The use of natural Mn oxide-containing wastes as a contaminated land remediation strategy and their effects on soil microbial functioning

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Abstract

The viability of using natural manganese oxide (MnOx)-containing wastes as amendments for contaminated land remediation was examined. The success of MnOx as a viable strategy was determined via the impact that their addition had upon microbial soil functioning, in addition to their ability to immobilise and/or transform inorganic and organic contaminants within industrially polluted soils.

Contaminated soils were obtained from two former industrial sites that are polluted with PAHs and metals. The intrinsic microbial functioning of these soils was assessed using a suite of microbial indicators reportedly sensitive to contamination [basal respiration (BR), potential nitrification (PNR), denitrification enzyme activity rates (DEA), microbial biomass carbon (MBC), metabolic quotient (qCO_2), microbial quotient (*qmic*)]. The diversity and community structure of key populations related to microbial indicators were assessed using culture-independent community analysis [polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE)] to target 16S rRNA and functional genes (*amoA*, *nirS*/*K*). These measurements were combined with a suite of physico-chemical analyses [pH, total organic carbon (TOC), soil organic matter (SOM), moisture content (MC), water holding capacity (WHC), extractable metal and PAH concentrations] to provide a combined geochemical and molecular microbial approach.

Contaminated soils were compared to a range of soils from land types defined as non-contaminated to provide a robust evaluation and define suitable microbial indicators for use in assessing soil microbial function in contaminated land and its remediation. Results showed that long term metal and PAH pollution had resulted in a microbial populations exhibiting extremely suppressed rates of BR and DEA, indicative of pollutants being bioavailable within the soil. Functional gene profiling revealed that inherent denitrifying and ammonia-oxidising community structures were significantly affected by contamination. Microbial functional processes of BR, DEA and PNR were determined to be superior indicators through their ease of use with standardised rapid and high throughput methods that could infer contaminant availability.

A preliminary assessment of MnOx addition upon microbial soil functioning was investigated though 6 month microcosm trials employing BR, DEA and PNR as

indictors. Microcosms employed two natural MnOx-containing wastes (mine tailings and coated sands) in a range of 0-30 % total MnOx, which were added to a low level metal contaminated soil. MnOx was found to stimulate PNR and DEA. Results implied that MnOx is not detrimental to soil microbial functioning and is capable of removing inhibitors of N-cycling, via a chemical rather than biological mechanism, ascribed to the immobilisation of bioavailable toxic metal ions by the MnOx.

The potential of MnOx amendment as a viable remediation strategy was investigated through the use of 9 month outdoor lysimeter trials. Measurement of extractable PAHs, along with extractable and bioaccessible Pb and As in metal, PAH and mixed contaminated soils showed no positive effects of using a 10 % by weight MnOx-coated sand amendment for remediation. Analysis of soil microbial functional indicators (BR, DEA, PNR) showed that MnOx amendment had no detrimental effects upon the function of microbial populations in the aforementioned soils.

Mn(II)-oxidising bacteria were isolated from contaminated soils and MnOx-containing wastes. This suggested that sustained and biologically enhanced redistribution of MnOx was possible in MnOx-amended soils, which may play an important role in pollutant transformation. This study provided the first demonstration of species within the genera *Amycolatopsis*, *Sacchorothrix*, *Lentzea* and *Micromonospora* as being capable of Mn(II) oxidation.

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List of Abbreviations

amoA, amoB, amoC	Ammonia monooxygenase genes
1-λ'	Simpson's diversity index
AMO	Ammonia monooxygenase
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising archaea
AR	Aqua regia
ATP	Adenosine 5-triphosphate
BARGE	Bioaccessibility Research Group of Europe
BARGE-UBM	Bioaccessibility Research Group of Europe-Unified Bioaccessibility Method
BGS	British geological society
BLAST	Basic local alignment search tool
bp	Base pairs
BR	Basal respiration
BSI	British standards institution
BTEX	Benzene, toluene, ethylbenzene and xylene
CFE	Chloroform fumigation extraction
CFI	Chloroform fumigation incubation
d	Margalef's richness index
DCS	Double-corner-sharing
DEA	Denitrification enzyme activity
Defra	Department for Environment, Food and Rural Affairs
DES	Double-edge-sharing
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DQRA	Detailed quantitative risk assessment
Eh	Reduction potential
EPMA	Electron Probe micro analysis
EPS	Extracellular polymeric substances

EU	European union
EXAFS	Extended X-ray Absorption Fine Structure
FISH	Fluorescence in situ hybridization
FYM	Farm yard manure
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GQRA	Generic quantitative risk assessment
GW	Green waste
H'	Shannon's diversity index
HAO	Hydroxylamine oxidoreductase
HF	Hydrofluoric acid
HMW	High molecular weight
HSD	Honestly significant difference
ISO	International Organisation for Standardization
J'	Pielou's evenness index
KMF	Kalahari manganese field
LBB	Leucoberbelin blue
LMW	Low molecular weight
LOI	Loss on ignition
MBC	Microbial biomass carbon
MC	Moisture content
MCO	Multi-copper oxidase
MDS	Multi-dimensional scaling
MEGA	Molecular evolutionary genetics analysis
MnOx	Manganese oxides
MOB	Manganese-oxidising bacteria
MPN	Most probable number
N	Total individuals
NAPL	Non-aqueous phase liquids
nirK	Cytochrome cd1 nitrite reductase
nirS	Copper nitrite reductase
NOB	Nitrite-oxidising bacteria
NPK	Nitrogen, phosphorus, potassium

NWSS	Northumbrian water scientific services
OM	Organic material
PAH	Polycyclic aromatic hydrocarbon
PAS	Publicly available specification
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
PFLA	Phospholipid fatty acid analysis
pH	Negative logarithm of the concentration of hydrogen ions
PNR	Potential nitrification rate
PZC	Point of zero charge
pε	Electron activity
qCO2	Metabolic quotient
qmic	Microbial quotient
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RDP	Ribosomal database project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Total species
SGV	Soil guideline value
SIR	Substrate induced respiration
SMB	Soil microbial biomass
SOM	Soil organic matter
SSCP	Single-strand conformation polymorphism
SSU rRNA	Small subunit ribosomal ribonucleic acid
TCA	Tricarboxylic acid
TCS	Triple-corner-sharing
TEM	Transmission electron micrograph
TES	Triple-edge-sharing
TOC	Total organic carbon
TPAH	Total polycyclic aromatic hydrocarbons

TRFLP	Terminal restriction fragment length polymorphism
US EPA	United states environmental protection agency
WHC	Water holding capacity
WHO	World Health Organisation
WTW	Water treatment works
XRF	X-ray fluorescence

Chapter 1. Introduction

1.1. Overview

The industrial past of the UK has left an inheritance of contaminated land in prime urban locations (Environment Agency, 2003). Therefore, research into appropriate remediation strategies for the redevelopment and reclamation of this land is now a key objective for environmental engineers. Population stresses upon cities in the UK has resulted in the need to reuse this land for applications such as housing and recreation. Consequently, government targets now call for 60 % of brownfield land to be regenerated for housing (DCLG, 2004).

Microorganisms play key roles in many soil processes and the delivery of essential ecosystem services. Thus, the maintenance of functioning microbial populations in soils is pertinent for the restoration of brownfield sites for the aforementioned end uses. It is of key importance that any remediation strategy not only aims to remove pollutants, but strives to maintain soil microbial populations and their essential functions, insuring no negative side effects on soil processes. Current research highlights microbial diversity and its activity as superior indicators in soil monitoring. This is due to the ability of microorganisms to respond rapidly to environmental changes (Avidano *et al.*, 2005; Ritz *et al.*, 2009). For instance, coupling functional measurements (e.g. nitrification) with modern culture independent techniques (PCR-DGGE) allows for a quick and robust quantification of community diversity and their metabolic processes in natural and engineered soils (Dickinson *et al.*, 2005; Malik *et al.*, 2008).

Mn oxides (MnOx) are naturally abundant in soils and the mediation of soil contamination is, in part, attributed to their ubiquitous occurrence (Toner *et al.*, 2005). This conclusion is based upon the fact that MnOx are one of the strongest naturally occurring oxidising agents in the environment, that have been shown to be capable of immobilising heavy metals and transforming organic contaminants into less harmful end products (Tebo *et al.*, 2004; Tebo *et al.*, 2005). Furthermore, the production of biogenic MnOx via microbial Mn cycling in the soil environment also transform contaminants, at rates up to several orders of magnitude faster than abiotic processes

(Miyata *et al.*, 2007; Tebo *et al.*, 2004). Therefore, MnOx present a unique material for solving remediation problems, having the potential to be an 'all round' amendment strategy for soils contaminated with both organic and inorganic pollutants.

MnOx have rarely been considered as a viable for contaminant removal in the past, as cheap sources have not previously been accessible. However, large quantities of natural 'waste' MnOx can now be sourced from the Mn mining (Clarke *et al.*, 2010) and water treatment industries. Both these materials have little commercial value or alternative use and could present a new contaminated land remediation product.

The research reported in this thesis was conducted in conjunction with Durham University and focuses on the viability of natural waste MnOx amendments as a remediation strategy. The overall aim was to understand whether natural waste MnOx can be used to remediate land contaminated with either metals, PAHs or a combination of both. Research at Durham University focused upon the chemical interactions of natural MnOx-containing wastes with pollutants, whereas the work conducted at Newcastle University centred upon the effects that natural MnOx-containing waste amendments had on soil microbial functioning.

1.1.1. *Scope, overall aim and objectives of this study*

This research has been the first to explore the use of natural Mn oxides as a contaminated land remediation strategy. To the author's knowledge this is also the first development of a remediation approach which has taken effects upon soil microbial functioning into account as a major factor in determining whether the strategy is successful. As such, the use of Mn oxides for *in situ* remediation could potentially provide a novel use for hundreds of thousands of tonnes of MnOx-containing wastes. Furthermore, it served to aid knowledge into interactions of Mn oxides in soils and their role in pollutant transformations. The overall aim of this study was to assess what affects the addition of natural Mn oxide-containing wastes as a contaminated land remediation strategy had upon soil microbial functioning. The overall objectives of this study were:

1) To use a combined geochemical, functional and molecular microbial ecology approach to determine suitable indicators to assess soil microbial functioning in contaminated land and its remediation (Chapter 2).

2) To employ the key functional indicators identified in objective 1 to determine the effect of Mn oxide addition upon microbial functioning, using a long term metal contaminated soil as an initial model (Chapter 3).
3) To assess the remediation potential of natural Mn oxide-containing wastes and the effects their addition has on microbial functioning in metal, PAH and mixed (metal and PAH) contaminated soils (Chapter 4).
4) To identify indigenous bacteria associated with microbial Mn(II) oxidation in long term contaminated soils, and MnOx-containing wastes employed in this study (Chapter 5). The presence of such organisms may be critical for enhanced geochemical cycling of Mn and hence the effectiveness of Mn oxides in remediation.

Please note more specific aims, objectives and hypotheses are presented within the introduction sections of subsequent chapters.

1.2. Soils

1.2.1. *Soils and their function as a microhabitat*

Soils, in comparison to other environmental mediums, have been described as "the most complex system on Earth" (Haygarth and Ritz, 2009). The study of soils is termed 'soil science' which is an interdisciplinary subject comprising physics, chemistry, pedology and biology (Bone *et al.*, 2010; Haygarth and Ritz, 2009). The formation of soils, termed 'pedogenesis', is driven by the integrated effects of climate and living organisms acting upon parent material, as conditioned by topography, over time (Bone *et al.*, 2010; Brady and Weil, 2008). A full description of soil formation is out of the scope of this study, although a comprehensive review of the processes involved can be found in Jenny (2009).

Many definitions and descriptions of soils exist such as:

- "the zone where plants take root, the foundation for terrestrial life, and the basis for a large amount of economic production, which varies in depth from a few centimetres to several metres" (Environment Agency, 2004b)

- "porous media that forms a thin layer across the surface of the majority of the terrestrial landmass, and are comprised of a wide variety of solid, semi-solid, liquid and gaseous constituents" (Brady and Weil, 2008)

Although as highlighted by Haygarth and Ritz (2009) many of these definitions do not clearly state that soils are dynamic, reactive and living entities which teem with a huge variety of life. Soils contain a wide range of organisms including the macrofauna (e.g. earthworms, vertebrates, snails, ants, termites and beetles), mesofauna (mainly microarthropods e.g. collembola), microfauna (e.g. mites, nematodes and protozoa) and microorganisms (bacteria, fungi and archaea) (Bot and Benites, 2005).

To put the size and diversity of soil microbial populations into perspective it has been estimated that one gram of forest soil contains 4×10^7 of bacterial cells, whereas one gram of cultivated grassland soil can have up to 2×10^9 bacteria, 10^5 protozoa, 10^2 nematodes and 1 km of fungal hyphae (Daniel, 2005; Paul and Clark, 1996; Richter and Markewitz, 1995; Young and Ritz, 2005).

The soil environment itself can be viewed simplistically as a combination of inorganic ('mineral' e.g. clay, silt, sand) and organic (containing carbon) particles in various stages of decomposition (Daniel, 2005; Haygarth and Ritz, 2009). The organic phase is made up of non-living or living matter (see Table 1-1 for more details), while the mineral phases of soils at the outset are derived from the underlying geology (Haygarth and Ritz, 2009). The inorganic and organic constituents form clay-organic matter complexes and aggregates comprised of clay, sand and silt particles; generally considered as the dominant structures within soils (Daniel, 2005).

Table 1-1: Summary of soil organic matter fractions taken from Hopkins and Gregorich (2005).

Component	Compartment	Fraction
Living	Plant roots	
	Invertebrates	Macrofauna Mesofauna Microfauna
	Fungi	Mycorrhizas Non-mycorrhizas
	Protists	Protozoa Algae
	Prokaryotes	Bacteria Archaea
Non-living	Non-humic substances	Nucleic acids Peptides and amino acids Sugars and polysaccharides Lignin Lipids
	Humic substances	Humic acids Fulvic acids Humin
Living and non-living	Nuclear magnetic resonance (NMR) defined fractions	Alkyl-C and methyl-C O-alkyl-C Aromatic-C Carbonyl-C
	Infrared (IR) defined fractions	
	Kinetically defined fractions	Passive C Active C Inert C
	Functionally defined fractions	Decomposable plant litter Resistant plant material Biomass Physically protected organic matter Chemically protected organic matter
	Physically defined fractions	Size fractions Density fractions Dissolved organic carbon

In the soil environment bacteria tend to live as single cells or microcolonies embedded in a matrix of extracellular polymeric substances (EPS) which consists mainly of polysaccharides (Giri *et al.*, 2005; Vu *et al.*, 2009). EPS are excreted by bacteria, but also archaea and fungi, which allow initial attachment to different

substrata, protection against environmental stress and dehydration (Vu *et al.*, 2009). Thus, soil microorganisms can strongly adhere to individual soil particles and clay-organic matter complexes (Daniel, 2005; Giri *et al.*, 2005). Additionally, they can inhabit the interior and exterior surfaces of soil aggregates, as well as the pore spaces between (Daniel, 2005; Giri *et al.*, 2005). Not only does the soil structure provide a habitat for microbial populations it also regulates the movement of gases, liquids, solutes, particles and organic material (Haygarth and Ritz, 2009).

It is important to highlight that while soil is a huge reservoir for microbial diversity, it is by its nature, extremely spatially and temporally heterogeneous. To put this into context Young and Ritz (2005) calculated that based upon a realistic surface area of one gram of soil being 20 m^2, at the scale of microbes, they themselves cover less than 0.0001 % of the total surface area available for colonisation. Thus, suggesting that microbial life in soil should be thought of as "oases surrounded by dessert" (Young and Ritz, 2005).

Soils have been described as the as the "beholder of life" (Verstraete and Mertens, 2004) and the "biological engine of the Earth" (Haygarth and Ritz, 2009). It is the presence of microorganisms that are the underlying catalysts of many processes in the soil (Garbeva *et al.*, 2004). Microbial populations play a key role in soil structure and formation, providing food for other organisms, the decomposition of organic matter, degradation of anthropogenic pollutants, suppression of soil borne plant diseases, promoting plant growth, water holding capacity, water infiltration rates, crusting and erodibility, susceptibility to compaction, the cycling of carbon, nitrogen, phosphorus and sulphur (Garbeva *et al.*, 2004; Winding *et al.*, 2005). Thus, the presence of active microbial populations is the key to keeping the soil biological engine running.

1.2.2. *Soils as a provider of ecosystem services*

Soils have been described as the ecological life-support system for civilisation due to the wide variety of ecosystem services that they provide for mankind (Costanza *et al.*, 1997). Recently Haygarth and Ritz (2009) summarised the most important ecosystem services supplied by soils in the UK which are presented in Table 1-2.

Table 1-2: The most critical ecosystem services and functions appropriate to soils and land use within the UK, taken from Haygarth and Ritz (2009) based upon Costanza *et al.* (1997).

Role	No.	Ecosystem service	Soil function	Example
Supporting	1	Primary production	Support for terrestrial vegetation	Support for principle photoautotroph's
	2	Soil formation	Soil formation processes	Weathering of rock and accumulation of organic material
	3	Nutrient cycling	Storage, internal cycling and processing of nutrients	Nitrogen-fixation, nitrogen and phosphorus mineralisation and cycling
Provisioning	4	Refugia	Providing habitat for resident and transient populations	Burrows for soil macro fauna
	5	Water Storage	Retention of water in landscape	Retention of water in pore network, modulates soil biochemical processes
	6	Platform	Supporting structures	Supporting housing, industry, infrastructure
	7	Food Supply	Provisioning plant growth	Provisioning for crops and livestock for farming
	8	Biomaterials	Provisioning plant growth	Producing timber, fibre, fuel
	9	Raw materials	Provisioning source materials	Topsoil, mineral, aggregates extraction
	10	Biodiversity and genetic resources	Sources of unique biological materials and products	Medical products, genes for resistance to pathogens and pests
Regulating	11	Water quality regulation	Filtration and buffering of water	Potable water for human consumption and good ecological status of rivers, lakes and seas
	12	Water supply regulation	Regulation of hydrological flows	Flood control where surplus, irrigation where deficit
	13	Gas regulation	Regulation of atmospheric chemical composition	CO_2/O_2 balance, O_3 for UVB protection, and SO_x levels
	14	Climate regulation	Regulation of global temperature, precipitation and other biologically mediated climatic processes	Greenhouse gas regulation
	15	Erosion control	Soil and colloid retention within an ecosystem	Retention of soil on hill slopes and in wetlands
Cultural	16	Recreation	Providing a platform for recreational activities	Eco-tourism, sport
	17	Cognitive	Opportunities for non-commercial activities	Aesthetic, education, spiritual, scientific value
	18	Heritage	Holds archaeological record of terrestrial occupancy and civilisations	Preservation/destruction of archaeological record

A full review of these services is out of the scope of this research. However, it is vital that they are highlighted to illustrate how dependent humans are upon soils and the need to protect them from any future degradation. This becomes more pertinent when it is realised that soils are non-renewable on a human time scale (Jenny, 2009). This is validated by the fact that it takes on average 100-400 years for a centimetre of

soil to form (Doran and Safley, 1997). Thus, the thin layer of soil covering the surface of the earth really does represent the difference between survival and extinction for most land-based life (Doran and Zeiss, 2000). Neglecting soil and the ecosystem services it provides for the human population may ultimately compromise the sustainability of humans in the biosphere (Bone *et al.*, 2010; Costanza *et al.*, 1997). Furthermore, soil is an extremely valuable economic resource. Costanza *et al.* (1997), who were the first to review ecosystem services provided by terrestrial environments, estimated that the services supplied represented an economic value that is twice that of the gross national product. Based upon this, Guimarães *et al.* (2010) approximated that a functioning soil providing vital ecosystem services represents a total value in the order of €10,000 per inhabitant, per year.

The significance of the ecosystem services supplied by soils has been emphasised by the European Commission who have put forward legislation to protect soils within Europe (Commission of the European Communities, 2006a; Commission of the European Communities, 2006b). In addition, ecosystem services have been used to pinpoint requirements for the safeguarding of soils within the UK (Defra, 2004a; Environment Agency, 2004b; Ritz *et al.*, 2009; SEPA, 2001).

1.2.3. *Soil monitoring within the UK*

In 2004, The Department for Environment, Food and Rural Affairs (Defra) carried out scientific research into developing biological indicators for the national monitoring of soil within the UK (Defra, 2004c). The aim of the project was to provide a final list of robust, practical, policy relevant, flexible and cost effective bio-indicators (Defra, 2004c).

The results of the study were later published by Ritz *et al.* (2009), who out of 183 candidate biological indicators, proposed 21 suitable for national monitoring (see Table 1-3). The 21 proposed indicators are based upon the maintenance of soils to support the key functions of food and fibre production, environmental interactions and habitat/biodiversity preservation; all of which are supported by the presence of microbial populations. However, out of these 21 indicators only 13 were classified as being fully deployable at present. The remaining 8 would require months or years of additional method development before they could be used. A full review of the

method for selecting indicators is out of the scope of this study but the reader is directed to Ritz *et al.* (2009) for further information.

This project was only the first step toward the deployment of a national soil monitoring scheme in the UK. Ritz *et al.* (2009) report that the suite of indicators identified, offers the strongest potential candidates and standard operating procedures. However, it was also noted that their inherent sensitivity, ability to discriminate between soil: land use combinations and ecological interpretation all need to be confirmed. Thus, a definitive national soil monitoring plan for the UK is still some time off.

Table 1-3: Biological indicators selected for national soil monitoring within the UK, adapted from Ritz *et al.* (2009).

Indicator	Method	Indicator descriptor
(a) Fully Deployable		
Ammonia-oxidisers/denitrifiers	TRFLP[a]	Genetic profile
Community composition	PFLA[b] profiles	Composition
Fungal community	TRFLP	Genetic profile
Multiple SIR[C]	GC[d]	Activity capability
Nematodes	Baermann extraction procedure	Numbers, composition and size
Bacteria	TRFLP	Genetic profile
Microarthropods	Tullgren dry extraction	Numbers, composition and size
Flora and fauna	On-site visual recording	Numbers estimate
Multi-enzyme	Microplate fluorometric assay	Enzyme potential activity
Archaea	TRFLP	Genetic profile
Methanogens/methanotrophs	TRFLP	Genetic profile
Invertebrates	Pitfall traps	Numbers, composition and size
Actinomycetes	TRFLP	Genetic profile
(b) Ready in the short term		
Nematodes	TRFLP	Genetic profile
Multiple SIR	Microresp™	Activity capability
Protozoa	TRFLP	Genetic profile
Arbuscular mycorrhiza Fungi	qPCR[e]	Genetic profile
(c) Not ready		
Functional gene arrays	Not developed	Genetic profile
Phylogenetic gene arrays	Not developed	Genetic profile
FISH[f]-keystone species	Not developed	Genetic profile
Soil proteomics	Not developed	Phenotypic profile

[a] terminal restriction fragment length polymorphism
[b] phospholipid fatty acid analysis
[C] substrate induced respiration
[d] gas chromatography
[e] quantitative polymerase chain reaction
[f] fluorescence in situ hybridization

1.3. The use of microbial indicators in soil monitoring

Microorganisms have been described as the most sensitive of all biological indicators as they are highly susceptible to any chemical or physical perturbation of the soil (Kennedy and Smith, 1995). Due to their presence in exceptionally high amounts and their rapid growth and death, they make the most dynamic measurement of all biological indicators, owing to their key roles in many processes and the delivery of essential ecosystem services (Bloem and Breure, 2003; Garbisu *et al.*, 2011; Peixoto *et al.*, 2006). Furthermore, they have intimate contact and interaction within the soil amplified by their small size and high surface to volume ratio (Bloem and Breure, 2003; Giller *et al.*, 1998; Winding *et al.*, 2005).

Measurements of microbial activity have the ability to reflect the sum of chemical, physical and biological factors (Bloem and Breure, 2003; Elliot, 1997). Thus, assessing microbial activity in soils can provide an integrated assessment which cannot be always obtained with physical and chemical measures, or analyses of higher organisms (Nielsen and Winding, 2002; Winding *et al.*, 2005). Furthermore, the community composition and diversity of soil microbial populations is thought of as insurance for resistance against perturbations (Wessén and Hallin, 2011). Changes in microbial community composition or diversity via a perturbation such as pollution, can alter the functional capability of soil microorganisms, which has lead to loss of microbial diversity, which is a major threat to soil (Haygarth and Ritz, 2009). Consequently, the use of microbial functions, community composition and diversity as indicators in soil monitoring allows for the microbial community to be assessed both as a native feature of the soil, or, how it may respond to perturbation.

The use of microbial indicators in soil monitoring has been questioned by Broos *et al.* (2005), who drew attention to their sensitivity, as often functions have large natural changeability between different soil types, and therefore ask if microbial indicators are indeed robust. It must also be remembered that the full extent of the diversity of microbial populations in soils and their precise functions are still not fully understood. Soils are often treated as 'black box' processes by scientists due to their complexities (Kennedy and Smith, 1995). However, the use of microbes as indicators has long been utilised and proven to be successful in soil monitoring, especially within the field of agriculture, having been shown to provide early warnings of soil

deterioration (Bloem and Breure, 2003; Hayat *et al.*, 2002; Jordan *et al.*, 1995; Peixoto *et al.*, 2006; Stenberg, 1999).

Doran and Safley (1997) suggested that a microbial indicator should be "linked and/or correlated with ecosystem processes and function, integrated with soil physical, chemical and biological properties and selected due to relative to ease of performance and cost effectiveness". Furthermore, Nielsen and Winding (2002) reported that a microbial indicator should represent "properties of the environment or impacts to the environment, which can be interpreted beyond the information that the measured or observed parameter represents by itself". Recently, Ritz *et al.* (2009) reported that, based on a Web of Knowledge search, there have been in excess of 17, 500 papers published on the use of biological indicators in soils between 1970-2008 the majority of which include microbial parameters. Thus, an assessment of indicators has grown out of the scope of a conventional literature review (Ritz *et al.*, 2009).

For the purposes of this study commonly used microbial indicators assessing community composition, functional activity and diversity were chosen (Winding *et al.*, 2005). Indicators included a suite of properties which the literature has identified to be reflective of a wide range of ecological functions and metabolic activities. These are of major importance in soils and give a reliable indication of overall microbial functioning (Garbisu et al., 2011). Brief outlines of the microbial indicators used in this study are outlined within the following sections.

1.3.1. *Bacterial diversity and community composition*

Research into bacterial diversity and community composition is based upon molecular microbial ecology which uses what is termed as 'modern culture-independent molecular techniques' that rely on the characterisation of nucleic acids (DNA and RNA) extracted directly from environmental samples such as soils (Malik *et al.*, 2008). The use of modern molecular techniques has become favoured by microbiologists because of the 'great plate count anomaly' which highlighted the inability for culturing to adequately represent community composition (Staley and Konopka, 1985). Modern methods allow for excellent quantification of soil microbial communities through their ability to measure both diversity and community composition in soils (Avidano *et al.*, 2005; Dickinson *et al.*, 2005). Furthermore, these techniques also permit the analysis of functional genes which allows for community

composition and diversity to be directly linked to functional measurements (see sections 1.3.5 and 1.3.6).

In soil microbial ecology, often the terms diversity and community composition are used interchangeably (Garbeva *et al.*, 2004), however, it is important to highlight that the terms are different and represent dissimilar meanings within this study. Microbial diversity can be defined as "the number of different species and their relative abundance in a given community in a given habitat" and microbial community composition relates to the structure of different recognisable taxa within the given habitat (Garbeva *et al.*, 2004).

In microbial ecology the ribosomal (rRNA) gene, particularly the bacterial 16S rRNA has been the most widely used molecular marker to identify microbial populations (Head *et al.*, 1998; Muyzer, 1999). Pace *et al.* (1986) were the first to realise that this phylogenetic framework of rRNA sequences could be used to design primers and probes to target genes. Their work was built upon by the seminal findings of Woese (1987) who determined that all organisms can be classified by a comparative sequence analysis of their small subunit rRNA (Head *et al.*, 1998; Muyzer, 1999). The use of rRNA molecules revolutionised microbial ecology, and there are many reasons for targeting probes to the 16S rRNA gene of the small subunit of the ribosome (SSU rRNA), which are outlined below (Amann and Ludwig, 2000; Case *et al.*, 2007; Gutell *et al.*, 1994; Head *et al.*, 1998; Olsen *et al.*, 1986; Van de Peer *et al.*, 1996).

- highly conserved sequence domains interspersed with more variable regions
- essential rRNA domains conserved across all phylogenetic domains, thus, 'universal' tracts of sequences can be identified
- a large amount of rRNA in most cells
- not been affected by lateral gene transfer
- good sequence length of about 1500 base pairs (bp) that provides sufficient sequence information to permit statistically significant comparisons
- contains the key elements of the protein-synthesising machinery
- availability of huge rRNA databases for comparative sequence analysis

Although the 16S rRNA is by far one of the most frequently used genes for bacterial community composition and diversity analysis, it must be highlighted that

methods in molecular microbial ecology are not limited to this gene (see sections 1.3.5 and 1.3.6) (Case *et al.*, 2007).

The use of modern molecular methods is based upon the extraction of DNA directly from soil which in theory is representative of all indigenous soil microorganisms (Daniel, 2005). Further analysis of microbial community structure and diversity can then be carried out by the use of polymerase chain reaction (PCR) amplification of selected genes, followed by a community fingerprinting method (Daniel, 2005). There are many microbial community fingerprinting methods that have been developed to examine the diversity and community composition of 16S rRNA sequences from PCR amplified soil DNA such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), temperature gradient gel electrophoresis (TGGE) (Rosenbaum and Riesner, 1987), terminal restriction fragment length polymorphism (TRFLP) (Liu *et al.*, 1997), single-strand conformational polymorphism (SSCP) (Lee *et al.*, 1996) and automated ribosomal intergenic spacer length polymorphism (ARISA) (Fisher and Triplett, 1999). Within this study PCR-DGGE was utilised to examine microbial diversity and community composition in soils as it is one of the most commonly used fingerprinting techniques (Nannipieri *et al.*, 2003; Winding *et al.*, 2005). A brief review of PCR and DGGE has been provided below.

Following DNA extraction, genomic DNA is subjected to PCR amplification using primers designed to amplify 16S rRNA or genes from a particular group of organisms (Head *et al.*, 1998). PCR results in an exponential increase in the number of copies of the initial target gene that produces millions of copies of the desired gene in a few hours (Malik *et al.*, 2008; Nakatsu, 2007). This method provides a way of amplifying target nucleic acid sequences from a complex environmental mixture of DNA and RNA, from many sources such as bacteria, archaea and fungi, that are generally too low in abundance to be applied to a subsequent fingerprinting method (Theron and Cloete, 2000). PCR can be summarised into three main stages: 1) the DNA is melted to convert double-stranded DNA to single-stranded DNA, 2) two primers are annealed to the target DNA fragment of interest, and 3) the DNA fragment of interest is extended by nucleotide addition from the primers by the action of DNA polymerase, such that synthesis proceeds across the region between the primers, replicating that DNA segment (Steffan and Atlas, 1991). This process is repeated numerous times in what is called cycles. The product of each cycle is complementary to and capable of binding to

primers so the amount of DNA synthesised is theoretically doubled in each successive cycle (Bej *et al.*, 1991).

Following PCR, the amplified fragments of the target nucleic acids of the same length but different base sequence compositions, can be resolved electrophoretically by DGGE, which provides a snapshot of community composition and diversity (Head *et al.*, 1998; Malik *et al.*, 2008). The DGGE gel is basically comprised of a linear gradient of denaturant, in which PCR amplified fragments migrate under the influence of an electric field (Valášková and Baldrian, 2009). When the fragment reaches a position in the gradient in which the strands separate, migration ceases, with sequences of different base pair compositions visualised as bands (Nakatsu, 2007; Valášková and Baldrian, 2009). The DNA strands are stopped from completely separating by adding a high GC sequence (GC-clamp) which is attached to the end of one PCR primer (Nakatsu, 2007). The number of bands in a DGGE profile is therefore theoretically proportional to the number of dominant species in the sample and their relative abundance which is represented by band intensity (Malik *et al.*, 2008; Nakatsu, 2007).

There are many problems associated with the use of modern molecular techniques that arise in DNA extraction, PCR amplification and also in the choice of fingerprinting technique (Nannipieri *et al.*, 2003). Many reviews have been published on these topics e.g. Head *et al.* (1993); Malik *et al.* (2008); Robe *et al.* (2003); van Elsas and Boersma (2011); Wintzingerode *et al.* (1997); therefore, only a summary of the main problems inherent to all steps has been provided in Table 1-4. It is important to note that all community fingerprinting techniques are prone to these problems. However, the use of DGGE can be advantageous over other methods due to the ability to excise individual bands which can then be re-amplified and sequenced, providing an opportunity to identify the microbial community (Head *et al.*, 1998; Malik *et al.*, 2008; Nakatsu, 2007; Nannipieri *et al.*, 2003).

Table 1-4: Advantages and disadvantages of culture-independent PCR-based microbial community fingerprinting methods adapted from Garbeva *et al.* (2004).

Advantages	Disadvantages
▪ Dependence on efficient cell lysis in DNA extraction and not on the physiological status of cells	▪ Incomplete lysis in DNA extraction of some species, notably gram-positive spore-formers
▪ Direct picture of the diversity of dominant microbial types, including the unculturables	▪ Possible biases in DNA extraction and PCR amplification, inhibition by soil compounds
▪ Direct assessment of time shifts or differences in microbial community structure/composition	▪ Possible presence of one particular sequence or band in different organisms
▪ Ease in handling. Simultaneous analysis of high sample numbers	▪ Heterogeneous bands that may originate from one bacterial strain due to heterogeneity in the gene sequence
▪ Reproducible results	▪ Phylogenetic information only is usually obtained, and the link to functional information is difficult unless functional genes are targeted
▪ Generation of sequences resulting in identification and specific probes to track the specific organism in the ecosystem	

1.3.2. *Carbon cycling*

Soils are a critical regulator of the global carbon (C) budget and contain more than three-fourths of the earth's terrestrial C (Johnston *et al.*, 2004; Lal, 2004). One of the key processes of the C-cycle in soils is aerobic respiration. This process releases carbon dioxide (CO_2) from the soil via the various pathways of root respiration, fauna respiration and the microbial decomposition of litter and soil organic matter (Luo *et al.*, 2006). Aerobic respiration is directly related to ecosystem productivity, soil fertility in addition to regional and global carbon cycles (Luo *et al.*, 2006). Out of the aforementioned pathways, it is the activity of soil microorganisms decomposing organic matter that is of the most importance (Nielsen and Winding, 2002). These organisms which are, in general, heterotrophic are reliant on inputs of C as energy from outside the microbial community (Coleman *et al.*, 2004; Nielsen and Winding, 2002). Thus, the measurement of aerobic respiration represents investigations of C-

cycling at an ecosystem level and also provides a good estimate of microbial activity as a whole (Nielsen and Winding, 2002; Winding *et al.*, 2005).

Aerobic microbial respiration is a series of metabolic processes that break down (or catabolise) organic molecules to liberate energy, water and carbon dioxide (CO_2) in a cell, primarily through the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle and the Krebs cycle) (Luo *et al.*, 2006). The catabolism of organic molecules by microbial populations under aerobic conditions generates energy by the oxidation of sugars (glucose or other carbohydrates) to CO_2 as in Equation 1-1. This process is carried out through glycolysis, the pentose phosphate pathway, the aforementioned TCA cycle and the electron transport pathway; however, a full review of all of these processes is out of the scope of this research (Luo *et al.*, 2006).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

Equation 1-1: Overall reaction for the oxidation of glucose to CO_2 in microorganisms.

It must also be highlighted that microbial respiration is not limited to aerobic pathways alone. Respiration can also occur under anaerobic conditions where inorganic compounds (e.g. NO_3^-, Mn^{4+}) serve as the terminal electron acceptors instead of oxygen (Madigan *et al.*, 1997). However, aerobic topsoil's (0-10 cm depth) are the subject of this thesis which, when regulated to 60 % of their maximum water holding capacities, are expected to result in an aerobic system. It is worth noting that positions of inorganic respiratory compounds in the electron tower decrease sequentially in the order of $O_2 > NO_3^- > Mn^{4+} > Fe_3^+ > SO_4^{2-} > CO_2$, resulting in different energy yields for bacterial populations (Madigan *et al.*, 1997). While anaerobic respiration is energetically less efficient than aerobic respiration it may be an important process in natural soils amended with Mn oxides undergoing wetting and drying cycles and throughout the deeper soil profile into anaerobic zones. In the instance of flooding, anaerobic respiration would rapidly result in the reduction of Mn oxides being the third most favourable electron acceptor. Concurrently, the envisaged pollutant reductive capacities of Mn oxides would be inhibited by bacterial reduction with a release of toxic Mn(II) (see section 1.5 for further details). These processes are out of the scope of this study but constitute an interesting research avenue.

Aerobic microbial respiration is highly influenced by temperature, soil moisture content, availability of nutrients and soil structure. This process is often measured in the laboratory under controlled and standardised conditions to manage for these previously mentioned variables (Joergensen and Emmerling, 2006; Winding *et al.*, 2005). Respiration can either be measured as CO_2 production or O_2 consumption. CO_2 production is favoured due to the low atmospheric concentration of this gas compared to O_2 (Joergensen and Emmerling, 2006; Winding *et al.*, 2005). It is worth noting that other CO_2 production processes within the soil environment include the anaerobic fermentation of glucose to organic acids, the anaerobic conversion of acetate to equal amounts of methane and CO_2 by acetoclastic methanogens and the aerobic actions of methanotrophic bacteria which oxidise methane (Luo *et al.*, 2006; Schink, 1997). Although, as previously mentioned these are not expected to result in high CO_2 emissions in aerobic topsoil's.

The measurement of aerobic soil respiration in this way is termed basal respiration (BR) and is in general reported as an activity rate i.e. the use of catabolic substrates by soil organisms over time to produce CO_2 (Joergensen and Emmerling, 2006). It is worth mentioning that field measurements of BR are generally not favoured due to difficulties in separating CO_2 production from microbial and plant origins, the uncertainty in assigning a rate measured per area volume and the inherent variability due to shifting climatic factors (Joergensen and Emmerling, 2006; Winding *et al.*, 2005). BR is an old but frequently used technique for quantification of microbial activities in soil. It has been successfully used in many soil monitoring programmes and has been shown to be an effective indicator of perturbation to soil (Brookes, 1995; Nielsen and Winding, 2002; Winding *et al.*, 2005).

1.3.3. *Biomass*

Biomass can be defined as the living tissue of soil microorganisms that are metabolically active (Joergensen and Emmerling, 2006). The measurement of the soil microbial biomass (SMB) has been described as a key parameter of any soil environment as it relates directly to soil fertility, organic matter content, cycling of nutrients, decomposition, biochemical transformations, soil structure and stabilisation (Bailey *et al.*, 2002; Winding *et al.*, 2005). However, soils contain both living, dead and dormant cells, which can create problems for accurately measuring the active

populations (Joergensen and Emmerling, 2006). There are many methods for determining SMB. They include direct measurements such as live-dead staining and fluorescence in situ hybridization (FISH) (Joergensen and Emmerling, 2006). However, indirect methods are more commonly used. These include PLFAs (phospholipid fatty acids) and the most abundantly used methods of chloroform fumigation incubation (CFI), chloroform fumigation extraction (CFE) and substrate induced respiration (SIR) (Joergensen and Emmerling, 2006; Winding *et al.*, 2005). All methods, whether direct or indirect, have been reported to give similar estimates of SMB and only one biomass indicator is typically employed in soil monitoring schemes (Beck *et al.*, 1997; Joergensen and Emmerling, 2006).

It is important to highlight that the SIR and CFE/CFI methods do report different kinds of SMB and therefore a brief outline of these methods have been given. CFE/CFI is based upon chloroform vapour dissolving cell membranes which releases cell constituents from both dead and alive microorganisms (Bloem and Breure, 2003). The size of the SMB is calculated from quantification of respired CO_2 which is released as a result of germinating microbial spores, or, via the quantification of extractable carbon; both involving a conversion factor (Bailey *et al.*, 2002; Winding *et al.*, 2005). In contrast the SIR procedure measures SMB based upon the response of the biomass to the fresh addition of a readily available substrate, most commonly glucose (Anderson and Domsch, 1978). After the addition of the substrate, the soil respiration rate is measured and SMB calculated using a conversion factor (Anderson and Domsch, 1978). Therefore, SIR measures the response of the SMB to freshly added substrate and estimates the 'active' SMB. On the other hand, CFE/CFI methods measures the (chloroform-sensitive) 'total' SMB (Bailey *et al.*, 2002). A limitation of CFE is that extraction of lysed cells with a salt solution does not yield information about the SMB beyond its size (Bailey *et al.*, 2002). In this study it was deemed that the measuring 'active' SMB was a more powerful approach for determining effects on soil functioning. Furthermore, the information from SIR can be used to calculate other indictors as outlined below.

Additional information about C-cycling, biomass and the use of substrates by the soil microbial population can be gathered from the measurement of eco-physiological ratios of the metabolic quotient (qCO_2) and the microbial quotient (*qmic*) (Anderson, 2003; Joergensen and Emmerling, 2006; Winding *et al.*, 2005). The qCO_2 is

also called the specific respiratory rate and is defined as the microbial respiration rate (measured as evolution of CO_2) per unit microbial biomass (Anderson and Domsch, 1990). This ratio is measured by determining the SMB (via the SIR method) and the basal respiration (BR) rate (Anderson and Domsch, 1990). The qCO_2 has been used in many studies and has been described as one of the most straightforward indicators of microbial functioning as it can infer stress, the maturity of the soil ecosystem and if substrates are labile (Anderson, 1994; Bastida *et al.*, 2008; Winding *et al.*, 2005). The measurement of *qmic* allows for the amount of microbial biomass to be related to the total organic carbon content. The *qmic* has been suggested as a sensitive indicator of microbial functioning due to the fact that microbial biomass in a soil responds more rapidly to change than the total organic matter (Anderson and Domsch, 1990; Bastida *et al.*, 2008). This ratio allows an assessment of soil organic matter dynamics, the availability of organic matter and avoids problems of comparing soils with different organic matter contents (Sparling, 1997; Winding *et al.*, 2005).

1.3.4. *Nitrogen cycling*

The nitrogen (N) cycle is one of the most important nutrient cycles in terrestrial ecosystems. N-cycling involves four microbiological processes of nitrogen fixation, mineralisation, nitrification and denitrification; a summary of which is presented in Figure 1-1 (Hayatsu *et al.*, 2008). Within the N-cycle the microbial processes of nitrification and denitrification have been identified as the two main routes that affect the fate of N in soils. Both nitrification and denitrification have been routinely assessed in soil monitoring studies and are therefore concentrated upon in this study (Winding *et al.*, 2005). Reviews of nitrification and denitrification have been presented in the two subsequent sections.

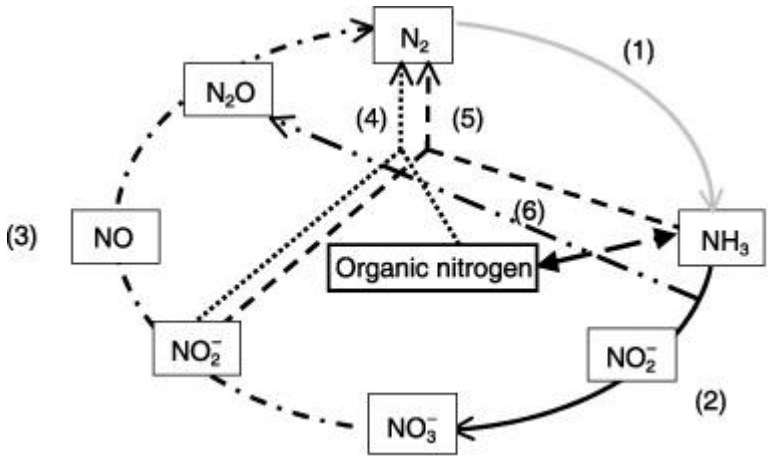


Figure 1-1: Microbiological processes in the nitrogen cycle taken from Hayatsu *et al.* (2008). (1) Nitrogen fixation; (2) bacterial nitrification, archaeal nitrification and heterotrophic nitrification; (3) aerobic and anaerobic bacterial denitrification, nitrifier denitrification, fungal denitrification and archaeal denitrification; (4) and (5) co-denitrification (by fungi); (5) anammox (anaerobic ammonium oxidation); (6) N_2O production during nitrification (ammonia oxidation).

1.3.5. *Nitrification*

Nitrification (Figure 1-1, line 2) is the biological conversion of reduced nitrogen (N) in the form of ammonia (NH_3), ammonium (NH_4^+) or organic N, to oxidised N in the form of nitrite (NO_2^-) or nitrate (NO_3^-) (Hayatsu *et al.*, 2008). The conversion of the cation, NH_4^+, to an anion (NO_2^- or NO_3^-) determines the mobility of N through the generally negatively charged soil and therefore strongly influences the fate of N in terrestrial systems (Norton and Stark, 2010).

Microbial ammonia oxidation, the conversion of NH_3 to NO_2^-, is the first and rate-limiting step of nitrification. It has been proposed as a sensitive parameter as only a small number of microorganisms (described later) are involved, which respond to perturbation of the soil through mechanisms such as contamination (Jia and Conrad, 2009; Kowalchuk and Stephen, 2001b; Visser and Parkinson, 1992; Winding *et al.*, 2005). Therefore, it was decided to concentrate upon ammonia oxidation as an indicator for nitrification within this study.

In soil, nitrification is controlled by the availability of the substrates (NH_4^+/NH_3, CO_2 and O_2), moisture content, temperature and pH (Norton and Stark, 2010). The process of nitrification is generally measured as a rate in soils and can be

experimentally determined as net, gross or potential (Norton and Stark, 2010). The most common measurement of nitrification is by determining its potential via the ammonia-oxidising assay, also called the potential nitrification rate (Belser and Mays, 1980). In this method soil is generally shaken in an aerobic slurry with an excess of substrates and at a controlled pH which ensures complete aeration of the sample and promotes uniform distribution of substrates, thus, minimising diffusion limitations of substrate supply (Belser and Mays, 1980; Norton and Stark, 2010). Chlorate is added to the soil slurry which is an inhibitor of chemolithotrophic nitrite oxidation and blocks the conversion of NO_2^- to NO_3^-, thus, the measurement of NO_2^- over time gives a potential nitrification rate (Belser and Mays, 1980).

Nitrification in soils is carried out by chemolithotrophic (autotrophic nitrification) and chemo-organotrophic (heterotrophic nitrification) bacteria and also the archaea (De Boer and Kowalchuk, 2001; Francis *et al.*, 2007). Autotrophic nitrification is a two step process which firstly consists of the conversion of NH_3 to NO_2^- by the ammonia-oxidising bacteria (AOB) according to Equation 1-2 (Norton and Stark, 2010):

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

Equation 1-2: Conversion of ammonia (NH_3) to nitrite (NO_2^-) by AOB.

Secondly, the conversion of NO_2^- to NO_3^- occurs by the nitrite-oxidising bacteria (NOB) in accordance to Equation 1-3 (Norton and Stark, 2010).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

Equation 1-3: Conversion of nitrite (NO_2^-) to nitrate (NO_3^-) by NOB.

AOB are aerobes, able to use NH_3 as a sole source of energy and CO_2 as a sole source of carbon (Kowalchuk and Stephen, 2001a). This trait until recently (see below), has been only found in a narrow range of a monophyletic groups within the β-subclass of the Proteobacteria. The currently accepted classification recognises only two genera

within this group, *Nitrosospira* and *Nitrosomonas* (Head *et al.*, 1993). A single genus within the γ-subclass of the Proteobacteria, *Nitrosococcus*, also exhibits ammonia oxidation, but appears to be restricted to marine environments (De Boer and Kowalchuk, 2001; Teske *et al.*, 1994).

The conversion of NH_3 (which is generally accepted as the substrate, not NH_4^+) to NO_2^- by AOB is unique and involves a compound reaction that proceeds via hydroxylamine (NH_2OH), as seen in Figure 1-2 (Kowalchuk and Stephen, 2001b). A membrane bound, multi-subunit enzyme, ammonia monooxygenase (AMO) catalyses the first reaction; while a periplasm-assosiated enzyme, hydroxylamine oxidoreductase (HAO), catalyses the second (Figure 1-2). The energy liberated by these reactions is used by the AOB as the sole source of energy for CO_2 fixation and growth (Hooper *et al.*, 1997).

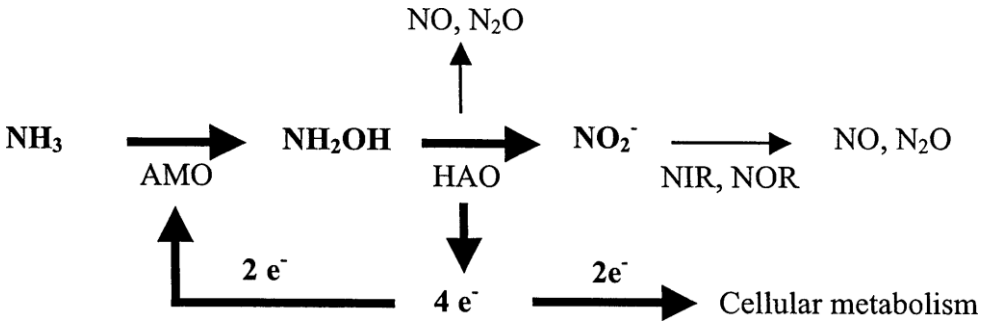


Figure 1-2: Pathway of ammonia oxidation in AOB taken from Arp and Stein (2003). Bold arrows and nitrogen compounds indicate major fluxes. AMO = ammonia monooxygenase, HAO= hydroxylamine oxidoreductase, NIR= nitrite reducatse and NOR= nitric oxide reducatse.

The diversity and population dynamics of AOB communities can be investigated through the use of molecular methods. The AOB represent one of the groups where amplification and analysis of the 16S rRNA gene has been successful in revealing more about their ecology and physiology, due to their concentration into two distinct monophyletic lineages (Hayatsu *et al.*, 2008; Junier *et al.*, 2010). However, the use of the 16S rRNA gene also has dangers. Sequence identification of amplified PCR fragments has shown that the primers used are not specific to the AOB alone and also target non-AOB-like sequences (Junier *et al.*, 2008; Junier *et al.*, 2010; Kowalchuk *et al.*,

1998; Phillips *et al.*, 1999). Therefore, genes encoding key enzymes (e.g. AMO) which are involved in a specific pathway have provided a superior alternative (Junier *et al.*, 2010).

The AMO enzyme has three genes which encode it, organised in a cluster of *amoC-amoA-amoB* (Arp and Stein, 2003). Out of these three genes it is the *amoA* that has been identified as the subunit which contains the active site of NH_3 oxidation (Arp and Stein, 2003). Consequently, primers have been designed which successfully target the *amoA* gene and have been utilised in the study of AOB communities in many investigations (De Boer and Kowalchuk, 2001; Stephen *et al.*, 1999). The use of *amoA* is more powerful as a molecular tool because of its high specificity and the fact that it encompasses both functional and phylogenetic traits (Hayatsu *et al.*, 2008; Junier *et al.*, 2010). Furthermore, the *amoA* gene contains more sequence variation than the 16S rRNA gene and allows for a greater degree of discrimination between closely related AOB populations (De Boer and Kowalchuk, 2001).

In contrast to the AOB, heterotrophic nitrification is carried out by a wide phylogenetic range of bacteria (e.g. *Paracococcus denitrificans* /*Pseudomonas putida*) and fungi (Hayatsu *et al.*, 2008). However, unlike the autotrophic AOB, heterotrophic nitrification is not linked to cellular growth (De Boer and Kowalchuk, 2001). Two pathways of ammonia oxidation have been proposed for heterotrophic nitrifiers (De Boer and Kowalchuk, 2001). Firstly, it is known that they can oxidise both organic and inorganic forms of N to hydroxylamine, nitrite and nitrate via AMO and HAO enzyme pathways like the autotrophic AOB (Daum *et al.*, 1998; De Boer and Kowalchuk, 2001; Moir *et al.*, 1996; Nishio *et al.*, 1998). However, the AMO genes of the heterotrophic nitrifiers only share a partial sequence similarity to that of autotrophic AOB (Hayatsu *et al.*, 2008). Secondly, in some heterotrophic nitrifiers ammonia oxidation is linked to aerobic denitrification under conditions where aerobic respiration is limited, which allows them to maintain a high growth rate when energy substrates are in excess (De Boer and Kowalchuk, 2001; Kuenen and Robertson, 1994; Wehrfritz *et al.*, 1993). The production of N-oxides by heterotrophic nitrifiers that are not capable of aerobic denitrification has been suggested to inhibit the growth of competing organisms (De Boer and Kowalchuk, 2001). The potentials of autotrophic and heterotrophic nitrifiers can be distinguished with the use of specific inhibitors (De Boer and Kowalchuk, 2001; Hayatsu *et al.*, 2008). However, it is thought that autotrophic and archaeal nitrification

(described below) is the main mechanism of nitrification in most environments (Lam and Kuypers, 2011). The role of heterotrophic nitrification has received considerably less attention due to technical challenges to differentiation between the lithotrophic and heterotrophic variants with molecular methods (Lam and Kuypers, 2011).

The most recent development within the study of nitrification in soils has been the discovery of ammonia-oxidising archaea (AOA). Initially some archaea were proposed to be capable of ammonia oxidation from the discovery of the *amoA* gene from uncultured crenarchaeota in two different studies (Treusch *et al.*, 2005; Venter *et al.*, 2004). The definitive link between the presence of the *amoA* gene and ammonia oxidation was discovered by Könneke *et al.* (2005) who showed that *Nitrosopumilus maritimus* contained the putative AMO genes *amoA-amoB-amoC*, and was able to convert ammonia to nitrite with bicarbonate as a sole carbon source. Quantification of *amoA* gene expression levels in microcosms has shown that the AOA are active in soils (Treusch *et al.*, 2005) and also in oceans (Francis *et al.*, 2005). Further to these discoveries, analysis of *amoA* sequences in the AOA has shown that there are many diverse and distinct members that possess this trait within varying habitats (Francis *et al.*, 2007). Phylogenetic analysis of *amoA* genes has also indicated that the crenarchaeotal AOA form five major monophyletic clusters. Within these clusters the AOA soil inhabitants form a separate Group called the 1.1b 'soil' lineage or *Nitrososphaera* cluster (Pester *et al.*, 2012).

The AOA like the AOB employ the same functional *amoA* gene, which catalyses the first step in ammonia oxidation. However, the bacterial and archaeal genes are sufficiently divergent to be distinguished by their sequences. Therefore, studies looking at the diversity and community structure of AOA in natural environments have targeted the AOA *amoA* functional gene (Wessén *et al.*, 2010). As the AOA do not from a distinct monophyletic clade as the AOB do, and belong to different lineages within the Crenarchaeota, the use of 16S rRNA gene primers has not been viewed as a promising approach (Junier *et al.*, 2010).

The quantification of AOA and AOB *amoA* genes has indicated that the AOA outnumber the AOB in most habitats by a factor of 10 to 1, 000; with *amoA* gene copy numbers of the AOA found to be 3,000 fold higher in some soils (Junier *et al.*, 2010; Leininger *et al.*, 2006). Consequently, studies have suggested that the AOA play a bigger role in nitrification than the AOB. Another body of research suggests that the

AOB are still the dominant ammonia-oxidisers in soils (Jia and Conrad, 2009; Okano *et al.*, 2004). At present it is still unresolved as to whether bacterial or archaeal nitrification is the main contributor to nitrification activity in soils. No experimental method presently exists that can specifically inhibit either the AOA or AOB, and distinguish their relative contributions (Hayatsu *et al.*, 2008).

Furthermore, there is evidence to suggest that the AOA are not strict autotrophs and may function as mixotrophs (Francis *et al.*, 2007). Evidence for this has been uncovered by Hallam *et al.* (2006a) and Hallam *et al.* (2006b) who found genes predicted to encode autotrophic carbon assimilation i.e. a near complete oxidative tricarboxylic acid cycle in an *amoA* containing AOA. Consequently, this means that the AOA have the potential to utilise both CO_2 and organic material as carbon sources and they may not therefore always function in the environment as ammonia-oxidising organisms (Hallam *et al.*, 2006b).

1.3.6. *Denitrification*

Denitrification is the process which completes the N-cycle and involves the biological stepwise reduction of nitrate (NO_3^-) to dinitrogen (N_2) gas (Figure 1-1, line 3). In general denitrification occurs where the substrates of NO_3^- and C are available and where oxygen (O_2) is scare or absent (Davidson and Seitzinger, 2006). This process is an alternative facultative respiration pathway carried out by many phylogenetically diverse microorganisms that are described in more detail later (Philippot and Hallin, 2005).

Denitrification in soils is affected by many abiotic factors such as C availability, temperature, moisture and thus O_2 availability, pH, predation and physical disturbances such as freeze/thaw and wetting/drying (Philippot *et al.*, 2007; Wallenstein *et al.*, 2006). Consequently, as this process is sensitive to many environmental parameters it has long been utilised in studies assessing perturbations to soils and their effects on microbial function (Winding *et al.*, 2005). There are many methods available to measure denitrification such as the use of ^{15}N tracers, direct N_2 quantification, mass balance approaches and stable isotopes (Groffman *et al.*, 2006). However, in general denitrification in soils is a problematic function to accurately measure due to the difficulty in the quantification of N_2 (Groffman *et al.*, 2006). Measurement of denitrification requires the ability to detect small changes in N_2,

which is challenging due atmospheric background levels being 79 %; thus, minor changes in its concentration are difficult to accurately quantify (Groffman *et al.*, 2006).

Consequently, the use of the acetylene (C_2H_2) inhibition technique, whereby the reduction of nitrous oxide (N_2O) to N_2 is inhibited by C_2H_2 and the terminal product N_2O is measured by gas chromatography, is favoured by many researchers (Groffman *et al.*, 2006; Philippot *et al.*, 2007; Smith and Tiedje, 1979; Winding *et al.*, 2005). The C_2H_2 inhibition method is the most commonly applied as it presents a sensitive measurement, with N_2O easily quantified due to its low atmospheric concentrations (Groffman *et al.*, 2006). However, it has received criticism as C_2H_2 inhibits the production of NO_3^- via nitrification and can lead to an underestimation of rates in terrestrial environments with small NO_3^- concentrations (Groffman *et al.*, 2006; Seitzinger *et al.*, 1993). To combat this issue the measurement of denitrification potential is employed via the use of the denitrification enzyme activity (DEA) assay (Groffman *et al.*, 1999). In the DEA assay, NO_3^- and C substrates are added in excess to soil under strict anaerobic conditions, thus, combating the issue of low NO_3^- and possible underestimation (Groffman *et al.*, 2006; Groffman *et al.*, 1999; Winding *et al.*, 2005).

Research on the diversity, abundance and community composition of denitrifying populations in the environment has focused on bacteria, however, this trait is also known to be carried out by some archaea and fungi (Philippot and Hallin, 2005; Wallenstein *et al.*, 2006). Within the bacteria, the functional trait of denitrification is found within more than 60 genra that represent up to 5 % of the total soil microbial community (Philippot *et al.*, 2007). It has been hypothesised that the wide spread ability in unrelated bacteria to denitrify is due to lateral gene transfer (Bothe *et al.*, 2000). As bacterial denitrification is believed to be the most dominant in the environment it has been concentrated upon in this study (Philippot *et al.*, 2007; Throbäck *et al.*, 2004; Wallenstein *et al.*, 2006). Denitrification involves four enzymatically catalysed reductive steps (Figure 1-3) of NO_3^- reduction, NO_2^- reduction, NO reduction and N_2O reduction. Denitrification has been described a 'community process' as many bacteria do not actually possess the full suite of enzymes and thus they may work together as a consortium (Wallenstein *et al.*, 2006; Zumft, 1997). It must be remembered that denitrification enzymes are created only under anaerobic

conditions and are not functional under aerobic conditions, even though they are present in bacterial populations (Winding *et al.*, 2005).

Due to the diversity of bacteria capable of performing this function research on denitrifying populations has focused on functional genes rather than species composition via 16S rRNA analysis (Throbäck *et al.*, 2004; Wallenstein *et al.*, 2006). In general the nitrate reducatse genes have not been targeted as they are also present in bacteria that do not denitrify, such as the dissimilatory reducers of nitrate and nitrate respirers (Chèneby *et al.*, 2003; Philippot *et al.*, 2002; Wallenstein *et al.*, 2006).

The most common molecular markers for the analysis of the bacterial denitrifying community are the nitrite reductase genes. This is a key step in the denitrification cascade that that leads to a gaseous intermediate. Additionally, the reduction of NO_2^- to NO distinguishes denitrifiers from other nitrate respiring bacteria (Groffman *et al.*, 2006; Philippot and Hallin, 2005; Philippot *et al.*, 2007; Throbäck *et al.*, 2004). The reduction of soluble NO_2^- to nitric oxide (NO) is catalysed by two evolutionary unrelated enzymes that differ in terms of structure and prosthetic metals: the cytochrome cd_1 (encoded by the gene *nirS*) and the copper-containing nitrite reductases (encoded by the gene *nirK*). (Groffman *et al.*, 2006; Philippot *et al.*, 2007). Bacteria carry either *nirS* or *nirK* but the two enzymes are functionally equivalent (Glockner *et al.*, 1993; Philippot *et al.*, 2007). These genes contain highly conserved DNA regions which has led to the successful development of primers to study denitrifier community structure in soil environments (Bothe *et al.*, 2000). A primer targeting both *nirS* and *nirK* has not been developed due to the fact these structurally different enzymes do not share similar sequence homology, thus studies into denitrifier community structure assesses both genes (Bothe *et al.*, 2000).

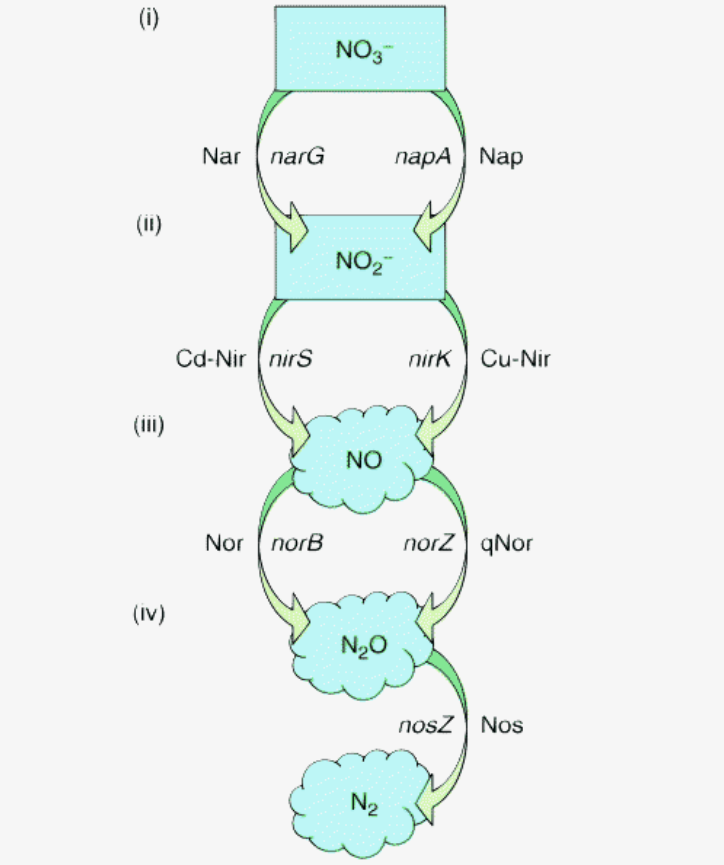


Figure 1-3: Denitrification pathway taken from Philippot and Hallin (2005). (i) Reduction of soluble nitrate (NO_3^-) to nitrite (NO_2^-) is catalysed either by a membrane-bound (Nar) or a periplasmic nitrate reductase (Nap). (ii) Reduction of soluble NO_2^- to nitric oxide (NO) gas is catalysed by either a copper (Cu-Nir) or a cytochrome cd1 nitrite reductase (Cd-Nir). (iii) Reduction of NO to nitrous oxide (N_2O) is catalysed by either a two-component type (Nor) or single-component type (qNor) of nitric oxide reductase. (iv)Reduction of N_2O to dinitrogen (N_2) gas is catalysed by the nitrous oxide reductase (Nos).

1.4. Soil contamination

1.4.1. *Soil contamination overview*

Any unwanted substance introduced into the environment is referred to as a 'contaminant' (Megharaj *et al.*, 2011). Detrimental effects or damages by the contaminants lead to 'pollution/contamination', a process by which a resource (in this case soil) is rendered unfit for use, more often than not, by humans (Megharaj *et al.*, 2011).

It is estimated that in Europe, potentially polluting activities have occurred at about 3 million sites, from which more than 8 % (nearly 250,000 sites) are in need of remediation and only 80,000 have been remediated (European Environment Agency, 2010). Furthermore, it is reported that by 2025, the number of sites requiring remediation may increase by more than 50 %. This is due to improvements in data collection and therefore increasing designation of contaminated sites, thus, soil contamination is an ever growing environmental issue (European Environment Agency, 2010).

The main sources of soil contamination in Europe have been identified as heavy metals and mineral oil (European Environment Agency, 2010). However, many other major contaminants exist such as polycyclic aromatic hydrocarbons (PAH), phenols, chlorinated hydrocarbons (CHC) and BTEX (benzene, toluene, ethylbenzene and xylene compounds) (Guimarães *et al.*, 2010). This study will concentrate upon both organic and inorganic contaminants, namely PAHs and heavy metals. Therefore, a brief outline of these pollutants has been given below.

1.4.2. *Polycyclic aromatic hydrocarbons*

Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons with two or more fused benzene rings formed during the thermal decomposition of organic molecules (Haritash and Kaushik, 2009). PAHs contaminate the soil via the burning of fossil fuels, manufacturing of gas and coal tar, wood processing, escaped automobile gasoline and incineration of waste (Peng *et al.*, 2008). PAHs are ubiquitously present and are of great concern due to their toxic, mutagenic, teratogenic and carcinogenic nature (Adonis *et al.*, 2003; Patnaik, 1992). Furthermore, they are difficult to degrade under natural conditions due to their non-polar nature causing high hydrophobicity in combination with their strong absorption to solid particles (Haritash and Kaushik, 2009; Peng *et al.*, 2008). As PAHs increase in molecular weight their water solubility decreases, melting and boiling point increase and vapour pressure decreases, thus, their persistence in the environment increases (Haritash and Kaushik, 2009). More than 100 PAH compounds actually exist in the environment; however, based upon their toxicity and abundance, 16 have been included in the United States Soil Protection Agency's list of priority pollutants as shown in Table 1-5. These 16 PAHs are

concentrated upon in this study (ATSDR, 2005; Keith and Telliard, 1979; Peng *et al.*, 2008).

Table 1-5: US EPAs 16 priority-pollutant PAHs and selected physical-chemical properties taken from Bojes and Pope (2007) based upon ATSDR (2005).

PAH[a]	Structure (# of rings)	Molecular weight (g/mole)	Solubility (mg/l)
Naphthalene	2	128.17	31
Acenaphthene	3	154.21	3.8
Acenaphthylene	3	152.20	16.1
Anthracene	3	178.23	0.045
Phenanthrene	3	178.23	1.1
Fluorene	3	166.22	1.9
Fluoranthene	4	202.26	0.26
Benzo(a)anthracene	4	228.29	0.011
Chrysene	4	228.29	0.0015
Pyrene	4	202.26	0.132
Benzo(a)pyrene	5	252.32	0.0038
Benzo(b)fluoranthene	5	252.32	0.0015
Benzo(k)fluoranthene	5	252.32	0.0008
Dibenz(a,h)anthracene	6	278.35	0.0005
Benzo(g,h,i)perylene	6	276.34	0.00026
Indeno[1,2,3-cd]pyrene	6	276.34	0.062

[a] PAHs that are highlighted in bold are probable carcinogens

1.4.3. *Heavy metals*

Heavy metals play an integral role in the life processes of microorganisms, animals and humans as essential micro-nutrients (Bruins *et al.*, 2000). However, at high concentrations they are well known to be toxic to all living organisms (Bruins *et al.*, 2000). The term 'heavy metals' in general includes both metal and metalloid elements with an atomic density greater than 6 g cm^{-3} (except As and Se) (Park *et al.*, 2011). They can be split into the biologically essential micro-nutrients (e.g. Cu, Cr, Mn and Zn) and non-essential which are considered as toxic (e.g. Cd, Pb and Hg) (Park *et al.*, 2011). However, even biologically essential heavy metals at high concentrations can also be toxic to living organisms (Adriano, 2001).

Heavy metal pollution occurs from multiple sources such as mining, smelting, atmospheric deposition, industrial processes, manufacturing and the disposal of domestic, agricultural and industrial waste materials (Brookes, 1995; Park *et al.*, 2011). Heavy metals cannot undergo microbial or chemical degradation within soil and remain for extremely long periods, having half lives of residence in the range of several thousands of years (Brookes, 1995; Guo *et al.*, 2006a). Thus, remediation strategies concentrate upon the minimisation of their 'bioavailability' which is defined subsequently.

1.4.4. *Defining bioavailability and bioaccessibility of contaminants*

Both bioavailability and bioaccessibility can be referred to in terms of either humans or microorganisms with several definitions existing depending upon whether the term is being used in pharmacology, toxicology or environmental science (Semple *et al.*, 2004). Thus, it has been said that defining either term is not a straight forward task (Semple *et al.*, 2004). Within this study the term 'bioavailability' is used to describe the availability of contaminants to microbial populations, whereas the term 'bioaccessibility' refers to the availability of contaminants to humans.

In this study microbial bioavailability is considered to be represented as a whole by the "maximum quantity of a contaminant available for uptake by an organism within a given time period" which includes processes covered by more specific definitions of bioaccessibility and bioavailability, seen in Figure 1-4 (Semple *et al.*, 2004; Semple *et al.*, 2007). The definitions for bioavailability and bioaccessibility are taken from Semple *et al.* (2004) who describes bioavailability as "that which is freely available to cross an organism's (cellular) membrane from the medium the organism inhabits at a given point in time" and bioaccessibility as "that which is available to cross an organisms' (cellular) membrane from the environment it inhabits, if the organism had access to it; however, it may be either physically removed from the organism or only bioavailable after a period of time" (Semple et al., 2004; Figure 1-4).

The definition chosen to represent human bioaccessibility within this study is taken from the Bioaccessibility Research Group of Europe (BARGE) whose methods of have been employed within Chapter 4. 'Bioaccessibility' refers to the fraction of a contaminant that is released from soil into solution by digestive juices. It represents

the maximum amount of contaminant that is available for intestinal absorption (Wragg *et al.*, 2011).

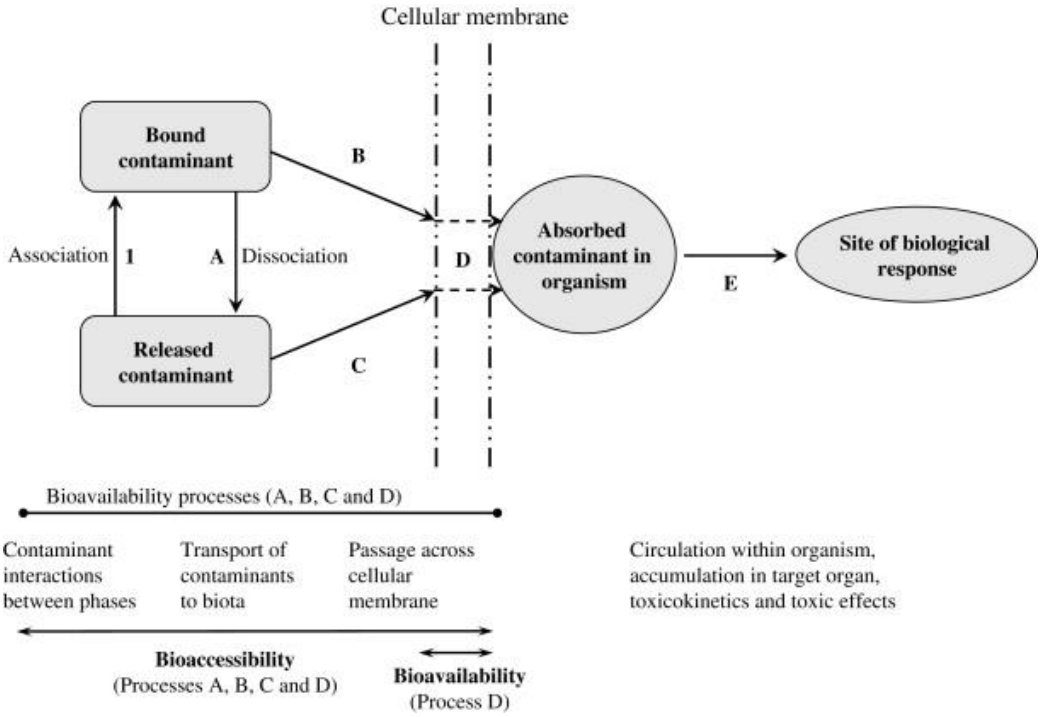


Figure 1-4: Bioavailability processes. Individual physical, chemical and biological interactions that determine the exposure of organisms to chemicals associated with soils and sediments taken from Semple *et al.* (2007). 1 = ageing, sequestration and/or chemical binding; A = release of bound/recalcitrant compound to a (more) labile state; B = transport of bound compound to biological membrane; C = transport of labile, soluble or dissolved compound to biological membrane; D = uptake across a physiological membrane; E = incorporation into a living system. Notes: (i) A, B and C can occur internally or externally to an organism. Process 1, which is not a bioavailability process *per se* but a contaminant fate/soil process, has been included for completeness.

1.4.5. *Soil contamination - a current threat to soil*

Many threats to the degradation of soil and its vital ecosystem services exist such as erosion, declining organic matter, acid rain, exhaustion by farming, landfill seepage, mining, highway construction, overgrazing, sealing, compaction, declining biodiversity, salinisation, floods, landslides and irrigation (Commission of the European

Communities, 2006b; Guimarães *et al.*, 2010). Over recent years the issue of soil contamination has received more attention as being a major threat to soils and ecosystem service degradation (Bone *et al.*, 2010).

Soil contamination has gained interest and has consequently become a major concern both at regional and European levels. In 2006, the EU developed a soil thematic strategy which was dedicated to the protection of soils with the overall aim of keeping the soils of Europe robust and 'healthy' (Commission of the European Communities, 2006a; Commission of the European Communities, 2006b). Principally there were two main objectives. Firstly, the preservation of soil functions by preventing degradation. Secondly, the restoration of soils to a level coherent with intended use taking into account cost through best available methods. Within the soil thematic strategy a specific section was dedicated to dealing with contamination and land management. Consequently, the European Commission has defined local and diffuse contamination as one of the major threats to soils and their services within Europe at present (Commission of the European Communities, 2006b).

Within the UK, The Department for Environment, Food and Rural Affairs (Defra) has also recognised contamination as a current threat to UK soils along with climate change, compaction, erosion, loss of biodiversity, loss of organic matter and sealing (Defra, 2004b). The long term sustainable management of soils was highlighted in the 'First Soil Action Plan' (2004-2006) whose vision was to ensure that England's soils will be protected and managed to optimise the varied functions that soils perform for society (Defra, 2004b). One of the central themes to the 'First Soil Action Plan' concentrated upon the evaluation and reduction of contaminants to land, taking into account the vital significance of soil biodiversity and its part in retaining ecosystem functions. The Environment Agency has produced a report on the state of soils in the UK (Environment Agency, 2004b), which highlighted the degradation of soils via pollution.

The sustainable management of soil is pertinent as pollution causes very serious environmental consequences for microbial populations, plants, animals and human health. Furthermore, the difficulties and cost associated with remediation of contaminated soils has severe socio-economic consequences, with current estimates of €2.4 – 17.3 billion/year linked to the cleaning up of polluted soil (European Environment Agency, 2010). Numerous reviews have detailed the fact that pollution of

soil can seriously affect the ability of soil microorganisms to perform their essential functions (Baath, 1989; Giller *et al.*, 1998; Ramakrishnan *et al.*, 2011). In some cases soils have been reported as becoming 'functionally dead' though pollution (European Environment Agency, 2010). Consequently, it is the effects that soil contamination and remediation strategies have upon soil microbial functioning, community composition and diversity that are centred upon within this study. The effects of heavy metals and PAHs upon the microbial indicators chosen within this study are discussed within Chapter 2 and are therefore not reviewed here.

1.4.6. *Contaminated land regulations in the UK*

In the UK, contaminated land is defined under Part IIA of the Environmental Protection Act (1990) as "any land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, on, or under the land , that (a) significant harm (to human health) is being caused or there is a significant possibility of such harm being caused, or, (b) pollution of controlled waters is being, or is likely to be, caused".

The determination of whether significant harm is being caused or whether there is a possibility of such harm, requires the use of site specific risk assessments. This process involves a three tiered approach with model procedures described in a technical framework called the Contaminated Land Report 11 (CLR 11) (Environment Agency, 2004a).

The three tiers used involve (1) a preliminary risk assessment, (2) a generic quantitative risk assessment and (3) a detailed quantitative risk assessment which assesses direct risk to human health (Environment Agency, 2004a; Ferguson and Denner, 1998). A preliminary risk assessment relies firstly upon the identification of a 'pollutant linkage' within the soil where at least one contaminant, pathway and receptor are present. The three essential elements of a 'pollutant linkage' are defined as (Environment Agency, 2009d):

- A contaminant - a substance that is in, on or under the land and has the potential to cause harm.
- A receptor - in general terms, something that could be adversely affected by a contaminant, such as people.

- A pathway - a route or means by which a receptor can be exposed to, or affected by, a contaminant.

It must be highlighted that without a pollutant linkage, there is no risk, even if a contaminant is present (Environment Agency, 2009d; Ferguson and Denner, 1998). If one or more 'pollutant linkage' is identified then the second step, a generic quantitative risk assessment (GQRA) takes place.

The second GQRA phase is based upon the use of soil guideline values (SGVs). SGVs are a screening tool of scientifically based generic assessment criteria that can be used to simplify the assessment of human health risks arising from long-term and on-site exposure to chemical contamination in soil (Environment Agency, 2009d). SGVs are derived from the Contaminated Land Exposure Assessment (CLEA) model that provides generic estimates of child and adult exposures to soil contaminants for those potentially living, working and/or playing on contaminated sites over long periods of time (Environment Agency, 2009a). Further information on the technical background of the CLEA model and the derivation of human toxicological assessments for soil contaminants is out of the scope of this study, but further information can be found in Environment Agency (2009a) and Environment Agency (2009c).

SGVs represent 'trigger values' where if the concentration of a contaminant is above the SGV level then it is possible that a significant harm to human health may exist at a site (Environment Agency, 2009d). There are different SGVs according to land use (residential, allotments and commercial). This is because people make use of land differently and this effects which humans and how they may be exposed to soil contamination (Environment Agency, 2009b). Current SGVs are outlined in Table 1-6, these values are only designed to indicate whether further site-specific investigation is needed (Environment Agency, 2009b; Environment Agency, 2009d).

Table 1-6: Soil Guideline Values (SGVs) published by the Environment Agency (2009b). All values reported are in mg kg^{-1} dry weight [1, 2].

Contaminant	Residential	Allotment	Commercial
Heavy metals			
Arsenic (As)	32	43	640
Nickel (Ni)	130	230	1,800
Elemental mercury (Hg^4)*	1.0	170	11
Inorganic Hg^{2+}	170	80	3,600
Methyl Hg^{+2}	11	8	410
Selenium (Se)	350	120	13,000
Cadmium (Cd)	10	1.8	230
BTEX			
Benzene	0.33	0.07	95
Toluene	610	120	4.4×10^3
Ethylbenzene	350	90	2.8×10^3
o-xylene	250	160	2.6×10^3
m-xylene	240	180	3.5×10^3
p-xylene	230	160	3.2×10^3
Dioxins	8	8	240
Phenol	420	280	3,200

[1] Based on a sandy loam soil with a soil organic matter (SOM) content of 6 %.
[2] Figures are rounded to one or two significant figures.
* SGV used in this study for Hg

However, many scientific researchers employ the use of SGVs in contaminated land studies to determine whether the land is contaminated, poses a significant threat to human health or if a proposed remediation strategy is successful e.g. Costa *et al.* (2011); Gbefa *et al.* (2011); Giusti (2011); Hartley *et al.* (2009); Okorie *et al.* (2010); Okorie *et al.* (2011). It must be highlighted that exceeding SGV levels does not necessarily mean that there is a risk to human health. To fully determine risk to human health a third further investigation, the detailed quantitative risk assessment (DQRA), is required. It must also be pointed out that SGVs were not explicitly derived to define remediation standards and are only meant to be used as a starting point for long-term human health risk assessment. Furthermore they do not assess risk to construction workers or non-human receptors (Environment Agency, 2009d).

A DQRA is to establish and use more detailed site-specific information and criteria to decide whether there are unacceptable risks to human health are present (Environment Agency, 2004a). This involves steps such as the further assessment of

the most significant pollutant linkages, determining whether SGV values are appropriate based upon the soil condition at the site e.g. different organic matter contents, determining bioaccessibility of contaminants and the development of tools and criteria to evaluate risk often through the use of computer models (Environment Agency, 2004a). Following these three assessment steps an appraisal can be completed that will choose the best available technique by representing available remediation options and consequently proposing a remediation strategy (Environment Agency, 2004a; Environment Agency, 2009d).

Current UK contaminated land law states that any remediation should result in land that is 'suitable for use', that is no longer contaminated and presents no significant harm to human health. To define a land as no longer contaminated then each pollutant linkage should no longer be significant by (i) removal or treatment of the (source) of the contaminant(s), (ii) removal or modification of the pathway(s) or (iii) removal or modification the behaviour of the receptor(s) which is assessed by the local authority (Environment Agency, 2009c; Environment Agency, 2009d).

1.4.7. *Contaminated land remediation technologies*

Contaminated land remediation and its technologies is an ever expanding field, with the range of remediation strategies available being extremely large and diverse (Liao and Li, 2012). These strategies can be broadly divided into traditional 'civil engineering' methods, 'natural remediation' methods and newer 'process based' methods (Mansfield and Moohan, 2002). Process based methods can be further divided into physical, chemical and biological treatment technologies (Scullion, 2006). However, remediation can incorporate a combination of methods to offer the most effective approach (Scullion, 2006; Strange *et al.*, 2008). A detailed review of all contaminated land remediation technologies is beyond the scope of this review. However, a summary of the main remediation strategies has been provided in Table 1-7, collated from the reviews of Hamby (1996); Liao and Li (2012); Scullion (2006) and Semple *et al.* (2001).

Table 1-7: Summary of the main physical, chemical and biological remediation strategies.

Classification	Technology
Physical*	Removal to landfill
	Capping
	Soil washing
	Physico-chemical washing
	Vapour extraction
	Electrokinetic remediation
	Incineration
	Vitrification
	Particle sorting
	Stabilisation/Solidification
	Thermal desorption
	Vapour Stripping
	Infilling with non-contaminated soil
	Air sparging
	Wash /pump and treat
Chemical	Solvent extraction
	Dehalogenation
	In situ flushing
	Surface amendments
	Oxidation
	Reduction
	Hydrolysis
	Dechlorination
	pH manipulation
Biological	***Microbial activity***
	Windrow turning
	Land farming
	Bioventing
	Bioslurry
	Biopiles
	Bioreactors
	Bioleaching
	Composting
	Biostimulation
	In situ bioremediation
	Plant activity
	Phytostabilisation
	Phytoextraction
	Phytodegredation
	Natural biological activity
	Monitored attenuation

* includes civil engineering based methods

Recent times have seen a change in the use of traditional techniques such as incineration, disposal to landfill and barrier techniques that controlled the pathway of the contaminant and concentrated on containment rather than treatment (Scullion,

2006). Today process based methods are the favored for remediation which strive to remove the contaminant (Liao and Li, 2012). These technologies may be applied *in situ* i.e. directly to the soil without prior removal, or *ex situ* where the treatment is applied following the excavation of the soil (Scullion, 2006; Strange *et al.*, 2008). In general, *in situ* technologies are favorable as they offer minimal disruption, although *ex situ* methods do have the benefit of offering better management and control (Scullion, 2006).

Over the past decade the use of organic and inorganic soil amendments has become a promising alternative to the aforementioned techniques (Tica *et al.*, 2011). This is based upon many materials being identified that are easily available or present as waste products from various industries. Due to the simplicity, *in situ* employment, low cost, ability to immobilise or transform various types of contaminants with reported high effectiveness, these strategies are now at the forefront of remediation research (Tica *et al.*, 2011).

1.4.8. *Assessment of soil functioning in contaminated land remediation*

At present contaminated land law, remediation and soil monitoring occupy separate legislative areas (Garbisu *et al.*, 2011). It must be highlighted that even though there may be a governmentally accepted level of pollutant reduction from remediation of contaminated soil, this does not also reflect a recovery of overall soil functioning (Brookes, 1995). For example, Hinojosa *et al.* (2004) found that a decrease of 84.4 % in the heavy metal contamination in soils from a pyrite mud spill only increased overall microbiological activity by 24.3 %. Thus, in modern remediation it is becoming apparent that just looking for pollutant reduction is no longer sufficient. Many studies have utilised microbial indicators in a contaminated soil setting. However, they tend to assess the detrimental effects of contamination upon microbial populations and their functions rather than assessing the impact of chosen remediation technologies e.g. Avidano *et al.* (2005) and Yang *et al.* (2006). In recent years there has been a move towards the consideration of biological indicators in conjunction with the remediation success (Dawson *et al.*, 2007). The need for biological indicators that assess microbial or biological functioning in the remediation of brownfield land has been explicitly stated by Dickinson *et al.* (2005).

It has been suggested that the characteristics of contaminated soils can never resemble that of a native non-polluted soil and this makes the assessment of soil functioning with regard to contaminated land difficult (Hartley *et al.*, 2008). Studies have used diverse microbiological indictors for determining soil functional capacity, however, there appears to be no prior assessment of microbial functioning in the polluted soil in question e.g. Hartley *et al.* (2008). Thus, the idea of assessing the impacts of soil contamination upon microbial populations is not new. Though there is little evidence in the literature of taking this extremely important factor into account when assessing if a remediation strategy may be viable. The assessment of suitable microbiological indicators for use in remediation in the UK has been only assessed by Hartley *et al.* (2008) and Dawson *et al.* (2007). It must be highlighted that the aforementioned studies did not exclusively test microbiological parameters; plant and earthworm assays were also included. Therefore, these aforementioned studies assessed soil biology as a whole, where this thesis only strives to look at the microbial aspect.

Hartley *et al.* (2008) assessed sites currently under phytoremediation, comparing them with non-contaminated urban soils. Within the study of Hartley *et al.* (2008) the microbiological parameters of respiration, microbial carbon, microbial activity by Adenosine 5-Triphosphate (ATP) and overall bacterial populations by MPN were assessed along with bacterial, nitrifier and fungal diversity by DNA presence and PCR solely. However, this form of assessment cannot actually deduce changes in soil functioning, diversity or community composition with remediation as no analysis was carried out from the native contaminated soil through the application of the remediation technology and thereafter. Consequently Hartley *et al.* (2008) found that the microbial parameters tested, provided relatively insensitive discrimination between contaminated and non-contaminated soils.

In a separate study by Dawson *et al.* (2007), biological indicators were assessed for hydrocarbon impacted soils undergoing various bioremediation treatments. Unlike Hartley *et al.* (2008), Dawson *et al.* (2007) reported that the microbiological parameters of microbial carbon, respiration and activity measurements via dehydrogenase activity were successful at differentiating the extent of soil remediation. Consequently, they reported that the inclusion of microbial indicators gave a clearer representation of ecological functioning than chemical data alone.

However, the study by Dawson *et al.* (2007) only examined hydrocarbon contaminated soils and the effectiveness of the indicators employed was not determined for the remediation of metal or mixed polluted sites. Furthermore Dawson *et al.* (2007) concentrated upon using microbial indicators to assess the effectiveness of remediation and did not explicitly examine if a remediation strategy itself had any effect on soil microbial function, community composition or diversity. The assessment of soil biology as a whole (i.e. including plant and animal indicators) after and due to remediation by specific technologies has gained more attention over recent year's e.g. Cébron *et al.* (2011); Hernández-Allica *et al.* (2006); Jones *et al.* (2012). Although, a set of robust indicators that assess soil microbial populations and their functioning in contaminated soils is still elusive.

1.5. Manganese oxide geochemistry

1.5.1. *Occurrence of manganese and its oxides in soils*

Manganese (Mn) is naturally abundant and is present in varying background levels within the soil environment. It is the second most common heavy metal in the Earth's crust after iron (Fe), occurring at levels of around 0.1 % in the lithosphere (Tebo *et al.*, 2004). Due to the weathering of igneous and metamorphic crustal rock via pedogenesis, Mn has become a natural and ubiquitous component of soils (Post, 1999; Tebo *et al.*, 2004). World soils are found to have a wide range of Mn, with levels between 80-1300 mg kg^{-1} (McBride, 1994). Within the UK, Mn is present at mean levels of 612, 502 and 713 mg kg^{-1} in rural, urban and industrial soils respectively (Barraclough, 2007). As Mn is easily oxidised it gives rise to more than 30 Mn oxides, abbreviated as MnOx within this study. The term MnOx is used to collectively include oxide, hydroxide and oxyhydroxide minerals, all of which are commonly found in terrestrial environments (Post, 1999).

MnOx occur in most soils as discrete particles and also as coatings on other soil and mineral grains (Post, 1999; Sparks, 2003). While Mn exists in a broad range of oxidation states, from −3 (e.g. $[Mn(NO)_3(CO)]^{3-}$) to +7 (e.g. permanganate), there are only three possible oxidation states in the soil environment: II, III and IV (McBride, 1994; Moens *et al.*, 2010). Several MnOx minerals have been identified in soils that range from manganous oxide (MnO) to manganese dioxide (MnO_2) (White, 1997). The

most stable form of MnOx is pyrolusite (β-MnO_2) although it is uncommon in soil (Sparks, 2003). The major MnOx minerals reported in soil are lithiophorite ($LiAl_2(Mn_2^{4+}Mn^{3+})O_6(OH)_6$), hollandite ($Ba_x(Mn^{4+},Mn^{3+})_8O_{16}$) and birnessite ($(Na,Ca)Mn_7O_{14}\cdot 2.8H_2O$) which are amorphous (i.e. poorly crystalline). Of these three minerals it is the birnessite group ([Na,Ca,Mn(II)] $Mn_7O_{14}\cdot 2.8H_2O$) that is the most common MnOx bearing, found in soils (Sparks, 2003).

1.5.2. *The importance of Mn and its oxides in soils*

Mn plays a critical role in aerobic life on earth as this element is an essential micronutrient for most organisms (Brouwers *et al.*, 2000b; Moens *et al.*, 2010; Tebo *et al.*, 2007). It is vital to the oxygen evolving complex in photosystem II, where it is present as a Mn_4O_xCa complex, which is responsible for the oxidation of water to oxygen (Najafpour and Govindjee, 2011; Philouze *et al.*, 1994). Extraordinarily, the Mn_4O_xCa complex is thus far nature's only solution to efficient water oxidation (Moens *et al.*, 2010). Therefore, it is fundamental to the production of molecular oxygen and is of great importance for carbon fixation by plants and other photosynthetic microorganisms (Moens *et al.*, 2010; Tebo *et al.*, 2007).

Additionally, Mn is a cofactor for numerous enzymes such as superoxide, dismutase, catalase and Mn dependent ribonucleotide reductase (Christianson, 1997; Tebo *et al.*, 2007). The importance of Mn in enzymatic processes is well exemplified by superoxide dismutase (SOD) in which it is one of the important metal cofactors. Mn-SOD evolved specifically to protect cells against oxidative damage and regulate cellular concentrations of superoxide (O_2^-) which is an extremely powerful oxidant and an unwanted by-product of cellular metabolism, that is highly toxic to cells (Christianson, 1997).

Furthermore, MnOx have been shown to oxidatively lyse large refractory pools of organic matter i.e. humic and fulvic acids, that cannot be enzymatically attacked by microbial populations (Sunda and Kieber, 1994). This oxidation produces a suite of low molecular weight (LMW) compounds that can be utilised for microbial growth (Sunda and Kieber, 1994). On the other hand, MnOx have also been identified as stimulators of humification in soils. MnOx have been shown to promote two humic substance forming reactions: 1) the Maillard reaction which involves condensation reactions between sugars and amino acids; 2) the polyphenol model of whereby polyphenols

and amino acids are transformed abiotically to nitrogen containing polycondensates (Jokic *et al.*, 2001; Jokic *et al.*, 2004; Shindo and Huang, 1982). However, at present it is still unclear as to why MnOx at times either oxidise or humify organic substances. Thus, MnOx could potentially enhance carbon sequestration, soil fertility, stabilisation of aggregates, restoration/maintenance or improvement of organic matter content in soils along with the ability to promote the sorption of contaminants (Lal, 2007; Peña-Méndez *et al.*, 2005).

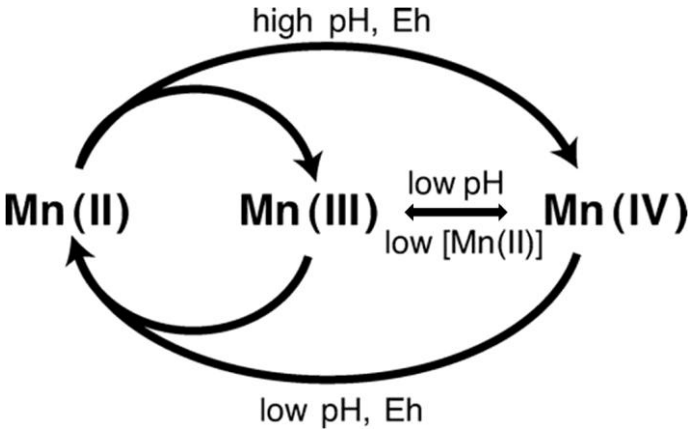
Owing to their redox properties (see section 1.5.5) MnOx are one of the most reactive components of soils playing a major role in the biogeochemical cycling of iron, nitrogen, carbon, sulphur and several nutrients (Tebo *et al.*, 2005). Furthermore MnOx have high sorption capacities for many heavy metal cations such as Ni, Zn, Cu, Co, Mn, Pb and Cd (Miyata *et al.*, 2007; Tebo *et al.*, 2004; Tebo *et al.*, 2005), and are natural oxidants of reduced metals and metalloids such as As(III) and Se(IV) (Sparks, 2003). Thus, the combined high adsorption capacities and scavenging capabilities of MnOx means that they are one of the primary controls of heavy metals and other trace elements in soils (Post, 1999). Not only are MnOx important in the sorption and oxidation of metals, they are also known to be strong oxidants of organic compounds e.g. hydroquinone, phenols and PAHs (Clarke *et al.*, 2012; Kang *et al.*, 2008; Stone, 1987b; Stone and Morgan, 1984a; Stone and Morgan, 1984b) which are discussed in more detail later.

Even though MnOx are only present in small total amounts in soils they exert a chemical influences far out of proportion to their relative abundance due the large surface areas , small particle size and the redox chemistry they exhibit (Bartlett, 1999; Post, 1999). It is the aforementioned affinity that MnOx possess for environmental pollutants, which will be described in more detail within this chapter, which is of upmost importance to this research.

1.5.3. *General chemistry of Mn oxides in soils*

Mn oxidation state in soil is controlled by redox potential and pH (see Figure 1-5). Of the three possible oxidation states that Mn can have when present in soils (II, III and IV), the occurrence of Mn(II) in the environment is thermodynamically favoured in the absence of oxygen at low pH, whereas Mn(III) and Mn(IV) are favoured under aerobic conditions at high pH (Tebo *et al.*, 2004).

a)



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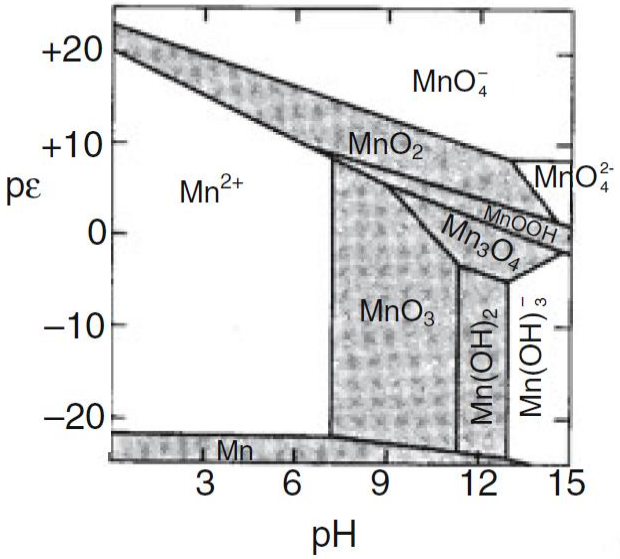


Figure 1-5: (a) The Mn cycle of oxidation states found in nature, diagram adapted from Tebo *et al.* (2004). (b) pε-pH diagram for the Mn-CO_2 system at 25 °C showing which species predominate under any given condition of pε or pH, diagram taken from Nealson (2002) which is based on that of Stumm and Morgan (1996); solid phases considered: $Mn(OH)_{2(s)}$ (pyrochroite), $MnCO_{3(s)}$ (rhodochrosite), $Mn_3O_{4(s)}$ (hausmannite), γ-MnOOH (manganite) and γ-MnO_2 (nsutite).

Mn(II) is the only stable oxidation state that exists in solution i.e. soil pore waters (McBride, 1994; Tebo *et al.*, 2004). In general Mn(II) occurs as a cation (Mn^{2+}) and is the most reduced form of soil Mn that exists (McBride, 1994; Spiro *et al.*, 2010). Below pH 6, Mn(II) is highly soluble, but above pH 6, solubility reduces and immobilisation occurs by interaction with soil organic matter, oxides and silicates

(McBride, 1994). However, at pH above neutrality precipitation can occur as insoluble phosphates, carbonates and hydroxides or as a minor constituent of other minerals (McBride, 1994; Tebo *et al.*, 2004).

In contrast to Mn(II), Mn(III) is a powerful oxidant that is thermodynamically unstable. Mn(III) tends to disproportionate to Mn(II) and Mn(IV) complexes, or oxidise water to liberate O_2 (McBride, 1994; Tebo *et al.*, 2004). It can be stabilised for a short period in solution via complexation with pyrophosphate, oxalate or sulphate (McBride, 1994). However, it is only truly stable in solid phase where it, along with Mn(IV), form various oxide minerals with a diverse range of structures (McBride, 1994).

It is these insoluble oxides that are regarded as the most important abiotic redox-active minerals in soils, with their effectiveness as electron acceptors having been described as unique among common soil minerals (McBride, 1989; Risser and Bailey, 1992; Sunda and Kieber, 1994).

1.5.4. *General redox properties of Mn oxides in soils*

MnOx, more specifically Mn(III)/Mn(II) and Mn(IV)/Mn(II) complexes have high redox potentials seen in Table 1-8, which clearly shows that they are one of the strongest oxidants in the soil environment. Reduction potentials reported in Table 1-8 are of standard states and thus in the real environment they would be subjected to differences in pH and Eh.

Table 1-8: Standard-state reduction potentials of half reactions that involving important elements in soils. Table taken from McBride (1994). Half reactions involving Mn oxides are highlighted in bold.

Reaction	E^o (volts)
$Mn^{3+} + e^- \leftrightarrow Mn^{2+}$	**1.51**
$MnOOH_{(s)} + 3H^+ + e^- \leftrightarrow Mn^{2+} + 2H_2O$	**1.45**
⅕ NO_3^- + 6/5 H^+ + e^- ↔ 1/10 $N_{2(g)}$ + 3/5 H_2O	1.245
½ $MnO_{2(s)}$ + 2 H^+ + e^- ↔ ½ Mn^{2+} + H_2O	**1.23**
1/4 $O_{2(g)}$ + H^+ + e^- ↔ ½ H_2O	1.229
$Fe(OH)_{3(s)}$ + 3H^+ + e^- ↔ Fe^{2+} + 3H_2O	1.057
½ NO_3^- + H^+ + e^- ↔ ½ NO_2^- +1/2 H_2O	0.834
Fe^{3+} e^- ↔ Fe^{2+}	0.711
½ $O_2^-{}_{(g)}$ + H^+ + e^- ↔ ½ H_2O_2	0.682
1/8 SO_4^{2-} + 5/4 H^+ + e^- ↔ 1/8H_2S + 3/5 H_2O	0.303
1/6 $N_{2(g)}$ + 4/3 H^+ + e^- ↔ 1/3 NH_4	0.274
1/8 $CO_{2(g)}$ + H^+ + e^-↔ 1/8 $CH_{4(g)}$ + ¼ H_2O	0.169
H^+ + e^- ↔ ½ $H_{2(g)}$	0.000

The oxidation of soluble Mn(II) to insoluble Mn(III)/(IV) oxides is one of the most important redox couples in soil (McBride, 1994). In general this reaction is only significant under aerobic conditions in highly alkaline soils where the pH is above 8, with a negative free energy of ~16kcal/mol (McBride, 1994; Nealson, 2002; Stumm and Morgan, 1996). If the aforementioned conditions are found in soil then the abiotic oxidation of Mn(II) to the insoluble Mn(III) and (IV) oxides will proceed via Equation 1-4 (McBride, 1994).

$$Mn^{2+}(\text{soluble}) \xrightarrow{OH^-} Mn(OH)_{2(s)} \xrightarrow{O_2} \begin{matrix} Mn_3O_{2(s)} \\ MnOOH_{(s)} \\ MnO_{2(s)} \end{matrix}$$

Equation 1-4: Abiotic oxidation of Mn(II) to Mn(III)/(IV) oxides.

However, often in the environment the formation of MnOx occurs at a pH below 8, which cannot be explained by the aforementioned process. If the pH is below 8 then the occurrence of formation of MnOx from Mn(II) is explained by the abiotic autocatalytic process of autooxidation (McBride, 1994). The first step in this process involves the oxidation of Mn(II) (Equation 1-5), this is then followed by selective adsorption of Mn(II) onto the freshly formed MnOx (Equation 1-6), following this the adsorbed Mn(II) is oxidised relatively quickly (Equation 1-7) (McBride, 1994).

$$Mn^{2+} + {}^{1}/_{2}\ O_2 \xrightarrow[slow]{} MnO_{2(s)}$$

Equation 1-5: Step 1 in the autocatalytic oxidation of Mn(II) to Mn(IV).

$$Mn^{2} + MnO_{2(s)} \xrightarrow[fast]{} Mn^{2+} \cdot MnO_{2(s)}$$

Equation 1-6: Step 2 in the autocatalytic oxidation of Mn(II) to Mn(IV).

$$Mn^{2+} \cdot MnO_{2(s)} + {}^{1}/_{2}\ O_2 \xrightarrow[fast]{} 2MnO_{2(s)}$$

Equation 1-7: Step 3 in the autocatalytic oxidation of Mn(II) to Mn(IV).

As highlighted in Equation 1-5, the initial oxidation of Mn(II) via chemical oxidation is slow, but thermodynamically favourable. It is worth noting that Mn(II) oxidation can occur in two main forms either by chemical means which is the mechanism described in this section, or via biological means which is described in section 1.5.7.

Estimates of the residence time of Mn(II) before its chemical oxidation have been widely different, ranging from hundreds of years, to several decades and months to weeks (Emerson *et al.*, 1982). However, an accurate estimation of this process has been difficult to determine due to the experimental time period required (Emerson *et al.*, 1982). The large activation energy required for Mn(II) oxidation means that is very

stable in the environment and is often found at high concentrations even in the presence of oxygen (Tebo *et al.*, 2004).

In contrast the chemical reduction of MnOx is mainly controlled by reductive dissolution under anoxic environments whereby oxidised Mn can be reduced by its oxidation of organic or inorganic compounds. This process can have a major affect upon the solubility and mobility of Mn (Sparks, 2003). As MnOx are envisaged to be active in these reactions within contaminated soils, it is these processes of MnOx reduction which are concentrated upon.

1.5.5. *Redox reactions of inorganic and organic pollutants with Mn oxides in soils*

As previously mentioned, MnOx are well known to be capable of the oxidation of inorganic compounds within soil due to their capacity to accept electrons**.** Out of all the metal oxides/hydroxides that exist in soil, MnOx are the strongest oxidants (Sparks, 2003). MnOx can oxidise Pu(III) to Pu(IV) (Crespo *et al.*, 1992), V(III) to V(V) (Bartlett and James, 1993), As(III) to As(V) (Scott and Morgan, 1995), Se(IV) to Se(VI) (Scott and Morgan, 1996), Cr(III) to Cr(VI) (Eary and Rai, 1987), Co(II) to Co(III) (Murray and Dillard, 1979) and synthetic chemicals such as endocrine disrupters (Daniel Sheng *et al.*, 2009). These reactions can occur as the potential of the oxidant half reaction is higher than the reductant half reaction, thus, the overall oxidation is thermodynamically favoured as shown in Figure 1-6.

It must also be highlighted that while the oxidation of harmful inorganic compounds is environmentally beneficial, these processes may also produce more harmful end products, specifically the oxidation of Cr(III) to Cr(VI). Upon oxidation insoluble Cr(III) forms Cr(VI) which is soluble and has been identified as a human carcinogen (Sedman *et al.*, 2006). While this process has been proven to occur in the laboratory, recent studies also confirm that it is an active process in the environment (Fandeur *et al.*, 2009; Ndung'u *et al.*, 2010).

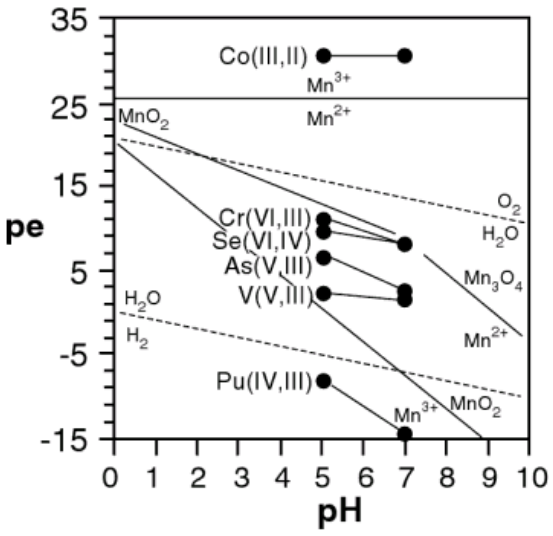


Figure 1-6: A pe-pH diagram for Mn^{3+}, MnO_2 and Mn_3O_4; as compared with reduction between pH 5 and 7 for Co, Cr, Se, As, V and Pu. Activity for ionic species is 10^{-4} M. Taken from Sparks (2003).

Additionally, MnOx can oxidise a range of organic compounds which include the microbial metabolites of oxalate and pyruvate, humic substances, phenols, polyhydroxy compounds, amines, hydrazines, hydrocarbons, heterocyclic compounds, catechols, hydroquinones, methoxyphenols and resorcinols (Clarke *et al.*, 2012; Fatiadi, 1976; Fatiadi, 1986; Stone, 1987b; Stone and Morgan, 1984a; Stone and Morgan, 1984b). Furthermore, MnOx are well known to be capable of oxidising persistent organic pollutants such as PCBs (Pizzigallo *et al.*, 2004), tricolsan (Zhang and Huang, 2003), antibiotics (Chen and Huang, 2011), chlorinated amines (Pizzigallo *et al.*, 1998) and pesticides such as atrazine (Cheney *et al.*, 1998).

The oxidation of inorganic and organic compounds, or indeed, the reduction of MnOx by these compounds, is termed reductive dissolution which occurs at the oxide mineral surface. The mechanisms for this process by MnOx have been studied in detail (Stone, 1987a; Stone and Morgan, 1984a; Stone and Morgan, 1984b) with the following steps determined:

1. Diffusion of the reductant molecules to the MnOx surface.
2. The formation of a surface complex between the reductant and MnOx surface.
3. Surface chemical reaction via inner sphere and precursor complex formation, electron transfer or breakdown of successor complex.

4. Desorption of the oxidised reductant.
5. Movement of Mn(II) from crystal lattice to the adsorbed layer.
6. Desorption of Mn(II) followed by autocatalytic oxidation of Mn(II) to Mn(IV).
7. Release of reaction products and diffusion away from the MnOx surface.

While MnOx are capable of all the aforementioned oxidation reactions, it must be highlighted that these have been proven using synthetic MnOx which have different properties to naturally occurring MnOx. Therefore, the use of naturally occurring MnOx to carry out these oxidation reactions in an inorganic or organic contaminated soil is a pertinent avenue of research.

1.5.6. *Sorption of metal cations to Mn oxides*

In addition to oxidation of inorganic and organic pollutants, MnOx also possess the ability for the sorption of a wide variety of toxic metal ions. Evidence for this was first established from sequential extraction data whereby substantial amounts of metals including Cu, Co, Cd, Zn, Ni, Sn, Pb, Ca, Fe, Ra, Hg, U, Pu, Po, As, Se and Th, were found to be held within the Fe/Mn oxide component of soils [data collated from a number of sources by Tebo *et al.* (2004)].

In general the sorption of heavy metal cations at mineral interfaces is largely controlled by the type and number of sorption sites, i.e. vacant sites, on the MnOx surface in question (Wang *et al.*, 2012). Due to their positive charge deficiency, vacant sites are the most important for heavy metal cation adsorption (Zhu *et al.*, 2010). Ordinarily MnOx consist of MnO_6 octahedron units arranged into tunnel or layer structures with interlayers or tunnels hosting water molecules and/or cations (Post, 1999). Both types of MnOx structure harbour vacant sites, however, it is the layer structured MnOx such as birnessite which dominate in the soil environment (Post, 1999).

Birnessite consists of stacked layers of edge-sharing MnO_6 octahedra separated by a monolayer of water molecules (Wang *et al*., 2012). It has a hexagonal symmetry layer structure that possesses a variable number of octahedral cationic Mn(IV) vacancies within each layer (Wang *et al.*, 2012). The number of layers within the mineral structure of birnessite is low and varies (Wang *et al.*, 2012). This along with the random staked fashion of the particles within this MnOx, results in vacancy sites

occurring within the interlayer regions (Wang *et al.*, 2012). In general, sorption of metal cations to MnOx such as birnessite follows three main mechanisms of (Miyata *et al.*, 2007; Wang *et al.*, 2012):

1. Complexation above and below structural vacant sites at interlayer regions.
2. Complexation at the MnOx structural sheet edge.
3. Incorporation into the vacant sites resulting in an isomorphical substitution for Mn(IV).

The aforementioned complexation of metals on MnOx surfaces occurs via four different means which are listed below and can be visualised in Figure 1-7 (Wang *et al.*, 2012):

1. Triple-corner-sharing (TCS) complexes above and below octahedral vacancy sites.
2. Triple-edge-sharing (TES) complexes above and below octahedral vacancy sites.
3. Double-corner-sharing (DCS) complexes at the mineral lateral sheet edges.
4. Double-edge-sharing (DES) complexes at the mineral lateral sheet edges.

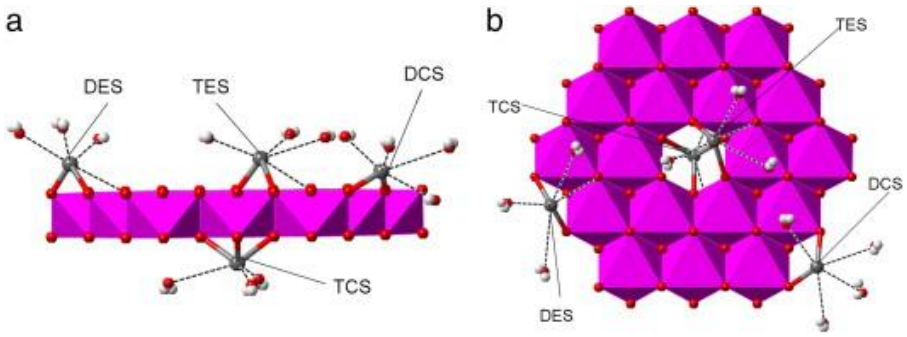


Figure 1-7: An example of Mn oxide surface complexation mechanisms showing Pb^{2+} sorption to birnessite. (a) Different complexation positions at birnessite particle edges (surface sites). (b) View of TES, DCS and DES perpendicular to the a–b plane, with TCS below the vacancy sites. Dashed lines show coordination with H_2O or latter oxygen. Colour scheme: red = O, gray = Pb, white = H and pink = Mn. Taken from Wang *et al.* (2012).

1.5.7. *Geomicrobiology of Mn(II) oxidation*

Owing to the high activation energy and pH required for Mn(II) oxidation it is thought that in the environment, the existence of MnOx is chiefly due to microbial not chemical processes. Consequently, most MnOx in natural environments are thought to be biological in origin (Tebo *et al.*, 2004). As previously mentioned in section 1.5.4, the chemical oxidation of Mn(II) in the normal pH range of soils in the environment is slow. While the oxidation of Mn(II) can also occur via other chemical means i.e. oxidation via other mineral surfaces such as Fe oxides (Davies and Morgan, 1989). It is the biological oxidation of Mn(II) by bacteria which is thought to be most significant (Tebo *et al.*, 2004). Mn(II) oxidation has been proven to occur via a diverse range of bacteria (Tebo *et al.*, 2004; Tebo *et al.*, 2005) and fungi (Cahyani *et al.*, 2009; Miyata *et al.*, 2006; Santelli *et al.*, 2011; Takano *et al.*, 2006) but has never been demonstrated by the archaea (Northup *et al.*, 2003; Tebo *et al.*, 2005).

The involvement of microbes in Mn(II) oxidation was first noted by Beijernick (1913). MnOx produced via these agents are termed 'biogenic' or 'biooxides'. Investigations into biological Mn(II) oxidation have found that it proceeds at rates up to five orders of magnitude faster than abiotic oxidation (Tebo *et al.*, 2005). This theory has been proved in a wide range of environments such as hydrothermal plumes (Cowen *et al.*, 1986; Mandernack and Tebo, 1993), fjords (Tebo *et al.*, 1984), lakes (Tipping, 1984), metal contaminated streams (Fuller and Harvey, 2000) and marine environments (Tebo, 1991; Tebo and Emerson, 1986; Van Cappellen *et al.*, 1998). Furthermore, biological Mn(II) oxidation has also being theorised as the chief explanation for MnOx occurrence in soils (Douka, 1977; Mann and Quastel, 1946; Nealson *et al.*, 1988; Sullivan and Koppi, 1992; van Veen, 1973). At present very little is known about this process in the soil environment which constitutes an interesting avenue for research (He *et al.*, 2008; Nealson *et al.*, 1988). Please note that bacterial oxidation of Mn(II) has been concentrated upon in this work and is reviewed below.

The phylogenetic diversity of bacteria capable of Mn(II) oxidation includes the alpha (α), beta (β) and gamma (γ) *Proteobacteria*; *Actinobacteria* and *Firmicutes*; which can be seen in Figure 1-8 (Tebo *et al.*, 2004; Tebo *et al.*, 2005).

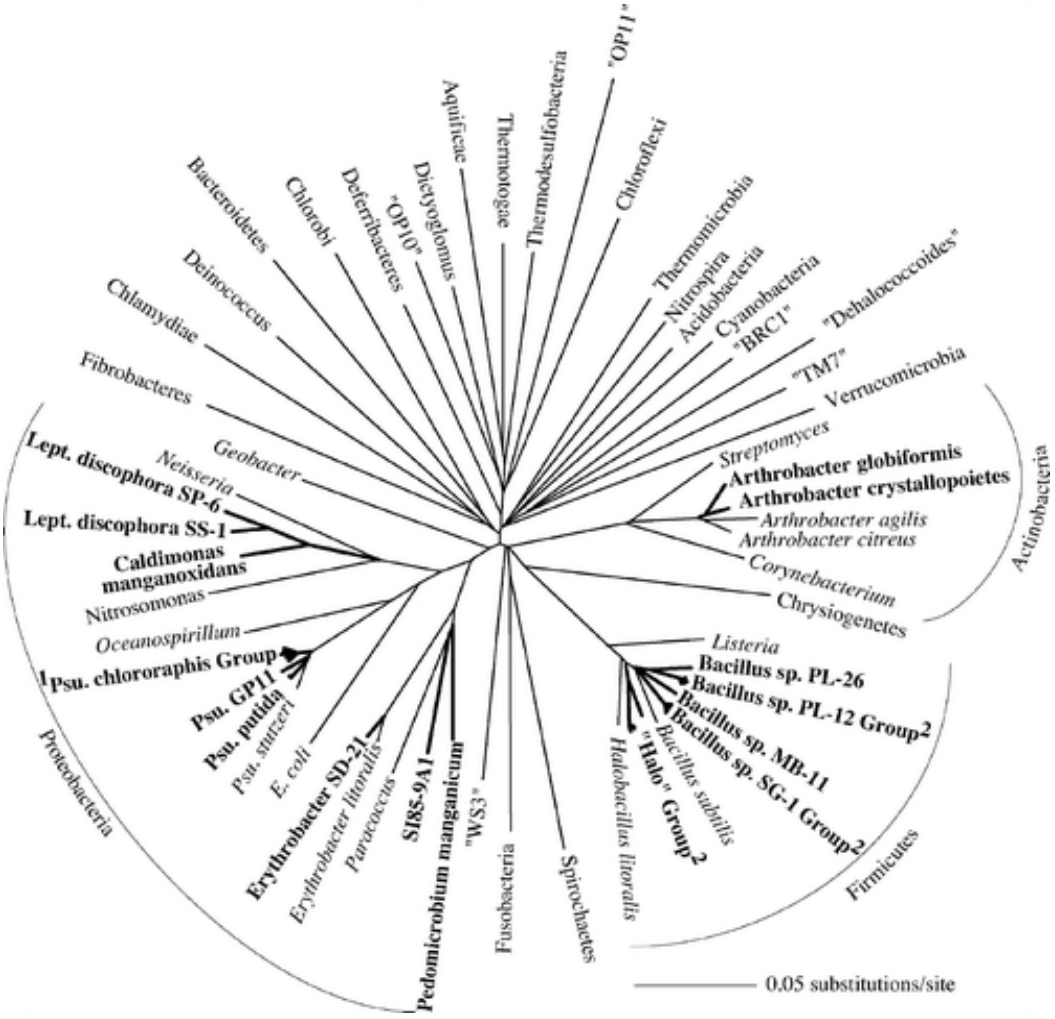


Figure 1-8: Location of Mn(II)-oxidising bacteria on a neighbour-joining, unrooted phylogenetic tree of the domain 'Bacteria'. Mn(II)-oxidising bacterial strains for which sequences appear in the RDP are shown in bold. Sequence similarity is only an estimate of evolutionary relationships and is expressed here as the distance between any two branch tips; sequences that appear next to each other are not necessarily close relatives. Phyla in quotes are considered taxa of uncertain affiliation and have few, if any, cultivated members. Abbreviations: *Lept., Leptothrix; Psu., Pseudomonas*. [1]The *Psu. chlororaphis* group includes the Mn(II)-oxidising strains ISO1, ISO6, GB-13, MG1 and PCP, but not all similarly close relatives of *Psu. chlororaphis* are known to oxidise Mn(II). [2]*Bacillus* species groups are labelled according to Francis and Tebo (2002). Taken from Tebo *et al.* (2004).

Even though this trait is common among bacteria that are ubiquitous across environments, the reason as to why they perform this physiological function remains elusive. Theories for the possession of this characteristic have been varied and thus far remain unproven. The most common explanations include the derivation of energy for growth, protection from harmful environmental situations such as UV radiation and heavy metal toxicity, or, the requirement of Mn(II) for intracellular reactive oxygen species ROS scavenging (Tebo *et al.*, 2005). An interesting possibility exists from the fact that MnOx are known to be capable of oxidising high molecular weight (HMW) humic substances into low molecular weight (LMW) compounds. If Mn(II)-oxidising bacteria are shown to use these LMW organic compounds for microbial growth then this trait would have a distinct evolutionary advantage (Sunda and Kieber, 1994; Tebo *et al.*, 2005). However, a common role for Mn(II) oxidation is still indefinable (Johnson and Tebo, 2008). It must be highlighted that while the oxidation of Mn(II) to Mn(III/IV) oxides is energetically favourable for bacteria, and it has been proposed that bacteria may drive energy for chemolithoautotrphic growth by this process, there is no proof that directly links Mn(II) oxidation to bacterial growth (Tebo *et al.*, 2004; Tebo *et al.*, 2005).

It is known that bacterial manganese oxidation occurs via the following reaction in the presence of oxygen (Tebo *et al.*, 2010):

$$Mn^2 + \frac{1}{2}O_2 + H_2O \leftrightarrow MnO_2 + 2H^+$$

Equation 1-8: Reaction for biological oxidation of Mn(II) to Mn(IV) oxides.

Of this diverse range of bacteria there are currently six well characterised Mn(II)-oxidisers which are often referred to as the 'model' organisms for this trait. These model organisms are outlined below with images of biogenic MnOx deposition where available shown in Figure 1-9:

1) ***Bacillus* sp. Strain SG-1:** is a Gram-negative marine bacterium isolated from near shore manganese rich sediment. This bacterium oxidises Mn(II) as metabolically dormant spores and not as growing or vegetative cells. Mn(II)

oxidation occurs on the outermost layer of the spores, the exosporium (De Vrind *et al.*, 1986; Tebo *et al.*, 2004).

2) ***Pseudomonas putida* strains MnB1 and GB-1:** are Gram-negative fresh water γ-*Proteobacteria* isolated from a MnOx encrusted drinking water pipeline and from the sediment of Green bay, Wisconsin; USA respectively. Biogenic MnOx accumulation begins in stationary phase on the outer surface of the cell, i.e. the extracellular organic matrix also known as the glycocalyx (Caspi *et al.*, 1998; Okazaki *et al.*, 1997; Tebo *et al.*, 2005).
3) ***Lepthothrix discophora* SS-1:** is a freshwater sheath forming β-*Proteobacterium* first isolated from a swamp. Biogenic MnOx formed by this bacterium are precipitated extracellulary around sheaths in stationary phase growth (Ghiorse, 1984; Ghiorse and Chapnick, 1983).
4) ***Pedomicrobium* sp. ACM 3067:** is a Gram-negative aquatic α-*Proteobacterium*, isolated from a water distribution system containing MnOx encrusted biofilms. These budding-hyphal bacteria accumulate biogenic MnOx in early to mid-exponential phase, on their extracellular polysaccharides upon the cell surface (Ridge *et al.*, 2007; Sly and Arunpairojana, 1987; Sly *et al.*, 1990a).
5) ***Aurantimonas manganoxydans* SI85-9A1:** are Gram-negative α-*Proteobacteria* isolated from a stratified Canadian fjord. It precipitates biogenic Mn(IV) oxides on its cell surface in exponential phase of growth (Anderson *et al.*, 2009a; Dick *et al.*, 2008).
6) ***Erythrobacter* sp. SD-21:** is a marine Gram-negative α-*Proteobacterium* isolated from surface sediments of San Diego Bay, USA. Mn(II) oxidation begins in logarithmic phase. In contrast to the other model Mn(II)-oxidisers the biogenic MnOx produced by this strain is soluble, developing as a halo in agar media. Later development of dark brown precipitates over colony surfaces has suggests that the initial oxidation product is a Mn(III) complex that undergoes abiotic disproportionation to Mn(II) and Mn(IV) (Francis *et al.*, 2001; Johnson and Tebo, 2008).

All of these model Mn(II)-oxidisers are from aquatic environments, and to date, no model Mn(II)-oxidisers have been described from the soil environment.

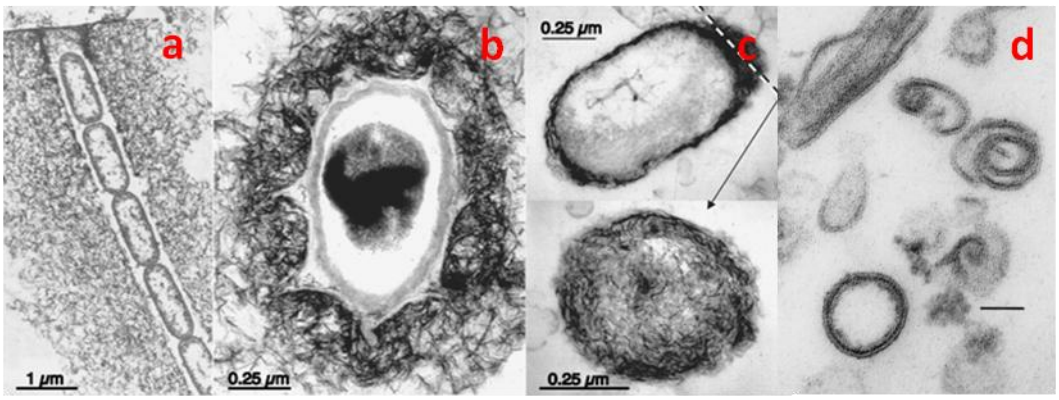


Figure 1-9: Transmission electron micrographs (TEMs) of the four Mn(II)-oxidising bacteria that have been studied in most detail. (a) Unidentified *Leptothrix* sp., (b) spores of the marine *Bacillus* sp. strain SG-1, (c) *Pseudomonas putida* strain MnB1,(d) thin sections of active Mn(II) oxidising membrane vesicles of *Pedomicrobium* sp. ACM 3067 (bar = 50 nm). TEMs shown in (a), (b) and (c) are taken from Tebo et al. (2004) while (d) is taken from Larsen et al. (1999).

As previously mentioned a universal pathway and mechanism by which bacteria oxidise Mn(II) remains ambiguous. However, it is known that the bacterial oxidation of Mn(II) occurs by both indirect and direct mechanisms (Tebo *et al.*, 2004). In general bacterial Mn(II) oxidation is thought of as a direct process attributed to enzymatic oxidation by putative Mn(II) oxidases, although to date no such oxidases have been purified or found to be over-expressed in active Mn(II)-oxidising environments (Tebo *et al.*, 2004; Tebo *et al.*, 2005). A large body of evidence over the years carried out by the Tebo group (Tebo *et al.*, 2004; Tebo *et al.*, 2010; Tebo *et al.*, 2005), has elucidated that Mn(II) oxidation is an enzymatic process which proceeds via two sequential one electron reactions. Firstly the oxidation of Mn(II) produces a Mn(III) intermediate, and secondly, Mn(III) is oxidised to Mn(IV) as shown in Figure 1-10 (Luther Iii, 2005; Tebo *et al.*, 2010; Webb *et al.*, 2005).

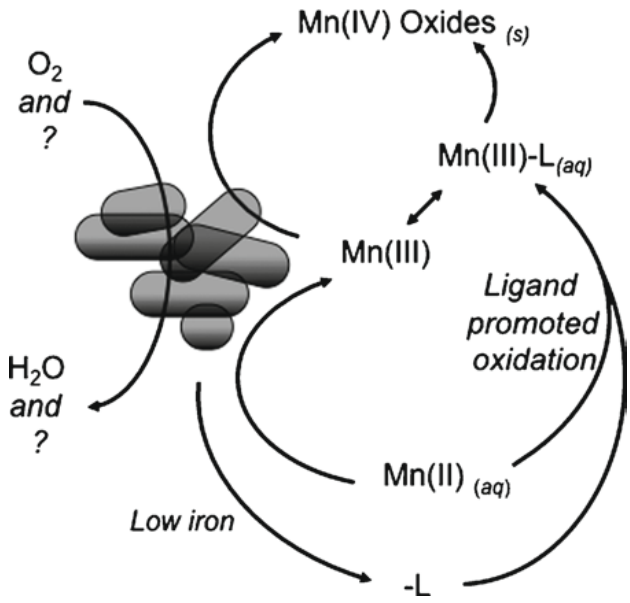


Figure 1-10: The current view of bacterial Mn(II) oxidation whereby enzymatic Mn(II) oxidation proceeds via two one-electron steps. In the presence of complexing organic or inorganic ligands (L) (e.g.,pyrophosphate or a siderophore), the Mn(III) intermediate can form a soluble Mn(III) complex (Mn(III)–L) which can be either stable in solution or undergo oxidation or disproportionation to Mn(IV) oxides. Under low iron conditions, some Mn(II)-oxidising bacteria and many non-Mn(II)-oxidising bacteria may produce organic ligands that can abiotically promote the oxidation of Mn(II) to Mn(III)-L. Taken from Tebo *et al.* (2010).

Furthermore the group of Tebo, through extensive genetic and biochemical studies, have identified two definite enzymes: 1) a multi-copper oxidase (MCO) encoding the putative Mn(II) oxidase and 2) a heme peroxidise, both of which are directly involved in bacterial Mn(II) oxidation (Tebo *et al.*, 2010). MCOs are a diverse family of proteins that oxidise their substrates via the use of multiple copper ions (Francis *et al.*, 2001). MCOs catalyse the four-electron reduction of dioxygen to water with the oxidation of the substrate (Solomon *et al.*, 1996). The oxidation of the substrate occurs via one electron transfers (Solomon *et al.*, 1996). The aforementioned MCOs can have either high or low substrate specificity (Solomon *et al.*, 1996). For example the well characterised MCO laccase, found in fungi, is known to have a broad substrate specificity for metals and phenolic compounds (Johnson and Tebo, 2008). Furthermore, the literature shows that MCOs play supporting a role in metal oxidation, with fungal laccases shown to oxidise Mn(II) to Mn(III) and other MCOs exhibiting the

ability to oxidise Fe (II) (Höfer and Schlosser, 1999; Huston *et al.*, 2002; Tebo *et al.*, 2010).

Three of the aforementioned model Mn(II)-oxidising bacteria have been proven to oxidise Mn(II) via an MCO, with each containing a gene that encodes a protein homologous with MCOs (Tebo *et al.*, 2010). These are *Bacillus* sp. Strain SG-1 containing the MnxG gene (Dick *et al.*, 2008; Van Waasbergen *et al.*, 1996), *Pedomicrobium* sp. ACM3067 containing the MoxA gene (Larsen *et al.*, 1999; Ridge *et al.*, 2007) and *Leptothrix discophora* strain SS-1 containing the MofA gene (Corstjens *et al.*, 1997). Addition of azide which is a potent inhibitor of MCOs in *Bacillus* sp. Strain SG-1 and *Leptothrix discophora* strain SS-1 has resulted in no Mn(II)-oxidising ability in these strains (Boogerd and De Vrind, 1987; Rosson and Nealson, 1982). Additionally, the disruption of the MoxA gene in a *moxA* knockout mutant in *Pedomicrobium* sp. ACM3067 resulted in no oxidation of Mn(II) (Ridge *et al.*, 2007). The disruption of the MnxG or the addition of Cu(II) to the aforementioned model organisms has shown a stimulated Mn(II) oxidation (Brouwers *et al.*, 2000a; Larsen *et al.*, 1999; Van Waasbergen *et al.*, 1996). Thus, all evidence provides conclusive verification that MCO's play an integral role in bacterial Mn(II) oxidation.

Previously *Pseudomonas putida* strains MnB1 and GB-1 were also believed to oxidise Mn(II) via an MCO (Brouwers *et al.*, 1999). However, this has since been discounted (Geszvain and Tebo, 2010). At present the gene responsible for Mn(II) oxidation in *Pseudomonas putida* remains unknown, although, it is speculated that an MCO and a heme peroxidise may be involved (Tebo *et al.*, 2010). A possible scheme for Mn(II) oxidation in bacteria by MCOs has been proposed by Spiro *et al.* (2010) and is shown in Figure 1-11.

In 2009, the first report emerged of a second possible enzymatic pathway for bacterial Mn(II) oxidation by heme peroxides (Anderson *et al.*, 2009b). Both model Mn(II)-oxidising bacterial strains *Aurantimonas manganoxydans* SI85-9A1 *and Erythrobacter* sp. SD-21 exhibited proteins tentatively named MopA (manganese-oxidising peroxidase). While this research is still in its infancy, it is known that Mn(II) is oxidised to Mn(IV) via an Mn(III) intermediate, which is consistent with known manganese peroxidase activity in fungi (Anderson *et al.*, 2009b). The fact that *Aurantimonas manganoxydans* SI85-9A1 produce a Mn(IV) oxide while *Erythrobacter* sp. SD-21 appear to produce a Mn(III) oxide indicates that the enzymatic pathway is

not the same in both strains (Anderson *et al.*, 2009b; Johnson and Tebo, 2008). Thus, it would appear that *Aurantimonas manganoxydans* SI85-9A1 undergo two sequential one electron transfers while *Erythrobacter* sp. SD-21 only require a one step one electron transfer (Tebo *et al.*, 2010). Anderson *et al.* (2009b) hypothesised that this may be linked to the fact that MopA from *Aurantimonas manganoxydans* SI85-9A1 has two heme peroxidise domains, while *Erythrobacter* sp. SD-21 only contains one. However, the second electron transfer step in *Aurantimonas manganoxydans* SI85-9A1 remains indefinable.

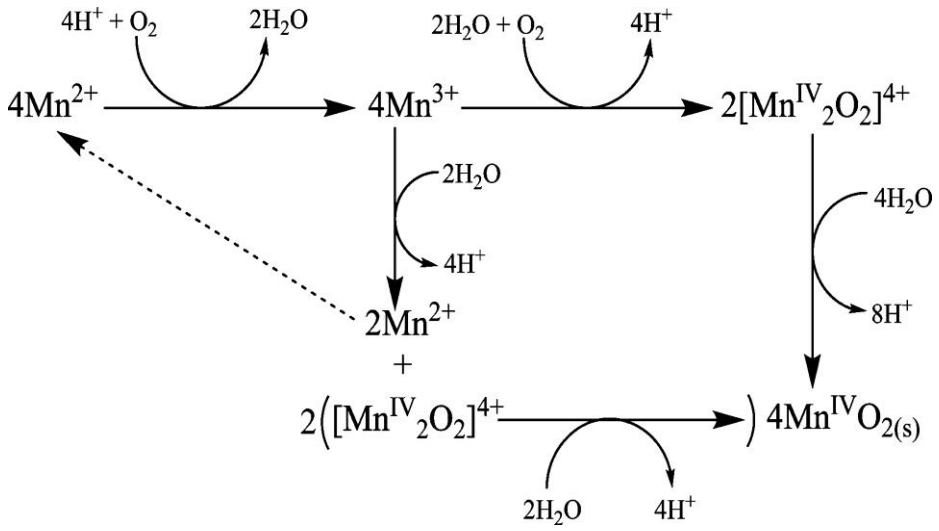


Figure 1-11: Proposed scheme for bacterial MnO_2 formation by multicopper oxidase-catalysed Mn^{2+} oxidation, followed by further oxidation (top) or by disproportionation of complexed Mn^{3+} (down arrow). Taken from Spiro *et al.* (2010).

In contrast to direct oxidation of Mn(II) by bacterial enzymes, it is important to highlight that indirect oxidation of Mn(II) can also occur by bacteria. Indirect mechanisms include the modification of redox environments which can occur via O_2 production, a pH increase due to CO_2 or acid consumption, or, ammonia release (Nealson, 2002). Recently Learman *et al.* (2011a) proved a new indirect pathway for Mn(II) oxidation to Mn(III, IV) oxides in the marine Mn(II)-oxidising bacterium *Roseobacter* sp. AzwK-3b. Mn(II) oxidation within this bacterium is now known to occur due to the bacterial enzymatic production of extracellular superoxide radicals. Thus providing evidence that Mn(II) oxidation may be an unintended secondary

reaction formed via metabolic processes unrelated to the metal (Learman *et al.*, 2011a).

The biogenic MnOx precipitated by the aforementioned 'model' bacteria are poorly crystalline and generally represent nanocrystallised H^+-birnessite or δ-MnO_2 (Miyata *et al.*, 2007). These freshly formed oxides are reportedly highly reactive and rapid scavengers of trace metal ions which will also oxidise Mn(II) (Tebo *et al.*, 2004). Furthermore, biogenic MnOx tend to exhibit much higher surface areas than their synthetic counterparts at 224 m^2g^{-1} and 58 m^2g^{-1} on average respectively (Miyata *et al.*, 2007). Sorption of toxic metal ions on biogenic MnOx proceeds through the same mechanisms as previously described in section 1.5.5 (Miyata *et al.*, 2007). Studies on biogenic MnOx have thus far centred upon metal sorption, from this previous research it is known that they have the ability for sorption of Pb(II), Zn(II) and Ni(II); with a higher affinity for Co(II), Cu(II) and U(VI) (Miyata *et al.*, 2007; Tebo *et al.*, 2004). More recently biogenic MnOx have been proven to be capable of the oxidation of pharmaceuticals such as diclofenac, ciprofloxacin and 17R-ethinylestradiol from waste-water (Daniel Sheng *et al.*, 2009; Forrez *et al.*, 2010; Zhang and Huang, 2005). This research suggests that Mn(II)-oxidisers and their biogenic MnOx precipitates might be a promising polishing technique for sewage treatment plant effluent. This has opened up a new avenue of research on environmental applications of Mn(II)-oxidising bacteria and their biogenic MnOx precipitates (Forrez *et al.*, 2011; Forrez *et al.*, 2010) Consequently the formation of biogenic MnOx presents an interesting avenue for research in contaminated soil settings.

1.5.8. *Toxic effects of Mn*

In general humans are exposed to Mn through food ingestion, drinking water, inhalation of Mn dust and ingestion of soil containing Mn compounds. It is ingestion via food which is the main route of human exposure to Mn (WHO, 1999). Mn is an essential nutrient for human health and the estimated safe and adequate daily intakes (1–5 mg) have been established based on the average amounts of Mn ingested through the human diet (WHO, 1999). However, oral ingestion of Mn minerals is a rare exposure route that is of low toxicity to humans (Dorman *et al.*, 2002; WHO, 1999). Above-average exposures to Mn have been reported to be most likely in people who work at or live near a site where significant amounts of Mn dust are released into the

air (WHO, 1999). In the event of chronic exposure, i.e. severe inhalation over 365 days or more, Mn can affect the lungs, nervous system and reproductive system. This has been reported to result in manganic pneumonia, while effects on the nervous system include neurological and neuropsychiatric symptoms that can culminate in a Parkinsonism-like disease known as manganism (Couper, 1837; WHO, 1999).

Current knowledge on inhibitory effects of Mn in the soil environment has centred upon toxicity to plant growth and soil invertebrates (Kuperman *et al.*, 2004; Paschke *et al.*, 2005). However, a review of the literature surrounding animal, invertebrate and plant toxicity is out of the scope of this study.

A toxic effect of Mn(II) to microorganisms has been also reported (Cabrera *et al.*, 2006). More recently Mn(IV) oxides have also been reported to inhibit PAH biodegradation in microbes grown under low oxygen conditions (Li *et al.*, 2011). These inhibitory effects are attributed to high concentrations of soluble Mn(II) derived from the rapid reduction of MnO_2 which is believed to hinder enzymatic activities (Li *et al.*, 2011). However, these studies were performed upon pure cultures with high concentrations of Mn(II) in solution and therefore are not reflective of interactions within the soil environment. Though, the release of water soluble Mn(II) is highly possible within the soil matrix through reduction of Mn(III)/Mn(IV) by manganese reducing bacteria or abiotic reduction reactions. The production of water soluble Mn(II) via chemical reduction generally occurs under conditions of low pH and Eh. However, release of soluble Mn(II) can also occur through other mechanism not related to reduction. Acidification can result in Mn(II) being released from MnOx through changes in their structure and chemistry (Banerjee and Nesbitt, 2001; Murray, 1974).

1.5.9. *The use of Mn oxides in contaminated soil remediation*

The structural properties that MnOx possess for sorption and sequestration of metals coupled to organic contaminant oxidation have lead them to be touted within the literature as a possible effective remediant for contaminated environments (Post, 1999; Spiro *et al.*, 2010; Tebo *et al.*, 2004; Wang *et al.*, 2012). Thus, the use of MnOx for contaminated soil remediation has received stimulated interest over recent years (Negra *et al.*, 2005; Spiro *et al.*, 2010). However, to date, few authors (outlined subsequently) have applied the use of MnOx in remediation treatments or carried out

a comprehensive study of their potential in this area. Current literature has centred upon the potential of MnOx for metal immobilisation in soils, with Pb and Cd being the most studied.

In experiments conducted by Varrault and Bermond (2011), MnOx (synthetic vernadite) was used as a binding phase for the remediation of four real Pb and Cd heavily polluted soils. They found that a 1 % (by weight) amendment of MnOx when mixed and stirred for 6 weeks in a 40 ml $NaNO_3$ 0.1 M solution, successfully decreased free soil concentrations of Pb and Cd by 24 % - 52 % and 40 % respectively. Chen *et al.* (2000) found that a 1 % synthetic MnOx amendment to Cd and Pb contaminated soils decreased the extractable concentrations of Pb and Cd, thus, significantly decreasing the uptake of these toxic metals in wheat shoots. Furthermore, it has been shown that MnOx addition to soils has protected rye grass from uptake of Cd and Pb more effectively than lime, phosphate, basic slag, iron oxides or alumino-silicates (Mench *et al.*, 1994). In addition, immobilisation of Pb has been observed in three types of soil by Hettiarachchi *et al.* (2000) using a synthetic Mn(IV) oxide *in situ*. Pb and Cd sorption to a synthetic birnessite has also been successful for sediments spiked with Cd and Pb, with 99 % of the Pb and Cd present after Mn(IV) amendment as non-extractable fractions upon the MnOx (Lee *et al.*, 2011).

Other research has shown that synthetic MnOx are effective treatments for decreasing available concentrations of Cu and Zn. In the experiments of Fawzy (2008) sequential fractionations indicated that the exchangeable fraction of Cu and Zn in contaminated soils can be transformed into unavailable forms after MnOx addition. However, metal remediation with MnOx amendment has also been shown to have negative effects. McBride and MartÍnez (2000) showed that synthetic MnOx (birnessite) addition to a Cu contaminated soil increased total soluble Cu concentrations. This effect was attributed to an increased soil pH from MnOx addition, which increased dissolved organic carbon, bringing more Cu into solution in organically complexed forms (McBride and MartÍnez, 2000). On the other hand, synthetic birnessite has been shown to sorb Pb and produce a very low (≈ 0) human bioaccessibility, making it an attractive candidate for Pb remediation (Beak *et al.*, 2007).

More recently the uses of MnOx as remediant has been examined for PCB contaminated soils. Synthetic MnO_2 amendment in combination with microwave

irradiation to soil contaminated with three PCBs, showed removal rates of 90 % and 40 %, which when compared to only a 10 % removal in the absence of MnO_2, indicated that MnO_2 is promising for the remediation of sites polluted with PCBs (Huang *et al.*, 2011).

Thus, the literature suggests that MnOx do have great potential for the immobilisation of metal contaminated soils. However, it is important to note that all the previous research has been carried out with synthesised MnOx mainly in artificially spiked soils. Other MnOx, i.e. naturally occurring or biogenic, may have different properties and may therefore interact differently in a real contaminated environment. Such oxides and contaminated settings are the subject of this thesis.

1.5.10. *Natural Mn oxide waste sources*

Previously, colleagues at Durham University identified significant amounts of MnOx-containing waste in the form of mine tailings material (Clarke *et al.*, 2010). This waste was generated during mining operations at the Hotazel Mn mine, which is no longer operational but still owned by the mining company BHP Billiton. The Hotazel Mn mine is situated outside the main Kalahari manganese field (KMF) in the Northern Cape Province of South Africa.

The KMF is the largest known land based Mn deposit (Beukes *et al.*, 1995). The supergiant deposits of the Kalahari Mn ore field are located in the eastern part of the Kalahari Desert, 60 km northwest of Kuruman, as depicted in Figure 1-12 (Kuleshov, 2011). The KMF harbours a metallic Mn resource estimated at more than 5 Gt and a Mn ore resource of 13.6 Gt (Kuleshov, 2011). As of 2010, South Africa held 75 % of the world's Mn resources (USGS, 2011). The ores of the KMF are estimated to be between 2200-2400 Ma old (Evans *et al.*, 2001).

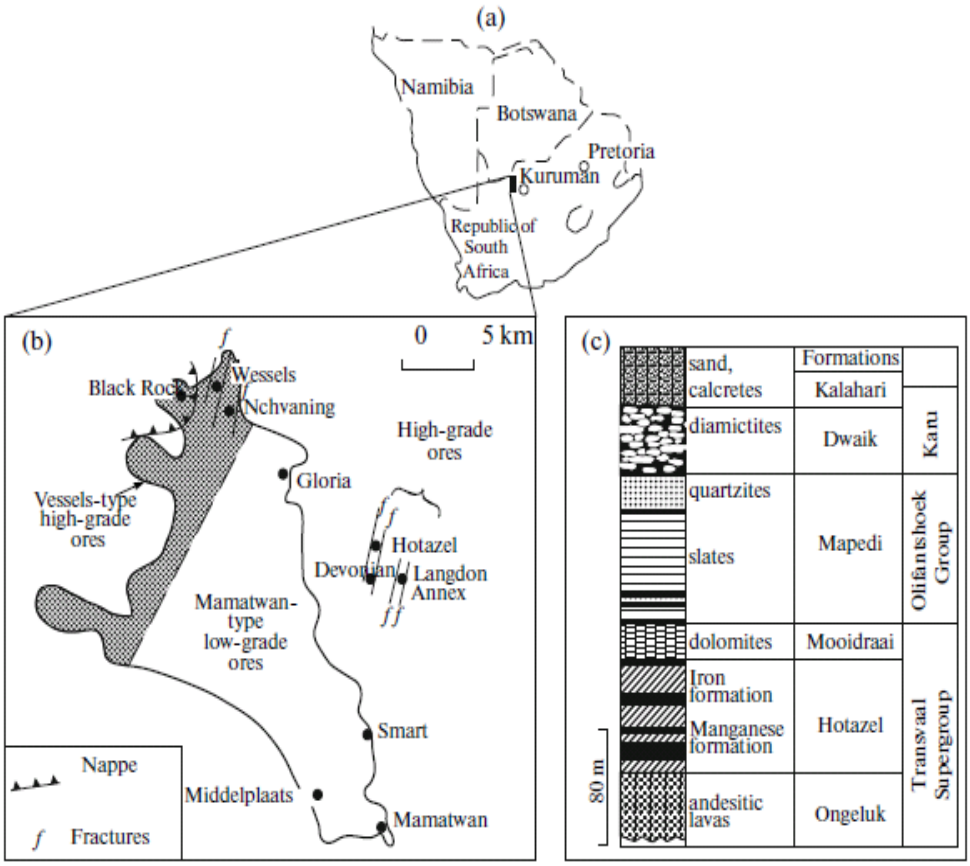


Figure 1-12: (a) Location of the Kalahari manganese ore field and (b) its deposits presented with (c) the stratigraphic position of the ore sequence in manganese deposits of the Kalahari manganese ore field. Taken from Kuleshov (2011).

The origin of the KMF ore body is still unknown; however, two main theories exist. The most popular hypothesis is that the ores were formed by chemical sedimentation in shallow water environments during fluctuating sea levels (Beukes, 1983; Boardman, 1964; Kuleshov, 2011; Tsikos and Moore, 1998). Although, another concept is that the ores are a result of submarine volcanogenic-exhalative activity (Cornell and Schütte, 1995). According to this theory Mn was originally derived from the upwelling of hydrothermal solutions from the underlying andesitic lavas of the Ongeluk Formation which were deposited in mid ocean ridges (Cornell and Schütte, 1995; Gutzmer and Beukes, 1996; Kuleshov, 2011). Further to these theories' it has also been suggested by Tebo *et al.* (2005) that the formation of the KMF may be related to an environmental or biological event that resulted in biomineralization of Mn 2.2 billion years ago. However, this theory is highly speculative and unproven. While the origin of the Mn ore body is still debated, the KMF deposit and its

mineralogy have been well characterised. The KMF comprises three laterally continuous, stratiform Mn beds which are interbedded with iron layers of the Hotazel formation, which can be seen in Figure 1-12c (Evans *et al.*, 2001).

The KMF contains two main Mn ores types (Figure 1-12b), a low-grade carbonate rich Mamatwan-type (30 and 39 wt %Mn) and a high grade oxide-rich Wessels-type (>42 wt % Mn), which were formed by metamorphism of the primary ore followed by hydrothermal alteration events (Evans *et al.*, 2001; Gutzmer and Beukes, 1996). However, the MnOx deposit of Hotazel represents a smaller outlining ore type to the main KMF ore body that is high grade and low in carbonate. Tailings were produced during ore extraction and milling processes when the mine was in operation. Due to the small particle size (<2 mm) and lower Mn content of tailings, they are regarded as a waste product within the mining industry. Consequently, they have undergone no chemical processing and are derived direct from the ore deposit. More specifically, Clarke *et al.* (2010) found that the Hotazel Mn tailings major mineral phases were manganite (MnOOH), branite ($Mn^{2+}Mn^{3+}{}_6SiO_{12}$), bixbyite ($(Mn^{3+}Fe^{3+})_2O_3$), hausmannite ($Mn_2O_3$), birnessite ($MnO_2$), todorokite ($MnO_2$), hematite ($Fe_2O_3$) and calcite ($CaCO_3$). Hotazel waste tailings are of a coarse texture (83.3 % sand size fraction) and have very low surface areas of 2.4 $m^2 \cdot g^{-1}$ (Clarke *et al.*, 2010). The net Mn oxidation state of the tailings was determined as 3+, with an O/Mn ratio of 1.5 (Clarke *et al.*, 2010). Within the study by Clarke *et al.* (2010) the waste tailings were shown to oxidatively break down acid azo dyes. The tailings were also determined to be suitable for water treatment due to their low trace element concentration which would not release high levels of toxic metals into solution. Due to this the waste tailings were also deemed suitable for addition to land and therefore for contaminated land remediation. It must be highlighted the Hotzael represent only one possible source of waste MnOx from South Africa. At present BHP Billiton and Assmang estimate that in Mamatwan and Wessels tailings dams contain approximately 1.6 and 1.5 million tons respectively of waste MnOx (Clarke, C., personal communication). Consequently, Wessels, Mamatwan, Gloria and Nchwaning Mn mines present additional sources of waste MnOx to those already identified at Hotazel.

A second source of waste MnOx termed ‘MnOx-coated sand’ has been identified as a by product from Mn(II)-removing water treatment plants (Hu *et al.*, 2004b). At levels above 0.1 mg/l soluble Mn(II) is unwanted due to the fact that it

produces drinking water of poor aesthetic quality via a black/brown discolouration and an undesirable taste (Sly *et al.*, 1990b). Furthermore, at levels as low as 0.02 mg/l it may result in the deposition of MnOx in pipelines which can restrict water flow (Sly *et al.*, 1990b). More importantly, Mn has been highlighted as being a threat to human health in potable water, and until 2011, a drinking-water guideline of 0.4 mg/l was set by the World Health Organisation (WHO, 2004). In 2011, this guideline limit was discontinued by the WHO, who ascertained that the value was well above concentrations of Mn normally found in potable water, so it was not considered necessary to derive a formal Mn guideline value (WHO, 2011a; WHO, 2011b). However, Mn has been reported to occur at levels above 0.4 mg/l in drinking water and in drinking-water supplies such as groundwater from aquifers, in 54 countries (Frisbie *et al.*, 2012).

Exposure to elevated Mn in drinking water is associated with neurotoxic effects in children, manganism and Mn-induced parkinsonism in adults, compulsive behaviours, emotional lability, hallucinations, attention disorders, low fetal birth weight and increased infant mortality (Aschner *et al.*, 2009; Beuter *et al.*, 1999; Bouchard *et al.*, 2007; Bouchard *et al.*, 2011; Bowler *et al.*, 1999; Collipp *et al.*, 1983; Ericson *et al.*, 2007; Frisbie *et al.*, 2012; Guilarte, 2010; Hafeman *et al.*, 2007; Henn *et al.*, 2012; Huang, 2007; Kim *et al.*, 2009b; Lucchini *et al.*, 2009; Menezes-Filho *et al.*, 2011; Perl and Olanow, 2007; Riojas-Rodríguez *et al.*, 2010; Rodríguez-Agudelo *et al.*, 2006; Sahni *et al.*, 2007; Solís-Vivanco *et al.*, 2009; Spangler and Spangler, 2009; Wasserman *et al.*, 2006; Zota *et al.*, 2009). Based on the aforementioned findings it has been recommended that a new Mn guideline is examined that is below 0.4 mg/l which has been reported to be too high to protect human health (Frisbie *et al.*, 2012; Ljung and Vahter, 2007).

The oxidation of soluble Mn(II) to Mn(III/IV) oxides in water treatment plants usually is achieved by a chemical oxidation step which consists of raising raw water pH to precipitate MnOx (Sly *et al.*, 1990b). MnOx are then sorbed onto silica or quartz sand surfaces during the process of rapid sand filtration, which results in the formation of MnOx-coated sands (Hu *et al.*, 2004b). The successful sorption of various toxic heavy metals ions in aqueous systems by MnOx-coated sands has been extensively studied in recent years (Boujelben *et al.*, 2009; Boujelben *et al.*, 2010; Chang *et al.*, 2008; Han *et al.*, 2006; Hu *et al.*, 2004a; Kim *et al.*, 2009a; Lee *et al.*, 2004; Lee *et al.*, 2010; Zou *et*

al., 2010). Thus, MnOx-coated sands from the water treatment industry could provide another valuable local source of MnOx waste.

Chapter 2. Assessing indicators of soil microbial functioning in contaminated land for implementation into remediation strategies

2.1. Introduction

Currently within the UK the development of soil biological indicators is aimed at nationwide monitoring to ensure functional activity is maintained for food and fibre production, environmental interactions and habitat/biodiversity preservation (Ritz *et al.*, 2009). However, these indicators were not designed to specifically or solely target soil microbial functions. Neither were they designed for application in contaminated land which occupies a separate legislative area, and will have different land use end points (Garbisu *et al.*, 2011). The success of soil remediation technologies is generally based upon reducing pollutant concentrations to a governmentally defined threshold whereby the pollutant no longer poses any significant harm to human health (Environment Agency, 2004a). However, this method of monitoring pollutant reduction does not uncover effects on microbiological activity.

Within soils, microbial populations are the underlying catalysts of a variety of ecosystem services, and the drivers of major biogeochemical cycles that are essential to envisaged end-uses of contaminated land for housing and recreation (Haygarth and Ritz, 2009; Verstraete and Mertens, 2004). Perturbation of soils in the form of contamination and/or remediation may result in changes in to microbial diversity, community composition and corresponding functions (Avidano *et al.*, 2005; Hinojosa *et al.*, 2004). Thus, it is of key importance that any remediation strategy monitors effects on soil microbial populations and their activities, in addition to pollutant concentrations. Consequently, there is a need to determine the intrinsic soil microbial function, and suitable indicators, of any contaminated soil. Such identified indicators can thus be employed to evaluate the key processes that may be under threat before and after remediation.

The impact of pollutants upon microbial activity within contaminated land or due to a pollution event has gained much attention in recent years (Baath, 1989; Bécaert and Deschênes, 2006; Bloem and Breure, 2003; Giller *et al.*, 1998; Hayat *et al.*, 2002; Ramakrishnan *et al.*, 2011). Some authors have explored potential microbial

indicators, for use in assessing the quality of remediated soils (Dawson *et al.*, 2007; Dickinson *et al.*, 2005; Hartley *et al.*, 2008). However, no studies have evaluated the effects of both long term organic and inorganic contamination upon microbial activities, placed this in context of comparisons with microbial functions of non-contaminated land, or used intrinsic activity of contaminated soils as a baseline for assessing impacts of remediation strategies.

2.1.1. *Aims and Objectives*

This chapter had two specific aims, firstly, to gain an understanding of the intrinsic microbial functioning within organic and inorganic contaminated soils that would be used within this thesis, concentrating upon the key processes of overall microbial activity, carbon and nitrogen cycling. Secondly, to provide a robust assessment of potential indicators of microbial functioning in contaminated land by comparison with a range of other land use types. Once an understanding of the baseline microbial functioning in contaminated land types was obtained and pertinent microbial indicators defined, this would be vital for comparison and assessment of the effects of MnOx addition (Chapter 3) and remediation (Chapter 4) upon microbial activity in these soils.

This was achieved through the following objectives:

(i) Determination of how microbial activity, specifically; overall activity, carbon and nitrogen cycling, has been affected in long term anthropogenic organic and inorganic contaminated soils by comparing with intrinsic microbial functional activities from a range of non-contaminated agricultural and garden soils.

(ii) Explore correlations between microbial functional activities, along with community composition and diversity of total bacterial, ammonia-oxidising and denitrifying communities through the use of culture-independent techniques. By combining this approach with soil physical and chemical characteristics the integrated nature of the soil environment is represented, providing a comprehensive assessment of microbial indicators.

(iii) Assess a variety of chosen microbial indicators that have been proven to be sensitive to contamination, and from these select specific high-throughput

indicators that best reflect the overall microbial functional status of the soils, and can be used to assess the potential benefits of remediation technologies upon microbial soil function.

Soil microbial indicators used in this study were chosen on the basis that they can be routinely measured with well developed methods that have been previously reported to be sensitive to contamination.

It was hypothesised that:

(i) Long term anthropogenic contamination has negatively affected soil microbial functioning, evidenced by suppressed activity rates in comparison to non-contaminated land types.

(ii) The community composition and diversity of bacterial, ammonia-oxidising and denitrifying populations will have been negatively impacted by long term contamination. Microbial community composition of contaminated land types will exhibit different structures and a reduced diversity in comparison to non-contaminated land types.

2.2. Materials and Methods

The materials and methods presented here are pertinent to this and other chapters in the thesis. Subsequent chapters will include additional methods specific to the work presented within each chapter, however, the main methods for soil analysis are only outlined once below.

2.2.1. *Site descriptions of PAH and heavy metal contaminated soils from St. Anthony's Tar and Lead Works*

Contaminated soils were selected from sites of historical anthropogenic PAH and heavy metal pollution. Samples were collected in February 2008 from two adjacent contaminated land sites, St Anthony's Tar (54°57°N - 1°32°W) and Lead works (54°57°N - 1°33°W). These sites are located on the Walker Riverside Park (Walker, Newcastle upon Tyne) situated on the North bank of the River Tyne, lying 3 miles east of Newcastle upon Tyne city centre (Figure 2-1).

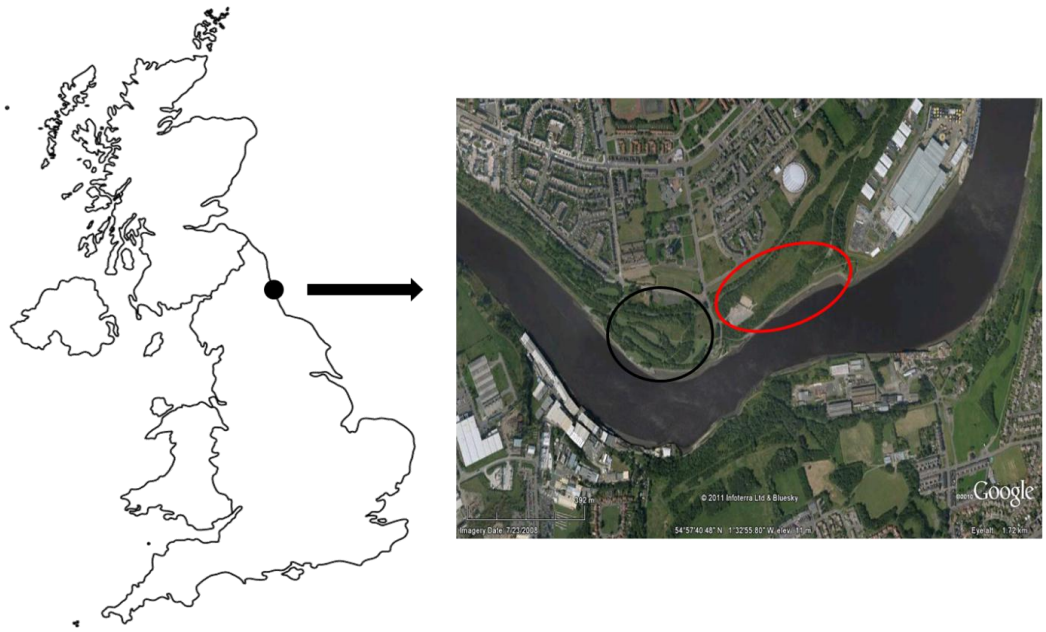


Figure 2-1: Position of Newcastle upon Tyne (left) and an aerial view of St. Anthony's Tar Works (red) and Lead works (black) on the North bank of the River Tyne (right) (map adapted from Google earth image).

In the 1920's St Anthony's Tar Works was established as a coal tar distillery which continued in operation until the early 1980's (ARUP, 2007). Coal tar is a highly viscous fluid that is made of a complex and variable mixture of polycyclic aromatic hydrocarbons (PAHs), phenols, sulphur, heterocyclic oxygen and nitrogen compounds, created from the carbonisation of coal at coking plants and coal gas works (Agency for Toxic Substances and Disease Registry (ATSDR), 2002).

Processing of coal tar at St. Anthony's resulted in the production of mixed hydrocarbons, benzole, naptha, creosote, pitch and road tar. Manufacture of purer organic chemicals such as anthracene, naphthalene, benzene, toluene, phenols, xylene, pyridine and caustic anti fouling paint were also carried out on site (ARUP, 2007). An amalgamation of the production of these hazardous compounds with relaxed health and safety regulations, careless disposal and repeated overfilling of coal tar in underground storage tanks has led to extensive soil contamination over the plant's 60 year operational period. Today visual evidence of contamination is clear (Figure 2-2) and the distinctive odour of hydrocarbons is unmistakable on the riverside footpath. In April 2008, the site was officially declared as Part IIA contaminated land after a site investigation by Ove Arup & Partners Ltd.

Figure 2-2: Evident pollution from St Anthony's Tar Works (photos taken by the author). (a) Solidified pitch permeating from soil. (b) Active pitch seepage from soil. (c) Seepage of hydrocarbons onto mudflats from quay wall into the River Tyne. (d) Public warning sign of contaminated land at the site.

Since the plant was decommissioned in 1982 by Tyne and Wear City Council, the site became incorporated into the Walker Riverside Park, making up its eastern portion. The park was designed to be used as recreational space for the community. However, the section that encompasses the old tar works is mainly unused by the public. Only the riverside path which lies to the south of the contaminated site is used by pedestrians, dog walkers and cyclists.

Previous attempts to remediate the site included surface remediation by infilling with non-contaminated soils and installing a tar interceptor, along with a

hydraulic barrier pump and treat system; all of which have been unsuccessful (ARUP, 2007).

Human health risks from the contaminated soil at the site were identified by Arup in 2007. Using source-pathway-receptor models they identified: (i) exposure to hydrocarbon contamination on the foreshore as an unacceptable risk to human health, (ii) a potential unacceptable risk to site users from benzo (a) pyrene, and (iii) a potential unacceptable risk through dermal contact and ingestion of hotspots of hydrocarbon contamination within the near surface soils (ARUP, 2007). It must be highlighted that contamination of the soils also has a vast impact upon the River Tyne through continual movement of groundwater contamination, dissolved phase hydrocarbons and non-aqueous phase liquids (NAPL) into its water (ARUP, 2007).

St. Anthony's Lead works were first established in 1846 by Locke, Blackett and Co, a Newcastle lead manufacturing company, in a large scale expansion of their lead manufacturing processes on Tyneside. The site location on the riverside allowed the company to process Spanish lead ores which were arriving on the Tyne in the late 19th century, and handle pre-smelted ores from the Durham dales (Rowe, 1983). Processing of lead on the site included smelting and de-silverising of the Spanish silver rich ores, a lead rolling mill for chemical sheet lead and pipe presses. Other specialities included the production of white lead, red lead and shot. Processing of lead at the site continued to around 1941 (Rowe, 1983).

The lead industry at the time used on-site landfills and lagoons for the disposal of by-products such as slag, dross, dust and waste water. Often waste material was used for new foundations and structures at plants (Environment Agency, 1995). This process is evident at St. Anthony's Lead Works as the soil contains rubble, former building foundations, brick fragments, tiles, ash, clinker, glass, coal and ballast; leading to it being termed 'made ground' and most probably containing toxic levels of heavy metals (Okorie *et al.*, 2011).

Today the former Lead Works site is situated in the western area of the Walker Riverside Park. The portion that accommodated the Lead Works comprises artificially created terraces constructed in the 1800s, to house factory buildings on the naturally occurring north-south oriented slope (Rowe, 1983). In the mid 1960's the terraces were landscaped by Newcastle City Council which involved a shallow surface dressing with topsoil, planting of trees on slopes and grassing flat terrace areas to create public

parkland (Okorie *et al.*, 2010; Okorie *et al.*, 2011). As a result of the landscaping the site is now covered by thriving grass and shrubbery which is navigated by footpaths lined with dense shrubs and small trees. This has led to the site being used by the public for a range of activities including fishing, walking, dog-walking, bike-riding and den-building by children (Okorie *et al.*, 2010; Okorie *et al.*, 2011).

Unlike St. Anthony's Tar works there is little visible evidence of pollution at the site. However, where bare ground is exposed under tree cover of slopes, ash and clinker debris is present (Okorie *et al.*, 2010). A survey of total metal concentrations (As, Cd, Cr, Cu, Mo, Ni, Pb and Zn) in 19 topsoil samples taken across the site by Okorie *et al.* (2010), identified that one or more of these elements were present in elevated concentrations in the majority of topsoil samples. The study by Okorie *et al.* (2010) concluded that parts of the site represented areas that posed a potential threat to human health. They found total concentrations of Pb ranging from 188-60 300 mg kg^{-1}, that exceeded UK Soil Guideline Values (SGVs) in 17 out of the 19 sites studied and levels of As that exceeded SGVs at 6 out of the 19 sites. This consequently led to Okorie *et al.* (2010) concluding that the site was significantly contaminated. Furthermore, the oral bioaccessibility of these metals and a generic quantitative risk assessment based on a residential land-use scenario was undertaken by Okorie *et al.* (2011). Their study found high oral bioaccessibility, 21 - 96 %, in all metals studied; and that Pb, As, and Cd posed a significant possibility of harm. Pb and As were highlighted as of particular concern due to a significant pollutant risk to humans via the hand to mouth route.

2.2.2. *Soil sample collection from St. Anthony's Tar and Lead Works*

Identification of locations at St. Anthony's Tar Works with differing PAH contaminant levels was accomplished by referencing PAH results from a previous site investigation preformed by Ove Arup & Partners Ltd in 2007 (ARUP, 2007). Soils were collected from the top 0-10 cm soil layer using an auger, with triplicate samples taken at least 2 m apart. Two soils were selected representing high and low PAH contamination.

A Niton field portable X-ray fluorescence (XRF) analyser was used to identify areas with differing soil metal contaminant levels across the St Anthony's Lead Works. The portable XRF is a tool for screening of contaminated sites for major metal and

metalloid contaminants. Total metal concentrations (in ppm) and respective standard errors are estimated by irradiating elements in the soil with a X-ray tube, which is achieved though the placing of the analyser in direct contact with the soil (CL:AIRE, 2010). Metals consequently emit X-rays; the energy and rate of these identifies the specific metal and its quantity respectively (CL:AIRE, 2010). Two soils were selected from XRF analysis across the Lead Works site to represent high and low heavy metal contamination. Soils were collected from the top 0-10 cm soil layer using an auger with triplicate samples taken at least 2 m apart.

2.2.3. *Soil sample collection and site description of agricultural soils*

Soils were collected from the Palace Leas hay meadow trial, at Cockle Park Farm, Northumberland, UK on the 13th November 2009. The hay meadow trials are the second oldest permanent grassland experiment in the world (Hopkins *et al.*, 2009). They were first established in 1897 to demonstrate the effect that a range of inorganic and organic fertilisers had upon the yield of hay from grassland (Shiel and Rimmer, 1984).

The experiment consists of fourteen unfenced, un-replicated plots, subjected to differing mineral fertiliser and manure treatments which lie within the same 1.5 ha field. Plots are parallelogram in shape and measure approximately 15 x 120 m each (Hopkins *et al.*, 2009). Fertiliser amendments have remained largely unchanged since their conception in 1897. Four plots were selected for this study to represent different agricultural soils as described in Table 2-1. Triplicate spatial samples were taken from the top 0-10 cm using an auger, across the length of each plot.

Table 2-1: Fertilisation treatments of selected plots from Palaces Leas hay meadow trial.

Plot	Treatments	FYM[a] (t ha^{-1} year^{-1})	Mineral fertiliser (kg ha^{-1} year^{-1})		
			N[b]	P[c]	K[d]
1	FYM + NPK[e]	20	17	30	34
2	FYM	20	0	0	0
6	Control[f]	0	0	0	0
14	NPK	0	100	66	100

[a] farm yard manure
[b] nitrogen (N) is applied in the form of ammonium sulphate [$(NH_4)_2SO_4$]
[c] phosphorus (P) is applied in the form of phosphorus oxide (P_2O_5)
[d] potassium (K) is applied in the form of potassium oxide (K_2O)
[e] nitrogen (N), phosphorus (P) and potassium (K) treatments applied
[f] control plot receives no fertiliser amendment

2.2.4. *Soil sample collection and site description of garden soils*

Two types of garden soils were collected on the 20th November 2009 from Moorbank Botanical Garden, Newcastle upon Tyne, UK. The garden is owned by Newcastle University, and occupies a 3 ha area used for a wide range of research purposes. It is located on the edge of 400 ha area of open pasture land, known as the 'Town Moor', in the city centre.

A representation of a typical garden soil was taken from the Northumberland bed (Figure 2-3). This soil was previously part of the Town Moor and has been untouched for c1000 years. It was converted into garden soil 7 - 8 years ago, without any fertiliser or management, and tends to be a clay loam (John Richards, Friends of Moorbank Garden, personal communication, November 2009). A second soil, taken from the perennial bed, was chosen to represent a typical garden soil with high organic material inputs (Figure 2-3). Here garden compost, leaf mould and mulch from the garden have been top-dressed onto the soil, leaving worms to work in the material (John Richards, Friends of Moorbank Garden, personal communication, November 2009).

Soils were collected from the top 0 - 10 cm soil layer of both beds using an auger, with triplicate samples taken at least 2 m apart.

Figure 2-3: The Northumberland bed representing a typical garden soil (left) and the perennial bed representing a garden soil with high organic material inputs (right).

2.2.5. *Soil sample storage for microbial, physico-chemical and molecular analyses*

All samples were transported back to the laboratory in sterile polyethylene bags (Fisher, UK) with free access to air. Soils were then processed and stored according to future experimental usage (see below). Extra care was taken with contaminated soils; dermal contact was avoided through the use of laboratory coat and gloves. Eye goggles and a face mask were also worn to prevent inhalation of harmful substances. Polluted soils were processed separately from other soil samples to avoid cross contamination.

Processing and storage of soil for microbial functional analyses (basal respiration, substrate induced respiration, denitrification enzyme activity, potential nitrification rates, moisture content and water holding capacity) was carried out in accordance to ISO 10381-6 (1993). Larger debris was removed from samples prior to passing through a 2 mm sieve (excluding clay soils which were crushed). At this point triplicate samples were homogenised into one bulk mass. Sieved soils were stored in the dark at 4 °C in loosely tied polyethylene bags (Fisher, UK). Each bulk soil sample was split amongst multiple, partially filled bags, to ensure aerobic conditions. Additionally, soils and were kept moist during pre-incubation until experimental use.

For physico-chemical analyses (pH, soil organic matter, total organic carbon, total metal and total PAH concentrations) soil sample processing was carried out in accordance to ISO 11464 (2006). Soils were air dried in the dark by spreading in thin layers on plastic trays at room temperature. Larger debris was removed from all

samples after air drying and samples were either passed through a 2 mm sieve or crushed (clay soils). At this point triplicate soil samples were homogenised into one bulk sample. Soils were stored in polyethylene bags at room temperature in the dark until experimental use.

Soils used for molecular analysis, i.e. DNA extraction, were stored immediately at -20 °C in sterile 20 ml universal tubes (Fisher, UK) on return to the laboratory.

2.2.6. *pH*

Soil pH was determined according to ISO 10390 (2005). Triplicate soil samples were suspended at a vol:vol ratio of 1:5 in deionised water. The slurry was mixed on a reciprocal shaker for 1 hour and left to stand overnight to equilibrate. The pH was measured using a Jenway 3020 pH meter (Jenway Ltd., UK), which was calibrated with standard pH solutions of 4 and 7.

2.2.7. *Moisture content*

The moisture content (MC) of soils was measured using ISO 11465 (1993). Triplicate samples of field moist soil (~1-2 g) in porcelain crucibles were dried to constant mass at 105 °C in a drying oven (Memmert, Germany). The MC was calculated on a dry mass basis and expressed as a percentage by mass according to Equation 2-1.

$$MC=\frac{m1\text{-}m2}{m2\text{-}m0}\cdot 100$$

Equation 2-1: Calculation of soil moisture content.

Where:

m0 = mass of the empty crucible (g)

m1 = mass of the crucible plus field moist soil (g)

m2 = mass of the crucible plus moist soil after drying to constant weight (g)

2.2.8. *Water holding capacity*

Water holding capacity (WHC) was determined according to annex A of ISO 14238 (1997). WHC of triplicate soil samples (~10 g) was performed using a cylinder with a perforated base (by means of filter paper; Whatman, UK). Briefly, cylinders

containing soil were covered, partially submerged in water for 2 hours followed by full submersion for 1 hour and then drained. The amount of water taken up by the soil was ascertained by weighing, drying to constant mass at 105 °C in a drying oven (Memmert, Germany) and reweighing. WHC was calculated using Equation 2-2. Values are expressed as a percentage of the dry mass of soil.

$$WHC = \frac{ms - mwf - mt - md}{md} \cdot 100$$

Equation 2-2: Calculation of soil water holding capacity.

Where:

ms = the mass of water-saturated solid + cylinder + filter paper (g)

mt = tare (mass of cylinder + filter paper) (g)

md = dry mass of soil (g)

mwf = mass of water absorbed by the filter (g)

2.2.9. *Soil organic matter*

Soil organic matter (SOM) was determined using the modified loss-on-ignition (LOI) method described by Nelson and Sommers (1996). Briefly, triplicate samples (~1-2 g) of field moist soil in porcelain crucibles were oven dried at 105 °C for 24 hours. After the weight was recorded, samples were heated to 500 °C in an AAF 1100 muffle furnace (Carbolite®, UK) for 16 hours and reweighed. Equation 2-3 was used to calculate SOM which is expressed as a percentage of the total mass.

$$LOI\ (\%) = \frac{Weight_{105} - Weight_{500}}{Weight_{105}} \cdot 100$$

Equation 2-3: Calculation of soil organic matter content.

Where:

$Weight_{105}$ = weight of soil sample after heating at 105 °C

$Weight_{500}$ = weight of soil sample after heating at 500 °C

2.2.10. *Total organic carbon*

Soil total organic carbon (TOC) was determined according to ISO 10694 (1995). The TOC of triplicate samples was analysed using a LECO CS244 carbon analyser (LECO Instrument Ltd., UK) with an infrared detection system. TOC was reported as a percentage of total mass.

2.2.11. *Soil heavy metal analysis*

Individual heavy metal concentrations for all soils were carried out by Northumbrian Water Scientific Services (NWSS) analytical laboratories (North Tyneside, UK). Metal concentrations were determined by aqua regia digestion followed by quantification using inductively coupled plasma-mass spectrometry (ICP-MS). Soils were analysed for priority heavy metals in relation to contaminated land i.e. mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), nickel (Ni), selenium (Se) and zinc (Zn). Analysis of manganese (Mn) and iron (Fe) was also carried out in all soils. All analyses were carried out to The Environment Agency's Monitoring Certification Scheme (MCERTS) for contaminated land. Please note only one bulk sample was analysed for each soil.

As published Soil Guideline Values (SGVs) only exist for Hg, As, Cd, Ni and Se (Chapter 1, Table 1.6); all other metal concentrations were compared to ranges of worldwide means for soils (Kabata-Pendias and Pendias, 1992; McBride, 1994) and contaminated land guidelines (Dutch list) used in the Netherlands (VROM, 2000). This allowed soil heavy metal levels within each land type be placed in context, giving an indication as to whether heavy metal levels were elevated (based on worldwide ranges), contaminated (based upon Dutch list values/SGVs) or typical (below worldwide ranges and Dutch list values/SGVs).

2.2.12. *Soil PAH analysis*

Polycyclic aromatic hydrocarbon (PAH) analysis of all soils was carried out by Northumbrian Water Scientific Services (NWSS) analytical laboratories (North Tyneside, UK), using soxhlet extraction followed by GC-MS analysis. Soils were analysed for the 16 priority PAHs listed by US EPA i.e. acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene,

benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene and pyrene. All analyses were carried out to The Environment Agency's Monitoring Certification Scheme (MCERTS) for contaminated land. Please note only one bulk sample was analysed for each soil.

Total PAHs (TPAHs) in soils were reported using the sum of all 16 EPA priority PAHs. Soils were classified as PAH contaminated if TPAH values exceeded the accepted Dutch contamination thresholds of 40 mg kg^{-1} for TPAHs (VROM, 2000).

2.2.13. *Basal respiration*

Basal respiration (BR) of soils was determined according to ISO 16072 (2002). Triplicate samples of field moist soil (10 g) were adjusted to 60 % WHC in 100 ml glass serum bottles (Wheaton science products, USA). Bottles were then sealed with septa and crimp closed (Sigma-Aldrich, UK). Gas samples were taken at 0, 20 and 24 hours using a 100 µl gas-tight, push-lock syringe (SGE, Australia) with CO_2 production measured by GC-MS. Calibrations were carried out by injecting a series of known volumes (100, 80, 60, 40, 20 and 10 µl) of a 1 % CO_2 standard gas (Scientific & Technical Gases Ltd, UK), which was also used to verify linear response.

Analysis of headspace CO_2 by GC-MS was conducted using a Fisons MD800 MS (electron voltage 70 eV, filament current 4A, source current 800 uA, source temperature 200 °C, multiplier voltage 500 V, interface temperature 150 °C) linked to a Compaq Deskpro computer, using Xcalibur software, in full scan mode (1.0-151.0 amu/sec). The 100 µl gas sample was injected in split mode and the GC program and MS data acquisition commenced. Separation of gases was achieved on a HP-PLOT-Q capillary column (30 m x 0.32 mm i.d) packed with 20 um Q phase. The GC was held isothermally at 35 °C with helium as the carrier gas (flow 1 ml/min, pressure of 65 kPa, split at 100 ml/min). The headspace CO_2 was determined using the parent ion at a mass to charge ratio (*m/z*) of 44. Chromatogram peaks of *m/z* 44 were integrated and quantified in Xcalibur and saved as excel files for further processing.

CO_2 values were calculated as $mgCO_2*g^{-1}$ dry soil, using the ideal gas equation (PV=nRT) modified to resolve into two component terms i.e. mass of CO_2 and molecular weight CO_2 (Equation 2-4).

$$PV*\text{mol weight } CO_{2\,(g/mol)}/RT = \text{mass of } CO_{2\,(g)}$$

Equation 2-4: Calculation of CO_2 values for the determination of basal respiration.

Where:

P = pressure of the gas standards (atm)

R = universal gas constant, 0.082 atm*l*(mol*K)$^{-1}$

T = absolute temperature, in this case 293 °K

V = volume of the gas, in litres

BR rates (mg CO_2*h^{-1}*g^{-1} dry soil) were then determined from the slope of the linear regression of plots of CO_2 production against sampling times.

2.2.14. *Denitrification enzyme activity*

Denitrification enzyme activity (DEA) was determined via a miniaturised acetylene block method as described by Patra *et al.* (2006) and Wertz *et al.* (2006). A few minor modifications were made to the method which is outlined below.

Field moist soil equivalent to 2 g of oven dried soil (as per Wertz *et al,* 2006), was placed in 10 ml serum bottles (Wheaton, Sigma-Aldrich, UK) and amended with 1.2 ml of distilled water containing potassium nitrate, KNO_3 (200 µg NO3– N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil). The original experiment was carried out in 150 ml plasma flasks (Wertz *et al.*, 2006), however, with a reduction in soil mass it was decided to reduce vessel volume to 10 ml in order to further miniaturise the experiment. Supplementary water was added to achieve 100 % WHC in all soils. Bottles were sealed with butyl rubber stoppers and crimp closed (Sigma-Aldrich, UK). The headspace of each bottle was then flushed with oxygen free nitrogen (N_2) gas (BOC Gases, UK) and subsequently replaced with 1 % acetylene (C_2H_2) in N_2 (CK Gas Products Ltd, UK). This provided inhibition of N_2O-reductase activity and ensured anaerobic conditions. In the original method of Wertz and colleagues, a 90:10 He–C_2H_2 mixture was used to flush headspace. However, this was unfeasible to employ due to safety reasons related with the stability of acetylene in the mixture. Instead a stable and safe mixture of 1 % acetylene C_2H_2 in N_2 was implemented within the assays of this study. The success of the application of the

lower percentage of C_2H_2 in N_2 provides a method which is more accessible to other researchers.

Experimental controls were carried out in triplicate, using the same weight of soil, but only flushed with N_2 to determine natural levels of N_2O emissions. Bottles were incubated at room temperature and headspace gas samples were measured at 4 and 6 hours to determine N_2O production by GC-MS.

As per section 2.2.13, 100 µl of headspace gas was extracted with gas-tight, push-lock syringe (SGE, Australia), that was flushed with N_2. After gas samples were extracted, 100 µl of N_2 was replaced into the headspace to maintain pressure. Concentrations of N_2O were determined using the major ion fragment of NO^+ at a mass to charge ratio (*m/z*) of 30. The parent ion of N_2O (*m/z* 44) was not used due to interference with any discharged CO_2. Calibration and linear response was checked using a gas standard of 0.988% N_2O in N_2 (Scientific & Technical Gases Ltd, UK) injected with volumes of 100, 80, 60, 40, 20 and 10 µl. GC-MS operational parameters were as per section 2.2.13 and the mass of N_2O produced was calculated as for CO_2 (Equation 2-4). DEA rates were expressed in µg $N*h^{-1}*g^{-1}$ dry soil following calculating rates based on linear N_2O to accumulation over time.

2.2.15. *Potential nitrification rate*

Potential nitrification rate (PNR) was measured using the sodium chlorate block method (Belser and Mays, 1980), according to the miniaturised version of ISO 15685 (2004), developed by Hoffman *et al.* (2007). For each sample 2.5 g of field moist soil, in triplicate, was incubated with 10 ml of 1 mM ammonium sulphate (Sigma-Aldrich, UK), and 5 µl of 1.5 M sodium chlorate (Sigma-Aldrich, UK) which blocks the conversion of NO_2^- to NO_3^-. The slurry was placed in an orbital shaker (Gallenkamp, UK) at 170 rpm and a constant temperature of 25 °C. After 2 and 6 hours, 2 ml of slurry was removed and centrifuged at 2,000 x g for 2 minutes (EBA12 centrifuge, Hettich Centrifuges, Germany). Centrifugal supernatant was removed and filtered through 0.45 µm filters (Nalgene Company, USA). Samples were frozen at -20 °C for subsequent nitrite (NO_2^-) analysis. Experimental controls used 1.25 ml of deionised water in place of soil.

NO_2^- production was measured photometrically according to the miniaturised protocol of Hoffman *et al.* (2007), in BD Falcon™ clear 96-well Microtest™ Plates (Becton Dickinson Labware Europe, France). Each reaction contained 150 µl of sample

plus 90 µl ammonium chloride (0.19 M) and 60 µl colour reagent (containing 0.06 M sulphanilamide acidified with hydrochloric acid and 1.72 mM N-(1-naphthyl)-ethyleneamine-dihydrochloride). After a reaction time of 15 minutes, absorbance was measured spectrophotometrically in a Multiskan Spectrum spectrophotometer (Thermo Scientific, UK) fitted with a microtiterplate reader at λ= 530 nm. Calibrations were carried out in triplicate using sodium nitrite (Sigma-Aldrich, UK) in a concentration range of 0 – 350 µM. PNR rates are expressed as µg NO_2-N*h^{-1}* g^{-1} dry soil.

2.2.16. *Substrate induced respiration*

Substrate induced respiration (SIR) was determined according to ISO 14240 (1997), based on the method developed by Anderson and Domsch (1978), to determine microbial biomass carbon (MBC). Firstly, optimal glucose concentrations for maximal respiration rates were determined for all soils. Six samples of each field moist soil (equivalent to 5 g of oven dry weight) were weighed into 100 ml serum bottles (Sigma-Aldrick, UK). Soils were then amended with a series of glucose solutions to give glucose amendments equivalent to 0, 0.5, 1, 2, 4 and 6 mg glucose g^{-1} dry soil, and were adjusted to 60 % WHC. Bottles were sealed with butyl rubber stoppers (Sigma-Aldrich, UK), crimp closed and incubated at room temperature. Every hour for 6 hours, 100 µl of headspace was removed and CO_2 concentration measured immediately by GC-MS, as per section 2.2.13, with the exception that a 9.977 % CO_2 in N_2 standard was used (Scientific & Technical Gases Ltd, UK).

To determine MBC values the same procedure as for SIR was carried out in triplicate using the optimal glucose concentration defined for each soil. Triplicate experimental controls were carried out in parallel without addition of glucose. MBC was calculated using Equation 2-5 as determined by Anderson and Domsch (1978).

$$X = 40*R + 0.37$$

Equation 2-5: Calculation of soil microbial biomass carbon.

Where:

X = microbial biomass carbon, in milligrams per kilogram of dry soil

R = maximum initial rate of CO_2 respiration, in millilitres per kilogram per hour

2.2.17. *Determination of metabolic quotient*

The metabolic quotient (qCO_2) for each soil was calculated from the ratio of basal respiration (BR): microbial biomass carbon (MBC) (Anderson and Domsch, 1990).

2.2.18. *Determination of microbial quotient*

The microbial quotient (*qmic*) for each soil was calculated from the ratio of microbial biomass carbon (MBC): total organic carbon (TOC) (Anderson and Domsch, 1989).

2.2.19. *Soil DNA extraction*

Genomic DNA was extracted from ~0.5 g of defrosted field moist soil using a FastDNA Spin Kit for soil (MP Biomedicals, UK) and a FastPrep Ribolyser (MP Biomedicals, France) in triplicate according to the manufacturer's protocol. A procedural blank was carried out using 250 µl of microbiological grade filter sterilised water (Sigma, UK) in place of soil to check kits were clear of contaminants. The presence of extracted DNA was checked by agarose gel electrophoresis (see section 2.2.21). DNA extracts were stored at -20 °C until further use.

2.2.20. *Polymerase chain reaction*

The polymerase chain reaction (PCR) was carried out in this study to assess the community composition and diversity of the predominant total bacterial, ammonia-oxidising and denitrifying communities in all soil samples. The diversity and community composition of the predominant total bacterial community was assessed by performing PCR reactions targeting conserved regions of 16S rRNA genes. Community

composition and diversity of the predominant denitrifiers involved PCR amplification of denitrification genes encoding nitrite reductase (*nirK* and *nirS*). Assessment of the community composition and diversity of the predominant ammonia-oxidising communities was carried out via amplification of the *amoA* gene which encodes a small subunit of ammonia monooxygenase in ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA). PCR amplifications from genomic DNA extracts were performed using the primers listed and protocols referenced in Table 2-2.

Table 2-2: Primers and PCR conditions used in this study.

Target gene	Primer	Sequence (5'-3')	Annealing site	Fragment length	Cycle details	Reference
Bacterial 16S rRNA	Primer 2 Primer 3[a]	ATTACCGCGGCTGCTGG GCCTACGGGAGGCAGCAG[a]	518-534[b] 341-357[b]	233*	95°C for 1min (95°C 30s, 65-53°C 30s, 72°C 30s) × 24 (95°C 30s, 53°C 30s, 72°C 30s) × 15 72°C for 10min	Muyzer *et al.*(1993) Muyzer *et al.*(1993)
AOB *amoA*	amoA-1f*[a] amoA-2r	GGGGHTTYTACTGGTGGT[a] CCCCTCKGSAAAGCCTTCTTC	332-349[c] 802-822[c]	530*	94°C for 10min (94°C 1min, 57°C 1min, 72°C 1min) × 42 72°C for 10min	Stephen *et al.* (1999) Stephen *et al.* (1999)
AOA *amoA*	Arch amoA-1F Arch amoA-2R	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	ND[f] ND	494	95°C for 10min (94°C 45s, 55°C 1min, 72°C 1min) × 30 72°C for 15min	Francis *et al.* (2005) Francis *et al.* (2005)
nirK	FlaCu R3cu[a]	ATCATGGTSCTGCCGCG GCCTCGATCAGRTTGTGGTT[a]	568-584[d] 1021-1040[d]	512*	94°C for 2min (94°C 30s, 57°C 1min, 72°C 1min) × 35 72°C for 10min	Throbäck *et al.* (2004) Throbäck *et al.* (2004)
nirS	Cd3af R3cd[a]	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA[a]	916-935[e] 1322-1341[e]	465*	94°C for 2min (94°C 30s, 57°C 1min, 72°C 1min) × 35 72°C for 10min	Throbäck *et al.* (2004) Throbäck *et al.* (2004)

[a] GC-clamp (5-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3')(Muyzer *et al.*, 1993) attached to the 5' end of the primer.
[b] Positions in the 16S rRNA gene sequence of E. *coli* (Brosius *et al.*, 1978).
[c] Positions in the *amoA* gene sequence of *Nitrosomonas europaea* (McTavish *et al.*, 1993).
[d] Positions in the *nirK* gene of *Alcaligenes faecalis* S-6 (Throbäck *et al.*, 2004).
[e] Positions in the *nirS* gene of *Pseudomonas stutzeri ZoBell* ATCC 14405 (Throbäck *et al.*, 2004).
[f] not described
* includes GC-clamp

PCR reactions were all conducted in 200 µl sterile eppendorf tubes (Starlab, UK) with a total reaction volume of 50 µl. Each reaction contained 47 µl MegaMix-Blue (Microzone Limited, UK), 1 µl of forward primer and 1 µl of reverse primer (both at a concentration of 10 pmol per µl) and 1 µl of template DNA. All primers used in this study were supplied from Thermo Scientific, Germany. Amplifications were carried out using an automated thermal cycler Techne TC-5000 (Bibby Scientific, UK) or a G-storm GS1 (GRI Ltd, Essex).

For each PCR a negative control (no template DNA, microbiological grade water only), control (procedural blank), and where possible a positive control (clone with target sequence, i.e. *E coli* for the 16S rRNA gene) were included. No clones with target sequences were available for *nirS*, *nirK*, AOB *amoA* or AOA *amoA* genes. Therefore, confirmation of PCR products was conducted through checking the correct fragment size had been achieved for all genes after agarose gel electrophoresis (see section 2.2.21). The success of PCR amplification was determined by checking for products in agarose gel electrophoresis as described below. PCR products were stored at -20 °C for subsequent analysis.

2.2.21. *Agarose gel electrophoresis of PCR products*

The presence of DNA in extracts and the certification of the correct size of gene fragments from PCR reactions were confirmed by agarose gel electrophoresis. DNA extracts were analysed in 0.7 % (w/v) agarose gels, and PCR products in 1 % agarose gels; both made in 1 x TAE (tris-acetate-EDTA) buffer (40 mM tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with 1.6 µl of ethidium bromide. For each genomic DNA sample, 5 µl of extract plus 2 µl of 6 x loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, and 30 % glycerol in filter sterile water; Sigma, UK) was run on the gel at 100 V constant current for 60 minutes. The same conditions were required for PCR product analysis, although the loading buffer was excluded as this was incorporated within the mastermix, additionally gels only needed to be ran for 45 minutes. 2 µl of λ-DNA Hind III Digest marker (Sigma, UK), heated to 60 °C for 3 minutes was run alongside DNA extracts to assess the relative yields of linear double stranded DNA molecules between 125bp and 23.1 kb. 2 µl of Hyperladder II DNA marker (Bioline, UK) which contains regularly spaced bands, ranging from 50 bp to 2 kb, was run alongside samples to ensure PCR fragments were the correct fragment length. All agarose gels

were visualised and photographed using a UV trans-illuminator (Fluor-S MultiImager, Bio-Rad, UK) and images were saved and viewed using the FluorS gel documentation software, Quantity One (Bio-Rad, UK).

2.2.22. *Denaturant gradient gel electrophoresis*

Microbial community fingerprinting was carried out by denaturant gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993). All DGGEs were carried out using the D-Code system (Bio-Rad, USA). DGGE consisted of polyacrylamide gels (acrylamide-N, N-methylenebisacrylamide ratio, 37.5:1; Sigma, UK) with chemical denaturant gradients of urea and formamide (Sigma, UK). Polyacrylamide gel percentage and denaturant concentration percentage varied depending upon the target gene being analysed. See Table 2-3 for optimal DGGE conditions for each gene targeted in this study. Gels were poured by the use of a persistalic pump (Model 520, Watson Marlow, UK) and a gradient former (Model 485, Bio-Rad, UK). All gels were 0.75 mm thick, and measured 16 by 16 cm.

Table 2-3: DGGE conditions for target genes used in this study**.**

Target gene	Polyacrylamide gel (%)	Denaturant concentrations (%)
Bacterial 16S rRNA	10	30 - 55
Bacterial *amoA*	5	30 - 60
Archaeal *amoA*	8	15 - 40
nirK	7	30 - 60
nirS	7	30 - 60

For each sample 11 µl of PCR product and 11 µl of 6 x loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in filter sterile water; Sigma, UK) was loaded onto the gel. Gel markers (containing the same mixture of cloned 16S rRNA gene fragments) which migrate throughout the gel, were included every 4^{th} or 7^{th} lane. This allows for normalisation and inter-gel comparison in subsequent gel analysis using Bionumerics 5.1 (Applied Maths, Belgium). Gels were electrophoresed for 4.5 hours at a constant voltage of 200 V (Bio-Rad PowerPac 3000, Bio-Rad, UK) and a constant temperature of 60 °C in 1 x TAE buffer (40 mM tris, 20 mM

acetic acid, 1 mM EDTA, pH 8.3). After electrophoresis, gels were stained for 30 minutes in 200 ml of 1 x TAE buffer plus 20 µl of SYBR Green (Sigma, UK) and visualised using a UV trans-illuminator (Fluor-S MultiImager, Bio-Rad, UK). Gel images were viewed and saved using the FluorS gel documentation software, Quantity One (Bio-Rad, UK).

2.2.23. *Statistical analysis of DGGE profiles*

Digital DGGE images were analysed using Bionumerics 5.1 (Applied Maths, Belgium). This allowed for different gels of the same target gene to be amalgamated. This task was facilitated by inclusion of the same marker profile (i.e. a universal fingerprint) in multiple regularly spaced lanes in all gels. Using these markers, the normalisation of individual gels was performed by the use of a universal fingerprint type that ensured any irregularities in gradients between and within DGGE profiles were corrected. Bands representing different components of the microbial communities in different profiles were detected automatically using the Bionumerics 'band detect' and 'band match' functions. Band assignments were then manually checked. Band presence/absence data and quantification values were exported from Bionumerics for further statistical analysis of community structure.

Multivariate and univariate analysis of DGGE profiles were performed in Primer v.6 (Primer-E Ltd., Plymouth, UK). Multivariate analysis of DGGE fingerprints from all soils was performed using non-metric Multi-Dimensional Scaling (MDS), based on Bray-Curtis similarities using presence/absence data of DGGE bands with 100 random restarts. MDS provides a graphical representation of community similarity in 2 dimensional (2D) space, where sample distance is based on relative similarity values based on the Bray-Curtis similarity matrix. The MDS algorithm generates a 2D stress value which denotes how accurately the dimensional relationships among samples are represented in the 2D output. Low stress values close to 0 are indicative of excellent representation, values of 0.1 showing good representation and stresses of 0.2 demonstrating potentially useful outputs. However, values close to and exceeding 0.3 indicate that placement of points in 2D space is close to random and should be discounted (Clarke and Warwick, 2001). Cluster analysis was performed using the CLUSTER method in Primer to identify any intrinsic groupings within microbial communities. This method uses hierarchical agglomerative clustering which in this

study was based upon the group average method where a new node takes the average similarity of the individual nodes. Clusters were then overlaid on 2D MDS plots graphically illustrated by contours specifying 20, 40, 60 and 80 % similarity.

Similarity between community compositions observed in MDS profiles were statistically assessed by the analysis of similarities (ANOSIM) function. This produces a sample statistic, R (Global R value), with an associated level of significance (p-value, based on Monte Carlo simulation). R values represent the degree of separation between test groups (Clarke, 1993). Values of approximately 0 indicate that samples between and within test groups will have the same community structure on average, while a value of 1 demonstrates that all replicates within sites are more similar to each other than any replicates from different sites (Clarke and Warwick, 2001). In Primer p-values are reported as percentages, therefore a p-value less than 5 or 1 % equates to a conventional p-value of 0.05 and 0.01 respectively. All ANOSIM calculations were based upon 250 permutations of the data set.

The BEST function in Primer was used to examine the 'extent to which the physico-chemical data is related to ('explains') the observed biological pattern' (Clarke and Warwick, 2001) using the BIO-ENV method (Clarke and Ainsworth, 1993). Environmental data were classified *a priori* into 3 groups (microbial functions, basic soil physiochemical properties, in addition to soil metals and PAHs) as too many variables existed to be tested simultaneously.

Environmental data were initially checked for co-linearity, requirement for transformation (e.g. to reduce skewness, and/or influence of outliers) and choice of transformation through the use of draftsman plots (i.e. pair-wise scatter plots of all variables). If two parameters gave a true correlation of 0.95 or above only one was included in BIO-ENV analysis. Correlation coefficients were always checked visually with scatter plots for validation that the coefficient was not misleading due to incidences of non-linear relationships or the presence of outliers. After inspection, data were appropriately transformed, where required, and normalised.

The BIO-ENV procedure compares a fixed Bray-Curtis similarity matrix of the bacterial, ammonia-oxidising and denitrifying community structures produced in this study, to a Euclidian distance similarity matrix of the selected environmental variables. Spearman's rank correlation coefficients are calculated between the matrices and the combination of environmental variables which produce the highest Spearman's rho (r)

are reported. Increasing Spearman's rho values (rho > 0) indicate increasing separation of microbial community composition, the significance of this separation is represented by a p-value. Environmental variables which best explained the microbial community structure were then visualised through the use bubble plots (superimposed over 2D MDS plots) to provide more insight into how they shape the microbial community.

Univariate community diversity analysis was conducted using the DIVERSE function in Primer. Quantification values, i.e. peak heights of the densitometric curves of each DGGE band, from Bionumerics was imported to calculate the indices outlined in Table 2-4.

Table 2-4: Diversity indices as calculated by the DIVERSE function in Primer v.6

	Diversity Index	Equation
S	Total species	Total number of bands
N	Total individuals	Total peak heights of all bands
d	Margalef's richness	$d = (S\text{-}1)/\log_e N$
H'	Shannon's diversity	$H' = -\Sigma\, P_i \log_e(P_i)$ [a]
J'	Pielou's evenness	$J'=H'/\log_e S$
1-λ'	Simpson's diversity	$1\text{-}\lambda' = 1- (\Sigma_i N_i(N_i\text{-}1))/(N(N\text{-}1))$ [b]

[a] where P_i is the abundance of the ith band divided by N
[b] where N_i is the abundance of the ith band

2.2.24. *Sequencing of DGGE bands*

Identification of bands of interest, which were clearly resolved in DGGE profiles were excised using sterile scalpel blades under UV light. The excised fragment was placed in 30 µl of microbiological grade water (Sigma-Aldrich, UK), mixed using a vortex, and incubated at 4 °C overnight to allow DNA to transfer into the H_2O. The excised fragment was then re-amplified by PCR using the relevant primer set and checked on 1 % agarose gels (section 2.2.21). PCR products were purified using with either ExoSAP-IT (USB Corporation, Denmark), or with a QIAquick PCR Purification Kit (Qiagen, UK) according to the manufacturer's instructions. Sequencing was carried out by DBS Genomics (Durham University, UK) using 6 µl of PCR product (DNA concentration was checked and diluted by DBS Genomics) and 3.2 pmol/µl of the

relevant forward primer at a volume of 3 µl per reaction using an ABI 3730 sequencer (Applied Biosystems, USA). All sequences were checked using the biological sequence alignment editor, BioEdit (Ibis Biosciences, Carlsbad, CA). Sequences of good quality (based on visual analysis) were compared to cultured and uncultured sequences in both the NCBI Basic Local Alignment Search Tool (BLAST), and in the EMBL-Bank database via Fasta-33.

2.2.25. *Phylogenetic analysis of recovered DGGE band sequences*

A phylogenetic analysis of sequenced DGGE bands was conducted using MEGA version 5 (Tamura *et al.*, 2011). Functional and 16S rRNA gene sequences from microbial lineages most closely related to DGGE derived sequences, including those from Type strains, other cultured and uncultured taxa as identified in section 2.2.24 were downloaded from the Ribosomal Database Project (RDP) Release 10 (Michigan State University, USA) or the NIH genetic sequence database, GenBank. These sequences along with DGGE band sequences of interest were opened in BioEdit and were trimmed to the same base pair length to import into MEGA. Sequences were aligned using ClustalW in MEGA and the aligned sequences were then used to construct trees. All trees were inferred using the Neighbour-Joining method (Saitou and Nei, 1987), clustered together and tested by bootstrapping using 1000 replicates. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2011).

2.2.26. *Statistical analysis*

Statistical analysis of measured microbial parameters (BR, DEA, PNR, MBC, *qmic* and qCO_2) and physico-chemical characteristics (pH, MC, WHC, SOM, TOC, soil metal and PAH concentrations) was conducted using SPSS 17 (SPSS, Inc. Chicago, IL). Soils were defined *a priori* into four land types: 1) PAH contaminated (high PAH and low PAH soils from St. Anthony's Tar Works), 2) metal contaminated (high metal and low metal soils from St. Anthony's Lead Works), 3) agricultural (FYM, FYM +NPK, NPK and control plots from Palace Leas Hay meadow trials at Cockle Park farm) and 4) garden (typical garden, garden + high organic material inputs from Moorbank Botanical Garden). Crossplots were used to visually determine if data required transformation, showing that only Log (base-10) transformations were necessary. Significant

differences between measured parameters of the four land type groupings was analysed using one way analysis of variance (ANOVA), followed by Tukey's HSD test. Box plots were used to visualise differences in microbial parameters, which represent the median, upper and lower quartiles of the distribution.

Correlation analysis between microbial and physicochemical factors was based on using the individual means of the ten soils sampled in this study (high PAH, low PAH, high metals, low metals, FYM, FYM + NPK, NPK, control, garden and garden OM). The mean value for each soil was calculated using Microsoft Excel 2007, based upon triplicate values. Correlations were determined using the bivariate two tailed Pearson correlation coefficient in the main, but also checked for non-parametric correlation using a bivariate two tailed Spearman correlation coefficient. All significant correlations ($p < 0.05$) were checked through the use of crossplots to determine if the relationship was true.

2.3. Results

Within this chapter soils have been summarised into four land types: PAH contaminated, metal contaminated, agricultural and garden (Table 2-5). Results reported are an average of values from specific sampling locations within each land type. Thus, for PAH contaminated, metal contaminated and garden land types, the value reported is an average of two different soil samples; while the agricultural land type is an average of four soil samples. The presentation of the data in this form was to provide a more realistic indication of parameters measured across different land types. Conversely, any correlation analysis (unless indicated) was based upon the mean value calculated from triplicate analysis of a bulk soil sample for the ten specific sampling locations. The PAH and metal contaminated land types have been collectively defined as 'contaminated', while agricultural and garden land types are together classified as 'non-contaminated'.

Table 2-5: Summary of the soils analysed within each land type of this study.

Land type	Geographical sampling location	Specific samples analysed from geographical sampling location
PAH contaminated	St. Anthony's Tar Works	1) High PAH 2) Low PAH
Metal contaminated	St. Anthony's Lead Works	1) High metals 2) Low metals
Agricultural	Palace Leas hay meadow plots, Cockle Park Farm	1) FYM 2) FYM + NPK 3) Control 4) NPK
Garden	Moorbank Botanical Garden	1) Northumberland bed = typical garden soil named 'garden' 2) Perennial bed = garden soil with added organic material (OM) named 'garden OM'

2.3.1. *Soil physico-chemical properties*

Mean soil pH values for each land type increased in the order agricultural (5.6) > garden (6.3) > metal contaminated (7.1) > PAH contaminated (7.9), with all significantly differing to each other (Table 2-6, one-way ANOVA, Tukey's HSD, $p < 0.05$).

Water holding capacity (WHC) values were largest for agricultural (180 %) soils which significantly differed to all other land types (Table 2-6, one-way ANOVA, Tukey's HSD, $p < 0.05$), and lowest for PAH contaminated soils (61 %). At the time of sampling moisture content (MC) values in the land types ranged from 52 % (garden) to 12 % (PAH contaminated). MC significantly differed were observed between all land types (one-way ANOVA, Tukey's HSD, $p < 0.01$), apart from metal and agricultural means (Table 2-6).

Mean soil organic matter (SOM) and total organic carbon (TOC) contents decreased in the order: garden > metal contaminated> agricultural > PAH contaminated, suggesting a potential correlation between these variables (Table 2-6). Statistical comparison of SOM and TOC showed that both of these variables significantly differed between all land types (one-way ANOVA, Tukey's HSD, $p < 0.05$), apart from metal and agricultural soils (one-way ANOVA, Tukey's HSD, $p > 0.05$).

Table 2-6: Mean soil physico-chemical properties for each land type in this study. Values are based upon the mean of 2 soils from PAH contaminated, metal contaminated and garden land types, and of four soils within the agricultural land type grouping.

	pH	SOM [a] (%)	TOC [b] (%)	WHC [c] (%)	MC [d] (%)
PAH contaminated	7.9 ± [e] 0.5	6.5 ± 0.3	2.3 ± 0.6	61 ± 13	12 ± 2
Metal contaminated	7.1 ± 0.4	14.0 ± 1.7	7.5 ± 0.7	109 ± 3	30 ± 1
Agricultural	5.6 ± 0.2	9.1 ± 1.0	3.2 ± 0.4	180 ± 20	38 ± 5
Garden	6.3 ± 0.4	15.4 ± 2.1	9.4 ± 0.4	128 ± 11	52 ± 4

[a] soil organic matter (SOM)
[b] total organic carbon (TOC)
[c] water holding capacity (WHC)
[d] moisture content (MC)
[e] ± 1 x standard error

Correlation analysis of all physico-chemical variables (Table 2-7), showed that SOM exhibited the strongest linear relationship with TOC where $r^2 = 0.95$, $p < 0.01$. Therefore, only TOC values were selected to represent both variables in further statistical analysis. Statistically significant correlations were also observed between WHC and MC ($r^2 = 0.68$, $p < 0.05$). Additionally, mean soil pH values showed a significant negative correlation with WHC ($r^2 = -0.73$, $p < 0.05$).

Table 2-7: Pearson correlation coefficient matrix of soil physico-chemical parameters based upon the average of 10 individual soil samples across garden, agricultural, metal and PAH contaminated soils.

	WHC	MC	pH	SOM	TOC
WHC	1	0.68*	-0.73*	0.13^{ns}	0.01^{ns}
MC	0.68*	1	-0.51^{ns}	0.62^{ns}	0.62^{ns}
pH	-0.73*	-0.51^{ns}	1	-0.04^{ns}	0.03^{ns}
SOM	0.13^{ns}	0.62^{ns}	-0.04^{ns}	1	0.95**
TOC	0.01^{ns}	0.62^{ns}	0.03^{ns}	0.95**	1

* Correlation is significant at the 0.05 level (based upon a 2-tailed bivariate Pearson correlation).
** Correlation is significant at the 0.01 level (based upon a 2-tailed bivariate Pearson correlation).
[ns] not significant

2.3.2. *Total soil heavy metal concentrations*

Analysis of total metal concentrations confirmed that soils within the metal contaminated land type were contaminated, with levels of As, Pb, Cd and Zn exceeding currently recognised contamination thresholds (Table 2-8). Mean levels of As (1615 mg kg^{-1}) exceed SGVs at residential, allotment and commercial land use levels (32, 43, 640 mg kg^{-1} respectively), with Cd concentrations (6 mg kg^{-1}) exceeding only the allotment SGV guideline value (1.8 mg kg^{-1}), and mean levels of Pb over 10 times the levels of Dutch list action values. Additionally, the metal contaminated land type was found to have elevated levels of Hg, Cu and Se.

Mean total metal values confirmed that PAH, garden and agricultural land types were not metal contaminated. Significant differences in metal concentrations were observed between PAH, agricultural and garden soils, however, as levels were either within usual worldwide ranges or below Dutch List action thresholds, they were not considered further in this study. Nonetheless, examination of mean Pb values within garden and agricultural land types along with their respective standard errors indicated that soils actually exceeded Dutch guideline limits. Inspection of Pb concentrations from each soil within these land types showed that the farm yard manure (FYM) applied plot of Cockle Park Farm, and the garden soil with addition of organic material (garden OM) from Moorbank Botanical Garden had Pb concentrations that exceeded Dutch guideline limits at 1100 mg kg^{-1} and 820 mg kg^{-1} respectively. These results indicate that Pb was being added to these soils through FYM and OM applications.

Total metal values for soils within the metal contaminated land type confirmed their classification of soils as low and high (Table 2-9). The high metals soil is contaminated with Pb, Hg, As, Cd and Zn, and has elevated concentrations of Cu. This soil exceeds SGVs for Hg and Cd at the residential land use levels, and As at the commercial land use level. Total concentrations of Zn at 1700 mg kg^{-1} are more than double Dutch List action levels, while Pb concentrations are exceptionally high at 11000 mg kg^{-1}, over twenty times the Dutch action level. In comparison, the low metals soil only shows elevated levels of Zn (190 mg kg^{-1}) and Pb (360 mg kg^{-1}). Interestingly, the levels of Pb and Zn within the low metals soil are comparable to that found in agricultural and garden soils (Table 2-8).

Table 2-8: Mean total heavy metal contents (mg kg^{-1}) of land types in this study. Values are presented with the range of worldwide soil means as taken from McBride (1994) and Kabata-Pendias and Pendias (1992), with threshold action values from the Dutch list as used in the Netherlands for contaminated land (VROM, 2000). Values are based upon the mean of 2 soils from PAH contaminated, metal contaminated and garden land types, and of four soils within the agricultural land type.

	PAH contaminated	Metal contaminated	Agricultural	Garden	Typical worldwide ranges	Dutch list
Hg	0.28 ±[a] 0.2	0.91 ± 0.6*	0.12 ± 0.0	0.16 ± 0.1	0.02 - 0.41[b]	10
As	8.50 ± 0.8	1615 ± 1585***	8.10 ± 2.7	9.40 ± 3.6	2.2 - 25[b]	55
Cd	0.29 ± 0.1	6 ± 5.2***	0.35 ± 0.2	0.50 ± 0.3	0.06 - 1.1[b]	12
Cr	112 ± 38	86 ± 8	147.5 ± 15	125 ± 5	7 - 221[b]	380
Cu	30 ± 2	114 ± 56*	28.75 ± 10	47.50 ± 25.5	6 - 80[b]	240
Fe	32000 ± 4000	18100 ± 14900	28500 ± 866	29000 ± 2000	5000 - 50000[c]	n/a
Pb	52 ± 4	5680 ± 5320**	302 ± 266*	431 ± 389*	10 - 84[b]	530
Mn	930 ± 370	325 ± 25	667.50 ± 174	875.00 ± 325	80 - 1300[b]	n/a
Ni	80 ± 20	57 ± 5	80 ± 7	74.50 ± 8	1 - 450[c]	210
Se	0.57 ± 0	17.44 ± 16*	0.69 ± 0.1	0.79 ± 0.2	0.05 - 1.27[b]	100
Zn	150 ± 10*	945 ± 755**	106.75 ± 42	146 ± 74*	17 - 125[b]	720

[a] ± 1 x standard error
[b] McBride (1994)
[c] Kabata-Pendias and Pendias (1992)
*Total metal concentrations that exceed typical worldwide ranges.
**Total metal concentrations that exceed Dutch list guidelines.
***Total metal concentrations that exceed UK Soil Guideline Values (SGVs).
Values underlined show that soil(s) within the land type averages exceeds Dutch guideline limits.

Table 2-9: Total metal concentrations for soils within the metal contaminated land type (mg kg^{-1}) carried out on a single bulked sample for each sampling location.

	Hg	As	Cd	Cr	Cu	Fe	Pb	Mn	Ni	Se	Zn
High metals	1.5	3200	11	94	170	33000	11000	300	62	34	1700
Low metals	0.31	29	0.56	78	58	3200	360	350	52	0.88	190

Correlation analysis based upon log transformed heavy metal concentrations from all soils showed high parametric and non-parametric relationships (Figure 2-4). This signifies that soils within land types which have elevated or contaminated levels of a heavy metal will inherently co-exhibit high levels of other heavy metals. No significant correlations were observed between physico-chemical characteristics (TOC, pH, WHC and MC) and total metal concentrations in soils, with the exception of Hg and pH where $r^2 = 0.70$, $p = 0.05$.

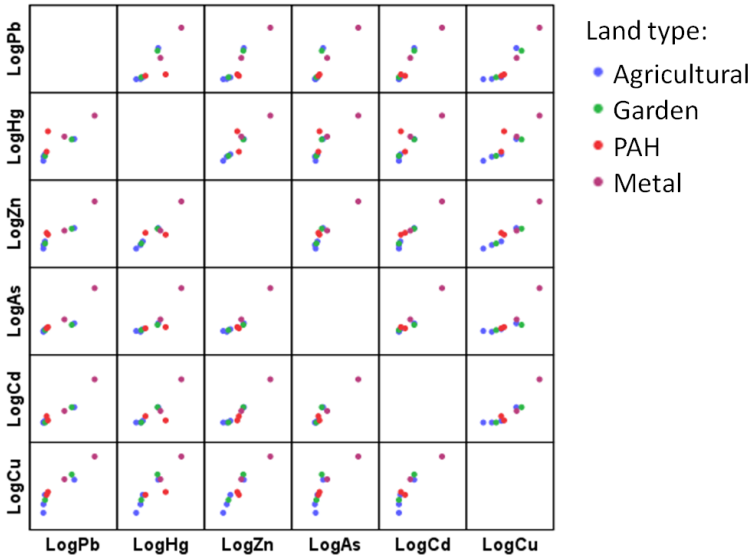


Figure 2-4: A selection of cross plots illustrating the parametric and non-parametric relationships between heavy metals concentrations (mg kg^{-1}) in the all soils studied.

2.3.3. *Total soil PAH concentrations*

The highest concentrations of the total 16 EPA priority PAHs (TPAHs) were found in the PAH contaminated land type (Table 2-10). Mean concentrations of TPAHs

ranged from 0.3 in agricultural soils to 85 mg kg^{-1} in PAH contaminated soils, but only PAH contaminated soils exceed the accepted Dutch contamination threshold of 40 mg kg^{-1} for TPAHs (Tim O'Hare Associates, 2002; VROM, 2000). Mean TPAH values and respective standard error from the metal contaminated land type indicates that one of the soils sampled also exceeds Dutch contamination thresholds. Inspection of individual metal contaminated soil TPAH concentrations showed that the high metals soil was in excess of the Dutch threshold at 44.8 mg kg^{-1}. This indicates that the metal contaminated land type may contain mixed pollution of both organic and inorganic origin. However, it must be noted that the Dutch threshold is based upon the sum of 10 individual PAHs, whereas PAH totals in this study are based on the sum of 16.

Table 2-10: Mean total PAH (TPAH) concentrations (mg kg^{-1}) of the land types in this study. Values are based upon the mean of 2 soil samples from PAH contaminated, metal contaminated and garden land types, and of four soils within the agricultural land type grouping.

	TPAH*
PAH contaminated	85 ± 52
Metal contaminated	29 ± 15
Agricultural	0.3 ± 0.2
Garden	31 ± 7

* based upon the sum of EPA 16 priority PAHs
[a] ± 1 x standard error

At the time of sampling the PAH contaminated land type, two soils were selected that potentially represented high and low TPAH contamination. TPAH results conclusively defined these soils as such (Table 2-11), with concentrations for the high PAH soil is far in excess of Dutch threshold limits (137.30 mg kg^{-1}) and the low PAH contaminated soil displaying TPAH levels below action limits (32.84 mg kg^{-1}). These results clearly display the variation in the level of PAH contamination across the PAH contaminated land type, that is reflected in its standard error (Table 2-10).

Table 2-11: TPAH concentrations for soils within the PAH contaminated land type (mg kg^{-1}) carried out on a single bulked sample for each sampling location.

	TPAH*
High PAH contaminated	137.30
Low PAH contaminated	32.84

* based upon the sum of EPA 16 priority PAHs

Correlation analysis demonstrated a significant positive relationship between log transformed TPAH values and soil pH where $r^2 = 0.85$, $p = 0.01$, and a significant negative relationship between log transformed TPAH and log transformed WHC where $r^2 = -0.83$, $p = 0.01$. No relationships were found between TPAH and metal concentrations.

2.3.4. *Comparison of soil microbial activity in soils characterised as contaminated and non-contaminated*

PAH and metal contaminated land types exhibited basal respiration (BR) rates which were significantly different and 340 times lower than rates observed in non-contaminated agricultural and garden land type soils (Figure 2-5a, one-way ANOVA, Tukey's HSD, $p < 0.000$). These results indicate that long term organic and inorganic pollution has negatively impacted microbial respiration. Maximum BR was found in garden (6.8 ± 1.2 $mgCO_2*h^{-1}*g^{-1}$ dry soil) followed by the agricultural land type soils (5.8 ± 0.8 $mgCO_2*h^{-1}*g^{-1}$ dry soil) which did not significantly differ from each other (one-way ANOVA, Tukey's HSD, $p > 0.97$). Metal contaminated soils had higher but not significantly different activities to PAH contaminated soils, at 0.022 ± 0.002 $mgCO_2*h^{-1}*g^{-1}$ dry soil and 0.009 ± 0.004 $mgCO_2*h^{-1}*g^{-1}$ dry soil respectively (one-way ANOVA, Tukey's HSD, $p > 0.19$).

Conversely, impacts of contamination upon microbial biomass carbon (MBC) were not apparent (Figure 2-5b). Metal contaminated soils contained the largest microbial biomass of all soils (1164 ± 219 mg $MBC*kg^{-1}$ dry soil), with PAH contaminated soils exhibiting the lowest (364 ± 234 mg $MBC*kg^{-1}$ dry soil). No statistical significant differences were detected between the soil MBC values of the different land types (one-way ANOVA, Tukey's HSD, $p > 0.2$).

Contaminated soils showed significantly lower metabolic quotient (qCO_2) ratios in comparison to non-contaminated land types (one-way ANOVA, Tukey's HSD, $p < 0.001$), thus, indicating that non-contaminated garden and agricultural land types are less microbially efficient than PAH and metal contaminated counterparts (Figure 2-5c). Mean qCO_2 ratios ranged from 20.3 ± 5.3 mg $CO_2*h^{-1}*g^{-1}$MBC in metal contaminated to 9049.5 ± 1018.1 mg $CO_2*h^{-1}*g^{-1}$MBC in agricultural land types.

Mean microbial quotient (*qmic*) ratios values ranged from 10.04 ± 2.36 mg $MBC*g^{-1}$TOC to 20.48 ± 8.82 mg $MBC*g^{-1}$TOC, increasing in the order: garden > PAH contaminated > metal contaminated > agricultural (Figure 2-5d). However, no significant differences were observed in *qmic* ratios between all land types (one-way ANOVA, Tukey's HSD, $p > 0.5$). This suggests that the microbial biomass makes the same contribution to organic carbon between the contaminated and non-contaminated soils and that the same proportion of TOC is available in all land types for biomass production.

Correlation analysis between BR, MBC, qCO_2, *qmic,* physico-chemical properties, TPAHs and total metal concentrations showed no significant correlations. Therefore, differences in BR rates and qCO_2 could not be attributed to any measured factor.

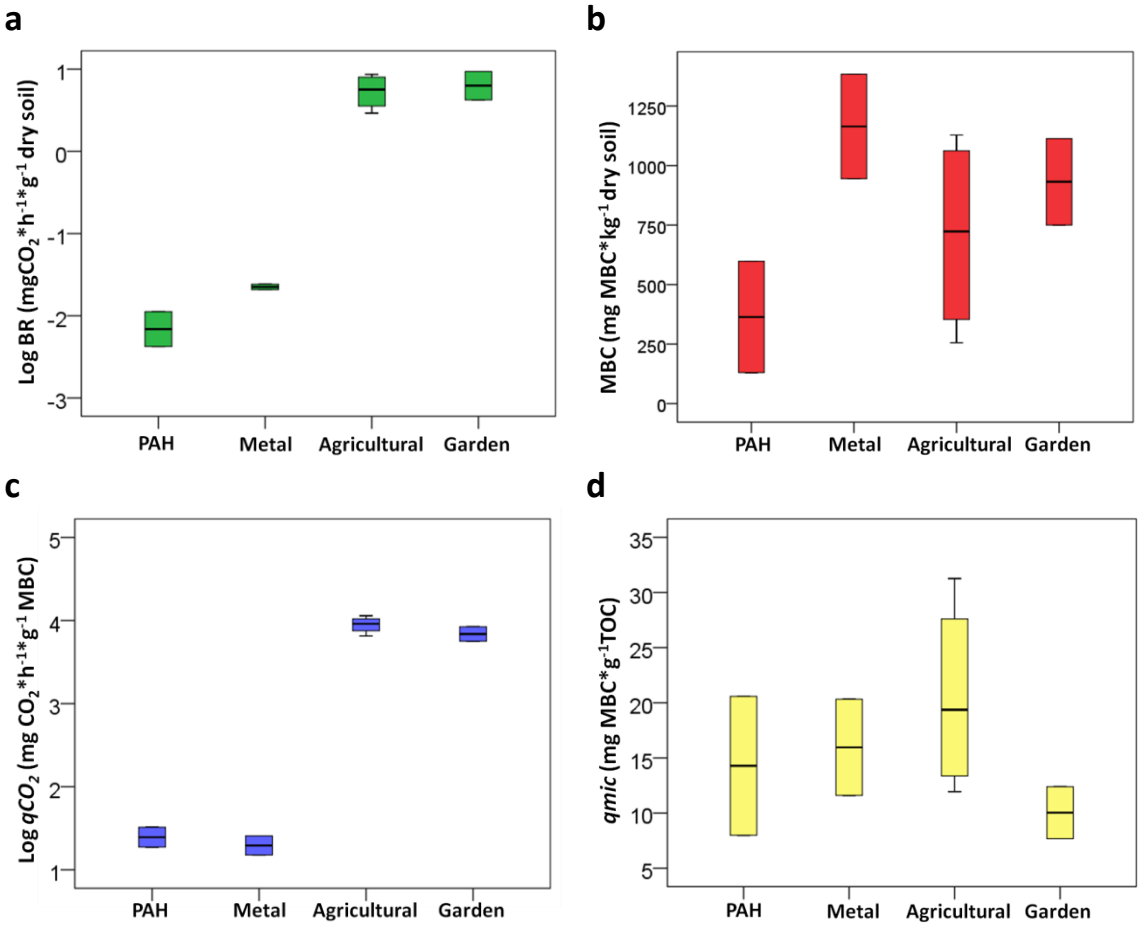


Figure 2-5: Box plots representing (a) logged values of basal respiration (BR), (b) non-logged values of microbial biomass carbon (MBC), (c) logged values of metabolic quotient (qCO_2) and (d) non-logged values of microbial quotient (*qmic*) across the different land types examined in this study. Box plots presented represent the minimum value, first quartile (25th percentile), median (50th percentile), third quartile (75th percentile) and the maximum value of each reported microbial parameter.

2.3.5. *Comparison of microbial community structure and diversity in soils characterised as contaminated and non-contaminated*

DGGE profiles of bacterial 16S rRNA PCR products showed that all soils within each land type harboured diverse predominant bacterial communities (Figure 2-6). Analysis of predominant bacterial community composition and diversity within the contaminated land type indicated that long term metal and PAH contamination had not adversely affected these parameters.

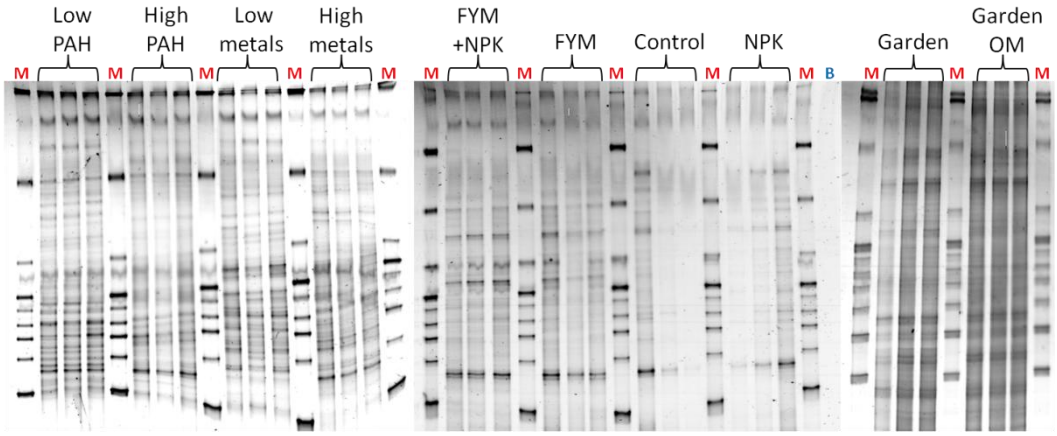


Figure 2-6: 16S rRNA DGGE profiles for PAH contaminated (low PAH, high PAH), metal contaminated (low metal, high metal), agricultural (FYM +NPK, FYM, Control, NPK) and garden (garden, garden OM) soils. Where **M** = marker lanes and **B** = procedural blank (carried through DNA extraction, PCR amplification and DGGE).

The predominant bacterial communities of contaminated soils were comparable and therefore not significantly different to garden soils in terms of Margalef's richness (*d*), Pielou's evenness (*J'*) and both Shannon's (*H'*) and Simpson's (*1-λ'*) diversity indices (one-way ANOVA, Tukey's HSD; $p > 0.3$, Table 2-12). Agricultural soils harboured significantly lower bacterial diversity indices in comparison to both contaminated and garden land types, indicating lower levels of the predominant community members [*d* (one-way ANOVA, Tukey's HSD, $p < 0.05$), *J'* (one-way ANOVA, Tukey's HSD, $p < 0.01$), *H'* and *1-λ'* (one-way ANOVA, Tukey's HSD, $p < 0.000$)]. Correlation analysis of diversity indices (*d*, *J'*, *H'* and *1-λ'*) showed no significant relationships with physico-chemical measurements, metal and total PAH concentrations, BR, MBC or eco-physiological ratios (*qmic* and qCO_2).

Table 2-12: Mean diversity indices derived from DIVERSE analysis of bacterial 16S rRNA DGGE profiles of the four land types analysed in this study. Indices were calculated based upon the total number of DGGE Bands (*S*) and the sum of relative band intensities (*N*), to give an estimate of Margalef's richness (*d*), Pielou's evenness (*J'*), Shannon's diversity (*H'*) and Simpson's diversity (*1-λ'*).

	S	***N***	***d***	***J'***	***H'***	***1-λ***
PAH [a]	25.50 ± 1.45	1292.05 ± 164.89	3.43 ± 0.15	0.93 ± 0.01	2.99 ± 0.07	0.94 ± 0.00
Metal [a]	26.83 ± 1.40	1283.35 ± 64.01	3.61 ± 0.20	0.95 ± 0.00	3.11 ± 0.06	0.95 ± 0.00
Agricultural [b]	18.25 ± 1.51	487.24 ± 81.08	2.84 ± 0.17	0.89 ± 0.01	2.54 ± 0.07	0.90 ± 0.01
Garden [a]	28.50 ± 1.41	727.77 ± 79.40	4.18 ± 0.15	0.95 ± 0.01	3.17 ± 0.05	0.95 ± 0.00

[a] Mean and ± 1x standard error based upon 6 samples i.e. triplicate samples of each 2 soil samples within defined land type.
[b] Mean and ± 1x standard error based upon 12 samples i.e. triplicate samples of each 4 soil samples within the agricultural land type.

Bacterial communities formed distinct clusters based on their geographical sampling locations (Figure 2-7), the MDS plot stress values was below 0.2 and therefore indicated a good spatial representation of the data. Analysis of Similarity (ANOSIM) showed that the strongest effect on predominant bacterial community composition was due to sampling location (Global R = 0.73, p = 0.1 %), followed by land type (Global R = 0.66, p = 0.1 %) and whether soils were urban or rural in location (Global R = 0.48, p = 0.1 %). Metal and PAH impacted soils showed similar community composition to each other (60 - 40 %) suggesting that different contaminant types do not impact bacterial community structure.

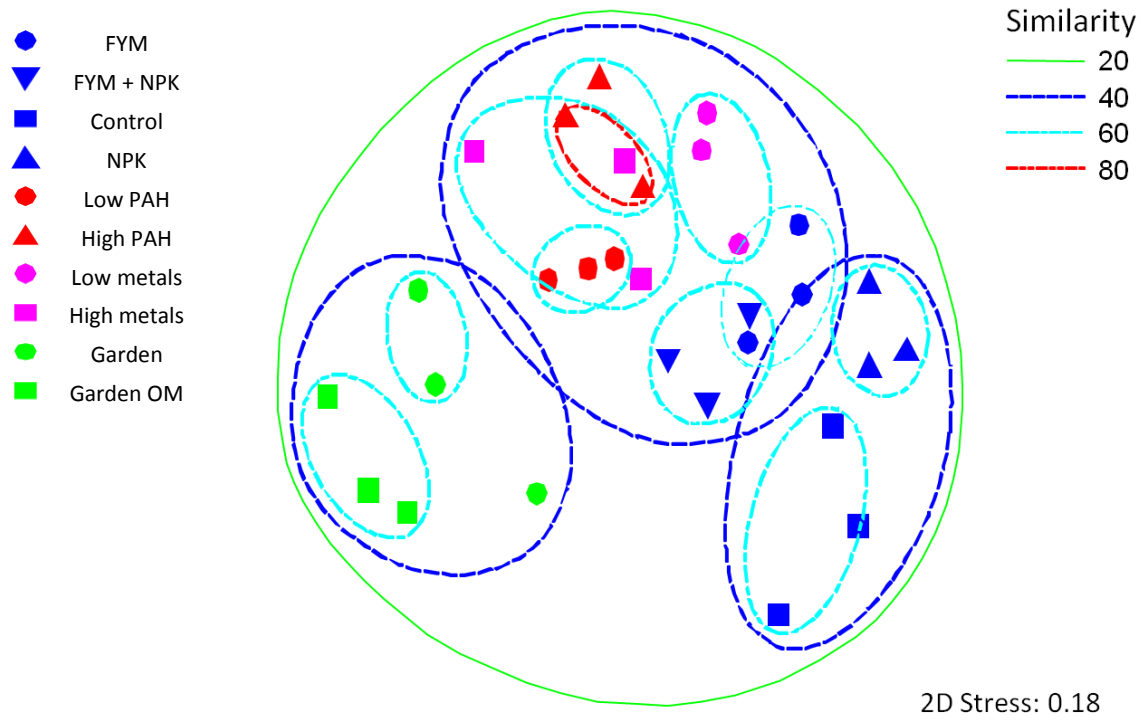


Figure 2-7: A 2D MDS plot showing a spatial representation of the similarity of predominant bacterial 16S rRNA community structures from DGGE profiles of agricultural (blue), garden (green), PAH (red) and metal (pink) contaminated land types. Similarity contour lines from cluster analysis are overlaid on the MDS plot.

BIOENV analysis indicated that TOC and pH were strongest and most statistically significant drivers of separation between the predominant bacterial community compositions in all soils (rho = 0.40, $p < 0.01$, Table 2-13). TPAH concentrations between all soils also showed a statistically significant correlation with community dissimilarity (rho = 0.26, $p < 0.05$). However, Spearman's rho correlation coefficients were weaker (i.e. closer to 0), suggesting that PAHs were not strongly influencing community composition between all soils. Interestingly, total metal and microbial activity parameters did not show any statistical influence on the spatial arrangement of bacterial community structures in all soils. Furthermore, BIOENV analysis of PAH and metal contaminated soils only, showed that total metal and PAH concentrations had no significant effect on the observed dissimilarities between predominant bacterial community structures. Instead, community dissimilarity between metal and PAH contaminated sites was found to be strongly and significantly driven by the physico-chemical variables of WHC and pH.

Table 2-13: Summary of the highest Spearman's rho correlations from BIOENV analysis determined between *a priori* defined environmental variables and bacterial 16S rRNA presence absence community data. Statistically significant parameters are highlighted in bold.

Soil	Variable type*	No. of variables	Best variable combinations	Spearman's rho	P-value
All	**Physico-chemical [a,1]**	**2**	**TOC + pH**	**0.40**	**0.001**
	Microbial [b,2]	1	BR	0.25	0.37
	TPAHs [c,3]	**1**	**TPAHS**	**0.26**	**0.03**
	Metals [d,3]	2	Cr + Cu	0.08	0.8
Contaminated only	**Physico-chemical**	**2**	**WHC + pH**	**0.57**	**0.01**
	Microbial	1	*qmic*	0.93	0.08
	TPAHs	1	TPAHs	0.58	0.12
	Metals	1	Fe	0.46	0.96

*All variables (excluding pH) were log transformed and normalised.
[a] Variables tested were pH, total organic carbon (TOC), water holding capacity (WHC) and moisture content (MC).
[b] Variables tested were microbial biomass carbon (MBC), basal respiration (BR), metabolic quotient (qCO_2) and microbial quotient (*qmic*).
[c] Variable tested was the sum of 16 EPA priority PAHs (TPAHs).
[d] Variables tested were Hg, As, Cd, Cr, Cu, Fe, Pb, Mn, Ni, Se and Zn.
[1] Analysis based upon individual physico-chemical results for each triplicate sample. Therefore, comparison of Bray Curtis and environmental Euclidean distance matrixes based upon all triplicate samples for each sampling location.
[2] Analysis based upon mean microbial activity measurements therefore comparison of Bray Curtis and environmental Euclidean distance matrixes which included one individual from within triplicate samples to avoid false replication.
[3] Analysis based upon a single value for TPAHs and individual metal concentrations therefore comparison of Bray Curtis and environmental Euclidean distance matrixes which included one individual from within triplicate samples to avoid false replication.

2.3.6. *Comparison of the potential nitrogen transformations of nitrification and denitrification on soils characterised as contaminated and non-contaminated*

Contaminated soils exhibited denitrification enzyme activity (DEA) rates which were over 50 times lower than that of non-contaminated soils (Figure 2-8a). DEA ranged from 0.21 ± 0.1 µg $N*h^{-1}*g^{-1}$ dry soil in the metal contaminated soils to 13.7 ± 5.0 µg $N*h^{-1}*g^{-1}$ dry soil in the agricultural soils. Significant differences in DEA were observed between contaminated and non-contaminated land types (one-way ANOVA, Tukey's HSD, $p < 0.01$), but not between soils within these land types (one-way ANOVA, Tukey's HSD, $p > 0.9$). Correlation analysis of DEA with physico-chemical properties and total metal concentrations showed no significant correlations. However, DEA did show a significant negative correlation with TPAHs at -0.66 where $p < 0.05$, with both values log transformed.

In contrast, no affect of contamination was apparent on PNR activities, as mean rates between the four land types did not significantly differ from each other (one-way ANOVA, Tukey's HSD, $p > 0.25$, Figure 2-8b). Correlation analysis showed that PNR significantly correlated with TOC ($r^2 = 0.7$, $p < 0.01$) and demonstrated that PNR was maximal at circum-neutral pH, with values above and below this showing an inhibitory effect upon rates (Figure 2-9). PNR also showed a positive correlation with Cu ($r^2 = 0.66$, $p < 0.05$), but no significant effects of other metals were apparent. No significant correlation was found between PNR and TPAHs ($p > 0.7$).

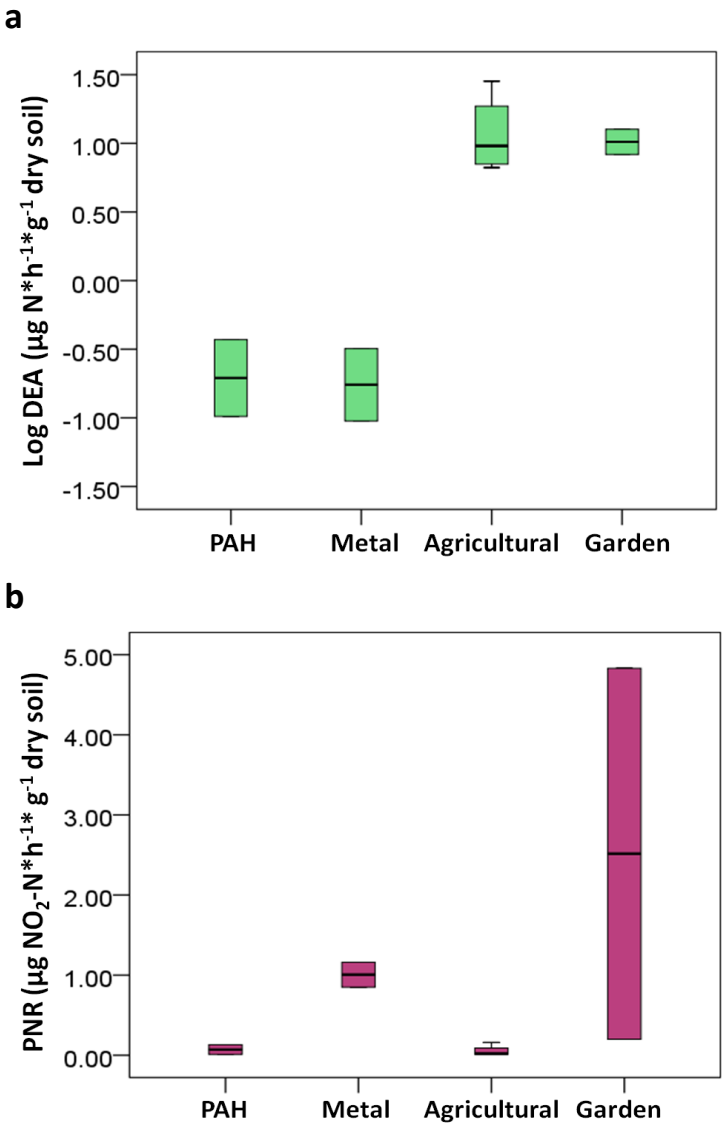


Figure 2-8: Box plots showing (a) log transformed average values for denitrification enzyme activity (DEA) and (b) non-logged average values for potential nitrification rate (PNR) between land types examined in this study. Box plots presented represent the minimum value, first quartile (25th percentile), median (50th percentile), third quartile (75th percentile) and the maximum value of each reported microbial parameter.

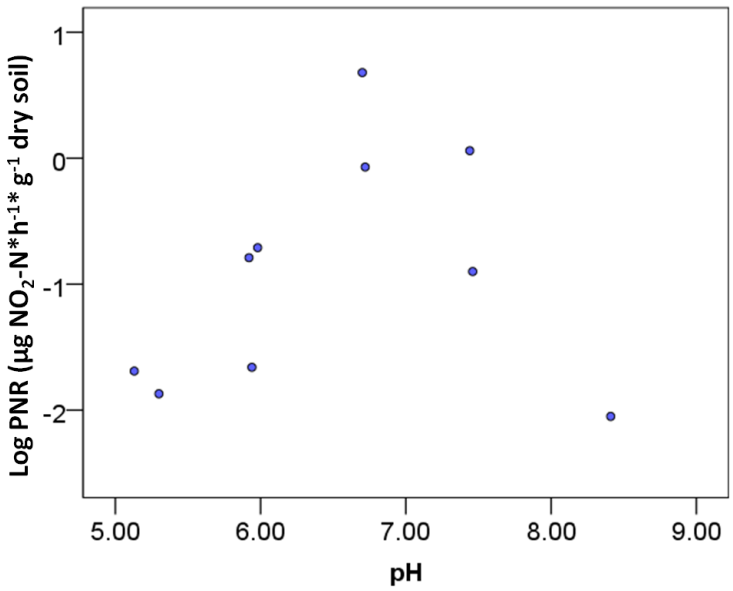


Figure 2-9: Relationship between pH and log transformed PNR values within the 10 soils sampled in this study.

2.3.7. *Comparison of potential nitrifying and denitrifying rates with associated functional group diversity and structure in contaminated and non-contaminated soils*

DGGE profiles of *nirS* and *nirK* PCR products showed that all soils within each land type harboured denitrifying populations (Figure 2-10 and Figure 2-11). MDS analysis of DGGE profiles showed a clear spatial separation of *nirK* genes between contaminated and non-contaminated sites (Figure 2-12a). However, the same spatial pattern was not evident for *nirS* genes (Figure 2-12b). MDS plot stress values were below or equal to 0.2 and therefore indicated a good spatial representation of the data. ANOSIM confirmed high similarity between triplicates for *nirK* (Global R = 0.83, p = 0.1%) and *nirS* genes (Global R = 0.97, p = 0.1%) for each sampling location. Predominant *nirS* community structure appeared to be mainly driven by land type (Global R = 0.65, p = 0.1 %), however, land type had a less strong effects upon *nirK* community structure (Global R = 0.64, p = 0.1%)

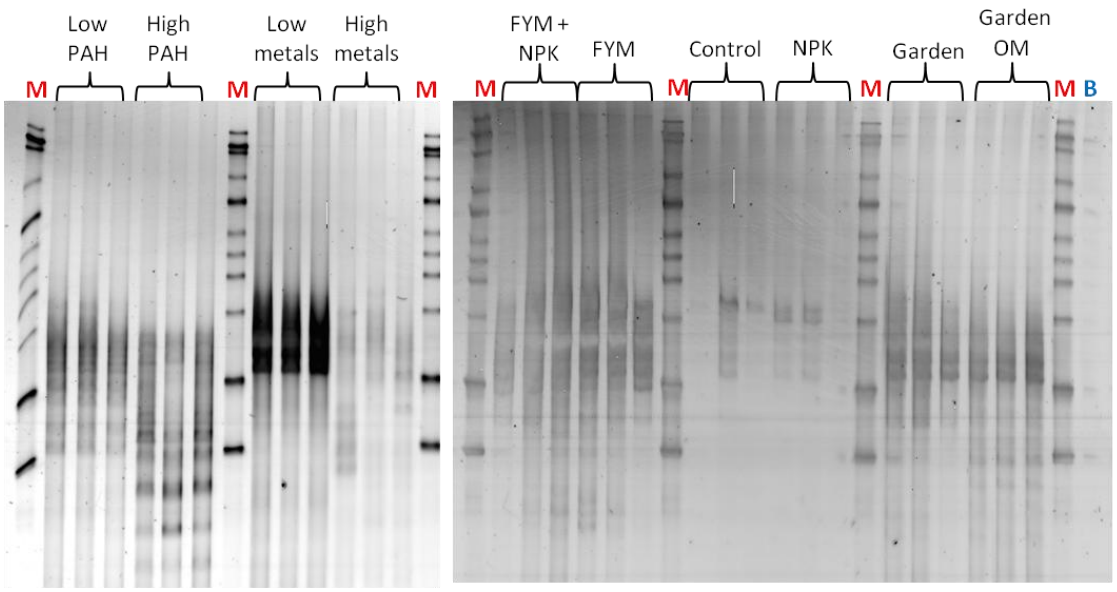


Figure 2-10: Nitrite reductase (*nirS*) DGGE profiles for PAH contaminated (low PAH, high PAH), metal contaminated (low metal, high metal), agricultural (FYM +NPK, FYM, control, NPK) and garden (garden, garden OM). Where **M** = marker lanes and **B** = procedural blank (carried through DNA extraction, PCR amplification and DGGE).

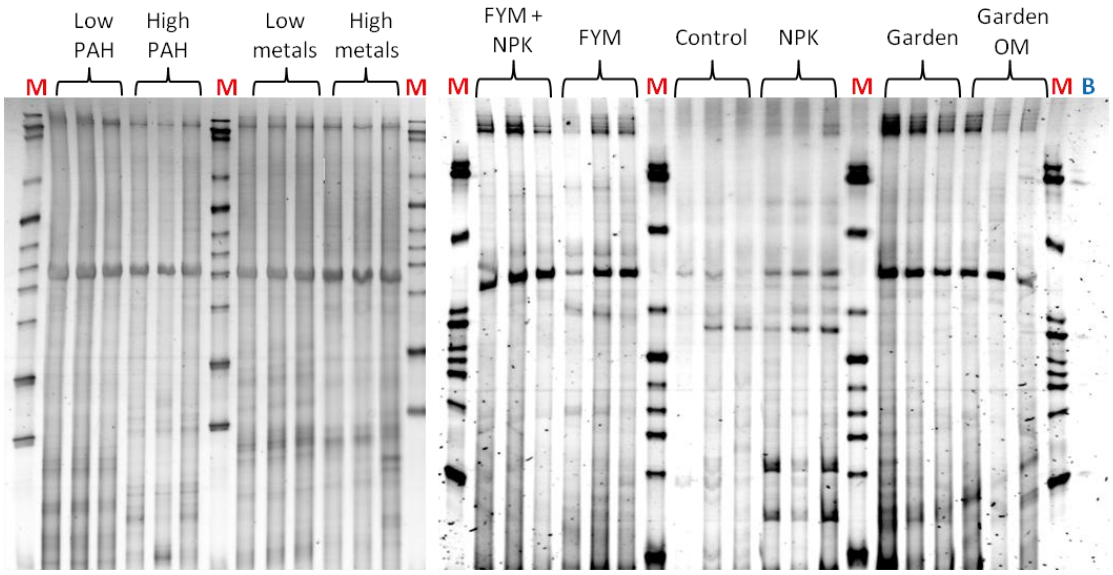


Figure 2-11: Nitrite reductase (*nirK*) DGGE profiles for PAH contaminated (low PAH, high PAH), metal contaminated (low metal, high metal), agricultural (FYM +NPK, FYM, control, NPK) and garden (garden, garden OM). Where **M** = marker lanes and **B** = procedural blank (carried through DNA extraction, PCR amplification and DGGE).

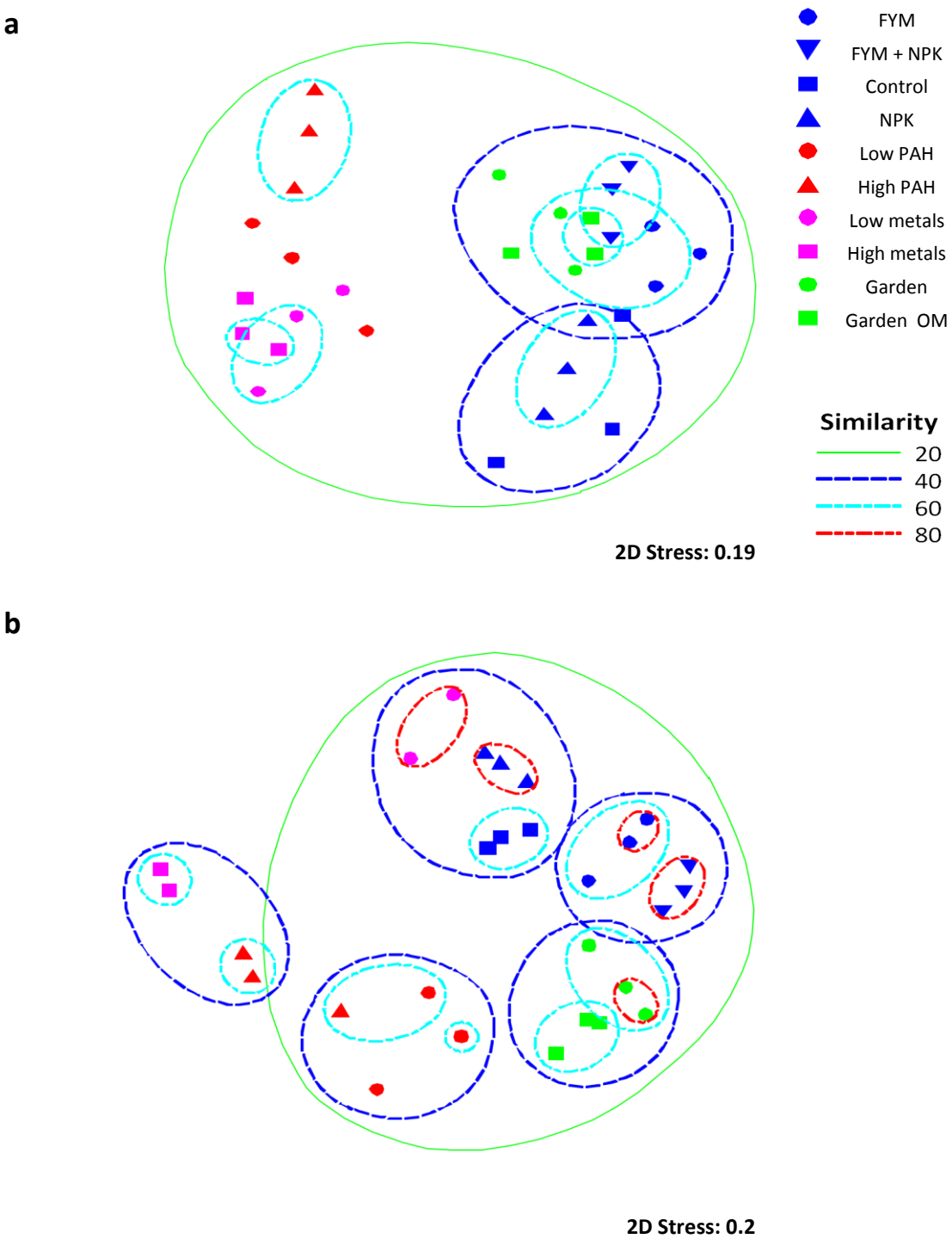


Figure 2-12: A 2D MDS plot showing a spatial representation of the similarity between DGGE derived community structures based on analysis of (a) *nirK* and (b) *nirS* gene fragments of agricultural (blue), garden (green), PAH (red) and metal (pink) contaminated land types. Similarity contour lines from cluster analysis are overlaid on both MDS plots. High and low metal soils are only represented by 2 points on MDS plot as third underlies one point (i.e. showing a 100 % similarity).

BIOENV analysis (Table 2-14) established that DEA was the main environmental factor defining the observed separation of *nirK* communities between contaminated and non-contaminated soils in MDS plots (rho = 0.63, $p < 0.01$). Therefore, indicating that potential activity is coupled to *nirK* community composition. The composition of *nirS* communities was found to be mainly explained by the combination of WHC and pH. DEA did show a statistically significant, although weaker, correlation with *nirS* community composition (rho = 0.47, $p < 0.01$). Both total metals and TPAHs did not show any statistical significance on the structure of the *nirK* or *nirS* community with p-values in excess of 0.05.

Table 2-14: Summary of highest Spearman's rho correlations from BIOENV analysis between *a priori* defined environmental variables, and *nirK* and *nirS* functional gene presence/absence community data. Statistically significant parameters are highlighted in bold.

Functional gene	Variable type*	No. of variables	Best variable combination(s)	Spearman's rho	p-value
nirK	**Physico-chemical [a, 1]**	**1**	**pH**	**0.56**	**0.01**
	DEA [b,2]	**1**	**DEA**	**0.63**	**0.01**
	TPAHS [c,3]	1	TPAHS	0.27	0.08
	Metals [d,3]	1	Cr	0.39	0.10
nirS	**Physico-chemical**	**2**	**WHC/pH**	**0.51**	**0.01**
	DEA	**1**	**DEA**	**0.47**	**0.01**
	TPAHS	1	TPAHS	0.21	0.08
	Metals	4	Hg/Cd/Fe/Se	0.62	0.06

*All variables (excluding pH) were log transformed and normalised.
[a] Variables tested were pH, total organic carbon (TOC), water holding capacity (WHC) and moisture content (MC).
[b] Variable tested was denitrification enzyme activity (DEA).
[c] Variable tested was the sum of 16 EPA priority PAHs (TPAHs).
[d] Variables tested were Hg, As, Cd, Cr, Cu, Fe, Pb, Mn, Ni, Se and Zn.
[1] Analysis based upon individual physico-chemical results for each triplicate sample. Therefore, comparison of Bray Curtis and environmental Euclidean distance matrixes based upon all triplicate samples for each sampling location.
[2] Analysis based upon individual DEA results for each triplicate sample. Therefore, comparison of Bray Curtis and environmental Euclidean distance matrixes based upon all triplicate samples for each sampling location.
[3] Analysis based upon a single value for TPAHs and individual metal concentrations. Therefore, comparison of Bray Curtis and environmental Euclidean distance matrixes based upon one individual from within triplicate samples to avoid false replication.

Bubble plots show that DEA increases from left to right on the X-axis for both *nirK* and *nirS* genes (Figure 2-13). This visually confirms the strong statistically significant separation of the *nirK* communities with DEA.

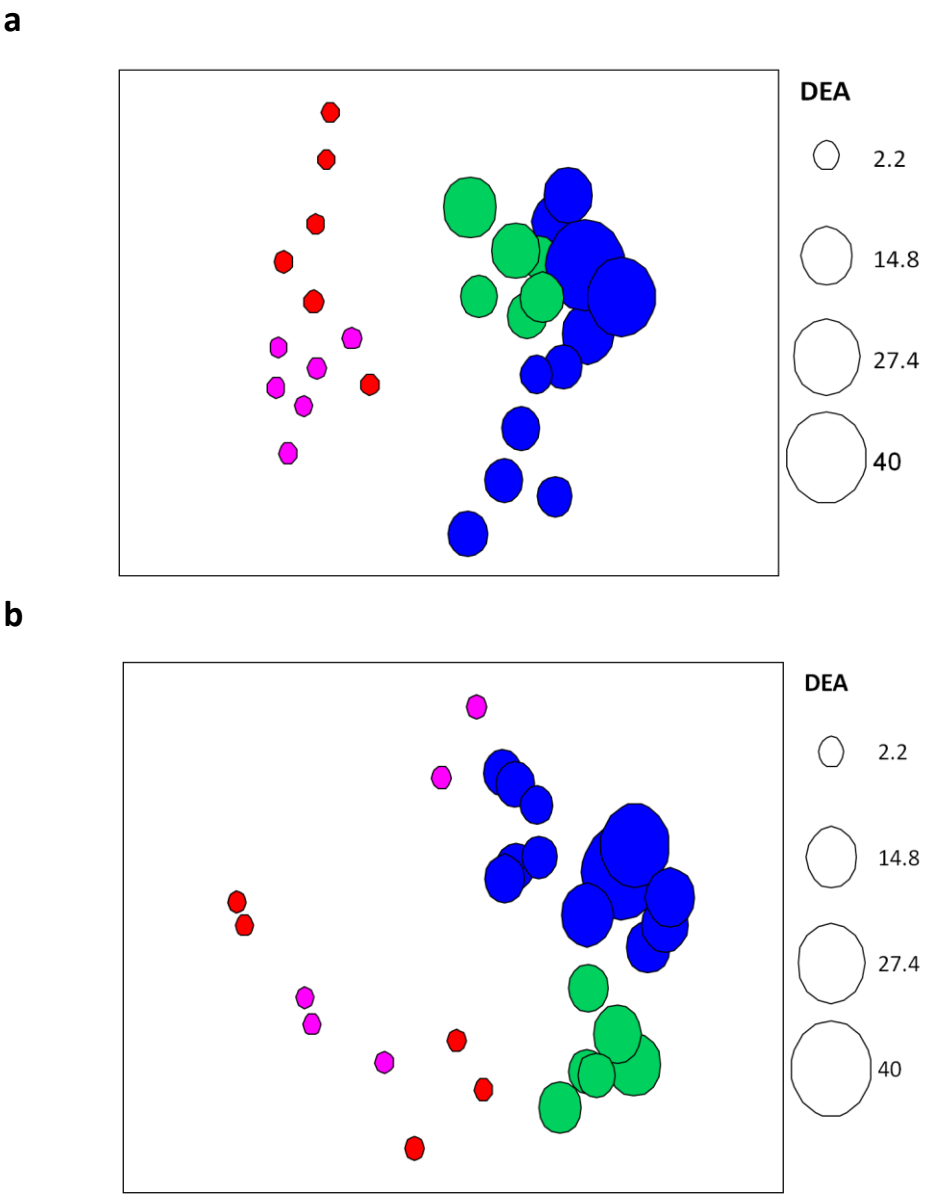


Figure 2-13: MDS ordinations of (a) *nirK* and (b) *nirS* community structure from all soils sampled, superimposed with bubble plots of denitrification enzyme activity (DEA). Size of bubbles indicates rate of DEA (µg $N*h^{-1}*g^{-1}$ dry soil) with fill colour representing land type: agricultural (blue), garden (green), PAH (red) and metal (pink) contaminated.

The predominant *nirK* denitrifying communities of contaminated soils showed significantly higher values of Margalef's richness (*d*) in comparison to non-contaminated soils (one-way ANOVA, Tukey's HSD, $p < 0.000$, Table 2-15). However,

they did not statistically differ to garden soils in terms of Pielou's evenness (*J'*), or Shannon (*H'*) and Simpson (*1-λ*) diversity indices (one-way ANOVA, Tukey's HSD, $p > 0.3$), but were significantly higher than agricultural soils (one-way ANOVA, Tukey's HSD, $p < 0.05$). Correlation analysis of *nirK* diversity indices showed no significant relationships with physico-chemical factors (pH, TOC, WHC and MC), TPAHs, DEA or total soil metal concentrations.

In contrast, predominant *nirS* diversity indices showed that contaminated soils possessed significantly lower values of *d*, *H'* and *1-λ*, in comparison to non-contaminated soils (one-way ANOVA, Tukey's HSD, $p < 0.05$, Table 2-15). However, contaminated soils expressed significantly higher values of *J'* in comparison to garden soils, showing less variation in *nirS* community composition (one-way ANOVA, Tukey's HSD, $p < 0.05$). Correlation analysis of *nirS* diversity indices with environmental parameters showed no relationships with total soil metal concentrations, TPAHs, DEA, WHC or pH. Only TOC showed a negative relationship with *J'*, where $r^2 = -0.77$, $p < 0.01$. Positive relationships were also observed between S, *H'*, *1-λ* and MC (where $r^2 = 0.76$, 0.64, 0.64 respectively; $p < 0.05$).

Table 2-15: Mean diversity indices derived from DIVERSE analysis of *nirK* and *nirS* DGGE profiles. Indices were calculated based upon the total number of DGGE bands (*S*) and the sum of relative band intensities (*N*), to give an estimate of Margalef's richness (*d*), Pielou's evenness (*J'*), Shannon's diversity (*H'*) and Simpson's diversity (*1-λ'*) .

		S	***N***	***d***	***J'***	***H'***	***1-λ***
nirK	PAH[a]	22.67 ± 2.78	316.03 ± 20.62	3.75 ± 0.44	0.86 ± 0.01	2.67 ± 0.13	0.89 ± 0.02
	Metal[a]	23.83 ± 1.62	373.80 ± 13.21	3.85 ± 0.25	0.87 ± 0.01	2.75 ± 0.09	0.91 ± 0.01
	Agricultural[b]	16.17 ± 0.72	999.03 ± 102.17	2.21 ± 0.08	0.85 ± 0.01	2.35 ± 0.05	0.87 ± 0.01
	Garden[a]	21.50 ± 0.72	1403.95 ± 69.13	2.83 ± 0.08	0.89 ± 0.01	2.71 ± 0.04	0.92 ± 0.00
nirS	PAH	9.00 ± 0.45	203.18 ± 24.63	1.51 ± 0.06	0.94 ± 0.01	2.06 ± 0.06	0.86 ± 0.01
	Metal	9.33 ± 0.61	351.63 ± 131.32	1.75 ± 0.31	0.89 ± 0.03	1.97 ± 0.05	0.84 ± 0.01
	Agricultural	13.83 ± 0.78	164.68 ± 20.94	2.56 ± 0.13	0.92 ± 0.00	2.40 ± 0.05	0.90 ± 0.01
	Garden	19.33 ± 0.61	296.80 ± 21.65	3.23 ± 0.12	0.84 ± 0.02	2.49 ± 0.05	0.89 ± 0.01

[a] Mean and ± 1x standard error based upon 6 samples i.e. triplicate samples of each 2 soil samples within defined land types.
[b] Mean and ± 1x standard error based upon 12 samples i.e. triplicate samples of each 4 soil samples within the agricultural land type.

Results for ammonia-oxidiser communities are presented by each soil sampling location rather than land type. Amplification of ammonia-oxidising bacteria (AOB) *amoA* gene fragments was only successful for metal and PAH contaminated soils. Ammonia-oxidising archaea (AOA) *amoA* gene fragments were successfully amplified from all PAH, metal and garden soils, however, only FYM + NPK and FYM soils from agricultural samples showed detectable AOA. Several additional attempts to amplify AOB *amoA* from garden and agricultural soils and AOA *amoA* from control and NPK soils proved unsuccessful. Results therefore indicated that ammonia oxidation in agricultural and garden soils is being dominated by AOA.

The inability to successfully amplify AOA/AOB *amoA* genes from the aforementioned soils may indicate that the abundance of these functional genes were below the detection limits of conventional PCR. However, it may also be due to poor primer coverage for AOA/AOB communities. It is likely that primers used in this study, and also all primers that have been designed to target AOA/AOB *amoA* functional genes, do not actually target all ammonia-oxidising populations present in the environment. It has been widely reported that actual AOA/AOB abundance and diversity may be underestimated and limited by the coverage of *amoA* based primers, particularly for AOA, as presently their actual coverage has not been well established (Hornek *et al.*, 2006; Mertens *et al.*, 2009). This is due to a limited number of full length *amoA* sequences from cultured AOB and AOA in existence, thus, it is impossible to determine what proportion of AOA/AOB communities current primers actually target (Hornek *et al.*, 2006; Stephen *et al.*, 1999).

DGGE profiles for AOA *amoA* gene fragments have been described through visual comparison. DGGE gel markers used in this study did not resolve in the gradient gel required for optimal band resolution of AOA *amoA* gene fragments (Figure 2-14 a, b). This meant that variations within and between gels could not be accounted for, thus, alignment of multiple gels was not possible. Therefore, AOA community structure and diversity could not be statistically interrogated with the established univariate and multivariate methods utilised in this study.

Visual analysis shows that both level and type of contamination within PAH and metal contaminated soils has had a very different effect on AOA and AOB ecology. Both soils from low contaminant areas (low PAH and low metals) clearly show strong and diverse communities of AOA and AOB present, which are both different to each

other, and to AOA found in garden and FYM/FYM+NPK soils (Figure 2-14 a,b,c). Highly PAH contaminated soils exhibited very similar predominant community compositions of AOA and AOB to low PAH soils, though band intensity is much weaker. This indicates that increased PAH contamination may have negatively affected both AOB and AOA population numbers. Furthermore, this is not believed to be an inhibition effect of PAHs upon PCR amplification. All other functional gene and total bacterial PCRs were successfully amplified for high PAH soil triplicates without a difference in PCR product band intensity observed. Additionally, dilution of template DNA to formally assess inhibition of PCR amplification only indicated weakened PCR product band intensity due to decreasing template (data not shown).

Profiling of AOA and AOB communities for highly contaminated metal soils indicated a clear difference in the relative dominance of AOA and AOB with increasing total metal concentrations. Strong and diverse AOA band profiles detected in low metals soils disappear in high metals soils. AOB community composition in high metals soils remains diverse with high band intensity. Nevertheless, banding patterns of high metals soils differ to low metals soils, signifying a difference in AOB community composition which was confirmed by MDS analysis of AOB communities (Figure 2-14d). MDS plot stress values were close to 0.0 and therefore indicated an excellent spatial representation of the data. ANOSIM analysis of the AOB MDS plot statistically confirmed significant differences between the contaminated soils rather than within soils (Global R = 0.79, p = 0.002).

BIOENV analysis (data not shown) of AOB communities within contaminated soils showed a moderately statistically significant relationship potential nitrification rates (PNR) (rho = 0.24, p = 0.06), and moisture content (rho = 0.40, p = 0.05). No significant relationships were observed between total metal concentrations (rho = 0.94, p = 0.4) or TPAHs (rho = -0.40, p = 0.7). Ecological diversity indices were also calculated for AOB (data not shown) but no significant differences were found between the contaminated soils (one-way ANOVA, Tukey's HSD, p > 0.3).

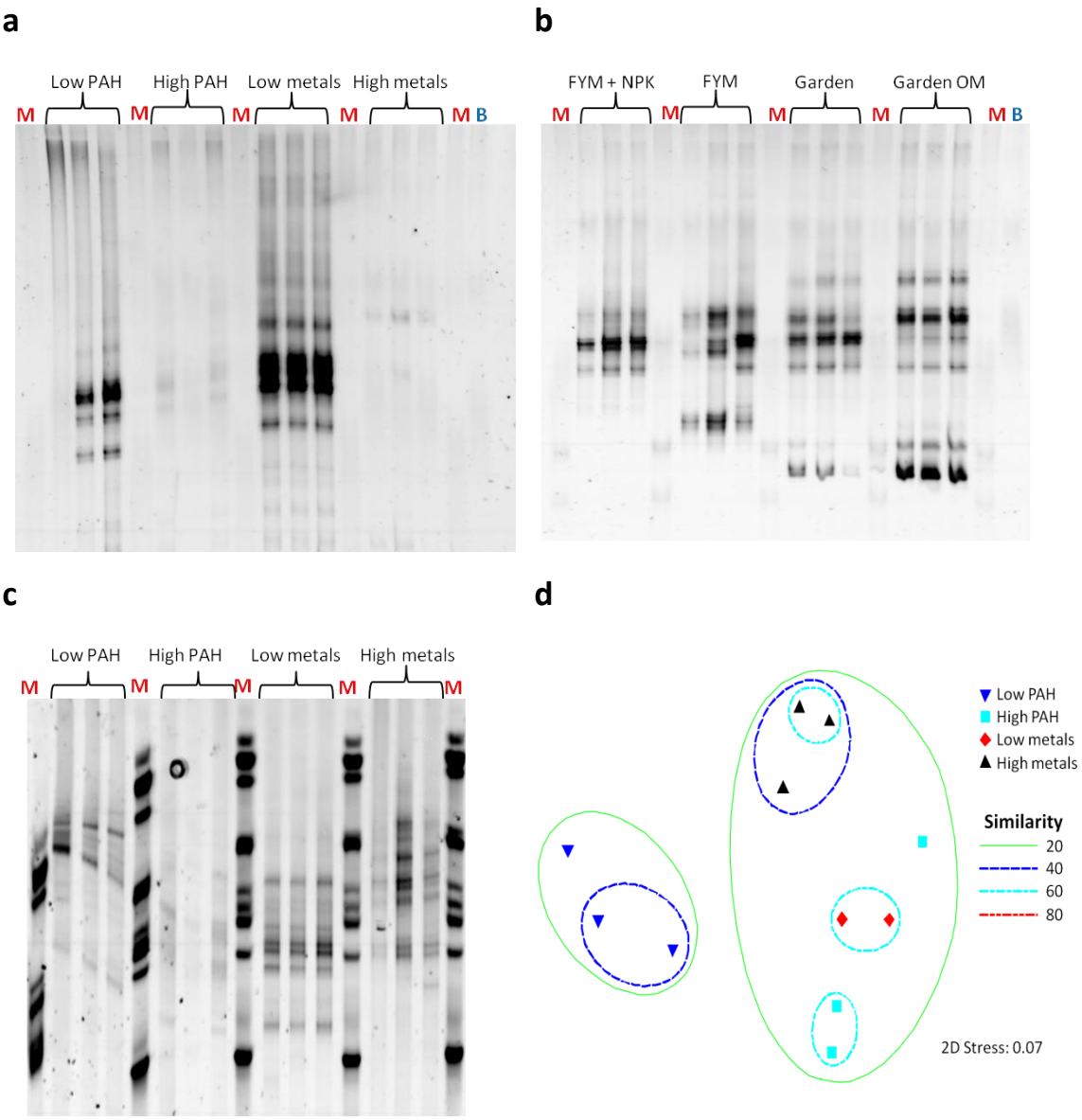


Figure 2-14: DGGE profiles of (a) AOA *amoA* gene fragments from PAH (low PAH, high PAH) and metal contaminated soils (low metals, high metals). (b) AOA *amoA* gene fragments from agricultural and garden soils (FYM +NPK, FYM, garden, garden OM). (c) AOB *amoA* gene fragments from PAH (low PAH, high PAH) and metal contaminated soils (low metals, high metals). **M** = represents marker lanes in profiles and **B** = procedural blank (carried through DNA extraction, PCR amplification and DGGE). (d) 2D MDS plot showing a spatial representation of the similarity between AOB *amoA* community structures from DGGE profile (c) using non-metric multidimensional scaling analysis. Similarity contour lines from cluster analysis are overlaid on both MDS plots. Low metals soil is only represented by 2 points on MDS plot as third underlies one point (i.e. showing a 100 % similarity).

Thirteen AOB bands were excised (five from low Pb metals, six from high metals, two from low PAH), all of which were of high quality. Sequences gave average read lengths of 450 bp, with one shorter read of 326 bp. Blast and Fasta sequence classifications confirmed that all excised bands were betaproteobacterial ammonia monooxygenase subunit A (*amoA*) genes. AOB *amoA* gene sequences obtained were closely related to the genus *Nitrosospira* (Figure 2-15). No closest cultured relatives were found, but gene fragments did relate to uncultured *amoA* genes from various soil environments, suggesting that AOB populations found are probably ubiquitous within soil environments (Figure 2-15). Furthermore, the phylogeny showed that the contamination level in metals soils does not select for a special high metal resistant AOB lineage.

Twelve AOA *amoA* DGGE bands were sequenced from the low metal and PAH contaminated soils (three from low PAH, nine from low metals). As agricultural AOA *amoA* DGGE profiles appeared to have different communities to long term contaminated soils it was hypothesised that AOA may form a distinct cluster, or, be phylogenetically affiliated to other low level contaminated environments. Sequences ranged from 310-561 bps of good quality, and were confirmed via Blast and Fasta analysis as partial crenarchaeote *amoA* genes. All sequences were distributed within the soil/sediment cluster and were affiliated to varying soil environments indicating that long term contamination did not select for any distinct cluster of AOA (Figure 2-16).

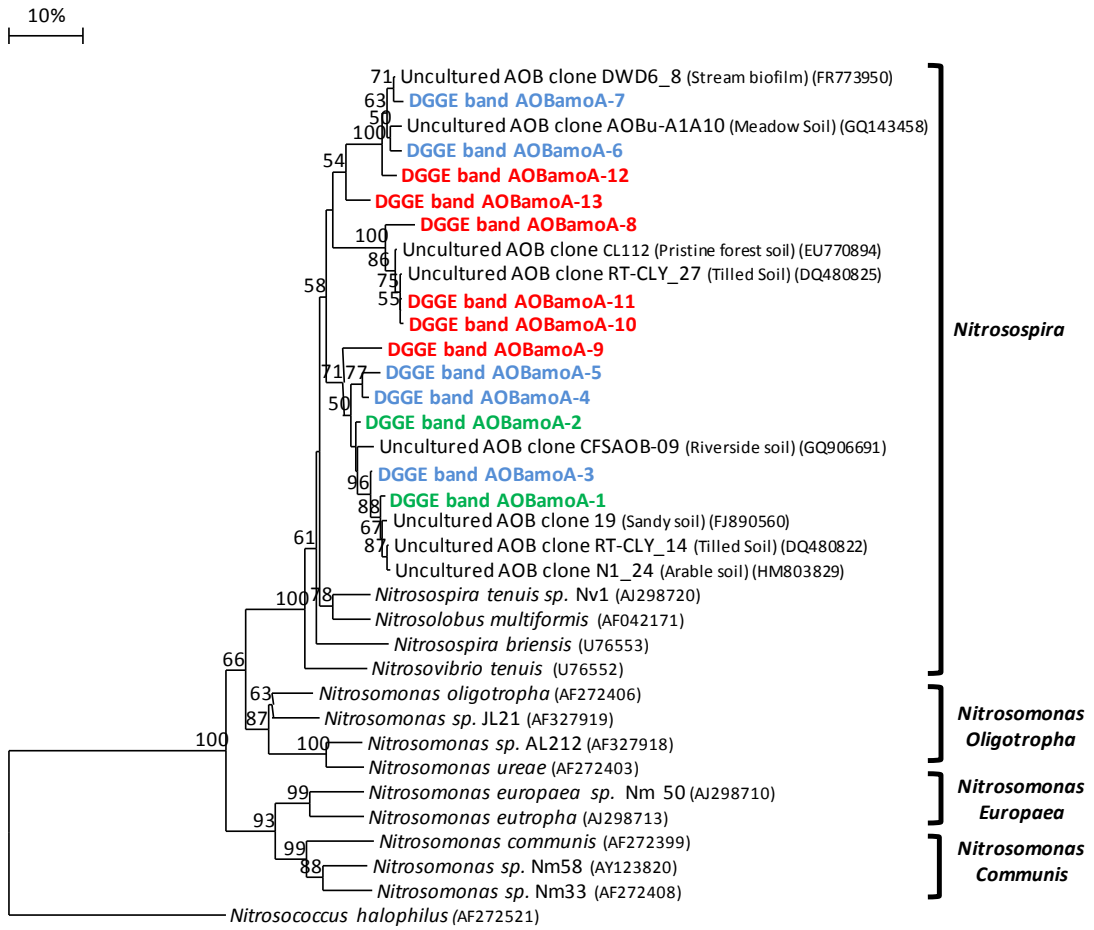


Figure 2-15: A neighbour joining phylogenetic tree showing excised ammonia-oxidising bacteria (AOB) *amo*A gene fragments from high metal (red and bold), low metal (blue and bold), and low PAH (green and bold) contaminated soils of this study. All sequences used to construct this tree were 405bp long (including gaps). The scale bar indicates a sequence divergence of 10 %, calculated using the Jukes and Cantor correction (Jukes and Cantor, 1969). Bootstrap values are given at nodes when equal to or greater than 50 % (1000 replicates). The root is '*Nitrosococcus halophilus*' a Gammaproteobacterial ammonia-oxidiser. Isolation sources are shown in parentheses followed by accession number.

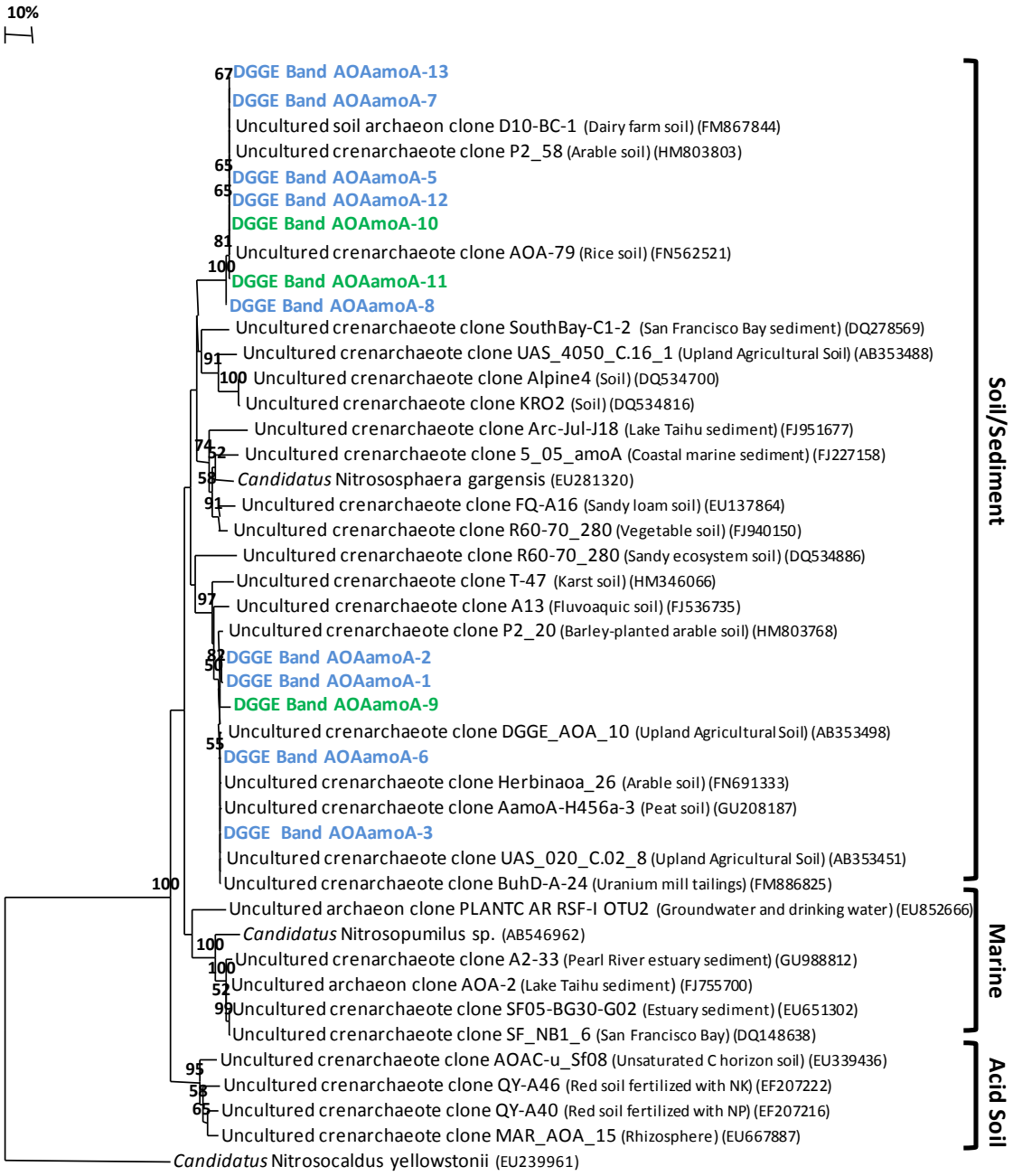


Figure 2-16: A neighbour joining phylogenetic tree showing excised ammonia-oxidising archaea (AOA) *amo*A gene fragments from low metal (blue and bold) and low PAH (green and bold) contaminated soils of this study. All sequences used to construct this tree were 316bp long (including gaps). The scale bar indicates a sequence divergence of 10 %, calculated using the Jukes and Cantor correction (Jukes and Cantor, 1969). Bootstrap values are given at nodes when equal to or greater than 50 % (1000 replicates). The outgroup is '*Candidatus* Nitrosocaldus yellowstonii' a thermophilic nitrifier of the autotrophic crenarchaeote. Isolation sources are shown in parentheses followed by accession number.

2.4. Discussion

The objective of the following discussion is to elucidate which measures of microbial activity, community composition and diversity are useful at discriminating the effect that metal and PAH contaminants have upon soil microbial functioning. While the end points of Ritz *et al.* (2009) for national soil monitoring were not concentrated towards microbial communities or contaminated land, their assessment of the effectiveness of indicators at a large scale, which are similar to indicators examined in this study, are still relevant. Additionally, this discussion aimed to identify any key processes that are at risk from current pollution levels within contaminated soils and therefore define suitable soil microbial functional indicators that could be employed within subsequent microcosm and remediation trials that assessed the effect of Mn oxide addition on microbial soil functioning (see Chapters 3 and 4).

2.4.1. *Total heavy metal concentrations and bioavailability*

Metal contaminated soils from of St. Anthony's lead works were determined to be contaminated with As, Pb, Cd and Zn. As a whole, the metal contaminated land type displayed total heavy metal concentrations in agreement with the range of values reported for As, Cd, Cr, Cu, Ni, Pb and Zn from 19 different locations across the site by Okorie *et al.* (2011). Total metal concentrations were measured as they are critical for defining if a soil is contaminated, and do indicate what level of metal stress the microbial populations in the metal contaminated land type have been historically subjected to. However, it is assumed that the total metal concentrations reported in this study are not reflective of actual concentrations accessible to microbial populations.

Total soil metal contents are known to be poor indicators bioavailable metal concentrations as they consist of metal forms and fractions that have different solubility's and availabilities (Giller *et al.*, 2009; Siebielec *et al.*, 2006). Bioavailability of soil metals is regulated by many factors including pH, cation exchange capacity, redox potential, clay and organic matter contents (Lazzaro *et al.*, 2006; Siebielec *et al.*, 2006). Furthermore, metal bioavailability in contaminated soils is expected to be reduced over time through diffusion, precipitation and occlusion processes (Lazzaro *et al.*, 2006). As the metal contaminated soils of St. Anthony's Lead Works have not received

any further pollution inputs from the closure of the Lead Works over 70 years ago, the availability of metals is presumed to have decreased over this time. This is further backed by Okorie *et al.* (2011) who theorised that the soils of St. Anthony's Lead Works would be naturally retentive of the metals due to the high cation exchange capacity (mean =28.6 $cmol_c$/ kg) and neutral to alkaline pHs.

Generally, the concentration of soluble metal fractions within soil pore waters is accepted as the most representative measure of metal available for uptake by microbial populations (Lazzaro *et al.*, 2006). However, dissolved metals can exist in a variety of chemical species such as soluble organic matter complexes, free metal species and inorganic ion pairs; it is the concentration of free metal species that have been found to explain toxicity to microbial populations (Lazzaro *et al.*, 2006; Sauvé *et al.*, 1997). Most metals in a contaminated system will be insoluble and bound to the solid phase (Sauvé *et al.*, 1998). Taking Pb, the major contaminant of metal contaminated soils of this study, as an example, Sauvé and colleagues (1997) have reported that only 1 - 0.01 % of total soil Pb is dissolved in the soil solution and, of that percentage, 60 - 80 % is complexed by organic matter. Thus, only a small fraction of the total metal concentrations are available to microbial populations as free metal species within soil water, even at high total concentrations.

Measurement of soil solution metal concentrations is, however, time consuming, difficult to analyse and not routine (Giller *et al.*, 2009). While soil solution metal concentrations have been reported to correlate with microbial toxicity, it has been recently reported that this is not the case (Giller *et al.*, 2009). In actual fact soil solution metal concentrations have proved to be substantially poorer than total concentrations in explaining metal toxicity to microbes (Giller *et al.*, 2009). The bioavailability of metals was not chemically assessed in this study, due to the fact that no agreed and standardised method exists. Instead metal bioavailability was inferred from impacts on soil microbial processes.

2.4.2. *Total PAH concentrations and bioavailability*

As expected the highest concentration of total PAHs (TPAH) were found in the PAH contaminated land type soils of St. Anthony's Tar Works, with values confirming its classification as PAH contaminated with regard to Dutch thresholds. Results also indicated that some areas of the metal contaminated soils from the adjacent Lead

Works may be impacted by both PAH and metal contamination. This is highly likely as coal tar was processed via distillation at St. Anthony's which will have undoubtedly resulted in contamination of nearby areas by atmospheric deposition from chimney emissions.

TPAH concentrations determined for the PAH contaminated soil of this study were compared to that from Lorenzi *et al.* (2010), the only published literature on St. Anthony's Tar Works soils. In their study mean TPAH concentrations were based upon the sum of the EPA 16 priority PAHs from 16 locations across the site. Soils were taken from 0 - 10 cm profiles and divided into two different particle size fractions: <250 µm (fraction A, which represents the grain size that is the primary course of human ingestion of pollutants) and >250 µm to <2 mm (fraction B). The study found that TPAH concentrations in the soils ranged from 9.0 to 1,404 mg kg^{-1} (soil fraction A) and from 6.6 to 872 mg kg^{-1} (soil fraction B). Lorenzi *et al.* (2010) concluded that the site warrants further investigation and remediation, as concentrations of TPAHs detected were notably high when compared with other PAH contaminated sites reported in the literature.

Furthermore, Lorenzi *et al.* (2010) reported that most polluted areas in the Tar Works were found to be close to the location of the former factory, which was also indicated by a 2007 ARUP investigation (ARUP, 2007). Samples taken to represent high PAH contamination in the present study were from this locale, adjacent to sampling site 1 of Lorenzi *et al*. (2010). The levels of TPAHs found for fractions A and B in site 1 were 123 ± 22 mg kg^{-1} and 234 ± 17 mg kg^{-1} respectively, and were in agreement with the value of 137.30 mg kg^{-1} reported in this study. While Lorenzi *et al.* (2010) reported lowest TPAH values from along the foreshore of the River Tyne, samples in this investigation were not taken from this location as they represented sediments rather than soils. Soils representing lower TPAH contamination, termed low PAH in this study, were taken nearby to sampling site 6 from Lorenzi *et al.* (2010). TPAH concentrations found for fractions A and B in site 6 were 46.4 ± 1.4 mg kg^{-1} and 39.9 ± 4.7 mg kg^{-1} respectively, and were in agreement with the value of 32.84 mg kg^{-1} reported in this study.

Interestingly, high levels of PAHs were detected in garden land type soils that are situated within the urban area of Newcastle upon Tyne city centre. According to the Environment Agency's UK Soil and Herbage Pollutant survey of 2007, median

concentrations of TPAH in urban areas are ~7 times those in rural locations (Barraclough, 2007). Urban garden soils had levels of TPAHs over 48 times that of rural agricultural values in this study, thus, exceeding UK differences. However, this is probably reflective of the small number of soils sampled within the study, although it is not certain.

Many of the factors pertinent to metal bioavailability are analogous to those affecting the availability of PAHs to microorganisms. As with total metal concentrations, TPAH values are critical for defining if a soil is contaminated. Though many studies have demonstrated that these values have little or no relation to the actual concentrations that microorganisms are exposed to within the soil matrix, and therefore tend to overestimate risk (Alexander, 2000; Cornelissen *et al.*, 2005; Ozretich *et al.*, 2000). As contact time between soil fractions and contaminants increase, interactions between both particles becomes stronger via what is known as the 'ageing' process, which may take anything from a few days to years to occur (Alexander, 2000; Stokes *et al.*, 2005). As St. Anthony's Tar Works has been decommissioned for over 30 years and soils have not received any further pollution inputs, the PAH contaminated soils collected from this location within this study are presumed to have undergone pollutant ageing.

The ageing process has been widely reported to reduce the bioavailability and toxicity of PAHs to soil microorganisms (Guerin and Boyd, 1992; Weissenfels *et al.*, 1992). Over time PAHs released from coal tar (the pollutant source in St. Anthony's Tar Works) become sequestered within the soil matrix, predominantly through the process of sorption to organic matter, but also via interactions with non aqueous phase liquids (NAPLs) and trapping within soil micro-pores (Johnsen *et al.*, 2005; Stokes *et al.*, 2005). In some cases adsorption is so powerful that no toxic effects at high total concentrations are apparent upon microbial populations (Weissenfels *et al.*, 1992). While TPAH toxicity and bioavailability are known to be reduced by ageing, it must be highlighted that risk of TPAH exposure to microbial populations will not be completely eradicated (Alexander, 2000). In the St. Anthony's Tar Works increased aging may also result in competitive sorption between the complex mixtures of PAHs from coal tar, often one contaminant can affect the sorption and bioavailability of another (Stokes *et al.*, 2005). However, it is assumed that the TPAH concentrations of St. Anthony's Tar

Works soil do not reflect the proportion of TPAHs that are available to the microbial population.

Interactions between microbial populations and PAH contaminants are limited to aqueous phases within the soil matrix. Concentrations within the aqueous phase have been found to better predict toxicity to microbes rather than bulk soil values, where PAH mainly exist as PAH-dissolved organic carbon (DOC) complexes or freely dissolved PAHs (Johnsen *et al.*, 2005; Neuhauser *et al.*, 2006; Ozretich *et al.*, 2000) . Due to the effect of reduced bioavailability over time, contact between microbes or extracellular enzymes and PAH contaminants will become dependent upon: solubility, desorption, mass transfer rates, microbial adhesion to contaminants and desorption through the production of biosurfactants and emulsifiers by indigenous microbial populations (Alexander, 2000; Park *et al.*, 2001; Stokes *et al.*, 2005).

In addition to the aforementioned interactions of aged TPAHs within the soil matrix, coal tar appears to be still actively moving through the soil profile within the soils of St. Anthony's Tar Works (Figure 2-2), which could have further impacts upon bioavailability and microbial interaction with pollutants. PAHs must first diffuse out of the complex organic coal tar matrix into soil aqueous phases before sorption and sequestration processes can occur, which is limited by mass transfer (Ahn *et al.*, 2005). In a study by Ahn *et al.* (2005), PAH release from the polymeric matrix of tar sludge within soil was found range from 4 - 22 % over 210 days. While release of PAHs through mass transfer is indeed slow and hinders bioremediation of organically contaminated soils, it is believed to be of importance within this study. The continual movement of coal tar through the soil profile may therefore be resulting in a continual release of PAHs into the aqueous phase in soils which are therefore available to microorganisms and their extracellular enzymes.

Bioavailability of TPAHs was not chemically assessed in this study, but as for metals, availability of PAHs was inferred via the range of measured soil microbial processes. The microbial indicators selected in this study should indicate if the microbial population as a whole are experiencing any toxicity and therefore give an indication as to whether TPAHs are bioavailable in the soil. This method of inferring bioavailability has been described as the 'worst case scenario' as microbes are inherently more accessible to contaminants than higher organisms, plants and humans (Alexander, 1995).

2.4.3. *Comparison of soil microbial activity in soils characterised as contaminated and non-contaminated*

In this study both metal and PAH contaminated soils showed basal respiration (BR) rates that were 340 times lower in comparison to relatively non-contaminated garden and agricultural soils. It has been widely reported and commonly acknowledged that contamination decreases basal respiration (BR) in soils, leading to it being considered as a good indicator of heavy metal toxicity and bioavailability to soil microbial populations (Brookes, 1995; Giller *et al.*, 1998; Hattori, 1992; Shi *et al.*, 2002; Shi *et al.*, 2005; Yang *et al.*, 2006). Therefore, results from this study indicate that metals and PAHs are bioavailable in the contaminated soils. Furthermore, as BR rates were uncorrelated to any other measured parameter it additionally suggests that contamination and its bioavailability, was the principle driver of the extremely suppressed rates observed. Consequently, it can be said that the microbial communities within the contaminated soil land types do not appear to have adapted a functionally tolerant community to either metal or PAH pollution over time. However, it is possible that these soils have inherently (i.e. regardless of pollution) lower rates of BR than the other soils investigated. A comparison of means by one-way ANOVA showed that low PAH contaminated soils exhibited significantly higher BR rates to that of highly PAH contaminated soils ($p < 0.01$). Conversely, high metal contaminated soils exhibited significantly higher BR rates to that of low metal contaminated soils (one-way ANOVA, $p > 0.01$). Therefore, the theory of inherently lower BR seems unlikely for soils within the PAH and metal contaminated land types.

BR is reported to be positively related to microbial biomass (Yang *et al.*, 2006). With microbial biomass carbon (MBC) renowned as a sensitive indicator of metal pollution (Brookes and McGrath, 1984; Chander *et al.*, 1995). MBC has been incorporated into numerous studies to determine the effects of pollutants on the functioning of microbial communities (Garbisu *et al.*, 2011). Long term heavy metal contaminated soils have been widely reported to harbour smaller MBC with elevated heavy metal concentrations (Barajas Aceves *et al.*, 1999; Fliessbach *et al.*, 1994; Kuperman and Carreiro, 1997; Yang *et al.*, 2006). MBC has also been reported as the most sensitive indicator for toxic effects of Pb contamination in soils containing multiple heavy metal contaminants (Yang *et al.*, 2006). The impact of hydrocarbon

contamination upon MBC has shown contradictory results within the literature. Presence of hydrocarbon pollution in soils has been shown to decrease MBC values (Dawson *et al.*, 2007; Lorenz and Kandeler, 2005; Megharaj *et al.*, 2000), or alternatively stimulate and increase the microbial biomass via more carbon sources being available, accessible from the contaminants (Blakely *et al.*, 2002; Caravaca and Roldán, 2003; Mosher *et al.*, 2006).

Results from this study are contrary to the common findings for metal contaminated soils. No significant difference was detected between the MBC of long term metal and PAH contaminated soils when compared to non-contaminated garden and agricultural values. However, they are in agreement with other studies where no differences were found in levels of MBC between contaminated and non-contaminated soils (Joynt *et al.*, 2006; Shi *et al.*, 2002), and where no correlations have been observed between metal concentrations and MBC (Wang *et al.*, 2009).

Negative effects of metal contamination upon the MBC may have been masked by a number of factors. Due to the length of time contamination has been present at St. Anthony's Tar and Lead Works, a metal tolerant community may have developed and any decrease in MBC may therefore have only been a temporary effect (Joynt *et al.*, 2006). Though, this could be contested as BR values appeared severely affected by the presence of contamination. Nevertheless, it may be possible that a metal tolerant community may still inherently operate with a lower respiration activity. Such a disparity between BR and MBC may be related to the concentration of bioavailable metal contaminants within the soil matrix. In studies by Aoyama and Nagumo (1996; 1997), both MBC and BR have been found to be controlled by metal speciation. MBC was found to be negatively affected due to high concentration of organically bound and slightly soluble metals, whereas BR was found to be reduced by high levels of water soluble and exchangeable metal forms (Aoyama and Nagumo, 1996; Aoyama and Nagumo, 1997). Therefore, it is hypothesised that in the soil pore water of the metal contaminated soils bioavailable metals exist in high concentrations as water soluble and exchangeable forms that has resulted in the greatly reduced BR rates observed. It could also be hypothesised that organically bound and slightly soluble metal concentrations are low, and therefore MBC does not appear to be reduced even though high concentrations of total heavy metals are present.

High microbial metabolic quotients (qCO_2) have been described as "a surprisingly common characteristic of contaminated soils" (Giller *et al.*, 1998). The use of qCO_2 as an indicator of stress or disturbance in heavy metal contaminated soils has been frequently found to show elevated values, as the microbial community spends more energy on the detoxification of metals, thus, switching energy use from production of biomass to maintenance (Stefanowicz *et al.*, 2010). The same phenomenon of increased qCO_2 via hydrocarbon contamination has also been observed (Franco *et al.*, 2004). However, results for this study showed that contaminated soils had significantly lower qCO_2 values then their non-contaminated counterparts. This indicated that the microbial community in the metal and PAH contaminated soils are energetically more efficient than microbial populations of non-contaminated garden and agricultural soils.

To put qCO_2 values into context, Anderson (2003) suggests that mature soils of neutral pH should ideally have qCO_2 ratios below 2.0 to define an energetically efficient microbial community. This guideline value can be applied to the metal contaminated soils of this study, as they reflect a long term contaminated environment of neutral pH. The mean qCO_2 ratios from the metal contaminated soil land type are above the threshold value of 2.0 for an energetically efficient microbial community, suggesting that contamination has had a negative impact on qCO_2. Therefore, qCO_2 values found in this study imply that while contaminated soil communities are energetically more efficient than those in non-contaminated soils, they are still stressed, using more energy for maintenance rather than growth. This may also partly explain why significant differences were not detected in MBC values.

The lower qCO_2 values observed between contaminated and non-contaminated soils has been described elsewhere, and may indicate enhanced dormancy or shifts in substrate utilisation due to contamination (Insam *et al.*, 1996). It is felt that the significantly higher qCO_2 values of the non-contaminated garden and agricultural land types may be partly explained by differences in pH and organic material inputs. Acidic soil pH values as found in the non-contaminated soils of this study, are known to exert additional stress upon microbial communities as energy requirements for maintenance are higher when soil pH differs from internal cell pH (Anderson, 2003; Anderson and Domsch, 1993). The qCO_2 has also been found to be higher in soils with organic material inputs that are more resistant to decomposition and in soils that are more

nutrient limited. In both cases the energetically expensive process of stable soil organic matter degradation has to occur for the microbial community to acquire adequate nutrients (Wardle and Ghani, 1995). This may be the reason for higher qCO_2 values within garden soils as leaf mould, applied to the garden OM soil has been reported to be a more resistant form of organic material. It is thought that leaf toughness due to anti-herbivore defence affects decomposition and consequently nutrient release (Cornelissen and Thompson, 1997; Gallardo and Merino, 1999).

The microbial quotient (*qmic*) is used to determine the availability of C substrates to soil microbes and also gives an indication of the contribution of microbial biomass to organic carbon. In general *qmic* ratios increase as the amount of available organic C increases, which reflects increased substrate availability and higher MBC via more available organic C for biomass production (Anderson and Domsch, 1989; Brookes, 1995). Consequently, lower *qmic* ratio values show limited availability of organic C in soil and therefore less available C for biomass production and higher BR rates (Anderson, 2003). Contamination has been found to express decreased *qmic* ratios which indicate a decreased amount of C that is available for growth and a less efficient use of C substrates in soil (Fliessbach *et al.*, 1994; Giller *et al.*, 1998).

No significant differences in *qmic* values were detected between contaminated and non-contaminated land types of this study. Therefore, neither PAH or metal contamination was found to show any detrimental effects upon C utilisation, a result consistent with Insam *et al.* (1996). This suggests that both contaminated and non-contaminated soils have the same contribution of MBC in the soil TOC. Critical *qmic* values of 2.0 for soils of neutral pH were also described by Anderson (2003). Values above this threshold are considered as being high and show an efficient use of C substrates in soils with a large amount of C available for growth. Values of *qmic* ratios for neutral metal contaminated soils in this study are above the critical value of 2.0. This implies that the microbes in these soils are not stressed and labile organic C is easily decomposed. The high *qmic* values obtained from all soils additionally suggests good substrate utilisation of C in all soils. This may further explain the high MBC values found in the contaminated soils of St. Anthony's Tar and Lead works.

In summary, results suggest that long term contamination of metal and PAH contaminated soils from St. Anthony's Tar and Lead Works has had a profound effect upon the microbial activity of BR with soils exhibiting qCO_2 values of a stressed

community that cannot be directly correlated with total heavy metal or PAH concentrations. While no correlation with total contaminant levels could be determined, findings did advocate that PAHs and metals are bioavailable with the soils of St. Anthony's. Therefore, it can be said that qCO_2 and BR are sensitive parameters for determining the status of soil microbial activity in long term contaminated soils. However, results did show that qCO_2 may only be valuable when comparing soils of similar physico-chemical characteristics, which support the views of Anderson and Domsch (1993) and Insam *et al.* (1996). This would greatly limit its use as an indicator of microbial activity in contaminated environments. The measurement of BR alone was deemed to be an ineffective biological indicator that does not provide useful discrimination by Ritz *et al.* (2009). However, this study has proved that for contaminated soils, BR is a sensitive and discriminative indicator for microbial activity. Ritz *et al.* (2009) suggest the potential use of multiple substrate induced respiration (MSIR) through the use of the MicroResp™ system (Campbell *et al.*, 2003). MSIR is still in its infancy with regard to soil monitoring and has not been rigorously tested in a variety of laboratories or even deployed against a range of land use or soil types (Ritz *et al.*, 2009).

Additionally, results from this study suggested that MBC and *qmic* were not sensitive to long term contamination, with these parameters comparable to relatively non-contaminated land types. Both MBC and *qmic* were deemed ineffective as indictors of soil microbial activity. MBC clearly reflects the complexity of the soil environment and its use as a sensitive indicator of the effects of soil contamination on microbial communities has been disputed (Martens, 1995). While being a key parameter of any ecosystem MBC, has also been described as not very sensitive in indicating soil microbial functioning (Winding *et al.*, 2005). Both the use of MBC and *qmic* have been proven to be more sensitive to effects of contamination upon microbial communities within the same soil over time, or in freshly spiked soils, but do not correlate well when compared with different land types as exemplified in this study (Broos *et al.*, 2007). Additionally, the determination of MBC and therefore qCO_2 and *qmic* via substrate induced respiration (SIR) is time consuming, labour intensive, with a limited amount of samples that can be analysed together. Therefore, as a routine monitoring tool these methods would not be practical or sustainable. Ritz *et al.* (2009) did not rate MBC as a top ranking indictor for biological soil monitoring. This

was due to the lack of discrimination that gross measurements provide, which this study is in agreement with. However, Ritz *et al.* (2009) do conclude that it is a valid baseline measure that can be achieved via the aforementioned top ranking but not fully approved or rigorously tested MSIR.

Overall, the functional capability of microbial respiration in the soils of St. Anthony's Tar and Lead works has been compromised. Therefore, BR is deemed the best indicator of microbial activity in these soils and consequently is suggested as good indicator for soil microbial functioning in contaminated land. Measurement of BR is also advantageous due to it being a rapid, high throughput and standardised technique that could be easily implemented into routine monitoring.

2.4.4. *Comparisons of bacterial community composition and diversity in soils characterised as contaminated and non-contaminated*

Genetic fingerprinting methods give two different ways of interpreting and understanding the effects of long term contamination upon microbial communities, in the form of diversity measures and community composition. Within this sub-section, while both diversity and community structure are derived from analysis of DGGE profiling, they will be discussed as separately.

Univariate diversity indices have been described as an elegant summary of complex bacterial communities (Blackwood *et al.*, 2007). Pollution within the soil environment has been well documented to decrease microbial diversity (Giller *et al.*, 1998). This decrease has been mainly attributed to microbial communities becoming dominated by organisms capable of either tolerating toxic metal contamination, or utilising PAHs as substrates. However, these conclusions have been mainly drawn from studies where soils have been artificially spiked and measured temporally. In real contaminated environments, microbial diversity can also be influenced by the duration of contamination and by which pollutants are present (Giller *et al.*, 1998; MacNaughton *et al.*, 1999).

Both metal and PAH contaminated soils examined within this study displayed diversity indices that were comparable to non-contaminated garden soils, and significantly higher than agricultural land types. This indicates that organic and inorganic contamination had no negative effect on diversity, which contrasts to the common theory of decreasing ecological indices with pollution. However, it should be

borne in mind that these diversity indices have been measured using DGGE. Many authors have found no differences in microbial diversity both between and within contaminated and non-contaminated soils by use of DGGE derived diversity indices, thus, the result from this study is not unusual (Andreoni *et al.*, 2004; Girvan *et al.*, 2005; Juck *et al.*, 2000; McCaig *et al.*, 2001; Müller *et al.*, 2002). Therefore, the use of DGGE profiling as a method to calculate diversity indices could be disputed.

The appropriateness of using ecological diversity indices on highly diverse microbial communities, as detected in this study, remains unclear (Hill *et al.*, 2003). While estimation of diversity indices based upon total number of DGGE bands and their relative intensities has been traditionally used in microbial diversity analysis of soils (Trevors, 1998). It has been highlighted that care must be exercised when interpreting these results (Kirk *et al.*, 2004). DGGE profiling has been reported to only detect the most dominant members of the microbial community at a level of 1 - 2 % (Kirk *et al.*, 2005). Therefore, the use of DGGE derived ecological indices on highly diverse communities is only an indication of the most predominant bacterial populations and not an absolute measure of the whole community (Marzorati *et al.*, 2008). These problems are not just inherent to DGGE, all molecular fingerprinting techniques (e.g. TRFLP, SSCP) provide such low resolutions that diversity of the soil habitat cannot be adequately described through their use (Bent *et al.*, 2007; Blackwood *et al.*, 2007; Dunbar *et al.*, 2000; Smalla *et al.*, 2007).

Limitations of DGGE profiling for accurate representations of soil microbial diversity are not restricted to detection levels alone. Problems in the accuracy of diversity measurements can occur prior to DGGE from the extraction of DNA and PCR amplification of 16S rRNA gene fragments. The DNA extraction method used is known to significantly affect fingerprints generated (Carrigg *et al.*, 2007). However, as the same extraction method was used for all soil samples in this study they are comparable and this factor can be disregarded. PCR amplification may affect DGGE fingerprints through to selective amplification of preferential targets due to small differences in the sequence of conserved regions, the formation of chimeras and heteroduplex molecules, non-specific amplification of non-targets and formation of single stranded fragments (Head *et al.*, 1998; Nakatsu, 2007).

The calculation of indices based upon the number of bands and their relative intensities also cannot give an accurate description due to inconsistencies arising

during electrophoresis. Individual organisms are known to produce multiple bands due to slight variations in sequences, or because of multiple heterogeneous rRNA operons in their genomes (Head *et al.*, 1998). Single bands may represent multiple species which differ in sequence but have co-migrated as a result of similar melting behaviours (Kisand and Wikner, 2003). Additionally, one band may represent two or more species due to identical sequences over the length of the partial gene amplified (Marzorati *et al.*, 2008). Often bands may merge or produce double bands with high concentration of species with similar melting properties further impacting reliable diversity estimations (Janse *et al.*, 2004). Smearing of bands is also a common product of DGGE profiling in very diverse communities, where many populations are present in equivalent concentrations (Nakatsu, 2007), this effect can be clearly seen in garden soil DGGE profiles within this study (Figure 2-6) which casts extreme doubt upon the reliability of diversity values calculated for this land type.

Due to all the aforementioned potential pitfalls of diversity estimates via molecular fingerprinting techniques it is deemed ineffective as an indicator of differentiating the effects of contamination upon soil microbial communities. Therefore, the use of DGGE derived ecological diversity indices is not suggested as an indicator of soil microbial functioning in contaminated land. Interestingly, diversity indices were not proposed by Ritz *et al.* (2009) as a potential indicator for national monitoring. To better describe bacterial diversity in the soils of this study it is felt that methods offering greater resolution such as next-generation sequencing e.g. Roesch *et al.* (2007), would be more beneficial.

Highly contaminated soils have been reported to harbour different microbial populations to soils from non-contaminated land uses, with estimates that anywhere from 10 – 100 % of the bacterial community in long term metal contaminated sites are metal resistant or tolerant (Konopka *et al.*, 1999; Macdonald *et al.*, 2011). Long term PAH contamination of soils has also been described as having a significant effect upon the composition of soil microbial communities (Maila *et al.*, 2006; Viñas *et al.*, 2005), although most literature centres upon community changes during bioremediation techniques (Muckian *et al.*, 2007; Muckian *et al.*, 2009; Viñas *et al.*, 2005). To the authors knowledge no other studies are available that compare both long term organic and inorganic contaminated bacterial community composition to non-contaminated soils from other land types as assessed in this study.

This study showed that predominant bacterial community structure in soil is most strongly affected by site-specific and physico-chemical factors rather than contamination. The most pronounced effect on bacterial community structure between contaminated and non-contaminated soils was due to geographical sampling location with community dissimilarity driven by total organic carbon (TOC) and pH. Both sampling site and/or land type along with physical soil properties, especially pH, are well recognised as being strong drivers of bacterial community structure, even within contaminated environments (Acosta-Martínez *et al.*, 2008; Dequiedt *et al.*, 2011; Drenovsky *et al.*, 2010; Jangid *et al.*, 2011; Lauber *et al.*, 2009; Lauber *et al.*, 2008; Macdonald *et al.*, 2011). Therefore, these results are consistent with the literature.

The fact that the bacterial community compositions of metal and PAH contaminated soils were not influenced by the pollutant types present may support the observation by Jangid et al. (2011), whereby microbial communities in previously disturbed soils tend to become more similar to those in the native soil with time. Consequently, it is thought that the use of DGGE profiling is probably best utilised to track temporal changes in soil communities after a pollutant stress has been applied, than assess the effects of long term contamination upon microbial community composition. The fact that predominant bacterial community dissimilarity of the metal and PAH contaminated soils was not influenced by total heavy metal or TPAH concentrations could be due to a number of reasons. Total pollutant concentrations are not reflective of actual concentrations of contaminants that are bioavailable; only the bioavailable fraction may correlate to community dissimilarity. Alternatively, microbial communities may have become adapted to available metals and have become resilient over time. However, TPAH concentrations do appear to influence community dissimilarity between different sites rather than within, suggesting that organic contamination has a more pronounced universal effect upon community structure than metals.

In summary, results suggest that long term contaminated soils investigated in this study did harbour significantly different predominant bacterial communities to non-contaminated garden and agricultural soils, although this was driven by geographical sampling location. Therefore, DGGE analysis of soil bacterial community composition does not appear to be an effective indicator for soil monitoring long term

contaminated soils. Furthermore, without band excision, which is impossible to accurately carry out for such diverse soil communities, the sequence based identification that would be necessary to assess the presence of lineages associated with known metal or PAH contaminated locations remains unknown. Ritz *et al.* (2009) suggested Terminal Restriction Fragment Length Polymorphism (TRFLP), as the most appropriate and best available current utility for the determination of soil community structure/composition for biological soil monitoring. However, TRFLP suffers from the same inherent problems as DGGE. As stated by Ritz *et al.* (2009) the best technology for the determination of bacterial community structure will undoubtedly change with the advent of high-resolution, faster and cheaper sequencing technologies. It is proposed that methods offering a greater taxonomic discrimination of soils e.g. next-generation sequencing technologies may provide a better insight into bacterial community structures of contaminated environments within the near future.

2.4.5. *Influence of metal and PAH contamination upon denitrification enzyme activities associated to impacts upon denitrifier diversity and composition*

Organic and inorganic contaminants are recognised inhibitors of denitrification (Guo *et al.*, 2011; Holtan-Hartwig *et al.*, 2002; McKenney and Vriesacker, 1985; Roy and Greer, 2000; Siciliano *et al.*, 2000; Throbäck *et al.*, 2007). It has been proposed that the denitrification pathway may be uniquely sensitive to metal exposure (Kandeler *et al.*, 1996; Sobolev and Begonia, 2008). As the genes involved in are located within the cytoplasmic membrane, in close proximity to the outer cell surface, which increases their vulnerability to bioavailable heavy metal and PAH contaminants (Sobolev and Begonia, 2008; Wallenstein *et al.*, 2006).

Results from this study are in agreement with the theory that pollution affects denitrification. Both heavy metal and PAH contaminated soils exhibited significantly reduced denitrification enzyme activities (DEAs) that were 50 times lower than non-contaminated garden and agricultural land types. While DEA was reduced, it is important to emphasise that the contaminated soils still have the functional capacity to denitrify. Although the significantly reduced DEA reflect an extremely hindered ability to perform this function in contaminated soils, the possibility exists that the denitrification in these soils is intrinsically low. However, this seems unlikely as a significant negative correlation was observed between log transformed DEA and TPAH

concentrations across all soils. Furthermore, examination of the individual DEA values for high and low metal and PAH contaminated soils (Figure 2-17), revealed that higher contamination levels resulted in significantly lower DEA (one-way ANOVA, Tukey's HSD, $p < 0.01$). Therefore, all evidence strongly suggests that heavy metal and PAH contaminated soil DEA potentials were inhibited by bioavailable pollutants. DEA findings are therefore consistent with results from respiration measurements.

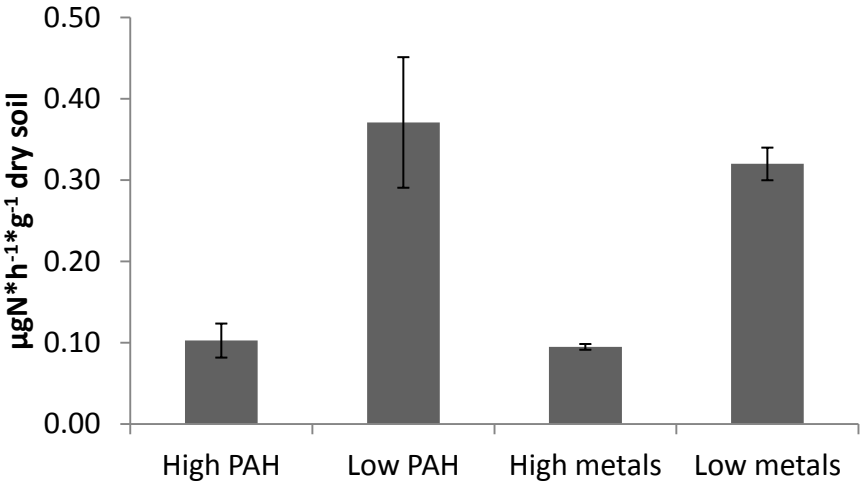


Figure 2-17: Denitrification enzyme activity (DEA) rates of low and high PAH and metal contaminated soils. Error bars represent 1 x standard error.

The use of *nirK* and *nirS* as molecular markers showed clear relationships between predominant denitrifier community structure and DEA. The community structure of *nirK* encoding denitrifiers showed a stark compositional difference in comparison to non-contaminated soils that was directly correlated to functional rates and therefore it is inferred bioavailable metal and PAH contamination. In contrast the *nirS* community structure appeared to be mainly driven by soil specific physico-chemical factors. However, DEA also showed significant relationships with predominant *nirS* communities, thus, bioavailable contamination is also inferred as having a significant, though lesser, effect on community composition.

Soil pH was identified as being a statistically significant driver of community dissimilarity in both denitrifier molecular markers. Results from this study may therefore suggest that pH selects for different assemblages of denitrifiers, playing a significant role in driving the community dissimilarity between contaminated and non-

contaminated soils, which in turn would impact DEA. Soil pH has been previously reported to affect the composition of denitrifiers (Deiglmayr *et al.*, 2004). However, often circum-neutral and slightly alkaline soil pH's are found to exhibit the highest rates of DEA (Šimek *et al.*, 2002). As both the heavy metal and PAH contaminated soils of this study are from circum-neutral to alkaline soils they could be expected to have high rates of DEA, but this is not the case. Also, the impact on community dissimilarity is probably not due to a pH effect on metal availability as the metal contaminated soils as they are circum-neutral to alkaline therefore availability of pollutants would be expected to be lower as discussed in section 2.4.1. However, the effects of pH upon denitrification rates and denitrifier communities are contradictory and no clear results have been reported (Enwall *et al.*, 2005; Šimek *et al.*, 2002).

The community structure of denitrifiers in the contaminated soils of this study are related to a lower but measurable potential DEA, and the community clearly shows a compositional difference from the non-contaminated soils. This could suggest adaption/tolerance of these communities to contamination. Mechanisms for this could either be attributed to the inhibition of contaminant sensitive denitrifiers followed by the enrichment of more tolerant species. The denitrification process has been reported to recover from a contamination event over time due to adaption of the denitrifying community. Soils which have been exposed to Zn illustrated recovery in denitrification from 2 to 12 months after exposure. This adaption is believed to occur relatively quickly due to the great diversity and fast growth rates of heterotrophic denitrifying populations (Holtan-Hartwig *et al.*, 2002; Ruyters *et al.*, 2010b). Sobolev and Begonia (2008) have suggested that heavy metal resistance via metal ion scavenging or pumping mechanisms would be ineffective in denitrifying microbes as this would place the metal in immediate contact with denitrification enzymes and severely disrupt the process. Instead Sobolev and Begonia (2008) suggest that any metal resistance that may be harboured by the denitrifying community will be driven by the production of metal resistant enzyme forms identified by gene sequence and genetic signature. Guo *et al.* (2011) have hypothesised that PAH contaminated soils may also have the capacity to recover denitrification through degradation and ageing of the organic contaminants, but no current proof of this exists for long term PAH contaminated soils. The effect of PAH contamination upon denitrification has not been well studied and has been highlighted as deserving more attention to understand the

response of the process to this type of organic pollutant (Guo *et al.*, 2011). Therefore, the literature suggests that long term contaminated soils have the capacity to recover denitrification via adaption of the denitrifying community.

If the microbial population has adapted and metal tolerant denitrifiers exist in the contaminated soils of this study, this does not explain why the potential enzyme activities that they exhibit are low. Measurements of community compositions give no indication of the actual size of the denitrifier community. To further determine why DEAs are lower in the contaminated soils denitrifier community abundance would need to be investigated. While the populations in these soils may to be adapted to contamination they may not be present in high numbers, which may explain low functional activities coupled to different community structures.

The diversity of denitrifiers in heavy metal and PAH impacted soils has not been well studied (Philippot *et al.*, 2007). Analysis of the ecological diversity of *nirS* and *nirK* genes was carried out in this study. However, the appropriateness of these indices relating to *nirS* gene diversity is highly questionable due to the reasons previously discussed, but additionally due to the extensive band merging and smearing as apparent in their respective DGGE profiles (Figure 2-10). Despite the fact that statistical analysis was performed and significant correlations were detected between *nirS* diversity indices and the physico-chemical properties of total organic carbon and moisture content it has been decided not to further discuss these observations. Instead this serves as a prime example in the caution needed to be exercised when interpreting results from DGGE profiling, along with the effectiveness of DGGE as a tool for the measurement of microbial diversity within contaminated land. In comparison, the *nirK* profiles (Figure 2-11) were better resolved, thus, it is believed that the ecological indices derived from these profiles are a better representation of predominant denitrifier diversity between the contaminated and non-contaminated soils of this study.

As previously reported (section 2.4.4) heavy metal and organic contaminants are renowned for decreasing microbial diversity. However, findings in this study for *nirK* and bacterial 16S rRNA genes did not support this hypothesis as contaminated soils showed evenness, Shannon and Simpson diversity indices comparable to non-contaminated garden soils and significantly higher than that of non-contaminated agricultural soils. Problems with the calculation of diversity indices via DGGE profiling

have also been highlighted in section 2.4.4. It is important to reiterate that these issues also hold true for diversity estimates for *nirK* encoding denitrifiers reported in this section.

Currently only one study exists by Throbäck *et al.*(2007), that investigated changes in *nirK* community structure due to heavy metal contamination. Throbäck *et al.* (2007) observed a decreased denitrification activity during a 90 day incubation of soil dosed with silver (Ag). This decrease in function co-occurred with reduced *nirK* abundance and increased diversity upon Ag contamination. The increased diversity of *nirK* was attributed to the intermediate disturbance hypothesis described by Giller *et al.* (1998). It postulates that stable environments with high numbers of competitive species have increased diversity because metal stress reduces the innate competitive exclusion between bacterial populations and induces enrichment of others. The diversity continues to increase until eventually the stress becomes so high that it will start to decrease. Therefore, results from this study appear to be in agreement with that of Throbäck *et al.* (2007), and it hypothesised that the reduced DEA observed from the contaminated soils will be due to decreased denitrifier abundance coupled to enrichment of metal tolerant species.

Ritz *et al.* (2009) proposed that denitrification as part of overall N-cycling is best measured via the combined use of denitrifiers via TRFLP, phospholipid fatty acids (PLFA) analysis and multi-enzyme profiling via fluorescently labelling substrates. However, Ritz *et al.* (2009) also highlighted that the aforementioned methods are fraught with problems when being applied to denitrifiers and their activity. Little work has been carried out on TRFLP for denitrifying communities, and it requires further work to identify suitable primers, along with the optimisation of PCR, restriction and fingerprinting steps (Ritz *et al.*, 2009). In comparison current DGGE methods have been well optimised and are widely used. The extraction of PLFAs as signature lipid biomarkers estimates biomass, though, this method is time consuming, currently has no standard operating procedures and requires measurement in more than one laboratory if many samples are to be analysed (Ritz *et al.*, 2009). This study did not take biomass of the denitrifying community into account. However, it is felt that emerging quantitative reverse transcriptase (RT) PCR (qRT-PCR) to measure the active mRNA levels of active denitrification genes will be a useful future tool in accurately measuring denitrifier abundance, e.g. Smith *et al.* (2007). Furthermore, the

measurement of biochemical process from multi-enzyme profiling is primarily used for enzymes involved in C-cycling (Ritz *et al.*, 2009). At present only phosphate and sulphatase fluorescently labelled substrates commercially available (Ritz *et al.*, 2009). While Ritz *et al.* (2009) ranked N-cycling as a top bio-indicator for national soil monitoring and stressed the importance of linking soil biology to soil functions; they have failed to actually do so in their recommendations.

Results from this study indicate that DEA is a sensitive indictor for determining the functional status of microbial populations in long term contaminated soils. Findings signify that PAH and heavy metal contaminants are bioavailable to the denitrifying community within St. Anthony's Tar and Lead works. The use of *nirK* and *nirS* as molecular markers showed a clear relationship between denitrifying community structure and potential function. This relationship is interesting, though, further research is important to fully understand how the denitrifying community is affected by long term contamination. For an initial analysis of soil microbial functioning in long term contaminated soils it is proposed that DEA be used. This is due to the rapid high throughput miniaturised method that can be employed for its determination which allows for quantification of many samples quickly.

2.4.6. *Influence of metal and PAH contamination upon potential nitrification rates associated to impacts upon ammonia-oxidising community structure*

Nitrification has long been reputed as one of the most sensitive indicators of both metal and hydrocarbon contamination, with many studies reporting the inhibitory effect of these pollutants not only upon potential activities, but also ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) population abundances and diversities (Broos *et al.*, 2005; Deni and Penninckx, 1999; Li *et al.*, 2009; Maliszewska-Kordybach *et al.*, 2007; Mertens *et al.*, 2006; Smolders *et al.*, 2001; Sverdrup *et al.*, 2002). PAH contamination has been reported to reduce nitrification rates through competitive co-oxidation of hydrocarbons by ammonia monooxygenase (AMO). This results in the replacement of ammonia (NH_3), the true substrate of AMO, by PAHs at its active site (Maliszewska-Kordybach *et al.*, 2007).

In contrast to these findings, results from this study demonstrated that potential nitrification rates (PNR) in contaminated soils were on par to those measured in non-contaminated soils, even though inhibitory effects were observed in other

microbial activities (BR and DEA). Recently, a large amount of literature has cast doubt upon the effectiveness of PNR as a sensitive indicator of contamination e.g. Sauvé *et al.* (1999). It is believed that the specific microbial communities involved in nitrification can become tolerant to this perturbation without compromising their functional capability, thus, producing disagreement within the scientific community (Diaz-Ravina *et al.*, 1994; Fait *et al.*, 2006; Kurola *et al.*, 2005; Siebielec *et al.*, 2006). Consequently, results indicate that the AOA and AOB in the contaminated soils of this study may have became tolerant to heavy metal and PAH contamination over time, in agreement with the aforementioned literature.

Within this study PNR was found to show a significant positive correlation with total organic carbon (TOC) and therefore soil organic matter (SOM), with a relationship with pH also observed. More commonly a negative relationship is found between TOC contents and PNR. This is an indication that soil C: N ratios are high with heterotrophic bacteria subjected to N limitation; therefore AOB are outcompeted for available ammonia, leading to a decrease in nitrification (Strauss and Lamberti, 2000; Verhagen and Laanbroek, 1991). Therefore, the positive relationship found between TOC and PNR potentials in this study may suggest that N limitation is not occurring, and that C: N ratios are low.

The PNR of both contaminated and non-contaminated soils were maximal at pH 7, with increasing and decreasing pH showing reduced activity. Soil pH is well documented to have a considerable effect on nitrification activity (Nicol *et al.*, 2008; Smolders *et al.*, 2001). Optimum soil pH for the nitrification process is reported to be between 7 and 8 with ammonia-oxidising activities reduced outside this range (Nieder and Benbi, 2008). Soil acidity is believed to affect ammonia oxidation through an exponential decrease in NH_3 availability with a lowering of the pH. At more acidic pH's ammonia (NH_3) is ionized to form ammonium (NH_4^+) therefore limiting the substrate available for nitrification (De Boer and Kowalchuk, 2001; Enwall *et al.*, 2007). At alkaline pH above 8.5, nitrification is documented to decrease through inhibition of nitrite-oxidisers via an increase in free ammonia therefore resulting in an incomplete ammonia oxidation reaction (Alleman, 1985; Smith *et al.*, 1997).

Interestingly, correlation analysis also showed a positive relationship between logarithmic values of PNR and copper (Cu). This may reflect Cu dependency in the metabolism of AOB and AOA and therefore nitrification activity. Genomic analysis of

AOA has revealed a highly copper reliant system for ammonia oxidation (Schleper and Nicol, 2010). While AOA metabolism is fundamentally different from their bacterial equivalents, genetic and biochemical studies have reported a copper metal centre in AOB enzymatic ammonia monooxygenase (Arp and Stein, 2003).

While no significant differences were detected between PNR of contaminated and non-contaminated soils, a clear distinction was apparent in the diversity of ammonia-oxidising microbial communities. Both contaminated soils harboured AOA and AOB, however, interesting differences were uncovered between their community structure and presence/absence between contaminant types and levels. Results for the metal contaminated soil suggested that high levels of contamination induce a difference in the ammonia-oxidising community from coupled domination of AOA and AOB, to AOB only. Consequently, high levels of metal contamination appear to select against the AOA whilst inducing a phylogenetic difference within the AOB community composition. The associated phylogenetic difference in the high metal contaminated soil was not attributed to a selection for a known lineage of metal resistant AOB, which has been previously reported to occur by Stephen *et al.* (1999). This indicates that the ability of the AOB to tolerate high metal concentrations in soil is ubiquitous throughout the *Nitrosospira*, as evidenced by phylogenetic tree analysis. AOB community dissimilarity was found to be driven by moisture content. Moisture is known to have an effect upon nitrifying communities and their function, as its availability can reduce hydration and activity of enzymes, and also restrict the substrate supply (Stark and Firestone, 1995). The significant reduction in AOA community between low and high heavy metal contaminated soils suggests that the AOA are extremely sensitive to high levels of metal contamination and do not have the ability to recover and adapt. Although it must be borne in mind that at present we do not accurately know the coverage of the AOA primers which could be vastly underestimate AOA diversity.

The findings of this study are corroborated by that of Ruyters *et al.* (2010a), Mertens *et al.* (2009) and Mertens *et al.* (2010). Mertens and colleagues (2009) reported that after 2 years of a soil being contaminated with zinc, nitrification recovered to pre-exposure levels, and this recovery was mediated by the AOB, which underwent a phylogenetic shift. This shift was also found to be widespread through the *Nitrosospira* lineage with no clustering with other tolerant members. The authors also reported the death of AOA after contamination, although after 2 years they

recorded significant numbers of AOA genes, virtually no *amoA* expression was observed. This was attributed to AOA population's surviving in dormant states or as spores. The results found by Mertens *et al.* (2010) were corroborated by Ruyters *et al.* (2010a). Furthermore, in 2010, Mertens *et al.*, also examined long term copper contaminated soils and found decreased numbers of AOA and differences in the composition of the AOB.

In the high PAH contaminated soils of this study, both AOA and AOB populations appeared severely decreased with different community compositions in comparison to the low PAH and metal contaminated soils. This suggests that high levels of PAH contamination negatively affected the diversity and composition of both AOA and AOB populations but not their nitrification potential. While differences in community composition could not be correlated to TPAH concentrations, they may be correlated with the bioavailable TPAHs, which were not measured. Furthermore, findings may mirror functional redundancy that is widely reported within metal contaminated environments. Little information is available on the impacts that PAH contaminants have upon diversity, composition and abundance of ammonia-oxidisers in soils. However, it has been reported that nitrification can be unaffected by PAH contamination (Deni and Penninckx, 1999) and contain stable *Nitrosospira*-like AOB populations (Kurola *et al.*, 2005). Even though PNR is maintained in high PAH contaminated soils, the reduced diversity may result in the ammonia-oxidising populations being vulnerable to additional perturbations.

In contrast, PNR in agricultural and garden land types appeared to be dominated by the AOA. The dominance of AOA in agricultural soils may be attributable to the soil pH. Low pH environments have been found to create a specific niche for AOA communities (Gubry-Rangin *et al.*, 2010; Lehtovirta-Morley *et al.*, 2011; Nicol *et al.*, 2008; Yao *et al.*, 2011). It is thought that the selection for AOA in acidic soils is due to AOA having a significantly lower half-saturation constant for NH_3 than their bacterial counterparts, or, that a low pH environment selects for specifically adapted AOA (Gubry-Rangin *et al.*, 2011; Martens-Habbena *et al.*, 2009). In contrast, garden land type soil pH ranged from acidic to neutral. Other studies have found that AOA are responsible for nitrification at neutral pH, which may be attributed to the availability of ammonia, differences in the physiology/metabolism in archaea or niche specialisation (Gubry-Rangin *et al.*, 2011; Yao *et al.*, 2011). A low but continual level of ammonia

supply from mineralisation of organic matter has been shown to select for AOA (Offre *et al.*, 2009; Verhamme *et al.*, 2011). Therefore, the dominance of AOA in the garden soils of this study at more neutral pH may also be ascribed to lower ammonia levels from the mineralisation of organic matter or organic material inputs.

Recently the abundance of ammonia-oxidising communities has been proposed as a possible cost effective bio-indicator for soil monitoring, as the literature suggests these populations have niche differentiations and are susceptible to environmental change (Wessén and Hallin, 2011). However, no precise guidelines, operating procedures or PCR primers were offered by the authors. Ritz and colleagues (2009) have also suggested the genetic profiling of AOB via TRFLP. However, the results from this study suggest that AOB profiling alone is insufficient because despite impacts on community composition; function may or may not be affected. While the abundance of AOA and AOB may be beneficial, it must be remembered that the exact contribution of the AOA in nitrification is still unknown (Mertens *et al.*, 2009). Any quantitative analysis must centre upon the transcriptional activity of *amoA* as it has been shown that while these genes exist in significant numbers they may not be actually being expressed, e.g. Mertens *et al.* (2009). Therefore, caution must be exercised, and the proposal of the using the abundance of AOA and AOB *amoA* as a bio-indicator alone is questionable.

In summary, PNR does not appear to be a sensitive indicator of PAH or metal contamination in long term polluted soils. Community profiling of AOB and AOA exposed strong negative effects of contamination upon community structure. This indicates that PNR measurements alone are inadequate in determining soil nitrification functioning in polluted environments. For nitrification to be included in assessing the intrinsic functional analysis of long term contaminated sites it is advised that any functional rate is coupled with functional gene profiling. Even though PNR is does not appear to be negatively affected by contamination the different AOA and AOB community compositions may result in these ammonia-oxidising populations being vulnerable to additional perturbations and thus, measurement of PNR is deemed as a worthy indicator of soil functioning when a remediation strategy is applied. Additionally, nitrification is intimately linked to denitrification, therefore, measurement of both DEA and PNR can give a good indication of the functional soil microbial N-cycling as a whole.

2.5. Conclusions

The current study indicates that despite high levels of PAH and heavy metal contamination within the soils of St. Anthony's Tar and Lead works diverse microbial populations are still present and are metabolically active. The long term pollution of these soils has resulted in a stressed microbial population that exhibits extremely suppressed rates of basal respiration and denitrification, indicative of pollutants being bioavailable within the soil matrix. Culture independent molecular analysis revealed a significant difference between the denitrifying populations of the contaminated soils in comparison to non-contaminated soils that were directly correlated to functional capability and thus contamination. Nitrification potential was unaffected by contamination as evidenced by rates equivalent with non-contaminated soils. However, community profiling revealed significant effects of contamination upon ammonia-oxidising community composition. Long term contamination of St. Anthony's Tar and Lead works soils did not affect bacterial community structure, diversity, microbial biomass or microbial quotients; with rates and values comparable to non-contaminated garden and agricultural soils. The study determined that denitrification enzyme activity (DEA) and basal respiration (BR) as the most sensitive indicators of microbial soil functioning within long term contaminated soils.

Chapter 3. The effects of natural Mn oxide-containing waste amendments upon key soil microbial functions in a long term metal impacted soil.

3.1. Introduction

Natural waste Mn oxides (hereafter referred to as MnOx which collectively includes oxide, hydroxide and oxyhydroxide minerals) have been proposed as a potential remediation strategy for contaminated soils (Tourney *et al.*, 2008). MnOx are one of the strongest naturally occurring oxidising agents in the environment after oxygen, facilitating the removal of organic contaminants and the immobilisation of heavy metals through processes such as surface adsorption, sequestration and oxidation (Tebo *et al.*, 2004; Tebo *et al.*, 2005). Consequently, natural pollutant degradation in soils is believed to be partly due to the ubiquitous occurrence of MnOx (Toner *et al.*, 2005).

The industrial past of the UK has left a legacy of contaminated land. Stresses such as rising populations in cities have resulted in the need to reuse this land for soft end applications such as housing and recreation. The maintenance of microbial populations in soil is pertinent for the restoration of contaminated sites for the aforementioned uses, via the protection of soil functions and processes. Consequently, in order for natural MnOx wastes to be effective as a remediation strategy they should not only remove pollutants but also strive to maintain soil microbial populations and their essential functions.

3.1.1. *Aims and Objectives*

While Mn is naturally abundant in soil, the impact of Mn upon microbial activity is unknown, in particular the impact of exogenous MnOx addition. The aim of the present study was to test whether applications of MnOx have any toxic effects on soil microbial activity in a historically metal impacted soil, and if so, what level of MnOx addition induces these effects. This study was designed to gain this understanding in a realistic application situation of natural MnOx sources added to soil with aged

contamination. The study further aimed to be a preliminary assessment as to whether natural waste MnOx were a viable remediation strategy that either promoted or maintained microbial functioning for future land applications.

The study objectives were:

(i) To use two forms of natural waste MnOx in microcosms, namely MnOx-coated sands from the UK water industry, and MnOx tailings from the South African Mining industry. These natural waste sources of MnOx are collectively termed as MnOx-containing wastes within this study and have been referred to as such hereafter.

(ii) To use a metal contaminated soil from St. Anthony's Lead Works, whose soil microbial functioning has been shown to be compromised (see Chapter 2).

(iii) To assess soil microbial functioning using three activity indicators: basal respiration (BR), potential nitrification activity (PNR) and denitrification enzyme activity (DEA).

(iv) To assess over time the effect that increasing concentrations of MnOx-containing wastes have upon soil microbial functioning in comparison with a non-MnOx-amended control through the use of microcosms.

(v) To assess the effect of sterile and non-sterile MnOx-containing wastes upon microbial functioning at one concentration level.

It was hypothesised that:

(i) MnOx addition would not have an overall negative effect on any measured soil microbial function as is has not been noted to have a high biological toxicity.

(ii) The addition of MnOx would not have any effect upon PNR within the heavy metal contaminated soil from St. Anthony's Lead Works.

- Results from Chapter 2 illustrated that nitrification appeared to be adapted to the heavy metal contaminated environment.

(i) The addition of MnOx would stimulate BR and therefore overall microbial activity through the sorption capacity that they have for inhibitory bioavailable metals, which would consequentially alleviate microbial metal toxicity.

- BR within St. Anthony's metal contaminated soils was determined to be intrinsically low in Chapter 2, appearing to have been suppressed by metal contamination.

(ii) That addition of MnOx to St. Anthony's metal contaminated soils will stimulate DEA through sorption of bioavailable metals onto the MnOx and mitigate metal stress upon denitrifying communities.

- DEA within St. Anthony's metal contaminated soils was previously determined to have been negatively impacted due to metal contamination (see Chapter 2).

3.2. Materials and Methods

3.2.1. *Soil sample collection, storage and analysis*

For this study soil was selected from an area of low metal contamination at the St. Anthony's Lead Works. A full description of St. Anthony's Lead Works along with sample collection, soil storage and preparation has been previously outlined in Chapter 2, sections 2.2.1, 2.2.2 and 2.2.5 respectively. This soil was selected as it represents a long term industrially polluted matrix, thus, making the risk assessment of MnOx addition to soil microbial functioning more realistic than their addition to a non-contaminated soil. Total metals analysis of this soil revealed that it contained elevated levels of Zn (190 mg kg^{-1}) and Pb (360 mg kg^{-1}). In other words, this soil has a relatively low level of metal pollution. It was envisaged that using a lower total metals concentration was more likely to result in immobilisation of bioavailable toxic metals in short term microcosm experiments. Therefore, this would make effects of MnOx upon soil functions measureable within the time period allocated. Furthermore, background levels of Mn within this soil were low at 350 mg kg^{-1} and an analysis of its microbial functioning has been previously described (Chapter 2). In Chapter 2 physico-chemical characteristics and microbial functions of were presented as mean values for both the high and low metal contaminated soils to represent a metal contaminated land type.

Please note that only values for the low metals soil are presented in this chapter which has been referred to as metal impacted hereafter.

3.2.2. *MnOx tailings site description, collection and storage*

Bulk MnOx tailing samples from Hotazel Mn Mine were collected by Dr Cathy Clarke (University of Stellenbosch, South Africa) in December 2008. A full description of Hotazel Mn Mine and its waste MnOx-containing tailings has been previously given in Chapter 1. Sampling was carried out by Dr. Clarke to ensure that samples used in this study as the same as previously described in Clarke *et al.* (2010). The tailings are stockpiled at the mine in four dry dumps, which are in places covered by grass. Samples were collected by Dr. Clarke from the top 0 - 10 cm of one tailings dump, from a position with no grass cover (Figure 3-1). Samples were sealed in sterile polyethylene bags (Fisher, UK) and couriered to Newcastle University. On arrival tailings were passed through a 2 mm sieve and were stored in the dark at 4 °C in loosely tied polyethylene bags (Fisher, UK) until further experimental analysis.

Figure 3-1: Photograph of one of four MnOx tailing dumps at Hotazel from which samples were taken. Picture is courtesy of Dr. Clarke (University of Stellenbosch, South Africa).

3.2.3. *MnOx-coated sand collection and storage*

MnOx-coated sands were locally sourced from Mosswood Water Treatment Works (WTW) (54°51°N - 53°59°W), which is owned and operated by Northumbrian Water Ltd. Mosswood WTW is situated 10 miles from Newcastle upon Tyne and lies 1.4 miles west of the village of Consett, Co. Durham. Mosswood WTW supplies, on average, 152 million litres of potable water to domestic and industrial customers within the Wearside and South Tyneside catchments and has been operational since 1968 (Northumbrian Water, 2008).

Untreated water for the Mosswood WTW is supplied from the Derwent Reservoir which is naturally high in dissolved Mn(II). Mn(II) removal at Mosswood follows the classical abiotic process of pH adjustment and filtration (Hu *et al.*, 2004b). During this the process water pH is adjusted to 9.2 by flash mixing with lime. At this pH soluble Mn(II) undergoes oxidation to insoluble Mn(IV) oxides. This is followed by rapid gravity filtration whereby the water passes through 12 rapid gravity filters arranged around a central gallery in 2 banks of 6. Each filter bed consists of graded silica sand aggregates topped by anthracite. During filtration Mn(IV) oxide is removed by attachment to sand particles, therefore, creating MnOx-coated sands.

MnOx-coated sands were collected in April 2008 from one of the 12 rapid gravity filter beds. One filter bed was taken out of operation and allowed to drain before Mosswood WTW staff augured cores across the top and bottom of bed to create a composite sample. MnOx-coated sands were collected in a sterile bucket and transported back to the laboratory where they were stored at 4 °C.

3.2.4. *Determination of the presence of MnOx*

Leucoberbelin blue (LBB; Sigma-Aldrich, UK) was used to test for the presence of MnOx in tailings, coated sands and the metal impacted soil. The colourless LBB reagent changes to blue via its oxidation with Mn(III) and/or Mn(IV) when present (Krumbein and Altmann, 1973). Spot tests were carried out by placing 1-2 grams of sample on filter paper. A few drops of 0.04 % LBB in 0.45 mM acetic acid were added to the sample and the presence of MnOx was confirmed when a blue colour change was observed. The filter paper used was checked for reactivity with LBB. As a control a synthetic MnO_2 (Sigma-Aldrich, UK) was checked for reactivity and colour change. In

addition, samples of MnOx-coated sands were sent to Professor Bradley M. Tebo at Oregon Health and Science University, USA, for Extended X-ray Absorption Fine Structure (EXAFS) analysis to determine the Mn oxidation state. The presence of MnOx and the oxidation state of Mn in the Hotazel mine tailings was previously determined by Clarke *et al.* (2010).

3.2.5. *Physico-chemical analysis of MnOx-containing wastes*

Physico-chemical analysis was carried out for MnOx-containing wastes included pH, moisture content (MC), water holding capacity (WHC) and organic matter (OM) content. Methods for these analyses have been previously described in Chapter 2, sections 2.2.6 to 2.2.9. Additionally for the MnOx-coated sands, metal and PAH analyses were performed, as described in Chapter 2 sections 2.2.11 and 2.2.12 respectively. Total metal contents of the MnOx tailings were previously characterised by Clarke *et al.* (2010).

3.2.6. *Microbial functioning of MnOx-containing wastes*

Both MnOx-containing wastes were tested in triplicate for the presence of basal respiration (BR), denitrification enzyme activity (DEA) and potential nitrification activity (PNR). A full description of these methods has been previously described in Chapter 2, sections 2.2.13 to 2.2.15.

3.2.7. *Molecular microbial diversity and structure of MnOx-containing wastes*

The community structure and diversity of the predominant bacterial, ammonia-oxidising and denitrifying communities associated with both types of MnOx-containing wastes were determined. Triplicate samples of each MnOx-containing waste underwent genomic DNA extraction, followed by PCR amplification of 16S rRNA, *nirS*, *nirK*, AOB *amoA* and AOA *amoA* genes with subsequent community fingerprinting by DGGE. All of the aforementioned analyses were carried out according to the methods previously outlined in Chapter 2, sections 2.2.19 to 2.2.23.

3.2.8. *Microcosm trial 1: Assessing the effects of increasing MnOx concentrations upon soil microbial functioning in a long term metal impacted soil*

Microcosms were performed with both MnOx-containing wastes to determine the effect of their addition upon soil microbial functioning using basal respiration (BR), denitrification enzyme activity (DEA) and potential nitrification rates (PNR) as indicators. While it was known that both coated sand and tailing wastes contain MnOx, the actual total percentage of MnOx was not determined. However, it is assumed that Mn is mainly present in oxide forms on both waste sources. Therefore, the calculation of final concentrations of MnOx applied to the metal impacted soil in microcosms was inferred using the total Mn concentrations for both wastes.

The total Mn concentration of both MnOx-containing wastes was determined by aqua regia extraction as described in Chapter 2 section 2.2.11. Both wastes contained different amounts of Mn, with MnOx-coated sands having 9000 mg $kg^{-1}$and MnOx tailings containing 38 000 mg kg^{-1}. To allow for direct comparisons in amounts of Mn applied to soil, additions were normalised to equate to the same total % weight Mn. Final concentrations of total % weight Mn, were 1, 3, 7, 12, 20 and 30 of the dry weight composition. Please note that total % weight Mn has been reported as % MnOx within this chapter as it assumed Mn is mainly present as MnOx. Soil microcosms comprising of the three lowest MnOx concentrations (1, 3 and 7 total % weight Mn) were constructed with both forms of MnOx-containing wastes, while higher MnOx concentrations (12, 20 and 30 total % weight Mn) were only tested using tailings.

To allow for direct comparisons between MnOx-containing waste microcosms the amount of soil in each microcosm was kept constant, at 10 g of field moist soil (< 2 mm) per assay. Thus, each microcosm had the same starting quantity of microorganisms discounting any possible variability in measured functional rates due to dilution of the soil microbial communities. Furthermore, the total mass of amended soil in each microcosm was 60 g per assay. This guaranteed that in each microcosm the microbial communities throughout the solid matrix were exposed to the same headspace and amounts of air. This additionally ensured that no differences in microbial processes could be attributed to oxygen depletion or deprivation, which could consequently induce changes in microbial community composition and functions.

As varying amounts of MnOx had to be added to achieve the respective total concentrations, the adjustment of the total mass to 60 g was carried out using inert sterile sand (Sigma-Aldrich UK) to make up the balance of solid. The sand was sterilised by means of dry heat sterilisation according to Trevors (1996). Sand was placed (1 – 2 cm deep) in glass beakers covered with aluminium foil and heated (200 °C) for 48 hours.

The mixing of the metal impacted soil, sterile inert sand and either MnOx-containing wastes naturally produced a new matrix with different water holding capacities (WHC). Therefore, prior to set up of the microcosms; soil, sand and MnOx-containing wastes were mixed in the same proportions required for each individual concentration being tested, so that their exact WHC could be determined following the protocol outlined in Chapter 2, section 2.2.8.

All microcosms were setup in triplicate using 100 ml sterile glass serum bottles (Wheaton, Sigma-Aldrich, UK). Initially, 10 g of field moist metal impacted soil was weighed and added to each serum bottle. Subsequently, the amount of required MnOx-containing waste for each MnOx concentration level was weighed and added. At this point both soil and MnOx-containing wastes were thoroughly mixed using a sterile spatula. Finally, inert sterile sand was added to all assays and given a final thorough mix. All microcosms were then adjusted to 60 % of their respective WHC using microbiological grade filter sterilised water (Sigma, UK). Control microcosms consisted of 10 g of field moist metal impacted soil with 50 g of inert sand, which underwent the same rigorous mixing. A constant WHC was maintained throughout the experiment by gravimetric means whereby water loss and replacement was determined on the basis of weight changes. Samples were stored at 4 °C and plugged with sterile cotton wool to allow for aerobic conditions but prohibit microbial contamination.

Microcosms were carried out for a 6 month period with samples taken at 0, 2, 4, 8, 16 and 24 weeks. The sampling regime consisted of firstly measuring BR so that disturbance of the microbial communities through sub-sampling would not impact respiratory activity. Microcosms were exposed to air by removing cotton wool caps for 15 minutes, then sealed with butyl rubber stoppers and crimp closed. Adjustment of moisture was not required as microcosms were already held at 60 % WHC. Measurement of BR was carried out as previously described in Chapter 2, section

2.2.13. Following BR experiments all microcosms were again exposed to air and sub-sampled for DEA and PNR assays. DEA and PNR were determined as in Chapter 2, sections 2.2.14 and 2.2.15 respectively. After sampling, cotton wool plugs were replaced and microcosms were returned to storage at 4 °C. Results for BR, DEA and PNR were normalised to show rates in per gram of dry soil.

3.2.9. *Microcosm trial 2: Assessing the effects of sterile and non-sterile MnOx-containing waste addition upon soil microbial functioning in a long term metal impacted soil*

Microcosms were performed with sterile and non-sterile MnOx-containing waste sources. This was to determine if any effects observed on microbial functioning due to MnOx addition was as a result of the addition of microbial populations associated with these natural wastes. Unless stated otherwise (see below) all microcosms were setup, incubated and sub-sampled as described in the previous section. Likewise, the effects on soil microbial functioning were inferred through the use of basal respiration (BR), denitrification enzyme activity (DEA) and potential nitrification rate (PNR) as microbial indicators, with the same metal impacted soil utilised.

Microcosms were carried out using only one concentration of MnOx. A total MnOx concentration of 7 % (i.e. 7 % total weight Mn) was used as it was the highest concentration that could be achieved with both MnOx-containing wastes. As in microcosm trial 1, total Mn percentages were used to calculate and infer MnOx concentrations. Within microcosm trial 2 the matrix only consisted of metal impacted soil and MnOx-containing waste. This was considered more reflective of the actual application of MnOx-containing wastes as a remediation strategy for contaminated soils where inert sand would not be included. This approach also allowed for determining whether the inclusion of inert sand in microcosm trial 1 had affected soil functioning. Nonetheless, the total mass in each microcosm was kept at 60 g to assure the same aerobic conditions.

Sterilisation of MnOx-containing wastes was performed by initially moistening and incubating shallow layers of both wastes in glass beakers covered with aluminium foil for 48 hours at 37 °C to encourage germination of spores. This was followed by autoclaving at 121°C for 20 minutes which was repeated 3 times on consecutive days

to ensure effective sterilisation (Trevors, 1996). After autoclaving the MnOx-containing wastes were checked for sterility by testing for microbial activity through the measurement of BR, DEA and PNR. Additionally, DNA extractions were performed on triplicate autoclaved samples with PCR amplification (as previously described in section 3.2.7) of bacterial, ammonia-oxidising and denitrifying genes to ensure that sterilisation was successful.

3.2.10. *Statistical analysis*

All statistical analyses were carried out using SPSS statistics for windows (SPSS v17, SPSS, Inc. Chicago, IL, USA). For both microcosm trials the overall effects of MnOx addition, time and the interaction between MnOx and time, were tested for each soil microbial function (i.e. basal respiration [BR], potential nitrification rate [PNR] and denitrification enzyme activity [DEA]) by means of a two-way analysis of variance (ANOVA). This was carried out using a univariate general linear model where the fixed factors for microcosm trial 1 were MnOx concentration (%) and time (weeks), the dependant variable was the measured microbial functional indicator (BR/DEA/PNR). For microcosm trial 2, fixed factors were type of MnOx addition (i.e. tailings sterile, tailings non-sterile, coated sands sterile, coated sands non-sterile) and time (weeks), the dependant variable was the measured microbial functional indicator (BR/DEA/PNR). Significance in the differences between concentration and type of MnOx addition along with time was tested using the Tukey HSD post-hoc test for pairwise comparisons. Additionally, pairwise comparisons for trial 1 microcosms were carried out to determine significant differences by comparing each MnOx concentration to the non-MnOx control at each sampling period. Significant differences between measured parameters of MnOx-containing wastes and the metal impacted soil were also determined using a one-way ANOVA followed by Tukey's HSD test, or Independent sample t-testing.

3.3. Results

3.3.1. *Presence of MnOx in the metal impacted soil, coated sands and mine tailings*

The leucoberbelin blue (LBB) test verified that MnOx were present in both coated sands and mine tailings, as the reagent underwent a colour change from clear

to blue upon contact with both samples (Figure 3-2a and b). The LBB assay also indicated the presence of some MnOx in the metal impacted soil, as evidenced by a very faint blue colouration around the edges of the sample (Figure 3-2c). LBB did not show any reaction with the filter paper used to contain the test material but showed a strong response with a synthetic MnO_2, which produced the same blue colouring as both MnOx-containing wastes and the metal impacted soil (Figure 3-2d). The Mn oxidation state of MnOx-coated sands was determined to be 4+ (Prof. B. Tebo, Personal Communication).

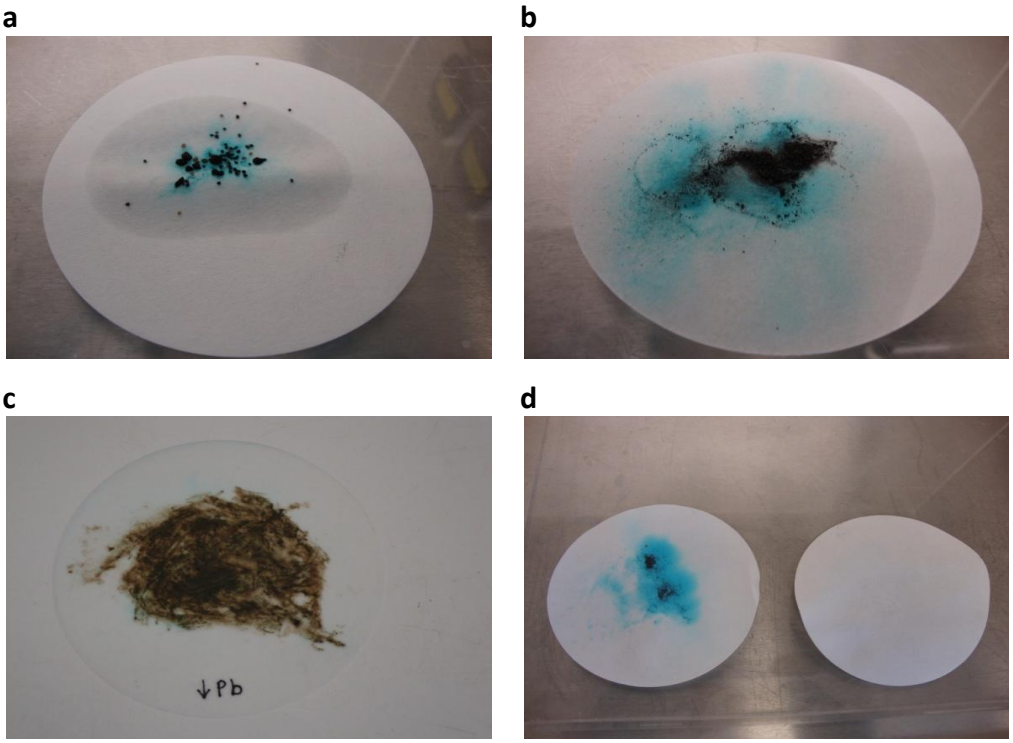


Figure 3-2: Images of LBB spot tests to verify the presence of MnOx in (a) coated sands, (b) mine tailings, (c) metal impacted soil and (d) synthetic MnO_2 (to left), filter paper control (to right).

3.3.2. *Physico-chemical analysis of MnOx-containing wastes and the metal impacted soil*

Comparison of means showed that all physico-chemical variables (pH, MC, OM and WHC) between MnOx-coated sands and MnOx tailings significantly differed from each other (one-way ANOVA, $p < 0.01$, Table 3-1). Both MnOx-containing wastes exhibited alkaline pH values. However, the tailings harboured a significantly higher pH

(8.6) in comparison to the coated sands (8.0). Conversely, coated sands showed higher values of MC, OM and WHC, in comparison to the tailings. In general, the metal impacted soil exhibited different mean physico-chemical properties to the MnOx-containing wastes (Table 3-1). This soil displayed a significantly lower pH (6.7) to both MnOx-containing wastes (one-way ANOVA, Tukey's HSD, $p < 0.01$, Table 3.1). The OM of the metal impacted soil was significantly higher than tailings (one-way ANOVA, Tukey's HSD, $p < 0.05$), but not different to the coated sands (one-way ANOVA, Tukey's HSD, $p > 0.2$). Additionally, WHC and MC were significantly higher for the metal impacted soil than that harboured by the MnOx-containing wastes (one-way ANOVA, Tukey's HSD, $p < 0.01$).

Table 3-1: Average physico-chemical properties for the MnOx-containing wastes and metal impacted soil used in this study. All values are based upon the mean of triplicate samples.

	pH	MC (%) [a]	OM (%) [b]	WHC (%) [c]
MnOx-coated sand	8.0 ± [d] 0.01	10 ± 0.8	24 ± 7	40 ± 1
MnOx tailings	8.6 ± 0.01	2 ± 0.3	3 ± 1	24 ± 2
Metal impacted soil	6.7 ± 0.01	32 ± 0.4	13 ± 1	106 ± 5

[a] moisture content (MC)
[b] organic matter (OM)
[c] water holding capacity (WHC)
[d] ± 1x standard error

Total metal analysis confirmed that Mn was the major metal present in both MnOx-containing wastes, with values far in excess of typical worldwide Mn concentrations found in soils (Table 3-2). Mn concentration in tailings was found to be four-fold higher than in coated sands, at 38 % and 9 % respectively. Both MnOx-containing wastes displayed typical levels of Hg, As, Cr, Cu and Se, which were below that of the metal impacted soil. However, the coated sands contain levels of Cd, Ni and Zn that would be classified as contaminated with regard to accepted soil guidelines. Concentrations of Zn (10,000 mg kg^{-1}) were in excess of Dutch list action value (720 mg kg^{-1}), with concentrations of Cd and Ni exceeding the UK soil guideline values (SGV) at

the residential land use level (10 mg kg^{-1}) and an allotment land use level (230 mg kg^{-1}) respectively.

In contrast, MnOx tailings only displayed elevated metal concentrations of Fe and Pb. Levels of Ni or Zn in the tailings were not determined by Clarke *et al.* (2010). It has been reported that ores within the Kalahari Mn field have mean Ni and Zn concentrations of 20 mg kg^{-1} and 62 mg kg^{-1} respectively (Gutzmer and Beukes, 1997). Therefore, it is assumed that levels of Ni and Zn are within typical worldwide ranges for soils. MnOx-coated sands were determined as having no significant levels of PAHs (< 1.0 mg kg^{-1} for each EPA priority PAH). PAH concentrations in the MnOx tailings were not determined.

Table 3-2: Total heavy metal contents (mg kg^{-1}) of MnOx-containing wastes presented with previously determined values for the metal impacted soil used in this study. Values are presented with the range of worldwide soil means for each heavy metal as taken from McBride (1994) and Kabata-Pendias and Pendias (1992), along with Dutch threshold action values for contaminated land (VROM, 2000). Values are based upon one sample for MnOx-containing wastes and the metal impacted soil, thus, no standard errors are reported. Values for MnOx tailings are taken from Clarke *et al.* (2010). All total metal concentrations were determined via aqua regia digestions.

	Metal impacted soil	MnOx-coated Sand	MnOx tailings	Typical Worldwide Ranges	Dutch thresholds
Hg	0.31	<0.06	0.1	0.02 - 0.41[a]	10
As	29	2.8	20	2.2 - 25[a]	55
Cd	0.56	32***	0.6	0.06 - 1.1[a]	12
Cr	78	4	42	7 - 221[a]	380
Cu	58	42	41	6 - 80[a]	240
Fe	3200	20000	140000*	5000 - 50000[b]	n/a
Pb	360*	44	110*	10 - 84[a]	530
Mn	350	90000*	380000*	80 - 1300[a]	n/a
Ni	52	1600***	-	1 - 450[b]	210
Se	0.88	<0.20	<0.2	0.5 - 1.27[a]	100
Zn	190*	10000**	-	17 - 125[a]	720

[a] McBride (1994)
[b] Kabata-Pendias and Pendias (1992)
*Total metal concentrations that exceed typical worldwide ranges.
**Total metal concentrations that exceed Dutch List guidelines.
***Total metal concentrations that exceed UK Soil Guideline Values (SGVs).

3.3.3. *Microbial functioning of the metal impacted soil and MnOx-containing wastes*

MnOx tailings showed no detectable levels of BR, DEA or PNR (Table 3-3). Similarly, MnOx-coated sands did not display any detectable levels of BR or DEA, however, they did produce measurable PNR rates at 0.27 ± 0.04 μg NO_2-N*g^{-1} dry solids*h^{-1} (Table 3-3). Independant sample t-testing confirmed that PNR of the MnOx-coated sands was signifcantly lower than that previously determined for metal impacted soil in Chapter 2 ($p < 0.01$).

Table 3-3: Mean values for basal respiration (BR), denitrification enzyme activity (DEA) and potential nitrification rates (PNR) for each MnOx-containing waste used in this study. Rates are presented with previously determined values for the metal impacted for comparison. All values are based upon the mean of triplicate samples.

	BR[a]	DEA[b]	PNR[c]
MnOx-coated sand	BD[d]	BD	0.27 ± [e] 0.04
MnOx tailings	BD	BD	BD
Metal impacted soil	0.021 ± 0.000	0.32 ± 0.02	0.85 ± 0.06

[a] basal respiration ($mgCO_2$*h^{-1}*g^{-1} dry solids)
[b] denitrification enzyme activity (μgN*h^{-1}*g^{-1} dry solids)
[c] potential nitrification rate (μgNO_2-N*h^{-1}*g^{-1} dry solids)
[d] below detection limit
[e] ± 1x standard error

3.3.4. *Molecular microbial diversity and structure of MnOx-containing wastes*

Both MnOx-containing wastes exhibited the presence of bacterial and denitrifying communities as evidenced by the amplification of 16S rRNA, *nirK* and *nirS* gene fragments. MnOx tailings showed no detectable AOA or AOB *amoA* genes. Interestingly, AOA or AOB *amoA* genes were undetected in MnOx-coated sands which displayed measurable PNR rates. On the other hand, denitrifying communities were detected in both wastes when no DEA was measureable. DGGE profiling of predominant bacterial and denitrifying communities illustrated that both MnOx-containing wastes harboured diverse communities (Figure 3-3).

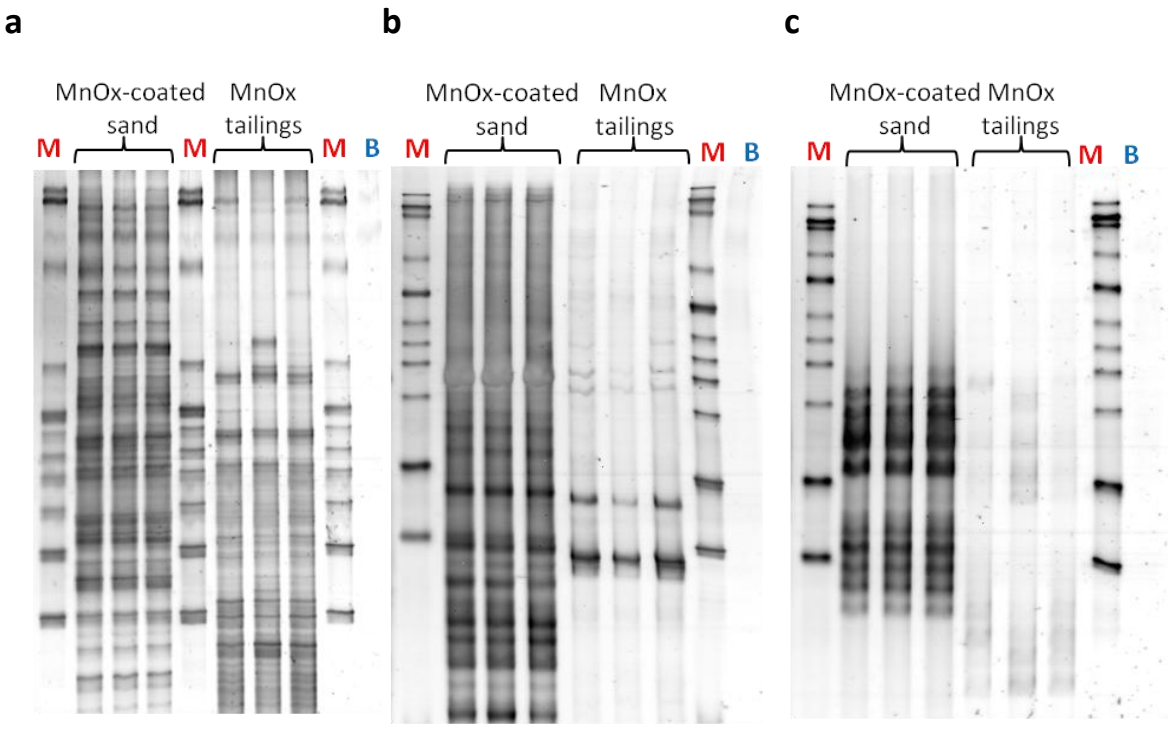


Figure 3-3: MnOx-containing waste DGGE profiles of (a) bacterial 16S rRNA, (b) *nirK* gene fragments and (c) *nirS* gene fragments. Where **M** = represents marker lanes in profiles and **B** = procedural blank (carried through DNA extraction, PCR amplification and DGGE).

Predominant bacterial and denitrifying communities formed distinct clusters for MnOx-containing wastes and the metal impacted soil (Figure 3-4). All MDS plot stress values were close to 0, indicating an excellent spatial representation of the data. Analysis of Similarity (ANOSIM) confirmed that differences between individual sample types were significant. Replicates of the predominant bacterial and *nirS* community compositions were more similar to each other within the metal impacted soil, MnOx-coated sand and MnOx tailings than between; the sands, tailings and the soil showed a high dissimilarity within their bacterial and *nirS* community structures (Global R = 1, $p < 0.01$). Furthermore, the predominant *nirK* community structure was significantly different between MnOx-containing wastes and the metal impacted soil (Global R = 0.92, $p < 0.01$).

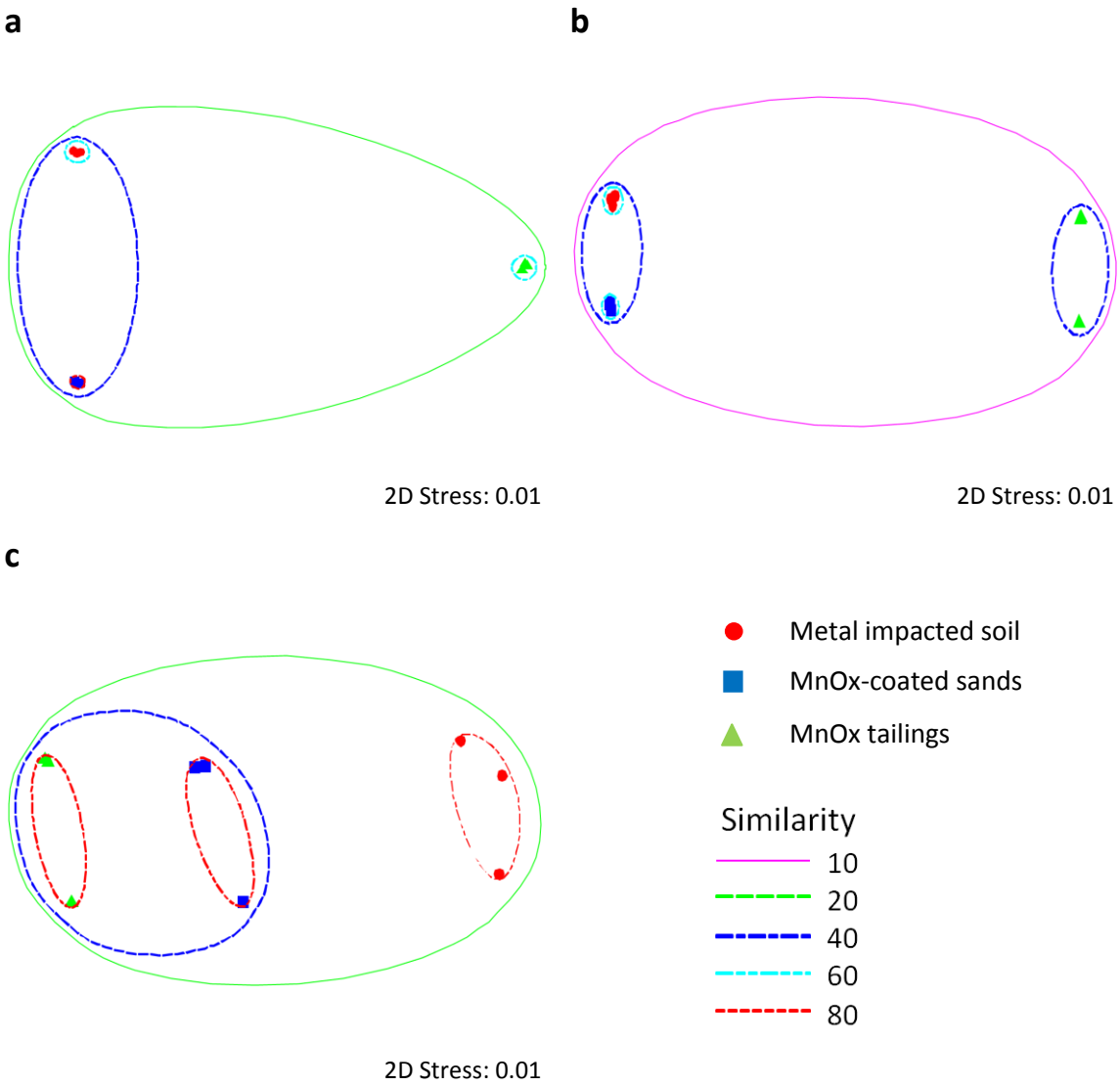


Figure 3-4: 2D MDS plots showing a spatial representation of the similarity between DGGE derived community structures based on analysis of (a) bacterial 16S rRNA, (b) *nirK* and (c) *nirS* gene fragments of MnOx-containing wastes and the metal impacted soil. Contour lines showing percentage similarity from cluster analysis are overlaid on all MDS plots.

The predominant bacterial communities of MnOx-containing wastes did not significantly differ to that of the metal impacted soil in terms of Margalef's richness (*d*), Shannon (*H'*) or Simpson (*1-λ*) diversity indices (one-way ANOVA, Tukey's HSD, $p > 0.06$, Table 2-12). Nevertheless, MnOx-containing wastes were significantly different to each other in term of *H'* and *1-λ* (one-way ANOVA, Tukey's HSD, $p < 0.05$), with MnOx tailings exhibiting significantly lower diversity index values. The predominant bacterial community evenness (*J'*) was found to be significantly lower in MnOx tailings in

contrast to both MnOx-coated sands and the metal impacted soil (one-way ANOVA, Tukey's HSD, $p<0.05$, Table 2-12), with the coated-sands and metal impacted soil displaying no significant differences to each other (one-way ANOVA, Tukey's HSD, $p > 0.01$).

Diversity indices (Margalef's richness, d; Pielou's evenness, J'; Shannon, H' and Simpson, 1-λ) of predominant *nirK* communities on MnOx tailings were significantly lower than those on MnOx-coated sands and the metal impacted soil (one-way ANOVA, Tukey's HSD, $p < 0.05$, Table 2-12). Diversity indices for the latter two environments were statistically indistinguishable (one-way ANOVA, Tukey's HSD, $p > 0.05$).

Diversity indices for predominant *nirS* communities were only determined for MnOx-containing wastes as DGGE profiles for the metal impacted soil were subject to extensive smearing (see Chapter 2, Figure 2-10) and were therefore considered unreliable. Overall, the diversity indices (Shannon, *H'* or Simpson, *1-λ*), of predominant *nirS* communities on MnOx-containing wastes were not significantly different from each other (one-way ANOVA, $p > 0.05$, Table 2-12). However, predominant *nirS* communities on MnOx tailings had significantly higher values of Margalef's richness (*d*) in comparison to MnOx-coated sands (one-way ANOVA, $p < 0.05$). Conversely, these communities on MnOx-coated sands were significantly more even (*J'*) than those on MnOx tailings (one-way ANOVA, $p < 0.05$).

Table 3-4: Mean diversity indices derived from DIVERSE analysis of triplicate bacterial 16S rRNA, *nirK* and *nirS* DGGE profiles of MnOx-containing wastes and the metal impacted soil. Indices were calculated based upon the total number of DGGE Bands (*S*), and the sum of relative band intensities (*N*), to give an estimate of Margalef's richness (*d*), Pielou's evenness (*J'*), Shannon's diversity (*H'*) and Simpson's diversity (*1-λ'*).

		S	*N*	*d*	*J'*	*H'*	*1-λ*
Bacterial 16S rRNA	Metal impacted soil	26.00 ± [a] 2.08	1354.90 ± 104.85	3.47 ± 0.28	0.95 ± 0.01	3.09 ± 0.09	0.95 ± 0.01
	MnOx-coated sands	32.33 ± 0.88	1711.03 ± 36.59	4.21 ± 0.11	0.97 ± 0.00	3.37 ± 0.03	0.96 ± 0.00
	MnOx tailings	25.00 ± 1.00	725.10 ± 47.99	3.64 ± 0.13	0.92 ± 0.01	2.95 ± 0.06	0.94 ± 0.01
nirK	Metal impacted soil	25.33 ± 3.28	372.77 ± 28.14	4.10 ± 0.51	0.88 ± 0.02	2.85 ± 0.18	0.92 ± 0.02
	MnOx-coated sands	25.67 ± 0.33	754.20 ± 26.54	3.72 ± 0.07	0.90 ± 0.01	2.92 ± 0.03	0.93 ± 0.00
	MnOx tailings	9.33 ± 0.67	298.27 ± 43.42	1.46 ± 0.08	0.69 ± 0.06	1.55 ± 0.18	0.69 ±0.07
nirS*	MnOx-coated sands	11.00 ± 0.00	675.80 ± 37.01	1.54 ± 0.01	0.95 ± 0.01	2.28 ± 0.01	0.89 ± 0.00
	MnOx tailings	14.00 ± 0.00	70.23 ± 14.52	3.10 ± 0.14	0.89 ± 0.01	2.35 ± 0.02	0.90 ± 0.00

[a] 1x standard error
* Metal impacted soil values excluded from *nirS* diversity indices due to smearing on bands in DGGE profiling.

3.3.5. *Microcosm trial 1: Assessing the effects of increasing MnOx concentrations upon basal respiration in a long term metal impacted soil*

MnOx-coated sand addition had no adverse impact on soil basal respiration (BR). Two-way ANOVA of MnOx-coated sand amended microcosms revealed that when time was controlled for, the addition of MnOx in this form had no significant effect upon metal impacted soil BR ($p > 0.5$, Figure 3-5a). Additionally, the interaction between MnOx-coated sand amendment and time was not significant (two-way ANOVA, $p > 0.1$).

In contrast, results suggested that the MnOx addition in the form of tailings had negatively affected BR in the metal impacted soil. When time was controlled for, MnOx tailing addition had a highly significant effect upon soil BR (two-way ANOVA, $p < 0.001$, Figure 3-5b). Pairwise comparisons showed that BR in all MnOx tailing amended microcosms significantly differed from the 0% MnOx control over the time course of the experiment (two-way ANOVA, Tukey's HSD, $p < 0.05$). Furthermore, the interaction between MnOx addition in the form of tailings and time had a highly significant effect

(two-way ANOVA, $p < 0.01$). The decrease in BR with MnOx tailing addition was most notable at 16 and 24 weeks (Figure 3-5b). At these time points soil BR in all MnOx tailing amended microcosms was found to be significantly lower than the control (two-way ANOVA, Tukey's HSD, $p < 0.05$).

Time had a highly significant effect upon soil BR in both coated-sand and tailing amended microcosms when MnOx was controlled for (two-way ANOVA, $p < 0.001$). Over the course of the experiment soil BR in the control and all MnOx-amended microcosms continued to increase (Figure 3-5). In all microcosms significantly higher soil BR rates were found at the end than the beginning of the experiment (one-way ANOVA, Tukey's HSD, $p < 0.01$). These results indicate that BR had been negatively affected during microcosm setup and that over time this function was still recovering. Soil respiration rates at 0 weeks in both MnOx-containing waste amended microcosms were significantly lower those previously determined for the metal impacted soil in Chapter 2 (one-way ANOVA, Tukey's HSD, $p < 0.05$). However, at 24 weeks BR in all microcosms became comparable and therefore not significantly different to that of the metal impacted soil (one-way ANOVA, Tukey's HSD, $p > 0.05$). This suggests that BR took 6 months to recover from the physical disturbance resulting from amendment, and by the end of the experiment the metal impacted soil had regained its original levels of microbial respiratory activity.

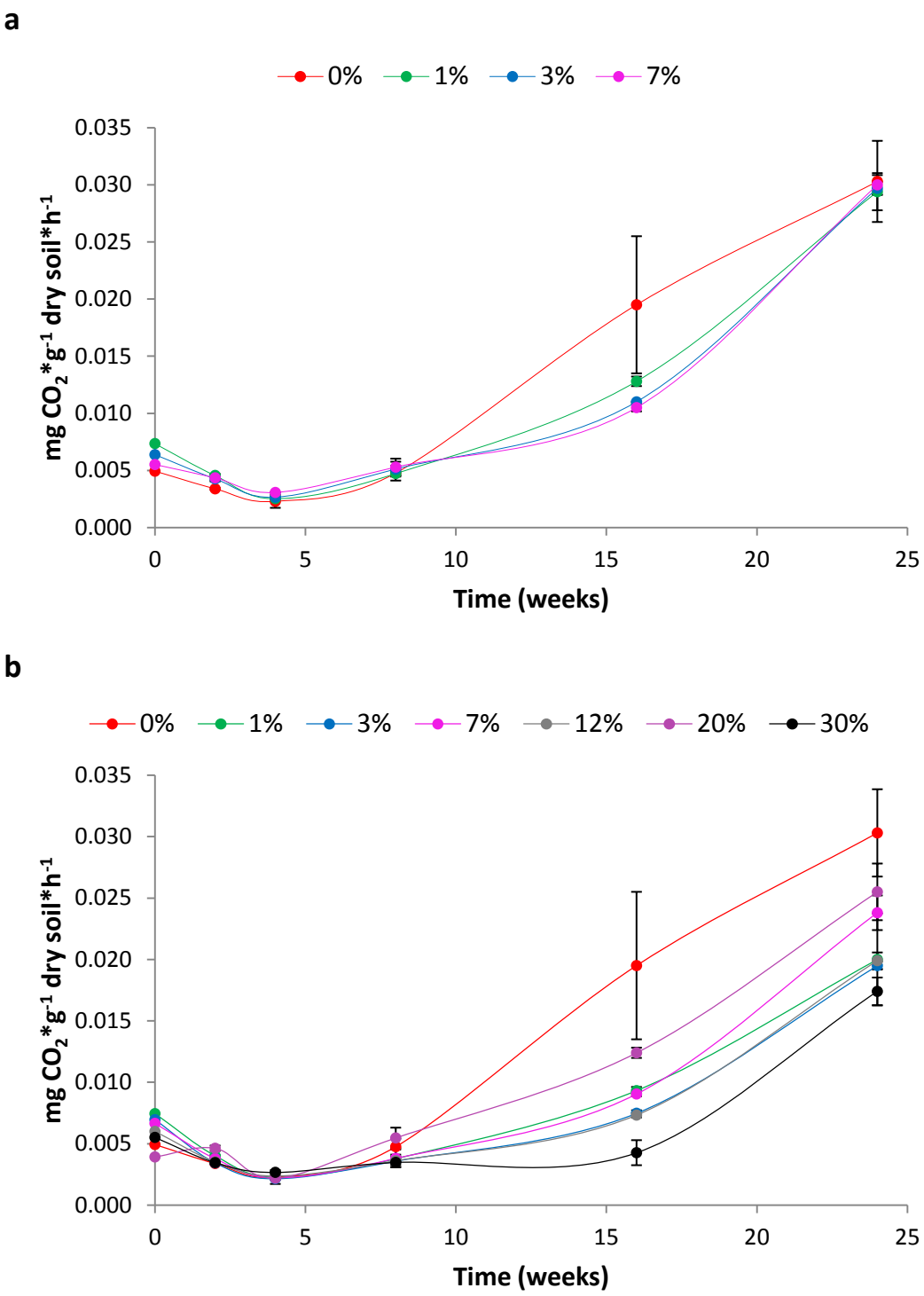


Figure 3-5: Basal respiration (BR) rates of the metal impacted soil in response to MnOx amendment via the use of two natural sources of MnOx-containing wastes: (a) MnOx-coated sands (b) MnOx tailings, over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 2, 4, 8, 16 and 24 weeks.

3.3.6. *Microcosm trial 1: Assessing the effects of increasing MnOx concentrations upon denitrification enzyme activity in a long term metal impacted soil*

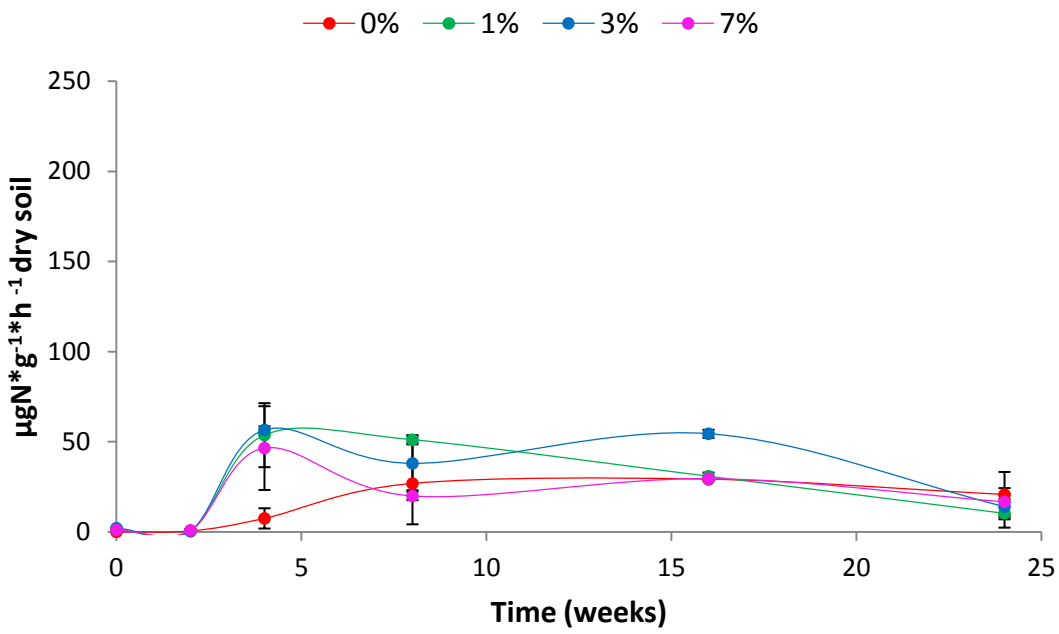
Results suggested that MnOx addition in the form of both MnOx-containing wastes induced a stimulation of denitrification enzyme activity (DEA) when added to the metal impacted soil. The addition of MnOx had a significant effect upon soil DEA when time was controlled for; interestingly coated sand amendment had a significant effect (two-way ANOVA, $p < 0.05$, Figure 3-6a), but, tailing amendment exhibited a highly significant effect (two-way ANOVA, $p < 0.001$, Figure 3-6b). Pairwise comparisons showed that 1 and 3 % MnOx-coated sands and 3, 7, 12, 20 and 30 % MnOx tailing amendments significantly differed from the 0 % MnOx control when time was accounted for (two-way ANOVA, Tukey's HSD, $p < 0.05$).

Time had a highly significant effect upon soil DEA within both MnOx-containing waste amended microcosms when MnOx was controlled for (two-way ANOVA, $p < 0.001$). Most notably, both coated sand and tailing amended microcosms exhibited the same pattern in DEA rates between 2 and 4 weeks, where DEA was stimulated in all amendments excluding 1 % MnOx mine tailing microcosms (Figure 3-6). Pairwise testing confirmed that all the aforementioned MnOx-containing waste concentrations (excluding 1 % MnOx tailings) resulted in a highly significant increase in DEA when compared to the control at 4 weeks (two-way ANOVA, Tukey's HSD, $p < 0.001$). However, in MnOx-coated sand amended microcosms, this stimulation decreased after 4 weeks. By the end of the experiment (24 weeks) the DEA of MnOx-coated sand amended assays were comparable to the 0% MnOx control (two-way ANOVA, Tukey's HSD, $p > 0.4$, Figure 3-6a). Furthermore, the overall interaction between MnOx-coated sand and time over the course of the experiment was not significant (two-way ANOVA, $p > 0.1$).

Conversely, the overall interaction between MnOx tailings and time was highly significant (two-way ANOVA, $p < 0.001$). From Figure 3-6b it is visually apparent that the stimulation in DEA observed at 4 weeks with MnOx tailings amendment levels of 3 - 30 % underwent a continual decrease until 16 weeks. However, from 16 to 24 weeks 7 - 30 % MnOx mine tailing amended microcosms showed a second spike in DEA. This second stimulation was supported by pairwise comparisons, which showed

significantly higher rates of DEA than the control (two-way ANOVA, Tukey's HSD, $p < 0.001$).

a



b

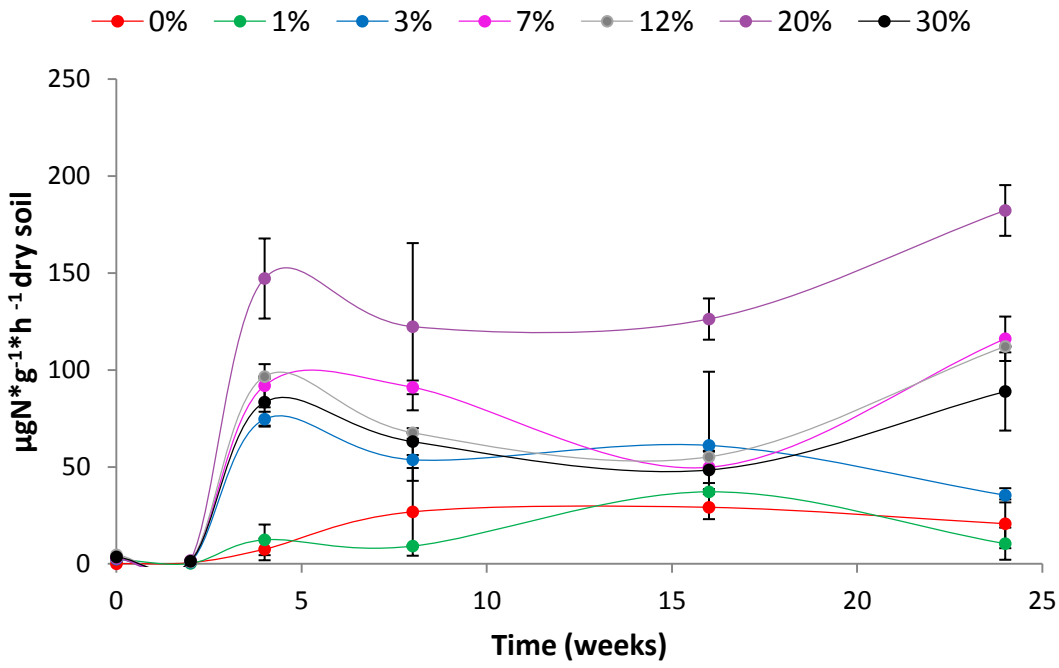


Figure 3-6: Denitrification enzyme activity (DEA) rates of the metal impacted soil in response to MnOx amendment via the use of two natural sources of MnOx-containing wastes: (a) MnOx-coated sands (b) MnOx tailings, over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 2, 4, 8, 16 and 24 weeks.

3.3.7. *Microcosm trial 1: Assessing the effects of increasing MnOx concentrations upon potential nitrification rate in a long term metal impacted soil*

The addition of MnOx-containing wastes, regardless of concentration level, stimulated potential nitrification rate (PNR) in the metal impacted soil. The addition of MnOx had a highly significant effect upon PNR when time was controlled for (two-way ANOVA, $p < 0.001$, Figure 3-7). Pairwise comparisons confirmed that all addition levels of MnOx-containing waste had significantly greater PNR than the control (two-way ANOVA, Tukey's HSD, $p < 0.01$). Time also had a highly significant effect upon PNR in both MnOx-containing waste amended microcosms when MnOx was controlled for (two-way ANOVA, $p < 0.01$). The overall interaction between MnOx-coated sand and time was not significant (two-way ANOVA, $p > 0.1$). However, MnOx tailing amended microcosms exhibited a highly significant interaction between MnOx and time (two-way ANOVA, $p < 0.001$).

The addition of MnOx-coated sands to concentrations of 3 and 7 % stimulated the metal impacted soil PNR, stimulation was longer for 7 % than 3 % amended microcosms (Figure 3-7a). At 2 and 4 weeks both 3 and 7 % coated sand amended microcosms showed a significantly increased PNR to the control (two-way ANOVA, Tukey's HSD, $p < 0.01$). From 8 weeks until the end of the experiment PNR decreased in the 3 % amended microcosms to levels not significantly different from the control (two-way ANOVA, Tukey's HSD, $p > 0.05$). Soil PNR in the 7 % MnOx-coated sand amended microcosms remained significantly higher than the control until 16 weeks (two-way ANOVA, Tukey's HSD, $p < 0.01$). From 16 to 24 weeks soil PNR values in these microcosms decreased and were therefore not significant different to the control (two-way ANOVA, Tukey's HSD, $p > 0.4$).

MnOx tailing amendments within the range of 1 – 20 % significantly differed to the control from 2 to 24 weeks (two-way ANOVA, Tukey's HSD, $p < 0.05$, Figure 3-7b). In contrast to MnOx-coated sand amended microcosms, MnOx tailing microcosms showed continued stimulation, albeit with decreasing PNR, to the end of the experiment (two-way ANOVA, Tukey's HSD, $p < 0.05$). The PNR rates in the 30 % amended microcosms were the only exception to this general pattern, exhibiting a continued increase that was significantly higher than the control over the 24 week experimental period (two-way ANOVA, Tukey's HSD, $p < 0.05$).

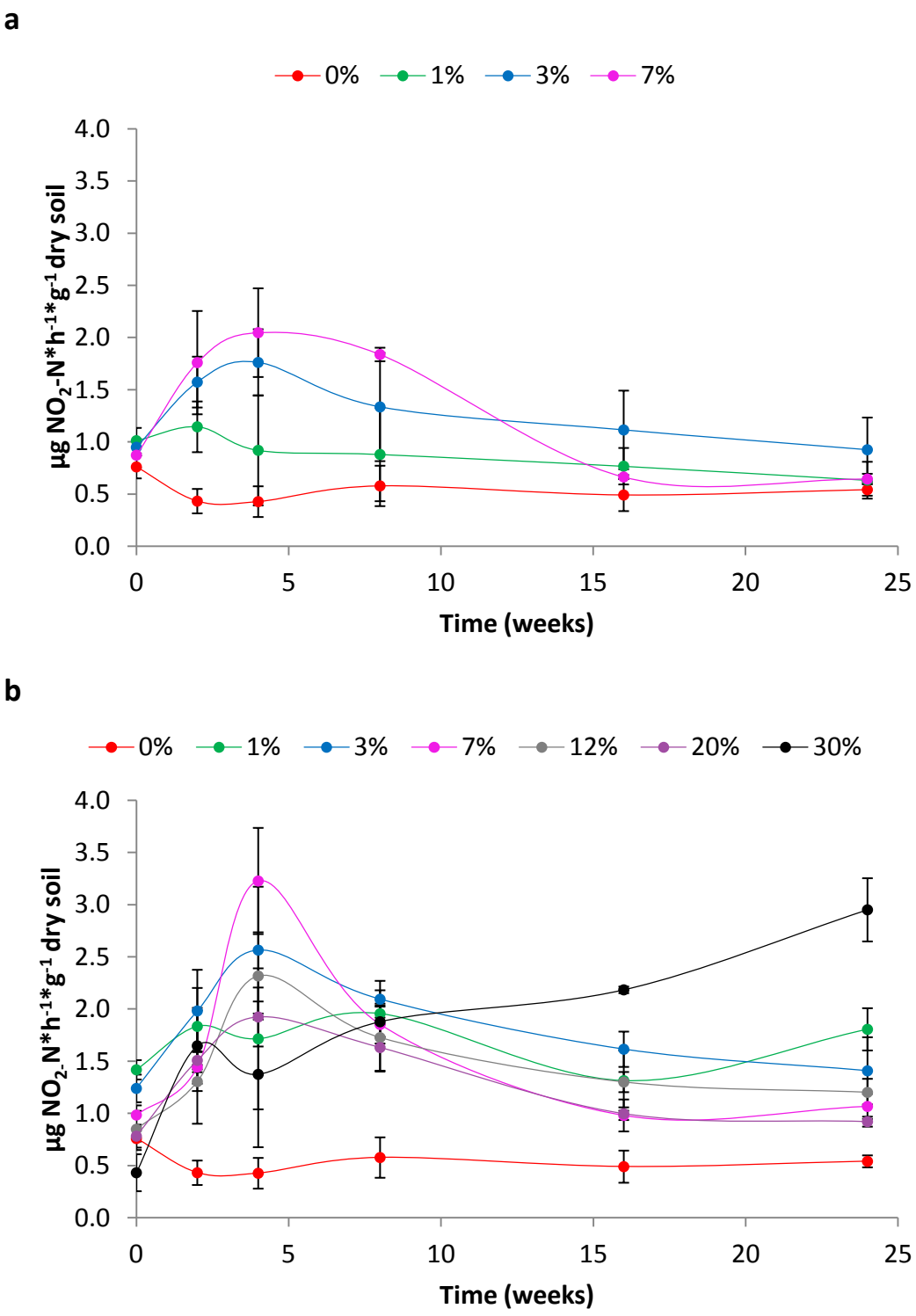


Figure 3-7: Potential nitrification rates (PNR) of the metal impacted soil in response to MnOx amendment via the use of two natural sources of MnOx-containing wastes: (a) MnOx-coated sands (b) MnOx tailings, over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 2, 4, 8, 16 and 24 weeks.

3.3.8. *Sterilisation of MnOx-containing wastes utilised in microcosm trial 2*

Both autoclaved MnOx-containing wastes showed no detectable levels of BR, DEA or PNR, thus, sterilisation had successfully removed any previously active microbial populations (Table 3-3). An alternative assessment of sterilisation was performed by checking for presence of DNA and post-PCR products from total bacterial and denitrifying communities. Ammonia-oxidising communities were not assessed due to their absence in previous PCR amplifications from live sands and tailings (discussed previously in section 3.3.4). Genomic DNA was undetected by agarose gel electrophoresis after autoclave sterilisation (Figure 3-8). However, DNA may have been present below the detection limits of this visualisation method. Therefore, to verify the success of sterilisation PCR amplification was carried out. This analysis revealed no amplification of bacterial 16S rRNA genes or denitrification (*nirS*/*nirK*) genes (Figure 3-9). Overall the absence of any microbial activity and amplified genes clearly demonstrates that sterilisation had been accomplished.

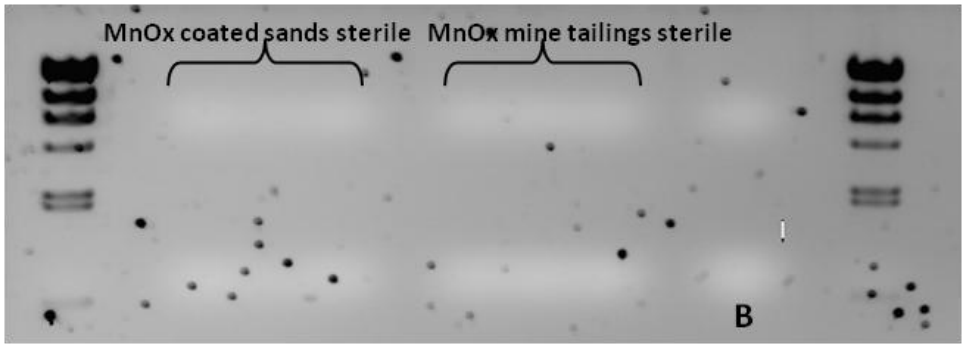


Figure 3-8: Electrophoresed agarose gel image showing genomic DNA generated from autoclaved sterile MnOx-containing wastes. First and last lanes are DNA markers (λ-DNA Hind III) with the procedural blank marked as B.

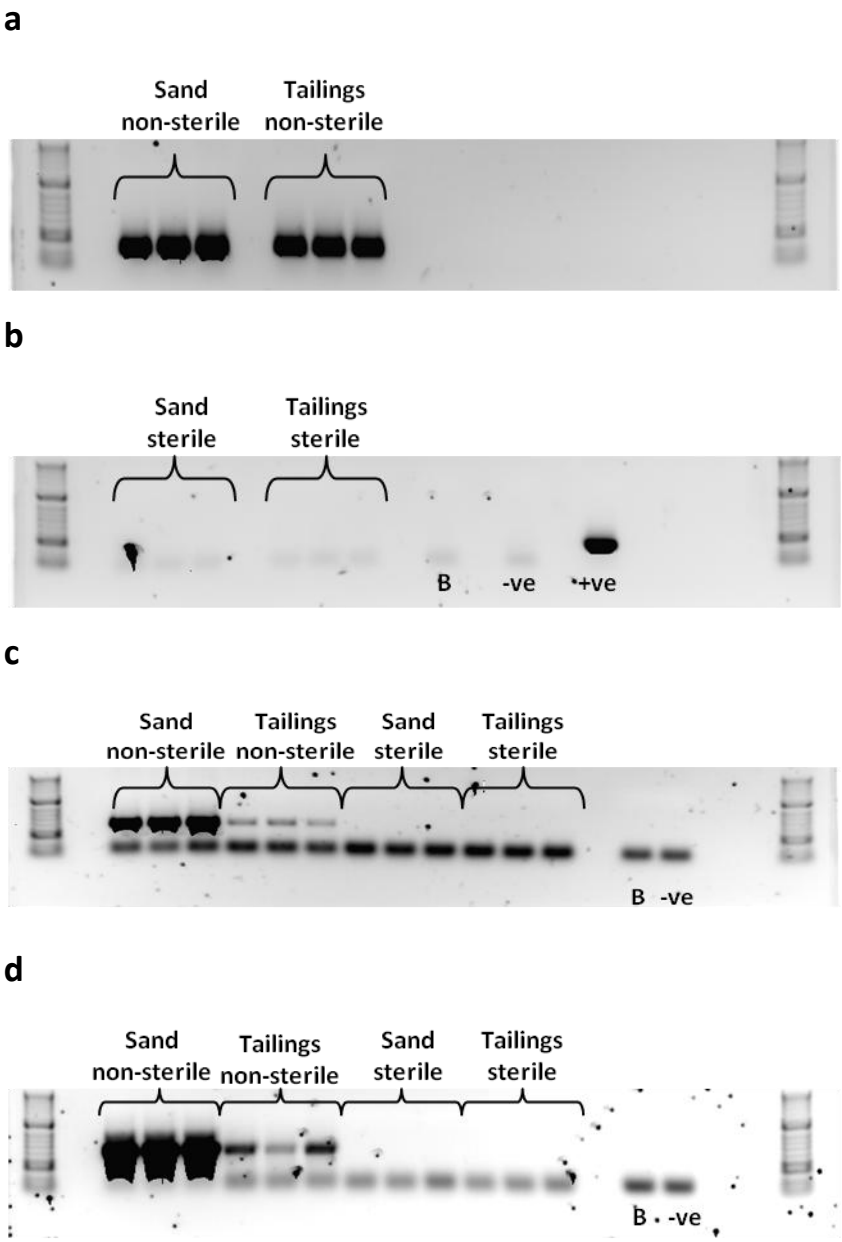


Figure 3-9: Electrophoresed agarose gel images showing PCR products of bacterial 16S rRNA (a, b), *nirS* (c) and *nirK* (d) genes generated from sterile and non-sterile genomic DNA of MnOx-containing wastes. Sands = MnOx-coated sands, Tailings = MnOx tailings. First and last lanes are DNA markers (Hyperladder II). Procedural blanks along with positive and negative controls are marked as B, –ve and +ve respectively.

3.3.9. *Microcosm trial 2: The effects of sterile and non-sterile MnOx-containing waste addition upon soil microbial functioning in a long term metal impacted soil*

The addition of microbial communities intrinsically associated with MnOx tailings had no additional effect on basal respiration (BR), since BR in the metal impacted soil with the addition of sterile and non-sterile tailings showed no significant differences (two-way ANOVA, Tukey's HSD, $p > 0.1$, Figure 3-10).

However, additions of sterile and non-sterile MnOx-coated sands were determined to result in significantly different soil BR (two-way ANOVA, Tukey's HSD, $p < 0.001$). Pairwise comparisons showed that the addition of sterile coated sands promoted significantly higher BR in the metal impacted soil when compared to its non-sterile counterpart at 0, 4 and 8 weeks (Figure 3-10, two-way ANOVA, Tukey's HSD, $p < 0.001$). By 16 weeks BR rates were statistically insignificant between sterile and non-sterile MnOx-coated sand amended microcosms until the end of the experiment (two-way ANOVA, Tukey's HSD, $p > 0.3$, Figure 3-10). The stimulation at 0 weeks with sterile MnOx-coated sand was unusual and suggests that autoclaving had induced an effect on the sands which caused a subsequent spike in BR. However, overall, the addition of microbial communities associated with MnOx-coated sands had little impact on respiratory activity in the metal impacted soil.

Both sterile and non-sterile MnOx-coated sands exhibited significantly higher BR rates when added to the metal impacted soil than that of sterile and non-sterile MnOx tailings (two-way ANOVA, Tukey's HSD, $p < 0.01$). Therefore, stimulation of microbial respiratory activity by the addition of MnOx-coated sands was not due to biology alone.

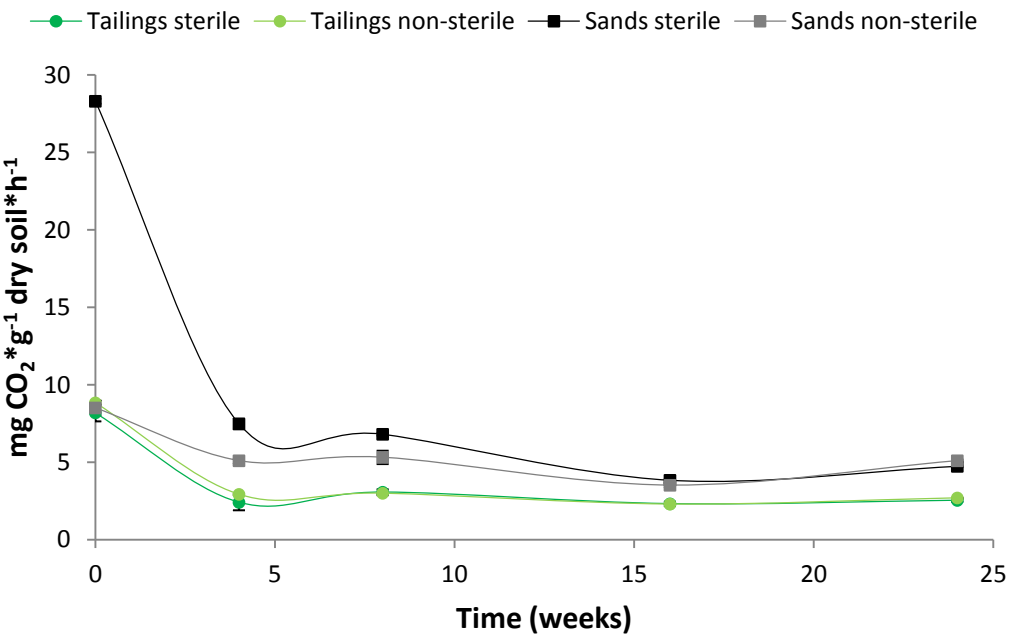


Figure 3-10: Basal respiration (BR) rates of a metal impacted soil in response to MnOx addition via the use of sterile and non-sterile MnOx-coated sands (sands) and MnOx tailings (tailings), over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 4, 8, 16 and 24 weeks.

Denitrification enzyme activity (DEA) rates in the metal impacted soil after the addition of sterile and non-sterile MnOx tailings were statistically indistinguishable from each other and from sterile MnOx-coated sands, when time was controlled for (two-way ANOVA, Tukey's HSD, $p > 0.5$, Figure 3-11). Thus, results suggest that the addition of microbial populations on MnOx tailings did not influence DEA in the metal impacted soil. Non-sterile MnOx-coated sand addition resulted in significantly lower DEA to all the aforementioned (two-way ANOVA, Tukey's HSD, $p < 0.001$, Figure 3-11). Furthermore, the sterilisation of MnOx-coated sands appeared to stimulate soil DEA rates. Time was also found to exhibit a significant effect upon DEA when differences in MnOx type were controlled for (two-way ANOVA, $p < 0.001$). Interestingly, all microcosms except for those with the addition of non-sterile MnOx-coated sands exhibited the same pattern in DEA rates over time as observed in microcosm trial 1 (Figure 3-6), whereby an initial stimulation was followed by decreasing potentials.

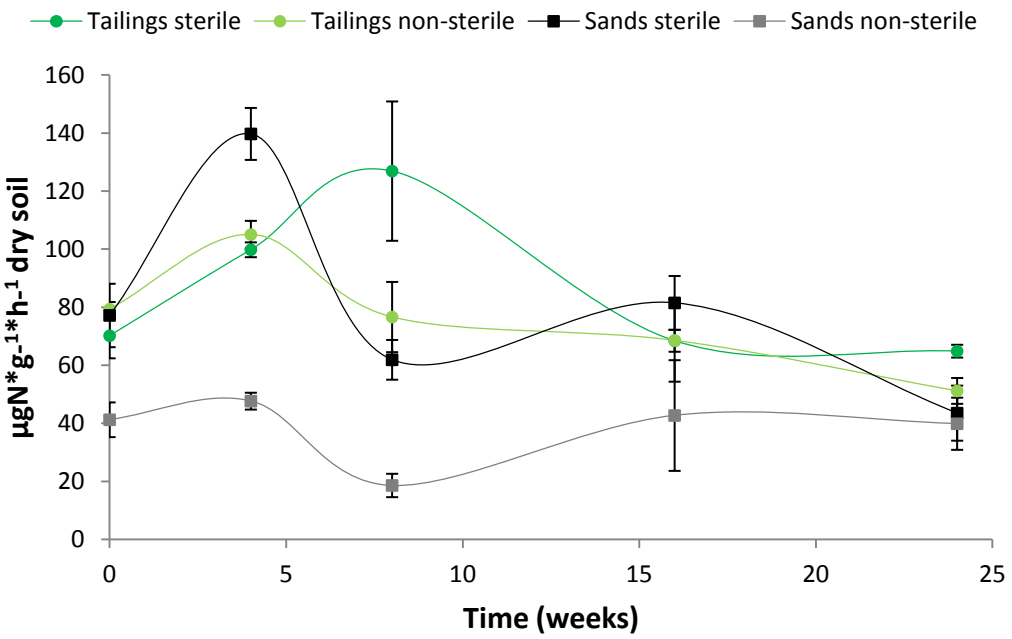


Figure 3-11: Denitrification enzyme activity (DEA) rates of a metal impacted soil in response to MnOx addition via the use of sterile and non-sterile MnOx-coated sands (sands) and MnOx tailings (tailings), over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 4, 8, 16 and 24 weeks.

Potential nitrification rate (PNR) in the metal impacted soil amended with sterile and non-sterile MnOx tailings were statistically indistinguishable (two-way ANOVA, Tukey's HSD, $p > 0.07$, Figure 3-12). Likewise, PNR in the metal impacted soil amended with of sterile and non-sterile MnOx-coated sands were also indistinguishable (two-way ANOVA, Tukey's HSD, $p > 0.7$). However, over the 6 month experimental period the addition of MnOx tailings consistently showed significantly higher soil PNR to MnOx-coated sand amended microcosms (two-way ANOVA, Tukey's HSD, $p < 0.01$). Soil PNR in both types of MnOx-amended microcosms fell over time, statistically confirmed by two-way ANOVA (Tukey's HSD, $p < 0.001$). Interestingly, this general pattern of decreasing PNR over time was also noted in microcosm trial 1 (Figure 3-7). However, the same stimulation of soil PNR observed in trial 1 between 0 to 4 weeks was not evident in trial 2.

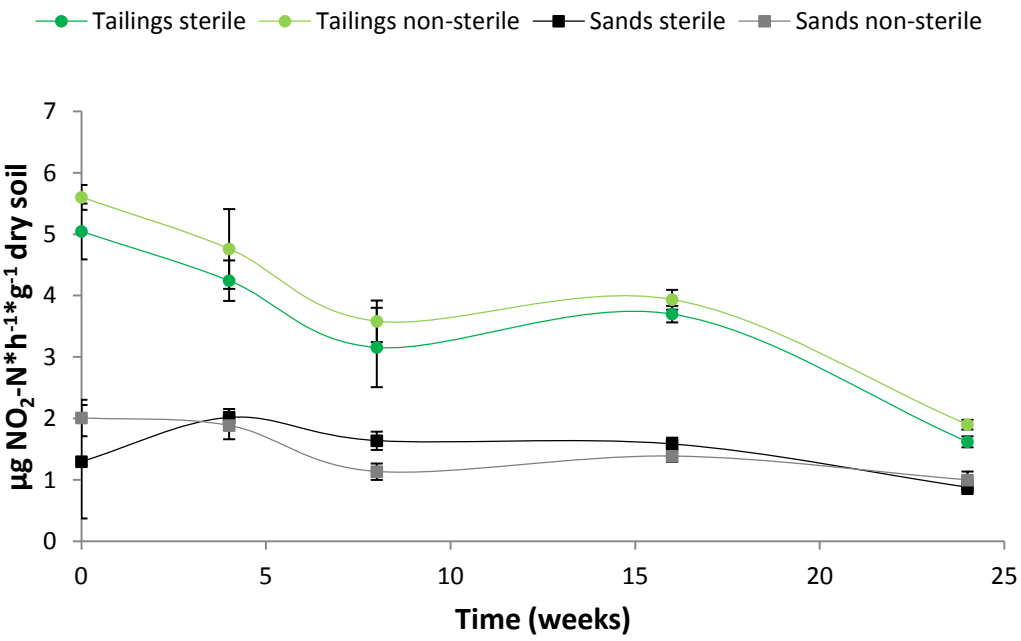


Figure 3-12: Potential nitrification rates (PNR) of a metal impacted soil in response to MnOx addition via the use of sterile and non-sterile MnOx-coated sands (sands) and MnOx tailings (tailings), over a sampling period of 6 months. Error bars represent 1 x standard error of triplicate microcosms. Samples were taken at 0, 4, 8, 16 and 24 weeks

3.4. Discussion

Many studies have looked at the effect various heavy metals (e.g. Cu, Ni, Cd and Zn) have on soil microbial activity and function, however, to date no literature exists upon effects of MnOx addition. The response of microbial activities to metal contamination has presented fundamentally diverse results, attributed to differences in metal bioavailability and the variability in the sensitivity of microorganisms to metal stress (Giller *et al.*, 2009). One of the greatest problems has come from the way in which short-term laboratory microcosms have been used. In general, microcosm based studies that assess metal toxicity to microbial populations have used a relatively uncontaminated soil that has been spiked with metal salts (Giller *et al.*, 2009). It is now accepted that short-term studies using metal salts cannot be used to infer effects of long term field exposure to metal toxicity (Giller *et al.*, 1998; Giller *et al.*, 2009; Renella *et al.*, 2002). This study attempted to overcome these complications by the addition of natural MnOx, not metal salts, to a long term industrially metal contaminated soil,

thereby producing a realistic situation in which the effects of MnOx addition upon microbial functioning could be inferred. The objective of the following discussion is to elucidate what impact the addition of natural MnOx-containing wastes has upon the soil microbial functioning and to present some possible explanations for observed impacts.

3.4.1. *Presence of Mn and MnOx in wastes and the metal impacted soil*

Total metal analysis of coated sands and mine tailings confirmed Mn as the major metal present in both wastes, with tailings having fourfold higher Mn contents than sands. These findings are consistent with the tailings being derived straight from a high grade Mn ore body, while MnOx only accumulates on the surface of sands during Mn(II) removal in water treatment.

Total metal analysis also showed that coated sands harboured levels of Cd and Ni that would be classified as contaminated with regard to UK Soil Guideline Values, and levels of Zn that exceeded Dutch contaminated land action thresholds. The levels are, therefore, of concern for MnOx-coated sand application to land as they could pose a risk to human health. Currently the only guidelines that exist on limits of total metal concentrations in waste materials that are to be applied to land are for compost (BSI PAS:100, 2011) and sewage sludge application (EU Sewage Sludge Directive 86/278/EEC, 1986). The Environment Agency promotes the use of waste in the restoration or improvement of land that has been previously subject to industrial or other man-made developments. However, no specific guidelines have been developed, and the use of wastes for land remediation is evaluated on a case by case basis via permit applications (Environment Agency, 2010).

While the levels of Cd, Ni and Zn are high in the MnOx-coated sands, a few points must be highlighted with regard to their application to contaminated land. Firstly MnOx addition to the soil would, in reality, be added at low levels e.g. ≤ 10 % of the total mass (see Chapter 4 for further details). Therefore, it would not be expected to make a major impact on total metal levels in soils that are already contaminated. If MnOx-coated sands were applied at 10 % of the total mass this would equate to additions of Cd at 3.2 mg kg^{-1}, Ni at 160 mg kg^{-1} and Zn at 1000 mg kg^{-1}. This would mean that addition levels of Cd are below the SGV for residential land uses (see Chapter 1, Table 1-6). However, Ni would still exceed the residential SGV (Chapter 1,

Table 1-6) and Zn would be in excess of Dutch thresholds. Secondly, although elevated Zn and Ni would still be present, they are envisaged to be tightly sorbed onto the MnOx and would therefore not be expected to be bioavailable or bioaccessible. Taking the total heavy metal results into account it could be said that MnOx tailings represent a better choice for remediation since they only exhibited elevated levels of Fe and Pb that were well below accepted contaminated land guideline values.

The LBB test was used to verify the presence of MnOx on both MnOx-containing waste samples being added to microcosms in this study. Both waste sources tested positive for presence of MnOx with LBB. However, it is worth noting that the LBB test cannot distinguish between oxidation states of Mn (Krumbein and Altmann, 1973). Although, it has been previously determined that Mn is present at an average oxidation state of 3+ on MnOx tailings (Clarke *et al.*, 2010), and the Mn oxidation state of MnOx-coated sands is 4+ (Prof. B. Tebo, Personal Communication). Additionally, LLB is known to react with Co(III) and synthetic Co(III) oxyhydroxides (Lee and Tebo, 1994). Even though levels of Co were not determined for either of the MnOx-containing wastes or soil used in this study it is assumed that Co(III) is present at low concentrations in comparison to the MnOx. The presence of some MnOx in the metal impacted soil was not unexpected as MnOx are commonly found in terrestrial environments (Post, 1999).

3.4.2. *Physico-chemical characteristics of MnOx-containing wastes and implications for their addition to a metal impacted soil*

One of the main concerns in adding a material to a metal contaminated soil is inducing a pH change. This is because as soil pH decreases, so does pH dependant charge, which causes an increase in metal solubility and thus, increased metal bioavailability to microbial populations. At lower soil pH metals are most commonly found as free ionic species or as soluble organo-metals complexes which places them in intimate contact with microbial populations (Maier et al., 2008; Yin et al., 2002). While both MnOx-containing wastes exhibited very different physico-chemical properties to each other, they were both of alkaline pH. This is important because the addition of these waste materials to soil would not cause a decrease in soil pH and result in the release of metals into soil pore water. The addition of MnOx wastes may, indeed, increase soil pH which would promote metal binding and reduce metal

solubility via a decrease in soil surface potential and proton competition (Maier *et al.*, 2008; Yin *et al.*, 2002). However, as the metal impacted soil is circum-neutral, most metals within the soil probably exist as insoluble mineral phosphates and carbonates, therefore, a small increase in pH would have a negligible effect upon metal bioavailability. It is also believed that the strong affinity for metals by MnOx would have a more powerful effect upon available metals in soil than any slight change in pH. Nevertheless the competition between MnOx and soil particles for metal ions during increasing soil pH is unknown.

MnOx-coated sands were shown to have significantly higher moisture, organic matter and water holding capacity contents than the MnOx tailings. Higher organic matter within sands is explained by that fact that rapid sand filtration at Mosswood WTW has a dual purpose to remove insoluble Mn(II), but also particulate organic matter from potable water. Differences in moisture contents are inherently due to the environments from which the wastes are found. As MnOx-coated sands are from an aquatic situation and MnOx tailings are piled in dry dumps, higher moisture levels in the coated sands were expected. Additionally, water holding capacities were also expected to be significantly higher for the coated sands due to the differences in their physical structures.

Based upon physico-chemical characterisation, the addition of MnOx-coated sands to the metal impacted soil present a superior source of additional organic matter and therefore carbon to the soil microbial community. This may be beneficial to microbial populations through the provision of more substrates for maintenance, growth and respiratory activity. However, the presence of organic matter on the MnOx-coated sand may also present competition on the MnOx reactive surface for metal sorption, or even reduced reactivity of the sand via binding of organic material to the MnOx mineral surface.

3.4.3. *Predominant microbial community structure, diversity and activity associated with MnOx-containing wastes*

Prior to this study the existence of microbial populations on both MnOx-containing waste sources was unknown. Both wastes displayed the presence of bacterial communities which have significantly different predominant community compositions to each other. MnOx-coated sands showed a higher predominant

bacterial diversity than that of MnOx tailings which may be attributed to more available carbon and generally a more heterogeneous structure to support a wider community. However, as discussed in Chapter 2 section 2.4.4, many issues are associated with the use of diversity indices from DGGE profiling, the values reported in this study only refer to approximately 1 -2 % of the most dominant communities therefore true diversity cannot be concluded.

If it is assumed that these communities are indigenous and active then it can be said that bacterial communities do exist in environments containing high levels of MnOx, a finding which would provide an initial assessment with regard MnOx toxicity to soil microbes. However, while bacterial communities were present they did not exhibit high levels of overall activity as evidenced by basal respiration rates which were below detection limits.

The discrepancy between activity and presence of bacterial communities may be for a number of reasons. For instance, microbial respiration rates may be intrinsically low as most energy is used for maintenance rather than growth, or activity levels may have below detection due to communities existing in low abundances. Additionally, the mere presence of amplified bacterial genes may not correspond with activity, as extra-cellular DNA can persist in soil for several months even when microorganisms are no longer present (Philippot and Hallin, 2005; Recorbet *et al.*, 1993). Thus, it is possible that the storage of the waste tailings and sands resulted in cell death consequently leaving DNA from communities which are no longer active.

Similarly a diverse community of denitrifiers was detected by targeting *nirS* and *nirK* functional genes in both MnOx-containing wastes. However, no potential denitrification enzyme activity was observed. Denitrification is an opportunistic growth mechanism and not necessarily a requirement for growth in soil (Wertz *et al.*, 2009). As aforementioned, genomic DNA extractions from soil do not distinguish between active and dormant organisms and the presence of a functional gene may not necessarily indicate that gene is being expressed *in situ* (Wallenstein *et al.*, 2006; Wallenstein and Vilgalys, 2005). As a result the detection of the functional gene does not always mean that denitrification is an active process in an environment. However, it is interesting that when the MnOx tailings were put into optimal conditions for denitrification in potential assays they still did not denitrify.

In contrast to microbial denitrifying populations, ammonia-oxidising (AOA/AOB *amoA*) communities from MnOx-containing wastes were not amplified. As MnOx-coated sands exhibited measurable PNR rates, results may indicate that the levels of functional genes present were below detection, or, that primers used do not target all ammonia-oxidising populations present on the coated sands. Issues pertinent to primer coverage of AOA and AOB communities have been previously discussed in Chapter 2, section 2.3.7. However, another possibility is that PNR measured was solely from abiotic nitrification, which would explain the presence of measurable rates with no active ammonia-oxidising populations. MnOx have been reported to be involved in nitrification; whereby MnO_2 is reduced by nitrite, thus, making nitrification possible in the absence of O_2 (Luther and Popp, 2002). However, the potential nitrification assay is aerobic in this study therefore abiotic involvement of MnOx in PNR seems unlikely. Although it is possible the anaerobic micro-sites existed in the soil slurry which may have induced abiotic nitrification by MnO_2, or that this reaction still occurs in the presence of O_2.

With regard to the addition of MnOx-coated sands to soil microbial functioning, the presence of nitrification and therefore the assumption that active nitrifying populations may be present could have a positive impact upon this function in soils. Overall results demonstrated that the addition of MnOx-coated sands to soil may have resulted in the addition of limited but potentially active microbial communities.

3.4.4. *The effect of MnOx addition upon the soil microbial functional indicator of basal respiration in a long term metal impacted soil*

Microcosm trial 1 revealed that the addition of MnOx-coated sand did not have any significant effect upon basal respiration (BR) in the metal impacted soil (Figure 3-5a). However, amendment of the same soil with MnOx tailings resulted in significantly lower BR (Figure 3-5b). Therefore, the addition of MnOx to soil in the form of coated sands maintained the same level of microbial respiration in the metal impacted soil as before their addition. However, the addition of MnOx in the form of tailings appeared to induce toxicity and stress on inherent soil microbial populations through the reduction of respiration and thus overall microbial activity and function.

It must be highlighted that the measurement of BR appeared to be compromised by the experimental set-up. Results from microcosm trial 1 clearly

showed that BR had been negatively impacted in both MnOx-containing waste amended microcosms at the beginning of the experiment, even after 6 months respiratory activity was still recovering (Figure 3-5). In this trial soil and MnOx-containing wastes were rigorously mixed with inert sands. It is this physical disturbance of the soil microbial communities that is thought to have caused the decreased respiratory activity. It has been well documented that microbial communities are sensitive to physical disturbance and do not rapidly recover to their original state, directly affecting ecosystem processes (Allison and Martiny, 2008). Disturbance of soils has been reported to increase microbial activity through the breaking down of soil aggregates which causes release of biological carbon substrates from the breaking up of organic matter, inducing a spike in respiration (Balabane and Plante, 2004; Shi *et al.*, 2002). CO_2 production from soil microbial communities is dependent upon the availability of soluble and labile C sources (Cleveland *et al.*, 2007). It is therefore hypothesised that the increase in BR over time is attributed to the breakup of soil aggregates that has released soluble and liable C sources, which in turn lead to a higher percentage of the microbial community having access to substrates for continued growth to recover from the initial physical disturbance. This theory is further substantiated by results from microcosm trial 2 (Figure 3-10), which showed when inert sand was not included in the microcosm matrix BR was not compromised. Furthermore, microcosm trial 2 did not exhibit the same pattern of increasing respiration over time as observed in trial 1 and the BR of the metal impacted soil was significantly higher than that measured in trial 1 (one-way ANOVA, Tukey's HSD; $p < 0.01$).

A longer experimental time would be required to fully assess how long it took for BR to recover from physical disturbance applied in the set-up of microcosm trial 1. It is consequently thought that the disturbance of the respiratory activity in these experiments may have masked the true effects of MnOx amendment to soil microbial activity. The significant differences observed between MnOx tailing amended assays and the control in microcosm trial 1 may just reflect a slower rate of recovery in these assays compared to the control. Thus, effects of MnOx addition upon BR could only be firmly concluded if this function had recovered. If the experiment was to be repeated again the inclusion of inert sand would not be carried out due the disturbance it had on BR. Unfortunately, microcosm trial 2 could not be used to infer the true effects, or

at least determine if reduced respiration was indicative of MnOx tailing addition, as the trial did not include a 0 % MnOx control.

Microcosm trial 2 did show that the addition of sterile MnOx-coated sands induced changes in BR. This was clearly evidenced by significantly higher soil BR at 0 weeks using sterile MnOx-coated sand amendments in comparison to rates observed using non-sterile MnOx-coated sand amendments (Figure 3-10). However, this initial higher respiratory activity was only transient, declining to a more stable rate after 4 weeks. Sterilisation by autoclaving has been commonly reported to stimulate CO_2 release, producing misleading respiratory results through the abiotic discharge of CO_2 leading to an overestimation of actual BR (Margesin, 2008). Autoclaving releases CO_2 that is free, loosely bound to soil particles, dissolved within soil pore water or entrapped within a soil inter-aggregate space such as cavities formed by organo-mineral complexes, thus, producing a stimulation of BR that is abiotic in origin (Margesin, 2008). Therefore, the stimulated respiration rates observed in microcosm trial 2 with the addition sterile of MnOx-coated sands can be explained by the method of sterilisation applied. Furthermore, it could be concluded that that the presence of microbial populations on MnOx-coated sands did not contribute to basal respiratory activity.

In contrast, the same abiotic stimulation of BR after autoclaving was not observed for sterile MnOx tailing amended microcosms (Figure 3-10). It is hypothesised that this was due to the dry nature of the tailing, whereby no CO_2 would have been dissolved within pore water. Additionally, the tailings are of crystalline structure with low organic matter content leading to little inter-aggregate spaces for entrapment of CO_2. Furthermore, microcosm trial 2 showed that the presence of microbial populations on MnOx-containing wastes did not contribute to basal respiratory activity which was not unexpected. Testing of both MnOx-containing wastes prior to their addition the metal impacted soil showed that neither exhibited measurable respiratory activity. Therefore, microcosm trial 2 provided more evidence that the DNA detected from MnOx-containing wastes through PCR amplification was probably from communities which were no longer active. However, it may be possible that these communities are in low abundance and their respiratory rates were below detection thresholds.

BR within the metal impacted soil was determined to be intrinsically low in Chapter 2, appearing to have been suppressed by bioavailable toxic metals. Consequently, at the beginning of this study it was hypothesised that the addition of MnOx would stimulate microbial respiration rates through the sorption capacity they have for inhibitory bioavailable/soluble metals, which would alleviate microbial metal toxicity. Independent batch metal adsorption experiments carried out by colleagues at Durham University assessed the ability of both natural MnOx-containing wastes for metal adsorption. MnOx-coated sands had higher capacities than tailings most likely due to their larger surface areas (Tourney, J., personal communication). At cirumneutral pH the tailings showed an ability to sorb 50 - 90 % of Pb in solution, while coated sands exhibited a higher Pb sorption capacity of 70 – 100 % (Tourney, J., personal communication). Sorption of Zn was lower for both wastes, however, MnOx tailings showed an ability to adsorb 5 - 30 % of total Zn, while MnOx-coated sands had a Zn sorption capacity of 30 - 80 % (Tourney, J., personal communication). The large differences in the sorption capacities determined was attributed to the heterogeneity of the natural samples, particularly the variation in surface areas (Tourney, J., personal communication). Additionally, EPMA analysis of MnOx-coated sands added to the metal contaminated soil utilised in this study showed that they are indeed capable of *in situ* Pb sorption within 9 month remediation trials. Please note that EPMA results showing Pb sorption are presented and discussed in Chapter 4, section 4.3.1.

While both waste MnOx sources were determined to be capable of metal sorption, no stimulation of BR was observed as a consequence of this process. However, as previously discussed the real effect of MnOx addition on the functional indicator of BR cannot be firmly concluded through the microcosm trials employed in this study. Thus, the original hypothesis cannot be definitively accepted or rejected.

3.4.5. *The effect of MnOx addition upon the soil microbial functional indicator of denitrification enzyme activity in a long term metal impacted soil*

Microcosm trial 1 showed that the addition of MnOx-containing wastes stimulated denitrification enzyme activity (DEA) in the metal impacted soil (Figure 3-6). Microcosm trial 2 (Figure 3-11) showed that the addition of microbial populations containing denitrification functional genes upon MnOx-containing wastes did not influence DEA, which was expected through the lack of activity associated with the

waste MnOx. Furthermore, trial 2 also exhibited the same pattern in DEA rates over time as observed in trial 1.

In Chapter 2, the metal impacted soil was found to exhibit significantly reduced DEA in comparison to non-contaminated soils, which was attributed to bioavailable toxic metals being present within the soil matrix. Thus, it was hypothesised that the addition of MnOx to this soil would be beneficial to DEA. This theory was based upon the assumption that soluble/available metals and/or other inhibitory substances present in the metal impacted soil would be sorbed to MnOx reactive surface, relieving stress on denitrifying populations and inducing a spike in DEA. As discussed in section 3.4.4, the MnOx-containing wastes utilised in these experiments were capable of metal sorption. Consequently, DEA results from both microcosm trials supported this hypothesis, and while sorption of bioavailable metals to MnOx-coated sands was not in fact assessed in this study, it can be inferred that this process was occurring through the stimulation in DEA.

It is worth noting that MnOx have been identified as an abiotic player within the nitrogen cycle and therefore the possibility of abiotic DEA stimulation via MnOx addition must be addressed (Luther *et al.*, 1997). For instance, MnO_2 is known to oxidise NH_3 and organic-N under aerobic conditions to produce N_2, short circuiting the conventional nitrification-denitrification pathway (Luther *et al.*, 1997). However, enhanced nitrous oxide (N_2O) production (as measured in the DEA assay) is not likely to have a resulted from such reactions because this experiment specifically tested the activity of the enzyme nitrous oxide reductase through its inhibition with acetylene. Likewise with other possible non-biological sources of N_2O, which include the chemical decomposition of nitrite (chemodenitrification) or hydroxylamine (NH_2OH) (Bremner, 1997) - with NH_2OH being more pertinent as MnOx are known to react with it to produce N_2O (Bremner, 1997; Mann and Quastel, 1946) - are not likely to have been measured via such a specific enzyme assay.

The addition of MnOx-coated sands displayed stimulatory but transient effects upon soil DEA microcosm trial 1 (Figure 3-6a), sterilised coated sands further enhanced this stimulation as evidenced in microcosm trial 2 (Figure 3-11).The transient nature of the DEA stimulus with MnOx-coated sand addition would suggest that the reactivity of the MnOx is being hindered somewhat. Soil DEA in trial 1 showed approximately a 6 week stimulation period, thus, it could be inferred that sorption of metals to the

reactive MnOx surface was only active during this time frame (Figure 3-6a). Additionally, results from trial 2 suggest that sterile MnOx-coated sands are actively sorbing metals for a longer time period (approximately 16+ weeks, Figure 3-11). Therefore, it is thought that the subsequent reduction in DEA within both trials may reflect the exposed reactive sites of the MnOx becoming reduced or covered in either adsorbed metals or organic material over time causing the surface to become less reactive. However, sterilisation of MnOx-coated sands further enhanced stimulation in DEA. This suggests that autoclaving rendered these sands better at metal sorption. Interestingly, independent infrared spectroscopic analysis of the MnOx-coated sand surface carried out by colleagues at Durham University indicated the presence of a C-H bond which is characteristic of the presence of organic matter (Tourney, J., personal communication). Additionally, the distribution of the organic matter was determined to be patchy, rather than a continuous layer upon the coating (Tourney, J., personal communication).

It is well documented that autoclaving releases organic carbon that is both trapped between and physically attached to soil particles (Serrasolsas and Khanna, 1995). However, heating organic material to 121 °C will not destroy all organic matter; instead it will only displace some bound organic particles from soil. While soils were not autoclaved in this study, it is assumed that the same effects are applicable to the MnOx-coated sand surfaces. Consequently, it is possible that autoclaving removed some of the organic matter that covered the reactive MnOx sand coating, thus, exposing more vacant sites on the MnOx for enhanced metal sorption, and thereby stimulating the DEA compared to the non-sterile MnO-coated sand. Additionally, autoclaving may have induced a chemical change to the MnOx coating thus enhancing sorption and stimulating DEA. Steam sterilisation is known to result in the partial oxidation of Mn(II) to insoluble MnOx which can then bind Mn(II) and catalyse its abiotic oxidation (Nealson, 2002). Therefore, it is possible that the process of autoclaving oxidised soluble Mn(II) leading to a release of fresh MnOx which were responsible for sorption of available metals.

MnOx tailing addition showed persistent and some cases increasing stimulation of the metal impacted soil DEA in trial 1 (Figure 3-6b), which stimulation attributed to abiotic rather than biotic mechanisms in trial 2 (Figure 3-11). Results for MnOx tailings in trial 1 suggested a threshold for stimulation of DEA somewhere between 3 and 7 %.

Intriguingly, the stimulation of DEA was strongest at 4 weeks when the total MnOx concentration were at 7 % or above; however, whether this increase would be sustained indefinitely was beyond the scope these relatively short term experiments. As with MnOx-coated sands it is also thought that the sorption of bioavailable metals from the metal impacted soil onto the MnOx-containing tailings caused the stimulation of DEA.

Results from microcosm trial 1 and 2 suggest that MnOx tailings may be more reactive than their coated sand counterparts due to their persistent and in some cases increasing stimulation of DEA (Figure 3-6 and Figure 3-11). Reasons for such a difference may include particle size, oxidation states of Mn, chemical composition or surface chemistry. Experimental analysis carried out by colleagues at Durham University determined that MnOx-coated sands possess a higher surface area (34 - 44 m^2/g) than the MnOx tailings (2.4 m^2/g), therefore, MnOx-coated sands would be expected to be more reactive than tailings. However, reactions carried out with hydroquinone, which is a mild reducing agent that gives an indication of the reactive Mn phases present (Bartlett and James, 1979), showed that tailings had a much higher composition of reactive MnOx phases than coated sands at 2.96 % and 0.01 % correspondingly.

While stimulation in DEA might be regarded as beneficial for the microbial soil functioning of the metal impacted soil, as this function has been severely compromised due to pollution in Chapter 2, it must be highlighted that this process is also the primary source of nitrous oxide (N_2O) in the environment. While the stimulation evident from the assay is a product of an artificial situation it may certainly have an effect under real world conditions. In comparison to CO_2, N_2O exhibits a global warming potential that is ~300 times higher and makes a total contribution of 6 % to the greenhouse effect (Philippot *et al.*, 2007). Soil alone is estimated to be responsible for 70 % of atmospheric N_2O emissions, thus, stimulation of DEA may have a knock on effect of increasing N_2O emissions (Philippot *et al.*, 2007). As denitrification and nitrification are de-coupled in their measurements in this study it is unclear whether in the actual soil environment an increased denitrification rate would be offset by an increased nitrification rate. Furthermore, the measurements of denitrification are, however, potentials measured under optimal conditions and may not be representative of actual rates. Therefore, a full examination of the complete

denitrification pathway and actual field N_2O emissions after MnOx addition are required to ensure that incomplete denitrification N_2O emissions are occurring. This could be determined via non-acetylene inhibited MnOx waste amended experiments. However, these were not carried out within this study and therefore no assessment of enhanced N_2O emission could be made.

Additionally, while the stimulation in DEA has been attributed to metal sorption by MnOx it begs the question as to why, if metals have been sorbed and are consequently not available to microbial populations, do DEA rates drop and stimulation is not sustained. It is well documented that MnOx are capable of oxidising complex humic substances to produce low molecular weight organic compounds (Sunda and Kieber, 1994). Therefore, it is possible that the organic matter in the metal impacted soil may have undergone oxidation by both waste sources of MnOx. If this is indeed the case it is likely that the oxidation of the soil organic matter has actually resulted in the release of toxic metal ions that were previously unavailable to the microbial community. As the reactive surface becomes covered in either sorbed metal ions or organic matter, and further metals have been released from soil organic matter, the DEA rate decreases as the microbial community encounters increased concentrations of bioavailable metals. Therefore, it is possible that the addition of MnOx to metal contaminated soils could possibly exacerbate metal toxicity to microbial populations.

3.4.6. *The effect of MnOx addition upon the soil microbial functional indicator of potential nitrification in a long term metal contaminated soil*

Microcosm trial 1 showed that the addition MnOx-containing wastes stimulated the potential nitrification rate (PNR) of the metal impacted soil (Figure 3-7). While amendment of the metal impacted soil with MnOx-coated sands stimulated PNR in trial 1, this effect was transient (Figure 3-7a). However, results demonstrated that increasing concentrations of MnOx by coated sand addition increased the PNR stimulation time period, as PNR was higher than the control for 8 weeks with 3 % amendment and for 16 weeks with 7 % amendment. Stimulation of soil PNR with MnOx tailing amendments (1 - 20 %) was also transient, but always significantly higher than the control (Figure 3-7b). The only exception to this trend was with the addition of 30 % MnOx tailings which showed a continual increase in soil PNR rates.

In Chapter 2 it was concluded that ammonia-oxidising microbial communities in the metal impacted soil had became tolerant to contamination over time as they exhibited a PNR on par with non-contaminated soils. The effectiveness of PNR as a sensitive indicator of contamination is in doubt due to the ability of ammonia-oxidising communities to become tolerant to contamination without compromising their functional capability (Sauvé *et al.*, 1999). Therefore, it was hypothesised at the outset of this study that addition of MnOx would not have any beneficial effect upon PNR in the metal impacted soil. However, results from this study suggest otherwise as there was a definite promotion of biological ammonia oxidation with MnOx addition.

It could be argued that PNR stimulation observed is due to increased NH_3 substrates released through oxidation of organic matter by MnOx. However, this is unlikely as these substrates were already present in excess in the PNR assay. In addition, it has been previously mentioned that MnOx are involved in nitrification; whereby MnO_2 is reduced by nitrite thus making nitrification possible in the absence of O_2 (Luther and Popp, 2002). However, the PNR assay is aerobic and the abiotic involvement of MnOx in seems unlikely. It is possible that anaerobic micro-sites existed in the soil slurry, which may have induced abiotic nitrification by MnO_2. However, the most compelling evidence that the stimulation of PNR is not biotic is that initial experiments with MnOx tailings showed no production of PNR (Table 3-3).

The stimulation of PNR cannot be due to the addition of ammonia-oxidising communities as; i) AOB or AOA *amoA* genes were undetected in either MnOx-containing waste, ii) no PNR was detected after sterilisation of sands and iii) microcosm trial 2 showed there were no significant differences between sterile and non-sterile MnOx amendments (Figure 3-12). It is more likely that while biological nitrification seemed to be well adapted to the previous contamination of in the metal impacted soil, that bioavailable toxic metal ions were still inhibiting the ammonia-oxidising communities, preventing them from reaching their full nitrification potential. Hence it is proposed that like DEA, the addition of MnOx to the metal impacted soil has resulted in sorption of toxic metal ions.

However, microcosm trial 2 showed that there was no spike in PNR, as observed between 0 and 4 weeks in trial 1; only a continual decrease in PNR over time with both types of MnOx amendment was recorded (Figure 3-12). As a 0 % MnOx control was not included within trial 2 it cannot be definitively concluded as to

whether soil PNR was stimulated. This discrepancy may be attributed to the inclusion of inert sterile sand in trial 1. Overall, the same pattern of decreasing soil PNR was found over time in both trials. Therefore, it is hypothesised that sorption of bioavailable metals in the metal impacted soil occurred faster in trial 2 without the inclusion of the inert sterile sand and the spike in soil PNR was missed.

Interestingly, results from both microcosm trials demonstrated that MnOx tailing amendments displayed higher soil PNR in comparison to that measured in MnOx-coated sand amended assays. This suggests that MnOx tailings may be more reactive than their MnOx-coated sand counterparts. Reasons for MnOx tailings being more reactive then MnOx-coated sands have been previously discussed in section 3.4.5. The fact that both microcosm trials indicated that DEA and PNR were superior in the metal impacted soil when amended with MnOx tailings provides further evidence supporting tailings being more reactive than sands.

Similarly stimulation in soil PNR in both MnOx-containing waste amended microcosms showed the same pattern of decreasing activity over time as observed with DEA experiments. Previously it was hypothesised that the MnOx-containing wastes had oxidised the organic matter in the metal impacted soil resulting in the release of toxic metal ions that were previously not available to the microbial community. While this seems less likely to affect the adapted ammonia-oxidising populations within the metal impacted soil, it may suggest that the community is only adapted to a certain level of metal contamination.

3.5. Conclusions

The current study showed that the application of natural MnOx-containing wastes did not have any direct toxic effects on soil microbial activity in a historically metal impacted soil. MnOx-containing wastes were found to be beneficial for soil microbial functioning through an enhancement of N-cycling occurring via a stimulation of nitrification and denitrification potentials. However, in some cases stimulation was followed by decreasing functional capability. This was ascribed to possible oxidation of soil organic matter by the MnOx resulting in the release of previously unavailable toxic metal ions. However, it can be said that MnOx does not decrease intrinsic microbial functioning and appears promising for maintaining microbial activity for future land

applications. Future work should fully assess impacts on trace gas emissions assessing real world (*in situ*) process rates, N processing in a wider range of contaminated and non-contaminated soils and interactions between the MnOx and soil organic matter in longer term experiments. MnOx-containing wastes are proposed as a viable remediation strategy on the basis that they do not harm intrinsic soil microbial functioning.

Chapter 4. The use of natural Mn oxide-containing waste amendments a viable contaminated land remediation strategy

4.1. Introduction

Most previous research on the use of Mn oxides (hereafter referred to as MnOx which collectively includes oxide, hydroxide and oxyhydroxide minerals) to remediate soil has investigated the application of synthetic MnOx. These studies have lead to MnOx being identified as a possible soil amendment that could remediate metal, PAH and mixed (metals and PAHs) contamination (Tourney *et al.*, 2008). This conclusion was drawn due to their ability to: immobilise metals such as Pb (Chen *et al.*, 2000; Hettiarachchi *et al.*, 2000; Lee *et al.*, 2011; Varrault and Bermond, 2011), effectively oxidise ions such as As (III) to As (V) (Chiu and Hering, 2000; He and Hering, 2009; Scott and Morgan, 1995), reduce risks posed to human health through a reduction in metal bioaccessibility (Beak *et al.*, 2007), transform PAH contaminants into less harmful intermediates (Clarke *et al.*, 2012) and enhance the humification of soil organic matter (Jokic *et al.*, 2001; Jokic *et al.*, 2004; Shindo and Huang, 1982).

The combination of all these factors also presents MnOx amendment as an approach that could help to protect not only human health, but also soil microbial function, via alleviating the exposure of both human populations and *in situ* microbial communities to harmful pollutants. Additionally, they could provide a sustainable and cost effective amendment, being present in large quantities as wastes from industries such as mining (Clarke *et al.*, 2010). However, reactions of pollutants with such natural MnOx may differ from their synthetic counterparts in the soil environment due to variations in their mineralogy, surface areas and the presence of organic coatings (Tebo *et al.*, 2004). Thus, research into the use of natural MnOx for contaminated land remediation requires further investigation.

4.1.1. *Aims and Objectives*

This current study was conducted as multi-disciplinary research carried out between Newcastle and Durham Universities. The study had two overall aims: 1) to assess the remediation potential of MnOx-containing wastes in highly metal, PAH, and

mixed (metal and PAH) contaminated soils, 2) to determine the effects of this prospective strategy upon soil microbial function. This research thus serves to determine whether MnOx-containing wastes could be a viable amendment that could be implemented within the contaminated land remediation industry.

The study objectives were:

(i) To use a lysimeter trial employing industrially metal and PAH contaminated soils, amended with and without a MnOx-containing waste; to assess the remediation potential and the effect MnOx has upon soil microbial function.
(ii) To use a second lysimeter trial employing the same conditions as in (i) but with the inclusion of young organic matter, to assess the competition between remediation and humification by MnOx-containing waste amendments in addition to the effect of MnOx upon soil microbial function.
(iii) To evaluate if using MnOx-containing wastes as amendments could be a viable remediation strategy by assessing metal/metalloid (Pb and As) and total PAH concentrations.
(iv) To assess the impacts of MnOx soil amendment upon human health via the use of bioaccessibility testing for Pb and As.
(v) To assess effects upon soil microbial function using three indicators: basal respiration (BR), potential nitrification activity (PNR) and denitrification enzyme activity (DEA).

The specific hypotheses tested were:

(i) The addition of MnOx to contaminated soils results in the immobilisation of Pb and As via sorption onto the oxide surface, which in turn reduces the bioaccessibility of these metals/metalloids to humans.
(ii) The addition of MnOx to soils containing PAH contaminants results in an overall reduction of total PAHs.
(iii) The addition of MnOx stimulates N-cycling and microbial respiration in PAH and metal/metalloid contaminated soils by the transformation of PAHs and relieving heavy metal stress on *in situ* bacterial communities via immobilisation.

- Results from Chapter 2 showed that PAH and metal contaminated soils had intrinsically low denitrification rates when compared to non-contaminated soils and this was attributable to the bioavailability of contaminants. Additionally, results from Chapter 3 illustrated that addition of MnOx to a low level metal contaminated soil enhanced nitrification and denitrification which was attributed to metal immobilisation.
- Basal respiration within contaminated soils was determined to be intrinsically low in Chapter 2 when compared to non-contaminated soils, and appeared to have been suppressed by metal and PAH contamination. Results from Chapter 3 were inconclusive with measured changes in respiration, credited to experimental set-up effects, thus, lysimeter trials would confirm effects of MnOx upon bacterial respiration.

(iv) The addition of MnOx to contaminated soils mixed with young organic matter would result in both remediation and humification reactions occurring simultaneously. This will further enhance remediation by coupled mechanisms: 1) direct contaminant transformation on the reactive MnOx mineral surface, 2) the indirect processes of contaminants binding to humic fractions produced from the abiotic MnOx humification of the young organic matter resulting in non-extractable, immobilised and detoxified products.

4.2. Materials and Methods

As this study was carried out in conjunction with Durham University some of the materials and methods presented in this chapter were conducted at and by Durham University. The lysimeter trials described herein were located at Durham University. However, design, construction and sampling were a joint effort between both Universities. Analysis carried out at Newcastle University was to assess the impact of MnOx amendment on soil microbial function.

4.2.1. *Contaminated soil collection, storage and analysis*

Contaminated soils were selected from sites of historical anthropogenic PAH and heavy metal pollution. Samples were collected from two adjacent contaminated land sites, St Anthony's Tar and Lead works, full site descriptions of both have been previously outlined in Chapter 2, section 2.2.1. Three different soil types from these sites were chosen to be utilised in lysimeter trials; a high metals soil from the Lead works (metals soil), a high PAH soil from the Tar works (PAH soil), and a low level metal polluted soil from the Lead Works (low metals soil). The soils were collected prior to the set up of lysimeter trials. A large amount of bulk soil was removed from each site by Durham University staff. At the time of collection soils were waterlogged due to extreme weather conditions, therefore to allow for efficient mixing with amendments all soils were air dried, sieved (> 2 mm), and crushed (only in the case of PAH contaminated clay soils) at Durham University. The intrinsic characteristics and soil microbial function of these soils have been previously described in Chapter 2.

4.2.2. *MnOx-containing waste collection, storage and analysis*

MnOx-coated sands were obtained from a local water treatment works (Mosswood WTW). A full site description of the Mosswood WTW has been previously described in Chapter 3, section 3.2.3. Briefly, three of the 12 rapid gravity filter beds were taken out of operation and allowed to drain before Mosswood WTW staff removed coated sands from the top layer of beds to create a composite sample. MnOx-coated sands were collected in sterile buckets and transported back to Durham University where they were stored at room temperature. The physico-chemical characteristics, along with microbial community profiling, diversity, function and total metal and PAH concentrations of the MnOx-coated sands have been previously described in Chapter 3.

4.2.3. *Young organic matter collection, storage and analysis*

The young organic matter used in this study was a green waste (GW) sourced by Durham University from Premier Waste Ltd (Co. Durham, UK). The GW is made from garden off cuts such as grass cuttings, shrubs, leaves, weeds and hedge trimmings (Premier Waste Ltd, Personal communication). The GW was collected by Durham

University as a bulk sample in sterile buckets and stored at room temperature. A sub-sample was transported to Newcastle University in a sterile polyethylene bag (Fisher, UK) where it was stored at 4 °C. The bulk sample was tested in triplicate for WHC, MC, pH, PNR, DEA and BR. The methods for these analyses have been previously described in Chapter 2 sections 2.2.6 to 2.2.8 and 2.2.13 to 2.2.15. One sample of the bulk GW was sent to Northumbrian Water Scientific Services (NWSS) analytical laboratories (North Tyneside, UK) by Durham University for analysis of Hg, As, Cd, Cr, Cu, Fe, Pb, Mn, Ni, Se, Zi and total PAHs, following the methods previously described in Chapter 2 sections 2.2.11 and 2.2.12.

4.2.4. *Lysimeter trial 1 construction and sampling*

The MnOx-coated sand amendment of contaminated soils carried out in lysimeter trial 1 is summarised in Table 4-1. Two levels of MnOx amendment were employed to determine the potential natural MnOx have for the remediation of metal, PAH, and mixed (PAH and metal) contaminated soils. MnOx amendments were carried out by adding either 5 % or 10 % MnOx by total weight. This therefore equates to adding an additional 450 or 900 mg kg^{-1} of MnOx to the soil. The higher limit of 10 % by weight MnOx addition was chosen to represent a practical level of amendment that could be added to a real contaminated site.

Table 4-1: A summary of the experiments performed within lysimeter trial 1.

Contaminated soil type	Soil source	Soil sample	0 % MnOx-coated sand	5 % MnOx-coated sand	10 % MnOx-coated sand
Metals	St Anthony's Lead works	High metals soil	x3*	x3	x3
PAH	St Anthony's Tar works	High PAH soil	x3	x3	x3
Mixed[a]	St. Anthony's Tar and Lead Works	n/a	x3	x3	x3
Low metal	St Anthony's Lead works	Low metals soil	x3	x3	x3

[a] mixed soil represents a 1:1 by weight mixture of metals and PAH soil
* replicate number of lysimeters

The soils from St. Anthony's Tar and Lead Works which were dried, sieved and crushed prior to lysimeter set up, were firstly rewetted to their original moisture

content at the time of collection using a cement mixer. Following the rewetting of the soils, where required, the MnOx-coated sand was added in the cement mixer and both soil and MnOx underwent mixing for 30 minutes. Each soil mixture was then divided into three and samples were collected. These samples were designated '0 month' time points. Subsamples for microbial analysis were collected from the samples into sterile polyethylene bags (Fisher, UK). The 0 month subsamples were then transported to Newcastle University with free access to air. On arrival the samples were stored and processed according to the methods described in Chapter 2, section 2.2.5. Samples were also collected by Durham University for the determination of pH, total PAHs, along with total and bioaccessible Pb and As, whose methods are described later in this section. After the samples had been taken the remainder of the various soil ± MnOx-coated sand mixtures were packed into polytetrafluoroethylene (PTFE) lined lysimeters (30 cm x 15 cm) and placed outside. A diagram showing the set up of a lysimeter is depicted in Figure 4-1.

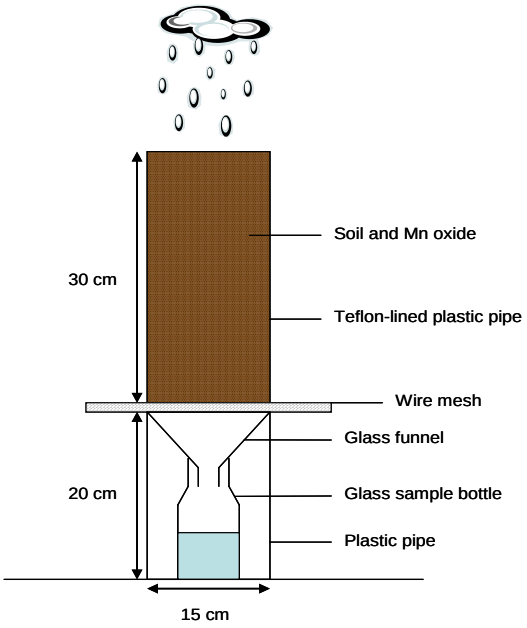


Figure 4-1: Lysimeter for trials 1 and 2. Diagram is courtesy of J. Tourney (Durham University). Glass sample bottle was used to collect and prevent pollution of outside area by contaminated leachate.

The trial was run for 9 months, and during this period any vegetation growth which occurred on the lysimeter surface was removed. During dry weather conditions,

artificial watering with rainwater was necessary in order to keep soils from drying out. After 9 months the lysimeters were destructively sampled. Samples were taken from the top, middle and bottom of the lysimeter pipes and bulked. Subsamples were taken from the bulk samples for soil microbial function analysis at Newcastle University. These samples represent the '9 month' samples. Further samples were taken by Durham University for the determination of pH, total PAHs, along with total and bioaccessible Pb and As.

4.2.5. *Lysimeter trial 2 construction and sampling*

The lysimeters within this trial consisted of a 1:1 mixture by weight of contaminated soil and green waste (GW) amendment. In this trial only a 10 % MnOx amendment by weight was applied to contaminated soils. The GW and MnOx-coated sand amendments of contaminated soils carried out in trial 2 are summarised in Table 4-2. The lysimeters were carried out for 9 months, and sampling was as previously outlined for trial 1. Please note that Durham University decided to carry out no formal assessment of humification within this trial.

Table 4-2: A summary of the experiments performed within lysimeter trial 2.

Treatment	Contaminated soil type	0 % MnOx-coated sand	10 % MnOx-coated sand
50 % green waste	Metals	x3*	x3
	PAH	x3	x3
	Mixed[a]	x3	x3
	Low metal	x3	x3

[a] mixed soil represents a 1:1 mixture of metals to PAH soil
* replicate number of lysimeters

4.2.6. *Lysimeter trial 1 and 2 physico-chemical analysis*

Samples were analysed for pH, moisture content (MC) and water holding capacity (WHC). MC and WHC were vital to perform soil microbial function assays. All analyses were performed in triplicate. The pH of the lysimeter soils was carried out by Durham University but followed the same method previously outlined in Chapter 2, section 2.2.6. MC and WHC analyses were carried out at Newcastle University using the methods previously described in Chapter 2, sections 2.2.7 to 2.2.10.

4.2.7. *Lysimeter trial 1 and 2 total PAH analysis*

Total PAH concentrations were determined for lysimeter samples that contained PAH contaminated soils by Northumbrian Water Scientific Services (NWSS) analytical laboratories (North Tyneside, UK). A description of the analyses carried out by NWSS has been previously described in Chapter 2, section 2.2.12.

4.2.8. *Lysimeter trial 1 and 2 Pb and As total soil concentrations and bioaccessibility*

Lysimeter samples were dried and sieved to a 250 µm size fraction and were sent to the British Geological Survey (Keyworth, Nottingham, UK) for analysis of extractable Pb and As via an aqua regia digestion, and additionally bioaccessibility by the BARGE-UBM method (Wragg *et al.*, 2011). In brief the BARGE-UBM method consists of a three stage ingestion simulation of: the mouth, stomach and small intestinal cavities, at a stomach pH of 1.2 and an intestinal pH of 6.3 under fasting conditions which produces two individual extracts: 'biostomach' and 'biostomach and biointestine' per test sample (Wragg *et al.*, 2011). The biostomach consists of an extraction solution simulating the mouth (i.e. salival fluid) and the stomach (i.e. stomach fluid) compartments (Wragg *et al.*, 2011). The biostomach and intestine consists of an extraction solution simulating the mouth, stomach and small intestine (utilising simulated saliva, stomach, bile and duodenal fluids) (Wragg *et al.*, 2011).

In addition to BGS aqua regia digestions; a subset of samples were also analysed by Hydrofluoric acid (HF) extraction for total Pb and As. Aqua regia digestions do not dissolve silicate minerals and therefore only give an estimate of metals which are potentially mobile, because of this they are often referred to as a 'pseudo' total (Naidu, 2008). For this reason Pb and As concentrations determined via aqua regia digestions will be referred to as 'extractable' Pb and As throughout this study. In contrast HF is viewed as a total dissolution method as it is able to dissolve metals bound in silicate mineral lattice structures (Kline and Fogler, 1981). Furthermore, prior to HF digestion, samples were incubated in hydrogen peroxide (H_2O_2) to ensure all soil organic matter was oxidised. HF extractions were carried out by Durham University using EPA Method 3052 (1995) with reference material CRM No. 7004 used for quality control. Briefly, soil samples that were air dried and sieved to <2 mm were further sieved to <250 µm using a mechanical shaker. The samples were then freeze-dried and

ball-milled. 5 ml H_20 and 6 ml H_2O_2 were added to 250 mg samples in Teflon tubes and left overnight. 9 ml of concentrated nitric acid (HNO_3) and 3 ml of concentrated hydrochloric acid (HCl) was added gradually. For total digestions, a further 1 ml of HF was added. The samples were digested for 20 mins using a MARSX microwave (EPA 3051X programme). Once cool, solutions were filtered into 100 ml glass volumetric flasks using Whatman 542 filter paper and made up to volume with deionised water. Analysis was carried out on a Spectra AA atomic absorption spectrometer for As and Pb using matrix-matched standards.

4.2.9. *Lysimeter trial 1 X-ray Diffraction (XRD), Electron Microprobe Micro-analysis (EPMA) and X-ray mapping*

A subset of samples from the high metals soil of lysimeter trial 1 was sent to Dr. Karen Hudson Edwards (Birkbeck, University of London) to determine whether immobilisation of Pb had occurred onto the MnOx-coated sand surface. Immobilisation of Pb was determined by XRD and EPMA analysis directly comparing MnOx-coated sand grains which had not been exposed to the metal contaminated soil with sand grains taken after 9 month reaction time within the metals soil of lysimeter trial 1. Powder XRD analysis was performed on a Phillips PW 1710 diffractometer using Cu-Kα radiation. EPMA was performed on a Jeol 8100 Superprobe (WDS) and an Oxford Instrument Inca system (EDS). Energy spectral data were collected in the 0-20 eV range. Analysis was carried out using an accelerating voltage of 15 kV, current of 2.5 mA and a beam diameter of 1 µm. The counting times for all elements were 20 seconds on the peak and 10 seconds each on the high and low backgrounds. The analyses were calibrated against standards of natural silicates, oxides and Specpure® metals with the data corrected using a ZAF program.

4.2.10. *Lysimeter trial 1 and 2 soil microbial functional analyses*

Lysimeter samples were tested in triplicate for basal respiration (BR), denitrification enzyme activity (DEA) and potential nitrification activity (PNR). A full description of these methods has been previously described in Chapter 2, sections 2.2.13 to 2.2.15. In this study microbial functional rates reported were not normalised to per gram of MnOx amendment. They are reported as per gram of dry matter to

assess real field emissions of CO_2, along with potential emissions of NO_2 and ammonia oxidation.

4.2.11. *Statistical analysis of lysimeter experiments*

Please note that while samples were taken for both lysimeter trials at 0 and 9 months with all levels of MnOx amendment, all samples were not analysed for Pb, As, pH and total PAHs by Durham University. For lysimeter trial 1 and 2, pH and total PAHs were only provided for metals, PAH and mixed soils, which had 0 % and 10 % MnOx-coated sand amendments from 0 and 9 month samples. In addition, only 9 month samples taken from 0 % and 10 % MnOx-amended metals, PAH and mixed soils of trial 1 and 2, were analysed for extractable and bioaccessible Pb and As.

All statistical analysis was carried out using SPSS statistics for windows (SPSS v17, SPSS, Inc. Chicago, IL, USA). Data provided by Durham University was analysed using a two tailed independent sample t-test to determine significant differences due to 10 % MnOx amendment via comparison with non-amended measured parameters for each contaminated soil type.

Soil function parameters from lysimeter trials 1 and 2 were analysed at 0 months, 9 months, to deduce any short term or long term effects of MnOx amendment upon functional activities. Measured microbial functions from lysimeter trial 1 and 2 were analysed using a two-way analysis of variance (ANOVA) to examine the overall effects of MnOx addition, contaminated soil type and the interaction between MnOx and soil type. This was carried out using a univariate general linear model. For trial 1 the fixed factors for were MnOx amendment (0 /5 /10 %) and contaminated soil type (low metals/ metals/ PAH/ mixed), the dependant variable was the measured microbial functional indicator (BR/DEA/PNR). Significance in the differences was tested using the Tukey HSD post-hoc test for pairwise comparisons. For trial 2 the fixed factors were MnOx amendment (0/ 10 %) and contaminated soil type (low metals/ metals/ PAH/ mixed), the dependant variable was the measured microbial functional indicator (BR /DEA /PNR). Significance in the differences could only be tested for contaminated soil type using the Tukey HSD post-hoc test for pairwise comparisons in trial 2, as MnOx amendment only contained two levels. Where a significant difference was detected in the two-way ANOVA for MnOx

amendment, Independent sample t-testing was employed to determine where the significant difference lay.

4.3. Results

Please note that results presented in sections 4.3.1-3 and 4.3.8-10 were generated by colleagues at Durham University, while results in sections 4.3.4-7 and 4.3.11-13 were carried out at Newcastle University. Both chemical and microbial results are presented and assessed to give a full picture of the effects of natural MnOx-containing wastes as a remediation tool.

4.3.1. *Lysimeter trial 1: Pollutant analysis of metal contaminated soils with and without MnOx amendment*

Aqua regia extracts for As and Pb showed that MnOx-amended metal soils had significantly higher extractable concentrations of these metals/metalloids than the unamended metals soil at 9 months (Independent t-tests, $p < 0.04$, Table 4-3 and 4-4). To verify these results using a different method, HF extracts were performed upon the same samples, which confirmed the higher concentrations of As and Pb at a highly significant level (Independent t-test, $p < 0.01$, Table 4-3 and 4-4), and showed no difference in concentrations of Pb and As between digestion methods (Independent t-tests, $p > 0.1$). Concentrations of extractable As in the amended and unamended metals soils were orders of magnitude higher than previously determined for the high metals soil (Chapter 2, 3200 mg kg^{-1}), while mean extractable Pb values tended to be lower than previously reported (Chapter 2, 11 000 mg mg^{-1}). Additionally, large standard errors were associated with extractable As and Pb concentrations in the metal lysimeter soils, thus, results indicate large pollutant heterogeneity.

No significant differences were found in bioaccessible As extracts between the MnOx-amended and unamended metals soil at 9 months (Independent t-test, $p > 0.3$, Table 4-3). However, a significantly higher Pb bioaccessibility was observed for both biostomach (Independent t-test, $p < 0.03$, Table 4-4) and biostomach and intestinal extracts with MnOx amendment at 9 months (Independent T-test, $p < 0.03$, Table 4-4). Over time the pH in the MnOx-amended metal soils did not change (Independent t-test, $p > 0.4$, Table 4-6). However, it did show a significantly lower soil pH than its

respective non-amended counterpart from the outset of the experiment (Independent t-test, $p < 0.02$).

Electron probe microanalysis (EPMA) was used to evaluate whether Pb was associated with the MnO-coated sand after 9 months in the 10 % metal amended soil. Figure 4-2 shows a representative MnOx-coated sand grain which had not been added to the metal contaminated soil. Figures 4-3 and 4-4 show a representative MnOx-coated sand grain after 9 months within the metal contaminated soil of lysimeter trial 1 with Pb enriched on the outer MnOx rich layer of the MnOx-coated sand. EPMA showed that no other metals to be associated with the outer layer of the MnOx-coated sand indicating no adsorption of As by the MnOx (data not shown).

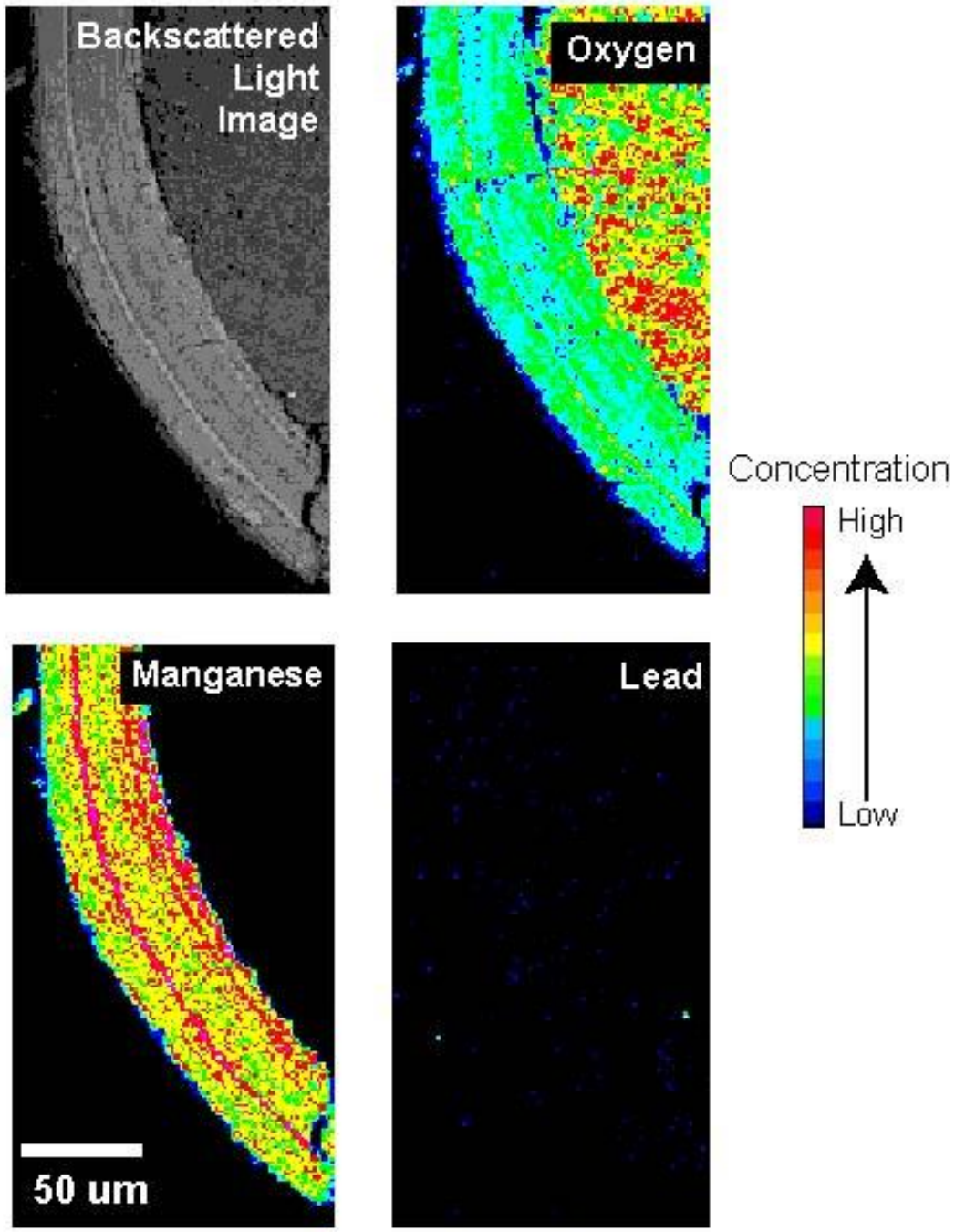


Figure 4-2: EPMA map of the Mn-oxide coated sand grain not added to the metal contaminated soil of lysimeter trial 1.

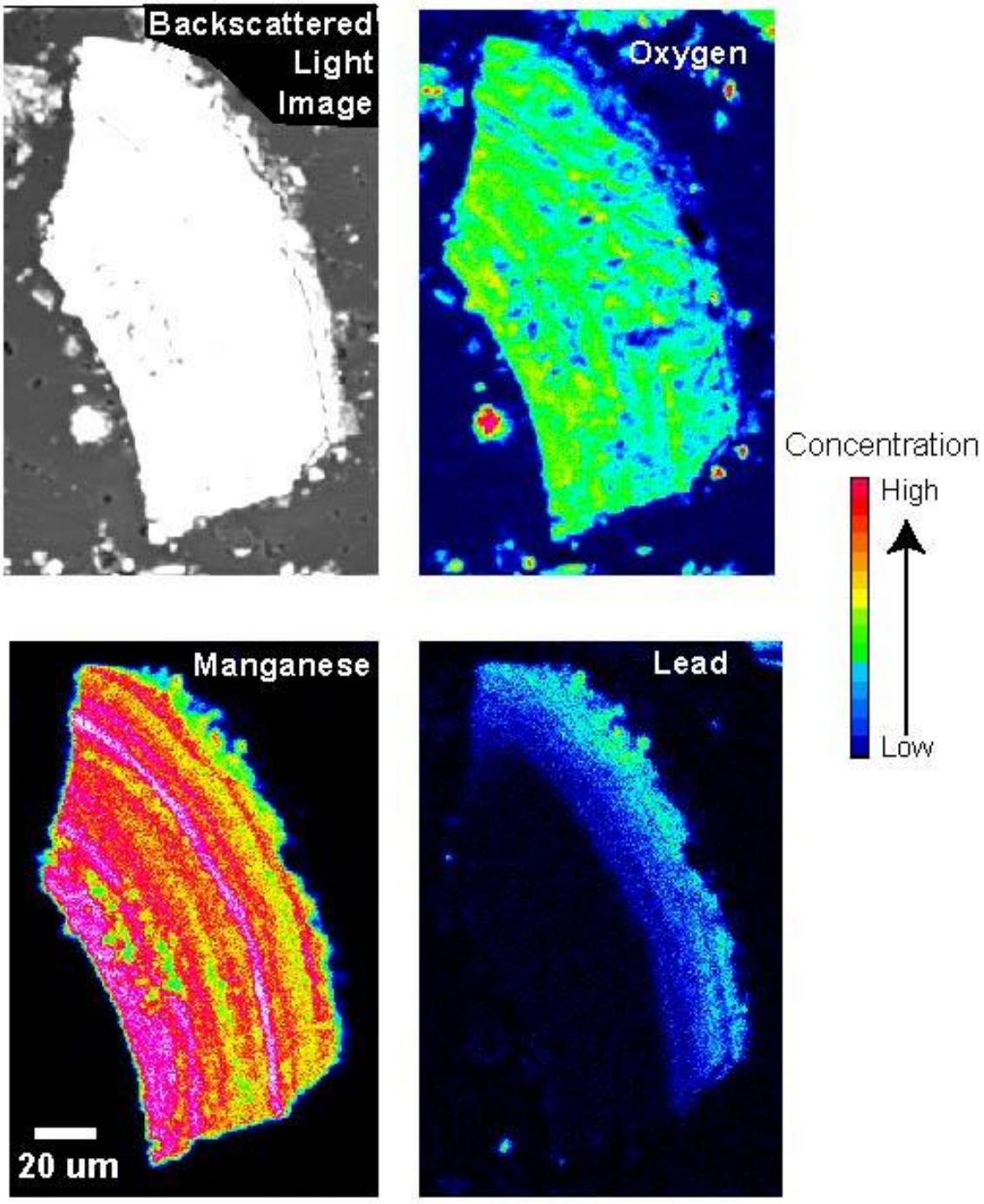


Figure 4-3: EPMA map of a section of the Mn-oxide coating of a MnOx-coated sand grain recovered after 9 months from the metal contaminated soil with 10 % MnOx-coated sand amendment of lysimeter trial 1.

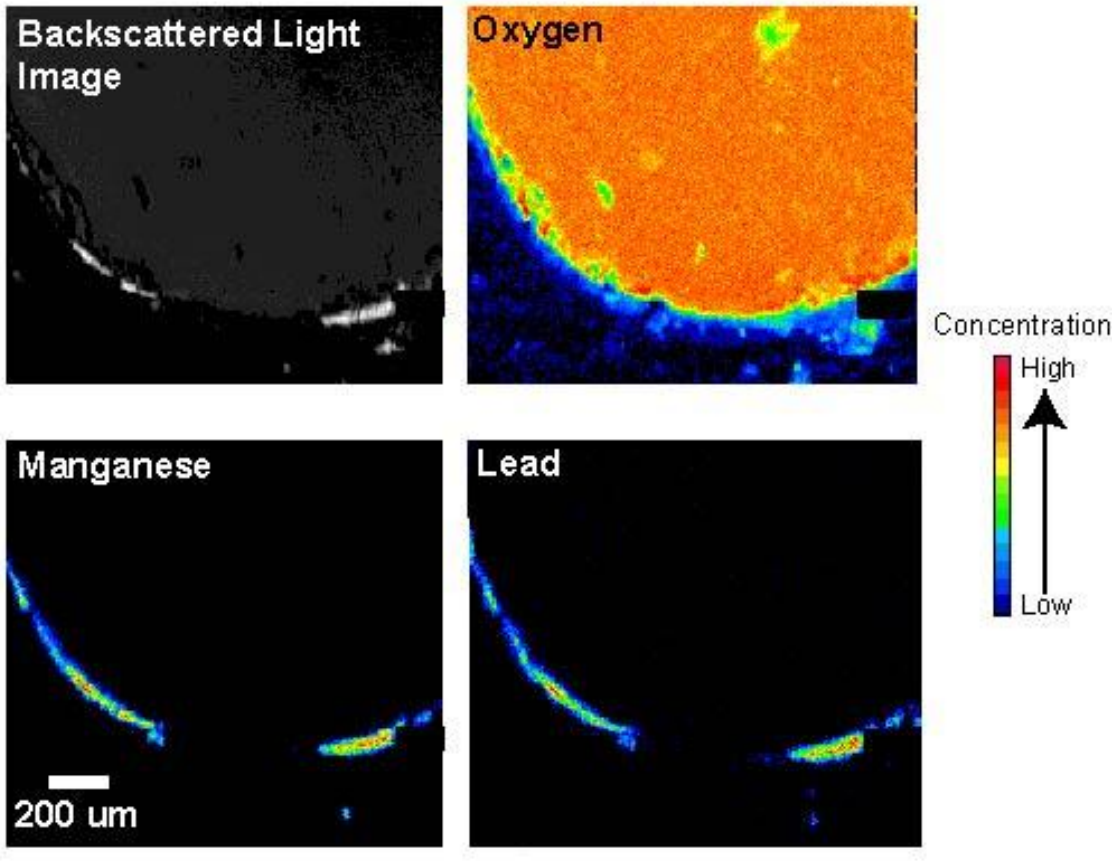


Figure 4-4: EPMA map of a complete MnOx-coated sand grain recovered after 9 months from the metal contaminated soil with 10 % MnOx-coated sand amendment of lysimeter trial 1.

4.3.2. *Lysimeter trial 1: Pollutant analysis of mixed contaminated soils with and without MnOx amendment*

The MnOx-amended mixed soil showed lower concentrations of extractable As at 9 months compared to its unamended counterpart (Independent t-test, $p < 0.04$, Table 4-3), although, the bioaccessibility of As was comparable between amended and unamended mixed soils (Independent t-tests, $p > 0.1$, Table 4-3). No significant difference was found in extractable Pb concentrations between MnOx-amended and unamended mixed soils (Independent t-test, $p > 0.08$, Table 4-4). However, Pb bioaccessibility was determined to be significantly lower in both extracts in the MnOx-amended mixed soil at 9 months (Independent t-test, $p < 0.05$, Table 4-4).

MnOx amendment did not have any effect on total PAHs (TPAH) or soil pH as no significant differences were found in extractable TPAH (Table 4-5) or pH values (Table 4-6), either over time, or, between amended or unamended mixed soils (Independent t-tests, $p > 0.05$).

As the mixed soil was a 1:1 combination of PAH and metals soils it would have been expected that the extractable concentrations of Pb and As within this soil would be half of that found in the unamended metals soil only, especially in light of the fact that the PAH soils had been previously found to have low values of extractable metal/metalloids (Chapter 2). This was however not the case, as mean values of As in the unamended mixed soil (7487 ± 830 mg kg^{-1}) were statistically comparable to the unamended metals soil (8277 ± 625 mg kg^{-1}, Independent t-test, $p > 0.4$), and mean values of Pb in the unamended mixed soil (9719 ± 3025 mg kg^{-1}) were also statistically comparable to the unamended metals soil at 9 months (4484 ± 400 mg kg^{-1}, Independent t-test, $p > 0.1$). Examination of the triplicate aqua regia extractable Pb values from the unamended mixed soil showed one triplicate that was double the other replicate values, consequentially explaining the large associated standard error, and clearly indicating large pollutant heterogeneity in the samples.

Additionally, mean TPAH values in the unamended mixed soils were around six-fold lower than that of the unamended PAH soil (please note the PAH soil is discussed in the subsequent section). Nonetheless, results were pertinent as the mixed soil was still representative of a cocktail soil harbouring high concentrations of Pb, As and PAH contaminants.

4.3.3. *Lysimeter trial 1: Pollutant analysis of PAH contaminated soils with and without MnOx amendment*

TPAHs concentrations in both MnOx-amended and unamended PAH contaminated soils did not significantly differ over the nine months of the experiment (Independent t-test, $p > 0.2$, Table 4-5). Additionally, no significant difference was observed in TPAH values at either 0 or 9 months with and without MnOx amendments (Independent t-test, $p > 0.1$, Table 4-5). The PAH soils amended with MnOx showed a significant increase in soil pH over time (Independent t-test, $p < 0.01$, Table 4-6), although, while this increase was significant, the soil remained alkaline.

Table 4-3: Mean values for extractable and bioaccessible As (mg kg^{-1} dry matter) from lysimeter trial 1, with and without 10 % MnOx amendment at 9 months. Values are based upon the mean of soil samples taken from each of the three lysimeter replicates.

	As			
	Aqua regia	**HF**	**Biostomach**	**Biostomach and intestine**
Metals	8277 ±[a] 625	7500 ± 70	2173 ± 70	2116 ± 207
Metals + MnOx	11 155 ± 344	10 516 ± 129	2227 ± 87	2334 ± 76
Mixed	7487 ± 830	ND[b]	1584 ± 215	1622 ± 197
Mixed + MnOx	4709 ± 181	ND	1228 ± 111	1222 ± 107
PAH	ND	ND	ND	ND
PAH + MnOx	ND	ND	ND	ND

[a] ± 1 x standard error
[b] not determined

Table 4-4: Mean values for extractable and bioaccessible Pb (mg kg^{-1} dry matter) from lysimeter trial 1, with and without 10 % MnOx amendment at 9 months. Values are based upon the mean of soil samples taken from each of the three lysimeter replicates.

	Pb			
	Aqua regia	**HF**	**Biostomach**	**Biostomach and intestine**
Metals	4484 ±[a] 400	5206 ± 307	1945 ± 114	597 ± 49
Metals + MnOx	9677 ± 1543	9080 ± 225	3491 ± 411	1125 ± 51
Mixed	9719 ± 3025	ND[b]	2879 ± 694	518 ± 55
Mixed + MnOx	2742 ± 118	ND	1175 ± 33	190 ± 34
PAH	ND	ND	ND	ND
PAH + MnOx	ND	ND	ND	ND

[a] ± 1 x standard error
[b] not determined

Table 4-5: Mean total PAH (TPAH) concentrations (mg kg^{-1}) from lysimeter trial 1 at 0 and 9 months with and without 10 % MnOx amendment. Values are based upon the mean of 3 soil samples from each lysimeter.

	TPAHS*	
	0 months	**9 months**
Metals	ND[a]	ND
Metals + MnOx	ND	ND
Mixed	43 ±[b] 27	98 ± 26
Mixed + MnOx	50 ± 7	134 ± 41
PAH	211 ± 50	569 ± 245
PAH + MnOx	230 ± 112	75 ± 18

* based upon the sum of EPA 16 priority PAHs
[a] not determined
[b] ± 1x standard error

Table 4-6: Mean pH values for lysimeter trial 1 at 0 and 9 months with and without 10 % MnOx amendment. Values are based upon the mean of 3 soil samples from each lysimeter.

	pH	
	0 months	**9 months**
Metals	6.94 ±[a] 0.09	7.26 ± 0.05
Metals + MnOx	6.23 ± 0.15	6.37 ± 0.07
Mixed	7.76 ± 0.02	7.74 ± 0.00
Mixed + MnOx	7.66 ± 0.05	7.84 ± 0.05
PAH	7.85 ± 0.03	7.98 ± 0.08
PAH + MnOx	7.91 ± 0.03	8.25 ± 0.01

[a] ± 1x standard error

4.3.4. *Lysimeter trial 1: Effects of MnOx amendment on basal respiration*

At the outset of lysimeter trial 1, MnOx amendment had no impact on basal respiration (BR) when contaminated soil type was controlled for (two-way ANOVA, $p > 0.8$, Figure 4-5). Furthermore, there was no interaction effect on BR between MnOx amendment and soil type (two-way ANOVA, $p > 0.1$). At 9 months MnOx had a significant effect on BR when contaminated soil type was controlled for (two-way

ANOVA, $p < 0.02$). Additionally, there was a significant interaction between MnOx and soil type (two-way ANOVA, $p < 0.01$). Pairwise comparisons revealed that 5 % MnOx significantly increased BR in the low metals soil (two-way ANOVA, Tukey's HSD $p < 0.01$), whereas addition of 5 and 10 % MnOx in the metals soil significantly decreased BR (two-way ANOVA, Tukey's HSD $p < 0.02$). When MnOx amendment was controlled for, soil type had a significant effect on BR at both 0 and 9 months which was expected (two-way ANOVA, $p < 0.01$). In general, BR rates in the contaminated soil types decreased in the order low metals > mixed > metals > PAH.

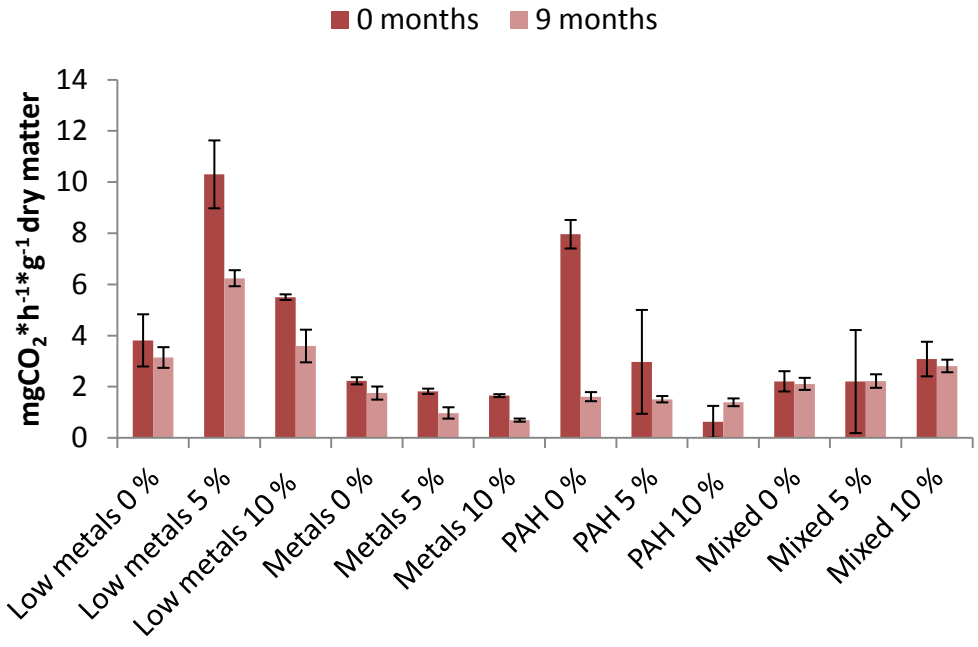


Figure 4-5: Basal respiration rates of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 1 with 0, 5 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.3.5. *Lysimeter trial 1: Effects of MnOx amendment on denitrification enzyme activity*

MnOx showed a significant effect upon DEA when soil type was controlled for at 0 months (two-way ANOVA, $p < 0.03$). Pairwise comparisons revealed that this significant difference only occurred in the low metals soils, where DEA were significantly higher for both 5 and 10 % MnOx amendments in comparison to the 0% MnOx control (two-way ANOVA, Tukey's HSD, $p < 0.05$, Figure 4-6). At 9 months MnOx

amendment showed no significant effect on DEA when soil type was controlled for (two-way ANOVA, $p > 0.7$). At both 0 and 9 months there was no significant interaction effect between MnOx amendment and contaminated soil types (two-way ANOVA, $p > 0.7$). When MnOx was controlled for, significant differences were apparent between the DEA of contaminated soils at both 0 and 9 months (two-way ANOVA, $p < 0.01$). Pairwise testing showed that DEA of the soil types decreased in the order: low metals > mixed > PAH > metals. At both time points the low metals soils exhibited significantly higher DEA rates to metals, PAH and mixed contaminated soils (two-way ANOVA, Tukey's HSD, $p < 0.01$, Figure 4-6).

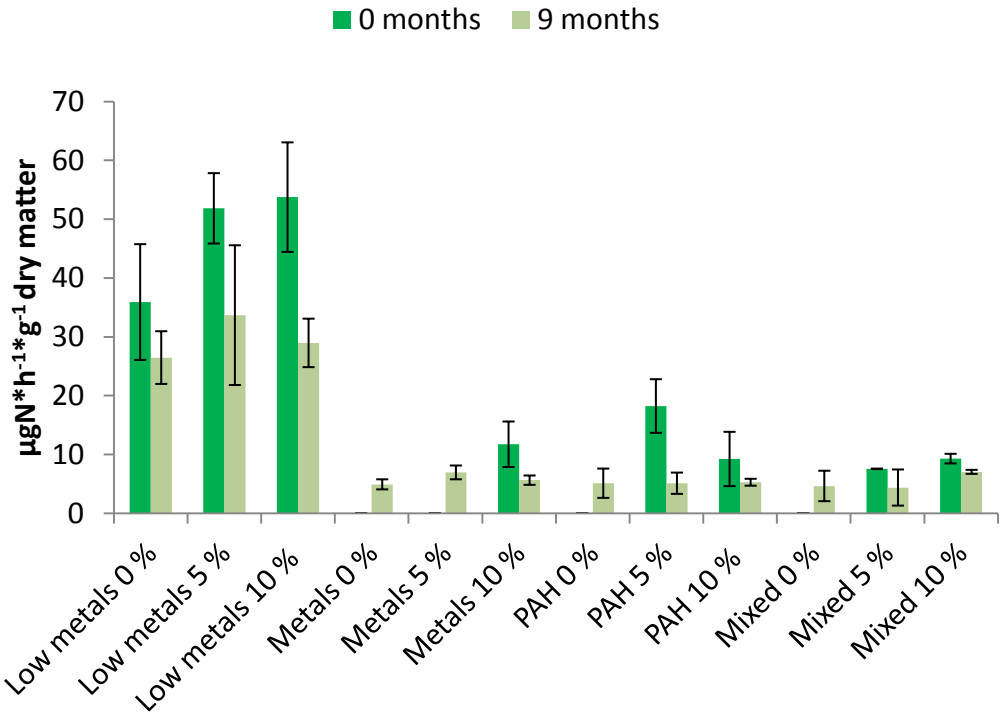


Figure 4-6: Denitrification enzyme activities of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 1 with 0, 5 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.3.6. *Lysimeter trial 1: Effects of MnOx amendment on potential nitrification*

At both 0 and 9 months MnOx amendment had no effect on PNR when contaminated soil type was controlled for (two-way ANOVA, $p > 0.1$, Figure 4-7), nor was there any interaction effect between contaminated soil type and MnOx amendment (two-way ANOVA, $p > 0.1$). At both time points PNR significantly differed between contaminated soil types (two-way ANOVA, $p < 0.01$). Pairwise comparisons

showed that PNR of the low metals soils were significantly higher than that measured for the metal, PAH and mixed contaminated soils types which did not significantly differ to each other (two-way ANOVA, Tukey's HSD, $p > 0.1$, Figure 4-7).

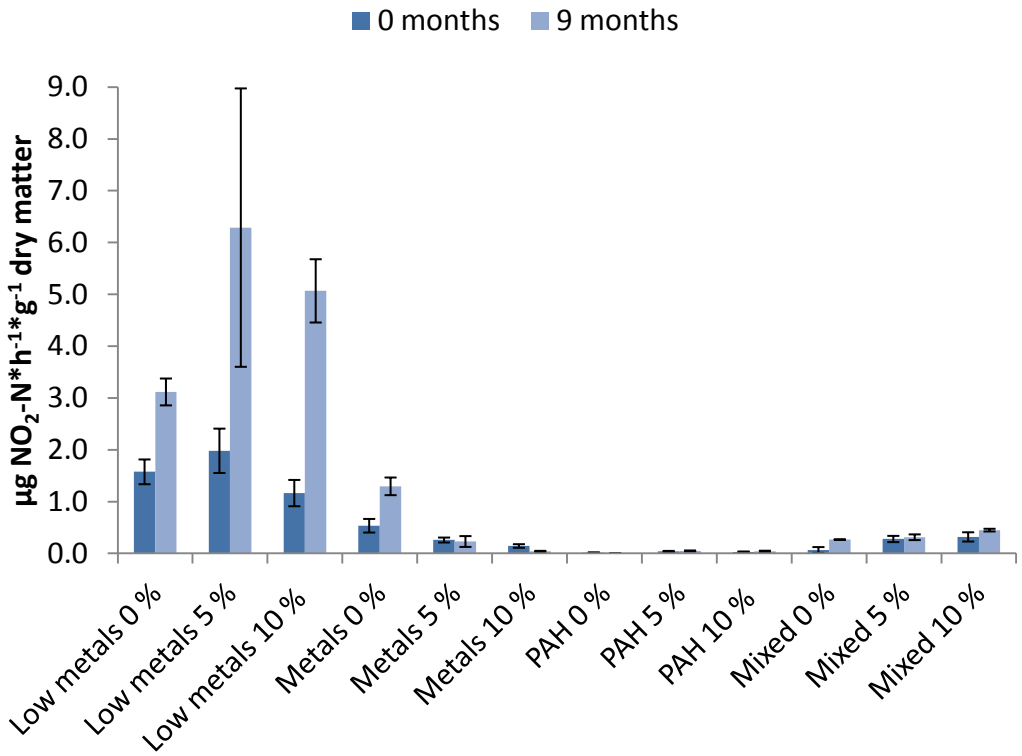


Figure 4-7: Potential nitrification rates of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 1 with 0, 5 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.3.7. *Analysis of green waste*

The green waste (hereafter referred to as GW) showed that concentrations of extractable metals that were under BSI PAS:100 critical thresholds for levels of Cd, Cr, Cu, Pb, Hg, Ni and Zn that are to be applied to land (Table 4-7). Therefore, with regard metal/metalloid concentrations the GW appears to be an acceptable soil amendment. Additionally, all values were under the lowest SGV land use (residential/ allotment/ commercial) thresholds for As, Ni, Hg, Se and Cd, that may cause significant harm to human health (please note SGV values have been reported in Chapter 1), and below Dutch thresholds for soil contamination. Levels of Pb and Zn were slightly elevated in comparison to typical concentrations of these elements found in worldwide soils; however, this is not of concern as they are below BSI PAS:100 critical thresholds. Levels

of TPAHs indicate that the GW may exceed Dutch contamination thresholds defined for soils, and therefore PAH values could be defined as high in the GW. However, it must be remembered that this analysis is based upon one sub-sample of a bulk GW sample and therefore the values only give an indication on potential metal/metalloid and PAH contaminants in the GW.

Table 4-7: Extractable heavy metal and total PAH contents (mg kg^{-1} dry matter) of the green waste (GW) young organic matter used in lysimeter trial 2. Values are presented with the range of worldwide soil means for each heavy metal as taken from McBride (1994) and Kabata-Pendias and Pendias (1992), threshold action values as used in The Netherlands for contaminated land (VROM, 2000), and upper PAS 100 limits for composted material to be applied to land (BSI PAS:100, 2011). Please note that only one bulk sample of the GW was analysed and for that reason there is no associated standard error.

	GW	Typical Worldwide Ranges	Dutch	PAS:100
Hg	0.25	0.02-0.41[a]	10	1.0
As	15	2.2-25[a]	55	n/a
Cd	0.61	0.06-1.1[a]	12	1.5
Cr	21	7-221[a]	380	100
Cu	53	6-80[a]	240	200
Fe	21000	5000-50000[b]	n/a	n/a
Pb	110*	10-84[a]	530	200
Mn	590	80-1300[a]	n/a	n/a
Ni	19	1-450[b]	210	50
Se	0.56	0.05-1.27[a]	100	n/a
Zn	180*	17-125[a]	720	400
TPAHs[c]	53	n/a	40	n/a

[a] McBride (1994)
[b] Kabata-Pendias and Pendias (1992)
[c] based upon the sum of EPA 16 priority PAHs
*total metal concentrations that exceed typical worldwide ranges

Physico-chemical analyses showed that the GW has an alkaline pH (7.65 ± 0.01), as do the PAH and metals soils it was mixed with (Table 2-6). Microbial analyses showed that the GW has a functioning microbial community present with detectable

BR, DEA and PNR. Measured values of DEA, PNR and BR were substantially higher than the PAH and metals soil used in the lysimeters. Thus, addition of GW was expected to significantly improve microbial functioning of the contaminated soils.

It should be noted that BSI PAS:100 guidelines do exist for microbial respiration. These guidelines state that microbial respiration rates for stable/mature composts should not exceed (16 mg CO_2 /g organic matter/ day) (BSI PAS:100, 2011). However, the GW used in this study represents a young composted waste material which is therefore not comparable. When BR rates are converted to the same units as BSI PAS:100 guidelines the GW would have a microbial respiration rate of 101 mg CO_2 /g organic matter/ day, which would be unacceptable for land application. Consequently, these results highlight the need for stabilisation and maturation of the GW if it was to be applied to a contaminated site.

Table 4-8: Mean microbial functions for the green waste (GW) organic amendment used in lysimeter trial 2. Values are presented with mean values from PAH and metals contaminated land types previously determined in Chapter 2 for comparison. Values are based upon the mean of 3 subsamples taken from a bulk GW sample.

	GW	PAH	Metals
DEA [a]	309.5 ± 6.2	0.1 ± 0.0	0.1 ± 0.0
BR [b]	14.0 ±1.9	0.004 ± 0.000	0.024 ± 0.000
PNR [c]	14.4 ± 0.5	0.01 ± 0.1	1.16 ± 0.1

[a] denitrification enzyme activity ($\mu gN*h^{-1}*g^{-1}$ dry matter)
[b] basal respiration ($mgCO_2*h^{-1}*g^{-1}$ dry matter)
[c] potential nitrification rate ($\mu gNO_2\text{-}N*h^{-1}*g^{-1}$ dry matter)
* ± 1 x standard error

4.3.8. *Lysimeter trial 2: Pollutant analysis of metal contaminated soils with MnOx amendment*

Extractable As and Pb showed no significant difference between MnOx-amended and unamended metal + GW soils at 9 months (Independent t-tests, $p > 0.3$, Table 4-9 and 4-10). However, bioaccessibility of As in MnOx-amended soils was significantly lower for the biostomach extract (Independent t-test, $p < 0.02$, Table 4-9), but not for the biostomach and intestine extract (Independent t-test, $p > 0.05$, Table 4-9). Pb bioaccessible extracts showed no significant differences between the MnOx-

amended and unamended metals + GW soils at 9 months (Independent t-test, $p > 0.05$, Table 4-10).

At 0 months the pH of metal + GW soils with and without MnOx amendment did not significantly differ (Independent t-test, $p > 0.09$, Table 4-12). Over time pH significantly increased in the MnOx metal + GW amended soil was therefore higher than its unamended counterpart at 9 months (Independent t-test, $p < 0.01$, Table 4-12). However, pH values remained in an alkaline range, with the increase being attributable to the alkalinity of the MnOx, and were therefore considered as being of minor importance.

4.3.9. *Lysimeter trial 2: Pollutant analysis of mixed contaminated soils with MnOx amendment*

The amendment of the mixed + GW soil with MnOx resulted in no significant differences in extractable As (Independent t-test, $p > 0.1$, Table 4-9), or either As bioaccessible extracts at 9 months (Independent t-tests, $p > 0.3$, Table 4-9). Furthermore, MnOx amendment of this soil showed no significant effects upon either extractable Pb or its bioaccessible extracts at 9 months (Independent t-tests, $p > 0.1$, Table 4-10). Examination of TPAH concentrations showed that MnOx amendment had no effect on TPAH concentrations over time (Independent t-test, $p > 0.3$, Table 4-11), nor was there any significant difference in TPAHs between MnOx-amended and unamended mixed + GW soils at 0 or 9 months (Independent t-tests, $p > 0.07$).

Soil pH values exhibited the same pattern as observed for metal soils in the previous section. At 0 months the pH both MnOx-amended and unamended mixed + GW was comparable (Independent t-test, $p > 0.2$, Table 4-12). Over time pH significantly increased in the MnOx-amended mixed + GW soil was therefore higher than its unamended counterpart at 9 months (Independent t-test, $p < 0.04$, Table 4-12). As previously discussed the increase resulted in pH values remaining alkaline and was therefore considered as being of minor importance.

4.3.10. *Lysimeter trial 2: Pollutant analysis of PAH contaminated soils with MnOx amendment*

Analysis of TPAH concentrations in the MnOx-amended PAH + GW soil over the time course of the experiment showed no significant difference (Independent t-test, $p > 0.2$, Table 4-11). Additionally, examination of the unamended PAH +GW soil also showed that no significant difference in TPAHs over time (Independent t-test, $p > 0.2$). As for both metal + GW and mixed + GW lysimeter soils, a significant increase was observed in PAH + GW soil pH with MnOx addition over the course of the experiment (Independent t-test, $p < 0.02$, Table 4-12), as before this increase resulted in the soil pH remaining within an alkaline pH range.

Table 4-9: A summary of mean values for extractable and bioaccessible As (mg kg^{-1} dry matter) from lysimeter trial 2, with and without 10 % MnOx amendment at 9 months. Values are based upon the mean of soil samples taken from each of the three lysimeter replicates.

	As		
	Aqua regia	**Biostomach**	**Biostomach and intestine**
Metals + GW[a]	5224 ±[b] 195	1232 ± 36	1203 ± 55
Metals + GW + MnOx	4942 ± 224	1121 ± 13	1062 ± 13
Mixed + GW	2571 ± 221	846 ± 38	814 ± 16
Mixed + GW + MnOx	3199 ± 187	833 ± 60	766 ± 43
PAH + GW	ND[c]	ND	ND
PAH + GW + MnOx	ND	ND	ND

[a] 1:1 mixture of contaminated soil and green waste (GW)
[b] ± 1 x standard error
[c] not determined

Table 4-10: A summary of mean values for extractable and bioaccessible Pb (mg kg^{-1} dry matter) from lysimeter trial 2, with and without 10 % MnOx amendment at 9 months. Values are based upon the mean of soil samples taken from each of the three lysimeter replicates.

	Pb		
	Aqua regia	**Biostomach**	**Biostomach and intestine**
Metals + GW[a]	5977 ±[b] 321	2494 ± 113	1152 ± 55
Metals + GW + MnOx	6316 ± 224	2621 ± 107	1376 ± 67
Mixed + GW	1600 ± 132	779 ± 54	359 ± 10
Mixed + GW + MnOx	2739 ± 546	1094 ± 251	524 ± 95
PAH + GW	ND[c]	ND	ND
PAH + GW + MnOx	ND	ND	ND

[a] 1:1 mixture of contaminated soil and green waste (GW)
[b] ± 1 x standard error
[c] not determined

Table 4-11: Mean total PAH (TPAH) concentrations (mg kg^{-1}) from lysimeter trial 2 at 0 and 9 months with and without 10 % MnOx amendment. Values are based upon the mean of 3 soil samples from each lysimeter.

	TPAHS*	
	0 months	**9 months**
Metals + GW[a]	ND[b]	ND
Metals + GW + MnOx	ND	ND
Mixed + GW	230 ±[c] 83	20 ± 6
Mixed + GW + MnOx	33 ± 14	16 ± 7
PAH + GW	136 ± 39	82 ± 13
PAH + GW + MnOx	127 ± 77	32 ± 3

* based upon the sum of EPA 16 priority PAHs
[a] 1:1 mixture of contaminated soil and green waste (GW)
[b] not determined
[c] ± 1 x standard error

Table 4-12: Mean pH values for lysimeter trial 2 at 0 and 9 months with and without 10 % MnOx amendment. Values are based upon the mean of 3 soil samples from each lysimeter.

	pH	
	0 months	**9 months**
Metals + GW[a]	6.91 ±[c] 0.08	7.02 ±[a] 0.00
Metals + GW + MnOx[b]	7.09 ± 0.02	7.29 ± 0.01
Mixed + GW	7.34 ± 0.03	7.24 ± 0.01
Mixed + GW + MnOx	7.45 ± 0.08	7.34 ± 0.03
PAH + GW	7.71 ± 0.08	7.75 ± 0.05
PAH + GW + MnOx	7.69 ± 0.10	7.97 ± 0.03

[a] 1:1 mixture of contaminated soil and green waste (GW)
[b] 10 % MnOx amendment
[c] ± 1 x standard error

4.3.11. *Lysimeter trial 2: Effects of MnOx amendment on basal respiration*

At 0 months MnOx amendment had a significant effect upon basal respiration (BR) when soil type was controlled for (two-way ANOVA, $p < 0.02$, Figure 4-8). Subsequent analysis revealed that MnOx amendment significantly decreased BR in mixed and PAH contaminated soils (Independent t-tests, $p < 0.01$), but not in metal or low metals soils (Independent t-tests, $p > 0.6$). Decreased BR was transient, as at 9 months MnOx amendment no longer had a significant effect when contaminated soil type was controlled for (two-way ANOVA, $p > 0.9$, Figure 4-8). At both 0 and 9 months there was no significant interaction between MnOx amendment and contaminated soil type (two-way ANOVA, $p > 0.1$). Contaminated soil type had a significant effect upon BR when MnOx amendment was controlled for at 0 and 9 months (two-way ANOVA, $p < 0.02$). Pairwise comparisons showed that BR in mixed contaminated soils was significantly lower in comparison to all others (Tukey's HSD, $p < 0.01$). However, low metals, metals and PAH contaminated soils exhibited BR comparable to each other at both time points (Tukey's HSD, $p > 0.05$).

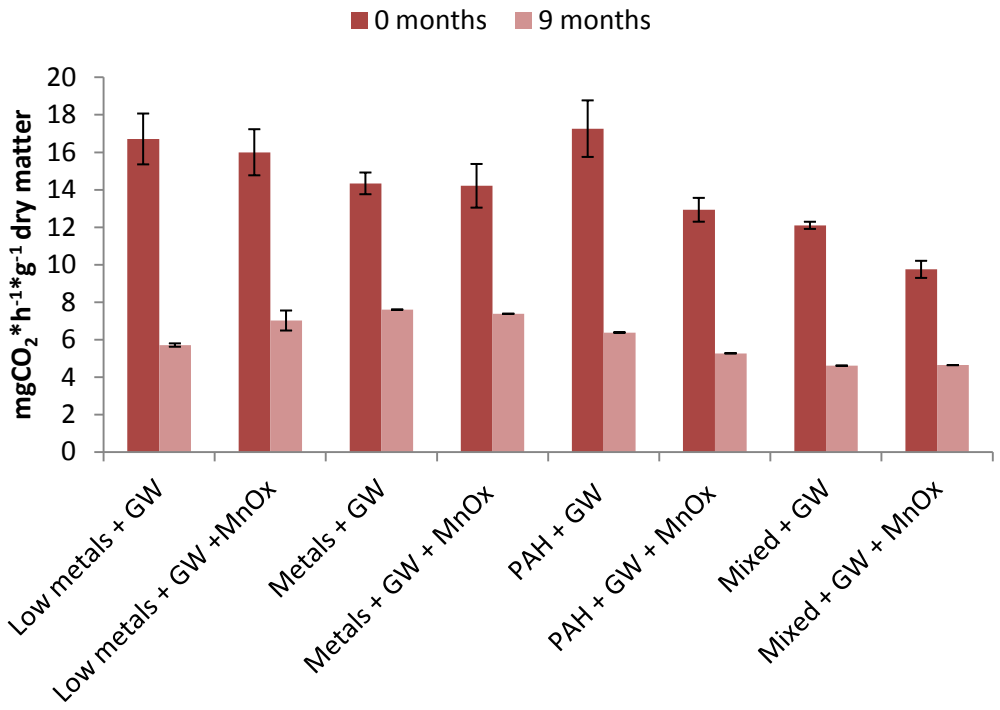


Figure 4-8: Basal respiration rates of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 2 which were mixed 1:1 with green waste (GW), with 0 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.3.12. *Lysimeter trial 2: Effects of MnOx amendment on denitrification enzyme activity*

MnOx amendment did not affect denitrification enzyme activity (DEA) when contaminated soil type was controlled for at both 0 and 9 months (two-way ANOVA, $p > 0.4$, Figure 4-9). Additionally, there was no interaction effect observed between MnOx amendment and soil type at either time points (two-way ANOVA, $p > 0.4$). At the outset of the experiment all contaminated soil types had comparable DEA (two-way ANOVA, $p > 0.4$, Figure 4-9). However, by the end of the experiment DEA significantly differed between contaminated soil types (two-way ANOVA, $p < 0.01$); with the low metals soil displaying significantly higher DEA to the other contaminated soils (Tukey's HSD, $p < 0.04$).

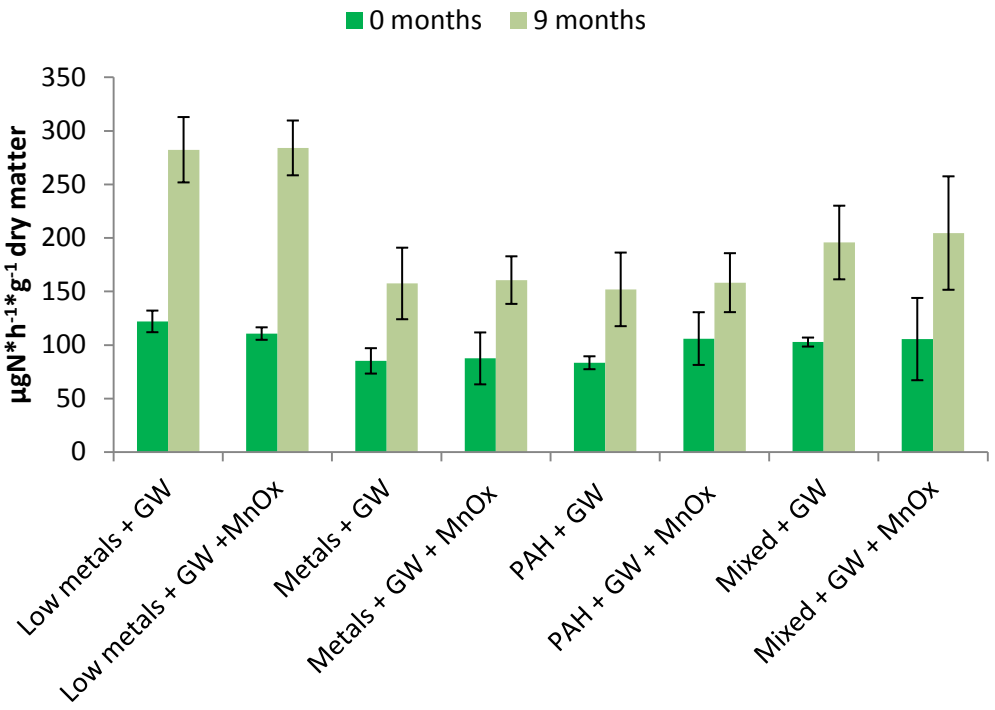


Figure 4-9: Denitrification enzyme activities of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 2 which were mixed 1:1 with green waste (GW), with 0 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.3.13. *Lysimeter trial 2: Effects of MnOx amendment on potential nitrification*

At 0 months MnOx amendment had no effect on potential nitrification rates (PNR) when contaminated soil type was controlled for (two-way ANOVA, $p > 0.3$, Figure 4-10), nor was any interaction observed between soil type and MnOx (two-way ANOVA, $p > 0.1$). PNR between the different contaminated soil types did significantly differ (two-way ANOVA, $p < 0.01$). Both low metals and PAH soils exhibited higher PNR rates (Tukey's HSD, $p < 0.01$) than that of metals and mixed soils (Tukey's HSD, $p > 0.4$). By the end of the experiment no significant differences were observed in PNR of the contaminated soils when MnOx was controlled for, or, was there any interaction effect between MnOx and contaminated soil type (two-way ANOVA, $p > 0.1$). However, at 9 months MnOx amendment did exhibit a significant effect on PNR (two-way ANOVA, $p < 0.01$). Subsequent testing showed that the only significant difference in PNR was between MnOx-amended and unamended low metal soils, where MnOx had significantly decreased PNR (Independent t-test, $p < 0.01$, Figure 4-10).

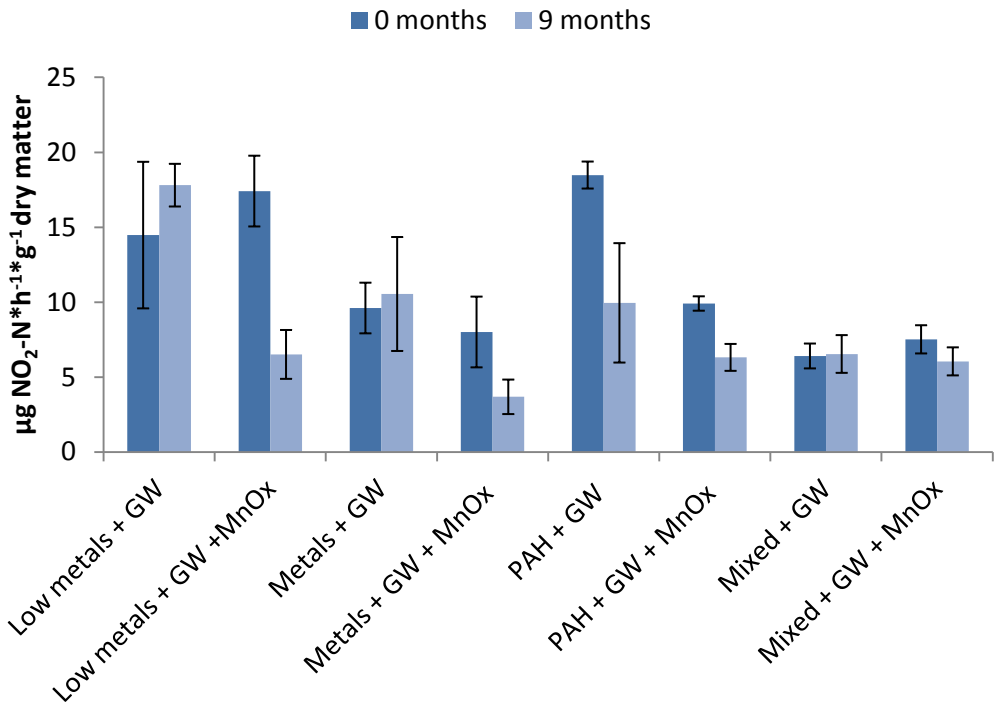


Figure 4-10: Potential nitrification rates of low metals, metals, PAH and mixed contaminated lysimeter soils of trial 2 which were mixed 1:1 with green waste (GW), with 0 and 10 % MnOx amendment at 0 and 9 months. Error bars represent 1 x standard error of triplicate lysimeter soils.

4.4. Discussion

Over recent years the use of organic/inorganic natural products and wastes such as: red mud, fly ash, zeolite and biochar, have received growing attention as soil amendments that are capable of the *in situ* stabilisation of metals in contaminated soils e.g. Beesley *et al.* (2010); Friesl *et al.* (2003); Guo *et al.* (2006b); Liu *et al.* (2011); Ram *et al.* (2011); Zhou *et al.* (2011). Organic amendments such as compost have been reported to accelerate the removal of PAHs from contaminated soils by the provision of more substrates for microbial growth, which in turn increases microbial activity and leads to the biodegradation of these toxic compounds (Fernández-Luqueño *et al.*, 2008; Wan *et al.*, 2003). However, to my knowledge, waste products other than MnOx, with the ability to chemically oxidise PAHs in soil thus far have not been discovered. To date, of these amendments, only biochar has been proven to be capable of reducing the bioavailability of both metal and PAHs, even in soils with mixed contamination

(Beesley *et al.*, 2010). Nonetheless, these soil amendments have received considerably less attention regarding their impacts on soil microbial function, with the exception of biochar (Bushnaf *et al.*, 2011; Jones *et al.*, 2012; Lehmann *et al.*, 2011). The objective of the following discussion was to deduce whether natural MnOx-containing wastes, which have been previously shown to absorb toxic metals (Chapter 3, section 3.4.4) and oxidise PAHs (Clarke *et al.*, 2012), could reduce the availability of metal and PAH pollutants but would not also negatively impact soil microbial function, and thus be a viable option for contaminated land remediation. To the author's knowledge this is the first study to assess the potential of natural MnOx-containing waste amendments as a remediation strategy for contaminated land.

4.4.1. *Lysimeter trial 1: MnOx amendment as a remediation strategy for metal contaminated soils*

Addition of MnOx to the metal contaminated soil did not show any reduction in extractable Pb or As when compared to the unamended control at 9 months. However, EPMA analysis strongly suggested that some Pb immobilisation had taken place upon the MnOx and indicated that MnOx-containing wastes may be a viable remediation strategy for Pb contaminated soils. Nonetheless, the amendment of these soils with MnOx was associated with higher concentrations of extractable As (35 %), extractable Pb (116 %) and bioaccessible Pb at 9 months in comparison to the unamended control.

These increases were apparent in both extractable and total digestions which showed that Pb and As were not bound to siliceous or other complex matrixes via MnOx amendment. Therefore, results suggest that the metal contaminated soils within lysimeter trial 1 contained significantly different total concentrations of Pb and As at the outset of the experiment, which was not measured. Contaminant results clearly showed that both Pb and As were not homogenous with the lysimeter soils, thus, a heterogeneous distribution of this metal and metalloid is very likely to result in significantly different total concentrations within the bulk soil. Furthermore, the higher concentrations of Pb and As could not be associated with addition from the MnOx itself. Previously, the MnOx-coated sand used as the amendment in lysimeters, was determined to have 2.8 mg kg^{-1} of As and 44 mg kg^{-1} of Pb (Chapter 3), consequently at an addition level of 10 % this would not have resulted in such dramatically higher Pb and As extractable concentrations.

MnOx have been shown to oxidise high molecular weight (HMW) humic substances to low molecular weight (LMW) compounds (Sunda and Kieber, 1994). It could be hypothesised that MnOx amendment of the metal contaminated soils resulted in the oxidation of HMW soil organic matter which released previously unavailable metals ions complexed to humic fractions that were not extracted during either AR or HF digests. Incomplete digestion of organic compounds by acid oxidants, particularly HNO_3, has previously been highlighted (Matusiewicz (2003) and references therein). As stated by Matusiewicz (2009) the extent to which oxidant acids in digestion methods actually destroy organic matrices has seldom been evaluated quantitatively. Although studies that have estimated the residual carbon content (RCC) as a measure of decomposition efficiency via the determination of carbon content before and after sample digestion clearly show incomplete digestion of organic materials (Kingston and Jassie, 1986; Nakashima *et al.*, 1988; Wurfels and Jackwerth, 1985). Other digestion methods often utilise additional H_2O_2 to enhance the destruction of organic matter in the soil, such as used in this study (Gaudino *et al.*, 2007). However, oxidising mixtures of H_2O_2 and HNO_3 have not been found to result in higher digestion efficiencies of organic matter than that of HNO_3 alone (Matusiewicz, 2003; Matusiewicz *et al.*, 1989).

It could be plausible that the oxidation of humic substances complexed within old refractory organic matter by MnOx has resulted in the release of previously unavailable metals, which in turn increased the extractability of Pb and As by both AR and HF digestions. This could also explain the higher Pb bioaccessibility, with As bioaccessibility unchanged due to the presence of Fe oxides, which have been found to be the primary control for As sorption in soils (Livesey and Huang, 1981; Pierce and Moore, 1982; Singh *et al.*, 1988). This would be consistent with the finding that the MnOx-coated sand grains contained Fe at around 2 % relative to MnOx at 9 % (Chapter 3). However, the significant differences in Pb bioaccessibility at 9 months between MnOx-amended and unamended soil are assumed to be an artefact of significantly different concentrations present at the outset of the experiment. Nevertheless, it would have been useful to monitor soil organic matter fractions throughout the lysimeter trials with a method such as thermogravimetry-differential scanning calorimetry (TG-DSC), and/or extraction of humic acids to conclusively prove whether

MnOx was involved in humification or oxidation reactions with soil organic matter (Dell'Abate *et al.*, 2003; Lopez-Capel *et al.*, 2005; Simpson, 2002).

It is also worth noting that As and Pb showed different patterns in biostomach (i.e. gastric) and biostomach and intestine (i.e. intestinal) extracts. In the gastric phase Pb bioaccessibility was 3-fold higher than in the intestinal fraction, whereas, As showed no difference between extracts (Table 4-3 and 4-4). This pattern was also observed in bioaccessibility extracts of the mixed soils, with Pb exhibiting a 6-fold increase in the gastric phase (Table 4-10 and 4-11). As EPMA analysis indicated Pb, not As, was associated with the MnOx it is hypothesised that the differing pH of the gastric (pH of 1.2) and intestinal (pH of 6.3) phase has an effect on MnOx-associated Pb. MnOx are renowned for their low point of zero charge (PZC), which generally falls between a pH of 2 to 5 (Gasparatos, 2012). Above this value, for example in a intestinal bioaccessibility extract, the MnOx has a negative surface charge and would result in the cationic sorption of divalent Pb^{2+}, which is the most common form of available Pb over the normal Eh and Ph range of soils (Gasparatos, 2012). Under these conditions Pb bioaccessibility would thus be low. However, if pH of a soil or solution falls below the PZC for MnOx, such as in a gastric bioaccessibility extract, the MnOx surface charge will change from negative to positive resulting in the desorption of cationic Pb. Therefore, it is theorised that Pb sorbed to the MnOx-coated sand underwent desorption within the gastric extract due to the pH falling below the PZC of the MnOx, which was followed by its re-adsorption in the intestinal phase, when the surface charge of the MnOx switched back to negative due to the solution pH being above the MnOx PZC.

While MnOx amendment did not show any difference in extractable Pb or As in comparison to the control at 9 months EPMA analysis showed that Pb immobilisation had occurred upon the outer MnOx layer of the MnOx-coated sand. EPMA has been traditionally employed to characterise mineralogical phases in environmental matrixes (Hudson-Edwards et al., 2005; Jacobson et al., 2007). However, it is also a commonly used method for determining the biosorption of metals on bacterial cells (Choi et al., 2009). While chemical based methods such as sequential extractions can identify changes in concentration of metals bound to MnOx phases, they cannot distinguish metals sorbed to intrinsic soil MnOx or added MnOx-coated sand. Thus, EPMA

presents a superior method for to verify metal adsorption to MnOx-coated sand in a soil matrix with multiple sorbents and intrinsic MnOx.

Consequently, it could still be possible that extractable concentrations of Pb, or indeed As if it has been immobilised by other sorbents such as Fe oxides, had decreased over time in the MnOx-amended soil. However, within this study determining whether metal immobilisation had occurred was based upon observing a reduction in extractable As and Pb via an aqua regia digestion at 9 months only. To my knowledge this method of digestion has not been reported to be incapable of extracting metals bound to MnOx. Consequently, it is possible that no reductions in extractable Pb would have been observed even though the MnOx had immobilised Pb. This suggests that the use of aqua regia here to determine immobilisation may have been incorrect. Instead it is proposed that the success of MnOx as a remediation strategy for metal contaminated soils in both lysimeter trials should have been complemented with methods to determine bioavailable Pb/As in leachate, such as sequential extractions coupled with EPMA analysis and biostomach and biostomach and biointestine bioaccessibility extracts to conclude whether metals were bound, however weakly, to MnOx over time (Mossop and Davidson, 2003; Quevauviller et al., 1997). Future work should determine whether aqua regia does indeed extract all heavy metals bound to reactive MnOx surfaces.

4.4.2. *Lysimeter trial 2: MnOx amendment as a remediation strategy for metal contaminated soils amended with young organic matter*

Trial 2 clearly showed that MnOx amendment of a highly metal contaminated soil mixed with young organic matter in the form of green waste (GW) had no effect on the extractable concentrations of Pb and As when compared to unamended metals + GW soils at 9 months. Consequently, it can be said that MnOx amendment did not have any positive effects as a remediation strategy and that addition of GW did not result in enhanced metal immobilisation via MnOx-induced humification.

However, at 9 months the MnOx-amended metals + GW soil exhibited lower bioaccessible concentrations of As but not Pb, when compared to an unamended metals + GW soil. The fact that As bioaccessibility was lower may suggest that some metalloid immobilisation may be taking place upon the reactive MnOx surface. It is also possible that As underwent sorption to the organic matter of the GW, although, it

is known that metal sorption by MnOx is superior to that of organic matter in soils (Adriano, 2001). Furthermore, it has been reported that organic matter would not provide significant sorption of As in soils especially in the presence of Fe oxides which would preferentially bind As in comparison to MnOx (Fitz and Wenzel, 2002). However, this effect was not found for Pb, thus it cannot be explicitly stated that metal immobilisation was occurring on the MnOx surface. Additionally, MnOx are known to be the dominant sorbent of Pb in soils, that have been reported to preferentially sorb Pb over As, exerting an irreversible binding of Pb that is 40 times greater than Fe oxides (Adriano, 2001; Beak *et al.*, 2007; Sahabi *et al.*, 2010). Thus, a decrease in Pb bioaccessibility would be expected to be linked to metal immobilisation upon the MnOx surface if it was occurring.

The fact that MnOx amendment to the metals + GW soil did not show any effect on Pb/As could be due to a number of reasons; (1) the use of aqua regia as a digestion method extracted metals bound to the reactive MnOx surface, (2) MnOx was added in too low a level to have an observable effect, (3) MnOx was humifying organic matter which was not resulting in the formation of non-extractable contaminants, (4) MnOx was oxidising organic matter, (5) reduction in pollutant concentrations could not be discerned due to reductions lying within the standard error, (6) MnOx had immobilised other pollutants than those measured, (7) the lysimeter soil contained heterogeneous contamination and therefore would have required a nested sampling regime to determine the number of samples required to provide precision and detection of significant differences, (8) extractable concentrations may have been reduced over time, however without 0 month data this could not be determined, (9) the large pollutant heterogeneity resulted in the reactive MnOx surface not being in contact with the pollutant, (10) the lysimeter trial was carried out over too short a time period to see an observable effect. However, the above speculative theories cannot be confirmed with the current dataset.

4.4.3. *Lysimeter trial 1: MnOx amendment as a remediation strategy for mixed metal and PAH contaminated soils*

MnOx amendment of mixed contaminated soils was associated with lower concentrations of extractable As and bioaccessible Pb at 9 months when compared to the unamended mixed soil. However, MnOx amendment had no significant effect upon

the extractable amount of total PAHs over time when compared to the unamended mixed soil. These results indicate that MnOx amendment is not a viable remediation strategy for mixed contaminated soils as it did not result in a reduction of both heavy metal and PAH contaminants.

As extractable and bioaccessible concentrations of Pb and As were not determined at 0 months a firm conclusion cannot be made as to whether the reductions observed at 9 months were; i) due to immobilisation by the MnOx, or, ii) if the unamended mixed soil contained significantly higher concentrations of Pb and As at the outset of the experiment. As noted within the results section, the mixed soils had large pollutant heterogeneity; therefore, it is possible that some samples analysed contained hotspots of Pb and As in the unamended control. It is hypothesised that concentrations were probably significantly different from the outset. Even though this could be the case it is still worthy of considering that there may indeed have been heavy metal immobilisation by the MnOx , especially in light of the fact that the MnOx-coated sands have been shown to immobilise metals (Chapter 3, section 3.4.4).

As the MnOx amendment did not cause any reduction in TPAHs it could be said that the MnOx appeared to reacting with the metals soil and not the PAH soil. It is worth noting that the metals soil originates from a site where there is extensive vegetation and leaf litter meaning that some of its SOM should be made up of relatively new material and LMW compounds. In contrast, the PAH soil is not vegetated so its SOM is likely to be aged and contain a higher percentage of HMW compounds. Therefore, it may be the case that MnOx interact differently with SOM of different ages and ratios of LMW: HMW compounds. If it is posited that MnOx amendment to the mixed soil of trial 1 resulted in heavy metal immobilisation via sorption onto the MnOx reactive surface then they most likely would have been extracted in aqua regia digests (as discussed in section 4.4.1), thus, a reduction in extractable As should not have been observed. As previously mentioned (section 4.4.1), aqua regia may not extract all heavy metals bound to SOM. Therefore, it could be hypothesised that the reduction in extractable Pb and As with MnOx amendment of the mixed soil was due to a reaction of the MnOx, such as humification, with the metals soil SOM that incorporated Pb and As into humic fractions forming non-aqua regia extractable compounds (Trevisan *et al.*, 2010; Wang and Mulligan, 2009). Furthermore, MnOx-humification of metal soil SOM may explain the observed

decrease in Pb bioaccessibility over maintained As bioaccessibility, as humic acids and soils have been found to have the highest affinity and therefore preferential sorption of Pb (Covelo *et al.*, 2004; Martyniuk and Więckowska, 2003). However, these explanations are only speculative as there was no evidence to suggest that humification had occurred.

MnOx did not appear to be a successful amendment that reduced total PAHs. Nonetheless it is proposed that it would have been beneficial to measure bioavailable PAHs during lysimeter trials using a method such as passive sampling e.g. Hale *et al.* (2010). As pollutants appeared to be heterogeneous in lysimeter soils it may be possible that MnOx amendment had resulted in oxidation of bioavailable TPAHs which may have not been detected due to concentrations lying within the associated standard error. Within the previous section reasons for MnOx not resulting in metal immobilisation were proposed, the same reasons are analogous to no reduction PAH contaminants being apparent with MnOx amendment, excluding point 1.

4.4.4. *Lysimeter trial 2: MnOx amendment as a remediation strategy for mixed metal and PAH contaminated soils amended with young organic matter*

MnOx-containing waste did not appear to be an effective remediation strategy for mixed contaminated soil amended with young organic matter in the form of green waste (GW). Furthermore, the addition of GW did not result in enhanced metal immobilisation or reduce TPAH extractability via MnOx-induced humification. This was evidenced by MnOx amendment not showing any effect upon extractable/ bioaccessible Pb/As or on total PAH concentrations. Reasons for MnOx amendments being unsuccessful in reducing extractable pollutant concentrations have previously discussed in sections 4.4.1 – 4.4.3, thus, they have not been presented again; however, all points are pertinent for mixed soils amended with GW.

4.4.5. *Lysimeter trial 1: MnOx amendment as a remediation strategy for PAH contaminated soils*

The amendment of PAH contaminated soil with MnOx showed no effect upon TPAH extractability over the time course of the experiment, therefore, it can be concluded from this study that MnOx amendment is not an effective for land contaminated by PAHs. As mentioned in section 4.4.3, the lysimeter soils containing

highly PAH contaminated soils would have benefited from measurement of bioavailable PAHs; reductions in which may have been missed due to the large experimental errors associated. Furthermore, examination of each individual priority EPA PAH may have uncovered reductions in specific PAHs over time with MnOx amendment. Although due to the large error associated with TPAH measurements neither of the aforementioned may have provided any further information. As previously mentioned a nested sampling regime would have been beneficial to determine the number of samples required to provide precision and detection of significant difference. Reasons for MnOx not reducing TPAH extractability cannot be deduced with the data from this study, however, possible explanations for MnOx not reacting with pollutants in the soil environment have been outlined in section 4.4.2 and are applicable to the MnOx-amended PAH contaminated soils also (excluding point 1).

While, it has been shown through pure mineral studies that natural MnOx-containing wastes have the ability to oxidise PAHs to less harmful and more bioavailable end products such as anthracene to anthraquinone (Clarke *et al.*, 2012), it appears that this may not be the main reaction mechanism between MnOx and PAHs within a contaminated soil environment. It has also been shown that MnOx can decrease TPAH extractability via MnOx induced humification reactions (see next section); however, this process also does not appear to be occurring with the MnOx-amended PAH contaminated soils of trial 1.

4.4.6. *Lysimeter trial 2: MnOx amendment as a remediation strategy for PAH contaminated soils amended with young organic matter*

MnOx-amended PAH contaminated soils mixed 1:1 with young organic matter in the form of green waste (GW) showed no reduction in TPAH extractability over time. Consequently, it can be said that MnOx amendment of a PAH contaminated soil that has been mixed with GW does not appear to be viable, and that addition of GW did not result in enhanced reductions in TPAHs via MnOx induced humification or oxidation.

Research has shown that addition of a synthetic MnOx (birnessite) in soils spiked with phenanthrene and pyrene, showed no interaction with parent PAH compounds during MnOx induced humification (Jung *et al.*, 2008). Instead synthetic MnOx have been shown to mediate the incorporation of PAH intermediates/

metabolites into soil organic matter via the formation of non-extractable residues with humic fractions through the oxidative coupling reactions (Jung *et al.*, 2008; Lee *et al.*, 2009). It is known that parent PAH compounds cannot be involved in abiotic humification reactions with MnOx due to the absence of hydroxyl functional groups that are required for the abiotic oxidative coupling reaction to occur (Jung *et al.*, 2008; Shindo and Huang, 1982; Stone, 1987b; Stone and Morgan, 1984b). The presence of PAH metabolites in the study of Lee *et al.* (2009) was only as a consequence of adding PAH degrading bacteria which successfully transformed the parent PAH (phenanthrene) to an intermediate (*cis*-phenanthrene dihydrodiol), that subsequently disappeared from the soil within 96 hours due to humification reactions with the MnOx and SOM.

It is therefore proposed that the reduction in extractable TPAHs in the PAH + GW soil or the PAH contaminated soils of trial 1, could occur via two distinct or coupled reactions: 1) the biodegradation of PAHs producing metabolites, or, the oxidation of PAHs by the MnOx (Clarke *et al.*, 2012); followed by 2) the humification of these metabolites and SOM by the MnOx. Within the current study it did not appear that TPAHs underwent any microbial degradation as evidenced by no significant differences being observed in the unamended PAH + GW soil over time. Consequently, it can be said that PAH degrading populations were not present in either the GW or PAH contaminated soils to degrade parent compounds into intermediates that would be required for a reduction in extractable TPAHs. Furthermore, this data also suggests that no oxidation of PAHs happened by MnOx. The work of Jung *et al.* (2008) and Lee *et al.* (2009) clearly showed that oxidation of PAHs was due to microbial transformation rather than MnOx. However, the work of Clarke *et al.* (2012) showed that abiotic PAH oxidation by MnOx is possible. Future work should assess whether the oxidation of PAHs observed in pure chemical studies occurs in the soil environment.

Other issues pertinent to examination of TPAHs within the lysimeter soils of this study have been outlined in the previous section and are applicable to trial 2 also. Furthermore, other possible reactions of MnOx in the soil matrix and suggestions as to why no pollutant reduction may have been observed in lysimeter trials have been outlined in section 4.4.2.

4.4.7. *Lysimeter trial 1: The effects of MnOx amendment as a remediation strategy on soil microbial function*

Pollutant results for low metal/metal/PAH/mixed contaminated soils indicated that MnOx was not a viable remediation strategy, as no decreases in Pb, As of TPAHs were observed. Thus, the original hypothesis of MnOx amendment stimulating carbon and nitrogen cycling via immobilisation of toxic contaminants can be rejected. However, analyses of soil microbial functions are of interest with respect to the use of MnOx as a soil amendment as no detrimental effects upon microbial function were apparent. Therefore, it can be inferred that MnOx does not have a high microbiological toxicity and is a viable remediation strategy at least with regard soil microbial functioning.

MnOx addition did not impact intrinsic soil microbial function when used as an amendment at levels of 5 and 10 % by weight, in PAH and mixed contaminated soils. This was evidenced by no negative or stimulatory effects upon basal respiration (BR), denitrification enzyme activity (DEA) or potential nitrification rates (PNR). Often microbial communities who have not been exposed to high concentrations of heavy metals, such as in the PAH contaminated soils, spend more energy on the detoxification of toxic heavy metals at the outset, switching their energy use from production of biomass to maintenance, thus resulting in decreased activity until tolerance had developed (Stefanowicz *et al.*, 2010). Consequently, it can be said that addition of MnOx did not disturb the microbial community of the PAH or mixed contaminated soils.

In contrast, MnOx amendment did show some effects in BR and DEA, but not PNR, in the two metal contaminated soils. Both 5 and 10 % MnOx amendments of the low metals soil was associated with an increase in DEA at the outset of the experiment. Although this stimulation was transient, as at 9 months rates in MnOx-amended soil were comparable to the unamended control. With regard BR, 5 % MnOx significantly increased this function in the low metals soil at 9 months. However, this effect was not observed for 10 % MnOx amendments therefore it did not appear to a solely due to MnOx addition. It is possible that the soil was not homogeneous and therefore subsamples taken have caused this indicator value to vary. This seems plausible as PNR for the 5 % MnOx low metal amended soil were associated with a large error (Figure 4-

7). In Chapter 3, the addition of the same MnOx to the low metal contaminated soil, showed a stimulation of N-cycling evidenced through spikes in DEA and PNR, suggesting that MnOx addition to the metals soil resulted initially in short-term metal immobilisation. While the same stimulation of PNR was not observed in lysimeter trials the theory of metal immobilisation by MnOx addition and subsequent DEA stimulation from Chapter 3 could not be confirmed as no pollutant data were provided by Durham University. As a result, the exact reasons for observed effects in DEA and PNR stimulations cannot be accounted for with the dataset in this study and require further investigation.

Both 5 and 10 % MnOx amendment to the high metal contaminated soil showed a decrease in BR at 9 months. It has been previously reported that Pb and As concentrations were significantly higher in the 10 % MnOx-amended soil at 9 months accredited to heterogeneous pollution and significantly different concentrations of heavy metals from the outset. The result of decreased BR in the high metal contaminated soil is therefore hypothesised as being coupled to the higher extractable heavy metal concentrations and heterogeneous nature of the soil and not as a direct result of MnOx addition. This can be further verified by the fact the no significant reduction was observed in BR at 9 months in the low metals soil.

4.4.8. *Lysimeter trial 2: The effects of MnOx amendment as a remediation strategy on soil microbial function*

Results for DEA, PNR and BR of trial 2 corroborated that of trial 1 whereby MnOx amendment appeared to have no detrimental effects upon soil microbial function at both 0 and 9 months. As in trial 1 MnOx amendment showed no positive effects as a remediation strategy with regard contaminant reduction in low metal/metal/PAH/mixed contaminated soils that were mixed with young organic matter (green waste-GW). Thus, the original hypothesis of MnOx amendment stimulating carbon and nitrogen cycling via immobilisation of toxic contaminants could be rejected. However, it is worth noting that the GW had its own highly stimulatory effects on activities. Within trial 2 both BR and PNR exhibited rates at least twice of that measured in trial 1 (BR – Figure 4-5, Figure 4-8; PNR – Figure 4-6, Figure 4-9), while DEA were over 4 times higher in contaminated soils when GW was added (Figure

4-7, Figure 4-10), consistent with the measurements of the raw material itself (Table 4-8).

Some variations in response were apparent between the different contaminated soil types with regard MnOx amendment. BR was found to decrease in mixed and PAH contaminated soils, however, this effect was short term and transient. Furthermore, MnOx amendment did not affect PNR or DEA in these soils. The reason for a decreased BR with MnOx addition is unclear. It did not appear to be a dilution effect as it would have been expected to see this within the low metal and metal soils at 0 months also. It is possible that the addition of MnOx disrupted the microbial community of the PAH contaminated soil through the addition of metals, thus, causing a disturbance. However, this effect was not observed in trial 1 and therefore seems unlikely. Furthermore, it did not appear to be due to MnOx suppressing activity of populations in the GW as if this was the case a decreased BR would have been observed in all soils at 0 months. However, on the whole the addition of MnOx did not permanently suppress respiratory activity, and after 9 months it was apparent that the addition of this metal oxide to a PAH and mixed contaminated soil was not detrimental to overall microbial activity.

At the end of trial 2 PNR was found to be decreased in MnOx-amended low metals + GW soils. The decrease in PNR suggests that there may have been an alternative substrate for the ammonia monooxygenase (AMO) gene. AMO enzymes are well documented as having a broad substrate range of over 40 compounds, that have been experimentally proven to be alternative substrates of this enzyme (Arp and Stein, 2003; Chang *et al.*, 2002; Keener and Arp, 1994; McCarty, 1999). As ammonia-oxidising bacteria (AOB) use ammonia (NH_3) as their sole source of energy for growth, the oxidation of an alternative substrate, will result in dramatic reductions in their nitrifying activity (PNR) and populations sizes as they are incapable of deriving energy from this process (Chang et al., 2002; Radniecki et al., 2011; Xu et al., 2000). However, this decrease in PNR was not observed in the metals soil in trial 2 or in both metal contaminated soil types in trial 1. Consequently it could be said that it did not appear that MnOx amendment was the cause of this decrease. As chemical analysis was not performed upon the low metals it is possible that another factor had influenced the suppressed PNR rates at 9 months such as changes in pH or concentrations of bioavailable metals. However, the exact mechanisms for PNR decreases cannot be

concluded with the data in this study. As PNR was unaffected by MnOx amendment in all other soils in both trials it was concluded that MnOx does not generally negatively affect this function.

4.5. Conclusions

The results from this study suggest that natural MnOx-containing wastes in the form of MnOx-coated sands are not a viable remediation strategy that can immobilise and reduce extractable Pb, As and TPAH pollutants in PAH or mixed contaminated soils. Mn-oxide coated sands did show evidence of *in situ* Pb sorption, but not As, in metal contaminated soils and may present a viable immobilisation strategy for Pb impacted sites. Soil microbial function analysis showed that MnOx amendment was not detrimental to the intrinsic microbial functioning of the aforementioned soils. Therefore, it is concluded that MnOx at the levels added does not have a high microbiological toxicity to soil microbial populations.

Chapter 5. Isolation and phylogenetic analysis of cultivable Mn(II)-oxidising bacteria associated with contaminated soils and Mn oxide-containing wastes

5.1. Introduction

Manganese (Mn) undergoes a microbial cycle, alternating between oxidised and reduced states, a process driven by phylogenetically diverse microbes (Tebo *et al.*, 2004; Tebo *et al.*, 2005). This cycle is known to strongly impact the transformation of contaminants in the soil environment (Tebo *et al.*, 2004). Mn(II)-oxidising bacteria are believed to accelerate the rate of Mn bio-mineralisation by up to several orders of magnitude faster than abiotic pathways and are thought to be the principle drivers behind redox cycling of this metal in nature (Spiro *et al.*, 2010; Tebo *et al.*, 2004). Therefore, the literature suggests that the presence of Mn(II)-oxidising bacterial populations in soils, can result in rapid recycling of Mn and production of biogenically formed Mn oxides (hereafter referred to as MnOx which collectively includes oxide, hydroxide and oxyhydroxide minerals). While Mn(II)-oxidising fungi commonly exist in nature and are known to contribute to Mn-cycling their identification was out of the scope of this study (Miyata *et al.*, 2007; Thompson *et al.*, 2005). Furthermore, Mn-reducing microbes were not examined because reduction of MnOx will happen abiotically via reductive dissolution reactions with pollutants, thus, looking for active biological reduction was less important in the application of MnOx-containing wastes for remediation.

5.1.1. *Aims and Objectives*

This chapter aimed to assess whether the bacterial communities present in MnOx-containing wastes and contaminated soils used previously in this study inherently contained Mn(II)-oxidising bacteria (MOB).

The objectives of this study were:

(i) To isolate MOB from MnOx-containing wastes and contaminated soils that were utilised in Chapter 3 and Chapter 4 of this study through the use of bacterial Mn(II)-oxidising enrichment cultures.
(ii) To identify MOB isolates through sequencing and determine any indigenous populations associated with the inocula.
(iii) To compare isolated MOB to other previously cultured representatives.

It was hypothesised that:

(i) MOB are present in contaminated soils and MnOx-containing wastes. In previous chapters (2, 3 and 4) both wastes and contaminated soils have been shown to contain diverse bacterial populations, oxidised Mn and associated organic matter, it is therefore possible that Mn-cycling and MOB exist.

5.2. Materials and Methods

5.2.1. *Inoculants used for Mn(II)-oxidising bacterial enrichment cultures*

Mn(II)-oxidiser enrichment cultures were carried out on three soils and two MnOx-containing wastes. The soils selected for analysis were from St. Antony's Tar (high PAH) and Lead Works (high and low metals), as used in Chapters 2, 3 and 4 of this study. Full descriptions of these contaminated sites have been given previously in Chapter 2. MnOx-containing wastes investigated were MnOx tailings from Hotazel Mn mine and MnOx-coated sands from Mosswood WTW as they represented the MnOx amendments investigated in this research. Descriptions of the site locations and sampling regime for both MnOx-coated sands and MnOx tailings have been previously given in Chapter 3 and Chapter 1 respectively. Soils were collected fresh from St Anthony's Tar and Lead Works the day before inoculation and stored aerobically at 4 °C overnight in sterile polyethylene bags. MnOx-containing wastes had been stored aerobically at 4 °C from initial collection as previously described (see Chapter 3, section 3.2.2 and 3.2.3).

5.2.2. *Mn(II)-oxidiser enrichment culture medium formulation*

Enrichment of Mn(II)-oxidising bacteria (MOB) was carried out on the K Medium (Table 5-1) which has been proven to support a phylogenetically diverse array of heterotrophic Mn(II)-oxidisers (Krumbein and Altmann, 1973; Nealson, 2002). Culture-dependent techniques are critical for the identification of MOB. Currently no primers exist that target these phylogenetically diverse populations due to no functional gene having been identified for Mn(II) oxidation.

Table 5-1: Formulation of the K Medium used to enrich Mn(II)-oxidising bacteria from MnOx-containing wastes, PAH and metal contaminated soils (Krumbein and Altmann, 1973).

Medium composition (per litre)	Carbon source (per litre)	Mn(II) source
10 mM HEPES buffer (pH 7.4)	2 g Peptone	15 g $MnSO_4$ (1 mM)
15 g agar	0.5 g Yeast extract	
Deionised water to 1000 ml		

Initially peptone, yeast extract and agar were added to 990 ml of deionised water and autoclaving was carried out for 20 minutes at 121 °C. After autoclaving, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and $MnSO_4$ were added to the medium by filter-sterilisation (0.22 µm). $MnSO_4$ can undergo oxidation during autoclaving leading to the formation of MnOx particles, which can bind to the remaining $MnSO_4$ and catalyse abiotic oxidation within the medium (Nealson, 2002). Therefore, the addition of the Mn(II) source after sterilisation ensures that no abiotic oxidation can take place within the medium which could be mistaken for biological production of MnOx (Nealson, 2002). HEPES buffer was added to adjust and regulate the pH of the medium to 7.0 - 8.0, thus, avoiding indirect Mn(II) oxidation due to possible pH changes over time (Nealson, 2002).

5.2.3. *Growth and detection of Mn(II)-oxidising bacteria*

To begin with 0.5 g of inocula was shaken in 5 ml of sterile tap water (i.e. 10 % w/v) and a dilution series from 10^{-1} to 10^{-5} was prepared. From each dilution, 0.1 ml was spread onto K plates. In addition, each inoculum source was rolled as a solid directly over the K plate surface to create a neat enrichment. After inoculation, the plates were incubated at 30 °C in the dark and examined daily for the appearance of Mn(II)-oxidising colonies. Screening for Mn(II)-oxidising colonies was based upon the appearance of a brown/black pigmentation around colony centres or edges, which putatively indicated MnOx formation. Each putative Mn(II)-oxidising colony was picked and streaked for isolation on fresh K plates. When adequate growth had occurred and the suspected Mn(II)-oxidisers had formed brown/black pigmentations, they were tested for presence of oxidised manganese.

5.2.4. *Colorimetric determination of oxidised manganese*

The presence of MnOx on or around suspected isolates was confirmed using the colorimetric dye leucoberbelin blue (LBB) (Krumbein and Altmann, 1973). A slightly modified LBB assay than that previously described in Chapter 3, section 3.2.4 was used. For suspected MOB, spot tests were carried out on streaked isolates by smearing a loopful of cells onto filter paper. A few drops of 0.04 % LBB in 0.45 mM acetic acid were added and an isolate scored as being Mn(II)-oxidising when a blue colour change was observed. The filter paper, disposable sterile loops and medium used were checked for reactivity with LBB. Previously LBB was reported to react with Co (Chapter 3, section 3.4.1) this possibility was discounted in enrichment experiments as no Co should be present in the medium.

5.2.5. *Storage of Mn(II)-oxidising isolates*

Plates with Mn(II)-oxidising bacterial isolates were stored at 4 °C, wrapped in parafilm and kept in plastic bags to prevent desiccation until molecular analysis was performed. A portion of positively identified Mn(II)-oxidisers were frozen at -80 °C by scraping cells directly from K plates into a 20 % glycerol solution.

5.2.6. *Microscopy of Mn(II)-oxidising isolates*

Using sterile scalpel blades a section of each Mn(II)-oxidising isolate was excised from K plates. A thin slice of agar was then taken from the top of the excised section and placed on a sterile microscope slide, secured by a drop of 50 % glycerol and a sterile cover slip was applied. The morphology of Mn(II)-oxidising isolates was viewed under light microscopy using a differential interference contrast microscope (Olympus, BH-2; Japan) using 100x oil immersion. Light micrographs were taken using an Olympus digital camera (E-400) with an OM adapter (MF-1) (Olympus, Pennsylvania, USA).

5.2.7. *Molecular identification of Mn(II)-oxidising bacteria*

DNA was extracted from the Mn(II)-oxidising isolates as described in Chapter 2, section 2.2.19. The only modification applied to the protocol was that no fixed weight of cells was used. Instead, cells were scrapped off K plates using sterile toothpicks and transferred directly to DNA lysing matrix tubes until they were ¾ full. The presence of DNA was verified by agarose gel electrophoresis (described in Chapter 2, section 2.2.21). PCR amplification was carried out as previously described (Chapter 2, section 2.2.20) except that the full length (~1534 bp) of the bacterial 16S rRNA gene was targeted using primer pair pA (5'- AGA GTT TGA TCC TGG CTC AG-3') and pH (5'- AAG GAG GTG ATC CAG CCG CA-3') (Edwards *et al.*, 1989). The PCR cycling conditions were as follows: after an initial denaturation step (95 °C for 3 minutes), 35 cycles of denaturation (95 °C for 1 minute), annealing (55 °C for 1 minute) and extension (72 °C for 1 minute) were followed by a final extension of 72 °C for 10 minutes. The correct size of gene fragments from the PCR reaction was confirmed by agarose gel electrophoresis (described in Chapter 2, section 2.2.21).

PCR products were purified using ExoSAP-IT and sequencing was carried out by DBS Genomics as previously outlined (Chapter 2, section 2.2.24). In order to obtain a substantial sequence coverage of the 16S rRNA gene, PCR amplification products of Mn(II)-oxidising isolates were sent with the forward primer pC which anneals to site 341 to 357bp on the 16S rRNA gene (5'-C TAC GGG AGG CAG CAG TGG G-3') (Edwards *et al.*, 1989). A phylogenetic analysis of sequences from Mn(II)-oxidising isolates was performed as previously described (Chapter 2, section 2.2.25).

5.3. Results

5.3.1. *Screening and isolation of Mn(II)-oxidising bacteria in MnOx-containing wastes and contaminated soils*

Mn(II)-oxidising bacteria (MOB) were successfully isolated from MnOx tailings, MnOx-coated sands, the high PAH soil and the high metals soil. However, the low metals soil showed no evidence of MOB.

A total of 21 putative Mn(II)-oxidising colonies were picked and streaked for isolation from the Hotazel MnOx tailings. However, only 6 tested positive for the presence of MnOx, these isolates were named H-15, H-16, H-17, H-18, H-19 and H-20 (Figure 5-1 and 5-2). Isolates H-15, H-17, H-18 and H-20 showed a strong reaction with LBB (i.e. strong blue colouration), while H-16 and H-19 showed a much weaker reaction (i.e. faint blue colouration). This may suggest that H-15, H-17, H-18 and H-20 were producing quantitatively more MnOx than H-16 and H-19. In all cases brown pigmentation occurred much later after colonies had fully developed.

Deposition of MnOx was apparent from all Hotazel MnOx tailing isolates on and below colonies but also spreading out from colonies onto the agar. Individual colonies initially exhibited aerial white mycelium with MnOx deposition occurring specifically within colony centres and dispersing into the agar from colony edges, thus, forming of a ring of MnOx. As time developed the MnOx deposition also occurred over white mycelium. Deposits were much darker underneath colonies which suggested that deposition was stronger and more concentrated in this local, an example of which can be seen in Figure 5-3a. Microscopic examination showed that they were composed of branching and fragmenting mycelia and hyphae (Figure 5-3b and c). MnOx deposition was also apparent on the top of aerial mycelium, which was visualised under the microscope by dark and dense areas of no distinct morphological shape (Figure 5-3d).

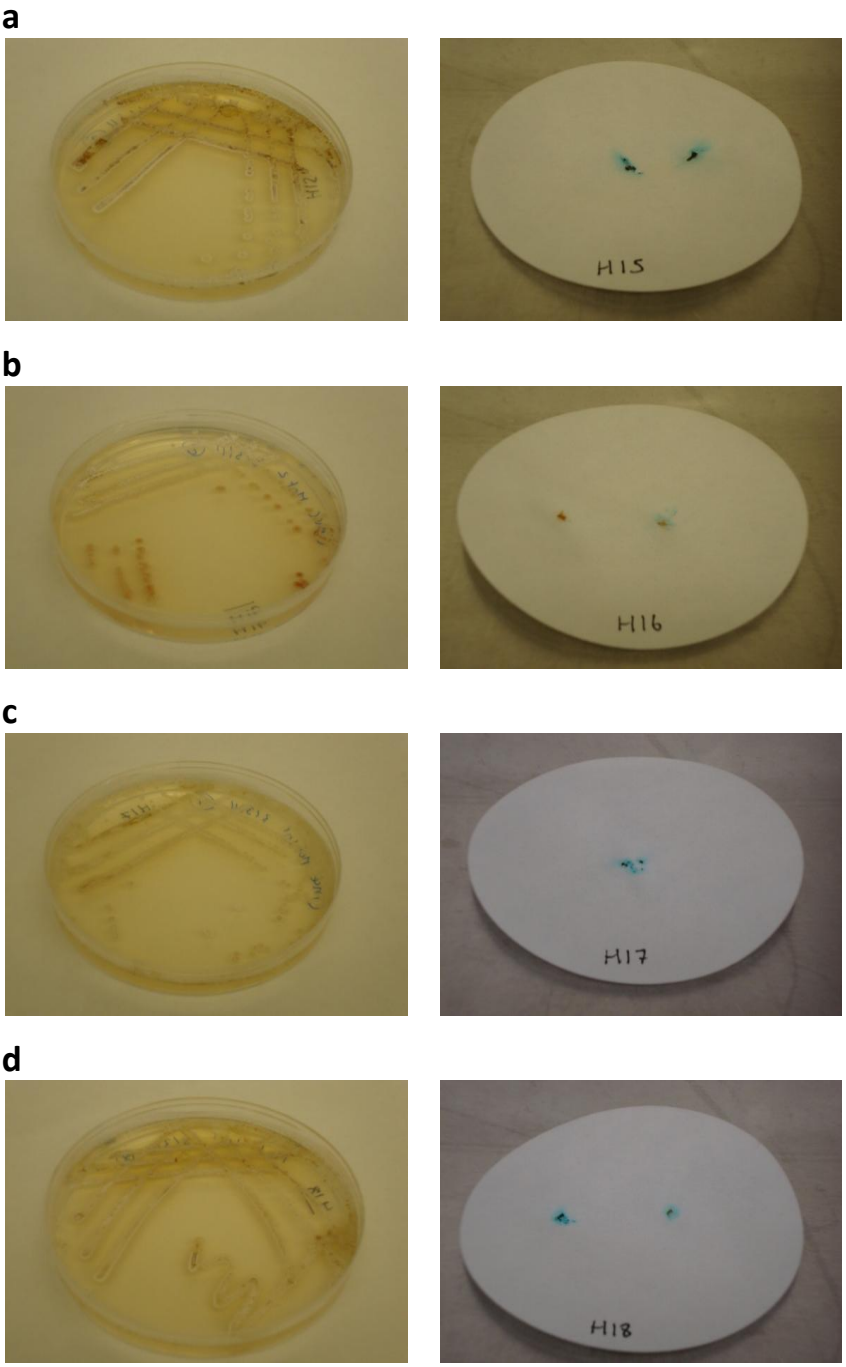


Figure 5-1: Photographs of Hotazel MnOx mine tailing Mn(II)-oxidising isolates (a) H-15, (b) H-16, (c) H-17 and (d) H-18. Pictures to the left are of single colonies which were picked and streaked for isolation on fresh K plates, with LBB spot tests for oxidised Mn from these colonies to the right.

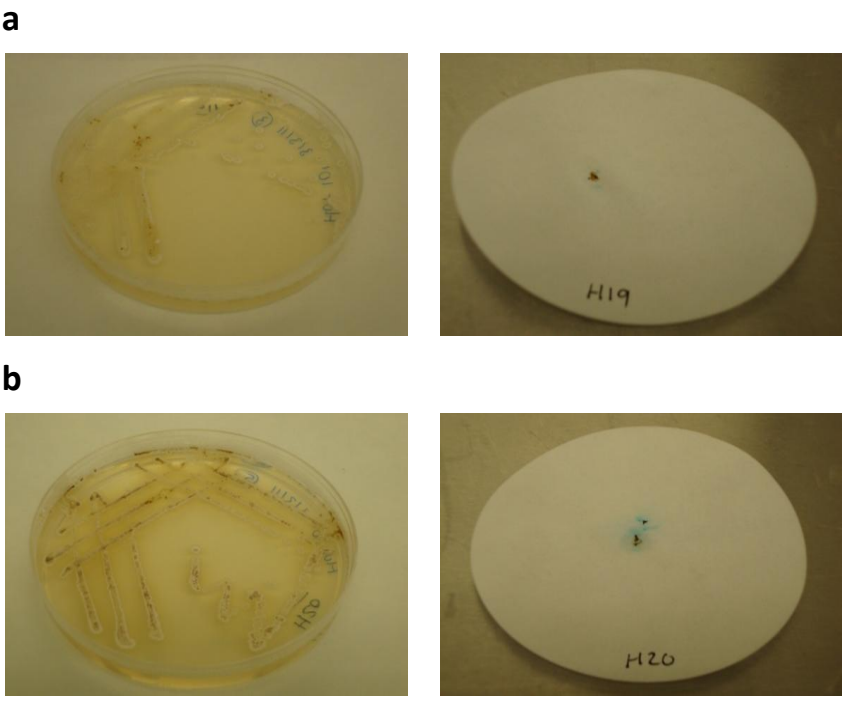


Figure 5-2: Photographs of Hotazel MnOx mine tailing Mn(II)-oxidising isolates (a) H-19 and (b) H-20. Pictures to the left are of single colonies which were picked and streaked for isolation on fresh K plates, with LBB spot tests for oxidised Mn from these colonies to the right.

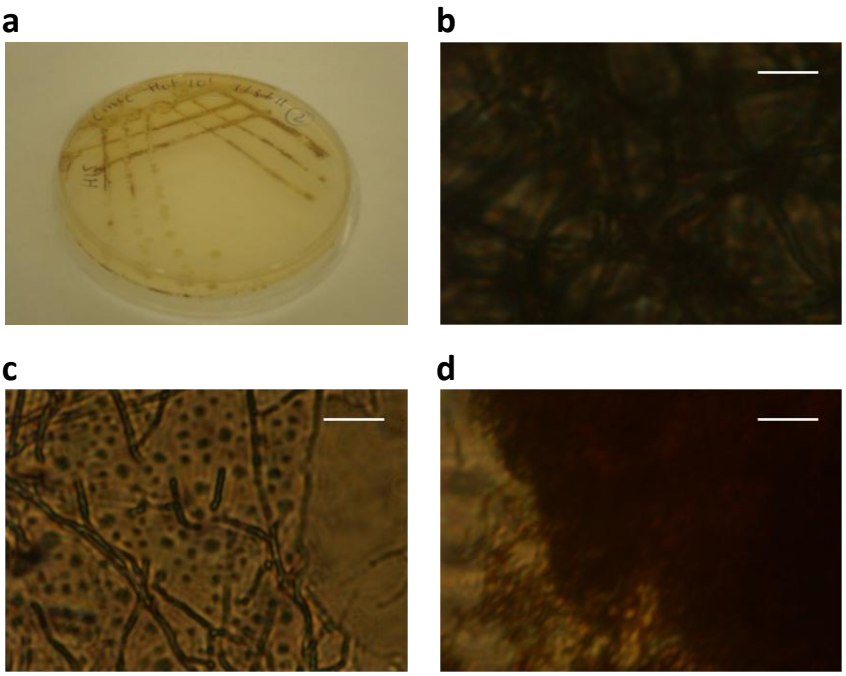


Figure 5-3: Light micrographs of (a) the underside of Mn(II)-oxidising bacterial isolate H-15 showing MnOx deposition, (b) branching mycelium, (c) hyphae and (d) MnOx deposition of Hotazel isolates. Scale bar represents 10 microns (µm).

A total of 13 putative Mn(II)-oxidising colonies were picked and streaked for isolation from MnOx-coated sands, out of which only 1 tested positive for the production of MnOx. This isolate was named MnS-7 (Figure 5-4).

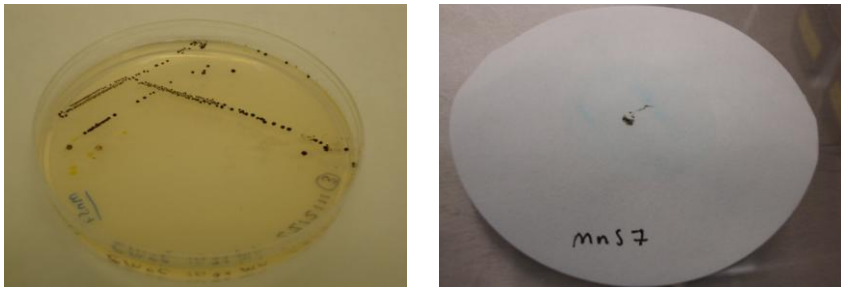


Figure 5-4: Photograph of MnOx-coated sand Mn(II)-oxidising isolate MnS-7. Picture to the left is of the single colony that was picked and streaked for isolation on a fresh K plate, with its respective LBB spot test for oxidised Mn to the right.

During the initial phases of growth, MnS-7 colonies exhibited a bright yellow colouration which re-occurred after re-plating. The dark brown colouration observed on the surface of colonies only developed after the streaked isolate had fully grown and was deemed to be in stationary phase. However, MnS-7 only displayed a very weak reaction with LBB, which suggested it was not oxidising large amounts of Mn(II).

The dark brown coloration was only evident over colony surfaces; these appeared as dry, shiny and hard surface coatings over the original wet yellow colony. Microscopic examination of MnS-7 revealed evidence of mycelium around colony edges (Figure 5-5a). The determination of any other morphological features was not possible as the dark brown precipitate completely enveloped all surfaces (Figure 5-5b).

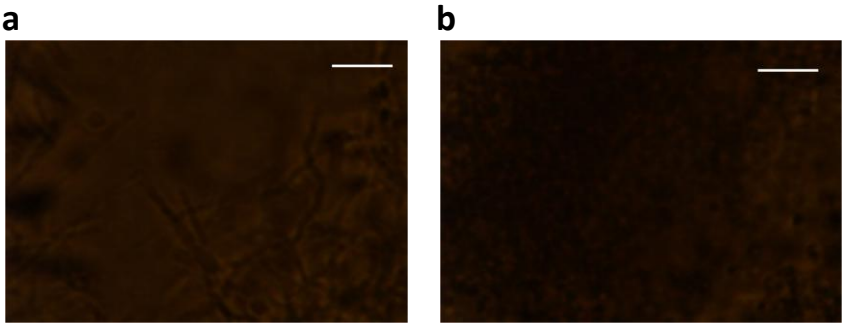


Figure 5-5: Light micrographs of Mn(II)-oxidising isolate MnS-7 showing (a) mycelium radiating from colony edges and (b) dark brown coating on top of a colony surface. Scale bar represents 10 microns (µm).

Enrichment cultures using St Anthony's high PAH contaminated soil produced 4 putative Mn(II)-oxidising colonies, however, only one tested positive for oxidised Mn. This isolate was named PAH-3, spot testing for oxidised Mn with LBB produced a strong reaction indicating substantial oxidation of Mn (Figure 5-6).

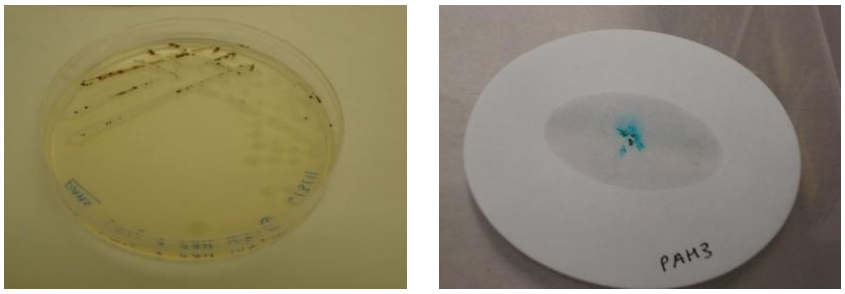


Figure 5-6: Photograph St. Anthony's high PAH contaminated soil Mn(II)-oxidising isolate PAH-3. Picture to the left is of the single colony that was picked and streaked for isolation on a fresh K plate, with its respective LBB spot test for oxidised Mn to the right.

MnOx deposition was concentrated on colony centres in isolate PAH-3. Colonies were white and had a chalky appearance. The development of a dark brown pigmentation which proved to be MnOx deposition only occurred when the colonies had fully developed. The production of oxidised Mn for isolate PAH-3 was much more rapid than that observed for MnS-7 or the Hotazel MnOx tailing (H) isolates.

Microscopic examination of PAH-3 revealed dark and dense areas of MnOx deposition that surrounded by hyphae and branched mycelium (Figure 5-7).

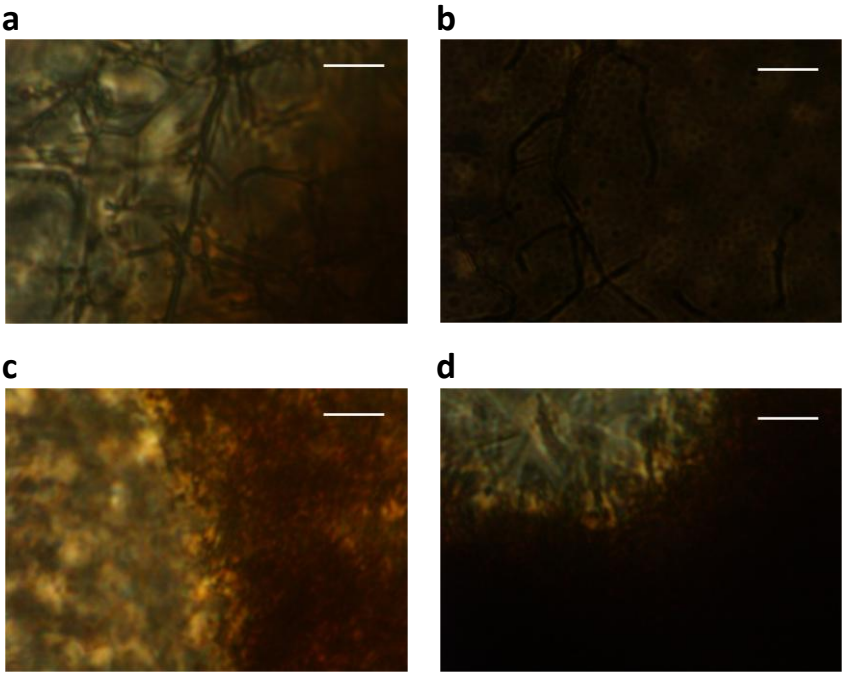


Figure 5-7: Light micrographs of Mn(II)-oxidising isolate PAH-3 showing (a) branching mycelium radiating from an area of MnOx deposition, (b) hyphae, (c) lighter MnOx deposition and (d) an area of strong MnOx deposition. Scale bar represents 10 microns (μm).

A total of 11 putative Mn(II)-oxidising colonies were picked and streaked for isolation from St. Anthony's high metals contaminated soil. However, LBB testing showed that only 9 were producing oxidised Mn. These isolates were named PB-1, PB-2, PB-3, PB-5, PB-6, PB-7, PB-8, PB-9 and PB-10 (Figures 5-8 to 5-10). Initially, all isolates formed smooth white colonies that developed over time to be powdery and chalky in appearance with aerial mycelium. Isolate PB-9 produced a yellow pigmentation which diffused into the K medium agar during growth. As with all previous enrichment cultures, Mn(II) oxidation occurred after colonies were fully developed. However, the growth of the Pb isolates and production of oxidised Mn was much more rapid than that of the Hotazel MnOx tailing isolates; being on a par with PAH-3 isolate Mn(II) oxidation. Isolates PB-2, PB-3, PB-5, PB-7, PB-8, PB-9 and PB-10 produced a very dark brown pigmentation from MnOx deposition, isolates PB-1 and

PB-6 showed a lighter brown deposition. Within the PB isolates there appeared to be 2 main forms of MnOx deposition: (1) covering all conies and surrounding mycelium (PB-1, PB-2, PB-3, PB-5, PB-7 and PB-10) and (2) underneath white aerial mycelium (PB-8 and PB-9).

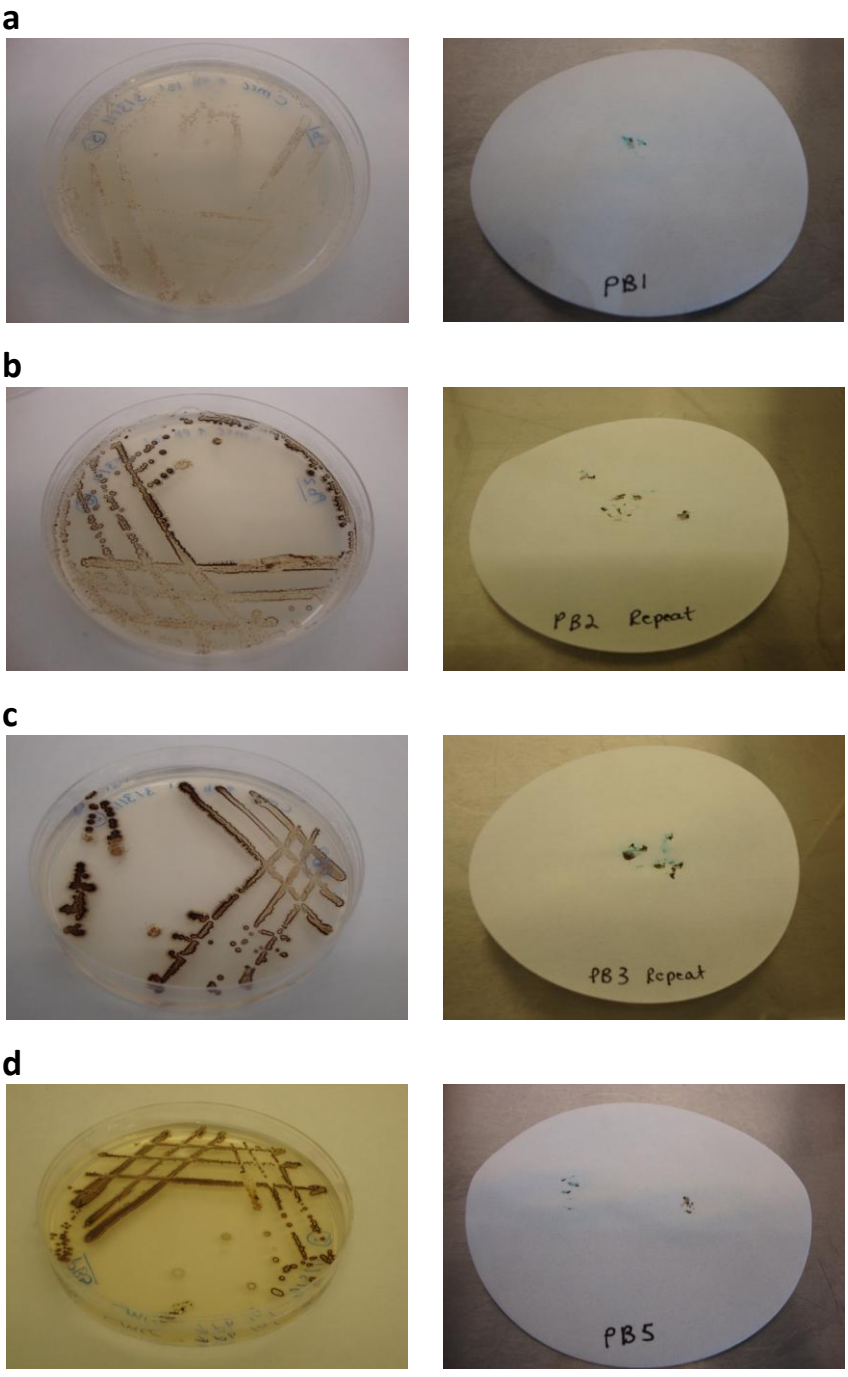


Figure 5-8: Photographs of St. Anthony's high metals contaminated soil Mn(II)-oxidising isolates (a) PB-1, (b) PB-2, (c) PB-3 and (d) PB-5. Pictures to the left are of single colonies which were picked and streaked for isolation on fresh K plates, with LBB spot tests for oxidised Mn from these colonies to the right.

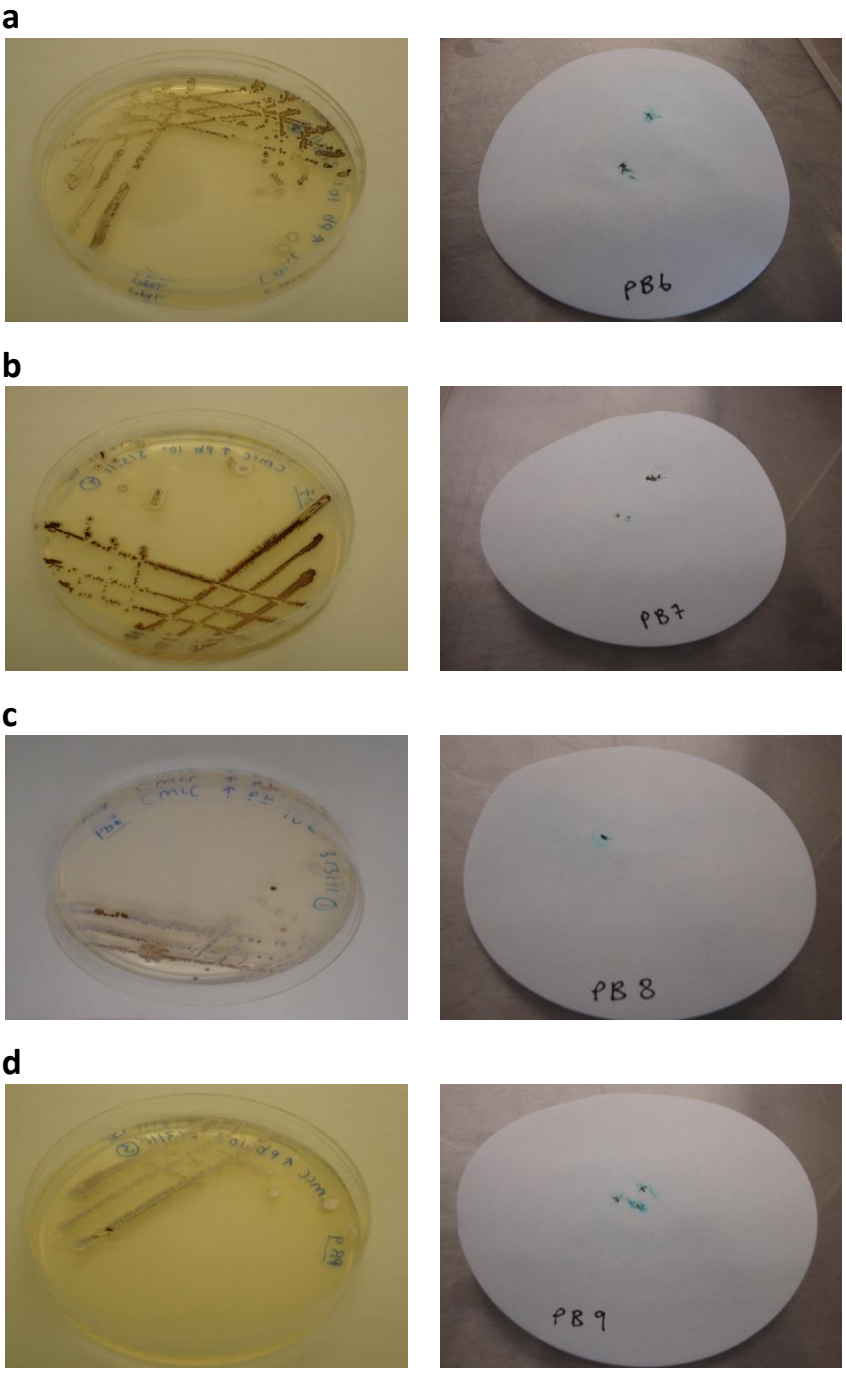


Figure 5-9: Photographs of St. Anthony's high metals contaminated soil Mn(II)-oxidising isolates (a) PB-6, (b) PB-7, (c) PB-8 and (d) PB-9. Pictures to the left are of single colonies which were picked and streaked for isolation on fresh K plates, with LBB spot tests for oxidised Mn from these colonies to the right.

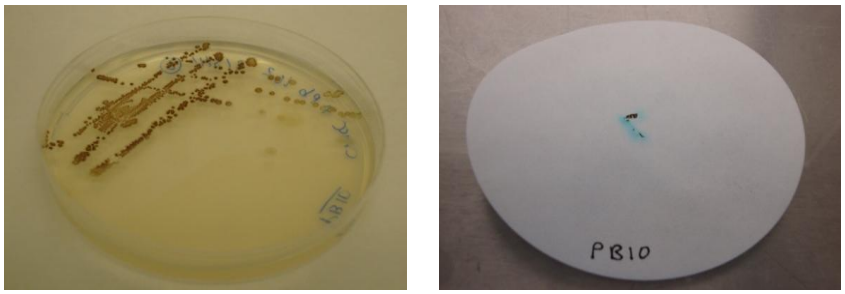


Figure 5-10: Photographs of St. Anthony's high metals contaminated soil Mn(II)-oxidising isolate PB-10. Picture to the left is of the single colony that was picked and streaked for isolation on a fresh K plate, with its respective LBB spot test for oxidised Mn to the right.

Microscopic examination of PB isolates showed that MnOx deposition generally occurred around mycelium, although, there was also evidence of patches of spores coated in MnOx. Separate colonies which merged together exhibited one large mass of dark brown/black MnOx under the microscope. All isolates demonstrated extensively branched mycelium. A selection of pictures taken from the microscopic examination of PB isolates is shown in Figure 5-11.

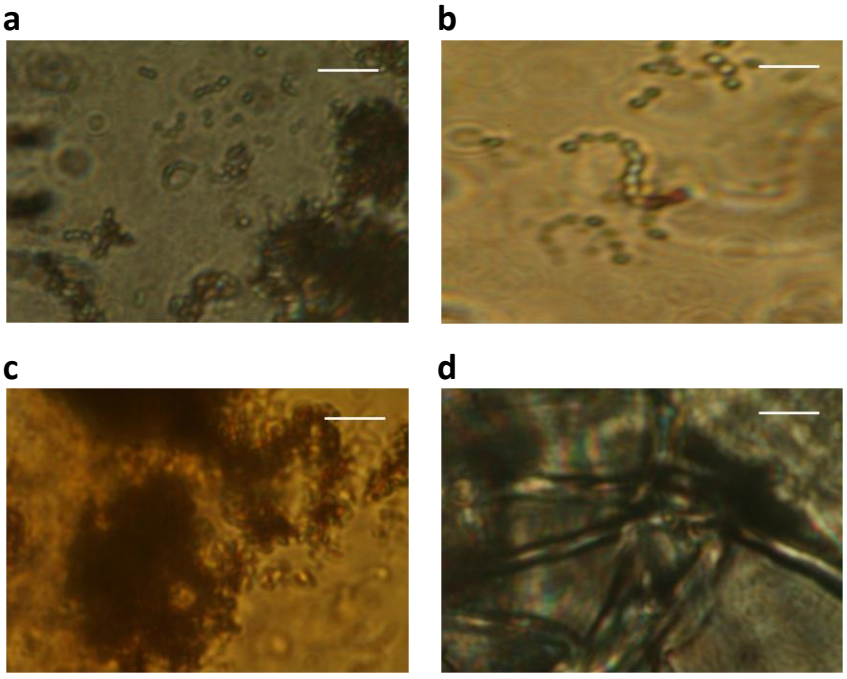


Figure 5-11: Light micrographs of Mn(II)-oxidising isolates from St. Anthony's high metals contaminated soil showing (a) edge of a colony coated in MnOx with spores on K plate agar, (b) a chain of spores, (c) spores coated in MnOx and (d) MnOx deposition around mycelium. Scale bar represents 10 microns (µm).

5.3.2. *Identification of Mn(II)-oxidising isolates in MnOx-containing wastes and contaminated soils*

Genomic DNA was successfully extracted from all 17 Mn(II)-oxidising isolates. PCR amplification of bacterial 16S rRNA genes was also successful for the 17 isolates and all were subjected to sequencing. Sequences generated were manually assessed for quality using BioEdit (Ibis Biosciences, Carlsbad, CA). Out of the 17 isolates sequenced, 13 were of high quality. Four of the PB isolates (PB-5, PB-7, PB-8 and PB-10) showed mixed bases in sequencing outputs, which indicated that more than one bacterial species was present in the enrichment. Consequently, these isolates were excluded from further phylogenetic analysis. The remaining 13 Mn(II)-oxidising isolates gave 16S rRNA gene read lengths ranging from 1061 bp (H-15) to 754 bp (PB-1) with an average read length of 876 bp.

Sequences were classified using the RDP Naïve Bayesian rRNA Classifier Version 2.2 (Wang *et al.*, 2007). Classification in RDP is derived from taxonomical hierarchy from the RDP training set 6, based on nomenclatural taxonomy and Bergey's Manual

of Systematic Bacteriology (Wang *et al.*, 2007). At a confidence threshold of 100 %, all 13 Mn(II)-oxidising isolates were classified as being from the phylum *Actinobacteria*. Additionally all fell within the order *Actionomycetales* at a confidence level of 100%. Isolate MnS-7, enriched from MnOx-coated sands, was identified as being from the genus *Micromonospora* (100 % confidence threshold). All five PB isolates (PB-1, PB-2, PB-3, PB-6 and PB-9) from St. Anthony's high metals soil were classified as being from the genus *Streptomyces* (100 % confidence threshold). Isolate PAH-3 enriched from St. Anthony's highly contaminated PAH soil was identified as being from the genus *Lentzea* (100 % confidence threshold). Five of the Hotazel MnOx tailing isolates (H-15, H-17, H-18, H-19 and H-20) were classified at 100 % confidence threshold limits as being from the genus *Amycolatopsis*, while H-16 was identified as being from the genus *Saccharothrix* (100 % confidence threshold).

The 16S rRNA sequences for all isolates were matched to closest cultured and uncultured sequences in the NCBI GenBank database using the nucleotide Basic Local Alignment Search Tool (BLAST®). Please note that differing % similarities relate to sequence assignment into different rank taxon groups. Similarities of ≥ 97 % indicate that sequences are from the same species as the cultured/uncultured representative, while similarities of 96- 95 % are assigned to the same genus, and similarities of 94- 92 % are assigned to the same family (Acosta-Martínez *et al.*, 2008; Quince *et al.*, 2009; Roesch *et al.*, 2007). A neighbour joining phylogenetic tree based on the order Actinomycetales, showing the 13 Mn(II)-oxidising isolates and their most similar cultured and uncultured relatives is presented in Figure 5-12. Details of the closest cultured and uncultured matches to the Mn(II)-oxidising bacteria isolated in this study are shown in Table 5-2 and Table 5-3 respectively. This comparison method was based upon the full length of recovered 16S rRNA sequences of all isolates.

Hotazel MnOx tailing isolates H-15, H-17, H-18, H-19 and H-20, were found to be closely related to other cultured *Amycolatopsis* strains mainly from metal contaminated and mine tailing environments at the species level (99 % similarity). However, further published information on these isolates was not available. Isolate H-16 was identified as a being related to a *Saccharothrix* strain isolated from a cave soil (publication not available) at the species level (99 % similarity). All of the Hotazel isolates did however share the same related uncultured Mn(II)-oxidising bacterium at

the family level (92-94 % similarity) which was isolated from Mn nodules in rice field subsoils (Cahyani *et al.*, 2009).

PB isolates from St. Anthony's high metals soil were related to other cultured *Streptomyces* species of soil environments at the species level (99 % similarity), excluding PB-9 whose isolation source was not described. Interestingly, all PB isolates shared the same uncultured *Streptomyces* species at the genus level (95-96 % similarity), which was isolated from a contaminated soil that was described within its GenBank entry as manganese peroxidise producing.

MnOx-coated sand isolate MnS-7 was 99 % similar, and therefore belonged to the same species, as a cultured *Micromonospora* from a coastal sediment environment. Additionally, this isolate was found to be belong to the same species (99 % similarity) to a suspected Mn(II)-oxidising *Actinobacteria* isolated from cave ferromanganese deposits (Northup *et al.*, 2003). This close uncultured relative was also shared by isolate PAH-3 at the same percentage similarity. The closest cultured relative of PAH-3 was found to be a type strain of *Lentzea albidocapillata* at the species level (99 % similarity), originally isolated from human tissue (Yassin *et al.*, 1995).

Figure 5-12: Phylogenetic tree of 16S rRNA genes of Mn(II)-oxidising isolates from enrichment cultures retrieved in this study (red and bold) and from other studies (blue). All sequences used to construct this tree were 755 bp long (including gaps). The scale bar indicates a sequence divergence of 10 %, calculated using the Jukes and Cantor correction (Jukes and Cantor, 1969). Bootstrap values are given at nodes when equal to or greater than 50 % (100 replicates). The out-group is '*Pseudomonas putida* MnB1'a γ-proteobacterium, model Mn(II)-oxidiser. Accession numbers are indicated in parentheses.

Table 5-2: Closest cultured relatives of the Mn(II)-oxidising isolates enriched in this study.

	Isolate	Fragment length (bp)	Closest cultured relative in GenBank	Accession	Source/ physiology of the closest cultured relative	% sequence identity	Reference
Hotazel MnOx tailings	H-15	1061	*Amycolatopsis orientalis* strain XM0301	DQ990889	Soil	99	Wang *et al.* (Genbank)[1]
	H-16	983	*Saccharothrix texasensis* strain PT749	FJ756556	Rare *Actinomycetes* from cave coils	99	Nakaew *et al.* (Genbank)[1]
	H-17	929	*Amycolatopsis* sp. K6-15	EF612300	Lead/zinc mine tailing site	99	Mendez et al. (2008)
	H-18	939	*Amycolatopsis* sp. IMER-B3-26	FJ772051	Mercury and chrome polluted soil	99	Whang *et al.* (Genbank)[1]
	H-19	960	*Amycolatopsis* sp. IMER-B3-26	FJ772051	Mercury and chrome polluted soil	99	Whang *et al.* (Genbank)[1]
	H-20	852	*Amycolatopsis* sp. IMER-B3-26	FJ772051	Mercury and chrome polluted soil	99	Whang *et al.* (Genbank)[1]
St. Anthony's high metals soil	PB-1	754	*Streptomyces tubercidicus* strain 14241	EF371435	Soil	99	Tian *et al.* (Genbank)
	PB-2	778	*Streptomyces* sp. Kkj5-1	GQ222217	Soil	99	Nonoh *et al.* (Genbank)
	PB-3	777	*Streptomyces tubercidicus* strain 14241	EF371435	Soil	99	Tian *et al.* (Genbank)
	PB-6	784	*Streptomyces tubercidicus* strain 14241	EF371435	Soil	99	Tian *et al.* (Genbank)
	PB-9	896	*Streptomyces* sp. N03-238	DQ787149	Bioactive *Streptomyces* strain	99	Zhang *et al.* (Genbank)
MnOx-coated sand	MnS-7	864	*Micromonospora* sp. N0073	AY221485	Coastal sediment	99	Zhao et al. (2004)
St. Anthony's high PAH soil	PAH-3	813	*Lentzea albidocapillata* strain IMSNU 21253^T	NR_041823	Type Strain	99	Lee *et al.* (Genbank)

[1] no accompanying journal publication

Table 5-3: Closest uncultured relatives of the Mn(II)-oxidising isolates enriched in this study. Please note that H isolates all had the same uncultured relative. Similarly PB isolates also shared the same uncultured relative.

	Isolate	Fragment length (bp)	Closest uncultured relative in GenBank	Accession	Source or physiology of the closest uncultured relative	% sequence identity	Reference
Hotazel MnOx tailings	H-15	1061	Uncultured Bacterium 1a-G6	AB354140	Manganese nodules in rice field subsoils	94	Cahyani *et al.* (2009)
	H-16	983				92	
	H-17	929				94	
	H-18	939				94	
	H-19	960				94	
	H-20	852				94	
St. Anthony's high metals soil	PB-1	754	*Streptomyces* sp. XKBH1 16S ribosomal RNA gene, partial sequence	GQ204110	Herbicide/dioxin contaminated soil. Produces manganese peroxidase	95	Nguyen *et al.* (GenBank)[1]
	PB-2	778				95	
	PB-3	777				95	
	PB-6	784				96	
	PB-9	896				96	
MnOx-coated sand	MnS-7	813	Uncultured bacterium clone EACUL1E6	AY186078	Ferromanganese deposits	99	Northup *et al.* (2003)
St. Anthony's high PAH soil	PAH-3	864	Uncultured bacterium clone EACUL1E6	AY186078	Ferromanganese deposits	99	Northup *et al* (2003)

[1] no accompanying journal publication

5.4. Discussion

The remediation of PAH and metal contaminated soils by amendment with natural MnOx-containing wastes has, thus far, in this research been discussed as a product of direct and indirect abiotic processes (Chapters 3 and 4). Such processes are proposed as being the main mechanism behind the initial remediation of polluted soils amended with MnOx-containing wastes. However, the literature (Tebo *et al.*, 2004; Tebo *et al.*, 2005) suggests that Mn(II)-oxidising bacteria are the principal drivers behind the cycling of Mn in terrestrial environments. Consequently, the presence of Mn(II)-oxidising bacteria in both contaminated soils and MnOx-containing wastes may result in the potential for regeneration and precipitation of 'biogenic MnOx' which could play a major role in remediation. After oxidative interactions with organics,

sorption of metals and oxidation/humification of organic matter, the MnOx reactive surface may become coated in organic and contaminants. Therefore, the MnOx reactive surface could be hindered if Mn(II) was not re-oxidised by autocatalytic processes on the pollutant coated MnOx surface. The objective of the following discussion is to consider the phylogeny of the bacterial isolates capable of Mn(II) oxidation and to elucidate the potential importance of biological Mn(II) oxidation when using natural MnOx-containing wastes as a potential remediation strategy for contaminated soils.

5.4.1. *The presence of Mn(II)-oxidising bacteria in MnOx-containing wastes and contaminated soils*

Results from this study showed that Mn(II)-oxidising bacteria (MOB) were present in the MnOx-containing wastes and the high PAH and metal contaminated soils as hypothesised. However, no MOB were isolated from the low metals soil, although, the detection of MOB in the high metals soil suggests that biological Mn(II) oxidation is present in soils of St. Anthony's Lead Works. The importance of confirmatory testing for oxidised Mn using the LBB assay in enrichment cultures must be stressed. Many colonies isolated in Mn(II)-oxidiser enrichments produced brown/black pigmentation around colony centre's or edges that could be falsely misconstrued as MnOx precipitation.

Previous studies have shown that MOB are phylogenetically diverse, widely abundant and distributed across many different geographic locations and environments (Tebo *et al.*, 2004; Tebo *et al.*, 2005). The presence of MOB in the contaminated soils and MnOx-containing wastes of this study, suggests that bacterially mediated Mn(II) oxidation can and may be actively occurring in such environments. Furthermore, it also supports previous reports that Mn(II)-oxidisers are found in diverse geographic locations and environments, as these isolates originated from both the north east of England and South Africa; and were from mine tailings, contaminated soils and water treatment sand filter environments.

On the other hand, all 13 MOB isolated from MnOx-containing wastes and contaminated soils in this study were from one phylogenetic lineage; the phylum *Actinobacteria*. Therefore, these isolates do not reflect the distribution of the MnOx phenotype from other known major bacterial phyla (Tebo *et al.*, 2004; Tebo *et al.*,

2005). Nevertheless, the *Actinobacteria* are one of the largest groups in the domain bacteria comprised of gram-positive bacteria with a high guanosine (G) and cytosine (C) content in their DNA, typically at around 65 - 75 % G + C (Miao and Davies, 2010; Ventura *et al.*, 2007). In general, the bacteria from this phylum dwell in many habitats including soils, the rhizosphere, marine and extremely arid environments (Miao and Davies, 2010). They are probably most well known as inhabitants of soil, where they carry out a vital function in the recycling of refractory biomaterials by decomposition and humus formation (Ventura *et al.*, 2007). However, *Actinobacteria* also exhibit diverse metabolic properties, such as the production of extracellular enzymes and the formation of a wide variety of secondary metabolites, most notably antibiotics (Ventura *et al.*, 2007). Additionally, the *Actinobacteria* demonstrate a wide variety of morphologies, from coccoid to fragmenting hyphal forms, or, permanent and highly differentiated branched mycelium with sporulating aerial mycelium (Ventura *et al.*, 2007).

It is of interest that only *Actinobacteria* were isolated from the different geographic locations and environment types examined in this study. It could be postulated that the enrichment cultures either: (i) selected for *Actinobacteria* or (ii) that the storage conditions of inocula favoured the survival of bacteria from this phylum. In general, *Actinobacteria* are renowned for being difficult to isolate (Hirsch and Christensen, 1983). This is due to the fact that they are characteristically slow growers relative to other bacteria (Hirsch and Christensen, 1983). Therefore, fast growing bacteria will outcompete and overgrow *Actinobacteria* in agar environments, unless prior enrichment and application of suitable selective pressures have been applied in medium formulation (Ensign, 1992; Goodfellow and Williams, 1983; Hirsch and Christensen, 1983). Examples of such measures include incorporation of antibiotics that inhibit fungi and non-actinobacterial bacteria, nutritional selection of nutrients preferentially utilised by *Actinobacteria* and drying of inocula to kill non-actinobacterial bacteria (Ensign, 1992; Hirsch and Christensen, 1983). Additionally, the *Actinobacteria* contain extreme desiccation resistance which allows them to survive in dry environments, which is ascribed to their high concentration of trehalose and the ability to form spores (Ensign, 1992).

In addition to the inherent difficulties in isolating *Actinobacteria*, all evidence from the literature points towards the fact that the K medium used for enrichment

cultures is not specifically selective for *Actinobacteria*. This medium did not contain any component specific for isolation of *Actinobacteria*. Consequently, if other bacterial Mn(II)-oxidisers were present such as *Bacillus*, *Pseudomonas putida* or *Leptothrix discophora,* they should have out-competed the *Actinobacteria*. This conclusion is supported by the fact that the K medium has been repeatedly shown to produce a phylogenetically diverse array of enriched MOB from various environments and provide a growth medium for other well characterised MOB e.g. Cerrato *et al.* (2010), Templeton *et al.* (2005), Francis *et al.* (2001), Wang *et al.* (2011), Dick *et al.* (2006), Francis and Tebo (2002), Learman *et al.* (2011b), Palanichamy *et al.* (2002), Lin *et al.* (2008) and Santelli *et al.* (2010).

It is unlikely that the storage conditions of inocula favoured the selection of *Actinobacteria*. This suggestion can be instantly discounted for contaminated soil samples from St. Anthony's as they were collected fresh on the day before enrichments began and were maintained at field moisture conditions. On this basis, it can be concluded that the *Actionbacteria* were simply the most common cultivable Mn(II)-oxidisers in these soils. MnOx-coated sands were refrigerated to maintain moist conditions from collection to inoculation and it is possible that refrigeration did lead to some desiccation. It is also possible that the storage of MnOx tailings at the Hotazel Mn mine has over time selected the desiccation-resistant *Actinobacteria*, due to the desert location of the mines on the edge of the Kalahari. Overall, it appears that these diverse environments contain MOB dominated by the *Actinobacteria*. Nevertheless, it may have been beneficial to enrich for Mn(II)-oxidisers on a variety of media such as PYGV (Ghiorse and Hirsch, 1979) or PC (Tyler and Marshall, 1967). These media specifically select for species such as *Hyphomicrobia*, *Pedomicrobium* and *Leptothrix,* this may eliminate culturing bias implicit from the use of one medium, and may have provided further evidence to conclude if contaminated soils and MnOx-containing wastes were dominated by *Actinobacteria*.

The *Actinobacteria* have been widely cited and identified as a bacterial phylum which contains oxidisers of Mn(II) (Tebo *et al.*, 2005). Results from the present study support the ubiquitous nature of these organisms. Many other studies have identified isolates from the *Actinobacteria* that are capable of Mn(II) oxidation, which further supports this premise (Bromfield, 1978; Bromfield, 1958; Bromfield, 1979; Bromfield and David, 1976; Cahyani *et al.*, 2009; Lin *et al.*, 2008; Northup *et al.*, 2003). Mn(II)-

oxidising *Actinobacteria* have also been isolated from diverse environments such as Arctic Ocean sediments (Lin *et al.*, 2008), acidic soils (Bromfield, 1978), rice field subsoils (Cahyani *et al.*, 2009) and caves (Northup *et al.*, 2003). Therefore, this study adds to the range of environments where Mn(II)-oxidising *Actinobacteria* are found.

5.4.2. *The phylogeny of Mn(II)-oxidising Actinobacteria isolated from MnOx-containing wastes and contaminated soils*

This study identified Mn(II)-oxidising bacteria (MOB) from five genra, namely, *Amycolatopsis*, *Sacchorothrix*, *Lentzea, Streptomyces* and *Micromonospora*. To the author's knowledge no other reports of Mn(II)-oxidising *Amycolatopsis, Sacchorothrix*, *Lentzea or Micromonospora* have been described, therefore, the isolates within this study provide the first demonstrated association of these organisms with biological Mn(II) oxidation.

Mn(II)-oxidising A*mycolatopsis* and *Saccharothrix* were isolated from the MnOx-containing wastes of Hotazel Mn Mine. The genus *Amycolatopsis* was originally proposed by Lechevalier *et al.* (1986) and is known to contain strains producing numerous antibiotics two of which, rifamycin and vancomycin, are currently commercially important (Malhotra and Lal, 2007). Additionally, members of this genus have been reported to metabolise aromatic compounds as the sole sources of carbon and energy (Malhotra and Lal, 2007). Currently, 45 species with validly published names constitute the genus *Amycolatopsis* (Everest and Meyers, 2011). These organisms are known to produce branched, fragmenting aerial and substrate mycelia of differing colours (Lechevalier *et al.*, 1986).

The genus *Saccharothrix* was first described by Labeda *et al.* (1984). *Saccharothrix* appear to be ubiquitous in soil and have a worldwide distribution (Labeda, 1992). This genus is characterised by the aerial mycelia, with both substrate and aerial hyphae that fragment into non-motile elements (Labeda, 1992). The genus *Saccharothrix* currently includes described 11 species, several of which have been shown to synthesise antibiotics and other bioactive secondary metabolites (Yan *et al.*).

Previously enrichments of microbial communities in cave ferromanganese deposits by Northup *et al.* (2003) revealed the presence of *Saccharothrix* isolates which were putatively described as Mn(II)-oxidisers. However, Northup *et al.* (2003) did not test these isolates for oxidised Mn, identification as to whether an isolate was

producing MnOx was solely based upon the development of black or brown bands of cells. As previously mentioned, this present study has found that testing for oxidised Mn was critical as many isolates produced a brown or black colouration which was not MnOx. While the findings of Northup *et al.* (2003) can be criticised, the results from this study back up their speculation that Mn(II) oxidation can be carried out by *Saccharothrix.*

Interestingly, the Hotazel MnOx tailings are thought to be biologically inert by the mining companies and this is the first microbial investigation that has been ever carried out on these samples. However, the presence of Mn(II)-oxidisers from Hotazel MnOx tailings suggests that microbial Mn-cycling could be an active process in waste tailings, thus, they may present a combined chemical and biological remediation approach. Most interestingly, the closest relatives of the Hotazel *Amycolatposis* isolates were found in metal rich environments such as soils and semi arid mine tailings (Mendez *et al.*, 2008). One of the numerous theories as to the potential benefits of bacterial Mn(II) oxidation is that by coating themselves in oxidised Mn, bacteria can protect themselves from heavy metal toxicity (Tebo *et al.*, 2005). Consequently, finding Mn(II)-oxidising bacteria from a heavy metal environment, that are related to other strains from similar environments, may suggest that protection from metal toxicity could be the reason for this trait in the *Amycolatposis* or *Saccharothrix* MOB.

Mn(II)-oxidising *Micromonospora* was isolated from MnOx-coated sands. This genus is widely distributed in nature, inhabiting environments such as coastal and marine sediments, peat swamp forests, floodplain meadows and plant rhizospheres (Hirsch and Valdés, 2010). At least 27 species have been described all of which are chemo-organotrophic aerobes, meaning they utilise organic compounds as energy sources (Hirsch and Valdés, 2010). The genus *Micromonospora* are best known for synthesising antibiotics such as gentamicin and netamicin, along with anti-tumor antibiotics, vitamin B1 and anti-fungal compounds (Hirsch and Valdés, 2010). Physiologically *Micromonospora* exhibit both substrate mycelia and spores, but no aerial mycelia, and their colonies can be a variety of colours, including white, orange, rose and brown (Hirsch and Valdés, 2010).

Mn(II)-oxidising *Lentzea* was isolated from St. Anthony's high PAH soil. This genus was first proposed by Yassin *et al.* (1995) and comprises mesophilic aerobes that

form abundant aerial hyphae which fragment into rod-shaped elements found in soil. In contrast, Mn(II)-oxidising *Streptomyces* were found in St. Anthony's high metals soil. This genus is one of the best known and studied within the *Actinobacteria* due to its wide distribution in soils, with hundreds of species recognised in *Bergey's Manual of Systematic Bacteriology* (Korn-Wendisch and Kutzner, 1992). *Streptomyces* produce metabolites called geosmins which give soils their characteristic odour, which in itself provides evidence of their ubiquity in this environment (Madigan *et al.*, 1997). However, *Streptomyces* are best known as being the most extensive antibiotic producers in this environment, they are also capable of producing a wide array of catabolic enzymes that degrade complex biological polymers, recalcitrant substrates such as cellulose and chitin and they produce antimicrobial compounds (Loria *et al.*, 2006). Furthermore, *Streptomyces* exhibit filamentous morphologies and form a mycelium of branching and aerial hyphae (Rigali *et al.*, 2008).

Streptomyces have been previously reported to oxidise Mn(II) in soil (Bromfield, 1978; Bromfield, 1979). Unfortunately, the 16S rRNA sequences from the aforementioned studies were not obtained as DNA sequencing was only being developed in the early 1970s. Thus, no sequences were available to allow for comparison with Mn(II)-oxidising *Streptomyces* isolated in this study. However, results from this study provide further evidence for Mn(II) oxidation in this genus and additionally provide 16S rRNA sequence data for future comparisons. Furthermore, previous studies (Bromfield, 1978; Bromfield, 1979) isolated Mn(II)-oxidising *Streptomyces* from acidic soils, however, enrichment cultures presented here provide evidence that biological Mn(II) oxidation by *Streptomyces* also occurs at circum-neutral soil pH.

Interestingly Mn(II)-oxidising *Streptomyces* in this study were found to be closely related to a species which produces manganese peroxide. As previously discussed within Chapter 1, thus far, only multi-copper oxidases (MCOs) and haem-containing Mn(II) oxidising peroxidases (Mop) have been linked to active Mn(II) oxidation in bacteria (Anderson *et al.*, 2011). In actual fact Mn(II)-oxidising haem peroxidases are most well known as the mechanism by which fungi biologically oxidise Mn(II). However, Mop are a relatively new group of proteins to be linked to bacterial Mn(II) oxidation (Anderson *et al.*, 2009b). Mn(II) oxidation by these peroxidases only oxidises Mn(II) to Mn(III), with production of Mn(IV) oxides requiring either further

enzymatic oxidation or a disproportionation step (Anderson *et al.*, 2011). So far biological oxidation of Mn(II) via Mop has only been proven for *Aurantimonas* and *Erythrobacter* species, and is believed to be involved in Mn(II) oxidation in *Mycobacterium and Arthrobacter* (Anderson *et al.*, 2011; Dubinina, 1978; Magliozzo and Marcinkeviciene, 1997). Recently it has been shown that organisms other than *Aurantimonas* oxidise Mn(II) via peroxidises (Anderson *et al.*, 2011). Therefore, it may be possible that Mn(II)-oxidising *Streptomyces* contain these peroxidises and further research into this may provide evidence for the mechanism of Mn(II) oxidation in the *Streptomyces*.

5.4.3. *Potential implications of the presence of Mn(II)-oxidising bacteria in the use of MnOx-containing wastes as a contaminated land remediation strategy*

The confirmation that Mn(II)-oxidising bacteria (MOB) exist in both contaminated soils and MnOx-containing wastes is of importance in this research. The presence of indigenous MOB for both MnOx-containing wastes and St. Anthony's contaminated soils provides the capability to recycle Mn and produce biogenic MnOx.

In MnOx-amended soils, it is envisaged that soluble Mn(II) will be available to these communities via the abiotic oxidative transformation of organic and inorganic pollutants by the added MnOx wastes. During oxidation of organic compounds by MnOx, Mn(II) is desorbed and therefore released into solution (Stone and Morgan, 1984a; Stone and Morgan, 1984b). In addition, during adsorption of metal ions to vacant sites on MnOx, Mn(II) is released (Lanson *et al.*, 2002; Matocha *et al.*, 2001; Zhao *et al.*, 2009). In general Mn(II) prefers to be sorped back onto the MnOx surface by autocatalytic oxidation processes. However, over time it is expected that the reactive MnOx surface of MnOx-containing wastes added to contaminated soils will become covered in either organic material or metals, therefore allowing Mn(II) to be released into soil pore water. The existence of MOB may therefore have an important role in the remediation environment by re-oxidising Mn (II); i) to provide more catalytic MnOx, and ii) to alleviate microbial communities from potential exposure to toxic Mn(II).The presence of MOB could therefore enable a more sustained transformation of pollutants in contaminated soils amended with MnOx-containing wastes by the production of biogenic MnOx and the continued re-oxidation and finer scale re-distribution of MnOx in the soil profile.

Biogenic MnOx are renowned for their high specific surface areas and reactivity (Sahabi *et al.*, 2010). Metal adsorption by biogenic MnOx, which are in general poorly crystalline or amorphous, have been shown to be greater than more crystalline abiotic MnOx minerals (Nelson *et al.*, 1999). Additionally, biogenic MnOx actively formed in heavy metal contaminated environments have been found to scavenge more toxic metal ions as the activation barrier required to incorporate metals into MnOx structures is removed or decreased (Tebo *et al.*, 2004). The superior characteristics of biogenic MnOx have lead to biological Mn(II) oxidation receiving enormous attention in the ever growing field of metal removal from contaminated water (Dong et al., 2000; Hennebel et al., 2009; Katsoyiannis et al., 2004; Miyata et al., 2007; Sahabi et al., 2010). To the author's knowledge no literature at present outlines any specific advantages to PAH oxidation with biogenic MnOx in comparison to abiotic MnOx. However, it is believed that due to the high specific surface areas and reactivity of biogenic MnOx, PAH transformation could be enhanced. Furthermore, to date no research has been carried out on the possible enhancement of pollutant transformation in contaminated soils by MOB; this may be an interesting avenue for future research.

5.5. Conclusions

This study provides evidence not only of the widespread distribution of Mn(II)-oxidising bacteria in the environment, but, also of the ability of a wide range of phylogenically diverse *Actinobacteria* to carry out this function. Furthermore, this study provided the first demonstration that species within the genera *Amycolatopsis*, *Sacchorothrix*, *Lentzea* and *Micromonospora* are capable of Mn(II) oxidation. The results from this study also indicate that bacterial Mn(II) oxidation takes place in both MnOx-containing wastes and contaminated soils from St. Anthony's Tar and Lead Works. Consequently, results suggest that the use of MnOx-containing wastes as a remediation strategy may result in sustained and biologically enhanced pollutant transformation through the existence of Mn(II)-oxidising bacterial populations.

Chapter 6. Conclusions and Recommendations

In the final section of this study, the main outcomes of each results chapter are summarised along with general conclusions. Additionally, recommendations that might lead to future work are suggested.

6.1. Conclusions

In this section the main objectives of each chapter are outlined followed by general conclusions and limitations.

6.1.1. *Chapter 2: Assessing indicators of soil microbial functioning in contaminated land for implementation into remediation strategies*

The main objective of this chapter was to characterise the intrinsic microbial functional status of long term metal and PAH contaminated soils, used throughout this study, by comparing measured microbial parameters to those obtained from non-contaminated land types. From this analysis suitable indicators for soil microbial functional assessment in contaminated soils and remediation strategies were proposed. Furthermore, interactions between community structure, diversity and function in contaminated soils were also investigated. This study has contributed to an expanding body of research into the effects of pollutants on soil microbial functioning. Specifically, it was found that:

- PAH and metal contaminated soils exhibited diverse microbial populations that are metabolically active. Long term contamination has, however, severely depressed denitrification enzyme activities (DEAs) and basal respiration rates (BR), indicative of bioavailable contamination.
- PAH and metal contamination had not suppressed microbial biomass carbon (MBC), microbial quotient (*qmic*), ecological diversity indices or potential nitrification rates (PNR).
- Bacterial community structure was not related to type or level of contamination. Instead it was found to be driven by geographical sampling locations with

dissimilarity related to the physico-chemical parameters of pH and total organic carbon (TOC).

- Community structure of *nirK* encoding denitrifiers was intimately linked to functional rates and therefore contamination.
- Metal contaminated soils are dominated by AOB. Metal contamination appears to select against AOA whilst also inducing a phylogenetic difference within the AOB community structure. In contrast, PAH contamination severely decreases both AOA and AOB populations. The impacts that PAH contaminants have upon diversity and structure of AOB and AOA communities in long term contaminated soils is an area where research is lacking, thus, these results are of importance.
- The microbial parameters of MBC and *qmic* were found to be insensitive indicators of microbial functional status as they did not exhibit any effects of contamination. These parameters along with qCO_2 comparisons were further discounted due to their time consuming, low throughput, labour intensive methods that would be unsuitable for routine monitoring.
- Molecular fingerprinting methods, i.e. DGGE profiling, of bacterial community structure and diversity was also deemed an ineffective indicator due to the low resolution they offer in soils and, thus, inadequate description of actual diversity and community structure.
- BR and DEA are proposed as superior indicators for the assessment of microbial function in contaminated soils through their sensitivity to the perturbation of contamination. PNR is also worthy of assessment as the community structure of ammonia-oxidisers was affected, thus, additional stresses or perturbations may harm this function which is intimately linked with denitrification. All functions offer rapid, high-throughput, standardised techniques that could be implemented in routine monitoring.

This study was limited by the range of contaminated and non-contaminated soils investigated. Aforementioned findings are only relevant for the contaminated soils of St. Anthony's Tar and Lead Works. Identical effects on microbial functions may not hold true for all contaminated soils. Therefore, a preliminary study on the status of soil microbial function is crucial for every contaminated land remediation investigation until sufficient comparisons can be made with the proposed indicators upon a wider

range of contaminated soils to allow more universal impacts to be inferred. However, this study was novel and scientifically relevant in the fact that no other research into soil microbial functional indicators has studied PAH and metal contaminated soils, and placed this in context of comparisons with the microbial function of soils from different land uses as a baseline for assessing impacts of remediation strategies.

6.1.2. *Chapter 3: The effects of natural Mn oxide-containing waste amendments upon key soil microbial functions in a long term metal impacted soil*

The main objective of this chapter was to be a preliminary assessment on the effects that MnOx-containing wastes (mine tailings and coated sands) had upon soil microbial function using a historically metal impacted soil, employing BR, DEA and PNR as indicators, in 6 month microcosm studies. It also served to elucidate if any MnOx effect upon soil microbial function was biotic or abiotic. Furthermore, this was the first formal assessment into the effect that MnOx addition has upon soil microorganisms. It was found that:

- Addition of MnOx-coated sands at levels of 1 - 7 % MnOx caused stimulatory but transient effects upon DEA, with the stimulation period lasting approximately 6 weeks. Addition of MnOx mine tailings showed a threshold for DEA stimulation between 3 - 7 % MnOx. MnOx tailing additions of 7 - 30 % showed a persistent and increasing stimulation of DEA lasting approximately 16 + weeks.
- Addition of MnOx-coated sands at levels of 3 and 7 % MnOx caused stimulatory but transient effects upon PNR, with a stimulation period of in the region of 6 weeks. Addition of MnOx mine tailings at levels 1 - 20 % MnOx showed a continual stimulation in PNR over a 22 week period with a spike in activity at 4 weeks from the outset. MnOx tailing addition at a level of 30 % showed a persistent and increasing stimulation of DEA over 22 weeks.
- The stimulation of DEA and PNR was attributed to sorption of bioavailable toxic metal ions by the MnOx although this could not be confirmed with the dataset. However, effects of MnOx on DEA and PNR were shown to be due to a chemical rather than biological mechanism from comparison of sterile and non-sterile additions.

- MnOx tailings appeared more reactive than MnOx-coated sands due to persistent and in some cases increasing stimulation of both DEA and PNR. The reasons for this could not be defined within this study. Furthermore, MnOx-coated sands contained levels of Cd and Ni which may cause significant harm to human health. MnOx mine tailings may present a better remediation amendment due to low levels of toxic heavy metals present and seemingly higher reactivity.
- There appeared to be an initial removal of metals via sorption onto the MnOx surface temporarily relieving metal toxicity and stimulating N-cycling. This was generally followed by a drop in stimulation which was ascribed to possible oxidation of soil organic matter by MnOx resulting in the release of toxic metal ions.

This study was limited by only one type of soil with a relatively low level of contamination. Assessment of the effects of MnOx-containing wastes on BR and therefore overall microbial activities were compromised by experimental set-up and inclusion of inert sand in microcosm trial 1 thus no conclusion could be made upon the effects MnOx had on this parameter. Additionally, these were relatively short term experiments and the stimulation of DEA and PNR in MnOx mine tailings could not be determined as being persistent or transient.

6.1.3. *Chapter 4: The use of natural Mn oxide-containing waste amendments as a viable contaminated land remediation strategy.*

The main objective of this chapter was to assess whether natural MnOx-containing wastes could be a viable amendment for contaminated land remediation. Using two 9 month outdoor lysimeter trials, soils with metal, PAH and mixed contamination were amended with a 10 % by weight MnOx in the form of MnOx-coated sands. Soil microbial function was assessed through measurement of BR, PNR and DEA. This was the first study to assess the use of natural MnOx-containing wastes for contaminated land remediation. It was found that:

- Amending PAH and mixed contaminated soils with 10 % by weight MnOx-coated sands did not show any positive effects as a remediation strategy, evidenced by no reductions in extractable/bioaccessible Pb, As and total PAHs.

- MnOx-coated sands showed evidence of Pb sorption in metal contaminated soils. These wastes may therefore be a possible contaminated land amendment that could result in *in situ* Pb immobilisation in Pb contaminated sites.
- Analysis of soil microbial function indicators (BR, DEA and PNR) showed that MnOx amendment had no detrimental effects upon the function of microbial populations in the aforementioned soils. Therefore, it is concluded that MnOx at a 10 % addition level does not have a high microbiological toxicity.

This study was limited by the analysis of only 9 month extractable and bioaccessible Pb /As and the methods selected to analyse contaminant concentrations. Furthermore, lysimeter trials would have benefited from a longer experimental time period, other forms and higher addition levels of MnOx-containing waste amendments being investigated. Mixed soils were artificially created, and the effects of MnOx amendment upon a historically industrially mixed polluted soil would have been beneficial. Furthermore, only Pb and As were assessed which does not allow for a comprehensive assessment of the immobilisation potential of MnOx for metal contaminated soils.

6.1.4. *Chapter 5: Isolation and phylogenetic analysis of cultivable Mn(II)-oxidising bacteria associated with contaminated soils and Mn oxide-containing wastes.*

The aim of this chapter was to enrich, isolate and identify Mn(II)-oxidising bacteria (MOB) from MnOx-containing coated sands and mine tailings, along with the metal and PAH contaminated soils used throughout this research. This study was conducted to determine the potential for the regeneration and precipitation of biogenic MnOx and hence the possibility for sustained biologically enhanced remediation and the finer scale distribution of MnOx. It was found that:

- MOB are present in MnOx-containing wastes and contaminated soils.
- MOB isolated were from five different genera: A*mycolatopsis* (MnOx tailings), *Saccharothrix* (MnOx tailings), *Micromonospora* (MnOx-coated sands), *Lentzea* (PAH contaminated soil) and the *Streptomyces* (metal contaminated soil).
- Mn(II)-oxidising *Streptomyces* were closely related to a species which produces manganese peroxide thus this species may oxidise Mn(II) via peroxidises.

- Isolates from the *Amycolatopsis, Saccharothrix, Micromonospora,* and *Lentzea* described in this study presented the first definitive evidence of Mn(II) oxidation in these genera.

While culture dependent approaches are inherently biased this remains the only option for the identification of MOB until the gene for Mn(II) oxidation has been successfully classified. Further work is required to determine the abundance of isolated Mn(II)-oxidisers in contaminated soils and MnOx-containing wastes, along with the capacity their biogenic MnOx precipitates have for metal sorption and PAH transformation.

6.1.5. *General conclusions*

The main aim of this study was to assess the effects that natural MnOx-containing waste amendments had upon soil microbial function. Investigations carried out within this thesis have shown that addition of MnOx-containing wastes to metal, PAH and mixed contaminated soils appear to have no direct negative effects on contaminated soil function. Indeed, MnOx-containing wastes could be beneficial for soil microbial function via an indirect stimulation of N-cycling. Furthermore, this study has shown that the proposed indicators of PNR, BR, and DEA are effective indicators of soil microbial function both in contaminated soils and also when determining the effects of a remediation strategy upon soil microbial function.

6.2. Recommendations

The aforementioned conclusions and other work conducted in this study have highlighted a number of possibilities for further work which are outlined below.

- The assessment of soil microbial function in contaminated soils would benefit from methods offering greater resolution and taxonomic discrimination such as 16S bar coded or whole metagenome pyrosequencing. Such approaches could provide better assessments of microbial community structures, ecological diversity indices and function. Thus, providing a more holistic assessment of the effects of contamination and remediation strategies upon soil microbial communities.

- The proposed soil microbial functional indicators of BR, PNR and DEA should be tested on other types of contaminated soils (e.g. BTEX, phenols) and their concurrent remediation strategies to further demonstrate their applicability and suitability for routine microbial soil function monitoring.

- Quantitative and transcriptional activity analysis of AOA *amoA*, AOB *amoA*, *nirS*, and *nirK* functional genes would be of great relevance especially to relate DEA and PNR process measurements to microbial community structure and diversity in contaminated soils. This approach coupled to investigations of their diversity, structure and abundance in a wider range of contaminated and non-contaminated soils would provide a better understanding of the link between diversity and function in polluted environments.

- Mn(II) oxidation rates, and the presence of heme peroxidises or multi copper oxidases (MCOs) should be determined for Mn(II)-oxidising isolates from this study. These MOB isolates may provide a suitable model strain for Mn(II) oxidation within the *Actinobacteria* and could also be the first model MOB described for soil or MnOx rich environments.

- In depth examination of stimulation of N-cycling with MnOx addition to a wider range of soils. Measurements of denitrification and nitrification in this study are potentials measured under optimal conditions and may not be representative of actual *in situ* rates. Therefore, a full examination of the complete denitrification and nitrification pathway and actual *in situ* process rates is required. N_2O emissions after MnOx addition are necessary to assess incomplete denitrification and enhanced emission of this greenhouse gas.

- The determination of whether and how metals, PAHs and SOM compete for the reactive MnOx surface needs to be uncovered. Competition experiments are required to understand the reaction sequence that MnOx surfaces have for processes such as oxidation, humification, metal sorption, metal oxidation, PAH oxidation/ humification in the soil environment.

- While the mineralogy of the MnOx-containing waste tailings utilised in this study have been well described, further research needs to be conducted to fully characterise the mineralogy of the MnOx-coated sands. X-ray photoelectron spectroscopy (XPS) could be used to identify combinations of Mn(II), Mn(III) and Mn(IV) on the surfaces of the filtration media sands, which provides a feasible means of accurately determining the oxidation state of MnOx in complex heterogeneous environmental samples. The composition and structure of MnOx in natural samples is hard to characterise due to complex mixtures of small, disordered minerals that are generally considered to be X-ray amorphous. Thus, specialised Synchrotron (SR)-based X-ray diffraction (XRD) and spectroscopy methods, i.e. SR-based XRD, Mn X-ray near-edge structure (XANES) and Mn extended X-ray absorption fine-structure spectroscopy (EXAFS) should be employed to accurately characterise the structure and Mn oxide phases present in MnOx-coated sands.

- Additionally, the point of zero charge (PZC) of MnOx-coated sands needs to be determined to conclude whether desorption of MnOx-associated Pb from bioaccessibility studies is due to this factor. Future work should also include BARGE bioaccessibility testing of various metals sorbed to MnOx-coated sands in batch chemical studies and from remediation trials. This would determine how strong metal sorption is to natural MnOx which would in turn allow for a conclusion to be made upon how effective they would be as amendments for *in situ* metal immobilisation in contaminated land remediation.

- Transmission electron microscopy (TEM), selected area electron diffraction (ED) and neutron diffraction (ND) techniques should be used to further characterise biogenic MnOx produced by Mn(II)-oxidising isolates from this study.

- X-ray absorption spectroscopy (XAS) should be performed to confirm bonding and therefore sorption between surface MnOx on MnOx-coated sands and Pb as shown in EPMA micrographs.

- If lysimeter trials were to be repeated Pb and As immobilisation should be concluded from reductions in available Pb and As in leachate over time and not from aqua regia extracts at nine months only.

- Enrichment cultures of Mn(IV) reducers should be performed for contaminated soils and MnOx-containing wastes to confirm that a microbial Mn cycle exists. In addition, the release of Mn(II) in MnOx-amended soils should be monitored to ensure abiotic reduction of MnOx is occurring in conjunction with metal sorption. Proving that both biotic and abiotic Mn(IV) reduction occur in MnOx-amended soils where Mn(II) oxidisers exist will provide further evidence to support the theory of continued MnOx re-oxidation and precipitation over time within a MnOx-amended contaminated soil profile.

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