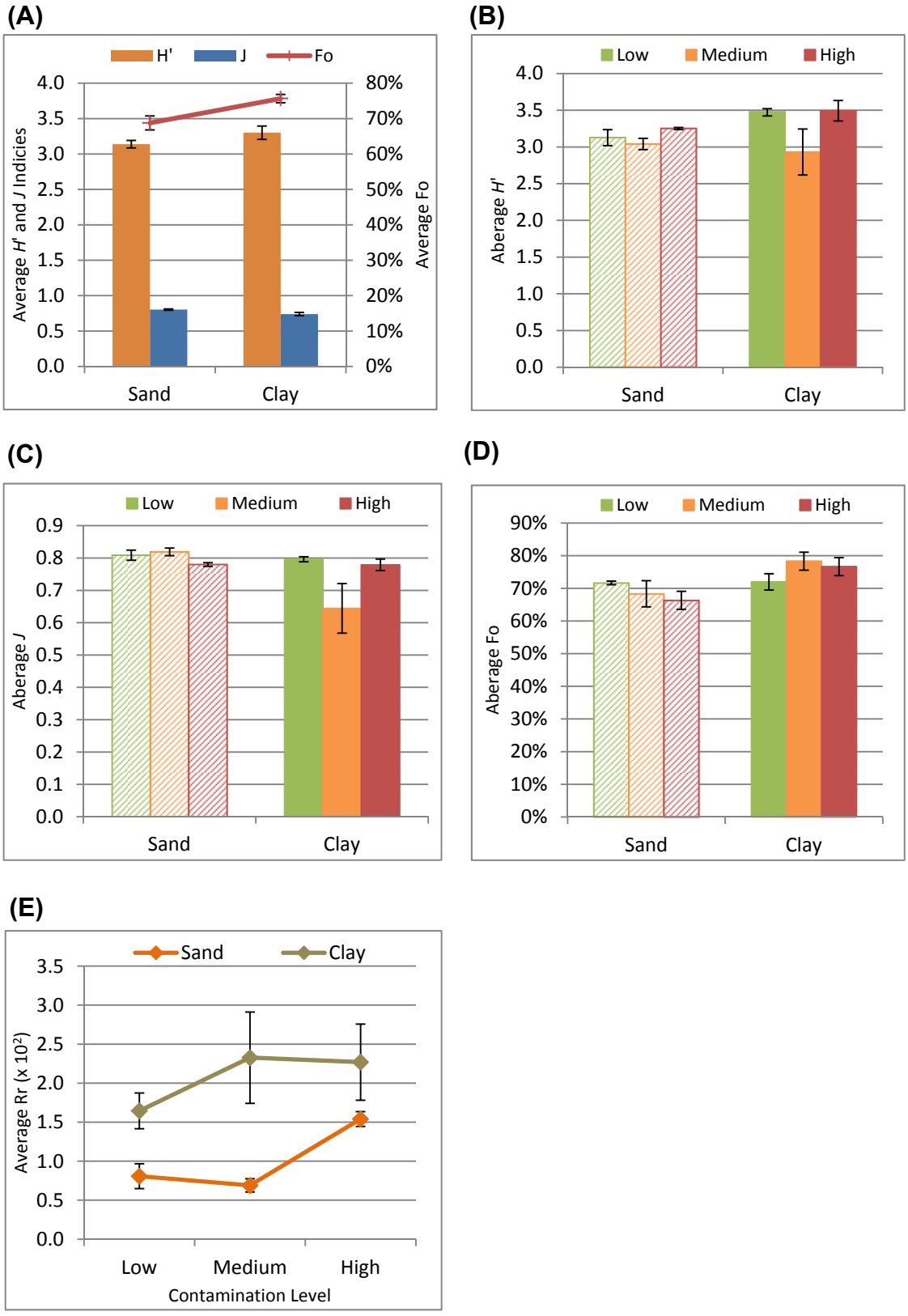

Figure 4.10 Comparison of fungal community dynamics in sandy and clayey soils. (A) Average Shannon Weaver diversity and Equitability and Functional organisation based on soil type, n=9; (B) Average Shannon Weaver diversity Indices dependent on contamination level n=3; (C) Average Shannon Weaver Equitability Indices dependent on contamination level, n=3; (D) Average functional organisation value dependent on contamination level, n=3; (E) Average range-weighted richness dependent on contamination level, n=3.

4.4 Discussion

4.4.1 Sandy Soil

The physio-chemical properties of the soils investigated from the area near the wharf were typical of sandy type soils with generally low moisture content. The pH of all sandy soils was slightly alkaline with no significant difference depending on contamination level. The analytical testing of soils demonstrated that TP30 was the soil with the highest contaminant level with TPH contamination above the NEPM HIL guideline and elevated concentrations of total PAHs. The NEPM HIL guideline exceedance indicates that there are concentrations at levels that could cause human health effects. There was evidence of TPH contamination in TP9, although at lower concentrations hence the assignment of the medium contamination level for this test pit.

Investigation of the fungal community revealed that the diversity of the most contaminated soil was significantly higher at 1.0 m compared to the other soils; no other statistically significant or strong trends were observed within the community dynamics. However, in general the most contaminated soil (TP30) had a higher diversity and the highest capacity to support a broader diversity of species (carrying capacity) compared to the other soils. The fact that the most contaminated soil had the highest diversity and was the most habitable environment is converse to common thought, as it is generally agreed that a reduction in microbial diversity and creation of a restrictive colonisation environment is induced by perturbation events such as petroleum hydrocarbon contamination (MacNaughton *et al.*, 1999; Bordenave *et al.*, 2008; Marzorati *et al.*, 2008). This observation is further supported by the fact that the sample with the highest level of contamination from medium contaminant level soil (TP9 2.0 m) had the highest community diversity and carrying capacity.

Investigation of the dominant fungal species present in the various communities identified that the majority (60%) of the species present were from the class Eurotiomycetes, which includes many recognized species capable of degrading petroleum based compounds such as *Aspergillus niger, A. versicolor* and

Penicillium chrysogenum (Cerniglia, 1992; Juhasz and Naidu, 2000; Garapati and Mishra, 2012). The fungi *Aspergillus fumigatus and organisms related to the Eurotiales and Mycosphaerella genus* were identified from bands that were present in all contamination level samples at all depths. *A. fumigatus and members of the Eurotiales* genus. have been identified in the degradation of TPH, PAHs (especially anthracene and phenanthrene) and waste effluent containing contaminants such as cyanide (Qiang *et al.*, 2009; Ye *et al.*, 2011; Sabatini *et al.*, 2012; Vanishree *et al.*, 2014). The genus *Mycosphaerella* is a member of the *Dothideomycetidae* order which is a group of ascomycetes that tolerates surprisingly harsh conditions prevailing such as rock surfaces (*Ruibal et al*., 2009). These organisms are rarely found in complex microbial populations and often occur in extreme environments or under poor nutrient conditions (Harutyunyan *et al.*, 2008) with other similar stress resistant organisms such as lichens (Onofri *et al.*, 2007) and cyanobacteria (Sterflinger, 2006).

Another *Asperigillus sp.* was identified across the medium contaminant level soil and the deeper sample of the low contaminant level soil, which may suggest that these species have a role other than hydrocarbon degradation in pristine or low perturbation soils.

One species, similar to *Plastismatia glauca*, was found in only the deep samples of the highest contaminant level soil. A review of the literature did not identify any instances where the petroleum hydrocarbon degrading properties of this epiphytic lichen were investigated. The majority of literature relating to *P. glauca* and other similar lichens was centred on their use as biosensors in detection of air and water pollution (Belnap and Harper, 1990; Antonelli *et al.*, 2005) and as antioxidant and antimicrobial agents (Gulluce *et al.*, 2006).

Generally, the fungal community of the sandy soils investigated contained fungi that were capable of hydrocarbon degradation. The dominance of key hydrocarbon degrading species *Aspergillus* and *Eurotiales* appeared to not change depending on contaminant level. This perhaps suggests that as the sampling location is close to a loading dock where small hydrocarbon spills may have occurred regularly over the life of the oil refinery use, the natural fungal

community has become skewed towards species that are capable of utilising hydrocarbons. This could also explain why the highest contaminant soil had the highest species diversity.

It is of note that the majority of sequences represent species belonging to taxonomic groups are of probable terrestrial origin, which is surprising given the location. An influencing factor may have been the time when samples were taken (e.g. low tide) causing a selection to these type of organisms. Alternatively, organisms of marine origin may have been present as spores and not particularly active and therefore not easily detected using these methods. This will be further discussed in research Chapter 5, where this problem can be circumvented through the use of Stable Isotope Probing (SIP).

4.4.2 Clayey Soils

The physio-chemical properties of the soils investigated from the tank farm area was typical of clayey type soils with medium to high moisture content (> 15%) which generally decreased with depth; the pH of all soils tested was alkaline. FTF6 (the highest contaminant level) had the lowest moisture content across all depths and contaminant levels. The analytical testing of soils demonstrated that FTF6 had the highest contaminant level as TPH, BTEX and Total PAH contamination was above the NEPM HIL guideline, FTF5 also had TPH and Total PAH contamination above the HIL guideline but at lower concentrations compared to FTF6.

Investigation of the fungal community demonstrated that the most diverse and equitable communities with the lowest functional organisation were at the surface, with the exception of the high contaminant level soil (FTF6). The 2.0 m sample from FTF6 had the highest diversity, which also had the highest TPH, BTEX and PAH concentrations for all clayey soils tested. This is similar to what was observed in the sandy soils, where the samples with the highest contamination level also had the highest diversity, again converse to common thought regarding the effects on microbial communities after perturbation events. However, this was not the case in the medium contamination level soil (FTF5). There was a considerable decrease in species diversity and equitability in

the 1.5 m sample which corresponded to the highest TPH and BTEX concentration in that test pit. The decrease was so large that this sample had the lowest diversity of all clayey soils tested. The functional organisation was the lowest at the surface for all test pits and generally increased with depth. The majority of Fo results were above 70% meaning the organisation of the communities was high and thus communities were dominated by a few key organisms, and the dominance of these species increased with depth.

Although there was a statistically significant difference in community diversity and evenness between the most contaminated soil (FTF6) and the other test pits there was no significant clustering based on sample depth or contamination level observed in the UPGMA analysis. This tends to suggest that something other than contamination level, such as nutrient level or organic/moisture content, may have been acting on the clayey fungal communities to cause a change in the functional organisation and diversity (Reverchon *et al.*, 2010; Porras-Alfaro *et al.*, 2011; Pereira e Silva *et al.*, 2012). Testing of the soil samples for things such as nutrient level was not the priority of this project and therefore only limited data was available and did not allow for further exploration.

Investigation of the dominant fungal species present in the various communities identified that all species were from the phylum *Ascomycota*, the largest phylum of the kingdom fungi (Spatafora *et al.*, 2006). One band (band 2) sequenced was present in all contaminant levels and was identified as *Pezizomycotina sp*.. This species is known as a decolourizing agent of synthetic dyes in wastewater (Ahlawat and Singh, 2011; Singh *et al.*, 2012).

The most dominant species in both the low and medium level contaminated soils across all depths (band 1) was identified as an member of the *Aspergillus* genus. Members of this genus are well known hydrocarbon degrading organisms (Cerniglia, 1992; Cerniglia, 1997; Juhasz and Naidu, 2000). Interestingly, this species was not present in any of the high contamination samples. An organism similar to *Plastismatia glauca* (band 4) was also only present in FTF1 and FTF5. As discussed in Section 4.4.1, this species has not been identified as a hydrocarbon degrader in literature. Given the low similarity score (79%) of the

sequence it is likely that this organism is a novel or previously uncharacterised hydrocarbon degrader. Unlike in the clayey soils, this species was only present in the deeper sandy soil samples from the highest contaminant level test pit (TP30). Since this species was not identified in FTF6 which had a high level of PAH contamination and TP30 and FTF5 were contaminated with TPH and BTEX, it could be concluded that this species may be involved in the degradation of shorter chain TPHs and not large aromatic hydrocarbons.

Identified species that were only present in the two contaminated samples across all depths were a *Mycosphaerella* species (band 3) and *A. fumigatus* (band 5), both of which were identified in the sandy soils investigation. As discussed previously *A. fumigatus* is a known degrader of TPH, PAH and cyanide (Qiang *et al.*, 2009; Ye *et al.*, 2011; Sabatini *et al.*, 2012; Vanishree *et al.*, 2014) and *M. pyri* is often present in extreme or nutrient poor conditions (Harutyunyan *et al.*, 2008).

The fungal community of the clayey soils investigated contained fungi that were known hydrocarbon degraders or based on their presence and absence in a particular test pit were likely to have the capacity to utilise hydrocarbons. Some of these key hydrocarbon degraders, i.e. *Aspergillus sp.* were identified in samples with low contamination concentrations, suggesting that these species may have another role other than degradation; or possibly the soils were contaminated in the past and the concentration of contaminants have decreased to concentrations that were no longer detectable in laboratory testing, resulting in a population that is skewed to and dominated by hydrocarbon degraders. This is certainly possible given the location of test pits.

Caution in interpretation of these results should be used, as there is some indication that some fungal species may be present as spores and not particularly active. Therefore we may be drawing incorrect conclusions on the ability of the soil to respond to a contamination event. Furthermore, the database utilised for fungal identification may not be reliable. This further emphasises the importance of SIP to identify the purely active members of a soil community.

4.4.3 Soil type comparisons

The physio-chemical properties of the two soils types were consistent with expectations given that one soil was clay and the other sand. The clayey soils had higher concentrations of TPH, BTEX and PAHs present and the contamination was more widespread across sample depth, which was reflective of the sample locations.

The clayey soils had a higher diversity but lower equitability and a corresponding higher functional organisation compared to all sandy soils, regardless of contaminant level. This was also observed in the MPN investigation completed as part of the initial method development experiments (Figure 3.5, Chapter 3). The fact that the clay soils had an overall higher contamination level and broader range of contaminants (i.e. TPH and PAHs) may explain why there was a higher diversity such that the contaminant level caused the community to be skewed towards a broader range of organisms that could utilise a range of hydrocarbons. Even though the community in clayey soil may have been skewed towards organisms that could utilise multiple types of hydrocarbons, the dominant organisms identified in both soil types had several species in common, namely *A. fumigatus*, and members of the *Mycosphaerella* and *Plastismatia* genus.

The differences in diversity across contamination levels were similar in both soils types, with the medium contaminant level having the lowest diversity. A similar pattern was observed in the equitability for clayey soils; however in sandy soils the medium level contaminated soil had the highest evenness. This suggests that the medium contaminant level soils appear to be unable to adapt to the stress of perturbation as well as the high contaminant level resulting in the decreased diversity.

The functional organisation of the clayey soils tended to be high, whereas sandy soils were medium to high. This could indicate that sandy soils have a better ability to adapt to contamination given the high functional capacity still existing, regardless of contamination history.

A measure of the habitability of a community is the range-weighted richness, the clayey soils were higher compared to sandy soils across all contamination levels. This suggests that the clayey soils have the potential to support a wider range of species and are unrestrictive to colonisation.

This higher range-weighted richness along with higher species diversity in the clayey soils tested may be related to the difference in pore size as described in a theory linking the physical property of low pore connectivity to high diversity in bacterial soil communities (Tiedje *et al.*, 2001). The same could be equally applied to fungal communities. Clays are defined by having finer grain sizes and therefore have smaller pores sizes and often lower pore connectivity or permeability, compared to sandy soils with larger grain sizes (Heath, 2004). The lower permeability and pore connectivity is thought to hinder the capacity of competitive organisms to decrease substrate availability, due to the low diffusion rates of substrate and motility of organisms under conditions of low water content, creating protective microhabitats (Gleason *et al.*, 2012) thereby enabling a broader community range and diversity (Tiedje *et al.*, 2001; Carson *et al.*, 2010; Chau *et al.*, 2011). Furthermore, fungal hyphae tend to stabilised clay soils by entangling the finer clay soil particles (Bell *et al.*, 2006), improving the creation of these microhabitats. The increased diversity in clay may also be related to the soil's higher water holding capacity and often higher organic carbon content compared to sandy soils (Gonzalez-Quinones *et al.*, 2011).

4.5 Conclusions

For sandy soils, the most contaminated test pit had the highest fungal diversity. This is not what was expected as it is widely thought that a reduction in microbial diversity is induced by perturbation events. The sandy soil may also be able to adapt better to these events, given their overall lower functional organisation. Sequencing of fungal profiles revealed that the majority of the dominant species were from the class *Eurotiomycetes* and included the well-known hydrocarbon degrading species of *Aspergillus* and *Eurotiales*. However, the dominance of these species did not change depending on contamination

level, suggesting a level of adaptability to multiple carbon sources or the fact that even the low contaminant level samples used in this project had some history of contamination.

A similar pattern of diversity was not observed in the clayey soils and there appeared to be no correlation between species diversity and contaminant levels. It is possible that other factors may have been contributing to changes in diversity such as nutrient levels. Although investigation to other contributing factors was outside the scope of this body of work, concentrations of nutrients and other factors would be a worthwhile focus of future experiments.

Similar dominant fungal species were identified in the clay communities to those found in the sandy soils and all species identified in the clay communities were part of the phylum *Ascomycota*.

There was higher species diversity and range-weighted richness for all clayey soils compared to sandy soil, which may be a result of the *pore connectivity theory* which suggest that low water connectivity in soils can promote diverse communities through creation of microhabitats (Tiedje *et al.*, 2001; Gleason *et al.*, 2012).

Given that the diversity of the clayey soils did not appear to be dependent on contamination level and the fact that a robust method for DNA extraction without PCR amplification could not be developed (see Chapter 3 for further detail), it was decided that the sandy soil type would be used for all remaining experimentation into the prediction of a soils capacity to adapt to perturbation events. This is further investigated in Chapter 5.

5. Prediction of mineralisation of secondary contaminants

5.1. Introduction

Investigation and identification of soil's microbial diversity can assist in the development of site specific remediation programs; however determining how a soil will adapt and cope in a polluted environment is challenging as the presence of an organism is not necessarily an indication of its degradative capacity (Adetutu *et al.*, 2012). Coupling the organisms' presence with its actual role within the community is the main focus of this chapter, using a technique called stable isotope probing (SIP).

A number of physical, chemical, biological or environmental factors may influence PAH degradation, including microbial capacity, moisture content, oxygen availability, pH, essential nutrient availability, temperature and contaminant bioavailability (Juhasz and Naidu, 2000; Torsvik and Øvreås, 2002; Ding *et al.*, 2010). The aim of the study was to determine if a prediction could be made about a soils' ability to respond to a contamination event based on the microbial capacity component, more specifically the soils baseline microbial community profile and to determine if a pristine soil, defined as a soil that has not be exposed to hydrocarbon contamination, would better adapt to a contamination event compared to a previously contaminated soil.

This chapter reports on work investigating the hypothesis that a pristine soil should be able to respond to a contaminant more effectively given that the baseline microbial community has not been skewed towards specific degrading organisms that do not have the ability to adapt quickly to a new contaminant. Two sandy soils with different contamination histories will be the subject of this investigation; one soil with no known historical contamination which will be referred to as the pristine soil (TP20) and the other soil which has been exposed to petroleum hydrocarbon contamination and will be referred to as the contaminated soil (TP30).

The investigation into the ability to predict a soils response to a contamination event, will focus on the degradation of phenanthrene a model 3-ring polycyclic

aromatic hydrocarbon. This study will involve identification of key organisms, both fungal and bacterial, in the baseline (or unamended) community. Determination of the soils ability to adapt to a secondary contaminant will be analysed through PCR and TGGE using 'prediction primers' that target the unique ring-hydroxylating dioxygenase (RHD_{α}) or initial dioxygenase involved in the bacterial degradation of PAHs, enabling the study of the 'key players' in the degradation process (Margesin *et al.*, 2003; Singleton *et al.*, 2009; Ding *et al.*, 2010).

Simulation of a contamination event through application of phenanthrene to soil will enable analysis of the soils microbial response and detection of the active degrading microbes to determine if predictions were accurate and the dominant organisms in the contaminated (or pulsed) community were responsible for adaption to the secondary contaminant.

5.2. Methods

5.2.1 Sampling and nucleic acid extraction

Soils used in this experiment were sourced from the same wharf location as in Chapter 4. The selection of soils was based on the results obtained in that chapter. TP30 was selected as the contaminated soil and was located just south of the wharf. TP20 was selected for the pristine environmental sample as it was located on the far end of the beach, well above the high tide area (Figure 5.1).

Test pits were excavated using a backhoe and 1.0 kg samples from every 0.5 m were collected from the bucket. The samples were then mixed in the laboratory to produce a representative sample of 0–1 m and 1–2 m for both test pits. Samples were stored at -80^{o}C until RNA and DNA were extracted using mechanical lysis and precipitation as described in Chapter 2.6.1.

Figure 5.1: **Locations of Test Pits for Sandy Soils**

5.2.2 Physio-chemical analysis of soils

Sub-samples of soils from varying depths within each test pit location were stored in laboratory supplied containers and transported to a NATA accredited laboratory for chemical analysis. All soil samples were tested for the presence of heavy metals, TPH, BTEX, halogenated aliphatic and aromatic compounds, phenolic compounds and PAHs using methods governed by Australian Standards and the NEPM guidelines (National Environmental Protection Council, 1999). The

following depths were analysed for each test pit: TP20 (0.5 m, 1.5 m); TP30 (1.0 m, 1.5 m, 2.0 m). It should be noted that as this testing was completed as part of a different project not every depth that was used in community profiling was analysed for contaminant concentration.

The results of the laboratory testing were used to score (low or no, medium and high) the relative contamination level of the soil compared to the other soil samples of similar type. Soils were also tested for pH, moisture content and classified according to the USCS system and potential hydrocarbon contamination indicators noted (Chapter 2.1).

5.2.3 Community Profiling

Bacterial and Fungal

Nested PCR amplifications targeting the fungal 5.8S rRNA ITS region were carried out using primer pairs EF4 with ITS4 and ITS1F with ITS2. A 40 bp GC clamp was included on the ITS1F primer. Community DNA template was used at a dilution of 1:10 for round one of the nested PCR. Template was diluted to reduce the effect of PCR inhibitors co-extracted during DNA extraction. Amplifications targeting the 16S rRNA gene were also completed using primer pair 341F and 518R. Again a GC clamp was included on the forward primer (refer to Chapter 2.6.4 for specific cycling conditions).

Five to ten microlitres of PCR products were loaded in triplicate (volumes loaded were dependent on band intensity in an agarose gel) and electrophoresed for 8 h using a temperature gradient of 50.0°C to 58.0°C for fungal amplicons and 55.0°C to 68.0°C for bacterial amplicons. The resultant gels were silver stained and scanned for diversity analysis (Chapter 2.6.6 and 2.6.10). Shannon Weaver diversity and Equitability Indices were calculated as well as Pareto-Lorenz evenness curves and UPGMA dendograms, constructed from similarity matching data (Dice-Sorenson Index) using the TotalLab Software package (Chapter 2.6.12). Representative bands of interested were excised from a re-run TGGE stained using SYBR Gold and sent for sequence analysis.

PAH-RHD$_\alpha$ Prediction Primers

Nested PCR amplifications targeting the conserved Rieske domain of the alpha subunit of the Ring-Hydroxylating Dioxygenase (RHD) were carried out using primer pairs PAH-RHD$_\alpha$ GN-F and GN-R for Gram-negative bacteria and PAH-RHD$_\alpha$ GP-F and GP-R for Gram-positive bacteria. A 40 bp GC clamp was included on each forward primer in the second round of PCR. Community DNA template was used at a dilution of 1:10 for round one of the nested PCR (refer to Chapter 2.6.4 for specific cycling conditions). Template was diluted to reduce the effect of PCR inhibitors co-extracted during DNA extraction.

Five to ten microlitres of PCR products were loaded in duplicate, volumes loaded were dependent on band intensity in an agarose gel, and electrophoresed for 8 h using a temperature gradient of 51.0°C to 60.0°C. The resultant gels were silver stained and scanned (Chapter 2.6.6 and 2.6.10). UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) was produced using the TotalLab Software package (Chapter 2.6.12). Representative bands of interested were excised from a re-run TGGE stained using SYBR Gold and sent for sequence analysis.

Amplification of key fungal PAH degrading enzymes (lignin peroxidase, manganese-dependent peroxidase and laccase) was attempted using primer pairs LnP (Reddy and D'Souza, 1998), MnP (Bogan *et al.*, 1996) and *lcc* (Bogan *et al.*, 1996) but were unsuccessful due to unspecific primer binding resulting in multi-banded profiles with bands of the correct size not being the most dominant.

5.2.4 ^{14}C-phenanthrene mineralisation

Soils (5.0 g) were first supplied with 0.75 mg.ml^{-1} of phenanthrene (non-radio labelled) and the water holding capacity of soil was adjusted to 40–60% using minimal media (Dandie *et al.*, 2010). Sodium hydroxide (0.5 ml 1M NaOH) and 1 µC of ^{14}C-phenanthrene were then added to the vessel and the lid sealed. Soils were incubated at room temperature for 25 days and the NaOH trap from each incubation was collected and scintillation counts performed on days 1, 2, 3, 4, 7,

9, 11, 16, 18, 23 and 25. A biocidal treated control (sodium azide) was run in parallel to ensure phenanthrene degradation was a result of biotic processes alone. Test samples were run in duplicate. A cumulative evolution of $^{14}CO_2$ was then calculated from the time points, as described in Chapter 2.4.

5.2.5 SIP

Soil samples (6.0 g) were incubated for 5 days at 25°C with 20 µg.g^{-1} of ^{13}C-labelled phenanthrene or unlabelled phenanthrene (^{12}C-phenanthrene) and minimal media. The length of incubation time was determined from the results of the ^{14}C mineralisation study, above. The incubation time was considered an appropriate length of time to enable sufficient incorporation of the label (^{13}C) into the nucleic acids of organisms metabolising phenanthrene but not enough time to skew the results due to cross feeding (Lueders *et al.*, 2004a; Manefield *et al.*, 2004; Manefield *et al.*, 2007). Refer to Chapter 2.5 for further details on incubation methods.

Nucleic acids (RNA and DNA) were extracted according to the method in Chapter 2.6.1.1 and subjected to isopynic centrifugation in the presence of caesium chloride (CsCl; for DNA) and caesium trifluoroacetate (CsTFA; for RNA). Gradients were fractionated and nucleic acids precipitated from fractions within the target buoyant density of 1.75 g.ml^{-1} (DNA) and 1.795 g.ml^{-1} (RNA).

Precipitated nucleic acids were then amplified using fungal and bacterial universal primers for community profiling as described above in Section 5.2.3.

5.3. Results

5.3.1. Physio-chemical analysis of soils

Results of pH and moisture content testing of the soils are provided in Figure 5.2. These results were used to assist in the classification of the test soil based on the dominant soil type, during which the appearance and indication of potential contamination were noted. See Table 4.1 for classification of the test soils.

The pH was similar in both test pits and across the two depths; the pH was slightly basic ranging from 8.6 to 8.9 (Figure 5.2A). The moisture content was

considerably lower in the pristine soil compared to the contaminated soil, although the moisture content in both soils increased with depth.

Table 5.1 Classification of test soils

Sample	Major Soil Group	Description	Potential contamination indicators	Contamination Level
TP30	Sand	Yellow/brown medium grained, well graded sand	Dark black staining present ≥ 1.5 m. Strong hydrocarbon odour	Contaminated
TP20	Sand	Yellow medium–fine grained, well graded sand	None observed	Pristine

NB: Contamination level was determined using chemical analysis results and scored relative to other samples of the same soil type (see Table 5.2).

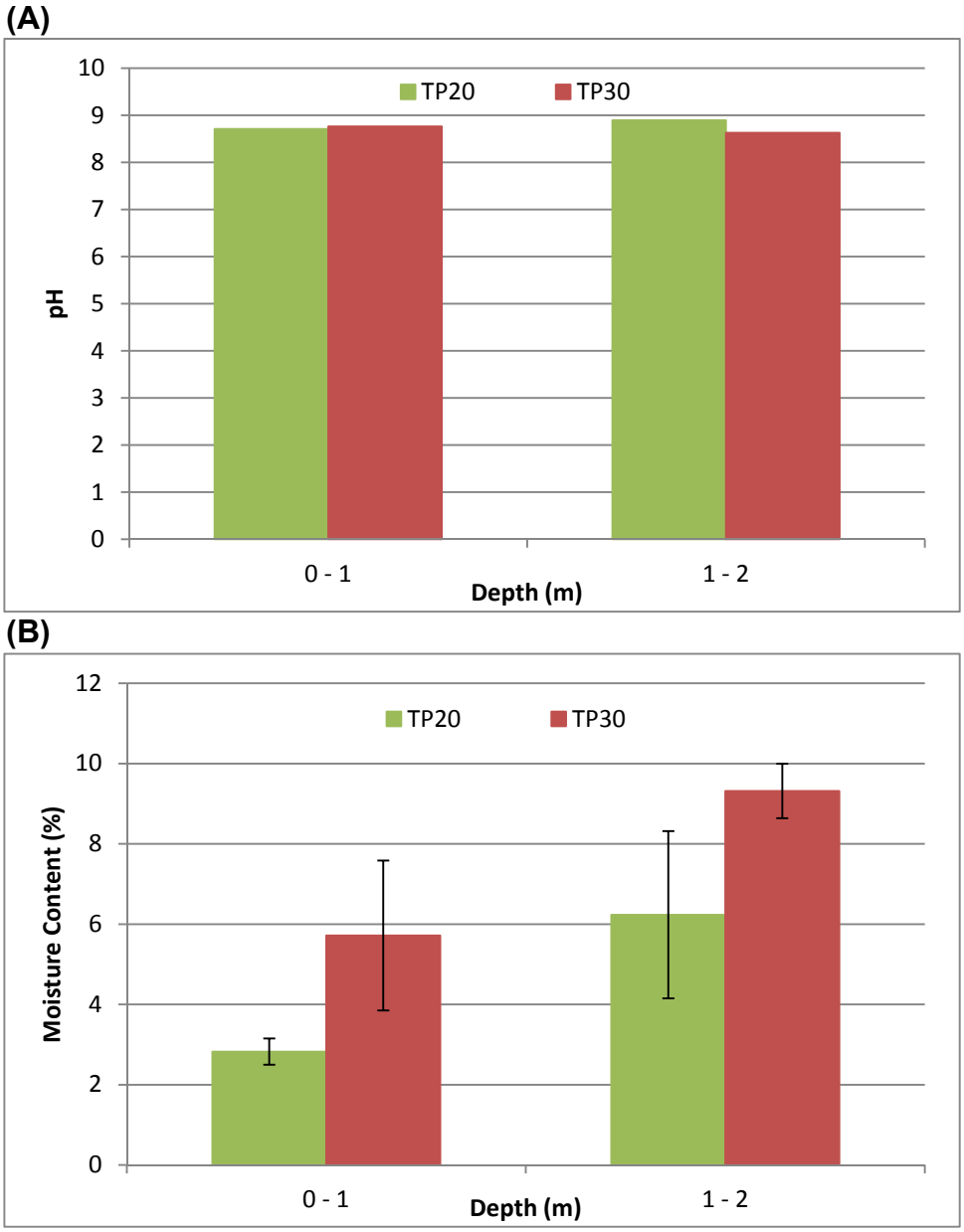

Figure 5.2 Comparison of pH and moisture content for soils across varying sample depths. n=3 (A) pH of soils, (B) moisture content of soils.

Results of the laboratory chemical testing are provided in Appendix A.2 with results compared against current legislative contamination guideline levels (the NEPM health and ecological values for industrial sites) (National Environmental Protection Council, 1999). A summary of the key contaminant levels (TPH, BTEX and PAHs) within the test soils is provided Table 4.2; all other analytes (metals, phenols and other hydrocarbons) were generally below the limit of laboratory reporting (LOR).

Table 5.2 Summary of key contaminant levels (mg.kg^{-1}) in all test soils

Sample	Depth (m)	TPH					B	T	E	X	Total PAH
		C_6-C_9	C_{10}-C_{14}	C_{15}-C_{28}	C_{29}-C_{36}	C_{10}-C_{36}					
TP20	0.5	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	1.5	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
TP30	1.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR
	1.5	**34**	**500**	**1230**	<LOR	**1780**	<LOR	<LOR	<LOR	<LOR	**1.2**
	2.0	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR	<LOR

NB: LOR limit of reporting;-not analysed; shaded boxes are values above the NEPM HIL guidelines.

The 1.5 m sample from the contaminated soil was the most contaminated with detectable levels of TPH and PAH. The TPH concentration was above the NEPM Health Investigation Guideline levels for industrial soils (relevant National soil guideline), indicating that samples have concentrations of petroleum based hydrocarbons at levels that could present a health risk.

5.3.2. Baseline Microbial Community Profiles

Fungal Community

The TGGE gel of the test soils across varying depths is provided in Figure 5.3 and the resultant UPGMA and diversity are provided in Figure 5.4 and Figure 5.5, respectively. The fungal profiles of the two soils were very similar with several common dominant species across the sample depths.

UPGMA analysis (Figure 5.4) showed that the samples formed two clusters (1 and 2) largely dependent on depth rather than contamination level. One sample (TP20 1-2 m replicate B) had a different banding pattern and therefore did not cluster with other samples; this was considered an outlier and not included in further analysis. The fungal communities within the clusters shared a similarity of 65% to 77%.

The Shannon Weaver diversity and Equitability Indices were relatively stable across sample depths in the contaminated soil, whereas there was a

considerable difference in diversity in the pristine soil. The deeper pristine sample had the lowest diversity index (*H'*=0.99±1.22) of all soils tested and the shallow pristine sample had the greatest diversity (*H'*=2.4±0.7) (Figure 5.5A). There was a similar trend in the equitability of the pristine soil. The equitability of the contaminated samples was again similar across depths. Statistical analysis of the diversity and equitability revealed that there was no significant difference in fungal community profiles between the pristine and contaminated soils (Students *t*-test $p > 0.05$).

Functional organisation of the different samples was interpreted using PL evenness curves (Figure 5.5C). The Fo for all communities was considered medium and ranged from 31% (shallow contaminated) to 44% (deep pristine). This suggests that all soils had only a few dominant species with the majority of the community in even concentration. The contaminated soils tended to be more even than the pristine soils, given the slightly lower PL curve value.

The range-weighted richness (Rr) of each sample was calculated and averaged to determine the habitability of the community (Figure 5.5D). All samples had a high range-weighted richness with values ranging from 56(±39) (deep pristine) to 198(±45) (shallow pristine). The carrying capacity of the shallow pristine sample was almost double that of the contaminated sample, whereas the deeper samples had a very similar Rr. The high Rr for all soils suggests that the soils have a capacity to support a wide variety of species with very few dominant species.

After analysis of the diversity of the test pits, nine representative bands of interest were excised and sequenced to determine the identity of the dominant organisms present in the samples (bands in red boxes in Figure 5.3). Sequences were matched with the highest identity score in NCBI (Table 5.3). Final sequences of excised bands can be found in Appendix A.3. It should be noted that identity matches below 99% are considered ambiguous and therefore the identities may represent a related species to those matched. Furthermore, the species sequence may in fact be representative of novel or previously uncharacterised organisms.

All excised bands were present in both the pristine and contaminated samples with the exception of bands F1 and F2 (Figure 5.3). Band F1 (*Mortierella*-like species.) was present in all samples except for the deeper pristine sample, and band F2 identified as *Penicillium griseofulvum* was unique to the deeper pristine sample. The majority of identified species (56%) were from the phylum *Basidiomycota*.

Table 5.3 Summary of sequence identifications of bands excised from fungal TGGE gel

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Taxonomic Phylum/ Class
F1	HQ533832.1	*Mortierella sp.* UFMGCB 3717	86	*Mortierellomycotina/ Mortierellales*
F2	JN032682.1	*Penicillium griseofulvum* strain LC2	99	*Ascomycota/ Eurotiomycetes*
F3	AB915387.1	*Cryptococcus sp.* DMKU-SP85	88	*Basidiomycota/ Tremellomycetes*
F4	JX310560.1	*Rhodotorula sp.* VITJzN03	98	*Basidiomycota/ Microbotryomycetes*
F5	KC243965.1	Uncultured *Davidiellaceae sp.* clone Z1D_O34	99	*Ascomycota/ Dothideomycetes*
F6	JN003620.1	*Rhodosporidium babjevae* isolate BD19	99	*Basidiomycota/ Microbotryomycetes*
F7	FN428883.1	*Cryptococcus nemorosus*	95	*Basidiomycota/ Tremellomycetes*
F8	JX996998.1	*Penicillium chrysogenum* strain DTO 102B4	99	*Ascomycota/ Eurotiomycetes*
F9	GU327539.1	*Papiliotrema bandonii* strain CBS9107	98	*Basidiomycota/ Tremellomycetes*

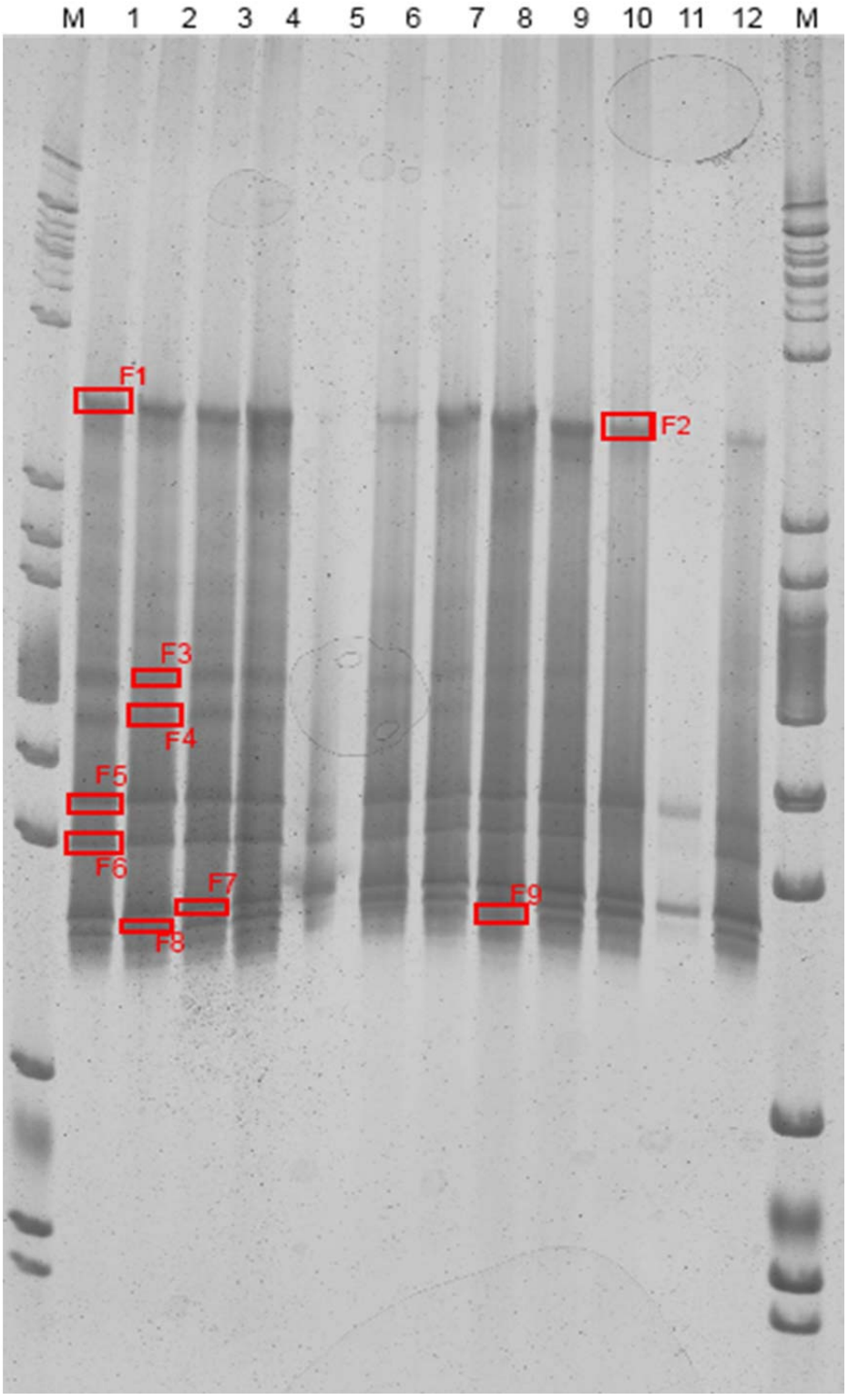

Figure 5.3 TGGE (55.0–58.0°C) of fungal community from test soils. n=3 M: Marker. Lane 1: TP30 0-1m Rep A; Lane 2: TP30 0-1m Rep B; Lane 3: TP30 0-1m Rep C; Lane 4: TP30 1-2 m Rep A; Lane 5: Lane 4: TP30 1-2 m Rep B; Lane 5: Lane 4: TP30 1-2 m Rep C; Lane 7: TP20 0-1 m Rep A; Lane 8: TP20 0-1 m Rep A; Lane 9: TP20 0-1 m Rep C; Lane 10:TP20 1-2 m Rep A; Lane 11: TP20 1-2 m Rep B; Lane 12: TP20 1-2 m Rep C. Bands in red boxes were excised for sequencing.

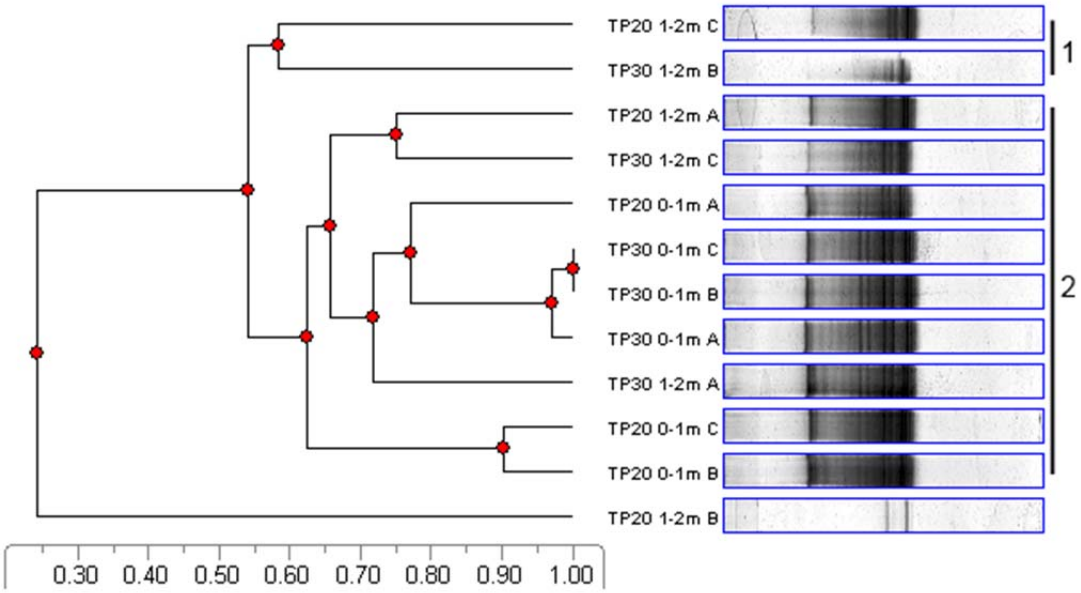

Figure 5.4 UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of rRNA ITS amplification from test soils. The scale bar represents similarity as a proportion of 1 while numbers 1 to 2 represent different community clusters.

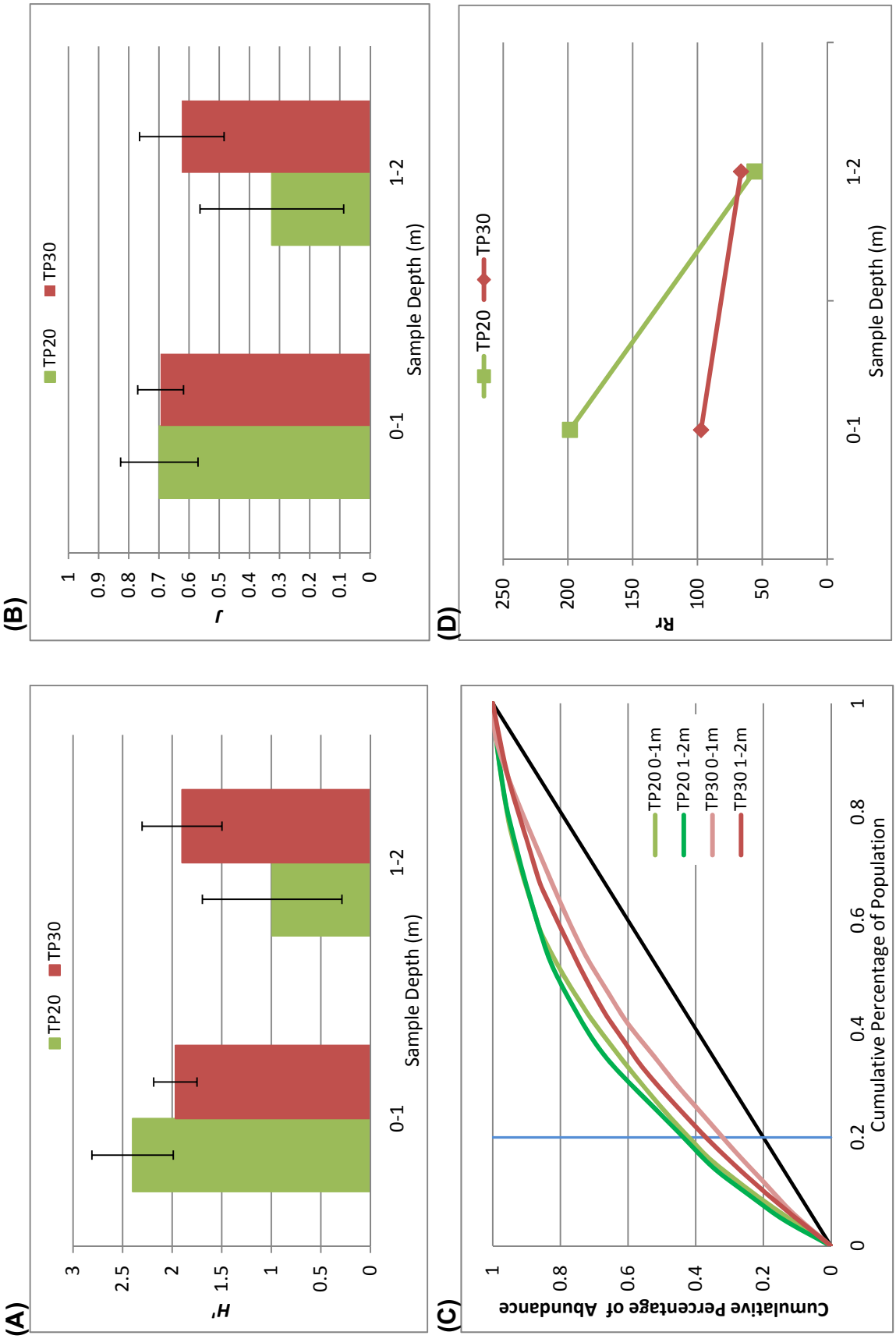

Figure 5.5 Shannon Weaver Indices and Functional Organisation curves for fungal community in test soils. n=3 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve; (D) Range weighted richness (Rr) of test soils. The black 45° line in C indicates the perfect evenness line of a population.

Bacterial Community

The TGGE gel of the test soils across varying depths is provided in Figure 5.6 and the UPGMA dendogram and diversity are provided in Figure 5.7 and Figure 5.8, respectively. The bacterial profiles of the two soils had a similar banding pattern with a few unique less dominant bands present in the different contamination histories.

UPGMA analysis (Figure 5.7) showed that the samples formed two distinct clusters dependent on contamination level, with the exception of one sample (TP20 0–1 m replicate A). Within the two clusters, samples grouped dependent on sample depth. The bacterial communities within the two large clusters shared a similarity of 54% to 63%.

The Shannon Weaver diversity and Equitability Indices were relatively stable across sample depths in the pristine soil, whereas there was an increase in diversity with depth in the contaminated soil. The pristine soil had a higher species diversity of the shallower samples (H'=3.63±0.02) and the contaminated soil had the highest diversity for the deeper samples. This sample also had the highest diversity of all samples tested (H'=3.65±0.13) (Figure 5.8A). The equitability remained constant for the pristine soils (J=0.79) and slightly increased with depth in the contaminated samples (J=0.83±0.07 for shallow sample and 0.85±0.03 for deeper sample) (Figure 5.8B). Statistical analysis (Students *t*-test) of the diversity and equitability revealed that there was a significant difference in bacterial community profiles between the pristine and contaminated soils (p=0.04 and 0.03 for H' and J, respectively).

Functional organisation of the different samples was interpreted using PL evenness curves (Figure 5.8C). The Fo for the majority of the communities was considered medium and the shallow pristine community had a high organisation; Fo ranged from 62% (TP30 1-2 m) to 74% (TP20 0-1 m). This suggests that the majority of the soils had only a few dominant species with the majority of the community in even concentration. The contaminated soils tended to be more

even than the pristine soils, given the slightly lower PL curve value. The same trend was observed in the fungal communities' investigated above.

The range-weighted richness (Rr) of each sample was calculated and averaged to determine the habitability of the community (Figure 5.8D). The carrying capacity of the soils was considered to be high and appeared to be similar based on the depth of the sample with the deeper samples of both contamination histories having the highest Rr (deep pristine Rr=162±4; deep contaminated Rr=162±1). The high Rr for all soils suggests that the soils have a capacity to support a wide variety of species with very few dominant species; the carrying capacity increased with depth.

After analysis of the diversity of the test pits, ten representative bands of interest were excised and sequenced to determine the identity of the dominant organisms present in the samples (bands in red boxes in Figure 5.6). Sequences were matched with the highest identity score in NCBI (Table 5.4). Final sequences of excised bands can be found in Appendix A.3. It should be noted that identity matches below 99% are considered ambiguous and therefore the identities may represent a related species to those matched. Furthermore, the species sequence may in fact be representative of novel or previously uncharacterised organisms.

The majority of excised bands (60%) were present across all depths and contamination levels. One dominant band was unique to the pristine soil, band B3 (similar to *Thiocapsa sp.*), and band B8 (member of the *Alcanivorax* genus) was only identified in the deeper samples from both contamination histories (i.e. TP20 1.0-2.0 m and TP30 1.0-2.0 m). The identity of the excised bands was dominated (80%) by *gamma-Proteobacteria* (*γ-Proteobacteria*), three of which (bands B5, B7 and B8) were identified as members of the *Alcanivorax* genus. The other identified bacteria belonged to the classes *alpha-Proteobacteria* (*α-Proteobacteria*) (band B10, similar to uncultured *Sphingomonadaceae* bacterium) and *Firmicutes* (band B1, similar to *Pontibacillus halophilus*).

Table 5.4 Summary of sequence identifications of bands excised from bacterial TGGE gel

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Physiology	Taxonomic Class
B1	NR_044532.1	*Pontibacillus halophilus* strain JSM 076056	90	Halophilic bacterium	*Firmicutes*
B2	HF675135.1	*Pseudomonas stutzeri*	91	Deep bore water isolate	*γ-Proteobacteria*
B3	HE863776.1	Uncultured *Thiocapsa sp.*	85	Anoxygenic phototrophic bacterium	*γ-Proteobacteria*
B4	KF032637.1	*Alcanivorax borkumensis strain WSL14*	98	Marine bacterium	*γ-Proteobacteria*
B5	JF330773.1	*Methylophaga lonarensis* MPL	91	Halo-alkaliphilic methyltroph	*γ-Proteobacteria*
B6	AM501673.1	Uncultured *Chromatiales* bacterium	90	Marine bacterium	*γ-Proteobacteria*
B7	HM171243.1	*Alcanivorax sp.* clone M81C61	99	Oil degrading marine bacterium	*γ-Proteobacteria*
B8	JF727668.1	Uncultured *Alcanivorax sp.* clone bac669	98	Halophilic bacterium	*γ-Proteobacteria*
B9	KF911342.1	*Marinobacter hydrocarbonoclasticus* strain HME9331	96	Oil degrading marine bacterium	*γ-Proteobacteria*
B10	HM171137.1	Uncultured *Sphingomonadaceae* bacterium	95	Marine bacterium	*α-Proteobacteria*

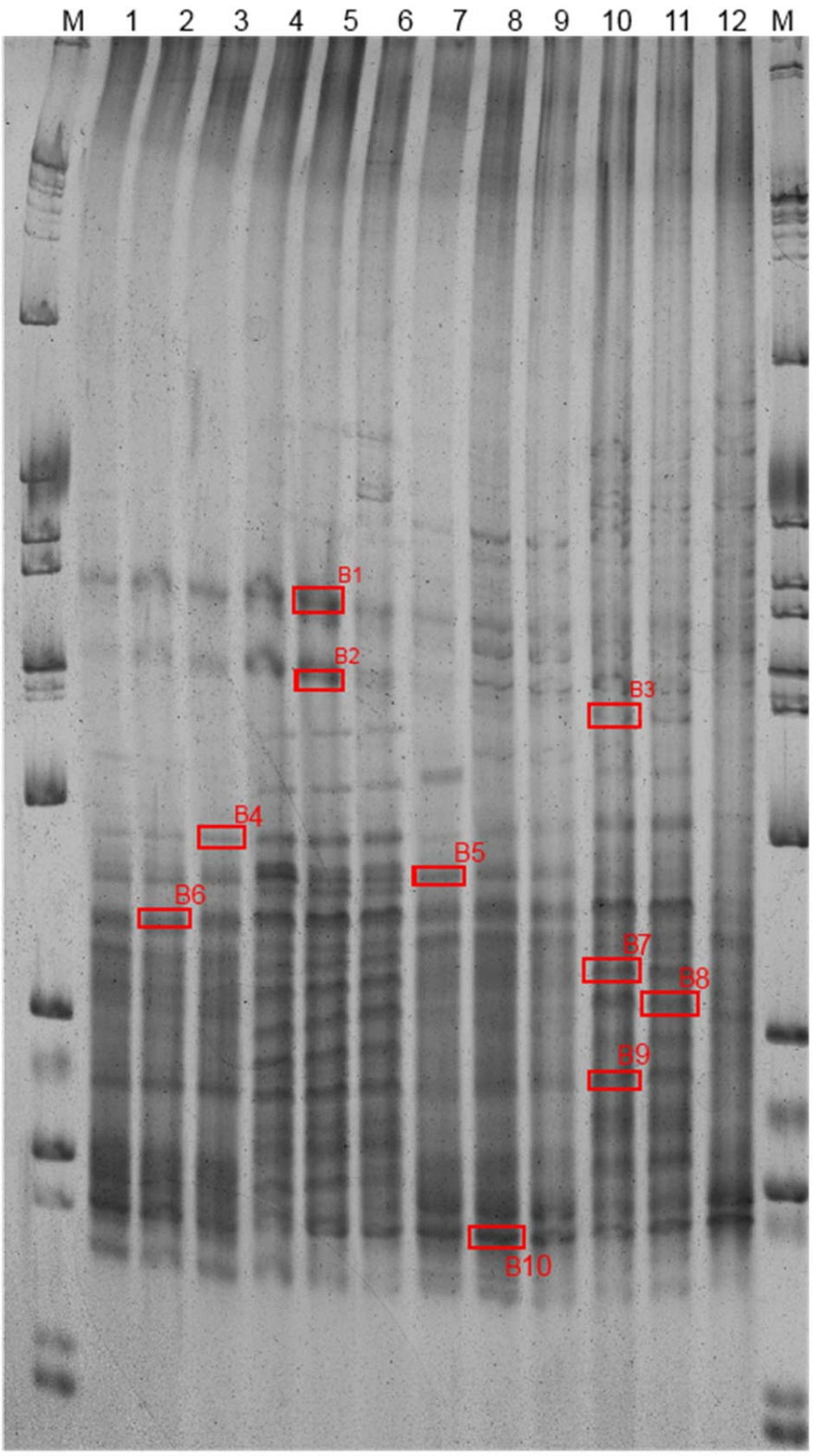

Figure 5.6 TGGE (55.0–68.0°C) of bacterial community from test soils. n=3 M: Marker. Lanes 1-3: TP30 0-1m; Lanes 4-6: TP30 1-2 m; Lanes 7-9: TP20 0-1 m; Lanes 10-12: TP20 1-2 m. Bands in red boxes were excised for sequencing.

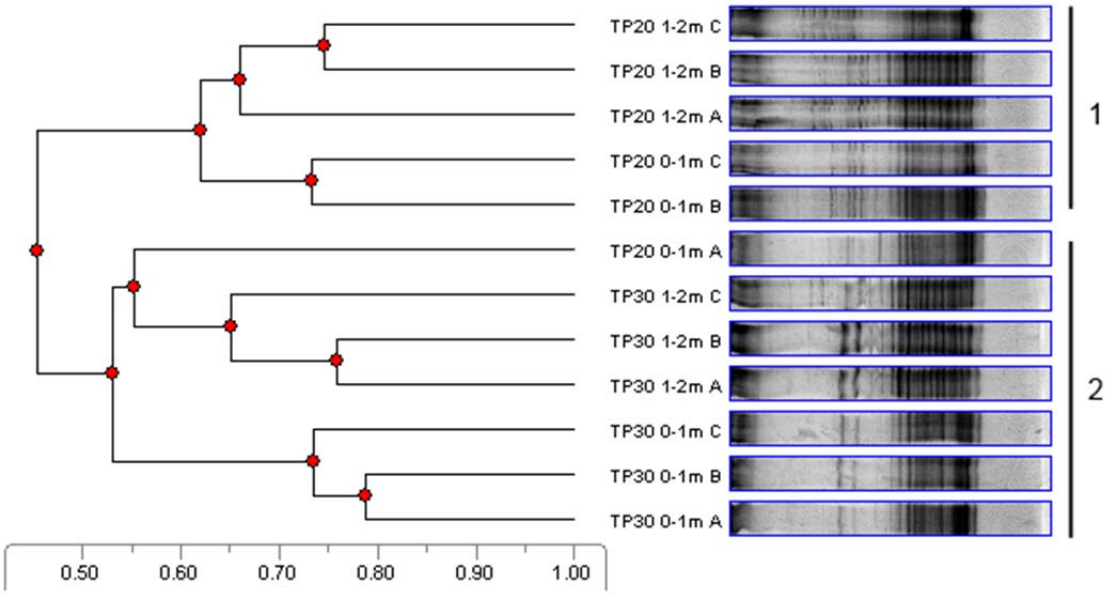

Figure 5.7 UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of 16S rRNA amplification from test soils. n=3 The scale bar represents similarity as a proportion of 1 while numbers 1 and 2 represent different bacterial community clusters.

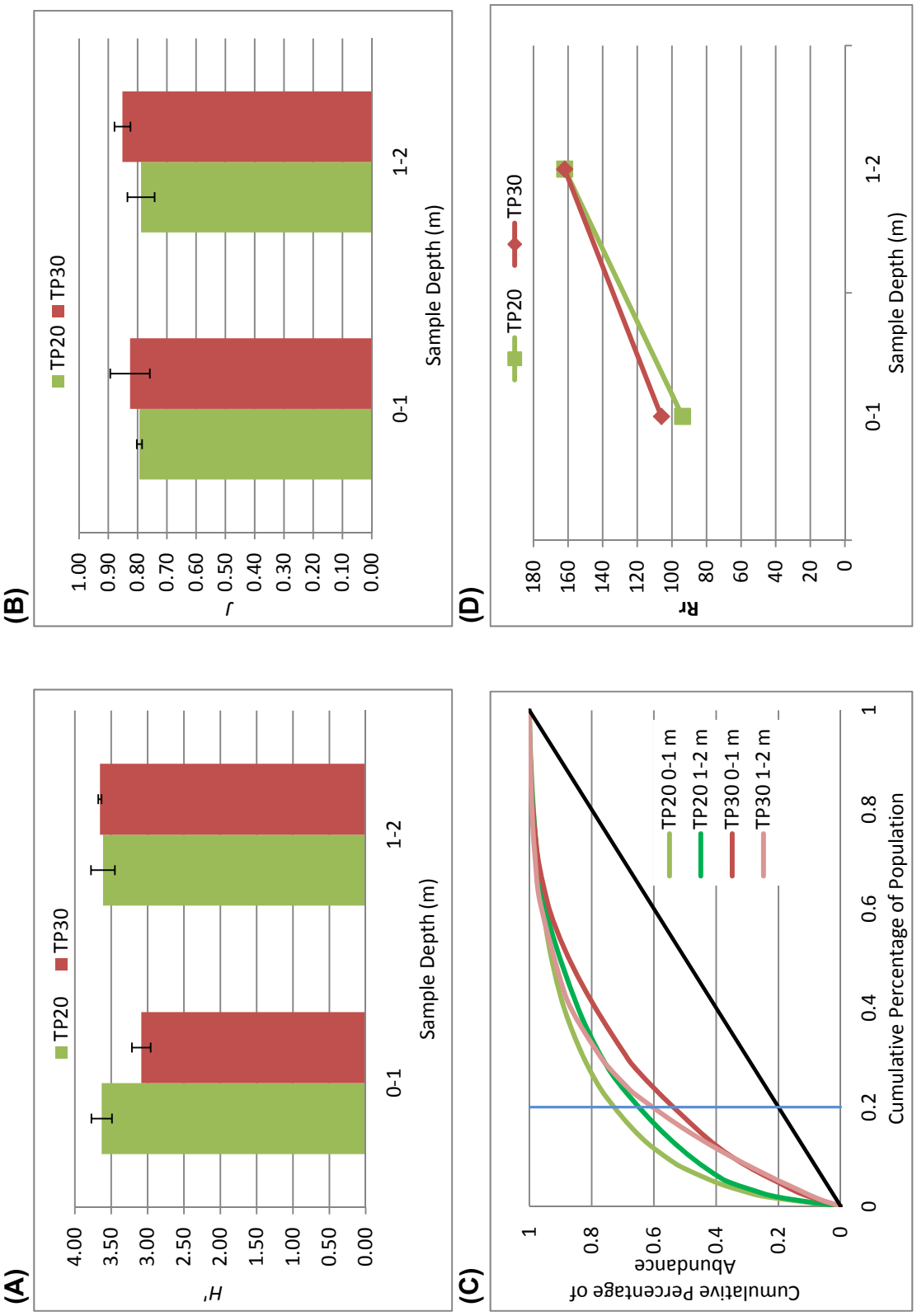

Figure 5.8 Shannon Weaver Indices and Functional Organisation curves for bacterial community in test soils. n=3 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve; (D) Rr of test soils. The black 45° line in C indicates the perfect evenness line of a population.

RHD Prediction Primers

Attempts to utilise prediction primers targeting key fungal PAH degradation enzymes (lignin peroxidase, manganese-dependent and laccase) were unsuccessful mainly due to non-specific primer binding resulting in multi-banded profiles even when template DNA was extracted from pure culture (data not shown). Therefore, investigation into the prediction of the soils ability to degrade PAH was undertaken on the bacterial community only.

The TGGE gel of the Gram-positive and Gram-negative RHD primers from both test soils is provided in Figure 5.9. The figure shows that there were very few species present that had the RHD gene present. A total of eight Gram-positive bacteria species and four Gram-negative bacteria were identified as having the capacity to degrade PAHs. The shallow pristine sample and deep contaminated samples both had the most number of Gram-positive or Gram-negative potential degraders, suggesting that these soils would respond best to a contamination event.

A representative of all RHD positive bands were excised and sequenced to determine their identity (bands in red boxes in Figure 5.9). Sequences were matched with the highest identity score in NCBI (Table 5.5). Final sequences of excised bands can be found in Appendix A.3. It should be noted that identity matches below 99% are considered ambiguous and therefore the identities may represent a related species to those matched. Furthermore, the species sequence may in fact be representative of novel or previously uncharacterised organisms.

The Gram-positive species were dominated by Actinobacteria (bands R1 to R7) and the Gram-negative species were all *γ*-Proteobacteria (bands R9 to R12), three of which were *Pseudomonads*. Band R7 (member of *Thermomonospora* genus), band R8 (member of *Sulfobacillus* genus), band R9 (similar to *Alcanivorax dieselolei*) and band R12 (similar to *Pseudomonas putida*) were present only in the contaminated soil, with band R7 and R8 found in the shallow sample and band R12 only in the deeper sample.

Band R1 (similar to *Sanguibacter keddieii*), band R3 (similar to *Clavibacter michiganensis*) and band R4 (similar to *Streptomyces davawensis*) were unique to the pristine soil, with the last two species only present in the shallow sample.

When comparing the bacteria identified as potential degraders with those that were dominant in the baseline community (Table 5.4) there were no two species that were the same. However, members of the genus *Alcanivorax and Pseudomonas* were identified in both.

Table 5.5 Summary of sequence identifications of bands excised from RHD TGGE gel

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Taxonomic Class
R1	CP001819.1	*Sanguibacter keddieii* DSM 10542	89	Actinobacteria
R2	CP002593.1	*Pseudonocardia dioxanivorans* CB1190	88	Actinobacteria
R3	HE614873.1	*Clavibacter michiganensis*	89	Actinobacteria
R4	HE971709.1	*Streptomyces davawensis* strain JCM 4913	88	Actinobacteria
R5	CP003876.1	*Nocardia brasiliensis* ATCC 700358	88	Actinobacteria
R6	CP007155.1	*Kutzneria albida* DSM 43870	83	Actinobacteria
R7	CP001738.1	*Thermomonospora curvata* DSM 43183	82	Actinobacteria
R8	CP003179.1	*Sulfobacillus acidophilus* DSM 10332	89	Firmicutes
R7	CP006966.1	*Phaeobacter gallaceciensis*	84	α-Proteobacteria
R8	CP002083.1	*Hyphomicrobium denitrificans*	96	α-Proteobacteria
R9	CP003466.1	*Alcanivorax dieselolei*	82	γ-Proteobacteria
R10	CP004143.1	*Pseudomonas denitrificans*	84	γ-Proteobacteria
R11	CP007224.1	*Pseudomonas aeuroginosa*	82	γ-Proteobacteria
R12	CP000926.1	*Pseudomonas putida*	89	γ-Proteobacteria

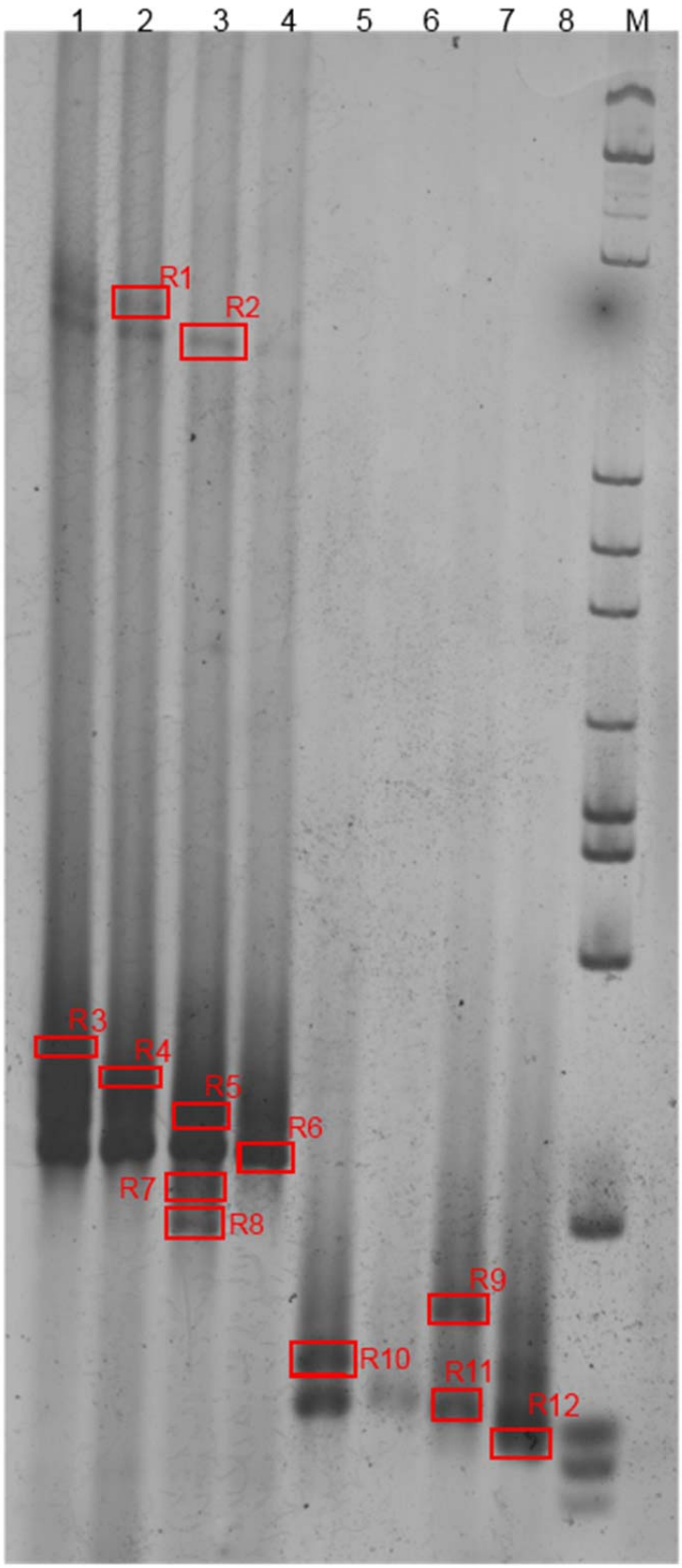

Figure 5.9 TGGE (50.0 – 58.0 °C) of RHD prediction primers from bacterial community. n=1
M: Marker. Lane 1: TP20 0-1m Gram-positive; Lane 2: TP20 1-2 m Gram-positive; Lane 3:TP30 0-1 m Gram-positive; Lane 4: TP30 1-2 m Gram-positive; Lane 5: TP20 0-1m Gram-negative;
Lane 6: TP20 1-2 m Gram-negative; Lane 7: TP30 0-1 m Gram-negative; Lane 8: TP30 1-2 m Gram-negative. Bands in red boxes were excised for sequencing.

5.3.3. ^{14}C mineralisation

The ability of the soils to degrade (or mineralise) a secondary contaminant, phenanthrene, was tested over a 25 day period using ^{14}C-phenanthrene (Figure 5.10).

The control (sodium azide treated sample) confirmed that any mineralisation observed during the experiments was due to biotic respiration, not abiotic processes.

After 4 days, a minimum of 10% of the phenanthrene had been mineralised in all test soils. The mineralisation rate of the shallow contaminated soil and the deeper pristine soil were similar and both were higher than the other two soil types, i.e. deeper contaminated and shallow pristine soils. The degradation in these two samples rapidly increased until around day 10 where around 70% of the phenanthrene had been utilised. After this the mineralisation gradually slowed to have an overall mineralisation of 87% and 81% for deep pristine sample and shallow contaminated sample after 25 days, respectively. The other samples had similar slower mineralisation rates, after 10 days only 20–30% of the phenanthrene was utilised, which was around half that of the deep pristine and shallow contaminated samples. The final percentage of phenanthrene mineralised after 25 days was 41% for the deep contaminated sample and 33% for the shallow pristine sample.

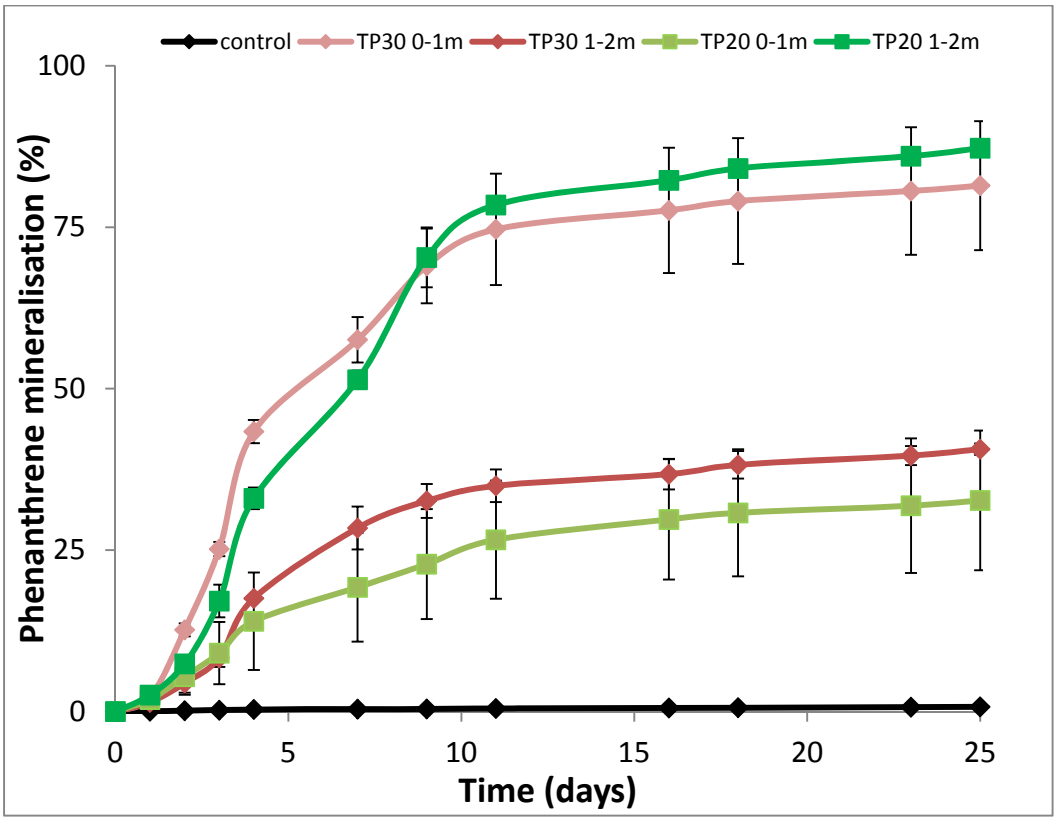

Figure 5.10 ^{14}C-phenanthrene mineralisation of Pristine and Contaminated Sandy Soils incubated in the presence of nutrients. ^{14}C-phenanthrene mineralisation is also shown for sodium azide-killed control. Average mineralisation percentage of triplicate experiments are shown.

5.3.4. Stable Isotope Probing

Based on the results of the ^{14}C-phenanthrene mineralisation study, mescosoms were incubated (or pulsed) with ^{13}C-labelled phenanthrene or ^{12}C-phenanthrene for 5 days to allow for sufficient incorporation of the labelled substrate into the biomarkers (DNA and RNA) of the metabolising organisms but not enough incorporation to dilute results through cross-feeding.

After incubation, biomarkers were extracted from triplicate samples and pooled to account for natural variability in sampling of non-homogenous soil. Samples of the total community DNA and cDNA were then amplified using universal fungal and bacterial primers.

Samples of the extracted biomarkers from the contaminated (or pulsed) soil samples were then centrifuged in the presence of CsCl or CsTFA to produce a density gradient. The gradients were fractionated and weighed to determine which fractions would be targeted for further downstream amplification.

Figure 5.11 provides an example of the gradient fractions and highlights the target weights for labelled DNA (A) and RNA (B).

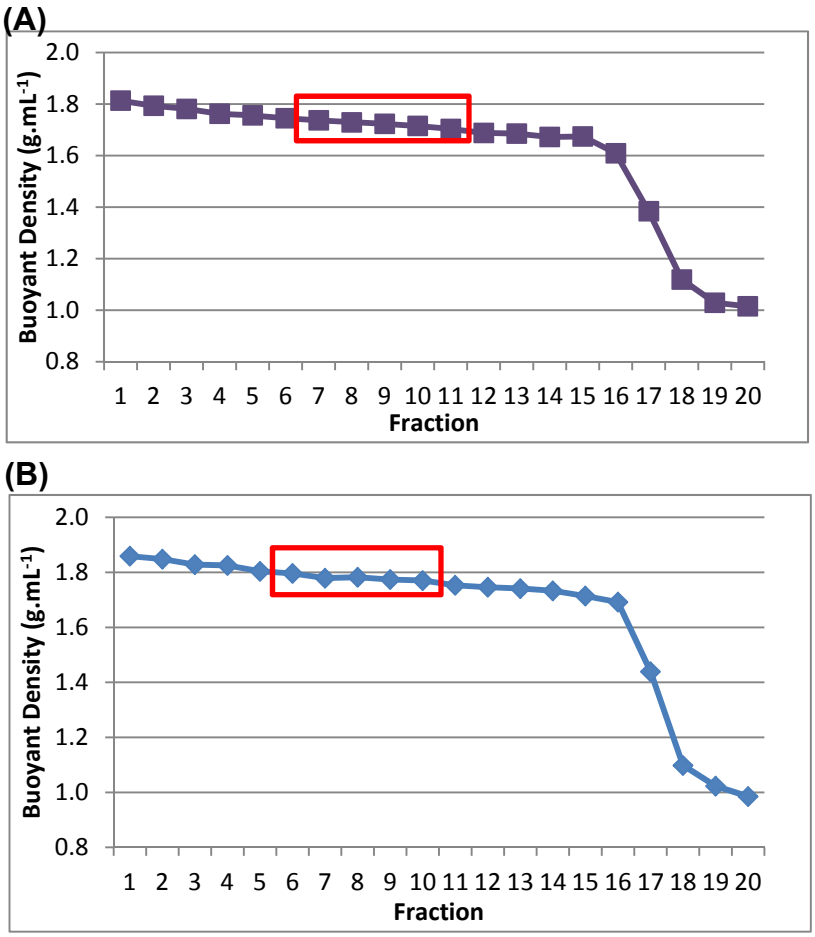

Figure 5.11 Example buoyant density of gradient fractions from SIP of TP20 1–2 m (A) DNA SIP; (B) RNA SIP; target density of labelled nucleic acid

The target fractions were amplified using the universal primers and the PCR products separated using TGGE, along with the baseline and pulsed total community samples for comparison. Figure 5.12 provides an example of a TGGE from TP20 0–1 m for both bacteria (A) and fungi (B).

TGGE separation gels were run for all test samples, and representative bands of interest were excised and sequenced to identify key microorganisms. For ease of presentation, the results for the bacterial and fungal communities will be discussed in separate sections.

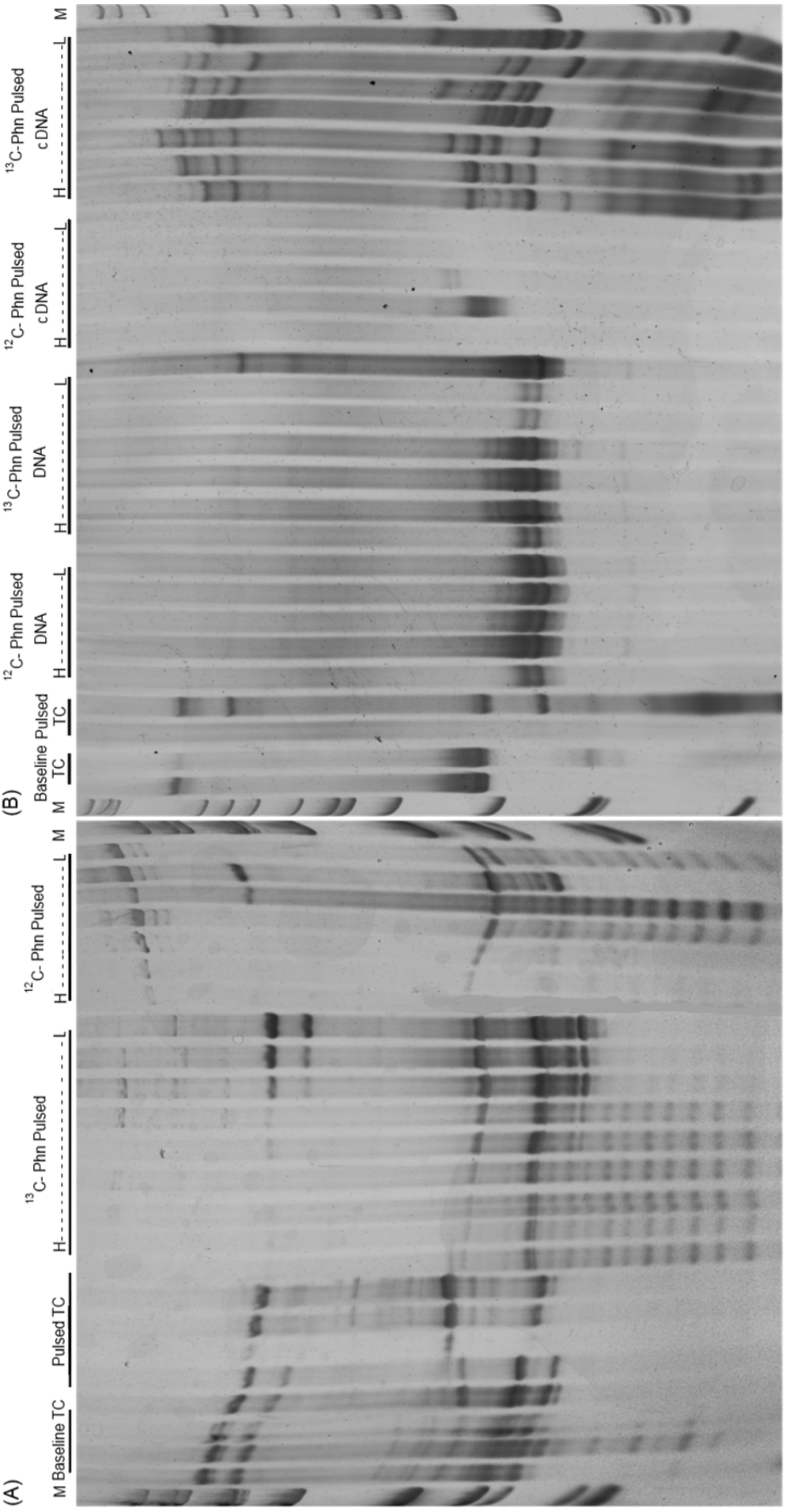

Figure 5.12 TGGE of bacterial (A) and fungal (B) community from TP20 0-1 m. M: Marker; TC: total community; Phn: phenanthrene; H: heavy fraction; L: light fraction. NB: Figure 5.12A does not contain cDNA samples; however cDNA was tested for all samples just not displayed in this image. Temperature gradient; A: 55-68ºC; B: 55-58ºC.

Fungal Community Dynamics

Figure 5.13A & B compares the Shannon Weaver diversity and Equitability Indices of the total fungal profile from the baseline and pulsed communities. The pulsed community diversity was amplified from cDNA extracted from the incubated mesocosm after 5 days, thus targeting the active degraders within the community.

In both the contaminated and pristine soils the species diversity decreased after incubation compared with the baseline, with the exception of the deeper pristine sample where the diversity within the pulsed cDNA was higher (*H'*=0.99 increasing to *H'*=1.24). A similar trend was observed with the equitability and functional organisation (Figure 5.13C), demonstrating that the incubation of test soils with phenanthrene as the sole carbon source caused a large shift and decrease in diversity with more dominant species present for most of the communities investigated. The exception to this was the deep pristine sample which increased its diversity and became more even.

The similarity in community profile of each soil ranged from 20% to 48% (Figure 5.13D), with the most similar communities being those from the shallow pristine soil and the deeper contaminated soil. These soils also had similar lower mineralisation rates during ^{14}C-phenanthrene studies (Section 5.3.3).

To discover the identity of the fungi that were responsible for the initial degradation, DNA and RNA-SIP was employed to characterise the key degraders. To ensure that identification of degraders was accurate, several fractions within the target buoyant densities were amplified and run using TGGE, with dominant representative bands excised and identified via sequencing (Table 5.6 provides a summary of the sequence identities. All sequences of excised bands can be found in Appendix A.3). A comparison gel is presented in Figure 5.14 with representative samples of the various densities compared to the baseline and total pulsed communities for all soils.

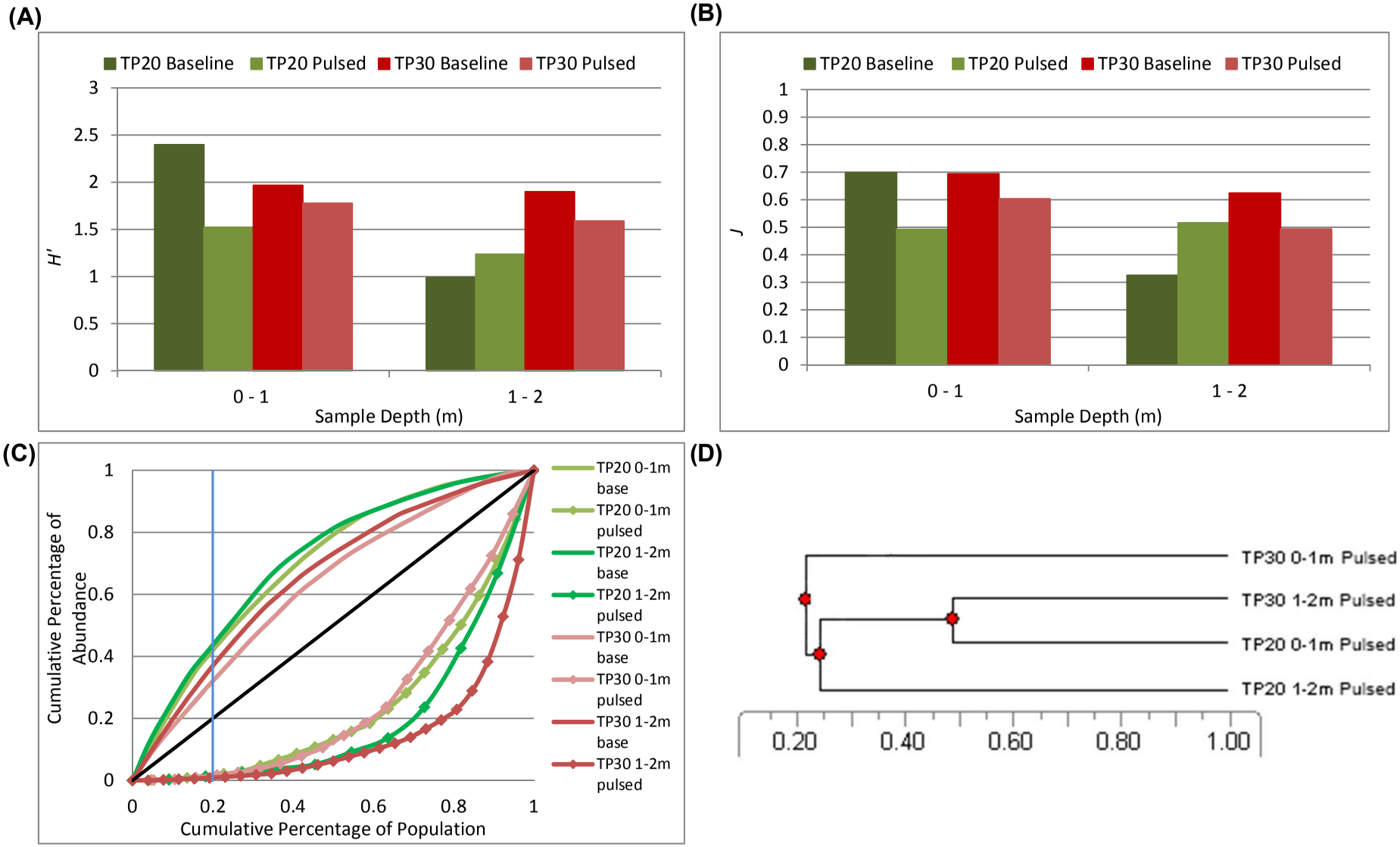

Figure 5.13 Shannon Weaver Indices and Functional Organisation curves for fungal community in test soils. n=1 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve, the black 45° line indicates the perfect evenness line of a population; (D) UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of ITS amplification from pulsed soils. The scale bar represents similarity as a proportion of 1.

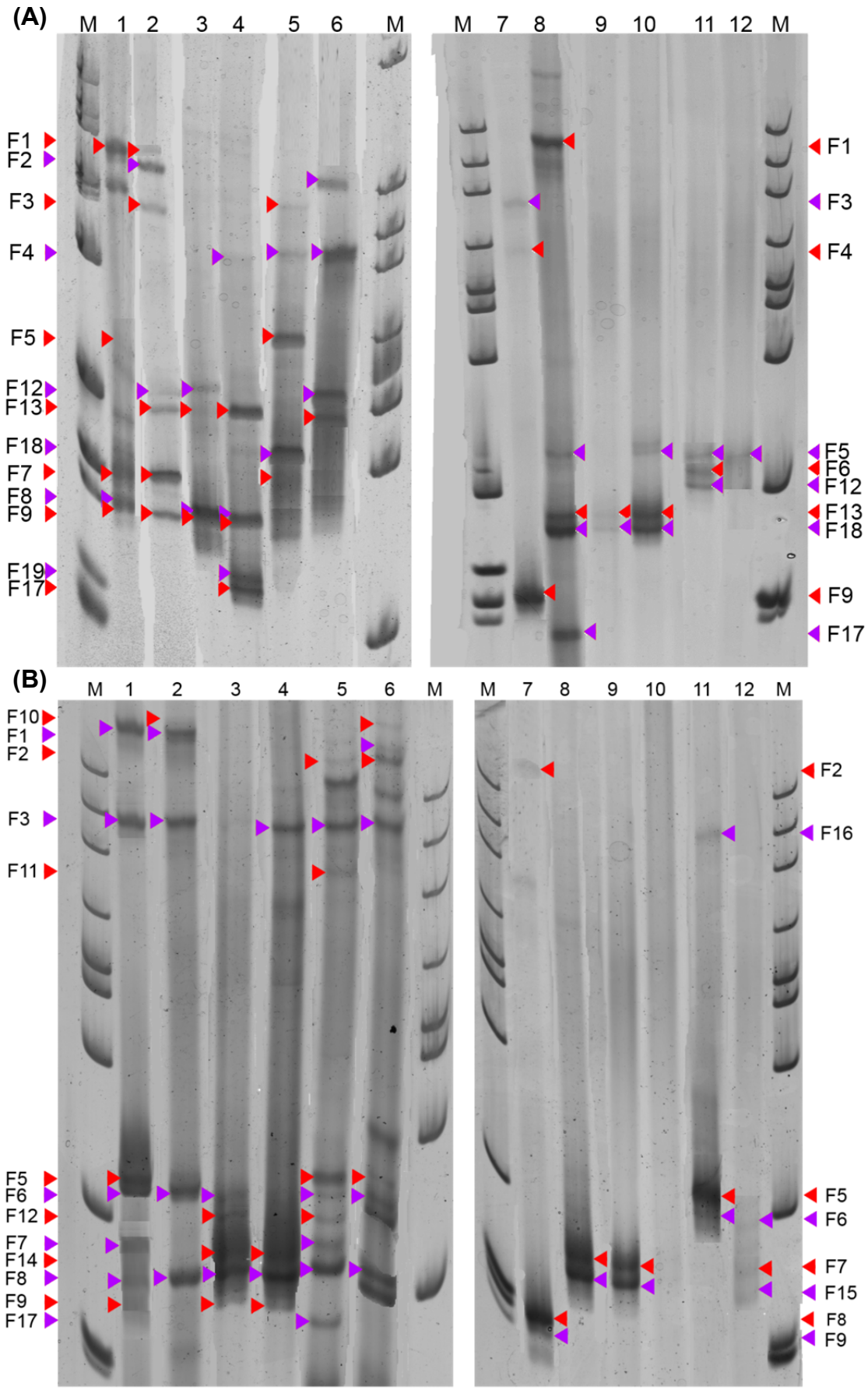

Figure 5.14 Comparison TGGE of Fungal communities after contamination. M: Marker. (A) Contaminated Soil (TP30); (B) Pristine Soil (TP20); Lanes 1 & 7: Baseline total community; Lanes 2 & 8: Pulsed total community; Lanes 3 & 9: Representative profile of active degraders from heavy DNA- SIP fraction; Lanes 4 & 10: Representative profile of light DNA- SIP fraction; Lanes 5 & 11: Representative profile of active degraders from heavy RNA- SIP fraction; Lanes 6 & 12: Representative profile of light RNA- SIP fraction. Arrows indicate sequenced bands.

Table 5.6 Summary of sequence identifications of bands excised from fungal DNA-SIP and RNA-SIP fractions and pulsed total community.

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Taxonomic Phylum/ Class
F10	KF468222.1	*Rhodotorula mucilaginosa* strain E228	99	*Basidiomycota/ Microbotryomycetes*
F11	AB105353.1	*Cryptococcus pseudolongus*	98	*Basidiomycota/ Tremellomycetes*
F12	EF060778.1	*Tremellales* sp. LM477	86	*Basidiomycota/ Tremellomycetes*
F13	AF472627.1	*Cryptococcus perniciosus* strain VKM Y-2905	89	*Basidiomycota/ Tremellomycetes*
F14	KC525814.1	Uncultured *Candida* clone EF1-834	98	*Ascomycota/ Saccharomycetes*
F15	NR_111084.1	*Auriculibuller fuscus* PYCC 5690	92	*Basidiomycota/ Tremellales*
F16	KC525576.1	Uncultured *Cladosporium* clone CSL1-1620	94	*Ascomycota/ Dothideomycetes*
F17	AF444390.1	*Cryptococcus sp.* CBS 8363	96	*Basidiomycota/ Tremellomycetes*
F18	GQ219851.1	Uncultured *Basidiomycota* clone SC_ITS_048	99	*Basidiomycota/* unknown
F19	EU002949.1	Uncultured *Sporidiobolales* clone 2d	89	*Basidiomycota/ Microbotryomycetes*

All of the dominant baseline species were identified in the pulsed total communities although not at the same dominance or in the same samples as at baseline. For example, *P. griseofulvum* (band F2) was present only in the deep pristine baseline community but after the pulse the species was not identified in the pulsed community but was a dominant species in the shallow contaminated community.

A total of 10 fungal species were identified as active degraders of phenanthrene due to the bands presence in only the heavy RNA- or DNA-SIP fractions (Table 5.7). The phenanthrene degraders were dominated (73%) by members of the phylum *Basidiomycota*, with the majority coming from the order *Tremellomycetes*.

Three of the active degraders were the dominant baseline species in the community (*Davidiellaceae sp.* in the deep pristine sample (band F5, 99% similarity), *R. babjevae* in the shallow pristine and deep contaminated sample (band F6, similarity 99%) and organism similar to *C. nemorosus* in the shallow contaminated and both pristine samples (band F7, 95% similarity). Of the dominant bands present in the total pulsed communities only three species were actually degraders (*R. babjevae* in the deep contaminated and shallow pristine samples, *Cryptococcus* sp. in the shallow contaminated and both pristine samples and *A. fuscus* in the deep pristine sample).

Only one of the active degrader species was identified by both RNA- and DNA-SIP, and five degraders (F6, F7, F9, F12 and F15) would not have been identified had only RNA-SIP been employed as the bands appeared in both the heavy and light cDNA fractions. *Tremellales sp.* and *Cryptococcus* sp. were the most common degraders as they were found in contaminated and pristine sample soils.

Table 5.7 Presence of active degrader bands in heavy fractions of DNA- and RNA-SIP.

Band	Similarity/Identity	Contaminated Soil		Pristine Soil	
		Shallow	Deep	Shallow	Deep
F3	*Cryptococcus sp.*	RNA	-	-	-
F5	*Davidiellaceae sp.*	RNA	-	-	RNA
F6	*Rhodosporidium sp.*	-	RNA	DNA^{+}	-
F7	*Cryptococcus sp.*	RNA	-	RNA	DNA^{+}
F11	*C. pseudolongus*	-	-	RNA	-
F12	*Tremellales sp.*	DNA^{+}	RNA	RNA / DNA	-
F15	*Auriculibuller sp.*	-	-	-	DNA^{+}
F16	*Cladosporium* sp.	-	-	-	RNA
F17	*Cryptococcus sp.*	-	-	RNA	-
F18	*Basidiomycota sp.*	RNA	-	-	-

NB: RNA -band present in heavy fractions of RNA-SIP; DNA -band present in heavy fractions of DNA-SIP; +-band present in heavy fraction in DNA-SIP, but present in both fractions in RNA-SIP

Bacterial Community Dynamics

Figure 5.15A&B compares the Shannon Weaver diversity and Equitability Indices of the total bacterial community from baseline and after incubation with phenanthrene.

As was seen in the fungal communities, the species diversity, equitability and functional organisation decreased in both the contaminated and pristine soils after contamination compared with baseline; the deeper contaminated sample was the least affected by the phenanthrene with only a slight decrease in diversity and an increase in equitability (Figure 5.15A-C). With the exception of the deeper contaminated soil, incubation with phenanthrene as the sole carbon source caused a large shift in the community diversity such that only a few species were present which dominated the community. In the baseline community the similarity in community profile was based on the level of soil contamination (Figure 5.4); however after the contamination event the shift in diversity was such that the contaminated soils and deep pristine soil had the

highest similarity (around 50%) and the pristine deeper soil was the least similar (Figure 5.15D).

Identification of the key bacterial degraders of phenanthrene using SIP was completed using the same DNA and RNA that was isolated for the fungal identification. 16S rRNA universal primers were used to amplify target fragments from the fractionated biomarkers, separated using TGGE and representative dominant bands excised and identified via sequencing (Table 5.8 provides a summary of the sequence identities. All sequences of excised bands can be found in Appendix A.3). A comparison gel is presented in Figure 5.16 with representative samples of the various densities compared to the baseline and total pulsed communities for all soils.

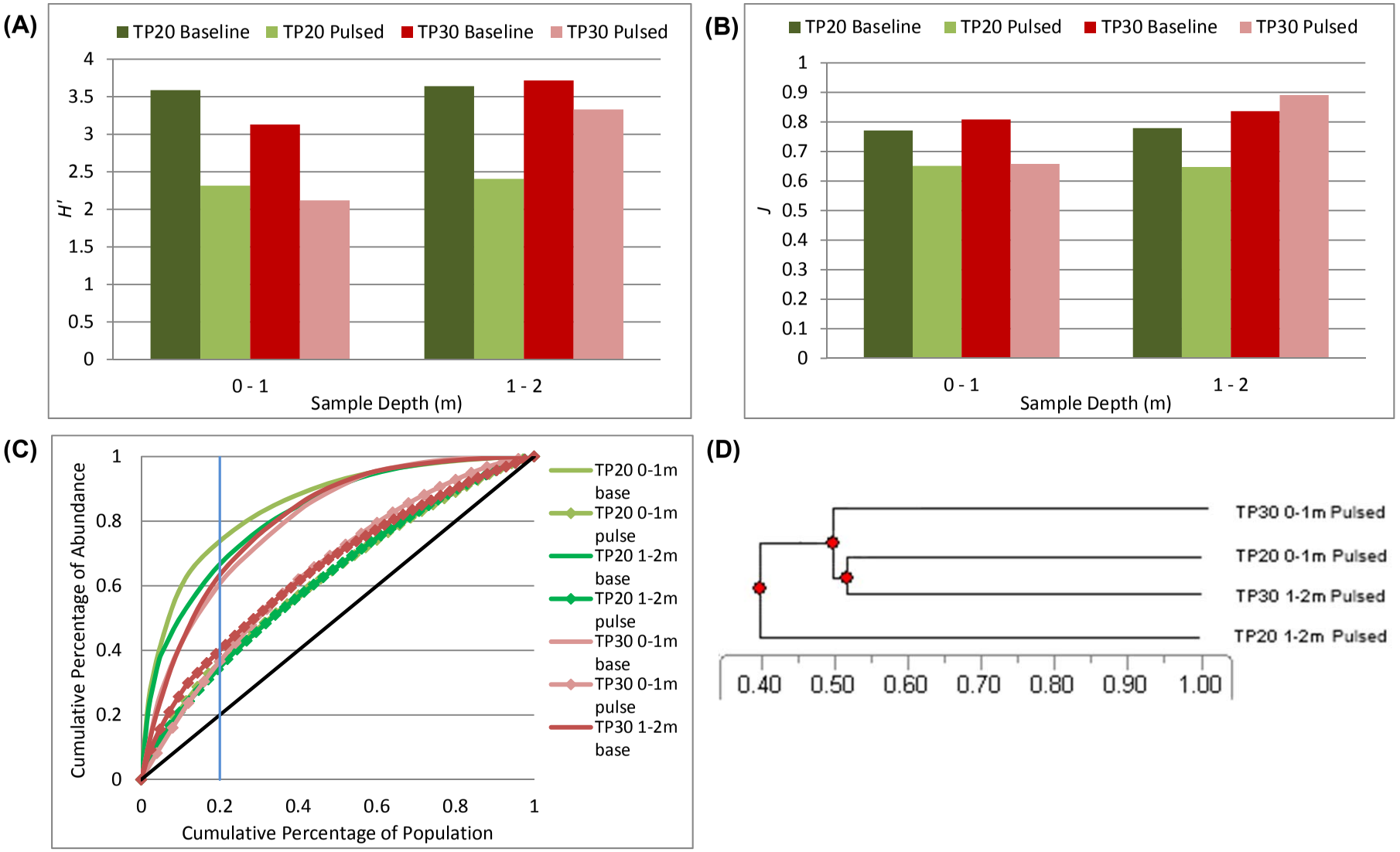

Figure 5.15 Shannon Weaver Indices and Functional Organisation curves for bacterial community in test soils. n=1 (A) Shannon Weaver diversity Indices, *H'*; (B) Shannon Weaver Equitability Indices, *J*; (C) Fo curve, the black 45° line indicates the perfect evenness line of a population; (D) UPGMA analysis constructed from similarity matching data (Dice-Sorenson Index) produced from TGGE profile of 16S rRNA amplification from pulsed soils. The scale bar represents similarity as a proportion of 1..

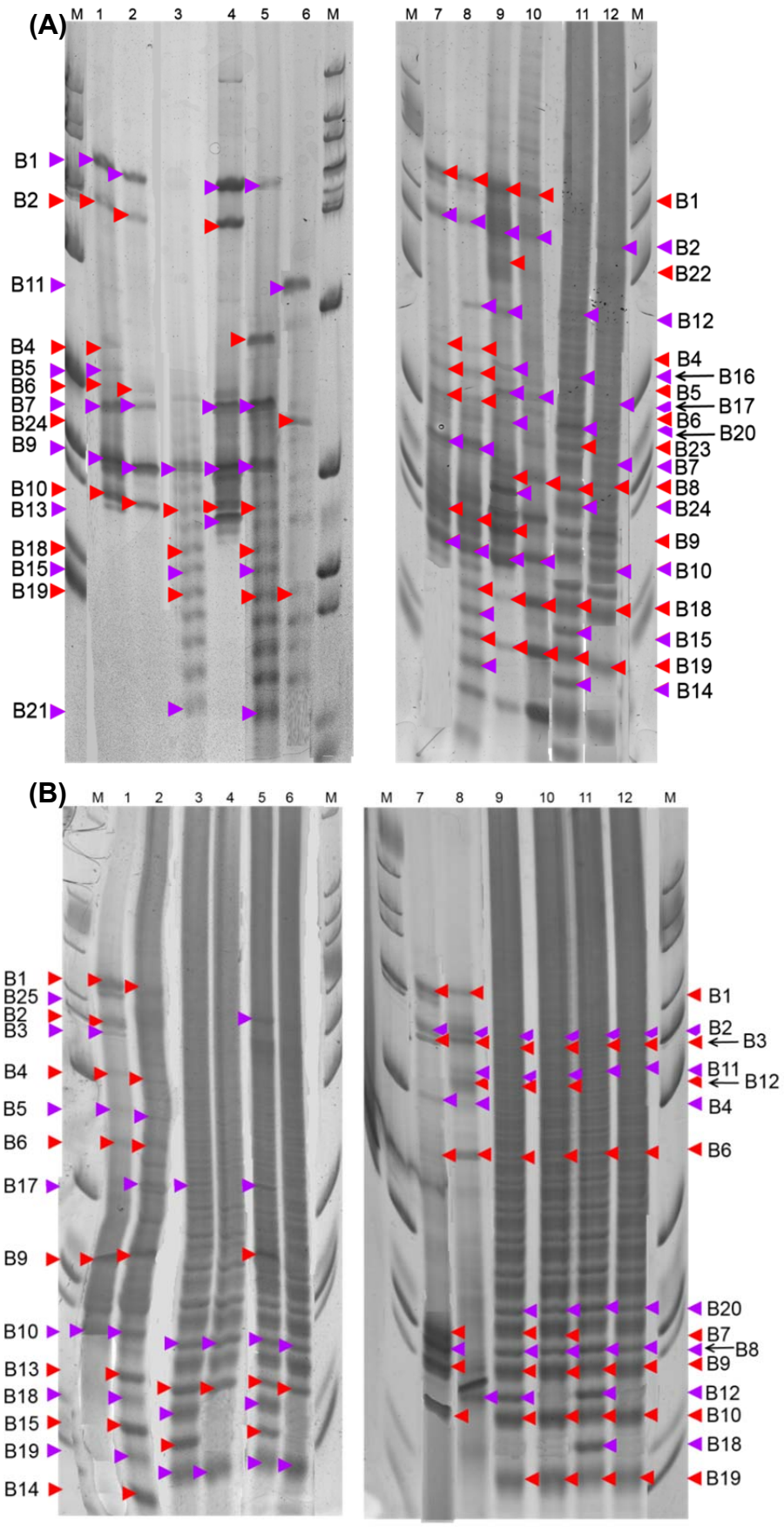

Figure 5.16 Comparison TGGE of Bacterial communities after contamination. M: Marker. (A) Contaminated Soil (TP30); (B) Pristine Soil (TP20); Lanes 1 & 7: Baseline total community; Lanes 2 & 8: Pulsed total community; Lanes 3 & 9: Representative profile of active degraders from heavy DNA- SIP fraction; Lanes 4 & 10: Representative profile of light DNA- SIP fraction; Lanes 5 & 11: Representative profile of active degraders from heavy RNA- SIP fraction; Lanes 6 & 12: Representative profile of light RNA- SIP fraction. Arrows indicate sequenced bands.

Table 5.8 Summary of sequence identifications of bands excised from bacterial DNA-SIP and RNA-SIP fractions and pulsed total community.

Band	Accession Number	Closest Match (NCBI Database)	Similarity (%)	Physiology	Taxonomic Class
B11	HF558623.1	Uncultured bacterium	99	Isolate from mine tailings	Unknown
B12	NR_114886.1	*Thiodictyon syntrophicum*	92	Sulfur reducing bacterium	*γ-Proteobacteria*
B13	KC445196.1	*Nocardiopsis sp.* SMVB7	99	Thermophilic bacterium	*Actinobacteria*
B14	NR_109065.1	*Spongiibacter borealis* strain CL-AS9	99	Marine bacterium	*γ-Proteobacteria*
B15	GQ471873.1	Uncultured *Enterococcus sp.* clone G1-5	99	Unknown	*Firmicutes*
B16	Kf909005.1	*Marinobacter sp.* BTCZ2	95	Marine bacterium	*γ-Proteobacteria*
B17	JX531089.1	Uncultured *Alcanivorax sp.* clone C114500069	85	Marine bacterium	*γ-Proteobacteria*
B18	EU841705.1	*Streptomyces flavidofuscus* strain HBUM173408	99	Thermophilic bacterium	*Actinobacteria*
B19	GQ471877.1	Uncultured *Klebsiella sp.* clone G2-4	95	Pesticide waste water sludge isolate	*γ-Proteobacteria*
B20	JN791398.1	*Microbulbifer sp.* OM03	99	Marine bacterium	*γ-Proteobacteria*
B21	AY394880.1	*Alcanivorax sp.* EPR 27W	99	Marine bacteria	*γ-Proteobacteria*
B22	EF521194.1	Uncultured bacterium isolate DDGE gel band D3	90	Isolate from compost biofilter	unknown
B23	JQ429490.1	*Sphingomonas sp.* 6PNM-8	94	Isolate from mine tailings	*α-Proteobacteria*
B24	JF512534.1	Uncultured *Actinomyces sp.*	99	Marine bacterium	*Actinobacteria*
B25	JX236683.1	*Sphingopyxis sp.* M32	94	Isolate from dioxin contaminated soil	*α-Proteobacteria*

Similarly to the fungal dynamics, all of the dominant baseline species were identified in the pulsed total communities although not at the same dominance or in the same samples at baseline. Only one of these species (band B1, *P.* stutzeri) was dominant in all four soil samples after the contamination.

A total of 15 bacterial species were identified as active degraders of phenanthrene (Table 5.9) which were mostly *γ-Proteobacteria* (9 of 15) with other degraders belonging to the classes *α-Proteobacteria* (2 species), *Actinobacteria* (2 species) and *Firmicutes* (1 species).

Only one of the active degraders was a dominant baseline species (band B9–similar to *M. hydrocarbonoclasticus* in the deep contaminated and shallow pristine soils); this species was also dominant in the pulsed communities. Only one other degrader was dominant within the total pulsed community, band B15 (*Enterococcus sp.*) in the shallow pristine sample.

The majority of degraders were identified using RNA-SIP and DNA-SIP. However band B19 (similar to *Klebsiella sp.*) would not have been identified as a degrader had only DNA-SIP been used. One species, a member of the *Sphingopyxis* genus (band B25) was unique to pristine soil whereas several species were unique to the contaminated soil (bands B4, B14, B16, B19, B22–B24). The *Enterococcus sp.* (band B15) and *S. flavidofuscus* (band B18) were the most common degraders.

Table 5.9 Presence of active degrader bands in heavy fractions of DNA- and RNA-SIP.

Band	Species	Contaminated Soil		Pristine Soil	
		Shallow	Deep	Shallow	Deep
B4	*A. borkumensis*	RNA	-	-	-
B9	*M. hydrocarbonoclasticus*	-	DNA	RNA	-
B12	*T. syntrophicum*	-	RNA / DNA	-	RNA / DNA
B14	*S. borealis*	-	RNA	-	-
B15	*Enterococcus sp.*	RNA / DNA	RNA	RNA / DNA	-
B16	*Marinobacter sp.*	-	RNA / DNA	-	-
B17	*Alcanivorax sp.*	-	-	RNA / DNA	-
B18	*S. flavidofuscus*	RNA / DNA	-	RNA / DNA	RNA
B19	*Klebsiella sp.*	DNA^{+}	-	-	-
B20	*Microbulbifer sp.*	-	RNA / DNA	-	-
B21	*Alcanivorax sp.*	RNA / DNA	-	-	-
B22	*Uncultured bacterium*	-	DNA / RNA	-	-
B23	*Sphingomonas sp.*	-	RNA	-	-
B24	*Actinomyces sp.*	-	RNA / DNA	-	-
B25	*Sphingopyxis sp.*	-	-	RNA	-

NB: RNA -band present in heavy fractions of RNA-SIP; DNA -band present in heavy fractions of DNA-SIP; +-band present in heavy fraction in DNA-SIP, but present in both fractions in RNA-SIP.

5.4. Discussion

5.4.1. Baseline Community Profile Investigation

The physio-chemical properties of both soils were typical of sandy type soils with generally low moisture content. However, the moisture content of the contaminated soil was considerably higher compared to pristine soils. This increased moisture content may be a result of the test pit location, as TP30 was below the high tide line on the beach and as a result exposed to more regular wetting whereas TP20 was well above the high tide mark and unlikely to be effected by the ocean. The moisture content of both soils increased with depth which may be indicative of an increase in clay content. The pH of all soils was slightly basic with no significant difference depending on contamination history. The analytical testing of soils demonstrated that TP30 was the soil with the highest contaminant level with TPH contamination above the NEPM HIL guideline and elevated concentrations of total PAHs, most of the contamination was below the 1.0 m depth. The NEPM HIL guideline exceedance indicates that there are concentrations at levels that could cause human health effects.

Investigation of the baseline fungal community revealed that there was no significant difference between the profiles of the contaminated and pristine soils with several dominant species present across all depths and contamination levels. The species diversity of the contaminated soil was very similar across the two depths, whereas the shallow pristine soil had the highest diversity of all samples tested but the diversity of the deeper sample was the lowest. The shallow pristine sample also had the highest range-weighted richness value which was almost double that of the contaminated sample, suggesting that the shallow pristine soil had the capacity to support a wide variety of species with a general evenness across the community. UPGMA analysis of the fungal communities revealed that the similarities in community profile were dependent on depth rather than contamination level.

Analysis of the dominant baseline fungal species present in the various communities identified that most were from the phylum *Basidiomycota* and

were present in all communities. *Penicillium griseofulvum* was the only dominant species identified that was unique to one soil, the deep pristine sample. However, this fungi has been associated with polychlorinated biphenyl and PAH (especially pyrene) degradation in contaminated soil (Ravelet *et al.*, 2000; Han and Tian, 2011). This suggest that the fungal community may already be skewed towards contaminant degraders as several other fungi identified in the community profiles have been shown to degrade various petroleum-based compounds and other common contaminating compounds. *P. chrysogenum* has been shown to degrade various aromatic hydrocarbons including benzo(a)pyrene (Zang *et al.*, 2007) and naphthalene (Juhasz and Naidu, 2000) as well as phenolic compounds (Leitão *et al.*, 2007; Wolski *et al.*, 2012) both in soil and aqueous environments. *Cryptococcus sp.* and other members of the phylum *Basidiomycota* (which includes other fungi identified in this community profiles *such as Rhodotorula sp., Rhodosporidium sp.* and *Papiliotrema sp.*) have been known to degrade herbicides (Das *et al.*, 2012), pesticides (Abdul Salam *et al.*, 2013), phenolics (Cea *et al.*, 2010) and other similar compounds through the production of enzymes such as laccase, lignin peroxidase and manganese-dependent peroxidase. Furthermore, members of the *Dothideomycetes* class (*Davidiellaceae sp., Ascomycota*) have been identified in the composting of abattoir wastes (Xu *et al.*, 2013) and degradation of crude oil (Al-Nasrawi, 2012).

Baseline bacterial community diversity investigation demonstrated that there was a statistical difference in profiles of the contaminated and pristine soils due to contamination history. The pristine soil had a relatively stable diversity and equitability across the two depth profiles, with the functional organisation being slightly lower in the deeper sample. The diversity of the pristine soil was high and the functional organisation was also the highest suggesting that although there was a high number of species present, the community was not even. The carrying capacity of the soils was similar, with the deeper samples from both contamination histories being more accommodating to colonisation (i.e. higher range-weighted richness), although all communities were considered to have a high carrying capacity. Interestingly, the most contaminated sample (the deeper

contaminated soil) had the most diverse, even community and had the potential to support more colonisation. This phenomenon was observed in Chapter 4 when investigating the fungal diversity of different soil types, and as discussed previously this is converse to what is expected to occur after contamination.

Analysis of the dominant baseline bacterial species present in the various communities identified that most were present across all contamination histories and depth with the exception of one species, *Thiocapsa sp.*, which was unique to the pristine soil. Most of the bacteria identified have been isolated from marine or hyper-saline environments and were members of the *γ-Proteobacteria* class. The dominance of salt tolerant bacteria is more than likely reflective of the coastal environment from where the samples were taken.

Organisms similar to *Pseudomonas stutzeri* and *Pontibacillus halophilus* were present across all samples but were the most dominant in the contaminated soils. *P. stutzeri* is known to degrade aromatic hydrocarbons such as phenanthrene (Grimberg *et al.*, 1996) and naphthalene (Bosch *et al.*, 1999) and other complex hydrocarbons such as tetrachloroethene (perchloroethylene) (Ryoo *et al.*, 2000). Members of the Genus *Pontibacillus* have also been associated with degradation of linear hydrocarbons and simple aromatic compounds through production of biosurfactants in oil-contaminated systems (Kheiralla *et al.*, 2013). The bacteria similar to *Alcanivorax sp.*, *Alcanivorax borkumensis* and *Marinobacter hydrocarbonoclasticus* were dominant in the deeper samples of both contamination histories, although their presence in the pristine soil is unexpected given that members of both the *Alcanivoraz* and *Marinobacter* genus have been referred to as obligate hydrocarbonoclastic bacteria and are recognized as playing significant roles in the biological removal of petroleum hydrocarbons from polluted marine water (Gauthier *et al.*, 1992; Schneiker *et al.*, 2006; Yakimov *et al.*, 2007).

Generally, both the fungal and bacterial baseline communities contained organisms that were capable of hydrocarbon degradation and other common anthropogenic contaminants, possibly indicating that the pristine soil is not truly unaffected by contamination. Given that the test pit was located in the small

cove close to the loading dock it is possible that the natural communities have become skewed; alternatively this could demonstrate the fact that many hydrocarbon-degrading microbes are ubiquitous in all environments regardless of previous exposure (Atlas, 1981; Greenwood *et al.*, 2009).

5.4.2. Community Response to Contamination Event

A simulated contamination event of the test soils was enacted via incubation with ^{14}C- and ^{13}C-phenanthrene. The addition of the secondary contaminant caused a decrease in diversity in all bacterial communities as well as fungal diversity except for the deeper pristine sample. The exposure to the secondary contaminant caused a shift in the baseline communities to become dominated by a limited number of bacterial and fungal species resulting in higher function organisation and low evenness.

The pre-dominant phylum of fungi present in both contaminated and pristine pulsed communities was *Basidiomycota* with many members of the *Tremellomycetes* class. The baseline fungal communities had been dominated by the same phylum. Interestingly, only two fungi were dominant in more than one community; a *Mortierella sp.* was found in the shallow pristine and deep contaminated soil and an organism similar to *C. nemorosus* was present in the deep pristine and shallow contaminated soil. This paring of shallow contaminated with the deep pristine sample and the other two samples was replicated in the results of the mineralisation study, discussed in further detail below.

The bacterial pulsed community was dominated by *γ-Proteobacteria*, as it was in the baseline community. There were four dominant species in the contaminated soils across both depths, all of which were present in the baseline community, suggesting that *Pontibacillus halophilus*, *Alcanivorax sp.*, *Marinobacter hydrocarbonoclasticus* and an uncultured *Sphingomonadaceae* bacterium are involved in the degradation of phenanthrene. All of these genera have been identified as having an involvement in hydrocarbon degradation, although often only the simpler compounds, such as n-alkane, and not complex aromatics such

as phenanthrene (Juhasz and Naidu, 2000; Seo *et al.*, 2009; Kanehisa Laboratories, 2014). Six species (mainly *γ-Proteobacteria*) were dominant in the pristine soils and only two of those were present in the baseline community, *Thiocapsa sp.* and *M. hydrocarbonclasticus*.

Mineralisation rates of the deep pristine sample and the shallow contaminated sample were almost double that of the other samples, suggesting that the structure of these communities were such that they were able to cope better or were more suited to utilising phenanthrene. The profile of the total pulsed fungal community of these two samples was very similar. It is noted that although the deep contaminated sample diversity decreased after the contamination the decrease was minimal. The dominant fungi in the total pulsed community of these samples were identified as *P. griseofulvum, C. nemorosus, P. bandonii* and *A. fuscus*, although only *C. nemorosus* was present in both communities. The pulsed total bacterial profiles were however were not a close match and both communities suffered a considerable decrease in diversity after the addition of phenanthrene. This perhaps supports the suggestion that fungi play a pivotal role in the initial degradation of PAHs (Anderson and Cairney, 2004; Scullion, 2006) as although the bacterial diversity decreased the fungal community was unaffected allowing for the more rapid initial degradation of phenanthrene observed in these samples.

Furthermore, it is possible that the species with homology to *Cryptococcus nemorosus* is a key degrader of phenanthrene given its presence in both soil samples. The presence of this fungi has not been previously associated with phenanthrene degradation but other members of the genus have been shown to be involved in the degradation of aromatic hydrocarbons such as fluoranthene (*C. albidus*) and methylnaphthalene (*C. neoformans* and *C. gatti*) (Juhasz and Naidu, 2000; Kanehisa Laboratories, 2014). This result helps to futher elucidate their (*Cryptococcus* sp.) role in degradation of hydrocarbon contaminants within a marine environment.

The similarity in mineralisation rates of phenanthrene was somewhat unexpected as it was hypothesised that soils of similar contamination histories

would have similar responses to a contamination event due to an almost anamnestic response and that a pristine soil would respond better given its assumed broader diversity (MacNaughton *et al.*, 1999; Bordenave *et al.*, 2008; Marzorati *et al.*, 2008). In addition, based on 'prediction primer' results it was thought that the shallow pristine and deep contaminated soils would respond the best, given the high number of RHD positive bacteria. It has been demonstrated that this is not the case, as the mineralisation study showed two distinct groupings which were not related to contamination history nor were they related to sample depth. It is true that a pristine soil sample did have the highest mineralisation rate but a contaminated sample was also very similar, suggesting that predicting a soils response to anthropogenic activity is not as simple as the structure of its microbial community. Soil is complex, containing communities for multiple trophic levels, complex substrate transport mechanisms, extreme temporal and spatial heterogeneity and a myriad of internal and external feedbacks (Bissett *et al.*, 2013), which all interact with varying complexity to determine community structure and ultimately community function. This suggests that investigation of the community structure alone is not sufficient to fully assess the hypothesis relating to soil response and therefore future studies could benefit from taking a more holistic approach to investigating such as complex question.

5.4.3. Identification of active phenanthrene degrading fungi

Stable isotope probing was successfully employed to track the fungi that were actively degrading the phenanthrene in the different soils. Of an interesting side note, both DNA- and RNA-SIP was completed on soil samples and each method produced differing results. In both the bacterial and fungal investigations several species were identified as being present in the heavy DNA fractions and therefore assumed to be active degraders, but these species were present in both the heavy and light fractions in RNA results suggesting that they were not active degraders and instead possibly benefitting from breakdown products. This discrepancy is most likely due to the differing rates of turn-over between the two biomarkers. Given that RNA turn-over is independent of cellular replication

and is often increased in periods of activity (Whiteley *et al.*, 2007), such as a contamination event, the label is incorporated much quicker into the biomarker. This further demonstrates the need to ensure that experiments are run over appropriate time frames to prevent cross-feeding.

Investigation of the pulsed fungal total community suggested that several species were involved in phenanthrene degradation due to their dominance in these communities; however this was not the case as only three of these dominant species identified in the total community were active degraders. The explanation for dominance and not degradation ability is possibly due to these species benefiting from the production of by-products of phenanthrene degradation but not undertaking the initial breakdown. The phenanthrene degrading fungi of both contamination histories was dominated by members of the *Basidiomycota* phylum, as they had in both the baseline and total pulsed community. Previous works have identified other members of this phylum as important organisms in PAH degradation process (Cerniglia, 1997; Jasalavich *et al.*, 2000; Arun *et al.*, 2008; Dai *et al.*, 2009), which is strengthened by the work completed in this chapter.

It was thought that *Cryptococcus nemorosus* would be a key degrader given it was the only fungi in common in the pulsed total community profiles of the high mineralisation rate soils (deep pristine and shallow contaminated). The SIP work did confirm this hypothesis, adding strength to the conclusion that *C. nemorosus* is a fungus that can rapidly degrade phenanthrene in consort with other fungal such as *Rhodosporidium babjevae* and *Auriculibuller fuscus.*

The comparison of the dominant organisms present in the total community with species traced to degradation via SIP revealed that only 20% of the species identified at baseline were responsible for degradation. Therefore any assumption relating to key degraders based on total community investigations could underestimate a soils capacity to adapt to a contamination event.

5.4.4. Prediction and identification of phenanthrene degrading bacteria

Prediction of the bacteria that would be responsible for the degradation of the secondary contaminant (phenanthrene) was accomplished by using primers targeting the alpha subunit of the multicomponent RHD involved in the initial oxidation of PAHs. Profiling of the species using Gram-positive and Gram-negative RHD primers detected 12 organisms that would potentially play a role in degradation across all soil samples after a contamination event, the majority of which were Gram-positive bacteria, which have been known to dominate communities in historical PAH-polluted sites (Uyttebroek *et al.*, 2006; Cébron *et al.*, 2008). All twelve species were known to degrade various aromatic hydrocarbons utilising dioxygenases (Seo *et al.*, 2009; Kanehisa Laboratories, 2014), including three well known phenanthrene degrading species *P. aeuroginosa, P. putida* and *S. davawensis* (Juhasz and Naidu, 2000; Seo *et al.*, 2009; Kanehisa Laboratories, 2014). Interestingly, none of these bacteria were dominant in the baseline community.

Analysis of the pulsed bacterial total community suggested that there were up to nine different species that were involved in the degradation of phenanthrene, based on their dominance. SIP identified that there were in fact fifteen active degraders although only two of these had been dominant in their respective pulsed profiles (*M. hydrocarbonoclasticus* and *Enterococcus sp.*). The majority of the identified degraders were known to be hydrocarbonoclastic bacteria and if not known they were members of a genus known to contain these type of bacteria (i.e. *Alcanivorax, Enterococcus, Marinobacter and Sphingomonas* (Juhasz and Naidu, 2000; Seo *et al.*, 2009)).

Comparison of the twelve species that were predicted to be involved in the degradation of phenanthrene (Table 5.5) with those that had been identified using SIP (Table 5.9) demonstrated that none of the predicted organisms were involved in degradation of phenanthrene in any soil tested. The only similarity between those that were thought to degrade and those that did was a member

of the *Alcanivorax* genus was identified in both groups, although there were not the same species.

The inability to predict the bacteria that would degrade may in part be due to the primer target of the prediction primers. Given the lack of success in designing primers that targeted phenanthrene-RHD (see Chapter 3), more general degenerate primers were used. Bacteria identified using the 'prediction primers' were known to include genes encode hydroxylating dioxygenase but perhaps these primers lacked specificity for PAH dioxygenase. Or possibly, given that the microbial diversity of soil is so large, the predicted organisms may have a preference for particular aromatic hydrocarbons and only under certain conditions will they degrade phenanthrene. Furthermore, the lack of similarity in detecting the active degraders with the 'prediction primers' may be a case of limited abundance, especially as it is was shown that the majority of the active degraders were not identifiable in the baseline or pulsed total community suggesting a low copy number thereby limiting detection.

5.5. Conclusions

Initial investigations into the baseline communities of the soils demonstrated that there was very little difference in fungal diversity but a significant difference in bacterial diversity dependent on contamination history. The pristine soil had the highest fungal diversity at baseline, although the contaminated soil had the highest bacterial diversity. Identification of the dominant fungal and bacterial species highlighted the presence of organisms capable of degradation of various petroleum-based compounds (i.e. alkanes, phenanthrene, and naphthalene) and other anthropogenic compounds (i.e. phenols, pesticides, herbicides) regardless of contamination history, suggesting that the pristine soil may have been previously exposed to contamination or that these type of organisms are ubiquitous in the environment.

Community response after the simulated contamination event identified that the deep pristine and shallow contaminated soil were the most able to adapt to

the presence of phenanthrene. This was contradictory to the project hypothesis. It was thought that the shallow pristine and deep contaminated soils would respond the best given the high number of species able to degrade PAHs, demonstrating that the microbial response to anthropogenic disruption is complex and observation of the community structure alone is not sufficient to make a prediction.

Similarity in the microbial community structure of the well adapting soils (deep pristine and shallow contaminated) demonstrated that an increased fungal community was less affected by the contamination event, suggesting that due to a highly adaptable fungal community these soils were able to rapidly respond to the introduction of phenanthrene, further enforcing the importance of fungal diversity when investigation a soil microbial community response to PAHs. This work identified that the fungal species, *Cryptococcus nemorosus*, previously not identified as a phenanthrene degrader was pivotal in this rapid adaptation. The degradation activity of this species was later confirmed with SIP.

Stable isotope probing identified 10 fungal and 15 bacterial species that were involved in the degradation of phenanthrene. The fungal degraders were dominated by the phylum *Basidiomycota* and *Ascoymcota* including members of the genus *Crypotococcus*and *Tremellales*. Bacterial degraders include members of the genus *Alcanivorax*, *Marinobacter* and *Enterococcus*, and were dominated by γ-Proteobacteria. Bacterial and fungal species that have previously not been associated with phenanthrene degradation were also identified.

The work completed as part of this research chapter was working towards determining if a prediction could be made about a soils capacity to degrade a contaminant dependent on the structure of its baseline community. Given that there was little synergy between dominant baseline microbes, predicted degraders and those that were determine to be actually degrading the contaminant it appears that a prediction cannot be made. This work has demonstrated that there are many complex interactions that occur once a soil is exposed to a contaminant and that a blanket approach to remediation is unlikely to be successful. The work has however shown the effectiveness of tracking the

active contaminant degraders through the process of stable isotope probing which could be implemented in large-scale remediation projects to help develop more efficient and effect remediation strategies.

6. General Discussion

The main aim of this study was to assess the microbial community dynamics of soil to determine if targets for remediation strategies can be predicted based on baseline community structure alone.

To do this, molecular techniques were utilised to investigate the microbial communities without the bias of culture-dependent techniques. Since the advances in next generation sequencing enable high throughput sequencing, the evaluation of soil microbial communities using metagenomics and other 'omics' technologies have become increasingly popular. At the commencement of this project investigation utilising this technology was still cost prohibitive and as a previous study by Ding *et al.* (2010) demonstrated many key degradative microbial genes or species will remain undiscovered in soil metagenomics unless enrichments are performed to increase the abundance of rare populations. Thus, PCR based techniques were selected for investigation of the project aims.

As part of Chapter 3, two novel nucleic acid extraction techniques were developed from clayey and sandy soils. Unfortunately the clay extraction process was unable to extract product of high enough concentration to be utilised for the main downstream application, stable isotope probing. Since the first publication by Muyzer *et al.* (1993) denaturing gradient gel electrophoresis has become one of the main tools used world-wide for the profiling of microbial communities. However, given the reproducibility limitations of DGGE an alternative profiling system, TGGE, was successfully trialled and optimised for this investigation. During the optimisation process a comparison between profiles and subsequent community dynamics produced using the two techniques was undertaken. It was shown that although there was a different banding pattern of communities within the two profiling techniques, there was no statistical difference in the results of the diversity, thus, strengthening the argument for use of TGGE over DGGE, especially due to the greatly reduced experimental run times and requirement of smaller sample volume. To the author's knowledge there are no other published comparative studies of the two systems. Although two studies

tried a similar comparison of DGGE to similar separation technique called Temporal Temperature Gradient Electrophoresis or TTGE (Bùrresen-Dale *et al.*, 1997; Farnleitner *et al*., 2000). TTGE uses a gradual temperature gradient, through heating of the running buffer, with a constant chemical denaturant to separate amplicons (Farnleitner *et al*., 2000). These studies found that the resolution of TTGE compared to DGGE was considerably lower. Furthermore, TTGE suffered from a lack of focus as there was no fixed gradient for bands to migrate to, making comparison of complex environmental sample almost impossible. The result of these studies gives further strength to the effectiveness of TGGE, given that it using a fixed gradient.

The optimised TGGE analysis method enabled the rapid profiling of the fungal communities of sandy and clayey soils with different contamination histories. It was thought that the history of the soil would affect the future microbial function (Keiser *et al.*, 2011) such that pristine soil would have a higher microbial diversity and therefore be able to respond to contamination events better. However, as was shown in Chapter 4, for sandy soils the more contaminated soil had the higher fungal diversity. It was also shown that the dominance of known hydrocarbonoclastic microbes did not change in soils with different contamination histories. This suggests either a level of innate adaptability to multiple carbon sources or the pristine samples used in this project had some history of contamination. A similar uncertainty in degradation was shown in a recent publication by Hamamura *et al.* (2013) which tested the response of the microbial communities of three distinct soils after exposure to different hydrocarbon mixtures (crude oil, diesel and kerosene). They found that it was not clear whether the contaminants were degraded by specialists whose populations may have been selected for by the different mixture types, or generalists with capabilities to degrade multiple mixtures.

Interestingly, it was determined that the clay soil had a higher diversity and capacity to support diverse organisms compared to the sandy soil, which may be a result of the *pore connectivity theory* which suggests that low water

connectivity in soils can promote diverse communities through the creation of microhabitats (Gleason *et al.*, 2012).

The final stage of this project was to further investigate the prediction of contaminant mineralisation utilising PAH degradation 'prediction' primers and SIP. Based on the higher number of potential PAH degraders present in the baseline communities, it was thought that the shallow pristine and deep contaminated soil samples would have the capacity to respond more rapidly to a contamination event. However, as shown in the mineralisation study in Chapter 5 these soils were slowest to respond and after 25 days had utilised around two times less phenanthrene than the deep pristine and shallow contaminated soils. Published studies on the effects of hydrocarbons on community dynamics suggested that the more diverse soil would exhibit a greater resistance and resilience to perturbation stresses (Girvan *et al.*, 2005; Hamamura *et al.*, 2006); however the work completed as part of this thesis would tend to support the conclusion of Bell *et al.* (2013) which suggested that diversity may be important in the initial selection of organisms, but less important for actual hydrocarbon degradation.

Several key organisms in the degradation of phenanthrene were identified using SIP. The fungal degraders were dominated by the phylum *Basidiomycota* and included key degraders such as *Crypotococcus nemorosus*, *Tremellales sp.* and other organisms affiliated to the *Crypotococcus* genus. Bacterial degraders were dominated by γ-Proteobacteria and included members of the genus *Alcanivorax*, *Marinobacter* and *Enterococcus*.

Tracking of the active degraders within the microbial communities revealed that around 10 to 20% of the dominant microbes in the baseline communities were responsible for the degradation of phenanthrene regardless of the contamination history of the soil. This suggests that the assessment of the baseline community would grossly underestimate the ability of a soil microbial community or potentially identify inaccurate remediation targets. Therefore it can be concluded that due to the complex nature of soil, a simplistic investigation of the microbial community is not sufficient to determine how a

soil will respond to a contamination event. A more systems based approach is required to tease apart the overall community function, a sentiment that was echoed in a recent publication by Bissett *et al.* (2013). The authors identified the importance of soil biota for ecosystem function and that we have a limited ability to predict and manage soil microbial community responses to change. Therefore we need to better understand this relationship between community structure and function using integrative studies that reflect the "dynamic and interactive" nature of microbial systems which focus on how environmental variables, functional groups and biogeochemical processes drive ecosystem function.

This thesis started with the purpose of determining if it is possible to predict a soil's response to contamination and thereby identify key targets for bioremediation strategies. Although this work determined that a prediction was not possible, it did however add strength to the argument that it is necessary to characterise the actual pollutant degraders to determine targets for bioremediation strategies (Bell *et al.*, 2013; Yang *et al.*, 2014). Currently the use of bioremediation as a clean-up strategy in Australia is limited, even more so in South Australia where there continues to be a preference towards excavation and disposal of contaminated soils to landfill ('dig and dump'). The most recent estimate of contaminated soil being disposed of to South Australian landfills was around 87,000 tonnes per annum (SKM, 2013). This practice of 'dig and dump' is clearly un-sustainable, and as legislation and economic interests shift, so too will the need to develop alternative remediation methods. Globally, the dig and dump approach has largely been cast aside and remediation strategies are switching to more technological based approaches such as *in situ* treatment and re-use, utilising a mixture of chemical and biotic remediation processes. The application of SIP during treatment and re-use programs would be invaluable as it would enable development of more targeted, time-efficient, cost-effective remediation strategies. This will not only benefit the industries responsible for contamination but aid in reducing the reliance of landfills.

This thesis has strengthened the use of stable isotope probing as an invaluable tool to better understand the complex microbial processes involved in successful bioremediation. Future work involving the coupling of SIP with metagenomics could be used to better understand the degradation of other priority environmental contaminants, such as halogenated solvents and recalcitrant heavy molecular weight PAHs. The application of *in situ* stable isotope probing has endless potential for scientific research and commercial application. There is already one US based company offering *in situ* stable isotope probing of BTEX compounds using phospholipid fatty acids as biomarkers to determine if biodegradation is occurring in contaminated groundwater. Therefore, further work into the commercial application using other biomarkers, such as RNA, which target different priority contaminants in the terrestrial environment, would be invaluable.

This thesis has also highlighted the importance of fungi through a unique method of investigation and demonstrates that further investigation of species such as *Crypotococcus nemorosus*, *Tremellales sp.* and other organisms affiliated to the *Crypotococcus* genus will further advance the knowledge into bioremediation of high molecular weight PAHs.

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Appendices

A.1 Multiple Sequence Alignments used for primer design

Provided on attached CD

A.2 Analytical Chemistry Results

			Inorganics	Field	Nutrients					Metals																
			Moisture (Lab)	pH (Lab)	Nitrate (as N)	Nitrite (as N)	Nitrogen (Total)	Reactive Phosphorus as P	Sulphate as S	Antimony	Arsenic	Barium	Beryllium	Cadmium	Chromium (III+VI)	Cobalt	Copper	Lead	Manganese	Mercury	Molybdenum	Nickel	Selenium	Tin	Vanadium	Zinc
			%	pH_Units	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
NEPM (1999) Soil HIL for Industrial Use										410[#1]	500[#3]	190000[#1]	100[#3]	100[#3]	600000[#6]	500[#3]	5000[#3]	1500[#3]	7500[#3]	75[#3]	5100[#1]	3000[#3]	5100[#1]	610000[#1]		35000[#3]
NEPM (1999) Soil EIL											20[#4]	300[#4]		3[#4]	400[#7]		100[#4]	600[#4]	500[#4]	1[#4]		60[#4]			50[#4]	200[#4]
Location	**Sample Depth (m)**	**Sampled Date**																								
Sandy Soil																										
TP4	1.5	25/10/2007	13.8	-	320	<0.1	320	262	-	<5	7	10	-	<1	7	2	10	<5	-	<0.1	<2	4	<5	<5	13	12
TP4	2	25/10/2007	13.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-
TP9	1.0	25/10/2007	8.9	-	<20	<0.1	<20	206	-	<5	6	70	-	<1	5	<2	9	<5	-	<0.1	<2	3	<5	<5	13	65
TP9	1.5	25/10/2007	9.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-
TP9	2	25/10/2007	14.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-
TP20	0.5	25/10/2007	10.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-	-
TP20	1.5	26/10/2007	5.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<5	-	-	-	-	-	-	-	-
TP30	1.0	26/10/2007	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<5	-	-	-	-	-	-	-	-
TP30	1.5	26/10/2007	17.4	-	420	<0.1	420	571	-	<5	<5	<10	-	<1	<2	<2	<5	<5	-	0.9	3	<2	<5	<5	<5	<5
TP30	2	26/10/2007	14.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<5	-	-	-	-	-	-	-	-
Clayey Soils																										
FTF1	0.4-0.5	10/06/2008	20.8	-	-	-	-	-	-	<5	<5	100	<1	<1	11	5	9	<5	130	<0.1	<2	12	<5	<5	12	8
FTF1	0.9-1.0	10/06/2008	22.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-	-	-	-	-	-	-
FTF1	1.9-2.0	10/06/2008	14.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-
FTF5	0.4-0.5	10/06/2008	22.5	8.8	<0.1	<0.1	<0.1	<0.1	<50	<5	<5	100	<1	<1	12	4	8	6	130	<0.1	<2	10	<5	<5	13	8
FTF5	1.5-1.7	10/06/2008	22.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-
FTF5	2.9-3.0	10/06/2008	21.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	-	-	-	-	-	-	-
FTF6	0.0-0.1	10/06/2008	19.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41	-	-	-	-	-	-	-	-
FTF6	0.4-0.5	10/06/2008	20	8.1	0.838	0.804	1.64	<0.1	60	<5	<5	100	<1	<1	11	5	8	7	131	<0.1	<2	12	<5	<5	10	9
FTF6	2.4-3.0	10/06/2008	15.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-
FTF10	0.9-1.0	11/06/2008	22.7	8.8	<0.1	<0.1	<0.1	<0.1	<50	<5	<5	90	<1	<1	12	4	7	8	116	<0.1	<2	11	<5	<5	16	8
FTF10	1.9-2.0	11/06/2008	17.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16	-	-	-	-	-	-	-	-

Comments
#1 USA EPA (2011) Region 9 Industrial
#2 NSW EPA (1994) Service Station
#3 NEPM (1999) HIL (F)
#4 NEPM (1999) EIL
#5 MHSPE (2000) Dutch Intervention Values
#6 In absence of Total Cr guideline, the Cr(III) guideline has been adopted, as NEPM HIL (F) assumes the valence state of Total Cr to be Cr (III)
#7 In absence of Total Cr guideline, the Cr(III) guideline has been adopted, as NEPM EIL assumes the valence state of Total Cr to be Cr (III)

			BTEX					TPH					PAH																				
			Benzene	Toluene	Ethylbenzene	Xylene (o)	Xylene (m & p)	Xylene (Total)	C6 - C 9 Fraction	C10 - C14 Fraction	C15 - C28 Fraction	C29 - C36 Fraction	C10 - C36 Fraction	Benzo(a) pyrene	Benzo(b)fluoranthene	Benzo(g,h,i)perylene	Benzo(k)fluoranthene	Acenaphthene	Acenaphthylene	Anthracene	Benz(a)anthracene	Chrysene	Dibenz(a,h)anthracene	Fluoranthene	Fluorene	Indeno(1,2,3-c,d)pyrene	Naphthalene	Phenanthrene	Pyrene	Total PAH
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
NEPM (1999)	Soil HIL for Industrial Use		1 [#2]	130 [#2]	50 [#2]	3000 [#1]			65 [#2]				1000 [#3]	5 [#3]	2.1 [#1]		21 [#1]	33000 [#1]		170000 [#1]	2.1 [#1]	210 [#1]	0.21 [#2]	22000 [#1]	22000 [#1]	2.1 [#1]	18 [#1]		17000 [#1]	2.1 [#1]
NEPM (1999)	Soil EIL		1 [#2]	1.4 [#2]	3.1 [#2]									1 [#2]																
Location	**Sample Depth (m)**	**Sampled Date**																												
Sandy Soil																														
TP4	1.5	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP4	2	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP9	1.0	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	44	180	640	<100	870	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP9	1.5	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP9	2	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP20	0.5	25/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP20	1.5	26/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP30	1.0	26/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP30	1.5	26/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	34	500	1230	<100	1780	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.2	<0.5	1.2
TP30	2	26/10/2007	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Clayey Soils																														
FTF1	0.4-0.5	10/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF1	0.9-1.0	10/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF1	1.9-2.0	10/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF5	0.4-0.5	10/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF5	1.5-1.7	10/06/2008	<0.2	3.2	0.6	1	3	4	65	50	150	<100	150	<0.5	<0.5	0.6	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	7.8	<0.5	<0.5	8.4
FTF5	2.9-3.0	10/06/2008	<2	39.6	34.7	266	323	589	1420	2790	2890	<100	2890	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF6	0.0-0.1	10/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	24.6	<0.5	<0.5	25.2
FTF6	0.4-0.5	10/06/2008	43.2	734	168	372	923	1295	4580	4260	4360	<100	4360	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6	-	-	-
FTF6	2.4-3.0	10/06/2008	<0.2	<0.5	<0.5	0.7	<0.5	0.7	<10	440	540	<100	540	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF10	0.9-1.0	11/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF10	1.9-2.0	11/06/2008	<0.2	<0.5	<0.5	<0.5	<0.5	<0.5	<10	<50	<100	<100	<100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Comments
#1 USA EPA (2011) Region 9 Industrial
#2 NSW EPA (1994) Service Station
#3 NEPM (1999) HIL (F)
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#5 MHSPE (2000) Dutch Intervention Values
#6 In absence of Total Cr guideline, the Cr(III) guideline has been adopt
#7 In absence of Total Cr guideline, the Cr(III) guideline has been adopt

			VOCs																																
			1,1,1,2-tetrachloroethane	1,1,1-trichloroethane	1,1,2,2-tetrachloroethane	1,1,2-trichloroethane	1,1-dichloroethane	1,1-dichloroethene	1,2,3-trichlorobenzene	1,2,3-trichloropropane	1,2,4-trichlorobenzene	1,2-dibromoethane	1,2-dichlorobenzene	1,2-dichloroethane	1,3-dichlorobenzene	1,4-dichlorobenzene	Bromodichloromethane	Bromoform	Carbon tetrachloride	Chlorobenzene	Chlorodibromomethane	Chloroethane	Chloroform	cis-1,2-dichloroethene	cis-1,3-dichloropropene	cis-1,4-Dichloro-2-butene	Dibromomethane	Hexachlorobutadiene	Iodomethane	Pentachloroethane	TCE	Tetrachloroethene
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
NEPM (1999) Soil HIL for Industrial Use			9.3[#1]	38000[#1]	2.8[#1]	5.3[#1]	17[#1]	1100[#1]	490[#1]	0.095[#1]	99[#1]	0.17[#1]	9800[#1]	2.2[#1]		12[#1]	1.4[#1]	220[#1]	3[#1]	1400[#1]	3.3[#1]	61000[#1]	1.5[#1]	2000[#1]		0.035[#1]	110[#1]	22[#1]		19[#1]	6.4[#1]	2.6[#1]
NEPM (1999) Soil EIL																																			
Location	**Sample Depth (m)**	**Sampled Date**																																	
Sandy Soil																																			
TP4	1.5	25/10/2007	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP4	2	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP9	1.0	25/10/2007	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP9	1.5	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP9	2	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP20	0.5	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP20	1.5	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP30	1.0	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP30	1.5	26/10/2007	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
TP30	2	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clayey Soils																																			
FTF1	0.4-0.5	10/06/2008	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF1	0.9-1.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF1	1.9-2.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF5	0.4-0.5	10/06/2008	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF5	1.5-1.7	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF5	2.9-3.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF6	0.0-0.1	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF6	0.4-0.5	10/06/2008	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<500	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
FTF6	2.4-3.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF10	0.9-1.0	11/06/2008	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FTF10	1.9-2.0	11/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Comments

#1 USA EPA (2011) Region 9 Industrial
#2 NSW EPA (1994) Service Station
#3 NEPM (1999) HIL (F)
#4 NEPM (1999) EIL
#5 MHSPE (2000) Dutch Intervention Values
#6 In absence of Total Cr guideline, the Cr(III) guideline has been adopt
#7 In absence of Total Cr guideline, the Cr(III) guideline has been adopt

			Chlorinated & Halogenated Hydrocarbons																Phenols												
			trans-1,2-dichloroethene	trans-1,3-dichloropropene	trans-1,4-Dichloro-2-butene	Trichlorofluoromethane	Vinyl chloride	1,1-dichloropropene	1,2-dibromo-3-chloropropane	1,2-dichloropropane	1,3-dichloropropane	2,2-dichloropropane	2-chlorotoluene	4-chlorotoluene	Bromobenzene	Chloromethane	Bromomethane	Dichlorodifluoromethane	2,4,5-trichlorophenol	2,4,6-trichlorophenol	2,4-dichlorophenol	2,4-dimethylphenol	2,6-dichlorophenol	2-chlorophenol	2-methylphenol	2-nitrophenol	4-chloro-3-methylphenol	Cresol Total	Pentachlorophenol	Phenol
			mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
NEPM (1999) Soil HIL for Industrial Use			690[#1]		0.035[#1]	3400[#1]	1.7[#1]		0.069[#1]	4.7[#1]	20000[#1]		20000[#1]	20000[#1]	1800[#1]	500[#1]	32[#1]	400[#1]	62000[#1]	160[#1]	1800[#1]	12000[#1]		5100[#1]	31000[#1]		62000[#1]	91000[#1]	2.7[#1]	42500[#1]
NEPM (1999) Soil EIL																															
Location	**Sample Depth (m)**	**Sampled Date**																													
Sandy Soil																															
TP4	1.5	25/10/2007	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP4	2	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP9	1.0	25/10/2007	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP9	1.5	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP9	2	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP20	0.5	25/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP20	1.5	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP30	1.0	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TP30	1.5	26/10/2007	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
TP30	2	26/10/2007	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
Clayey Soils																															
FTF1	0.4-0.5	10/06/2008	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF1	0.9-1.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF1	1.9-2.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF5	0.4-0.5	10/06/2008	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF5	1.5-1.7	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF5	2.9-3.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF6	0.0-0.1	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF6	0.4-0.5	10/06/2008	<50	<50	<50	<500	<500	<50	<50	<50	<50	<50	<50	<50	<50	<500	<500	<500	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	1.7
FTF6	2.4-3.0	10/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FTF10	0.9-1.0	11/06/2008	<0.5	<0.5	<0.5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<5	<5	<5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<1	<2	<0.5
FTF10	1.9-2.0	11/06/2008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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#3 NEPM (1999) HIL (F)
#4 NEPM (1999) EIL
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#6 In absence of Total Cr guideline, the Cr(III) guideline has been adopt
#7 In absence of Total Cr guideline, the Cr(III) guideline has been adopt

A.3 Sequences of TGGE excised bands

Chapter 4: Sandy Soils

Band 1 (252 bp)

GATACGGATCTATTGTCTTGCGCGGTAACTTATCCATTGGAGCTATGTGAAATGTTGAT
TGACTTCGGCGGTTACGCCGTTTTGACGGACGTTGTGTAGGCCTTGAGCGTCATGTTAT
TATAACGAAGAAGACTTTCAACAACGGAACTCTTGGTTCTCGAAAGTATGAAGAATGA
GTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATG
AAGAACGCAGCTGAAC

Band 2 (288 bp)

AATGGCATTCATGGTGATGAGCAACTGATAGGACAACAACCCGAACCGGTGGTTCCAT
AAAGCGTACCTGTGGTTTCTTCGGAGGGCGCGGGCCAGGTTTCGAAGGCCTCCGGGG
AGGCCTTGCGAAACCGGGCCCGGGCCCGCCGAAGACCCCAACAAGGTACGATGTACA
CGGGTGGGAGTATGGAGCCAGAGTTGACTCACTCGGTAATGATCCTTAAGAATTTCAA
CATACGGAAGTCTTGGTTCCGGCATCGATGAAAAACGCAGCAACATTGAGGCCTTTGA

Band 3 (255 bp)

GGCATTCGGAATCAGGCCCCGCGGAAACGTCCCACCAGGTGGTCATCGGTAAATAGTT
GGTGTAGGCGGGCAGGCCGTTTAGAAGGACGCCGTTGAGGACATGAGCCCCCGGGAC
GGCTGCGGCCGAAGGACCAAACAAGAACGCCGTTCCGAAAGTATGCAGTATGAGAAG
AAAAACGAAATCAGACAAAACTTTCAACAACGGATCTCATGGTACCGGCATCGATGAA
GAACGCAGAGAAAAATTGCGGGGGG

Band 4 (238 bp)

GTAAAAATTGGGTGAGGTCGGGCAGAACCCTTTAGGGGAAAGTCAGCATAGTAGAGT
AATGAAAGGGGAAGTACCATGGTTAGGTGTGTGTAGAGGGCGGGTTCTGCGCTGACA
CCAGTACAAGACCAAAAAGCCTTTCGAATAGCAGTCAGCGTCAGTGAACAACGTAATC
ATTAAAACTTTCAACAACGGATCTCTCGGTTACAGGCATCGAGGGAAACGCAGAGAAA
CGCAGCTA

Band 5 (243 bp)

TACGATCGGGATGGTGAAGGTTTACTGATTACGATAATCAACTCAGACTGCATACTTTC
AGAACAGCGTTCATGTTGGGGTCTTCGGCGGGCGCGGGCCCGGGGGCGCAAGGCCTC
CCCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCAACAAGGTACGATAGACACGGG
TGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGATCACCTA
CGGAAGTTTATA

Chapter 4: Clayey Soils

Band 1 (260 bp)

TTTACTGGAGGTACCATACGCGACTCAGACCGGCATAGTTTCAGGACAGTGGGCCTGT
TGTCGGAGCTTTGCCGGTTTGACGGGCCCGGGGGGCGCAAGGCCTTCCCGGCGGCCGG
CGAAACGGCGCGCCCGCCGAAGACCACAACGTACGATAGACACGGGTCTGAAAGTAT
GGACTCGGAGGGCACTCACTCGGTAATCAGCCTTCCGCAGGTTCACCAACGGAACGCT
TGGTTCCGGCATCGATAAAGCACGAAGCAAA

Band 2 (215 bp)

CTTCCGTAGGTGACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACC
TCCCACGCGTGTCTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCC
GGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTT
CTGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTA

Band 3 (250 bp)

GGCATTCGGAATCAGGCCCCGCGGAAACGTCCCACCAGGTGGTCATCGGTAAATAGTT
GGTGTAGGCGGGCAGGCCGTTTAGAAGGACGCCGTTGAGGACATGAGCCCCCGGGAC
GGCTGCGGCCGAAGGACCAAACAAGAACGCCGTTCCGAAAGTATGCAGTATGAGAAG
AAAAACGAAATCAGACAAAACTTTCAACAACGGATCTCATGGTACCGGCATCGATGAA
GAACGCAGAGAAAAATTGCG

Band 4 (242 bp)

GTAAAAATTGGGTGAGGTCGGGCAGAACCCTTTAGGGGAAAGTCAGCATAGTAGAGT
AATGAAAGGGGAAGTACCATGGTTAGGTGTGTGTAGAGGGCGGGTTCTGCGCTGACA
CCAGTACAAGACCAAAAAGCCTTTCGAATAGCAGTCAGCGTCAGTGAACAACGTAATC
ATTAAAACTTTCAACAACGGATCTCTCGGTTACAGGCATCGAGGGAAACGCAGAGAAA
CGCAGCTAGC

Band 5 (232 bp)

TACGATCGGGATGGTGAAGGTTTACTGATTACGATAATCAACTCAGACTGCATACTTTC
AGAACAGCGTTCATGTTGGGGTCTTCGGCGGGCGCGGGCCCGGGGGCGCAAGGCCTC
CCCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCAACAAGGTACGATAGACACGGG
TGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGATCACCTA
C

Chapter 5:

Fungal Extracts

Band F1 (255 bp)

GGTTCGTATCGTTGTGACCTGCGGATGGATCATTAACAATCCAGTGTTTCTATACCACTT
TCAAAAGGACAGGTTTACCACCGCGTGCACTGTGGGAACCTGAATGTCTTTGGTAGGG
TCTTGACTCAACCACTCCGTTGTTGAACTTTTTTTTTATTCCCAGATTTTGCTTCTATTCTG
AATAGGTTTAAGGAATTTGTGTGACGTATTTCGGGATAAATGTGTTAGGACAGAGGTC
GCTTCTCTCTTCCAACA

Band F2 (258 bp)

CCTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAA
CCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCG
GGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCTGAAG
ATTGTAGTCTGAGTGAAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTC
CGGCATCGATGAAGAACGCAGC

Band F3 (200 bp)

TCATTACCGATTGTTCGAAGCGTCCCCATCTTAACATCCCTCACCTCTGGGAACCGTTTG
ACCCCCGGGTCTAAGTAAACTAGACGGTTTCACATGTAACGAACGTCTTGTTATCTTAA
CAAAACAAAACTTTTAACAACGGATCTCTTGTCTCTCGCATCGATGATGAACGCAGCGT
CTCTGGAAAACACGACAAAAGC

Band F4 (273 bp)

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG
AAGGATCATTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTC
TAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACT
TATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAACG
GATCTCTTGGCTCTCGCATCGATGAAGAACGCAGAAA

Band F5 (273 bp)

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG
AAGGATCATTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTC
TAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACT
TATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAACG
GATCTCTTGGCTCTCGCATCGATGAAGAACGCAGAAA

Band F6 (236 bp)

CTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGAATCTAGGACGTCCAACTTAACTT
GGAGTCCGAACTCTCACTTTCTAACCCTGTGCATCTGTTAATTGGAATAGTAGCTCTTCG
GAGTGAACCACCATTCACTTATAAAACACAAAGTCTATGAATGTATACAAATTTATAAC
AAAACAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCAA

Band F7 (241 bp)

CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA
AGGATCATTAATGATTGACCGAAAGGTCCTAAATCTTAACATCCCTCACCTCTGTGAAC
CGTTTGACCCCCGGGTCTAACAAAACTATACAAACATCAGTGTAACGAACGTCTTGTTA
TCTTAACAAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACG
CAGCA

Band F8 (254 bp)

CTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAAC
CTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGG
GGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCTGAAGA
TTGTAGTCTGAGTGAAAATATAAATTATTCAAAACTTTCAACAACGGATCTCTTGGTTCC
GGCATCGATGAAGAACGC

Band F9 (241 bp)

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG
AAGGATCATTAATGATTGACCGAAAGGTCCTAAATCTTAACATCCCTCACCTCTGTGAA
CCGTTTGACCCCCGGGTCTAACAAAACTATACAAACATCAGTGTAACGAACGTCTTGTT
ATCTTAACAAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
GCAGCC

Band F10 (272 bp)

TCTTGGTCATTTAGAGGAAGTAAAAGTTGTAACAAGGTTTCCGTAGGTGAACGTGCGG
AAGGATCATTAGTGAATATAGGACGTCCAAAATAACTTGGAGTCCGAACTCTCTCTTTC
TAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACT
TATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAACG
GATCTCTTGGCTCTCGCATCAATGAAGAACGCAGCA

Band F11 (211 bp)

CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA
AGGATCATTAGTGATAAAACTATTATCTTAACACCTGTGAACTGTGAACCGAAAGGTTC
TTCCAAACATTGTGTAATGAACGTAATACATTATAAACAATACAACTTTCAACAACGGAT
CTCTTGGCTCTCGCATCGATGAAGAACGCAGCCA

Band F12 (185 bp)

TTAATTACAACGATGTTTGTATAATTTTGTTCCACCCGGGGGCCAAACCGTTCACGAAG
GTGAGGGATGTTCCGATTTGGGACCTTTCGGGCAATCATTAATGATCCTTCCGCAGGTT
CACCCTTCCAAACCTTCTCCCAACTTTAACTTCCTCAAACTGACCAAGACCCTAAATGAC
CAAGAAA

Band F13 (198 bp)

CATTACCGATTGATCGAAGGGTCGCCTGCTTAACATCCCTCACCTCTGTGAACCGTTTGA
CCCCCGGGTCTAAGAAAACTATACGACATCACATGTAACAAACGTCTTGTTATCTTAAC
AAAACAAAACTTTTAACAACGGATCTCTTGTCTCTCGCATCGATGATGAATCCGGCGTC
TATGAAAAACACAAAAAAAG

Band F14 (246 bp)

GAGTCTTGGTCATTTAGAGGAAGTAAAAGTTGTAACAAGGTTTCCGTAGGTGAACCTG
CGGAAGGATCATTAATGATTGACCGAAAGGTCCTAAATCTTAACATCCCTCACCTCTGT
GAACCGTTTGACCCCCGGGTCTAACAAAACTATACAAACATCAGTGTAACGAACGTCTT
GTTATCTTAACAAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAG
AACGCAGCATC

Band F15 (241 bp)

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG
AAGGATCATTAATGATTGACCGAAAGGTCCTAAATCTTAACATCCCTCACCTCTGTGAA
CCGTTTGACCCCCGGGTCTAACAAAACTATACAAACATCAGTGTAACGAACGTCTTGTT
ATCTTAACAAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
GCAGCA

Band F16 (181 bp)

GTTATAATAACATGACGTTCATTACACAATGTTTGTAAAATCAATTGACCGAATTCAATC
AACAGTTCACAGGTGTAGATGGATGTATTTAACCCTCAGAAAACAATTCACTAATGATC
CTTCCGCAGGTTCACCTAAGGAAACCTTGTTACTACTTTTACTTCCTCTAAATGACCAAG
AA

Band F17 (239 bp)

TTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA
GGATCATTAATGATTGACCGAAAGGTCCTAAATCTTAACATCCCTCACCTCTGTGAACC
GTTTGACCCCCGGGTCTAACAAAACTATACAAACATCAGTGTAACGAACGTCTTGTTAT
CTTAACAAAACAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCAATGAAGAACGA
AGCA

Band F18 (174 bp)

CTTCCGTAGGTGAACCTGCGGAAGGATCATTAGTGATAAAACTATTATCTTAACACCTG
TGAACTGTGAACCGAAAGGTTCTTCCAAACATTGTGTAATGAACGTAATACATTATAAA
CAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCA

Band F19 (206 bp)

TCATTATGATTTTAGAACTCCCCTTAACTTGTCCTCCCAATTCTCTTTTTCAAACCGTGCG
CACTTGTTTGGGATAGTAACTCTTTTAAGAGAGCGAACTCCTATTCACTTATAAACTCAA
TGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGC
TCTCGCATAAATGAAGAACGCAGCA

Bacterial Extracts

Band B1 (147 bp)

CGGAGCACTCCGCGTGAGTGATGAAGGCCTTCGGATCTGTAAAGCTCTGTTGTTAGGG
AAGAACAGCTACCGGCTTTACTGCTGGTACCTTGACGGTACCTAACCAAAAAGCCCCG
GCTAATTTCGTGCCAGCAGCCGCGGTATTTA

Band B2 (150 bp)

CTGATCCAGCATGCCGCGTGAGTGATGAAGGCCTTCGGATTGTAAAGCTCTTTTATTTG
GGAGGAAGGGGAGTAACTTAATACCTTGCTATTTTGACGTTACCGACACAATAAGCAC
CGGCTAACTTCATGCCAGCAGCCGCGGTAATAA

Band B3 (221 bp)

AGTGTCGTTTCGTAGTGACCTGCGGAAGGTCATTAATAAACGGCGAAGCTTGAAAGAG
CCAAGCTTTATATTATTCCATAACACCTGTGAACTATTTGCCTTCTTGTCATGAGAAGGT
ACAATCTATACAAACATAACTGTAATGAATGTAATCTATTATAACATAATACAACTTTCA
ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGACA

Band B4 (135 bp)

TATGATACTACGCTAGCTGCTGATCTTTACCTCACACCGAAATGTTTACAACCCGAAGG
CCTTCTTCCACACCCGGTATTGCTGGATCACGGTTGCCCCCATTGTCTAATATTCCCCAC
TGCTGCCTCCCGTAGG

Band B5 (197 bp)

TCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGGGCAACCCTGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAG
GCTTATCCTTAATACGGATGAGTACTTGACGTTACCTACAGAAGAAGCACCGGCTAATT
TCGTGCCAGCAGCCGCGGTAATGA

Band B6 (148 bp)

TGATGAGCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGTTGG
GAGGAAATACTTAATGTTAATAGCCTTGAGTCTTGACGTTACCTTCAGAAAAAGCACCG
GCTAACTCTGTGCCAGCAGCCGCTATAATAA

Band B7 (196 bp)

TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAG
GCTTATCCTTAATACGGATGAGTACTTGACGTTACCTACAGAAGAAGCACCGGCTAATT
TCGTGCCAGCAGCCGCGGTAATA

Band B8 (167 bp)

TGAAGGATTCGCCGCTGCTGGTACGTAGCGCTCACCGGTTATTACCCCTAACCCTTCCT
CCCTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGGAT
CAGGGTTGCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGA

Band B9 (160 bp)

GGAATACGCTACGTGAGAGAGACGTCGTTCTAATAAAAAATGTTGATTTGGGGTGAAG
GAAAGCAATCAGATAGGATCGTGCAACCAAGGTTTACAAGGATTATAAAGCCTTCCCC
ACCACTAATTTATCTTACATATTCATACCGATGTTCAAAATTGG

Band B10 (149 bp)

TGCGTACGTCGGCCTTATCGTAATTACCGATAAGCCTTCCTCCCTACTGAAAGTGCTTTA
CAACCCGAAAGCCTTCTTCACACACGCGGCATGGCTGGATCAGGGTTGCCCCCATTGTC
CAATATTCCCCACTGCTGCCTCCCGTAGGA

Band B11 (131 bp)

GATGCAGATATGCCGCGTGAGTGATGAAGCGCCTTAGGTATTGTAAACCTCTTTCACCA
GGGATGATAATGACGGTACCTGGAGAATAAACCCCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATAACTATT

Band B12 (147 bp)

GCTCAGCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCATTGGGG
AAGAACACCTGATGGTTAATATCCCTGAGGCTTGACGTTACCCTCACAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATTA

Band B13 (189 bp)

TCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCG
ACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTACCACCAACGCAGG
CCCGGAGTTCTCTTCGGGTTGACGGTAGGTGGGGAATAAGGACCGGCTAACTACGTGC
CAGCAGCCGCGGTAAT

Band B14 (178 bp)

CCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGACGAAAGTCTGACGCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTTTGGGTTGTAAAGCAGTTTCAATAATAGCATACGG
GAGGCAGCTGCCGCGGTAATAGCCTACGGGAGCCAGCAGCCGCGGTAATATCCTACG
GTATTT

Band B15 (175 bp)

TCCTACGGGAGGCAGCAGCGGGCTTACTTAGGGAAGGGAGCAGGATAGAATCCTACG
GGAGGCAGCAGCCGCGGTAATAGCCTACGGGAGGCAGCAGCCGCGGTAATAGCTACG
GGAGGCAGCAGCCGCGGTATAGCCTACGGGAGCCAGCAGCCGCTGTAATATCCTACG
G

Band B16 (145 bp)

CCGCGGTGTATGCACGGGAGTGACAACGCCTTCGGACTGTAACTCTGTTGTTAGGGAG
GAAGGCTTCCGGCTAATAACCCTGAGTACTTGACGTTACCTACCCAAAAAGCCCCGGCT
AATTTCGTGCCAGCAGCCGCGGTAAAA

Band B17 (219 bp)

GTTCGCTCGTAGTGACCTGCGGAGCACATTAATGATTGACCGAACGSTGCTAAATCTTA
GCATGCCTCACTGATGTGAACCGTTACCYYCGGGTCTAGTAAAGCTATACATACATCAG
TAGTGACGAACGTCTTATYCTCTAATACGAATGAGTACTTGACGTTACCTACAGAGCAA
TCACTTGGCTAATCTCGTGCCATCAGACGCAGTAATGAAGCA

Band B18 (189 bp)

TCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCG
ACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTACCACCAACGCAGG
CCCGGAGTTCTCTTCGGGTTGACGGTAGGTGGGGAATAAGGACCGGCTAACTACGTGC
CAGCAGCCGCGGTAAT

Band B19 (140 bp)

CAGCAGCCGCGGTATAGCCTACGGGAGCCAGCAGCCGCGGTAATAGCCTACGGGAGG
CAGCAGCCGCGGTAATAGCCTACGGGAGGCAGCAGCCGCGGTAATAGCCTACGGGAG
GCAGCAGCCGCGGTAATAGCTACGGA

Band B20 (197 bp)

TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGTGAGGAGGAAAA
CCTGATGGTTAATATCCATGAGGCTTGACGTTACTCACAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATTA

Band B21 (195)bp

CCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGGGCAACCCTGATCCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAGG
CTTAGGGCTAATAACCCTGAGTACTTGACGTTACCTACAGAAGAAGCACCGGCTAATTT
CGTGCCAGCAGCCGCGGTAATT

Band B22 (181 bp)

GACGGGCTCTCGCACAGCAGCCGCGGTATACCCTACGGGAGGGGGACCGCGGTAGAG
GCAAAGCTTAATGTTTGAGAGGCGATCTTCAGAAAGATGGGCGTCGAGAACCAAACAA
CCACACCGCTATTTCCACCCGCAGGACCCGCTTTCTTTCGAAAATCGACTAACATGGATA
AATTGT

Band B23 (179 bp)

CATTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTACCCGGGATGA
TAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
GAAGTA

Band B24 (142 bp)

CCTGAGCAGCACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTACCA
CCAACGCAGGCCCGGAGTTCTCTTCGGGTTGACGGTAGGTGGGGAATAAGGACCGGC
TAACTACGTGCCAGCAGCCGCGGTAAT

Band B25 (122 bp)

GTCTTATTAACCCGGGTAAGACATGAGCTTCTACAACCCTAGGGCCTTCATCACTCACG
CGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTA
GGA

RHD Primer Extracts

Band R1 (249 bp)

GTGTCTTCGCGACGCGCACGCATCCTGCGCACGCTCCCCGGATCGGTTGCTATCTGCGG
CAATCACGAGGAAAAGGCGCTCCGCTTCCACAAGGGCGGGAAGCATGACCAGTTCATC
GACGGTCACGCTCACCGTGAGTGGATTCGCAACGCGACCGATGAGGACTGGGCGTTCA
TCGCCGCGATGCCTCTGACGTGGTACGACCCGGGGCTGAACGTCCGCGTGATCCACGG
CACCGTGTTCCCCCCC

Band R2 (257 bp)

TCTTCGCGACGCGCAGCGCATCCTGCGCACGCTCCCCGGATCGGTTGCTATCTGCGGCA
ATCACGAGGAAAAGGCGCTCCGCTTCCACAAGGGCGGGAAGCATGACCAGTTCATCG
ACGGTCACGCTCACCGTGAGTGGATTCGCAACGCGACCGATGAGGACTGGGCGTTCAT
CGCCGCGATGCCTCTGACGTGGTACGACCCGGGGCTGAACGTCCGCGTGATCCACGGC
ACCATGTTCCCCAATATTTCCAACA

Band R3 (246 bp)

TCGTCCGAACCTGGAGCGTGCGCAGCTCCCGGACGGTTGCTTCTCGAACGACATCGAA
AACGCCCTCGGCTTCCCTGGCCGGCACGAAAGACCACTTCAACGACTGTCCCTCTCAGC
CTGAGTGGATTCTCATGGCCACCGACGACTACTGGGCGTTCATGGTCGCTATGCCTCTG
ACGTGGTACGACCCGGCGCTGAACGTCTTCGTGATCCTCGGCGCCATGTTGCCCCGTAT
TTCCACCAAGG

Band R4 (246 bp)

CAGCGCATCCTGCGCACGCTCCCCGGATCGGTTGCTATCTGCGGCATCACATCGAAAAG
GCGCTCCGCTTCCACAAGGGCGGGAAGCATGACCACTTCATCGACGGTCACGCTCACC
GTGAGTGGATTCGCAACGCGACCGATGAGGACTGGGCGTTCATCGCCGCTATGCCTCT
GACGTGGTACGACCCGGGGCTGAACGTCCGCGTGATCCTCGGCACCATGTTCCCCCGT
ATTTTCCACAGGG

Band R5 (241 bp)

GCGACGCGCAGCGCATCCTGCGCACGCTCCCCGGATCGGTTGCGTATCTGCGGCAATC
ACGAGGAAAAGGCGCTCCGCTTCCACAAGGGCGGGAAGCATGACCAGTTCATCGACG
GTCACGCTCACCGTGAGTGGATTCGCAACGCGACCGATGAGGACTGGGCGTTCATCGC
CGCGATGCCTCTGACGTGGTACGACCCGGGGCTGAACGTCCGCGTGATCCACGGCACC
GTGATCCCCC

Band R6 (225 bp)

TGCTACGGCGATGGTTGGCTCTACCAGATCAAGGTAGCTGACAAGGCGGATCTTGAAA
GCCTGCTCAGCGCCGAAGATTACGCCGCCCAGATCGAAGAAGACGCCTGACCTGACAC
GGAACCCCCATGCCCTATATTCCCCACACCCCGGAAGATGTGCAGTCCATGCTCGACGC
CATTGGCGTTGCCCGCATTGAGGATCTGTTCGACGAAATTGTCGGCGCCG

Band R7 (211 bp)

GTCTAGCACTCGAAGGCGAGGACCAATTCGCTCGCGAGTATGCCGTGGGCGGCCCGAT
CAATCCGAAGACGAGTGCGCCGTTCGGTCCGAACACCAAGGCCTTTGCCGAGTGGGCC
GCCAGCCACGGCAAGGACGTCCTGACCGACGCCCAACACGCACTGGTCCAGTTCATGG
CGGTGAACGTACGAACCCACAAAATTGTCGGCGCCGA

Band R8 (276 bp)

CGGCGCCGACAATTTTGTGGGTTCGTACGTTCACCGCCATGAACTGGACCAGTGCGTG
TTGGGCGTCGGTCAGGACGTCCTTGCCGTGGCTGGCGGCCCACTCGGCAAAGGCCTTG
GTGTTCGGACCGAACGGCGCACTCGTCTTCGGATTGATCGGGCCGCCCACGGCATACT
CGCGAGCGAATTGGTCCTCGCCTTCGAGTGCTAAACTGTGAACCGCGCGCCCGATTACC
TACGCCGGCCGGTCTTCATTCATGGCACCGTGTTCCCCTCCGC

Band R9 (176 bp)

ATCACGAATTTCTTGATCCCTTGGCGATAGTGACGTTTGGTTTAAAAGCTTTATCGTGCC
CAATACAACGCGGATTTCGTATCGAATTGCCCGCGATGTGCCGGAATTCCATGCCAATG
CGCGGTCGCGTCGTATTGCACTTTTGGCAACGGCACAGTCTTCCCGAACAACAGCTA

Band R10 (165 bp)

TGATGGCGAAGAAGCGCCCGCAGATCAGTACCGAAGGCCTGGATATTTCCCGCTACAG
CAATTCCCCGGAGAACGCCAAGAATGCCCATCCAGCGCCAGCACACTAACGAGCGTAT
GAGCCAGATCGTCGTCCACAACGGCACTGTCTTCCCGAACAACAGCTGA

Band R11 (164 bp)

AAGCGCCCGCAGATCAGTACCGAAGGCCTGGATATTTCCCGCTACAGCAATTCCCCGG
AGAACGCCAAGAATGCCCATCCAGCGCCAGCACACTAACGAGCGTATGAGCCAGATCG
TCGTCCACAACGGCACAGTCTTCCCGAACAACAGCAGAGACGAAAAAG

Band R12 (181 bp)

CCTTGGTCGATATGCGGGTTCTACTTGGCATTGAACTCGCGGACGAAGAGCATTGTTTT
TGCTCCATTTTCAACTACTCCACCGACCTGTTCGACGCCTCCACCGTGGAACGCCTGGCC
GGCCATTGGCGCAACCTGTTGCGCGGCATCGTCGCCAACCCACGTGGTATGCATCTCAA
CGC