Analysis of Microbial Diversity in Polluted and Non-Polluted Soils: A Comparison of Genetic, Functional and Culture Based Techniques.

By

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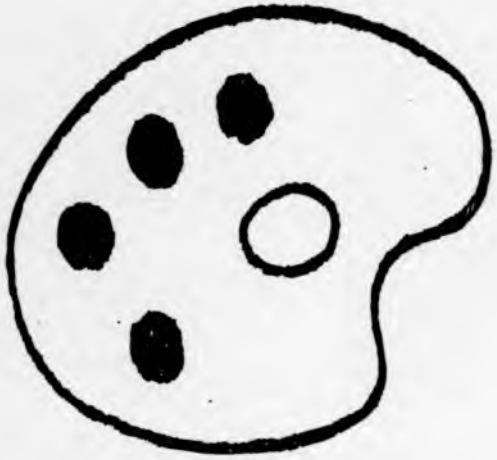
NUMEROUS ORIGINALS IN COLOUR



CONTENTS.

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SUMMARY.

The examination of the microbial communities from different soils was conducted by means of a polyphasic approach utilising traditional culturing methods, taxonomic analysis (DGGE) and potential metabolic activity assays (Biolog). To examine how representative the Biolog assay was of the total bacterial community DGGE analysis of selected wells from Biolog Gram positive and Gram negative plates which were incubated over 96 hours. The results indicated a considerable enrichment effect of the microbial community in comparison with the original soil community. Taxonomic analysis of Biolog wells identified *Pseudomonas* species as the dominant microorganism present. Despite this enrichment effect Biolog analysis could still be used to provide a reproducible measure of potential metabolic activity in the soil and to analyse temporal changes.

A wet sieving technique was used to examine the location of specific bacterial groups in Warwick soil. Wet sieving allowed reproducible separation of the soil into six different sized water-stable aggregates. The community analysis of these different sized soil fractions indicated that actinomycetes were located in higher numbers in the larger soil particles. Whereas pseudomonads were found in higher numbers in the smaller soil particles. Each aggregate fraction had a distinct and different microbial community. Correlations were found between the taxonomic diversity, culturable plate counts and metabolic potential.

The polyphasic approach was used to compare Warwick soil with polluted and non-polluted Doncaster soils obtained from a coal spoil site. Soil fractionation analysis established that different microbial communities were present in the polluted and non-polluted soil fractions . The polluted soil had reduced genetic diversity, metabolic activity, fewer culturable propagules, and a very different soil particle distribution pattern when compared with the two non-polluted soils. Microcosms containing differing proportions of Warwick and Doncaster polluted soil were prepared to study the impact of pollutants on soil microbial communities. Microcosms containing high proportions of polluted soil resulted in the reduction of microbial potential activity, taxonomic diversity and culturable numbers. In the microcosm systems containing low proportions of polluted soil, potential metabolic activity was stimulated; this was also reflected in changes in the genetic diversity.

Community analysis techniques were used to monitor a bioremediation field trial with industrial collaborators BG plc. (formerly British Gas). This field trial involved five soil treatment pits containing a complex mixture of polyaromatic hydrocarbons compounds, subjected to varying conditions designed to stimulate biodegradation rates. Analysis identified dominant bacterial groups present in the bioremediation treatment pits and provided a rigorous evaluation of the microbial diversity present. A combination of microbial analysis with physical and chemical data (provided by BG plc.) allowed the identification of treatments providing the highest rates of bioremediation. Polyaromatic hydrocarbon degradation in the different treatment pits did correlate with the highest potential metabolic activity, genetic diversity and culturable numbers.

ABBREVIATIONS.

A	Adenine
ANOVA	Analysis of variance statistical test
ATP	Adenosine 5-triphosphate
bp	Base pair
Biolog GN	Biolog Gram negative plates
Biolog GP	Biolog Gram positive plates
CFU	Colony forming unit
°C	Degrees centigrade
d	Day
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ddH_20	Double distilled water
EDTA	Ethylenediaminetetraacetic acid
g	Gram
G + C	Guanine and Cytosine
h	Hour
µ	Micro
µg	Micrograms
µg / g	Micrograms per gram
µg / ml	Micrograms per millilitre
mg	Milligrams
mg / g	Milligrams per gram
mg / ml	Milligrams per millilitre
min	Minute
ml	Millilitre
mM	Millimolar
M	Mole
NA	Nutrient agar
NB	Nutrient broth

ng	Nanograms
ng / ml	Nanograms per millilitre
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RASS	Reduced arginine salts solution
RNA	Ribonucleic acid
Rnase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
s	second
sp.	Species
TAE	Tris-acetate EDTA
TBE	Tris borate EDTA
TE	Tris-EDTA
TRIS	Tris (hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
V	Volts
v / v	Volume for volume
w / v	Weight for volume
x g	Centrifugal force

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself and all sources of information have been specifically acknowledged by reference.

Joy E. M. Watts

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PUBLICATIONS AND PRESENTATIONS.

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Presentations.

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For my Mum and Dad and in
memory of W. J. Clarke.

Chapter 1

General Introduction.

1. Introduction.

1.1. The Soil Environment.

Soil is a complex, constantly changing environment which, due to its high solid / liquid ratio, contains many diverse microhabitats for microorganisms (Paul and Clark, 1989). Soil is responsible for essential nutrient cycling and decomposition reactions that maintain the equilibrium of the ecosystem (Prosser, 1997). Good soil structure and fertility are requirements for high rates of crop production and resistance to soil erosion. Soil structure and fertility is of increasing importance due to food shortages around the world and increased levels of desertification (Chiras, 1994).

Soil formation is the result of a number of interactive forces including microbial activity, climate, topography and parent material (Paul and Clark, 1989). Soil consists of a number of different sized particles; >2 mm gravel, 2000-200 μm coarse sand, 200-20 μm fine sand, 20-2 μm silt and <2 μm clay (Richards, 1994). The relative proportions of each size component, combined with interactive factors, determine the texture and physical properties of the soil. The interactive factors produce many different types of soil, including podzols, brown earths, lateritic and chernozems (Gray and Willams, 1971). Each type of soil has distinctive characteristics and profiles, however, many soils exist as intermediate forms of the major groups (Paul and Clark, 1989).

Gradients will exist in the soil from the surface to the lower regions. These gradients will include abiotic factors, such as nutrients, oxygen, light and water (Paul and Clark, 1989). Often the highest levels of productivity and decomposition will be present in the upper horizons of the soil (Richards, 1994). Seasonal variations in abiotic factors can occur in the soil, for example temperature, although these changes are often only detected in soils which undergo extreme climatic changes, as the soil is well buffered against small changes (Gray and Willams, 1971).

Clay type and content has a considerable influence on the properties of the soil. Clay particles have a larger surface area than an equivalent weight of sand or silt (Nedwell and Gray, 1987). The clay type and content determines the water holding capacity of the soil and water availability. Clay particles carry a net negative charge and, hence, can interact with water and allow exchangeable ions to be replaced by H^+ (Richards, 1994) and this is defined as the soil's cation exchange capacity (C. E. C). As the clay particles have a net negative charge, a typical soil microorganism, at neutral pH will become attracted to clay colloids via electrostatic attraction and hydrogen bonding (Nedwell and Gray, 1987). Clay particles are not free in the soil, due to their charge and size they exist as coatings on larger particles, or through interactions with other clay particles that bridge domains between the larger particles in the soil (Stotzky, 1997).

The microbial community present in soil is in part determined by the nature of the nutrients available (Paul and Clark, 1989). Most substrates are found in the upper most layers of the soil or plant roots, which penetrate the soil (Liesack *et al.*, 1997). These nutrients, with the exception of root exudates, are enclosed in insoluble matrices which must be broken down before they can be utilised. Substrates will be unevenly distributed in the soil as a result of water leaching and transport by macroorganisms (Stotzky, 1997).

To fully appreciate the soil environment, the microscale is of uppermost importance (Hattori and Hattori, 1993). In the microenvironment, the spatial arrangement of soil particles into aggregates affects the overall integrity and subsequent fertility of the soil (Jastrow *et al.*, 1988).

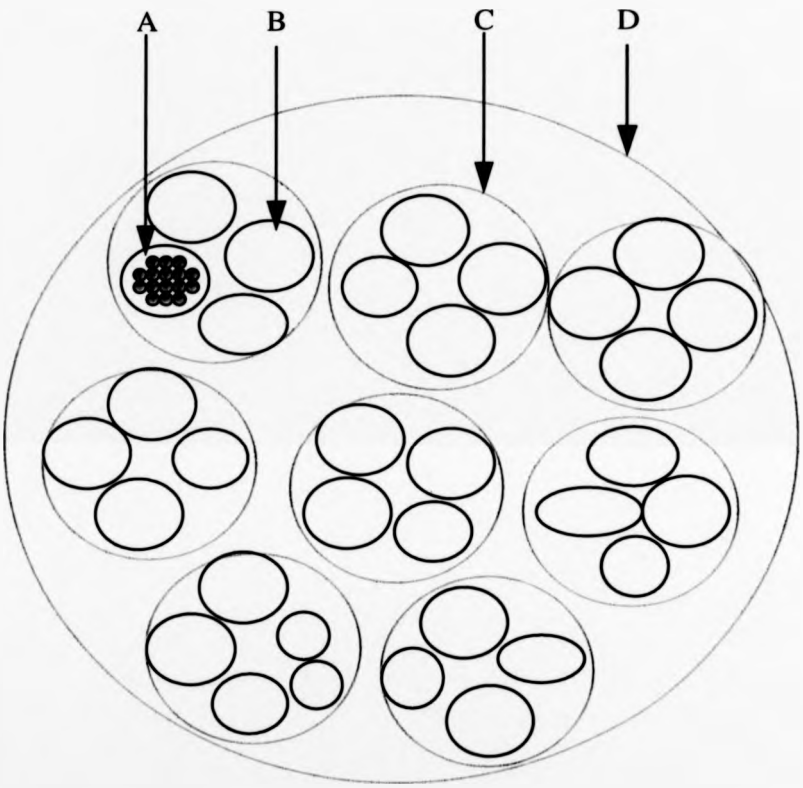
1.1.1. Soil Aggregates.

Aggregates have been defined as groups of soil particles that cohere more strongly to each other than to other adjoining particles (Kemper and Rosenau, 1986). Soil structure is essential in the functioning of the soil ecosystem (Jastrow *et al.*, 1988) and is one of the basic elements affecting soil fertility (Dabek-Szreniaeska, 1977). This is a result of aggregates affecting a range of soil properties which control crop establishment including water infiltration, resistance to erosion, aeration, drainage and creation of microhabitats for soil biota (Oades, 1993).

Microaggregates are more stable than macroaggregates (Oades and Waters, 1991) with their size and stability being a reflection of environmental conditions (Beare and Bruce, 1993). The effect of tillage on aggregate stability was examined by Kandeler and Murer (1993). They showed that continuous vegetation combined with the activities of soil microorganisms are preconditions of good aggregate stability. The relationship between environment and aggregate stability is due to aggregate formation being initiated by microorganisms and root exudates which combine soil particles and clays together (Hattori and Hattori, 1993).

Soil structure is one of the factors which provides microorganisms with protection from predation. Postma *et al.* (1990) suggested that small free-living amoebae were the main predators controlling bacterial populations in soils. A soil system contains numerous biopores formed by the action of macro and mesobiota (such as earthworms) and these biopores allow the movement of nutrients, gases, water and predators throughout the soil structure (Richards, 1994). Clays and other soil particles provide microorganisms with protection from predation due to a number of physical properties including charge, pore size inhibition and the increasing the distance between the microorganisms and the predators (Gestel *et al.*, 1996).

Figure 1.1. Concept of aggregate hierarchy (adapted from Oades, 1993).



A = Clay particles, B = Microaggregates, C = Macroaggregates, D = Soil crumb.

This diagram represents the hierarchial structure of aggregates. The clay particle complexes are bound by permanent bonds and are stable. These particles are bound together into microaggregates which are relatively stable, whilst macroaggregate and crumb structures, which are bound together by transient bonds, are relatively unstable. The soil structure is dynamic and the stable particle structures will be constantly reforming into other aggregate clusters.

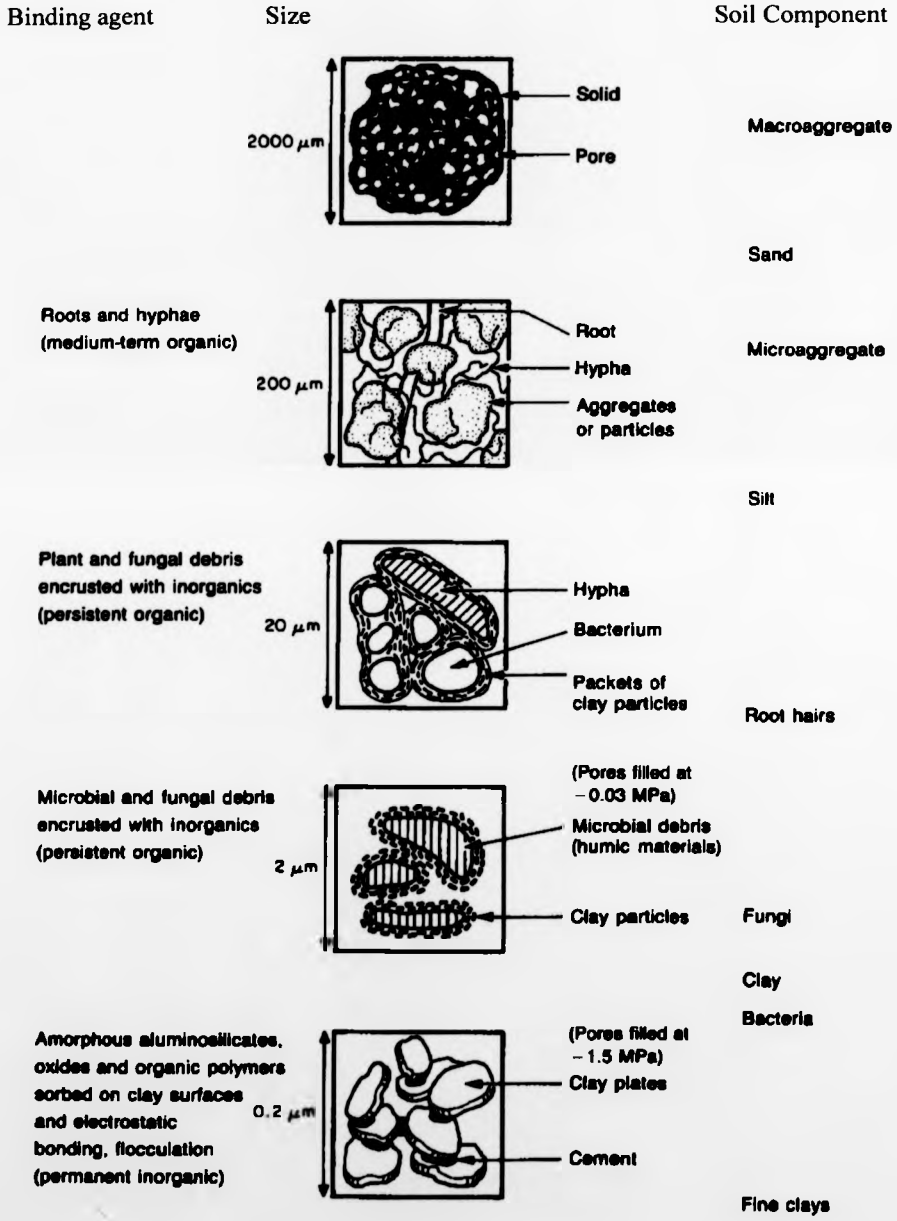
1.1.2. Aggregate Hierarchy.

Aggregate hierarchy was proposed by Tisdall and Oades (1982), who suggested that smaller soil aggregates have a greater tensile strength. Particles are bound together to form microoaggregates (Fig. 1.1). These particles are then combined by charge attractions and ionic bonds. The microaggregates are the building blocks of macroaggregates and are combined by filamentous microorganisms and roots (Fig. 1.2) which act as a 'sticky string bag' (Oades and Waters, 1991). This provides a mechanical framework for the initial formation and stabilisation of the macroaggregate (Oades and Waters, 1991).

Once initial stabilisation takes place, biological activity occurs within the structure. This involves substantial amounts of polysaccharides and other organic compounds being deposited, these serve to stabilise the macroaggregates (Tisdall and Oades, 1982). Microorganisms secrete insoluble viscous material of high molecular weight into the soil which is important in the adhesion of cells to clay / soil particles and stabilisation (Fig. 1.2). The macroaggregates combine together to form soil crumbs. These soil crumbs are formed when macroaggregates become bound together by root networks, and wetting and drying cycles (Tisdall and Oades, 1982). Crumbs can combine together to form peds. Crumb and ped structures are formed by physical forces such as drying, freeze-thaw, root growth, compaction and animal movement (Paul and Clark, 1989).

Stability of the soil structure is dependent on the type of bonding holding the aggregates together (Fig. 1.2). In microaggregates (<20 μm in diameter), a mixture of clay microstuctures, biopolymers and microorganisms are responsible for binding the particles together (Tisdall and Oades, 1982). These bonds are often strong electrostatic attractions or adhesive biopolymers. These interactions are often classed as permanent inorganic and persistent organic bonds and are relatively stable in the soil (Fig. 1.2).

Figure 1.2. Aggregate organisation showing the relative size of the components and the major binding elements (reproduced from Tisdall and Oades, 1982).



Microaggregates (20-250 μm diameter) are often based upon organic material which has been degraded leaving recalcitrant matter such as lignin (Oades, 1993). These aggregate structures have been stabilised by microbial biopolymers and other physical interactions to allow the aggregate to remain intact after the organic matter has been degraded (Oades, 1993). While macroaggregates (>250 μm) often have large cores of organic matter, these structures break into constituent microaggregates when the organic matter is degraded. These interactions are often referred to as temporary or transient bonds and are unstable in the soil environment (Tisdall and Oades, 1982).

Soil aggregates exist in dynamic equilibrium, with various forces combining together to create and destroy aggregate structures (Young and Ritz, 1998). Factors affecting the degradation of aggregates include rainfall, trapped air and the microbial decomposition of organic materials, such as polysaccharides and humics serving to combine particles together (Tisdall and Oades, 1982). The plasticity of the soil is closely dependent on the water and clay content (Paul and Clark, 1989).

1.2. Soil Microflora.

Microbial communities exist in a highly complex, physical framework of variable composition over many spatial scales (Stotzky, 1997). The soil provides substrates, water, oxygen and protection from predation for the microorganism (Paul and Clark, 1989). However, the structure and stability of the soil is itself affected by its biotic component (Young and Ritz, 1998). These interactions allow the formation of a soil with good aggregate structure and high fertility, capable of nutrient cycling and high decomposition rates.

The soil has a large biotic component, which has a major role in the formation and function of the soil ecosystem. The biotic component can be split into three major groups based on size: microbiota, mesobiota and macrobiota (Richards, 1994). The macrobiota consist of the larger soil organisms such as earthworms, molluscs, large enchytraeids and burrowing rodents. Mesobiota comprise of the smaller nematodes, springtails, arthropods and enchytraeids, whilst the microbiota consists of the protozoa, algae, fungi, bacteria and virus groups, each plays an essential role in the soil environment (Richards, 1994). The microbiota interact via various biotic relationships such as predation and competition; these biotic relationships exist between and within groups. The microorganisms in soil will comprise numerous groups, with communities of different composition observed, depending on the soil conditions and the detection method used. There are a number of predominant bacterial groups in soil including bacilli, actinomycetes and pseudomonads. These groups have been reported in numbers ranging from 5-20% of the total numbers, in different soils (Gray and Williams, 1971).

The soil microbial community is affected by a range of biotic and abiotic interactions. Due to the discreteness of the soil habitat, the biotic interactions, for example competition, may not be as strong as in the aquatic environment (Stotzky, 1997). As nutrients are heterogeneously distributed temporally and spatially in the soil, microorganisms will grow only when suitable nutrients are present (Willams, 1985). When nutrients are depleted, the microorganisms will either die or employ a dormancy mechanism, such as spore formation. The microorganisms will remain in the dormant state until nutrients return to that location in the soil, or until the microorganisms are transported to new areas containing nutrients, by indirect forces such as transport via macroorganisms and rainfall (Paul and Clark, 1989).

It is a general belief that microorganisms in soil can be divided into two groups; those that are oligotrophic (*K*-strategists) or copiotrophic (*r*-strategists). The oligotrophic organisms can be characterised by slow growth rates in low numbers. This allows greater diversity than in nutrient-rich copiotrophic environments with high competition levels. Copiotrophic microorganisms are often opportunistic

bacteria that can grow at rapid rates in the presence of nutrients, but will rapidly decline in numbers as a result of cell death or dormancy when the nutrient source is depleted (Stoztky, 1997).

Soil is often referred to as an unfavourable environment for microorganisms, due to its low nutrient status and its variation of abiotic factors over the microscale (Gray and Willams, 1971). The soil exerts a strong selective pressure for bacteria that can cope with this environment (oligotrophic microorganisms) and many are highly adapted to the ever changing conditions in the soil. Although soil is often labelled as a harsh environment, this may be a misconception as the soil contains more species of microorganisms (as yet cultured) than any other habitat (Stoztky, 1997).

1.3. Microbial Diversity.

Microbial diversity can be considered at three different levels (Atlas, 1984; O'Donnell *et al.*, 1994): genetic diversity (within species), species diversity (number of species) and ecological diversity (community diversity). Microbial community diversity describes the heterogeneity of the species present in an environment. To descibe diversity there are two main components; species number, which is the total number of species present and species richness, which describes the evenness or equitability of the distribution of microorganisms (Atlas, 1984). The relationship between diversity and ecosystem function and the effect of stress on diversity have not yet been fully elucidated in microbial ecology (O'Donnell *et al.*, 1994).

In macroecology, many paradigms exist for the relationship between diversity and the environment. When applying these models to microbial ecology care must be taken, due to the differences in size of the organisms and the relationships within the environment. Models in macroecology include the observation that plant diversity has been shown to increase when the community survives at intermediary levels of stress and disturbance (Wardle and Giller, 1996). It is often stated in macroecology, that the ability of an ecosystem to withstand anthropogenic disturbance may depend

partly upon the diversity or robustness of the community present (Fenchel, 1992; Lovejoy, 1994). However, few studies have been performed to elucidate the relationship between environmental conditions and diversity in microorganisms. It has been shown that there is a beneficial relationship between microbial diversity, soil function, plant quality and ecosystem sustainability (Kennedy and Smith, 1995). If an environment supports a high level of diversity, associated with this diversity will be levels of functional redundancy. This functional redundancy arises when a number of species conduct similar functions in the environment. If any groups of species are removed or decreased due to stress, then the overall soil processes will not be affected, since another species may fill this niche (Bianchi and Bianchi, 1995). This link between diversity and functional redundancy may be an essential factor in the resilience of soils to stress (Degens, 1998b).

The addition of toxic compounds to a microbial system have important affects on microbial diversity. It has been proposed that if a selectively toxic compound was added to the environment, it may increase diversity if it served to inhibit a dominant organism (Atlas, 1984). However, most pollutants are usually toxic to a wide range of microbes, and when added to a system, overall taxonomic and genetic diversity decreases (Atlas, 1984). Little is known about how microbial genetic diversity affects taxonomic diversity and how these both affect functional activity in the environment (Zak *et al.*, 1994). It has been hypothesised that biodiversity has genetic foundations as diversity arises due to different evolutionary pressures. The environment affects gene expression and ecological interactions between bacteria (Colwell, 1992).

If increasing rates of extinction in macroecology can be correlated with loss of species diversity in microbial ecology, then unknown biological resources may be lost (Lovejoy, 1994). This perceived reduction in diversity is due to a vast array of microorganisms in the environment have as yet, not been cultured and screened for novel metabolites and enzymes (Watts *et al.*, 1998). If diversity is reduced in the ecosystem then new potential products may be lost (Colwell, 1997). Bacterial secondary metabolites are in commercial use as antibiotics, antifungal, antiprotozoan

and antitumour agents. As a result of widespread antibiotic resistance new and novel antibiotics are required (Colwell, 1997). Bacterial enzymes are also used in many industrial products such as detergents, new enzymes with the ability to withstand temperature extremes are being isolated and optimised (Trombly, 1995).

To examine diversity and make comparisons between different habitats, diversity indices can be utilised. A number of diversity indices exist, for example Species Richness, Mehnhiniks' Index, Margalef's Index, Simpson's Index and the Shannon-Weiner Function (Shannon index). The Simpson and Shannon index are probably the most commonly used in microbial diversity estimation (Watve and Gangal, 1996). The diversity index measures two elements of diversity, species richness and species equitability. The indices are based on the premise that if the probability of two individuals being the same is low then the diversity is high. When using diversity indices it is important to define taxonomic groups, this is often problematic with microorganisms and these methods tend to underestimate diversity (O'Donnell *et al.*, 1994; Watve and Gangal, 1996). Other theoretical problems arise when estimating microbial diversity, this includes, species contribution to diversity, for instance, are there key-stone species in microbial communities, as in macroecology (Harper and Hawksworth, 1994).

It is important to assess diversity in the soil ecosystem as the soil is an essential and dynamic site for biogeochemical cycling and decomposition reactions (Richards, 1994). As soil systems are receiving increasing levels of pollutants and meeting higher demands in agriculture, it is important to assess if there is a link between microbial diversity and soil function (Chiras, 1994). Soil is a highly complex system where microbes can encounter a diverse mixture of solid, liquid and gaseous phases. These soil structures are temporally and spatially heterogeneous. This results in many discrete microhabitats with varying abiotic conditions which favour increasing levels of microbial community diversity (van Elsas *et al.*, 1998). Inherent in each soil system is a unique microbial flora which will alter with different environmental conditions. The changes in the microbial community are also affected by any stress

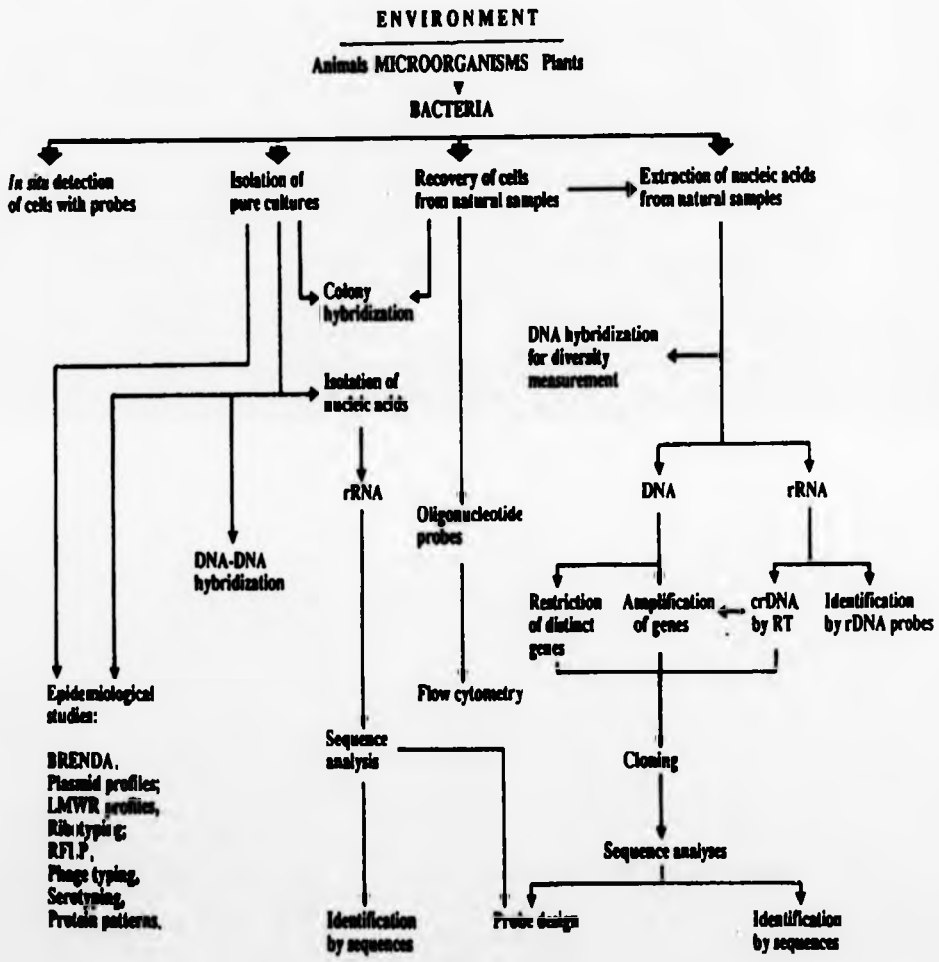
that is added to the environment (Bååth, 1998). The measure of diversity provides insight into the functional status of the community (Atlas, 1984).

1.4. Microbial Community Analysis Techniques.

When examining bacterial communities there are a number of questions that need to be addressed including which groups are present, their location, their function in the community and how the communities develop over time. A wide range of techniques have been developed to examine the microbial communities present. Fig. 1.3 shows some of the methods frequently used in microbial ecology. Two main approaches exist for the analysis of microbial communities, firstly the detailed examination of isolates obtained from an environment from enrichment and other culture based techniques. Secondly, the analysis of the total community by techniques such as nucleic acid extraction followed by further analysis such as probing or sequencing.

These two approaches often produce different estimations of diversity for a sample, but by combining the approaches a more rigorous assessment of microbial communities can be made. A combination of different techniques (polyphasic approach) can be taken to help overcome the limitations of using a single method (Stotzky, 1997).

Figure 1.3. Commonly used methods in microbial ecology, (reproduced from Stackebrandt, 1992)



1.4.1. Culturing and Isolation of Microorganisms From the Environment.

Traditional methods of community analysis involve culturing microorganisms on a range of media followed by characterisation of the isolates, allowing the detection of changes in culturable bacterial communities (Akkermans *et al.*, 1994). Many reports of the non-culturability of many bacterial species now exist (Wayne *et al.*, 1987; Ward *et al.*, 1995) and bacteria which have yet to be cultured have been detected by molecular methods (Rhiems *et al.*, 1996) and microscopy (Hartmann *et al.*, 1997). Torsvik *et al.* (1990b) detected very high levels of diversity in a soil system that has not yet been reflected by culturing methods. It has been estimated that less than 1% of bacteria from the environment can be cultured in the laboratory (Dykhuizen, 1998).

Media are often rich in nutrients, selecting for fast-growing copiotrophs rather than the slower growing oligotrophs which are dominant in soil samples (Liesack *et al.*, 1997). Plates are usually incubated for 2-3 days, but this does not normally provide adequate time for the slow-growing oligotrophs to adjust to the new conditions and if suitable, produce colonies.

DeLiej *et al.* (1993) described a culture-based method for the examination of microbial communities. This culture technique can be used to quantify the relative numbers of copiotrophic and oligotrophic organisms in an environment. This is similar to the ecocollections proposed by Hattori *et al.* (1997) which are collections of organisms from the same sample over time. Culture methods still play an essential role in the study of microbial diversity. By culturing a microorganism it is possible to elucidate its metabolic activities and maybe its role in the environment (Palleroni, 1997).

1.4.2. Analysing Microbial Communities Using Genetic Techniques.

Bacterial diversity can be evaluated by molecular techniques using the nucleic acid component of an environmental sample. This molecular analysis is based on the detection of signature molecules, such as DNA and RNA (Morgan and Winstanley, 1997). Specific DNA sequence detection allows the identification of an organism present in the environment. Detection of a specific sequence can be achieved in a number of ways, including PCR screening, probing and sequencing (Amann *et al.*, 1995). Techniques to detect mRNA are used to allow a measurement of activity, as only active cells will be transcribing mRNA (Felske and Akermanns, 1998). In microbial community analysis, rRNA genes have been routinely used particularly the 16S rRNA (Amann *et al.*, 1995).

The rRNA genes are ideal signature molecules as they contain a number of domains where sequence variation changes at different rates, leading to variation increasing with phylogenetic distance (Liesack, 1997). These molecules have conserved and variable domains. The conserved areas allow the determination of relationships between kingdoms, such as Archaea and Eubacteria, whilst the variable regions allow closely related species to be resolved (Woese, 1987).

16S rRNA analysis can be used for the prediction of function by close phylogenetic relationship to other species. This prediction of phenotype was achieved by Lonergan *et al.*, (1996), when *Pelobacter* was predicted to be capable of growth on Fe(III) due to its close relatedness to *Geobacter* and *Desulforomonas*. However, care must be taken when drawing inferences of phenotype from signature molecules, although it may sometimes allow the approximation of culture conditions for as yet uncultured organisms.

Many studies of bacterial diversity have focused on the detection and sequencing of bacterial isolates from the environment. This yields considerable genotypic information about the species present. This is a time consuming method and unless the species has already been cultured it gives no functional or activity information.

Fingerprinting techniques can be employed to rapidly assess temporal or spatial changes in a community. Fingerprinting techniques do not normally allow the identification of species or the determination of activity (unless using mRNA), but allow rapid screening and comparison of communities in different samples over time (Colwell, 1997).

1.4.2.1. Extraction of Environmental Nucleic Acids.

The first step in any genetic analysis of a community is the extraction of nucleic acids from an environment. For an accurate assessment of microbial diversity, the DNA extraction procedure is of paramount importance (Steffan and Atlas, 1991). The process of nucleic acid extraction has been extensively reviewed (Bakken, 1985; Ogram *et al.*, 1987; Holben *et al.*, 1988; Picard *et al.*, 1992; Smalla *et al.*, 1993; Zhou *et al* ., 1996)). No single DNA extraction method can extract all DNA molecules from the soil (van Elsas *et al.*, 1998). Therefore, DNA extraction and purification methods should be chosen appropriately for the environmental sample and its characteristics.

1.4.2.2. PCR Detection.

PCR has revolutionised molecular microbial ecology by the selective amplification of regions of DNA. Analysis by PCR can be used for the detection of bacterial groups using signature molecules or for the detection of functional genes in environmental samples (Watts *et al.*, 1998). Examples of functional gene detection includes detection of *merA* genes (Hart *et al.*, 1998), methanol dehydrogenase genes (McDonald and Murrell, 1997), polychlorinated biphenyl-degrading catabolic genes (Walia *et al.*, 1990) and naphthalene degrading genes (Herrick *et al.*, 1993). Sequencing cloned or direct PCR products, can be achieved easily and the sequence compared to a database for identification (Steffan and Atlas, 1991). This has greatly increased our knowledge of the microbial communities present in the environment.

PCR-based analysis does have limitations. For example, universal primer sets may not detect all of the bacterial species present in an environment. Other considerations include each primer set is affected by levels of G + C in the template DNA, the accessibility of the template to primer after denaturation such as secondary structures and gene copy number. Chimeric DNA molecules can affect the PCR reaction. These are produced in the reaction by partial length fragments of different 16S rRNA gene type annealing to the highly conserved regions. This may be followed by primer independent elongation to full length fragments. PCR can be inhibited by humic acids which are present in the soil environment (Tebbe and Vahjen, 1993), although the addition of bovine serum albumin may prevent this inhibition, (Liesack, *et al.*, 1997).

By using two different primer sets of identical predefined mixtures of DNA, Suzuki and Giovannoni, (1996) determined that PCR bias was positively correlated to the number of cycles. Heuer and Smalla, (1997b) used DGGE (Denaturing gradient gel electrophoresis) to examine PCR biases in a mixed community. This analysis indicated that when the template DNA was diluted, bands were detected that were not present in the higher template DNA concentrations, as a result of preferential amplification.

PCR provides a sensitive and rapid technique to detect species and functional genes in environmental samples if the limitations are recognised and appreciated. Correlation between phenotypic characterisation by molecular data will become more reliable as more species are identified and sequenced. Most molecular studies reveal the presence of an microorganism but not the level of its activity or its role is in the environment. RNA studies allow the combination of presence and activity in the environmental system to be examined (Felske and Akkermans, 1998).

1.4.2.3. Analysis of Communities Using Nucleic Acid Probes.

Phylogenetic or function specific probes can be utilised to assess the microbial community structure and function (Hartmann *et al.*, 1997). Probes can be long PCR products or oligonucleotidein length, labelled by radioactive or non-radioactive methods including digoxenin and fluorescence (Burlage, 1998). Numerous phylogenetic probes exist which bind to specific signature sequences on rRNA molecules. This can provide information on what bacterial groups are present in an environmental sample without a culture based step (Amann *et al.*, 1995). Probes can also detect functional abilities such as the *nah* gene for naphthalene degradation (Herrick *et al.*, 1993). Different types of probing can be used to screen either communities or isolates. For instance colony hybridisations can be used to examine the phylogeny or specific activities, by probing, in colonies grown on the membrane. Dot blots involve the purified DNA being placed onto the membrane and then hybridised with probes. This may allow quantification of the DNA if internal standards are used (Burlage, 1998).

1.4.2.4. Genetic Fingerprinting Techniques.

Molecular fingerprinting methods have recently been reviewed (Muyzer and Smalla, 1998; Muyzer, 1998). These include Low molecular weight (LMW) fingerprinting, Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Single Strand Conformational Polymorphism (SSCP), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Fluorescent Restriction Fragment Length Polymorphism (Flu-RFLP). Each fingerprinting method has its own advantages and limitations (see Table 1.1). Fingerprinting methods allow the rapid comparison of community structure over temporal and spatial studies. For any environmental study, these limitations should be considered and the method most suitable to the study applied.

Table 1.1. Frequently used microbial community genetic fingerprinting techniques.

Technique	Description of method	Advantages and limitations
LMW - fingerprinting, (Höfle 1988).	Separates the small subunit rRNA molecules on PAGE gels to receive a characteristic fingerprint.	No PCR amplification necessary. Small size of subunits yields only limited phylogenetic information. Reproducibility and resolution may be poor.
RAPD, (Novo *et al.*, 1996).	Low stringency PCR performed with short random primers, which produces a bar-code after binding to many sites on the DNA.	Don't need to design primers for specific organisms. Number of bands cannot be related to number of species. Reproducibility may be poor.
SSCP, (Lee *et al.*, 1996).	Separation of PCR products is based upon secondary structure formation in gels containing a denaturant	Number of bands is not comparable to number of species present. Some species can form over 15 bands on the gel (Ritz, pers. comm.).
Bis-benzamide (Muller *et al.*, 1997).	Allows the separation of PCR products of the same length but different base pair composition by preferentially binding to A + T regions, retarding migration in the gel	Resolution and reproducibility has still to be optimised.

Technique	Description of method	Advantages and limitations
RFLP and ARDRA (Bruce *et al.*, 1995; Martinez-Murcia *et al.*, 1995).	Involves the restriction of DNA (ribosomal in the case of ARDRA) and the separation of the fragments on PAGE gels	Number of bands doesn't correlate with number of species. Complex fingerprints may be obtained which are difficult to analyse.
Flu-RFLP or T-RFLP (Liu et al., 1997; Bruce, 1997).	One of the primers for the PCR reaction is labelled with a fluorescent dye. The amplified products are restricted and products are analysed on a DNA sequencing machine which detects the fluorescently linked fragments.	Requires access to a DNA sequencing machine. Patterns produced are a lot less complex and therefore easier to analyse in comparison with traditional RFLP analysis.
DGGE or TGGE (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998).	PCR products of the same length but different base pair composition are separated in a gradient of denaturant or temperature	As well as obtaining fingerprint patterns, bands can be excised and sequenced. The method is based on PCR and may have limitations from any biases of the amplification.

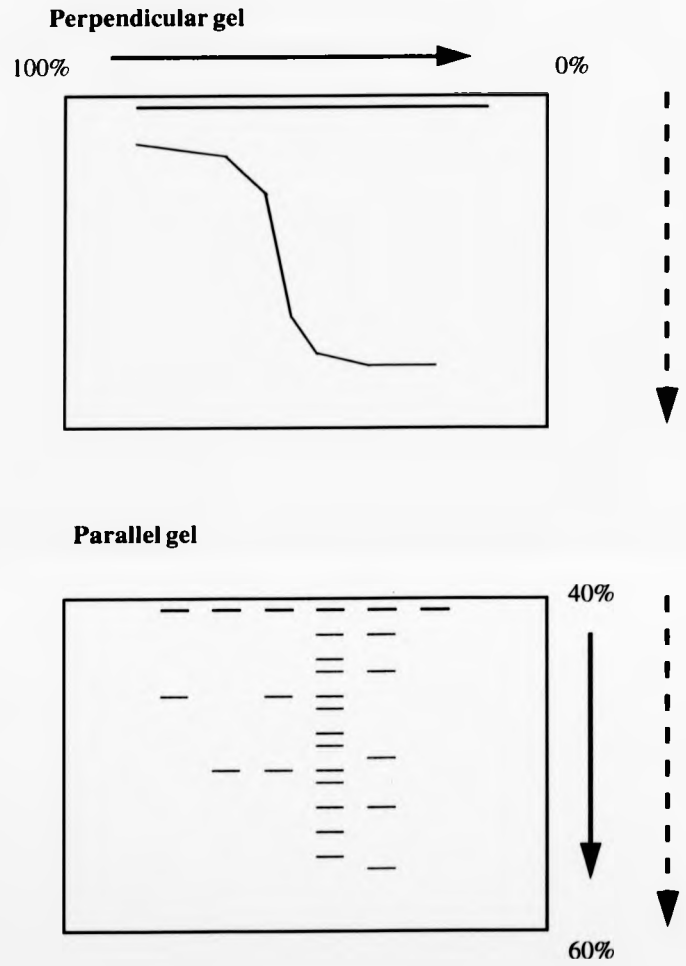
1.4.2.5. Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE).

DGGE and TGGE are genetic fingerprinting techniques that allow the profiling of complex bacterial communities. This is achieved by separating DNA molecules of the same length but with different base pair compositions (see Fig. 1.4). DGGE is based on the principle that an increasing gradient of denaturants will melt double stranded DNA in distinct domains and these melted domains will have reduced mobility in a gel. Whilst DGGE uses a denaturant to separate the dsDNA, TGGE uses temperature. To optimise the separation of PCR products, perpendicular gels are used (Muyzer *et al.*, 1993). Once the optimal gradients have been found, then, parallel gels are used for the routine separation of PCR products (see Fig 1.4 and Section 2.6.4.).

Although most studies have used DGGE, (probably due to the availability of the BioRad system) TGGE was found to produce comparable patterns to DGGE (Heuer *et al.*, 1997). To analyse communities by DGGE, a GC clamp is usually attached to one end of the DNA. This GC clamp consists of 30-40 G / C bases forming a high melting point region that ensures that the region of interest is in a lower melting domain and that the DNA will remain partially double stranded.

One of the major advantages of DGGE as a fingerprinting system is the ability to identify microorganisms present. This can be achieved by excising a band from a community pattern and sequencing it (Ferris *et al.*, 1996). This allows the rapid analysis of community structure and the identification of the species present. Probes can be produced and used in the original environment to identify whether the species identified by DGGE is present in the environment. Most studies have focused on the use of 16S rRNA operon analysis, although it is also possible to examine functional genes using this technique (Wawer *et al.*, 1995; Rosado *et al.*, 1998).

Figure 1.4. The Denaturing Gradient Gel Electrophoresis Technique, Parallel and Perpendicular Gels.



Perpendicular gels are used to determine optimal gradient conditions for PCR product separation. Once conditions have been optimised parallel gels can be used for the routine separation of PCR products.

Many species have been found to produce multiple bands by DGGE, possibly due to sequence heterogeneity of the 16S rRNA genes, multiple operons and the primers used. This is important when the number of bands is being used as an indication of increased microbial diversity. In environmental samples with large number of equally abundant microorganisms, highly complex banding patterns can inhibit analysis as a product smear is obtained (Baker, 1997; Heuer *et al.*, 1997; Heuer and Smalla, 1997b). DGGE bands are sequenced directly, and the need for cloning, which may impose another bias, is eliminated (Heuer and Smalla, 1997b). However, only limited sequence information can be produced by DGGE because PCR products separated in DGGE gels are normally less than 500 bp (Muyzer and Smalla, 1998).

1.4.3. Functional activity measurements.

1.4.3.1. Detection of Functional Activity in Environmental Samples.

The relationship between metabolic diversity and genotypic diversity is not fully understood (Zak *et al.*, 1994). Functional diversity arises from genetic variability, environmental effects on gene expression and ecological interactions between microbial populations. Analysis of the functional activity is based on the premise that metabolic activity reflects the microbial community. Two main approaches can be undertaken to examine functional activity in an environmental sample, specific activity measurements or a total sample approach. Total functional activity methods include Biolog (Garland and Mills, 1991) and *in situ* catabolic potential (Degens and Harris, 1997), biomass (Jenkinson *et al.*, 1975; Howarth and Paul, 1994) and ATP estimation (Martens, 1985). When using a total sample approach, neither individual species nor the activity of the individual organisms present can be detected. Care must be taken in the analysis when comparing different sample types as some methods can be affected by heavy metals and the type of soil present (Chander and Brookes, 1991).

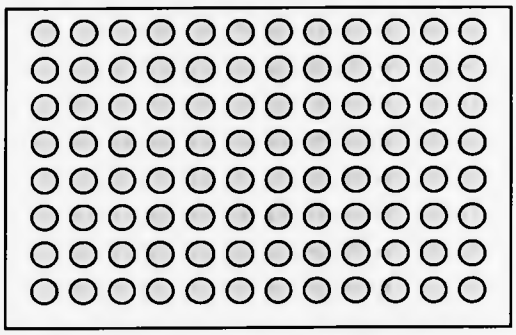
To examine activity at a higher resolution, specific activities can be measured. These specific measurements can include carbon transformations, nitrogen transformations, phosphorus and sulphur transformations (Prosser, 1997). Enzymes, such as oxidoreductases, hydrolases, dehydrogenases and transferases, have been used as indicators of the metabolic activities of soil microflora (Paul and Clark, 1989). Enzymes can remain in the soil combined to clays and this can bias activity measurements. Enzyme assays can often be affected by low levels of heavy metals which may rule out this analysis for contaminated soils (Prosser, 1997). Another approach to examine specific activity is the introduction of a marker into a population which will be detected when activity is occurring. An example of an activity marker is the *lux* bioluminescent reporter, this has been used successfully in many studies. Sayler (1998) reported that in a mesocosm containing a bacterial strain with a *lux*-marked naphthalene degradation pathway, light could be detected using fibre optics when naphthalene degradation was occurring.

1.4.3.2. Biolog analysis.

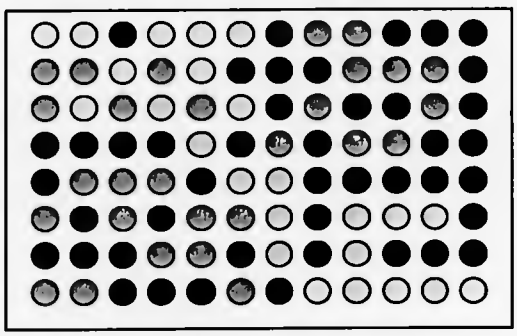
The Biolog system (Biolog Inc., Hayward, Calif.) was originally developed to allow identification of bacterial strains by monitoring sole carbon source utilisation patterns (Miller and Rhode, 1991; Klingler *et al.*, 1992.). The plates consist of 96 wells containing a basal medium, 95 of which contain additional carbon sources and a control well with no additional carbon source (See Fig. 1.5). The wells also contain a redox dye (tetrazolium violet) which changes colour when electrons are donated from NADH to the electron transport chain. This indicates that the carbon source is being oxidised (Bochner, 1989a). This colour change can be monitored by measuring changes in absorbance in the well.

Figure 1.5. Biolog Analysis of Soil Communities.

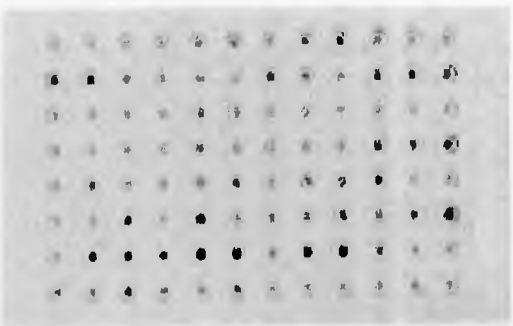
A.



B.



C.



A and B are diagrammatic representations of the Biolog plate before inoculation (A) and after incubation (B). Various colour responses can be identified in the 95 different carbon source wells. The Biolog plate is also shown C.

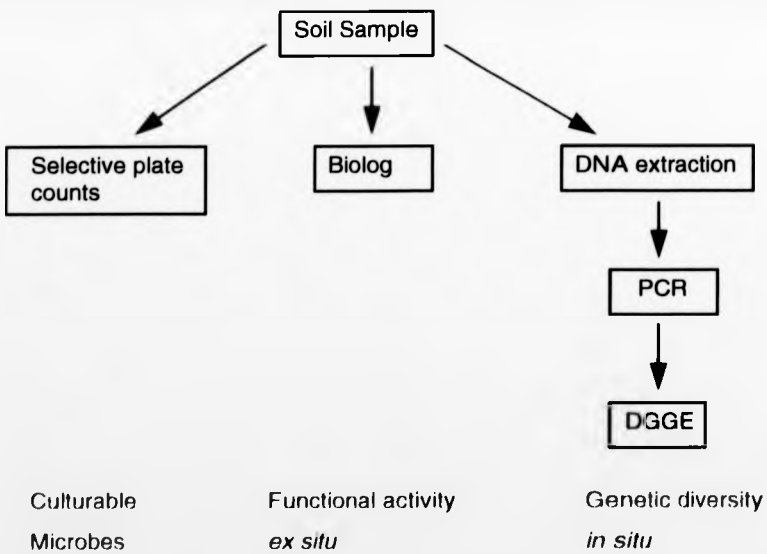
The Biolog system was adapted to perform community analysis by Garland and Mills (1991). The bacterial fraction from an environmental sample can be added directly to the wells and the carbon source utilisation patterns analysed. These patterns of respiration are thought to give a metabolic fingerprint of the sample, representing the functional attributes of the inoculated bacterial community with respect to a range of substrates (Bochner, 1989b). The Biolog plate is essentially 95 separate carbon source enrichments on one plate.

Biolog metabolic fingerprints have allowed the discrimination of different microbial communities in different environment systems including rhizosphere, inoculated rhizosphere and bioreactor monitoring (Garland and Mills, 1994). It has been used to analyse differences in metabolic activity in different size particles of the same soil, (Winding, 1994) and different plant phyllospheres (Heuer and Smalla, 1997a). Biolog fingerprints have been used to characterise shifts in microbial communities in soil when placed under heavy metal stress (Knight *et al.*, 1997), hydrocarbon pollution (Wünsche *et al.*, 1995), and when amended with genetically-modified plant materials (Donegan *et al.*, 1995).

1.5. Aims of the project.

A polyphasic approach will be taken to examine microbial communities in different soil types (Fig 1.6). It is hypothesised that the selective plates will provide information on specific culturable groups and large community shifts in overall numbers, whilst Biolog will allow an examination and comparison of potential metabolic activity in the different soils. Molecular techniques will also be utilised to examine the genetic diversity in the soil samples. The three methods will then be compared and contrasted in their ability to detect diversity. This approach will be applied to soil at the microscale level and allow the comparison of different soils. The effects of pollution on the microbial community will also be examined and the ability of the analysis methods to effectively assess bioremediation in a field trial.

Fig. 1.6 Polyphasic approach used in this study.



1.5.1. Summary of Aims.

* The distribution and diversity of bacteria in soil at the microscale will be estimated by using a combination of genetic, culture-based and metabolic activity techniques.
* A comparison of the diversity detected by the various techniques, to determine if there are differences in the sensitivity of the analysis methods.
* Examine the effects of heavy metal pollution on microbial communities in different sized soil aggregates.
* Ascertain if the community analysis techniques used are able to detect variations in microbial communities temporally and in different soils.
* Use the validated methods to monitor a bioremediation field trial.
* Investigate the ability of Biolog plates to effectively assess community diversity.

Chapter 2

Materials and Methods.

2.1. Soils.

Soils were obtained by taking ten random samples (approximately 100 g) from the top 10 cm of the soil (A horizon) from each site, (for site descriptions see Appendix 9.2). The samples were pooled to overcome field variability (van Elsas and Smalla, 1997). Warwick and Doncaster soils were air-dried, sieved through a 2 mm metal sieve and stored at room temperature for further analysis. Samples from the British Gas site were sieved and stored at 4°C.

2.2. Media and Solutions.

All media (Table 2.1) were made up to 1 litre with distilled water. The pH was measured, corrected, and solutions sterilised by autoclaving at 121°C for 15 min. Antibiotics were added from stock solutions to prepare selective isolation agar plates (Table 2.2). Chemicals were obtained from Sigma, BDH or Fisons, unless otherwise stated.

Table 2.1. Media.

Medium or Solution	Constituent(s) / l
Oatmeal agar	20 g fine oatmeal (boiled 20 min) 5 g yeast extract (Oxoid) 15 g Lab M agar (Amersham)
Reduced arginine, starch and salts agar (RASS) (Herron & Wellington,1990).	L-arginine 0.1 g Soluble starch 12.5 g Di-potassium phosphate 1 g Sodium chloride 1 g $MgSO_4.7H_20$ 0.5 g $MgSO_4.7H_20$ (1% w / v) 1 ml $FeSO_4.7H_20$ (1% w / v) 1 ml $ZnSO_4.7H_20$ (1% w / v) 1 ml $CuSO_4.7H_20$ (1% w / v) 1 ml Agar 15 g Adjusted to pH 8.0

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Tryptone soya broth plus sucrose	20 g Tryptone soya broth (Oxoid) 100 g Sucrose
Nutrient agar	28 g Oxoid nutrient agar
Pseudomonas Agar (Oxoid)	48.4 g Pseudomonas Agar Base 10 ml Glycerol
Starch casein agar (Horan, 1994)	10 g Soluble starch 0.3 g Vitamin free casein (Difco) 2 g Potassium nitrate 2 g Sodium chloride 2 g Di-potassium hydrogen phosphate 0.05 g Magnesium sulphate 0.02 g Calcium carbonate 0.01 g Iron II sulphate 20 g Lab M agar (Amersham)
Diluent Ringers Solution (¼ Strength)	2.25 g Sodium chloride 0.10 g Potassium chloride 0.12 g Calcium chloride 0.05 g Sodium hydrogen carbonate

Table 2.2. Antibiotic stocks.

Antibiotic	Stock solution (mg / ml)	Final concentration in media (μg / ml)
Cyclohexamide[1]	25	50
Nystatin[2]	50	50
Ampicillin[3]	100	50
Cetrimide[3]	50	10
Fucidin[3]	50	10
Cephalosporin[3]	50	50

All antibiotics were supplied by Sigma, except Nystatin which was supplied by BDH.
[1]Dissolved in distilled water and sterilised by autoclaving.
[2]Dissolved in methanol and 5% acetic acid
[3]Dissolved in distilled water and filter sterilised through Acrodisc PF filter (0.8-0.2 μm) Gelman Scientific.

The reagents and solutions used for molecular investigations are listed in Table 2.3. Other molecular biology reagents are as described in Sambrook *et al.* (1989).

Table 2.3. Buffers, reagents and solutions.

Solution / Reagent	Constituent(s) / l
50 x Phosphate Buffer	350 g Di-potassium hydrogen phosphate 100 g Potassium di-hydrogen phosphate
50 x TAE	242 g Tris base 57.1 ml Glacial acetic acid 100 ml 0.5M EDTA (pH 8.0)
TE	10 mM Tris-HCl 50 mM EDTA Adjusted to required pH with HCl
0% Stock Denaturant Acrylamide (100 ml)	25 ml 40% Acrylamide/Bis (37:5:1) 2 ml TAE (50 x)
100% Stock Denaturant Acrylamide (40%)	25 ml 40 % Acrylamide/Bis (37:5:1) 2 ml TAE (50 x) 40 ml Formamide (deionised with 2 h stirring with AG501-X8(D) resin) (Biorad) 42 g Urea
20 x SSC	175.3 g Sodium chloride 88.2 g Tri-Sodium citrate
Neutralising Solution	121.14 g Tris-HCl 87.66 g Sodium chloride (pH 8.0)
Denaturing Solution	87.66 g NaCl 20 g NaOH
Prehybridisation solution (50 ml)	12.5 ml SSC (20 x) 2.5 ml SDS 10 % (w / v) 5 ml Denhardts (50 x) 100 ml sheared non-homologous DNA (Herring Sperm, Sigma)

Oligonucleotide probe reaction mixture	4 µl Forward reaction buffer 1 µl T4 Polynucleotide kinases (GibcoBRL) 10 ng specific primer 12 µl ddH_2O
Denhardts (50 x)	10 g Ficoll 400 10 g Polyvinylpyrrolidone 360 10 g Bovine serum albumen (Sigma) Stored -20°C
Low stringency	10 ml SDS (10 % w / v) 300 ml SSC (20 x) (use only 150 ml / wash)
Medium stringency	10 ml SDS (10 % w / v) 100 ml SSC (20 x) (use only 150 ml / wash)
High stringency	10 ml SDS (10 % w / v) 10 ml SSC (20 x) (use only 150 ml / wash)
NDP	0.02 g Sodium desoxycholate 0.5 g Polyethylene glycol
Phenol chloroform (Sambrook *et al.*, 1989)	100 ml Phenol 100 ml Chloroform
Cell lysis buffer	25 mM Tris 25 mM EDTA 10 mg / ml lysozyme

2.3. Soil Analysis.

2.3.1. Air Dried Soil Fractionation.

Air dried, non-sieved soil (100 g) was placed into a 251 µm mesh sieve in an ethanol-sterilised plastic container, covered with 1500 ml sterile distilled water and shaken gently for 10 min on a IKA-Vibrax-VXR shaker. The sieve was removed from the soil suspension and left to drain for 2 min. The soil on the sieve was collected. The soil suspension poured onto a 63 µm mesh sieve and gently shaken

for 10 min. The sieve was removed from the soil suspension and left to drain for 2 min. This soil was collected and the suspension poured into a large glass vessel. This was left to sediment for a number of minutes determined from the sedimentation table. This was repeated twice to collect the soil fraction containing 20-63 μm sized soil aggregates. The remaining soil suspension was centrifuged at 3,000 g for 20 min at 4°C in a Beckman J2-21 centrifuge to obtain the 2-20 μm fraction. The supernatant was centrifuged at 10,000 x g for 30 min at 4°C to yield the 1-2 μm fraction. The resulting supernatant was decanted and 5 M $CaCl_2$ added to a final concentration of 0.025 M. After 4 h at 4°C, it was centrifuged at 15,000 x g for 1h. The soil fraction <1 μm was collected as the sediment at the bottom of the vessel. All soil fractions were weighed and samples were taken for total viable count determination (1 g), water content estimation and DNA extraction (3 g). To determine water content in the soil, the soil was weighed in an Eppendorf tube and placed uncapped in an oven at 80°C for 4 h. The tubes were removed from the oven, allowed to cool and the dry weight measured. The water content was calculated as wet weight minus the dry weight of the soil.

2.3.2. Soil Fractionation of Fresh Warwickshire soil.

Fresh soil was fractionated by the same procedure as dry soil (2.3.1.) with a few modifications. Soil (100g) was placed into an ethanol-sterilised box to which 250 ml sterile distilled water was added. The box was covered and placed at 4°C for 2 h to thoroughly wet the soil. The soil mixture was poured through a 2 mm sieve and the roots, pebbles and macrofauna were collected using sterile tweezers. The soil mixture which had passed through the 2 mm mesh sieve was separated into aggregates by wet sieving (Section 2.3.1.).

2.4. Selective Counts and Isolation Procedures.

2.4.1. Actinomycete, Pseudomonad and Total Counts.

From each of the soil fractions, soil (1 g) was mixed with 9 ml diluent and shaken vigorously on a shaker (Stuart Scientific) for 20 min. 10-fold serial dilutions were prepared and plated onto nutrient agar for total counts (2 d incubation) or selective media for actinomycetes (7 d incubation) and pseudomonads (24 h incubation). The actinomycete specific plates contained Reduced Arginine Starch and Salts (RASS) agar, cyclohexamide, nystatin and ampicillin. The pseudomonad selective agar base (Oxoid) also contained cyclohexamide, nystatin, cetrimide, fucidin, and cephalosporin (Table 2.2). All plates were incubated at 28°C.

2.4.2. Isolation of Actinomycetes from Soil.

Soil was treated as in section 2.3.2. In addition, when inoculating plates the soil suspension was plated in triplicate onto starch casein medium and RASS medium which are selective for actinomycetes. The plates were incubated for 7 d at 28°C. A plastic grid containing 64 squares was stuck onto the back of spread plates containing >100 colonies. The actinomycetes were isolated from predefined squares (using random number tables) using a sterile toothpick and plated onto RASS agar and incubated at 28°C for 3 d and examined for growth. The purity of each culture was checked by examining the plates visually.

2.4.3. Storage of Isolates.

Actinomycete isolates were grown on RASS plates for 7 days at 28°C. Mycelia were scraped from the surface using sterile loops and placed into bijoux containing sterile 10% (v / v) glycerol. These were kept until required in a -20°C freezer. For long term storage, pseudomonads were also kept at -20°C in 10% (v / v) glycerol.

2.5. DNA extraction and Purification.

2.5.1. DNA Extraction from Soil.

Soil (1 g) was placed into a bead beating bottle (Braun) along with 1 g Glaspergen glass beads (0.1-0.11 mm diameter) and 5 ml 1 x phosphate buffer, (pH 8). This was placed into a bead beater (Braun) and shaken for 5 min with carbon dioxide cooling. The soil solution was placed into universals and treated with lysozyme (10 mg / ml) for 30 min at 37°C. The sample was centrifuged (MSE Mistral 2000) at 400 x g for 15 min. The supernatant was collected, 0.2 volumes of 8 M potassium acetate added, and the sample placed on ice for 15 min. The pellet was re-extracted by resuspension in 4 ml phosphate buffer and shaken (Stuart Scientific) for 5 min. The resulting solution was centrifuged as above. The supernatant was added to the reserved supernatant and kept on ice for a further 15 min before centrifuging in a Beckmann J2-21 centrifuge at 4°C for 30 min at 10,000 x g. The pellet was discarded and 10 ml supernatant added to 10 ml PEG 6000 50% (w / v) with 125 ml of 5 M NaCl. Samples were left for 16 h at 4°C.

After centrifugation at 4°C for 30 min at 10,000 x g, the supernatant was discarded. The pellet was resuspended in 1 ml of TE, placed on top of a Sephadex G50 (Pharmacia) spin column (Sambrook *et al.*, 1989) and centrifuged for 10 min at 400 g (MSE Mistral 2000). The resulting elutrate was placed on top of a Chelex 100 (Biorad) spin column (Straub, 1994) and maintained at 4°C for 30 min prior to centrifugation for 10 min at 400 x g. The DNA was treated with 25µl spermine (350 mg / ml) and stored at room temperature for 15 min. The mixture was centrifuged at 16,060 x g for 20 min (Hereaus Biofuge) and 100 µl of TE added. DNA was stored at 4°C until required.

2.5.2. Quantification of Soil DNA.

Optical density readings were taken at 260 nm, 280 nm and 230 nm using a PU 8720 UV / VIS scanning spectrophotometer (Philips). An OD measurement at 260 nm of 1 was taken to be equivalent to 50 mg / ml DNA and OD ratios for 260 / 280 nm and 230 / 260 nm of 1.7 and above taken to be pure DNA (Sambrook *et al.*, 1989). The soil community DNA after extraction was found to be of sufficient purity to amplify PCR products using universal primers, pA and pH for the 16S rRNA gene (Edwards *et al.*, 1989).

2.5.3. Extraction of DNA from Actinomycete Cultures.

Each isolated actinomycete was grown in tryptone soya broth liquid medium plus sucrose (10% w / v) at 28°C for 3 days. Culture (1 ml) was centrifuged at 16,060 x g for 10 min in a bench minicentrifuge (Hereaus Biofuge). The pellet was resuspended in 500 µl cell lysis buffer and incubated at 37°C for 2 h. After incubation, 25 µl SDS (10%) was added and the mixture incubated at 65°C for 30 min. After incubation 215 µl of 5 M potassium acetate was added and the tube placed on ice for 30 min. The sample was centrifuged at 16,060 x g for 10 min. The resulting supernatant was added to an equal volume of isopropanol and left at room temperature for 10 min. The supernatant was centrifuged at 16,060 x g for 30 min and the supernatant was discarded. The pellet was washed with 500 µl of ethanol, centrifuged at 16,060 x g for 30 min and the pellet resuspended in 50 µl of TE and 1 µl RNase (50 mg / ml). The DNA obtained was incubated at 65°C for 2 h to redissolve and finally stored at -20°C.

2.5.4. Extraction of DNA from Non-actinomycete Cultures.

Bacterial cultures were grown up in Nutrient Broth at 30°C for 2 days. Culture (5 ml) was removed by centrifugation at 16,060 x g for 10 min (MSE Mistral 2000). The pellet was resuspended in 3 ml cell lysis buffer and incubated for 1 h at 37°C. SDS (10% w / v) was added (150 ml) to the mixture and incubated at 65°C for 30 min. The solution was added to 3.5 ml of phenol-chloroform solution, vortexed and centrifuged at 16,060 x g for 1 min. The aqueous layer was removed and the bottom layer discarded. The procedure repeated twice more to remove any trace phenol. A final step was performed with chloroform only. The aqueous layer was placed into an Eppendorf containing 400 µl of ethanol and 25 µl of 8 M sodium acetate and kept at -20°C for 16 h. The precipitated DNA was collected by centrifuging at 16,060 x g at 4°C for 40 min.

2.5.5. DNA Extraction from Agarose Gels.

A QIAquick gel extraction kit (Qiagen) was used according to manufacturers' instructions.

2.5.6. Polymerase Chain Reaction.

The polymerase chain reaction (PCR) was used to detect and amplify genes of interest from soil community DNA and isolated strains. See Table 2.4 for primers used in this study and the annealing and extension temperatures used. To try and overcome PCR bias, reactions were carried out in triplicate and then combined for further analysis.

2.5.6.1. Standard PCR Reaction.

The PCR solution was added to 100 ng of DNA (determined spectrophotometrically on a PU 8720 UV/VIS scanning spectrophotometer, Philips) in 500 µl Eppendorf tubes. Reactions were carried out in a total volume of 50 µl and overlaid with 50 µl DNase-free mineral oil (Sigma). Control reactions consisted of a negative containing no DNA and a positive containing type strain DNA. Samples were placed into a PCR machine (OmniGene, Hybaid) and the first cycle for 5 min at 94°C was initiated. The tube temperature was cooled to 82°C and 0.3 µl Taq polymerase (GibcoBRL) was added (hot start). The temperature was increased to 94°C for 1 minute, followed by temperature decrease to a specifically chosen annealing temperature for 1 minute (see table 2.4.). DNA extension was performed at 72°C for a specific time, dependent on the size of fragment being amplified. These three steps were repeated for 30 cycles before a final 10 min extension at 72°C.

2.5.7. Electrophoresis.

Agarose (GibcoBRL) was mixed with 1 x TAE to a final concentration of 0.8 % (w / v) and then boiled. The agarose was cooled to 50°C and ethidium bromide added to a final concentration of 0.25 µg / ml. A 1Kb DNA ladder (GibcoBRL) was used to size products and sample (5 ml) loaded into the wells with 1 ml of loading buffer. Gels were visualised using a short wave transilluminator (UVP International, Model TS35) and gel images were recorded using UVP Life Sciences Grab It 2.0 Synoptics Ltd.

Table 2.4. Primers and annealing temperatures used in this study.

Primer name	Target gene	Primer sequence	Target groups	Annealing temperature (°C)
PA	16S rRNA	5'- AGA GTT TGA TCC TGG CTC AG-3'	Universal (Edwards *et al.*, 1989)	62
PH	16S rRNA	5'-AAG GAG GTG ATC CAG CCG CA-3'	Universal (Edwards *et al.*, 1989)	62
FD2	16S rRNA	5'- AGA GTT TGA TCA TGG CTC AG-3'	Pseudomonads (A. Morgan, pers. comm)	68
FD2 Rev	16S rRNA	5'- ACT GCC CTT CCT CCC AAC TT-3'	Pseudomonads (A. Morgan, pers. comm)	68
A1	16S rRNA	5'-GGA TGA GCC CGC GGC CTA-3'	Actinomycete (F226-243) (Heuer *et al.*, 1997)	64
A2	16S rRNA	5'-CLAMP-CGG CCG CGG CTG CTG GCA CGT-3'	Actinomycete (R513-528GC) (Heuer *et al.*, 1997)	64
P3	16S rRNA	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3'	Universal (Muyzer *et al.*, 1993)	60

P2	16S rRNA	5'-ATT ACC GCG GCT GCT GG-3'	Universal (Muyzer *et al.*, 1993)	60
nahAc1	*nahAc*	5'-GTT TGC AGC TAT CAC GGC TGG GGG TTC GG-3'	Naphthalene dioxygenase Herrick *et al.*, (1993).	58
nahAc2	*nahAc*	5'-GCT CGC GTG GAG AGC TTC CAT GGC TTC ATC-3'	Naphthalene dioxygenase Herrick *et al.*, (1993).	58
nahAc3	*nahAc*	5'-TTC GAC AAT GGC GTA GGT CCA GAC CTC GGT-3'	Naphthalene dioxygenase Herrick *et al.*, (1993).	58

2.5.8. Sequencing.

An Applied Biosystems Model 373A automatic sequencer was used for DNA sequencing reactions were carried out by cycle sequencing with the Dye Terminator Kit of PE Applied Biosystems (Warrington, Cheshire,UK).

2.5.9. DNA Manipulation Programmes used on the World Wide Web.

To detect any similarities with previously published sequence the BLAST programme was used, while ENTREZ was used in retrieving sequence for primer design. Both programmes are available at the National Centre for Biotechnology Information at http://www.ncbi.nlm.nih.gov/.

For alignments of sequences the ClustalW Multiple Sequence Alignment programme was used which can be found at http://www2.ebi.ac.uk/clustalw/.

2.6. Denaturing Gradient Gel Electrophoresis.

2.6.1. Addition of High GC Clamp During PCR for DGGE Analysis.

For DGGE analysis of PCR products, a high GC oligomer was attached to specific primer pairs. This GC 'clamp' allows DNA denaturation to occur at the region of interest but clamps the two strands together so that they do not completely separate in the DGGE gel. The clamp was attached to a specific primer at the 3' end (Table 2.4). The other primer used in the amplification remained unchanged. The PCR protocol was unchanged and the PCR products were run on a agarose gel (0.8% w / v). If non-specific PCR products were observed then the PCR was repeated under more stringent conditions until only one band of the expected size was obtained.

2.6.2. Perpendicular Denaturing Gradient Gel Electrophoresis.

To establish the optimal gradients for melting of PCR products, perpendicular gradient gels were performed. A 0-100% denaturant gradient was established using a Model 475 Gradient Delivery System (Biorad) according to manufacturers' instructions.

2.6.3. Travel Denaturing Gradient Gel Electrophoresis.

Once optimum gradients had been established using perpendicular DGGE, parallel gels could be used to determine the optimal time for separation. Two different PCR products formed using the same primer set were mixed together (1:1) and an equal

volume of loading dye was added. The maximum volume which could be added to each well was 40 µl. Aliquots of these PCR product mixtures were added at specific time intervals (generally 30 min) until all 16 wells had been loaded. During this time the samples were electrophoresed at 150 V from top to bottom in 0.5 x TAE buffer at 60°C.

2.6.4. Parallel Denaturing Gradient Gel Electrophoresis.

Once optimum time and gradients for separation of different PCR products had been determined, it was possible to perform parallel DGGE for the samples of interest. Gels were cast according to manufacturers' instructions and left for 8-12 h to polymerise. The PCR product mixture was added to loading buffer and placed into specified wells. The samples were electrophoresed for 6 h at 150 V from top to bottom in 0.5 x TAE buffer at 60°C.

2.6.5. Denaturing Gradient Gel Staining.

The gels were stained using 1 µg / ml ethidium bromide in 0.5 x TAE buffer (100 ml) for 15 min on a horizontal shaker (Luckham, UK). This solution was removed and replaced with 100 ml distilled water and shaken for 15 min. The gel was visualised under the UV transilluminator (UVP Inc., California, USA) and photographed (Polaroid P665 film) with a 20 second exposure.

2.6.6. Sequencing Bands from DGGE gels.

Bands were excised from the gels and sequenced according to the method of Ferris *et al.* (1996).

2.7. Radioactive Probing Procedure.

2.7.1. Southern Blotting.

DNA was transferred from agarose and polyacrylamide gels by capillary transfer (Sambrook *et al.*, 1989) onto Hybond N+ membranes (Amersham). When Southern blotting of DGGE gels was performed the denaturation and neutralisation steps were carried out on the filter after the DNA had been transferred. The DNA was fixed to the filter using a short wave transilluminator (UVP International, Model TS35) for 4 min (2 min each side). If the filter was not used immediately, it was stored with 5 ml SSC at 4°C in sealed plastic.

2.7.2. Prehybridisation.

Filters were covered with nylon mesh and the filter sandwich rolled up and placed in a Hybaid tube, along with a small amount of 2 x SSC. The roll was unravelled until it stuck to the walls of the tube. The liquid in the tube was exchanged for 35 ml of the prehybridisation solution. The sealed tube was placed in a Hybaid oven at 50°C for 3 h.

2.7.3. Preparation of ^{32}P Labelled Oligonucleotide Probe.

T4 polynucleotide kinase (GibcoBRL) was used to end-label olignucleotide probes. 10 ng of oligonucleotide were labeled using the forward labelling reaction as per manufacturers instructions (GibcoBRL).

2.7.4. Hybridisation.

The prehybridisation buffer was removed and 15 ml of fresh prehybridisation solution was added to the hybridisation bottle. The radioactive probe was now added. The bottle was put in a Hybaid oven and incubated at 50°C for 18-24 h.

2.7.5. Stringency Washes.

The hybridisation mixture was poured out of the bottle. The filter was washed at low stringency for 1 h at room temperature followed by medium stringency for 45 min at 37°C and finally at high stringency for 30 min at 45°C. After stringency washes, radioactive filters were sealed in plastic bags.

2.7.6. Autoradiography.

The radioactive filters were placed in a Harmer film cassette with Fuji X-ray film and two intensifying screens. The cassette was left at -70°C for a sufficient time to obtain the best exposure. The exposed film was developed in an automatic developer Agfa Curix 60.

2.7.7. Radioactive Filter Stripping.

The radioactive filter was placed into a hybridisation bottle and stripped by adding 150 ml of boiling 0.1% (w / v) SDS. The filter was placed in the Hybaid oven and rotated at 25°C for 2 h. The filter was checked for any residual radioactivity and if still radioactive then the procedure was repeated. If the filter was not radioactive it was stored as in Section 2.7.1, until required.

2.8. Biolog Community Analysis.

2.8.1. Soil Treatment and Inoculation into Biolog Plates.

Biolog Gram negative (GN) and Gram positive (GP) plates (Biolog, Inc, U.S.A) were used for community analysis. Soil (5 g) was placed into a universal and 20 ml of NaCl (0.85 w / v) added The mixture was shaken vigorously for 10 min on a shaker (Stuart Scientific). The soil solutions were centrifuged for 3 min at 500 x g in a bench centrifuge (MSE Mistral 2000). The supernatant was collected and stored on ice. The soil pellet was resuspended in 10 ml of NDP and 1 g of Chelex 100 resin (Biorad). The resuspended pellet was shaken vigorously for 5 min on a shaker (Stuart Scientific) and incubated at room temperature for 5 min. This was repeated twice and the soil solution after shaking was centrifuged for 3 min at 500 x g in a bench centrifuge. The resultant supernatant was added to the original supernatant and stored on ice. After removal of the supernatent the soil pellet was again resuspended in 10 ml of NDP and shaken for 10 min on a shaker. The resulting soil solution was centrifuged again for 3 min at 500 g in the bench centrifuge, and the soil pellet was discarded. The supernatant was added to the original supernatant on ice. This soil extract was placed into Oakridge tubes and centrifuged at 5,000 x g for 20 min at 4°C to yield a bacterial pellet. This bacterial pellet was resuspended in 10 ml NaCl (0.85% w / v) and vortexed to ensure complete resuspension of the pellet. A ten-fold dilution was made of the soil suspension and 150 µl was added into each of the Biolog wells. The Biolog plates were incubated at 26°C for 48 h unless specified. This procedure was carried out in triplicate for each soil sample.

2.8.2. Biolog Plate Reading.

Absorbance of the Biolog plates was measured using a Multiscan® Plus MK11 (Labsystems) at 590 nm. These data were transferred into Microsoft Excel for statistical analysis.

2.8.3. Statistical Analysis of Biolog Plates.

Statistical analysis of Biolog plates was carried out using the methods of Garland and Mills, (1991). The Average Well Colour Development (AWCD) was determined for each plate.

$AWCD = [\Sigma (C - R) / 95]$

C = control well

R = mean difference of the 95 control wells

Data was expressed as transformed data, where the raw difference value is divided by the AWCD of the plate

$= (C - R) / [\Sigma (C - R)] / 95$

2.8.4. DNA Extraction from Biolog Plates.

The Biolog plates contain 11 different types of carbon source such as alcohols, amino acids, phosphorylated compounds. One of each carbon source type was chosen from the GP and GN plates and the control well (Table 2.5). The contents from the designated wells in the triplicate Biolog plates were collected and pooled. The pooled samples were treated with lysozyme (10 mg / ml) for 30 min at 37°C. Samples were treated with 100 µm SDS solution 10% (w / v) at 65°C for 30 min. This solution was added to 1 ml of phenol-chloroform solution and vortexed prior to centrifugation at 16,060 x g for 1 min. The aqueous layer was removed and the bottom layer discarded and the procedure repeated twice more. To remove any trace of phenol a final step was performed with chloroform only and the aqueous layer placed into an eppendorf with 400 µl of ethanol and 25 µl of 8 M NaAc and left 16 h at -20°C. The precipitated DNA was collected by centrifuging at 16,060 x g at 4°C for 40 min. The DNA was then used as a template for PCR (Section 2.5.6.1.) and examined by parallel DGGE (Section 2.6.4).

Table 2.5. The different carbon sources from GP and GN Biolog plates used for DGGE analysis.

GN	Carbon Source and Well Reference	GP	Carbon Source and Well Reference
1	a-D-glucose (B6)	1	tween 40 (A8)
2	mono-methyl-succinate (C12)	2	D-gluconic acid (B10)
3	tween 80 (A6)	3	maltose (C3)
4	p-hydroxyphenylacetic acid (E1)	4	D-alanine (G2)
5	glycerol (H9)	5	methyl pyruvate (F6)
6	putrescine (H6)	6	thymidine (H4)
7	glucose-6-phosphate (H12)	7	putrescine (G10)
8	L-phenylalanine (G5)	8	D-C-a-glycerol phosphate (H12)
9	urocanic acid (H1)	9	2,3,butanediol (G11)
10	bromo-succinic acid (F1)	10	succinamic acid (F10)
11	succinamic acid (F2)	11	a-D-glucose (B11)
12	control well (A1)	12	control well (A1)

2.8.5. Statistical Analysis.

All Biolog and selective plate count data are a result of three replicates for each sample and wet sieving for each soil was repeated five times.. Standard errors and means were calculated for the replicates. All data were subject to statistical analysis using Microsoft Excel Version 5, Analysis toolpack (Microsoft Corporation). A one-

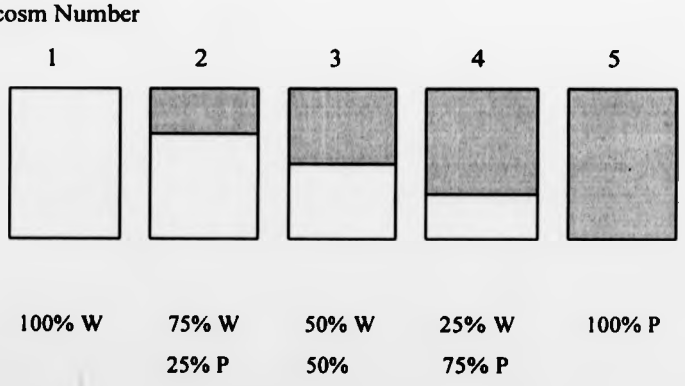
way analysis of variance (ANOVA) was calculated for each data set. The standard error for each data point is plotted on the graphical representations of the data, and the significance of the result is reported in the text.

2.9. Microcosm Experiments.

2.9.1. The Microcosm System.

Non-sterile batch microcosms were constructed using mixtures of Doncaster polluted and Warwick soil. The microcosms were constructed in sterile sandwich boxes with filters (Acrodisc PF, 0.8-0.2 μm, Gelman Scientific) placed in the top of the box to allow gaseous exchange. The soil which had been air dried and stored at room temperature was pulverised using a pestle and mortar before use, and passed through a 2 mm sieve. The appropriate amounts of each soil were mixed together for each microcosm (Table 2.5). Water was added to achieve a matric potential of -11 MPa (100 g soil 22.5 ml). This was kept constant by weighing the microcosms every 5 d and adding water to the required weight. This was adjusted as soil was removed for analysis.

Fig. 2.1. The different microcosm systems.



W = Warwick soil, P = polluted soil

Table 2.6. Amounts of Warwick and Doncaster polluted soil in the five different microcosm systems.

Microcosm Type	Warwick soil (g)	Polluted soil (g)	Matric Potential MPa	Water added (ml)
1	100	0	-11	22.5
2	75	25	-11	22.5
3	50	50	-11	22.5
4	25	75	-11	22.5
5	0	100	-11	22.5

2.9.2. Sampling the Microcosms.

The microcosms were sampled using a sterile core borer to take five random samples from the sandwich box. These samples were combined together. Samples were taken after 1, 2, 4, 8, 16, 32 and 80 d. Soil from each sampling date was used for inoculating selective agar plates (Section 2.4.1) and DNA extractions (Section 2.5.1). Biolog analysis was performed on the samples from days 1, 16 and 80 (Section 2.8.1).

Chapter 3

Warwick Soil Fractionation and Community Analysis.

3.1. Introduction.

3.1.1. Bacterial Location in Soil.

The location of the microorganism in the soil environment is a critical factor for its survival and activity, it also has important effects on the soil structure (Young and Ritz, 1998) as discussed in Section 1.1.2. The soil has a large number of discrete microhabitats. These microhabitats provide a wide range of abiotic conditions, which may allow higher levels of bacterial diversity to occur than in a more homogeneous environment (Stotzky, 1997).

Bacterial location in soil can be limited by pore size. Many pores afford protection to microbes by preventing the entry of predators. One study by Lugtenberg and deWeger, (1992) found that pseudomonad cells measured approximately 0.8 μm length in the soil, therefore due to their small size, they will be able to pass freely into most pores in the soil. The filamentous actinomycetes would be expected to have larger dimensions (Goodfellow and Cross, 1984). Size restrictions therefore prevent the filamentous organisms entering the smaller pores, although actinomycetes may be present in the small pore spaces during the spore stage of their life cycle.

Soil structure can also give protection against predation by providing physical obstacles for the predator to overcome before reaching the microorganism. Gestel *et al.* (1996) determined that, if soil has a fine texture, then predators would have to travel longer distances in order to gain adequate nutrients. Therefore a smaller predator population would exist.

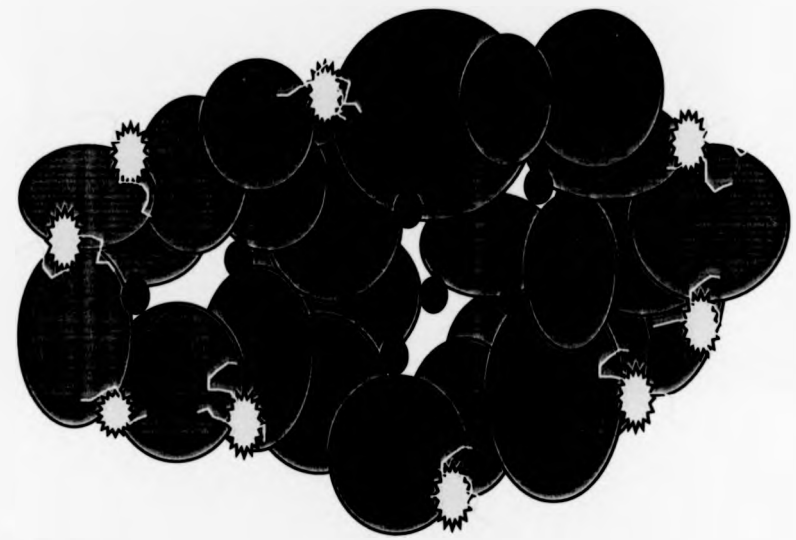
To examine bacterial distribution in soil aggregates, Hattori (1973), compared the mean number of cells in or on a particle with the mean diameter for each aggregate. Analysis of the larger aggregates (>250 μm) revealed that bacterial

numbers rapidly increased with particle size, indicating that bacteria may be able to live in high numbers in the inner regions of the aggregate.

It has been proposed that certain bacterial groups are located in different areas of the soil (Hattori, 1988). This distribution was examined by immersing aggregates in water and treating with gentle sonication to release bacteria on the surface. The aggregate was disrupted and the bacteria located on the inside released by sonication. The two communities were compared. It was determined that fungi were predominant on the outside of aggregates, actinomycetes were present in the inner and outer regions while Gram-negative bacteria were concentrated in the inner regions (Fig. 3.1). As nearly all microorganisms found on the outer regions of the soil were found to be spore formers this difference in location was proposed to be due to the microorganisms being able to survive long periods of desiccation. Actinomycetes were found in both locations but due to their ability to survive desiccation they may have a selective advantage on the outside of the particle.

Kandeler and Murer's study (1993) determined that high bacterial counts were associated with the 2-20 μm fraction of grassland soil, whilst in agricultural soil a more even distribution was observed. This distribution may be due to the high levels of C and N in this soil fraction (2-20 μm) or detached cells from the surface of other aggregates. In the grassland, where the bulk soil has a poor nutrient status the microaggregates may provide a stable environment with water and nutrients. However in the agricultural soil, which had been fertilised and tilled, the nutrients would be more freely distributed in the soil allowing bacteria to flourish in other locations.

Figure 3.1. Bacterial location in soil as proposed by Hattori (1988).



Gram-positive organisms

Gram-negative organisms

Hattori (1988) proposed that Gram-positive and Gram-negative bacteria have different locations in the soil. Gram-negative bacteria are thought to be in highest densities in the inner areas of the aggregate. While Gram-positive organisms with their resistance to desiccation are proposed to have a selective advantage on the outer regions of the aggregate.

Darbyshire (1993), observed that macroaggregates (>250 μm) had the highest levels of C and N and the rates of mineralisation in the macroaggregate were higher than in the microaggregates.

Kanazawa and Filip (1986), observed that there appeared to be more copiotrophic bacteria than oligotrophic bacteria in the 20-50 μm fraction than in the larger fraction. It was proposed that this was due to the accumulation of nutrients in this fraction.

Soil fractionation studies performed by Jocteur-Monrozier *et al.* (1991) suggested that water stable aggregates 2-20 μm in size, have higher bacterial densities due to high clay content and high microporosity. In larger aggregates smaller microbial populations were present and the majority of bacterial cells were found to reside in the pore space rather than on the surface of the aggregate. This could result from microorganisms growing within the aggregate on nutrients which have diffused through pores. The aggregate will also protect the microorganisms from desiccation as capillary water will be present. The finer pores in the smaller aggregates may enable nutrients, water and oxygen to flow into the inner regions of the aggregate whereas wider pores in the larger aggregates will have lower capillary flow (Hattori, 1988; Jocteur-Monrozier *et al.*, 1991). This suggests that larger soil aggregates may be anaerobic in the centre and may explain why anaerobic bacteria have been found in the upper horizons of the soil.

These studies have reported differing bacterial numbers in various aggregate sizes of the soil by selective plate counts, revealed conflicting evidence on bacterial location in soil aggregates. This may be due to differences in the soils used or because all previous studies have utilised traditional culturing techniques. It may be possible that by employing a combination of culturing, functional activity analysis and molecular methods, the location of microbial groups in soil aggregates may be elucidated.

3.1.2 Location of Specific Bacterial Groups in Soil.

To examine the distribution of microbes in the soil this study has focused on two main groups, the actinomycetes and the pseudomonads. Both of these groups are ubiquitous in soil but have differing life strategies and abiotic requirements. It is proposed that by comparing and contrasting these populations that further insight into the soil habitat will become possible.

3.1.2.1 Actinomycetes.

Actinomycetes are Gram-positive which have a high G + C content DNA (63-78%). Reproduction is via fragmentation of hyphae or the production of spores. Most species are chemoorganotrophic and aerobic. Many produce antibiotics which can have antibacterial, antifungal, antiprotozoa and antitumour properties.

Actinomycetes form a significant part of the microbial population of most soils with the majority of actinomycetes isolated being streptomycetes (Willams, 1978) and can be detected at high levels (10^5 - 10^6 CFU / g) in dry soils (Willams *et al.*, 1972). Lower population levels have been recorded in water-logged and anaerobic soils (Willams and Wellington, 1982). The majority of propagules in soil exist as spores and if conditions are suitable for growth, mycelial structures will be present growing on organic debris, such as dead plant roots (Mayfield *et al.*, 1972). The actinomycete mycelia in the soil are long, with few branching points, which allows growth between the crevices of soil aggregates (Cresswell, 1992).

3.1.2.2 Pseudomonads.

Pseudomonads are a diverse collection of microorganisms. The genus *Pseudomonas* has been revised many times, however, the taxonomy has now been clarified by the use of 16S rRNA gene sequencing. Pseudomonads have some general characteristics which can be used for classification. They are Gram negative, straight or slightly curved rods, often motile with polar flagella, aerobic and mostly organotrophic (Palleroni, 1984).

Pseudomonads have a wide substrate range with some species able to degrade over 100 compounds (Brock and Madigan, 1991). These compounds include sugars, amino acids, alcohols, aldose sugars, hydrocarbons, oils and many other pollutant compounds (Golovela *et al.*, 1992). Their ability to degrade toxic chemicals has led to many studies on their suitability for field release to enhance bioremediation. Pseudomonads have been extensively studied in the environment because of their interactions with plants in the rhizosphere. These interactions include beneficial relationships such as the ability of *Pseudomonas fluorescens* to produce a phenazine antibiotic that inhibits growth of *Gaeumannomyces graminis* var *tritici*, the organism responsible for "Take-All" disease of wheat (Thomashaw and Weller, 1988). Other strains have been described that can control seedling and plant diseases (Pierson and Pierson, 1996; Whipps, 1997). Not all associations with plants are beneficial, however, some species are phytopathogens which attack plants, for example *Pseudomonas syringae* is often isolated from plant chlorotic lesions.

Pseudomonads do not form spores but may enter a non-culturable dormant state in adverse conditions. Various *Pseudomonas* species have been reported in this state including *Pseudomonas putida* (Morgan *et al.*, 1989), *Pseudomonas fluorescens* (Binnerup *et al.*, 1993), and *Pseudomonas aeruginosa* (Binnerup *et al.*, 1995). In this study when referring to pseudomonads, the rRNA Group 1 species are

inferred. Group 1 contains the fluorescent pseudomonads such as *P. fluorescens, P. aeruginosa* and *P. putida.*

3.1.3 Soil Storage.

All experiments involving soil, unless performed in the field, will have a transportation and storage period which will affect the microbial community present and the aggregate distribution. Short term storage (up to three weeks) was found to result in minor changes in the microbial community (Peterson and Klug, 1994). However this was determined using fatty acid profiles which may not reveal all the changes in the soil microbial community. Long term storage resulted in significant changes whether the soil was air dried (Bartlett and James, 1980; Salanius, 1983; Hattori, 1988), refrigerated or frozen (Shishido and Chanway, 1998; Stenburg *et al.*, 1998). The population size of viable microorganisms declined significantly during cold storage at 4°C reducing the bacterial component up to a hundred fold, while freezing was preferred for the bacterial component (Stenburg *et al.*, 1998). The opposite trend was observed for eukaryotes, where cold storage was less damaging than freezing (Shishido and Chanway, 1998). Biolog analysis was also affected when the soils were stored, the same trends being observed in average well colour development (AWCD) but at lower levels. This trait was consistent so that if all soils were stored in the same way, comparisons could still be made.

The soil storage conditions can also affect the aggregate distribution. Hattori (1973), determined that air drying of soils resulted in a greater quantity of aggregates in the larger fractions. Although it was also determined that fresh and dried soils had the same distribution of differently sized aggregates (Hattori, 1988). If all of the soil samples are treated with the same storage conditions then valid comparisons can still be made between the samples.

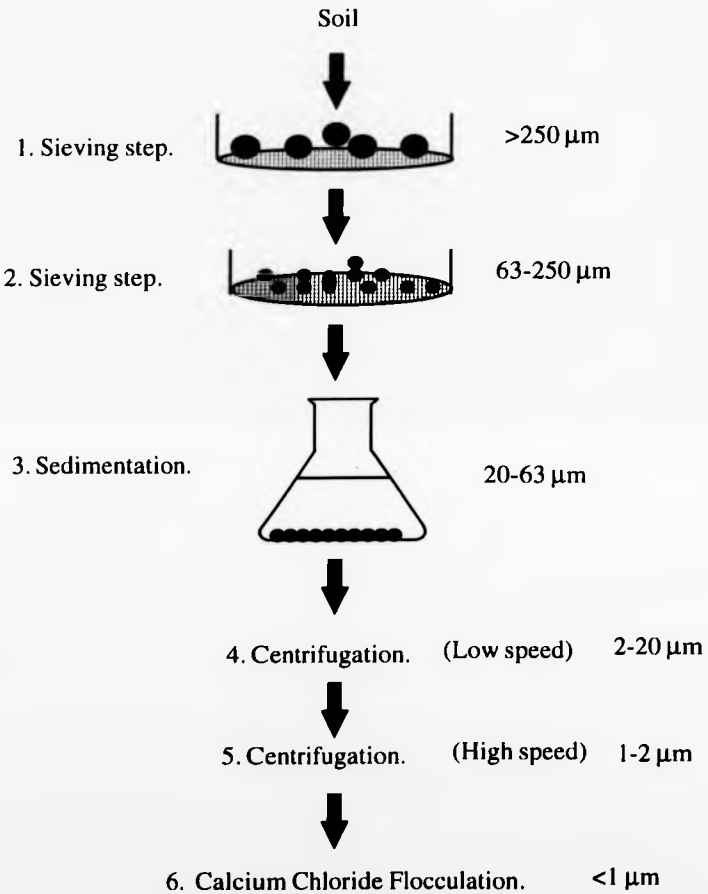
3.1.4. Techniques for Assessing Bacterial Location in Soil.

There are a number of techniques which are capable of separating the soil into different sized components, probably the most common being soil fractionation. Soil fractionation can be used in a number of ways for example wet sieving to obtain water stable aggregates (Jocteur-Monrozier *et al.,* 1991), or dry sieving to examine crumb structure (Christensen, 1992).

Wet sieving involves gentle shaking of the soil aggregates in water to disrupt any unstable structures on a defined sized mesh sieve (Fig 3.2). This shaking allows the collection of the water-stable aggregates while the unstable aggregates break down into smaller components and pass through the sieve. The smaller aggregates are collected by sedimentation and centrifugation (Jocteur-Monrozier *et al.*, 1991).

Other methods to determine bacterial location in soil include microscopic methods. This involves embedding soil with a resin and once the sample is set, cutting it into thin slices. Quantity and size of pores can be estimated and the location of bacteria can be observed using scanning electron microscopy (SEM) (Tippkötter and Ritz, 1996). The method of soil impregnation provides qualitative data and is dependent on the application of resin and the amount of sections examined.

Figure 3.2. The Wet Sieving Technique Used in this Study.



This is a modification of the techniques used by Jocteur-Monrozier *et al.* (1991). This wet sieving technique allows the separation of the soil into six different sized aggregate classes.

Another method used to investigate the distribution of pores to be examined is mercury intrusion porosimetry, which is often used to examine aggregate size and structure (Darbyshire, 1993). Both of these methods require specialist equipment and are labour intensive. Wet sieving provides a relatively rapid method to examine bacterial location in soil, allowing a high number of replicates of each soil to be processed.

3.1.5. Aims of the Warwick Soil Experiment.

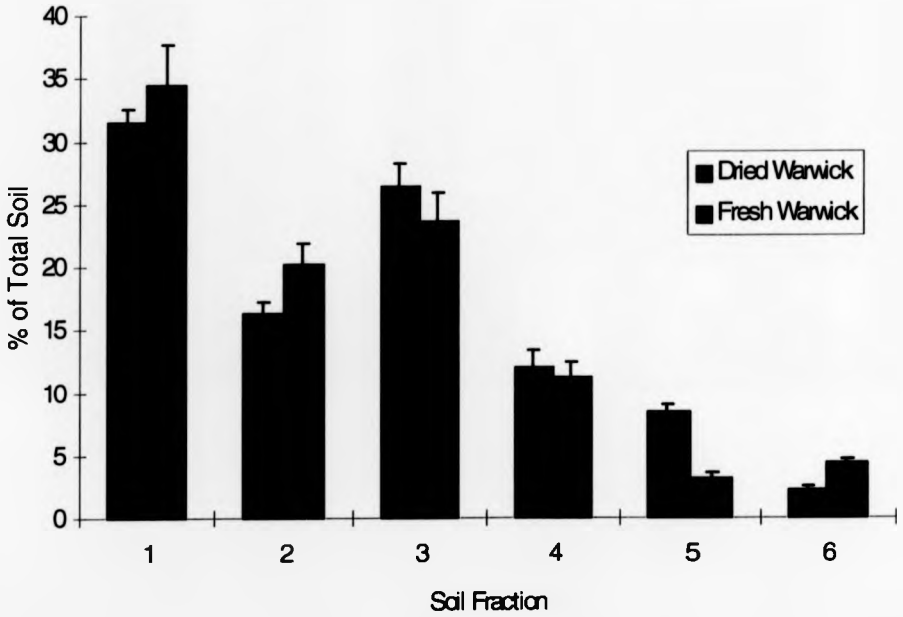
* To determine if different bacterial populations were located in different regions of the soil using molecular methods.
* To examine the bacterial community in the different sized soil aggregates using selective plates, genetic analysis and functional activity.
* To investigate the affects of drying the soil on aggregate stability.
* To assess community analysis techniques, do the three methods used provide similar results or are there anomalies between culturable counts, Biolog and genetic analysis.

3.2. Results.

3.2.1. Sieving Analysis.

Warwick soil was fractionated by a wet sieving technique (Jocteur-Monrozier *et al.*, 1991). This allowed the separation of soil water-stable aggregates into six size classes. Different sized aggregates are present in differing amounts in the soil (Fig. 3.3). Statistical tests (one-way ANOVAs) were performed to determine if these differences were significant in both air dried and fresh soil.

Figure 3.3. Percentage composition of different aggregate sizes in fresh and dried Warwick soil, fractionated by wet sieving.



Percentage composition of a 100 g of Warwick fresh and dried soils, size fractionated into 6 different size aggregates; soil fraction 1= < 250 μm, fraction 2= 63-250 μm , fraction 3= 20-63 μm , fraction 4= 2-20 μm , fraction 5= 1-2 μm , fraction 6= < 1 μm.

The error bars shown represent standard error of five replicates.

The results were significant for dried ($F_{5, 24} = 94.29$, $p = 2.621$) and fresh ($F_{5, 24} = 42$, $p = 2.621$). Both Warwick soils showed significant differences in their aggregate distribution.

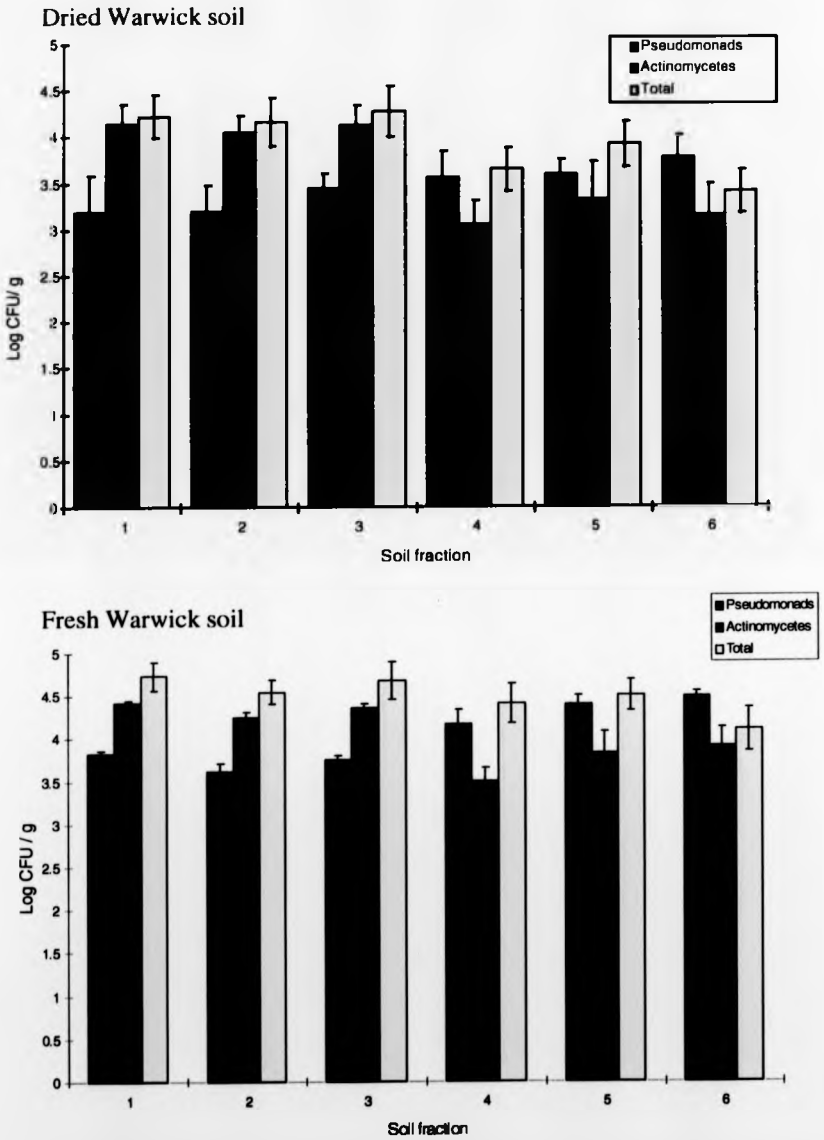
Aggregate distribution in the dried soil follows the same pattern as the fresh soil, with only minor non-significant differences in the largest four size fractions. These differences were only significant in the two smallest fractions.

The most abundant aggregate appears to be in the largest soil fraction (>250 μm) in both fresh and dried (32% and 34% respectively). The second most abundant fraction is the 20 - 63 μm soil aggregates, followed by the 63 - 250 μm size particles. In the fresh and dried Warwick soil, the largest three sized soil fractions are in the highest abundance. Recovery of the soil by this method was always higher than 96%. The unrecovered 4% was probably small clay particles that were left in the supernatant and also some soil particles left on the sieves.

3.2.2. Selective Plate Counts.

Selective plate counts were performed to enumerate pseudomonad, actinomycete and total culturable numbers in a Warwick soil. Fig. 3.4 shows that pseudomonads and actinomycetes occurred at roughly the same culturable population size (approximately 10^3 CFU / g) but numbers were higher on the overall culturable number plates (approximately 10^4 CFU / g). Some trends were observed throughout the plate count analysis. The culturable actinomycete numbers were highest in the largest three fractions, while the pseudomonads were more prevalent in the smaller soil fractions.

Figure 3.4. Selective plate counts using six different sized fractions of dried and fresh Warwick soil.



Selective plate counts using different aggregate sizes from fresh and dried Warwick soil. Fraction 1 = < 250 mm, fraction 2 = 63-250 mm, fraction 3 = 20-63 mm, fraction 4 = 2-20 mm, fraction 5 = 1-2 mm, fraction 6 = < 1 mm. The error bars shown represent standard error of five replicates.

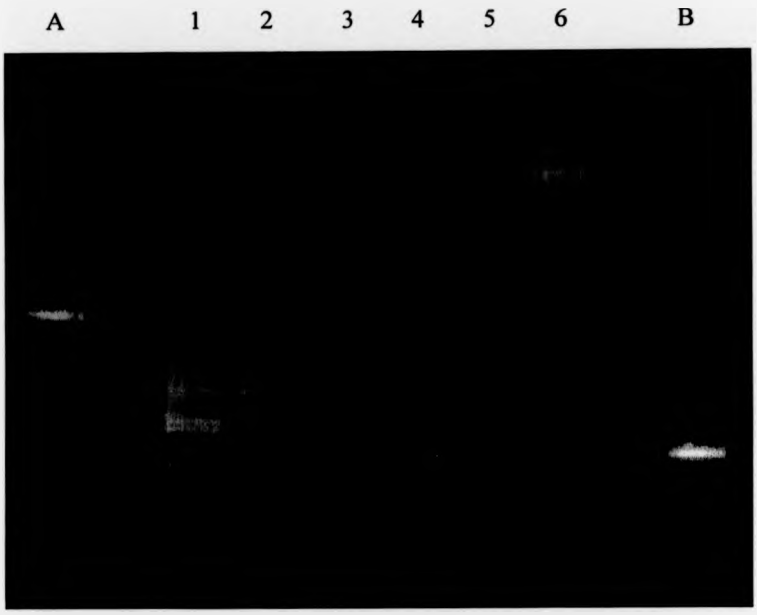
The plate counts were significantly different in the dried soil for the distribution of actinomycetes in different soil fractions ($F_{5, 24} = 3.39$, $p = 2.62$) but not for pseudomonads ($F_{5, 24} = 0.71$, $p = 2.62$) or overall culturable numbers ($F_{5, 24} = 2.5$, $p = 2.62$). In the fresh soil there were significant differences in the distribution in the six size soil fractions of actinomycetes ($F_{5, 24} = 5.44$, $p = 2.62$) and pseudomonads ($F_{5, 24} = 13.4$, $p = 2.62$) but not the overall culturable numbers ($F_{5, 24} = 1.2$, $p = 2.62$).

3.2.3. DGGE Analysis of the Warwick Soil Fractions.

To examine the bacterial communities present in the different size soil aggregates a genetic technique (DGGE) was used. This molecular analysis involved the DNA extraction from Warwick soil (fresh and dried) and the 16S rDNA amplified using universal primers P2 and P3 (Muyzer *et al.*, 1993). The PCR products were separated in DGGE gels (Fig 3.5) to examine the soil microbial diversity in each of the fractions. These DGGE gels revealed bands throughout the lanes resulting in a characteristic smear. This has been reported before with Warwick soil (Baker, 1997).

The patterns for fresh and dried soils were the same, however, the fresh soil produced brighter smears in comparison with the dried soil (data not shown). From the DGGE gel (Fig 3.5) distinct and different microbial communities can be observed in the various size Warwick soil aggregate fractions. There were strong bands present in the region of the *Streptomyces griseus* marker in the largest three soil aggregate fractions. These bands may represent an actinomycete or other high G + C organism due to their lower location on the gel, this could be confirmed by sequencing or probing. In the smaller soil fractions the bands are present but not at such intensity. Although PCR is not quantitative, a higher intensity band could be an indication of a larger population being present in that aggregate.

Figure 3.5. DGGE separation of 16S rRNA amplified using universal PCR primers P2 and P3, from the six different size fractions of dried Warwick soil separated by DGGE.



Universal PCR products separated on a DGGE gel. The gel was electrophoresed for 6 h at 150 V.

A = *Pseudomonas fluorescens*, B = *Streptomyces griseus*. Soil fraction 1 = < 250 μm, fraction 2 = 63-250 μm, fraction 3 = 20-63 μm, fraction 4 = 2-20 μm, fraction 5 = 1-2 μm, fraction 6 = < 1 μm.

The genetic analysis may correlate to the selective plate counts as the appearance of the brighter band also correlates with increasing numbers on the viable counts.

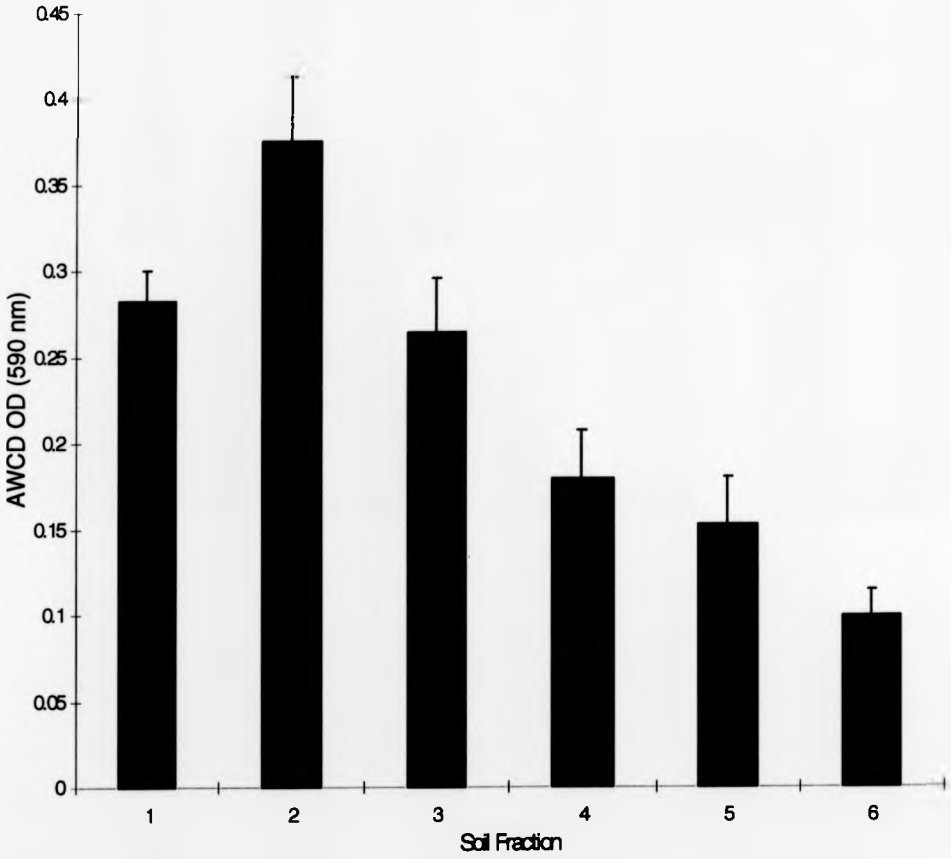
3.2.4. Metabolic Activity of the Different Sized Soil Fractions.

Biolog analysis was carried out on each of the different sized soil fractions and the AWCD (Section 2.8.3) was determined for each fraction (see Fig 3.6). Statistical analysis was carried out and a significant difference was found in metabolic potential in the 6 different soil fractions ($F_{5, 24} = 13.3$, $p = 3.1$). These data suggest that whilst metabolic potential is high in the largest soil fraction, higher levels of metabolic potential could be detected in the second fraction. The AWCD decreased with the next four sizes of soil aggregates. These data indicate that the second soil fraction (63-250 μm) had the most metabolically active, as it has higher levels of AWCD than any of the other fractions. Total unfractionated soil was also inoculated into Biolog plates (Fig 4.7) yielding an AWCD of 0.35 that is comparable to size fraction 2.

3.2.5. Analysis of the Biolog Plates by DGGE.

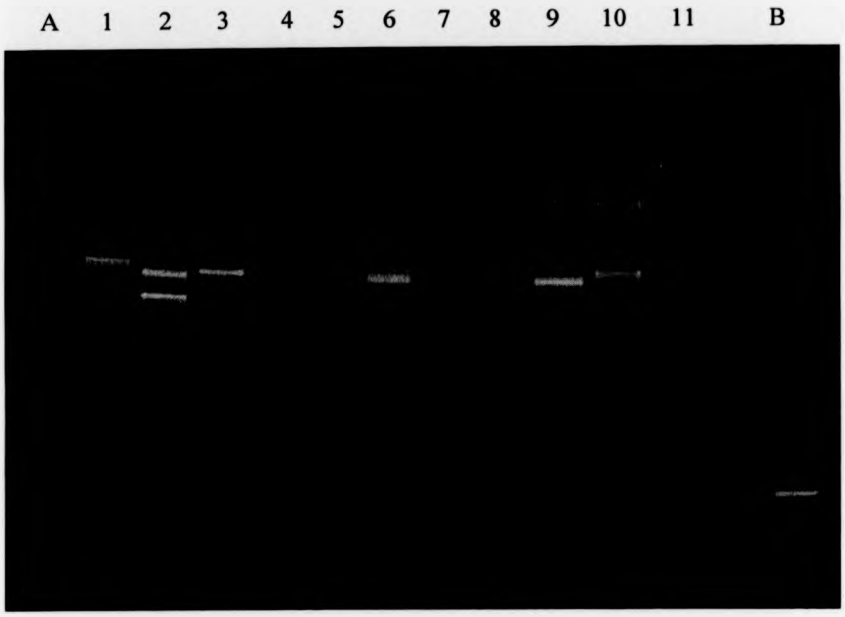
After Biolog analysis of the six size fractions had been completed the well contents were removed, DNA extracted and 16S PCR performed followed by DGGE. The analysis of the Biolog plates containing Warwick soil from size fraction 1 (Fig 3.7) had fewer bands than in the original soil sample. The communities in the Biolog plate appear to be more defined with different communities present in the different carbon sources. In many of the carbon sources no bands were detectable.

Figure 3.6. Average well colour development in Biolog GN plates using the six different Warwick soil fractions.



Biolog analysis of the six different size soil aggregates; fraction 1 = < 250 µm, fraction 2 = 63-250 µm, fraction 3 = 20-63 µm, fraction 4 = 2-20 µm, fraction 5 = 1-2 µm, fraction 6 = < 1 µm. The error bars shown represent the standard error of three replicates.

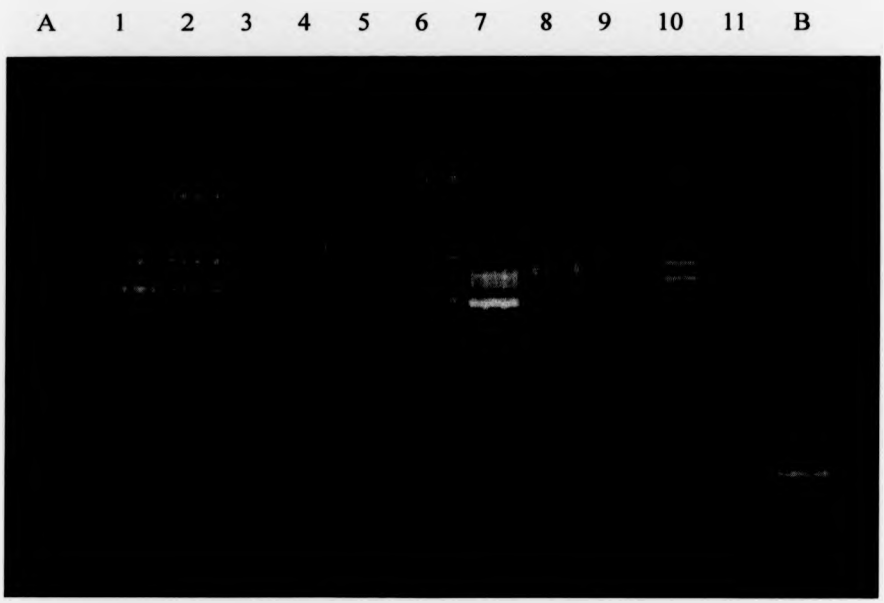
Figure 3.7. Warwick soil (fraction 1) 16S rRNA amplified using universal PCR primers (P2 and P3), from different carbon sources in Biolog GN plates separated in a (80-20%) DGGE gel.



DGGE gel separating universal PCR products obtained from DNA extracted from triplicate Biolog GN plates, after inoculation with Warwick soil fraction 1 (< 250 μm). Gels were electrophoresed for 6 h according to Section 2.6.4.

Each lane corresponds to a different carbon source on the Biolog plate; 1 = L-arabinose, 2 = Acetic acid, 3 = Tween 40, 4 = L-alanine, 5 = Methyl pyruvate, 6 = Bromo succinic acid, 7 = Inosine, 8 = Phenyl ethylamine, 9 = D, L-a glycerol phosphate, 10 = Glycerol, 11 = Succinamic acid. A = *Pseudomonas fluorescens*, B = *Streptomyces lividans*

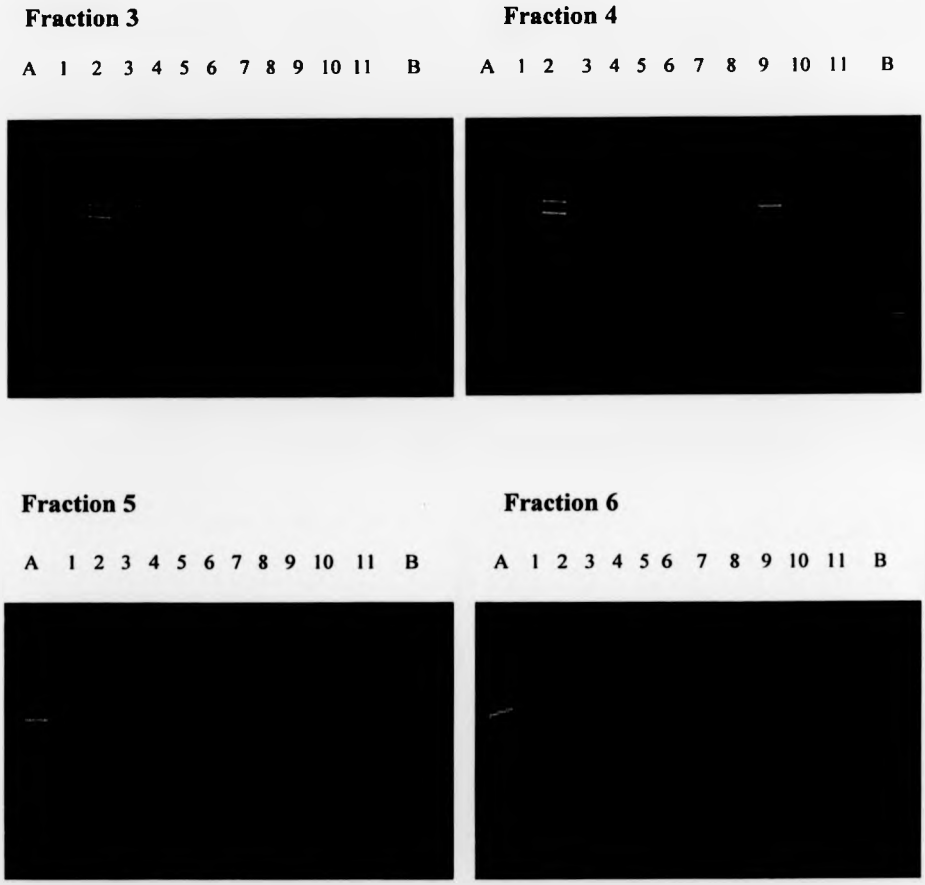
Figure 3.8. Warwick soil (fraction 2) 16S rRNA amplified using universal PCR primers (P2 and P3), from different carbon sources in a Biolog GN plates separated in a (80-20%) DGGE gel.



DGGE gel separating universal PCR products obtained from DNA extracted from triplicate Biolog GN plates, after inoculation with Warwick soil fraction 2 (63-250 μm). Gels were electrophoresed for 6 h according to Section 2.6.4.

Each lane corresponds to a different carbon source on the Biolog plate; 1 = L-arabinose, 2 = Acetic acid, 3 = Tween 40, 4 = L-alanine, 5 = Methyl pyruvate, 6 = Bromo succinic acid, 7 = Inosine, 8 = Phenyl ethylamine, 9 = D, L-a glycerol phosphate, 10 = Glycerol, 11 = Succinamic acid. A = *Pseudomonas fluorescens*, B = *Streptomyces lividans*

Figure 3.9. Different sized Warwick soil fractions, 16S rRNA amplified using universal PCR primers, from different carbon sources in Biolog GN plates separated in a (80-20%) DGGE gel.

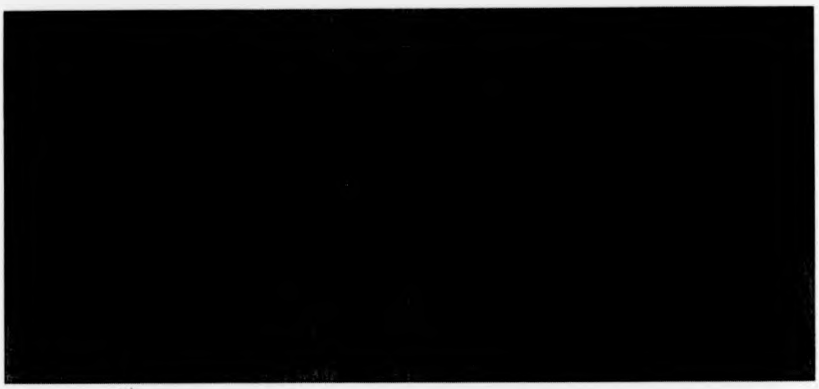


Each lane corresponds to a different carbon source on the Biolog plate; 1 = L-arabinose, 2 = Acetic acid, 3 = Tween 40, 4 = L-alanine, 5 = Methyl pyruvate, 6 = Bromo succinic acid, 7 = Inosine, 8 = Phenyl ethylamine, 9 = D, L-a glycerol phosphate, 10 = Glycerol, 11 = Succinamic acid. A = *Pseudomonas fluorescens*, B = *Streptomyces lividans*

Figure 3.10. Warwick soil fractions 1 and 2 DGGE gel hybridisation with universal probes.

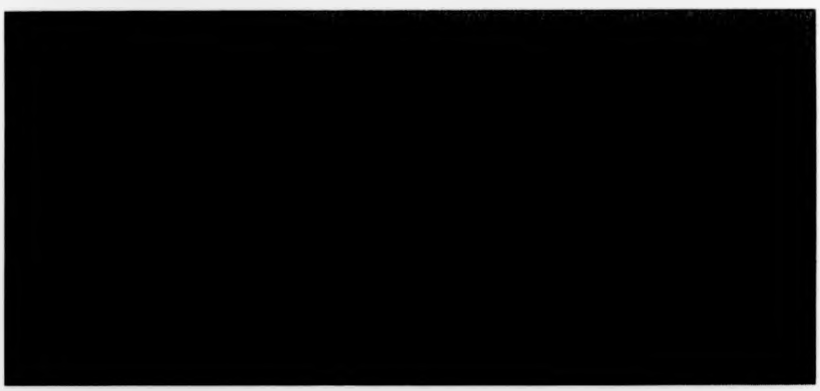
Fraction 1(>250 μm) Warwick soil, see Fig. 3.7.

A 1 2 3 4 5 6 7 8 9 10 11 B



Fraction 2 (60-250 μm) Warwick soil, see Figure 3.6.

A 1 2 3 4 5 6 7 8 9 10 11 B



Warwick soil fractions were inoculated into Biolog GN plates. DNA extractions were carried out on selected wells, and 16S rRNA amplified using universal PCR performed. The PCR products were separated using DGGE gels and Southern blotted (Section 2.7.1). Each lane corresponds to a different carbon source on the Biolog plate; 1 = L-arabinose, 2 = Acetic acid, 3 = Tween 40, 4 = L-alanine, 5 = Methyl pyruvate, 6 = Bromo succinic acid, 7 = Inosine, 8 = Phenyl ethylamine, 9 = D, L-a glycerol phosphate, 10 = Glycerol, 11 = Succinamic acid. A = *Pseudomonas fluorescens*, B = *Streptomyces lividans*

Chapter 5

The Use of Microbial Community Analysis Techniques to Monitor A Bioremediation Field Trial.

In Fig 3.8 fraction 2 was separated by DGGE and again there are fewer bands than in the original community. There does, however, appear to be more bands present in more of the carbon sources than in the fraction 1 sample. The next four size fractions had decreasing numbers of bands in the different carbon sources (Fig 3.9).

3.2.6. Probing Analysis of DGGE Gels.

To determine what bacterial groups were present in the DGGE gels, two probes were designed, one specific for Group 1 pseudomonads and the other specific for actinomycetes. The specificity of these probes was tested by hybridisations to PCR products from type strains. The probes proved to be specific for their target groups and hybridised giving strong bands. The DGGE gels were probed successfully with universal probes (Fig. 3.10). However, when the gels were stripped and probed with the specific probes, no bands could be detected, even on the pseudomonad-positive and actinomycete-positive controls. The gels were stripped and universal probes used again which successfully hybridised with the bands. This discrepancy in using specific probes on DGGE gels and agarose gels has been observed in other studies (G. Campbell, pers. comm.).

3.3. Discussion.

The wet sieving analysis of the dried and Warwick soils allowed reproducible separation of the different sized water stable aggregates. The most abundant sizes of the aggregates were the three largest sized aggregates indicating a good soil structure with high levels of macroaggregates present. The fresh and dried Warwick soil did have the same pattern of distribution of aggregates with some variation in the actual abundance of each size fraction.

There are some limitations to the wet sieving method. Sieving, sedimentation and centrifugation collect only the approximate size of soil particle, therefore the technique is not completely accurate. In the sieving steps, this can be due to some parts of the mesh being damaged by small stones allowing larger sized particles through. The sedimentation steps are based upon Stokes' law which assumes all particles will have the same density which is not the case in soil (Darbyshire, 1993). Sedimentation, however, is still used by the soil survey of Great Britain to separate soil into its component fractions. Wet sieving analysis is not a true representation of the soil *in situ*, however it allows the separation of the soil into its component size fractions. The essential factor is that all soils are treated in the same way before analysis to keep the comparisons standardised.

Different methods of sieving result in alterations to the distribution of aggregates (Beare and Bruce, 1993). Each method will have its own advantages and limitations, the best method has to be judiciously chosen to match the type analysis and level of accuracy required. When making comparisons between soil types it is important to ensure that the same method of soil sieving has been used.

Care must be taken to not damage the aggregate structure of the soil before wet sieving. The fast wetting of the aggregates may damage the structure and is possibly a limitation of this method. In future analysis, it would be advised that vapour or slow wetting of the soil before sieving would be advisable to stop disintegration of soil structures by differential swelling (Darbyshire, 1993).

In the selective plate analysis, there were significant differences in the distribution of actinomycetes and pseudomonads in the fresh soil. This may result from pseudomonads and actinomycetes having different environmental nutritional and spatial niches in the soil. It could be hypothesised that when one population was in high numbers in a location, the other species would have sub-optimal conditions.

With the culturable analysis, pseudomonads were in the highest levels in the smallest size fractions. This could be due to pseudomonads being strongly attracted to the clay particles. These small clay particles are carried through the wet sieving process and then collected in the smaller fractions with the attracted pseudomonas cells. Gestel *et al.* (1996) proposed that the <2 μm sized fractions are probably abraded from larger aggregate structures that have broken in the wet sieving process releasing the bacteria that were located in the inner areas of the aggregates.

The culturable plate counts were enumerated using selective agars with antibiotics that would have inhibited other microbial species. However, occasionally other species did appear on the plates and care was taken in the enumeration of numbers. Non-culturability will effect plate counts as not all actinomycete and pseudomonad species will be able to grow on the selective agar. Also the selective plates analysis is biased for fast growing bacterial species and therefore the counts will underestimate numbers. During soil preparation for culturable analysis, bacteria that are strongly attracted to soil particles or embedded in clumps of soil may be left in the soil matrix and not be enumerated. These limitations will occur in all of the soil fractions and valid comparisons between fractions can still be drawn.

The DGGE analysis suggested that there were different microbial communities in each of the soil fractions. There were numerous bands present in each of the lanes and this is indicative of a highly complex, diverse community with many bacterial species present at low levels (Heuer and Smalla, 1997b; Baker, 1997). This would be expected in a soil with high levels of heterogeneity and good aggregate structure. The bands present could be sequenced although when the band is present in such a complex DGGE pattern, it is often difficult to excise the band from the gel without collecting other DNA bands.

Recently molecular studies examining bacterial diversity over a macroscale have been performed (Felske and Akkermans, 1998; van Elsas *et al.*, 1998) which have concluded that bacterial diversity does not alter dramatically over field scale, depth, rhizosphere and non-rhizosphere conditions. These results are surprising as bacterial species are thought to be selected for by the environmental conditions present (Atlas, 1984).

There are many explanations for the results of the above studies it could be due to the heterogeneity of the soil system providing all possible conditions in a 100g sample. Or the methods used could very sensitive and detect all cells in the soil in very low number. However, the most likely explanation may be due to limitations of the PCR-DGGE technique, which may not be sensitive enough to detect changes in the smaller components of the community. The analysis of the macroscale may only focus on the predominant microorganisms present in the total soil. To examine the total diversity of an environment then microscale analysis, such as this study may be required

Analysis of the different size soil fractions revealed that they had significantly different levels of metabolic activity. Fraction 2 (63-250 μm) aggregates appear to have the highest levels of metabolic potential, this level being slightly higher than the total soil metabolic potential. This could be due to separation of the soil into the different sized aggregates allowing higher levels of release of the bacteria from the soil matrix. As the metabolic activity levels in the total soil and fraction 2 are similar it may be an indication that the Biolog soil preparation does not break the smaller microaggregates.

Another study used Biolog analysis to compare different soil size aggregates (Winding, 1994). This study determined that the metabolic activity was very similar in aggregates sized 2-20 μm, 20-63 μm and 63-250 μm. While the metabolic activity of aggregates sized >250 μm and <2 μm were different, they concluded indicating different bacterial communities in these fractions. This study reported the Biolog data as dendrograms of metabolic activities and so comparisons cannot be made for total activity with this study.

The Biolog system itself has a number of inherent limitations since the plate will consist of a nutrient rich, aquatic environment which is not representative of the soil system. There were a number of wells on the Biolog plate where no growth was detected whilst in the control well with no carbon source, growth could be observed. This lack of growth could be due to the carbon source being inhibitory or the nutrient rich conditions that are completely different to the soil. This system will measure metabolic potential rather than actual activity in the soil. It is a valid technique for the comparison of samples of the same type under the same conditions.

The DGGE analysis of the Biolog plates revealed that there was a considerable decrease of the number of bands present in the selected carbon sources compared to the original soil sample. This suggests that the Biolog plates are acting as an enrichment culture. This enrichment effect was expected as the Biolog plate provides completely different growth conditions compared with the soil environment. The high nutrient levels will probably select for fast growing copiotrophic organisms rather than the oligotrophic organisms that are dominant in soil (Stoztky, 1997). Different banding patterns can be observed in the different carbon source lanes on the DGGE gel possibly indicating that different populations are able to utilise different carbon sources.

With some carbon sources a colour change occurred in the Biolog plate but bands could not be detected on the DGGE gel. This lack of detection could be due to microorganisms being present at levels below the detection limit or that the DNA extraction method was not able to extract DNA from all of the microorganisms present.

There is a correlation between the Biolog analysis and the DGGE analysis of the Biolog plates. Fraction 2 was the most metabolically diverse on the Biolog plates and more bands were detected on the DGGE gel which indicates greater diversity. As the metabolic activity decreases then the genetic diversity also decreases on the DGGE gels.

It was possible (by a wet sieving method) to separate the soil into its constituent aggregate sizes. Fresh and dried Warwick soils were compared and the actual distribution patterns of aggregates was found to be the same, with some variation in the abundance of each size. Using a combination of methods, it was determined that actinomycete and pseudomonad populations are located in different regions of the soil. The different sized soil aggregates appear to have different bacterial communities. For the first time genetic molecular analysis was performed on different sized soil aggregates to compare bacterial diversity.

3.4. Future work

* To allow further analysis of the microbial community probing and sequencing of the DGGE gels would be optimised and used routinely.
* *In situ* hybridisations with actinomycete and pseudomonad specific bacteria in the soil to examine the location of the bacterial groups in the soil environment.
* The control wells from the Biolog plates could be analysed using DGGE to observe what growth could be supported just by the soil inocula.
* Different DNA extraction methods could be used to overcome the inherent biases in each of the methods.

Chapter 4

Comparison of Polluted and Non-Polluted Soils and Microcosm Studies.

4.1. Comparative Analysis of Diversity in Soils.

4.1.1. Examination of Microbial Diversity in Polluted Soils.

The examination of microbial communities in different soil samples allows comparisons of diversity and allows the determination of the distribution of certain bacterial populations in the environment. Analysis and the importance of microbial diversity in soil has already been discussed in Section 1.3. The soil microflora plays an essential role in the functioning of the soil ecosystem. A reduction in microbial diversity may have a detrimental effect on important processes such as decomposition and the cycling of nutrients which are both essential for soil fertility (Bianchi and Bianchi, 1995). Many different types of analysis exist for the comparative studies of the diversity of the different soil samples (reviewed in Section 1.4), including genetic analysis, such as fingerprinting techniques (Muyzer and Smalla, 1998), community DNA hybridisations (Torsvik *et al.*, 1990b) and %G + C profiles (Griffiths *et al.*, 1997). The activity of a community can also be determined to compare different samples. These techniques include biomass estimation (Howarth and Paul, 1994), Biolog (Garland and Mills, 1991) and ATP determination (Martens, 1985; Brooks *et al.*, 1986).

Community structure is determined by the interactions of populations existing together in a functional niche (Fenchel, 1992). The diversity of the microbial community is a reflection of the abiotic and biotic interactions present, which may enable indications of conditions in the environmental system (Bianchi and Bianchi, 1995). Pollution-induced community tolerance (PICT) profiles are based on the premise that when a community is under stress, a characteristic tolerance profile will be produced. This can then, be compared for different soil samples. The complete effects that heavy metals and other pollutants have on soils has not yet been fully elucidated (Hardman *et al.*, 1993). Usually, if a compound exerts selective toxicity on the microbial community the number of bacteria that survive will decrease. However, if the pollutant removes the dominant species, this may then increase eveness of the community. This selective toxicity is not common, and generally,

diversity will decrease when a polluting compound is added to the system. This is due to elimination of species resulting from the toxicity of the pollutant, or the enrichment of a resistant population occurs resulting in decreased eveness of the population (Bianchi and Bianchi, 1995). The examination of microbial diversity in a polluted soil can allow the identification of key bacteria which are resistant to toxic pollutants and may be able to biodegrade certain compounds. A reduction of biological activity was determined to be an indicator for the reduction of structure in soils (Garcia and Hernandez, 1997). Profiling of the microbial community in polluted soils may allow a greater understanding of which microbial genera are necessary for good soil structure and function (Heuer and Smalla, 1997b).

Heavy metals and other pollutants have two major effects on the soil microbial community. Firstly, the abundance or biomass of the community is reduced; and secondly, the diversity of the microorganisms decreases (Wuertz and Mergeay, 1997). A 50% decrease in community uptake of [^{3}H] thymidine incorporation was observed after soil was polluted with copper sulphate solution (Bååth, 1992). Toxic pollutants in the soil decrease various soil processes, including litter decomposition, carbon mineralisation, microbial respiration, nutrient cycling, nitrogen transformations and enzyme activities (Atlas *et al.*, 1991; Kandeler *et al.*, 1996).

Toxic pollutants in soils can be removed using a number of techniques, including traditional methods, such as landfill or incineration, and relatively new techniques, such as *in situ* bioremediation, land-farming and composting. These new techniques are based on stimulating the catabolic activities of the indigenous microorganisms present in the soil to degrade the pollutants (Eisermann, 1995). In land-farming systems, the polluted soil is often mixed with a non-polluted top soil and tilled on a regular basis to increase oxygenation and homogenisation of the soil. Nutrient additions and degradative inocula may be added to the system to optimise the efficiency of the process (Leung *et al.*, 1997). The addition of top soil has been found to increase populations of heterotrophic aerobic bacteria, actinomycetes, and fungi (Segal and Mancinelli, 1987). The addition of a high clay content top soil will be better able to buffer pH and may contain indigenous bacteria capable of degrading

organic compounds present (Hobbs, 1996). In Blaenavon, Wales, British Coal have employed a large scale land moving project, importing top soil in an attempt to remediate a polluted site (Humphries, 1984). To examine the effects of pollution on the natural environment a site was required that also had non-polluted areas of the same soil type, to allow comparison with the Warwick soil. A site was found at the Earth Centre, with highly polluted and non-polluted soil sites. The Earth Centre also had a number of physical and chemical surveys of the site performed.

4.1.2. The Earth Centre.

The Earth Centre is situated near Consibrough, South Yorkshire, on the site of the Cadeby and Denaby Main collieries (Section 9.3). These collieries were involved in the production of coal for over 80 years, but the Denaby Main pit was closed in 1967 and Cadeby colliery in 1986. There is a considerable pollution problem at these sites, with high levels of heavy metals and other organic compounds, such as petroleum present. The Earth Centre was established as a test area for new remediation technologies coupled with an 'ecological theme park' to educate people about recycling and other environmental issues (Fig 4.1). The effects of the high levels of pollutants were readily observed at the Earth Centre site. The soil was eroding in some areas, with evidence of gulling, soil compaction and the die-back of the already sparse vegetation (Fig 4.2). The Earth Centre was declared bankrupt in 1996, and this halted the clean-up of the area leaving large areas of polluted soil.

Colliery spoil waste is difficult to bioremediate. Usually as there are high levels of heavy metals and organic compounds present and this is often coupled with a low pH (Hobbs, 1996). Coal spoil has a low moisture content compared to agricultural soils and is also prone to compaction, which leads to a reduction in number and size of pore spaces. This reduction in pore space has a detrimental effect on water and air movement throughout the soil. The lack of pore spaces reduces the number of suitable habitats for microorganisms in the soil (Oades, 1993). There are often signs of slow combustion deep within the spoil heap and if high temperatures are reached, microbial action and survival can be inhibited.

Figure 4.1 The Earth Centre; education, entertainment and enterprise for a sustainable future.

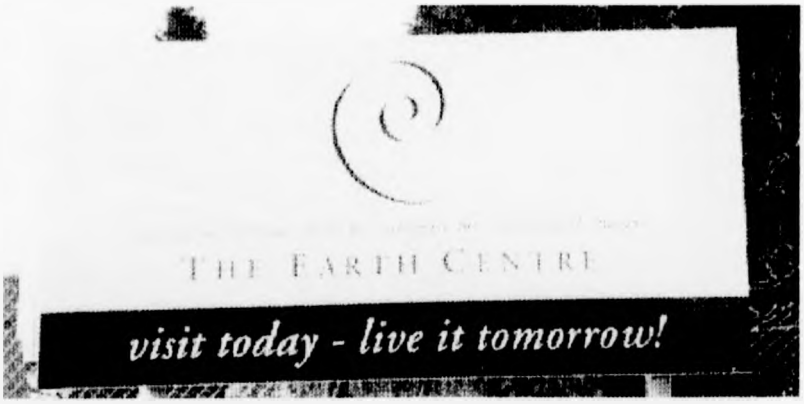


Figure 4.2. Top of the 'Whaleback'. Large patches of eroded top soil can be observed with high levels of soil compaction and the lack of vegetation.

At the Earth Centre site, most areas had a thin veneer of top soil which was rapidly being eroded, over mine-spoil. Two sites were chosen for microbial analysis, a highly polluted area called the 'Whaleback' (Fig. 4.3), and the non-polluted pony field which was located across the river Dearne (Fig. 4.4) [Section 9.2 for site descriptions]. Analysis of heavy metal content of the non-polluted site indicated that high levels of lead, iron and other heavy metals were present. This probably resulted from aerial deposits on the pony fields during the active life of the collieries (Section 9.1).

The Earth Centre was investigating ways to remediate the soil by means of bioremediation. The original purpose of this collaboration was to examine the microbial flora involved in the bioremediation of Earth Centre soil. A number of studies have been carried out to elucidate the physical properties of the site (Wild and Woodhouse, 1994; Dyckhoff *et al.*, 1995; Hobbs, 1996). But, before the bioremediation analysis began, further experiments for the validation of the methods used in Chapter 3 were performed. The analysis included the polyphasic approach being utilised to allow the discrimination between different sites, and microcosms systems were used to determine the ability to monitor temporal changes in mixtures of polluted and non-polluted soil. This allowed the development of the analysis techniques, however, due to the unforeseen closure of the site, the bioremediation monitoring was not performed.

Figure 4.3. The East side of the 'Whaleback', the polluted soil sample site.

Figure 4.4 The non-polluted soil sample site, the pony field, located opposite the mine over the river Dearne.

4.1.3. Aims

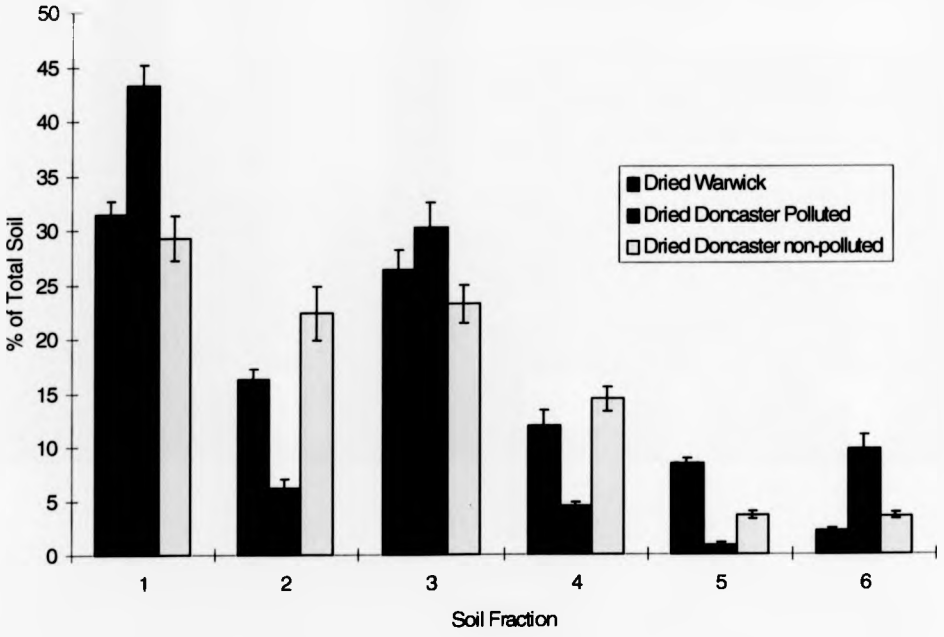
* To determine if community analysis techniques allow the discrimination between different soil communities.
* Compare and contrast the Warwick, Doncaster polluted and non-polluted soil microbial communities using culturable numbers, potential metabolic activity and taxonomic diversity.
* Examine the effects of pollution on soil structure and determine if this linked to a reduction in microbial diversity.
* Collect isolates from each soil type and examine using functional and group specific probes.
* Utilise microcosm analysis to determine how effective these methods are at monitoring temporal changes.

4.2. Results.

4.2.1. Sieving Analysis.

By means of wet sieving analysis, both Warwick soil and Doncaster non-polluted soils produced very similar distributions of water-stable soil aggregates, although the Doncaster-polluted soil had a very different soil particle distribution pattern (Fig 4.5). The Warwick soil and Doncaster non-polluted soils had the highest percentage of soil particles in the largest three fractions, with less soil in the smallest three size classes. The polluted soil had a different distribution pattern with the >250 μm and 20-63 μm fractions comprising 74% of the sample. All of the other fractions in the Doncaster-polluted soil were at low levels with the exception of the <1 μm soil fraction which accounted for 10% of the sample. This is a considerably higher percentage than observed with the other two soils for this particle size range.

Figure 4.5. Percentage composition of different sized aggregates in Warwick soil and Doncaster polluted and non-polluted soils.



Percentage composition of a 100 g of different soils, size fractionated into 6 different size aggregates; soil fraction 1= >250 µm, fraction 2= 63-250 µm, fraction 3= 20-63 µm, fraction 4= 2-20 µm, fraction 5= 1-2 µm, fraction 6= <1 µm.

The error bars shown represent the standard error of five replicates.

One-way ANOVA statistical tests were applied to the size fractionation analysis in order to determine whether significant differences exist between the soil particle distributions within each of the different soils. Warwick ($F_{5,24}$ = 94.29, p = 2.6), Doncaster polluted ($F_{5,24}$ = 145.9, p = 2.6) and non-polluted soils ($F_{5,24}$ = 46.58, p = 2.6) had significant differences in their soil particle distribution. One-way ANOVAs were used to test for significant differences between soil particle distribution in the Warwick Doncaster polluted and non-polluted soils (Table 4.1). A significant difference was observed between all soil fractions with the exception of the soil fraction measuring 20-63 μm. This analysis revealed that the different soils had different quantities in each of the size fractions and that five out of six particle sizes had significantly different amounts in each fraction.

Table 4.1. Tests for significant differences using one-way ANOVA to determine if the Warwick, Doncaster polluted and non-polluted soils have significantly different particle distributions at p< 0.05.

Soil fraction size (μm)	Difference between the Warwick soil, Doncaster polluted and non-polluted soils particle distribution.
> 250	18.1
63-250	26.1
20-63	***3.2**
2-20	22.4
1-2	93.9
<1	24.7

F critical value determined as $F_{2,12}$ = 3.9

* Non-significant result.

4.2.2. Selective Plate Counts.

Selective plate counts were used as described in Section 2.4.1, with pseudomonad, actinomycete, and total counts performed. The plate count data showed culturable numbers from the Warwick and the non-polluted soils were very similar (Fig 4.6 and Fig 3.4), whilst the polluted soil had a reduced culturable component (Fig 4.6). Some general trends could be observed in the distribution of pseudomonads and actinomycetes in the three soils (Fig 4.6 and Fig 3.4). The pseudomonads were present in higher numbers in the smaller fractions, whilst the actinomycetes were present in higher numbers in the larger size particles in all three of the soil types. Statistical tests were used to determine if a significant difference existed between the location of different bacterial groups in the different soil fractions (Table 4.2). From this analysis significant differences were detected in the distribution of both pseudomonads and actinomycetes in the polluted and non-polluted Doncaster soil. Only actinomycete distribution in the Warwick soil, was significantly different. No significant differences were observed in the total counts in the three soils.

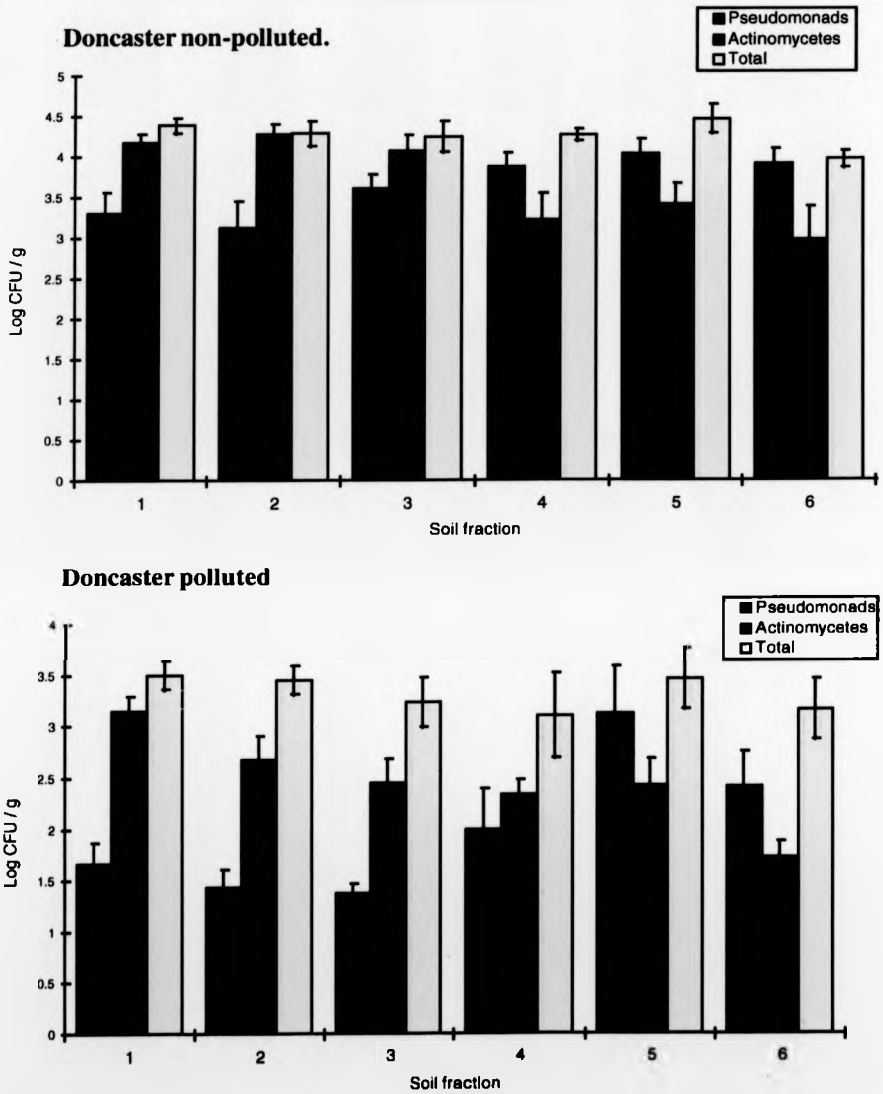
Table 4.2. Test for significant differences using one-way ANOVA to determine if the Warwick, Doncaster polluted and non-polluted soils have different bacterial distributions in different size soil fractions at $p < 0.05$.

Selective plates	Warwick soil	Doncaster non-polluted	Doncaster polluted
Pseudomonads	***0.7**	2.7	4.7
Actinomycetes	3.4	4.5	5.3
Total	***2.5**	***1.9**	***0.4**

F critical value determined as $F_{5,24} = 2.6$

* Non-significant results.

Figure 4.6 Selective plate counts using non-polluted and polluted Doncaster soil.



Selective plate counts using different aggregate sizes from the three different soils. Fraction 1 = >250 µm, fraction 2 = 63-250 µm, fraction 3 = 20-63 µm, fraction 4 = 2-20 µm, fraction 5 = 1-2 µm, fraction 6 = <1 µm. The error bars shown represent standard error of five replicates.

Statistical tests were used to analyse if there were significant differences between the distribution of each bacterial group within different size soil fractions (Table 4.3). Pseudomonad counts on selective agars were significantly different in all of the soil fractions with the exception of the smallest particle size. Actinomycete counts were significantly different in the three soils in each size fraction, with the exception of the 2-20 μm and 1-2 μm fractions. The total counts were significantly different in all three soils in all fractions with the exception of the 1-2 μm soil particle size fraction.

Table 4.3. Tests for significant differences using one-way ANOVA to determine if bacterial counts are different in different size fractions in the Warwick soil, Doncaster polluted and Doncaster non-polluted soils at $p< 0.05$.

Soil fraction size (μm)	Pseudomonad counts	Actinomycetes counts	Total counts
> 250	8.1	13.1	18.2
63-250	5.5	22.7	13.4
20-63	15.9	20.5	78.7
2-20	4.5	***3.5**	11.1
1-2	4.4	***3.0**	***2.3**
<1	***3.3**	5.9	9.9

F critical value determined as $F_{2, 12} = 3.8$

* Non-significant results.

4.2.3. Metabolic Activity Analysis of the Different Sized Soil Fractions.

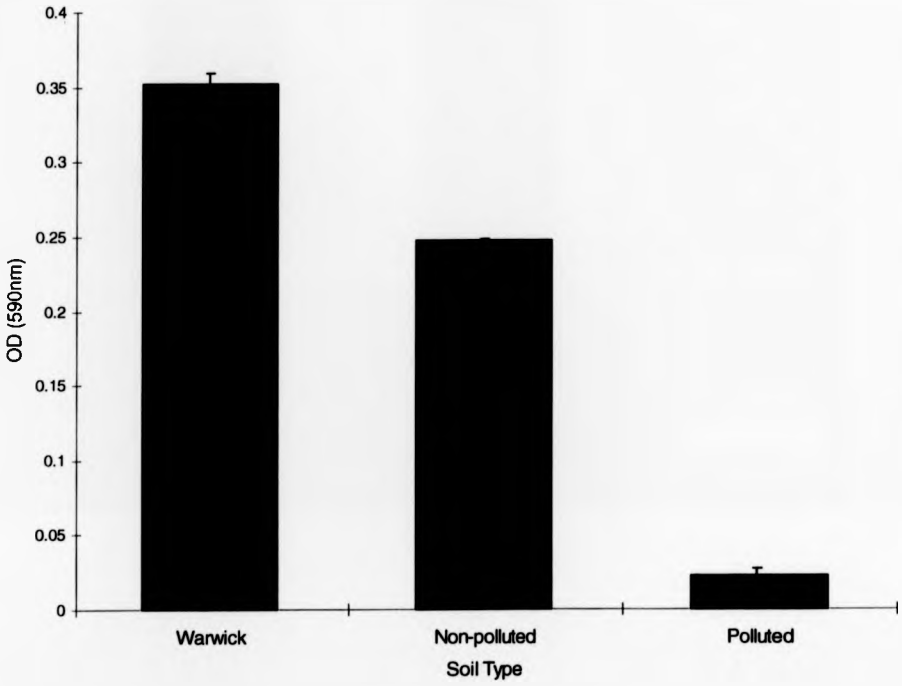
Potential metabolic activity was assessed in the three different soils by means of Biolog analysis, determining the AWCD for each sample. The Biolog analysis revealed very clear reproducible differences in the three soil types (Fig 4.7). The Warwick soil had a higher metabolic potential than the other soil types. The Doncaster non-polluted soil had lower rates of AWCD than the Warwick soil. While the Doncaster polluted soil revealed a limited metabolic potential with a much lower AWCD. The analysis was highly reproducible within the triplicate plates for each soil type (Fig 4.7). Statistical tests, employed to determine the significance of differences between the AWCD of the three different soil types, indicated that highly significant differences exist between the potential metabolic activities of the three soil types ($F_{2,6} = 955.4$, $p = 5.1$).

4.2.4. Genetic Diversity Analysis of the Six Different Size Fractions of the Warwick, Doncaster Polluted and Doncaster Non-Polluted Soils.

Bacterial communities within the different sized soil fractions in the three soils were examined for taxonomic diversity using molecular methods. The total community DNA was extracted from each of the fractions and then used as a template for 16S rRNA gene PCR using universal primers. These products were then separated by DGGE analysis. Warwick soil fractions have already been examined in Section 3.2.3 and Fig. 3.5. To summarise, the Warwick soil produces a high number of bands throughout the gel lane resulting in a smear. It was also determined that the different size fractions contained distinct and different bacterial communities.

The Doncaster polluted and non-polluted soil fractions were also examined by the DGGE technique (Fig 4.8). The Doncaster non-polluted sample was similar to the Warwick soil; a characteristic smear being produced with many bands detected.

Figure 4.7. The average well colour development on Biolog plates using Warwick, Doncaster polluted and non-polluted soils.



Biolog analysis of the three different soil types. The error bars shown represent the standard error of three replicates.

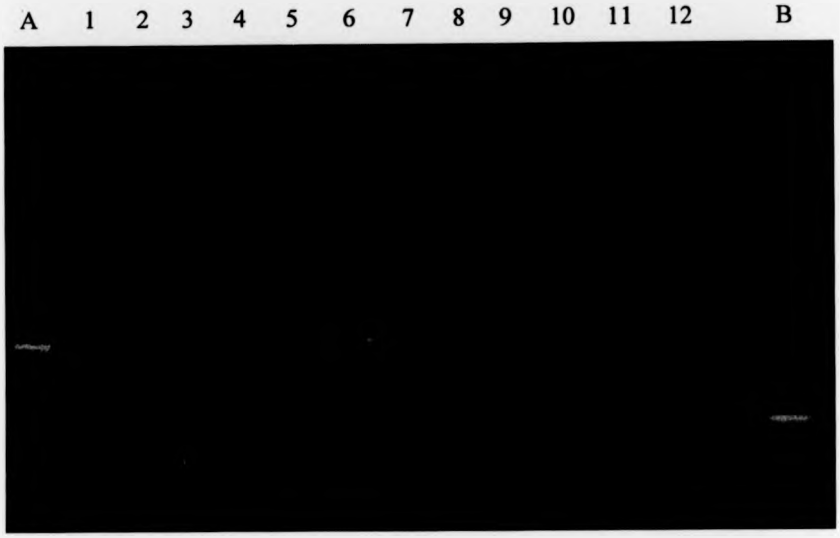
The Doncaster non-polluted soil produced considerably fewer bands, with only a few discrete bands being detected by DGGE analysis. The various soil size fractions in each of the three soil types were host to different microbial communities. The Doncaster non-polluted soil produced on DGGE gels high intensity bands which were located in the same region as the pseudomonad marker in the smallest size fraction of soil particles and bands parallel to the actinomycete marker in the largest soil fractions. Attempts were made to probe the DGGE gels with group-specific probes, but this was unsuccessful (Section 3.2.6).

Group specific PCR primers were used for the examination of actinomycetes and pseudomonads in the different soils and microcosm. The 16S gene products after PCR, were analysed by DGGE analysis. However, later analysis with type strains determined that the actinomycete-specific primers (Heuer *et al.*, 1997) were not specific. There were also considerable problems with the pseudomonad-specific primers and single bands for the PCR were often unobtainable for further DGGE analysis, in this study.

4.2.5. Analysis of Bacterial Isolates from the Different Soils.

From each of the three soil types, an isolate collection randomly collected from the actinomycete selective plates, was assembled consisting of 200 strains from each soil. DNA extractions were performed for forty randomly chosen strains from each soil. Universal 16S rRNA genes were amplified using universal primers for the three soil types. It was extremely difficult to extract DNA from the polluted soil isolates in comparison with the non-polluted and Warwick soils. Subsequent amplification with universal 16S rRNA primers was also unsuccessful for the majority of the strains from the polluted soil. Primers for the naphthalene dioxygenase gene (Herrick *et al.*, 1993) were used to amplify products from the Warwick and Doncaster polluted soil, but due to difficulties with the DNA extractions from the isolates originating in the polluted soil no comparisons could be made between the three soils.

Figure 4.8. DGGE of the six different sized aggregates in polluted and non-polluted Doncaster soil, 16S rRNA amplified using universal primers.



A = *Pseudomonas fluorescens*, B = *Streptomyces griseus*. Doncaster non-polluted soil 1 = > 250 μm, 2 = 63-250 μm, 3 = 20-63 μm, 4 = 2-20 μm, 5 = 1-2 μm, 6 = < 1 μm.

Doncaster polluted soil 7 = > 250 μm, 8 = 63-250 μm, 9 = 20-63 μm, 10 = 2-20 μm, 11 = 1-2 μm, 12 = < 1 μm.

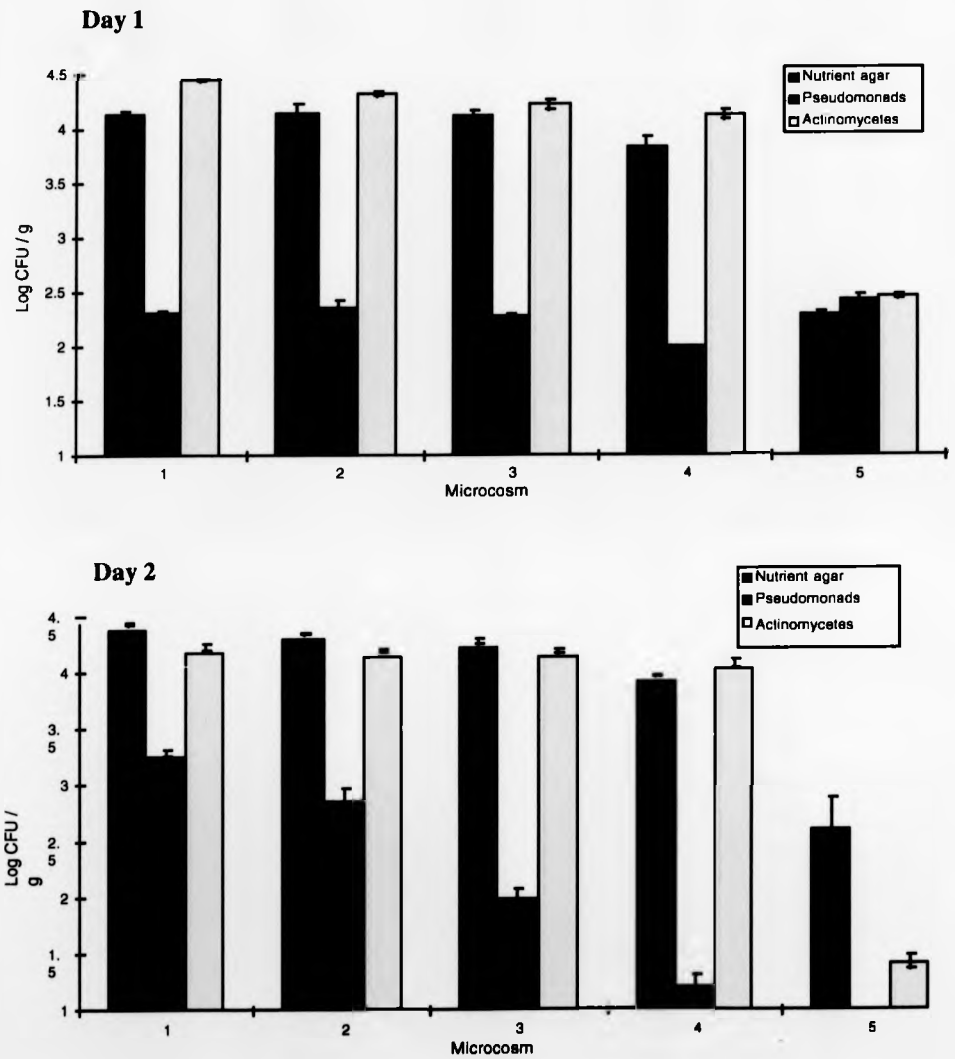
4.2.6. Microcosm Analysis.

Microcosms were prepared according to Section 2.9. Different amounts of polluted Doncaster soil and Warwick soils were mixed together (Fig 2.1). To establish the effects on the microbial community and to monitor any changes that may arise in the genetic diversity of the microcosm systems over time. It was hypothesised that different combinations of polluted and Warwick soil would result in the detection of changes in microbial communities, with a correlation being found between increasing amounts of polluted soil and decreasing diversity. It was postulated that potential metabolic activity would also decrease with increasing levels of polluted soil. The microcosms facilitated the examination of microbial communities in a laboratory based system where conditions could be manipulated and controlled.

4.2.6.1. Microcosm Selective Plate Analysis.

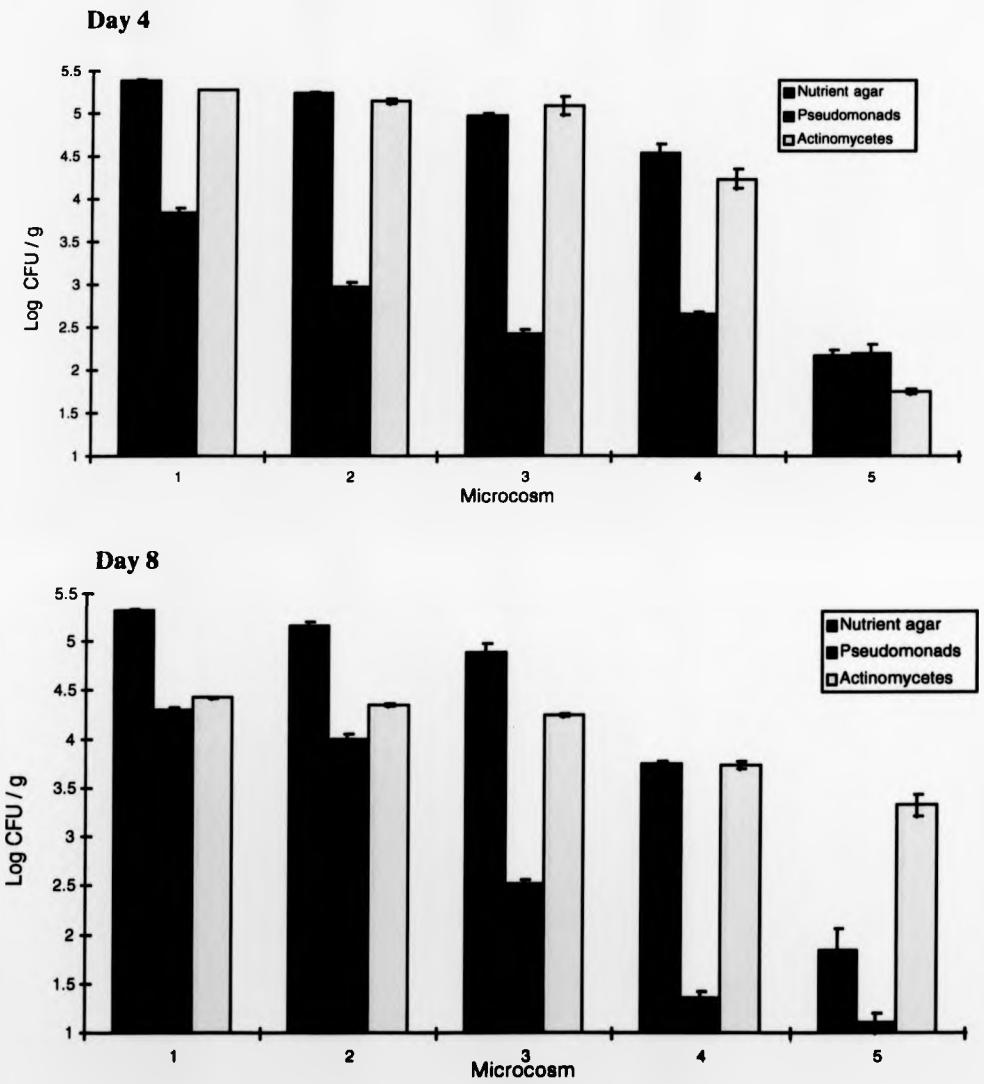
Selective plating was used to monitor changes in the microbial community present in the microcosm systems. On day 1, when the soil in the microcosms was first combined, the counts were very similar in all of the microcosms with the exception of M5, which contained 100% polluted soil (Fig 4.9). The total culturable and actinomycete-counts were very similar whilst pseudomonad-counts were lower in all the microcosm systems. Counts performed on day 2 were very similar to day 1, although in microcosms 4 and 5 a reduction in pseudomonad numbers was observed (Fig 4.9). On day 4, the numbers of culturable bacteria increased in all microcosms with pseudomonads being detected at higher numbers in microcosms 4 and 5 (Fig. 4.10). The samples taken on day 8, were similar to day 4 in culturable count numbers, although an increase in actinomycete propagules in microcosm 5 and pseudomonad numbers in microcosms 1 and 2 was observed (Fig 4.10). The samples taken on days 16, 32 and 80 were similar, with the same general patterns being observed at each date (Fig. 4.11 and 4.12).

Figure 4.9. Selective plate counts at days 1 and 2, on five microcosms containing differing amounts of Warwick and Doncaster polluted soil.



Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted. The error bars shown represent the standard error of three replicates.

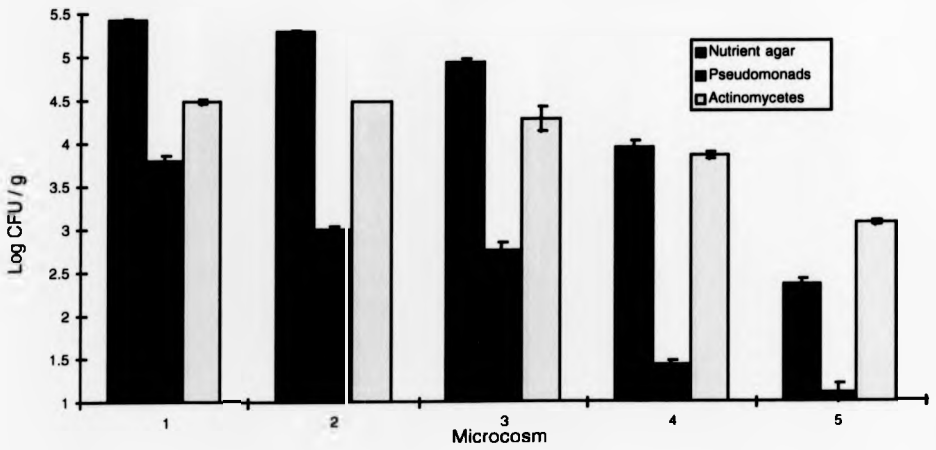
Figure 4.10. Selective plate counts on day 4 and 8, on five microcosms containing differing amounts of Warwick and Doncaster polluted soil.



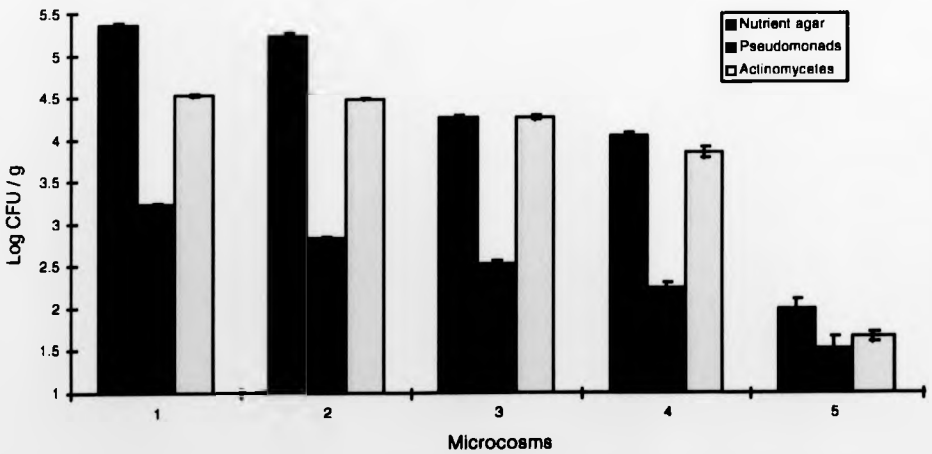
Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted. The error bars shown represent the standard error of three replicates.

Figure 4.11. Selective plate counts on days 16 and 32 in the five microcosms containing differing amounts of Warwick and Doncaster polluted soil.

Day 16

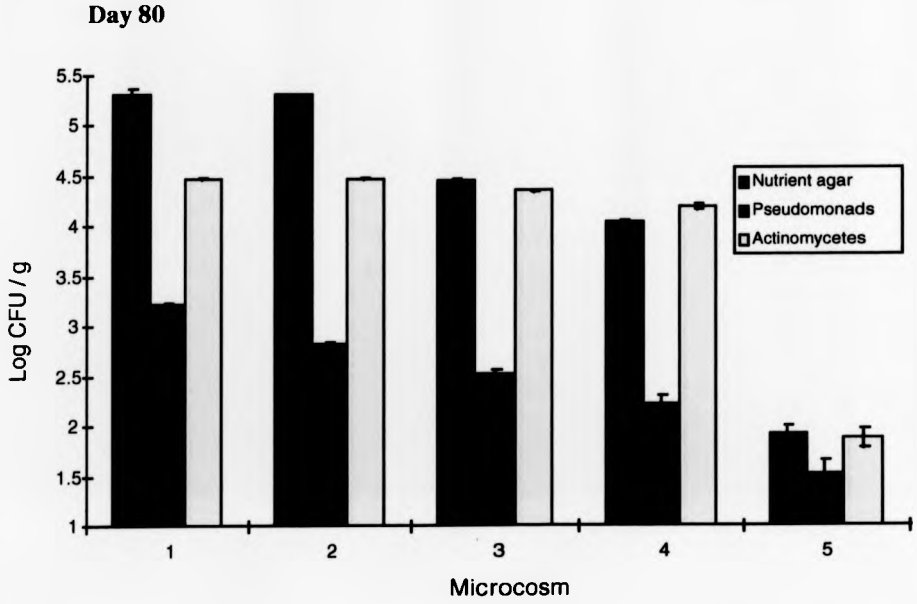


Day 32



Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted. The error bars shown represent the standard error of three replicates.

Figure 4.12. Selective plate counts on day 80 in the five microcosms containing differing amounts of Warwick and Doncaster polluted soil.



Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted. The error bars shown represent the standard error of three replicates.

These general trends included the number of total culturable microorganisms gradually decreasing with increasing levels of polluted soil and the actinomycete numbers also following this pattern with propagule numbers similar to the total counts. The numbers increase over time in microcosms 1, 2 and 3 in all three selective counts (Fig. 4.13).

Statistical analysis, was used to determine if there were significant differences over time in each microcosm and between the different microcosm types on the selective counts. There were no significant differences over time for each selective count within the different microcosms [pseudomonads ($F_{6,25,}$ = 1.1, p = 2.4), actinomycetes ($F_{6,25,}$ = 0.8, p = 2.4), and total counts ($F_{6,25,}$ = 1.2, p = 2.4)]. However, significant differences were observed in the culturable counts between the different microcosms systems (Table 4.4).

Further statistical analysis was employed to determine if there were significant differences between the three bacterial counts in each of the microcosms at each sample date. The one-way ANOVA tests revealed that there were significant differences in three out of the seven sample dates, with these being at days 1, 2 and 16 (Table 4.5).

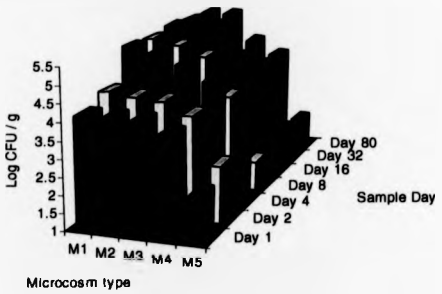
Table 4.4. Tests for significant differences using one-way ANOVAs to determine if the selective counts are significantly different between the five microcosm systems at p< 0.05.

Selective plates	Significant differences between plate counts in different microcosm systems.
Pseudomonads	14.1
Actinomycetes	37.2
Total	61.1

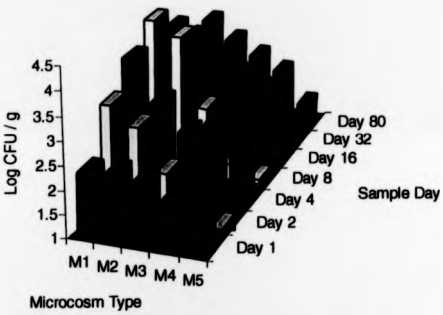
F critical value determined as $F_{4,30}$ = 2.7

Figure 4.13. Bacterial counts on selective agars using soil from different microcosms containing different levels of Warwick and Doncaster polluted soils.

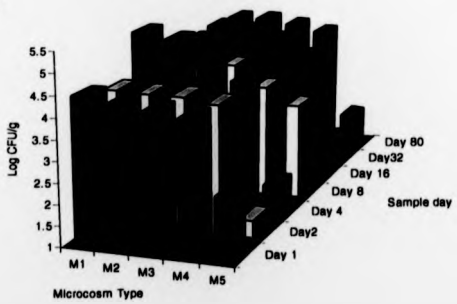
Total culturable counts



Pseudomonads



Actinomycetes



Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted

Table 4.5. Tests for significant differences using one-way ANOVA to determine if the three selective counts are different between each date in the different microcosm systems at $p < 0.05$.

Sample day	Differences between the three bacterial counts in the microcosms
1	8.9
2	4.8
4	***2.8**
8	***2.4**
16	5.1
32	***3.2**
80	***3.6**

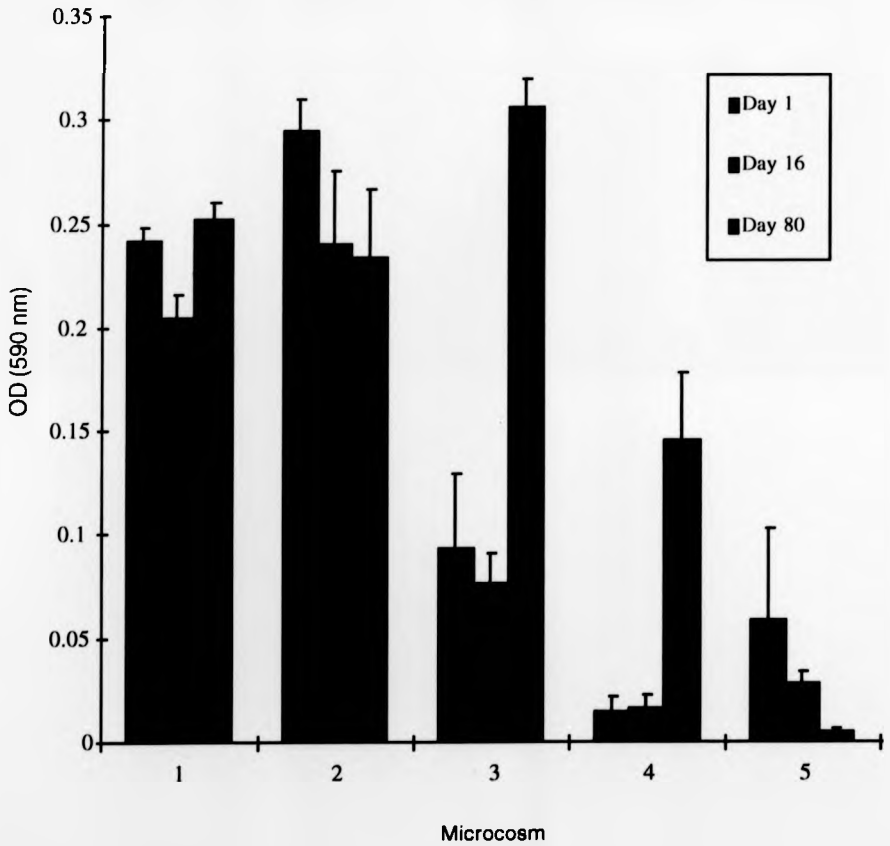
F critical value determined as $F_{2, 12} = 3.8$

* Non-significant results.

4.2.6.2. Microcosm Biolog Analysis.

Biolog analysis was used to examine the metabolic potential of the microbial communities in the microcosm systems. The AWCDs for the Biolog plates were recorded for days 1, 16 and 80 (Fig 4.14). The AWCDs at days 1 and 16 were similar in microcosms 1 and 2 with both having higher levels of metabolic activity than the other three systems. Microcosm 2 had a higher AWCD than microcosm 1 on both days 1 and 16. However, the day 80 sample analysis revealed a very different trend, microcosm 3 having the highest metabolic activity with a marked increase in comparison with days 1, 16 in microcosm 4. High levels of activity were still detected in microcosms 1 and 2 at day 80. Statistical tests to determine if significant differences existed between the microcosms at each date were performed (Table 4.6), indicated that the AWCD was significantly different in the three soils.

Figure 4.14. AWCD in Biolog Plates for Microcosms containing different amounts of Warwick and Doncaster Polluted soil.



Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted. The error bars shown represent the standard error of three replicates.

Analysis was also performed to determine if there were significant differences in each microcosm over time (Table 4.7), this indicated that there were significant differences in microcosms 3 and 4 over time, but not in 1, 2, or 5.

Table 4.6. One-way ANOVA tests performed to determine if the AWCD are significantly different in each microcosm systems at $p< 0.05$.

Sample day	Statistical differences in AWCD in the different microcosms
1	20.4
16	32.5
80	9.0

F critical value determined as $F_{4,10} = 3.5$

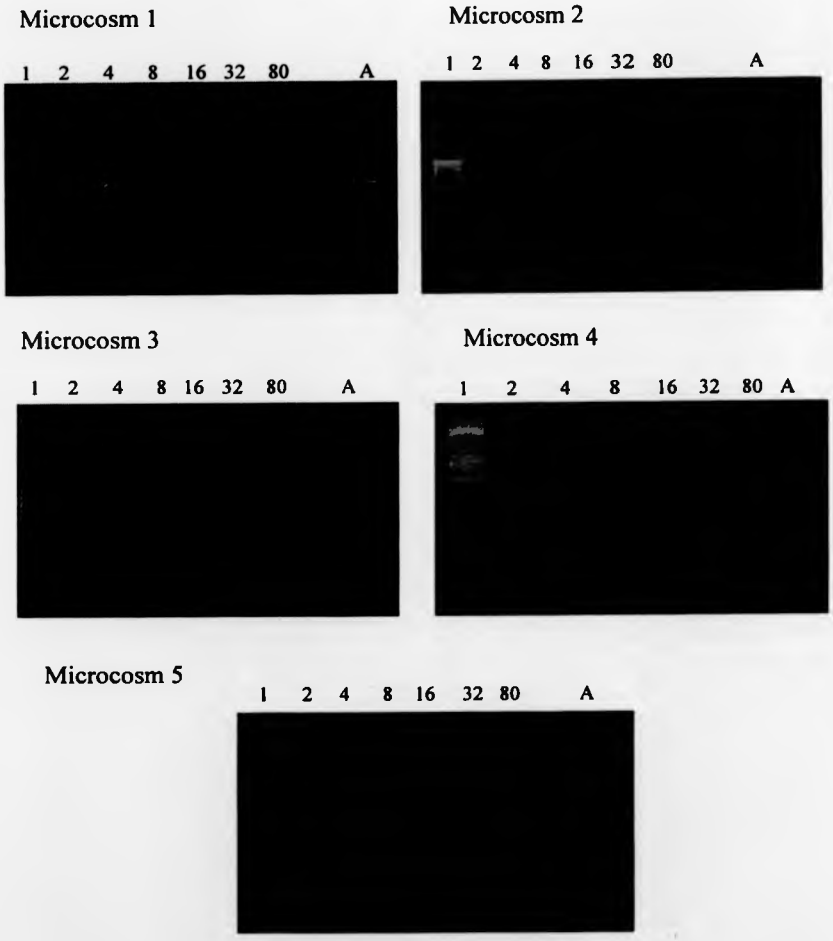
Table 4.7. One-way ANOVA tests performed to determine if AWCD changes significantly over time in each microcosm system at $p< 0.05$.

Microcosm	Statistical differences in AWCD over time
1	***0.3**
2	***1.3**
3	28.9
4	14.6
5	***1.1**

F critical value determined as $F_{2,6} = 5.1$

* Non-significant results.

Figure 4.15. 16S rRNA amplified with universal primers, from all five microcosms, sample days 1 to 80 separated on a (80-20%) DGGE gel.



A = *Pseudomonas putida* type strain. Microcosm type; M1 = 100% Warwick soil, M2 = 75% Warwick, 25% Doncaster polluted, M3 = 50% Warwick, 50% Doncaster polluted, M4 = 25% Warwick, 75% Doncaster polluted, M5 = 100% Doncaster polluted

4.2.6.3. Microcosm DGGE Analysis.

Total community DNA was extracted from the five different microcosm systems at each sample date. These DNA extractions were used as the template for 16S rRNA universal PCR reactions. The PCR products were separated on DGGE gels and compared (Fig. 4.15). Microcosm 1 had the highest number of discrete detectable bands in all samples. A decrease in the number of bands was observed in the microcosms with increasing ratios of Doncaster to Warwick soil, and a decrease was detected over time. This decrease in bands may have been due to a decrease in diversity with increasing amounts of the polluted soil. This decrease in bands was also apparent over time. Taxonomic diversity varies between the soil mixtures. Attempts were made to probe the gels with group specific probes, but this was unsuccessful (Section 3.2.6).

4.3. Discussion.

Wet sieving analysis of the three soils indicated that the Doncaster non-polluted soil and the Warwick soil had very similar distributions of water-stable aggregates, whilst the polluted soil had a very different pattern. It can be hypothesised that, bacteria play an essential role in the formation of aggregate structures by releasing polysaccharides and other adhesive compounds, which bind soil particles together. Therefore decreasing bacterial numbers may result in lower amounts of water-stable aggregates. The production of polysaccharides and other compounds is linked to the activity of the microorganisms which is, in part, determined by the microbes habitat in the soil (Young and Ritz, 1998). If soil structure is poor, therefore, the number of suitable niches will be lowered, reducing the numbers of active bacteria in the soil (Hattori, 1973). Garcia and Hernadez (1997), used biological indicators, such as enzyme activity, biomass and basal respiration to demonstrate a strong correlation between biological activity and good soil structure and function.

The polluted soil had a high proportion of large particles (mostly small stones and shale) or fine dust. The mid-range aggregates appeared to have been lost from this soil system. It has been previously shown that microbial activity in the mid-range aggregates was high (Kandeler and Murer, 1993). In the polluted soil, the low proportions of these mid-range aggregates is probably due to the poor nutrient status of the soil, combined with the lack of microaggregates to provide a suitable environment for microbial growth and activity (Young and Ritz, 1998). There are small differences in the distribution of the aggregates in Warwick and the non-polluted Doncaster soil which may result from low-level pollution being present in the non-polluted soil (Table 9.1). There is also likely to be differences in the chemical and physical characteristics of the two soils. The wet sieving technique had a number of limitations which must be considered, as discussed in Section 3.3.

The examination of the culturable fraction of the population revealed a number of trends in the three different soils. Namely that pseudomonads appeared to be in highest numbers in the smallest size fractions whilst actinomycetes were in the highest numbers in the largest soil particles (this was observed in Chapter 3). There were significant differences in the distribution of pseudomonads in all soils and actinomycetes in both Doncaster soils. This may result from pseudomonads and actinomycetes having different environmental, nutritional and spatial niches in the soil. It can be hypothesised that, when one population is in high numbers in a location, the other population would have sub-optimal conditions. The high levels of pseudomonads in the smallest fractions may be due to strong attraction / attachment to the clay particles. Wilcke and Kaupenjohann (1997), found that heavy metals were located on the outer regions of the aggregate rather than the interior. This may influence the location of the bacteria in soil, with sensitive bacteria receiving protection from the heavy metals inside the aggregate structure.

The plate counts were similar for the Warwick and the Doncaster non-polluted soil, whilst the polluted sample had considerably lower culturable counts. This drop in culturable propagules in the polluted soil was probably due to the toxic compounds exerting a selective pressure of the bacterial community, therefore bacterial numbers

were reduced. Culturable plate count analysis has a number of inherent limitations as discussed in Section 3.3. These include problems of non-culturability of soil microorganisms or the difficulties in releasing all cells from the soil matrix (Wellington *et al.*, 1997).

Biolog analysis examined the potential metabolic activity of the soil samples and allowed comparisons of AWCDs for the three soils. The Biolog results were reproducible and indicated that the Warwick soil appeared to have the highest metabolic activity in comparison with the two Doncaster sites. Of the two Doncaster soils the non-polluted soil had higher levels of activity.

The DGGE analysis of the six different fractions of the three different soils indicated that different bacterial communities are located in the different sized soil particles. The Doncaster non-polluted fraction had numerous bands, appearing as a characteristic smear of bands throughout the gel. This also was observed with the Warwick samples (Baker, 1997) and this study (Section 3.2.3). The Doncaster-polluted samples had far fewer bands present. In the Doncaster-polluted soil, there was a correlation between the selective plates and the DGGE analysis. A strong band in the region of the *P.fluorescens* marker was obtained in the smallest size soil particles by DGGE analysis, which corresponded to high culturable pseudomonad counts. Further sequencing or probing will be necessary to validate this. Taxonomic analysis indicated that diversity decreases with increasing levels of polluted soil. This may be due to increasing selection pressure for microorganisms which are capable of surviving in the presence of high levels of pollutant (Wuertz and Meargey, 1997).

Microcosms were used to determine the effects of mixing the Doncaster polluted soil with Warwick soil and examining the changes in the microbial community using the polyphasic approach developed in Chapter 3. It was important to assess whether the changes observed in the soil microcosm were due to abiotic or biotic factors of the soil. This could be examined by further microcosm experiments with sterile soils to determine which effects are abiotic and which are due to biotic factors.

Selective plate count analysis from the microcosms revealed the same general trends in all sample days, with increasing pollution resulting in a decrease of the amount of culturable bacteria in the sample. This was observed in all three counts, but the most pronounced reduction in numbers in the microcosms was that of pseudomonads. The reduction in numbers that were observed in all three counts in the five microcosm system were significantly different. This indicated that the levels of pollution had a detrimental affect on the numbers of culturable bacteria present in the soil. At day 2 in microcosm 5 (100% polluted soil) the numbers of actinomycetes and pseudomonads decreased rapidly. This was unexpected since the soil only had water added and not polluted soil. The reduction in numbers may be due to an anomaly in the plate counting procedure, or it is possible that the addition of the water had a detrimental effect on the bacterial population present, although this is unlikely. Overall pseudomonad counts appeared to be affected by the polluted soil more than the actinomycete or total counts. This may be due to actinomycete counts including spore-forming bacteria, which are dormant in the soil germinating when plated on suitable media. Or the high numbers may be due to the presence of pollution tolerant *Rhodococcus* species which may be able to degrade the pollutants (Finnerty, 1992).

The bacterial culturable counts on selective media in microcosm 1 were similar to those observed in Warwick soil (Chapter 3) indicating that numbers were not reduced due to the experimental system. In all three selective counts, there appeared to be a peak of culturable numbers in microcosms 1, 2 and 3 from day 8. This increase in numbers may have been due to the availability of water supporting increased growth and it would be expected that this would decrease when the nutrient sources became limiting. The microcosms were kept as representative of the environmental system as possible with no nutrient addition. It would, however be of interest to observe the effects of nutrient addition in the microcosm system, especially as many land-farming processes have a nutrient-addition system (Muller-Markgraf, 1995).

An unexpected result from the microcosm analysis was provided from the potential metabolic activity analysis. Days 1 and 16 had very similar patterns of AWCD in

each of the five microcosms. There was an increase in metabolic potential in microcosm 2 (which has 25% polluted soil) and this stimulation of metabolic activity may result from the polluting compound containing high levels of an essential inorganic compounds (such as iron) which may be of limited supply in Warwick soil (Neidhardt *et al.*, 1990). Samples from day 80 revealed a very different profile, with high levels of metabolic activity in microcosm 3, this microcosm having equal amounts of Warwick and polluted soil. This increase in metabolic potential may be due to members of the bacterial community becoming adapted to the new conditions and possibly gaining the ability to utilise the new carbon sources available (Frostegård *et al.*, 1993). There were significant differences in AWCD in the different microcosm systems at each sample date. The taxonomic analysis of the soil microcosm systems revealed a decrease in diversity with increasing amounts of polluted soil. The banding patterns also decreased with time over the 80 days. This was may be due to the decrease in nutrients available in the microcosms.

Soil microcosms can be used to study interactions in the soil under controlled conditions. However, it is these controlled conditions that prevent the microcosm system from being a realistic model for the soil environment. Various factors restrict microcosm utility, including that in the environment nutrient inputs are usually slowly released in low levels also in the microcosm system soil structure may be limited (Prosser, 1997). Soil insects and animals (such as earthworms) are not normally included in the microcosm systems, but these are essential for aeration and the movement of nutrients in natural systems (Bardgett and Griffiths, 1997). After dry sieving the soil to remove stones and break up the large aggregate structures, there will be an increase in the amount of nutrients available from fine roots, fungi and dead bacterial cells (Richards, 1994). Soils also undergo seasonal changes, with wetting and drying cycles and small changes in temperature. These factors must be considered when analysing the results from microcosm systems, before applying the results to environment (Paul and Clark, 1989). Before mixing the soil together for the microcosm systems, the soil was sieved through a 2 mm mesh sieve to remove any large aggregate structures. However, at the microscale, the soil may not be

completely mixed and so microbial communities may exist in microaggregates of the unmixed soil with little interaction with the other soil type.

Sieving analysis was not carried out on the microcosm systems, as the systems would have to be greatly increased in size to allow replicate analysis of each microcosm. This increase in size would cause the microcosm to becomes a less homogenous system resulting in difficulties with sampling representatively. The microcosm systems would not contain all of the required abiotic an biotic factors for soil structure formation, therefore, valid comparisons could not be made. In this experiment batch microcosms were used in triplicate and sampled at set time points. This enabled an examination of the microbial community over time. However, sampling disturbs the soil system and may alter the microbial community. At the onset of the experiment, there will be a different ratio of soil to microcosm surface and this will alter with the removal of soil for sampling. The addition of water also changed with sampling, at the start of the experiment with more soil in the microcosm water may be less evenly distributed. To overcome these problems destructive microcosm systems could be used although a large number of microcosm would have to be constructed to enable analysis over all the sample dates.

The three methods utilised in the polyphasic approach correlated in many cases. All of the methods indicated lower levels of microorganisms with increasing levels of polluted soil. Plate counts using selective media and the DGGE analysis both indicated that there were differences in the microbial communities present in the different sized soil fractions in the three soils. As in Chapter 3 there appeared to be a correlation between the selective plate counts and the genetic analysis although further analysis of the DGGE gels is required before these data can be substantiated. The microcosm analysis did reveal changes in the bacterial communities over time. There also appeared to be a stimulation of potential metabolic activity by the addition of low amounts of polluted soil to the Warwick soil. This may be due to the high levels of many inorganic compounds in the polluted soil, which may be limited in the Warwick soil. The polyphasic approach in this current study allowed the

discrimination and comparison of the three soils and the microcosm studies enabled the validation of these techniques to monitor temporal changes.

4.4. Future work

* The microcosm experiments could be repeated with sterile polluted soil to determine what effects were caused by the abiotic factors, rather than biotic relationships.
* To further analyse the DGGE gels sequencing could be performed to identify the dominant bacteria present in the system.
* The metabolic potential of the six different size fractions in the two Doncaster soils could be examined by Biolog analysis.
* PCR analysis of the isolate collections could be continued and screening of the isolates with specific and group probes, to allow a deeper understanding of the microbial community present.
* Microcosm studies using different conditions, including the addition of nutrients, this would provide further insight into diversity levels in the microcosm systems.

5.1. Bioremediation.

A large number of polluted areas now exist in Britain due to the increasing volumes of industrial wastes being produced. It was estimated that in 1989 there were 4800 current and disused toxic waste disposal sites in the UK (Rose, 1990). Traditional methods for the disposal of pollutants include landfill, incineration and other physical processes, which are rapid, predictable and inexpensive (Eisermann, 1995). Physical methods involve the extraction and concentration of the pollutant and its subsequent removal to another site, but not the breakdown of the pollutant (Head, 1998). An alternative to the physical techniques is the process of bioremediation, which can be defined as the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous (Ewis *et al.*, 1998). Bioremediation is an attractive process as it involves the destruction of many organic contaminants, and often less harmful intermediate / end point compounds are generated. The bioremediation process can provide a permanent resolution to contaminant problems for organic compounds. The process is economically viable, and when carried out *in situ* avoids problems which are inherent in transporting contaminated land to landfill (Muller-Markgraf, 1995). Bioremediation methods can be used to alter the valence state of some metals. Changes in valence state can reduce toxicity and solubility of many inorganic compounds (Madsen, 1998). This concentration of inorganic pollutants facilitates the removal of pollutants from the site to an area of waste storage (Ewis *et al.*, 1998).

Biodegradation can be defined as the partial or complete destruction of the molecular structure of an environmental pollutant by a physiological reaction performed by microorganisms (Madsen, 1998). These breakdown reactions are natural processes that is responsible for the removal of naturally occurring organic substances. With increasing amounts of industrial pollutants entering the environment combined with the population increase, the natural rate of degradation cannot match the rate of pollutant production and therefore, pollutants accumulate (Chiras, 1994). To alleviate this problem the natural ability of microorganisms to degrade polluting compounds can be harnessed by means of bioremediation projects (Hart, 1996).

Bioremediation optimises the conditions for the growth of microorganisms that are capable of degrading the polluting compound in the environment. These conditions are often determined in the laboratory and applied to the environment, although there are inherent difficulties when applying such conditions to the soil environment, due to the heterogeneity of the system. Optimisation involves a wide range of abiotic parameters including oxygenation, temperature, pH, water content and nutrient concentration / spectrum (Holden and Firestone, 1997). Increased oxygen levels in the soil can improve the rate of aerobic degradation of many compounds (Muller-Markgraf, 1995). Air can be forced through the soil via a sparging or bioventing system to achieve elevated oxygen concentrations. Similarly, hydrogen peroxide can be used although this method is limited due to its toxicity to microbes (EPA, 1996).

Water is the major transport medium in the soil system, bringing nutrients and the polluting compounds (if soluble) to the microbes. By maintaining water at optimal levels an increased biodegradation rate may be achieved. Microorganisms require a wide range of nutrients for growth, therefore the provision of these nutrients can increase growth rates (Hardman, *et al.*, 1993). pH conditions play a major role in the bioremediation process, since pH determines the solubility of many pollutant compounds and therefore their availability to the microorganism (Hardman, *et al.*, 1993). Biodegradation rates can increase with optimising temperature for a given microorganism, some composting reactions can reach temperatures of 60°C and above (Carpenter-Boggs *et al.*, 1998). At elevated temperatures volatilisation and solubility of the contaminant are increased (Muller-Markgraf, 1995). In temperate climates many bioremediation projects have a reduced efficiency over winter, when temperatures decrease below the optimum for many microorganisms.

When optimising conditions for the microbial flora in the soil environment a compromise has to be achieved as a variety of microorganisms may be present, each requiring differing optimal conditions. Optimisation of one abiotic factor can often have a detrimental effect on other environmental factors. For example, an increase of water content can have an adverse affect on oxygen concentration by increasing

anaerobic zones. Such relationships must, therefore, be considered when optimising bioremediation conditions.

The location of the pollutant and its availability to the microorganisms involved in its breakdown are important factors in biodegradation. Inaccessibility of the pollutant to the microorganisms can be due to spatial separation or the temporal affect of ageing (Bewley *et al.*, 1989; Head, 1998). Spatial partitioning can be reduced by the homogenisation of the soil before bioremediation begins. Ageing is a process which causes the availability of a pollutant to decrease with increasing time in the soil environment. Ageing is a complex process and is not yet completely understood. It has been proposed that it is a result of a number of processes including, the entry of pollutants into micropores in the soil and the pollutant becoming irreversibly bound to soil particles, minerals and humic compounds by ionic / electrostatic bonds (Knaebel *et al.*, 1994; Head, 1998).

The microbial breakdown of compounds will often be a part of a 'treatment train' whereby many processes are used sequentially to remove various components of the pollution present (Sayler, 1991). The use of bioremediation as a valid procedure for pollutant removal has been approached with caution because of problems with performance and reproducibility in the field and the lack of comprehensive field trials with adequate controls (Head, 1998).

5.1.2. Bioremediation Treatment Processes.

Bioremediation can be achieved *in situ* or *ex situ*. A number of factors are involved the determination of the type and location of treatment chosen for each site (Fig. 5.1). Once the pollutant and site have been characterised, then a choice can be made between *in situ* or *ex situ* bioremediation as well as how intensive the treatment system will be. *In situ* bioremediation involves the treatment of in the ground on site and therefore reducing the costs as transport and excavation are not required.

Figure 5.1. Contaminant and site factors to be considered before selection of a bioremediation strategy.

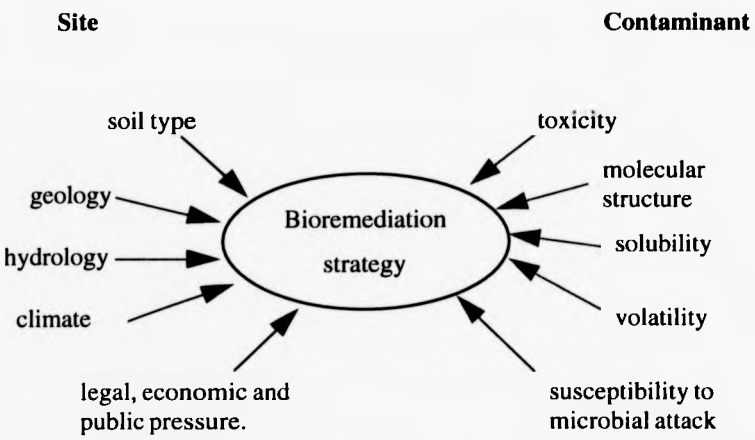
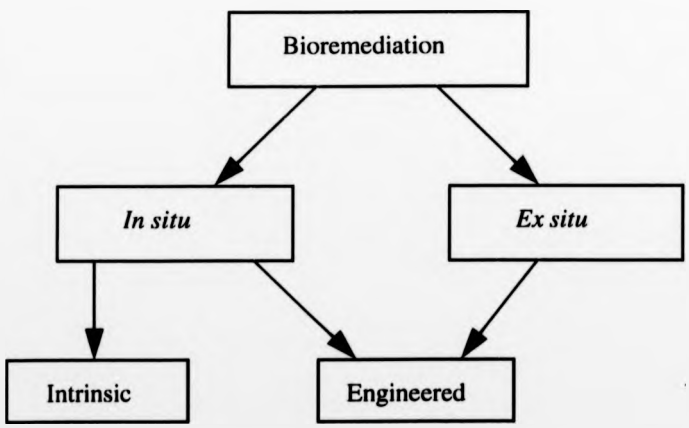


Figure 5.2. Bioremediation strategies (adapted from Madsen, 1998).



Ex situ bioremediation involves the physical removal of the soil and its subsequent treatment this can be on-site or at another location. The nature of the treatment system will have a bearing on the location of the treatment (*in situ* or *ex situ*) and vice versa. Treatments can be engineered or intrinsic strategies *in situ*, but only engineered strategies are employed in *ex situ* locations (Fig 5.2).

Engineered systems for biodegradation can be static or dynamic and include composting, bioreactors, and land-farming systems. Static systems mix the polluted soil with nutrient and / or other supplements for microbial growth, (some systems involve the addition of non-polluted soil) and provide high levels of ventilation and drainage. Dynamic processes involve the homogenisation of the soil, prior to further treatments. Large scale *ex situ* remediation is usually monitored closely and the bioremediation can be more controlled than *in situ* systems. *Ex situ* systems are relatively expensive due to the cost of transporting the soil to the bioremediation site. Dynamic processes are often labour intensive and therefore costly, but the process is rapid with more process control, offsetting the initial costs to some degree. These *ex situ* systems are most applicable to soils with a high concentration of a toxic pollutant (Hart, 1996).

Intrinsic bioremediation is a natural process allowing degradation of pollutants with little disruption to the site although it can take a considerably longer time than physical methods (Madsen, 1998). It is based on the premise that if the indigenous microorganisms can survive the presence of toxic compounds, then they must have resistance to these pollutants or the metabolic capabilities to degrade them (Leung *et al.*, 1997). Optimisation of the abiotic conditions in the environment allows increased levels of degradation by providing the optimal conditions for microbial activity (Muller-Markgraft, 1995). Careful monitoring procedures must be in place to ensure that bioremediation is occurring and that toxic intermediates are not being produced (Hardman *et al.*, 1993). During intrinsic bioremediation, mixed wastes may prove to be a problem as bacterial consortia may not be resistant to all types of pollution present (Head, 1998). Bioremediation may occur heterogeneously across a

site and process control is, therefore, a necessity to ensure that the whole site is remediated (Madsen, 1998). Intrinsic bioremediation may be aided by the addition of other organisms, for example by bioaugmentation (Muller-Markgraf, 1995) and phytoremediation (Wantanabe, 1997). Phytoremediation involves the use of plants for the *in situ* treatment of soil, sediment or ground water (Sadowsky, 1998). Bioaugmentation involves the addition of specialist microorganisms to the treatment system before or after bioremediation. In bioaugmentation systems, indigenous microorganisms can be isolated from the soil, grown up to high cell density in bioreactors and inoculated back into the original site, often with the addition of nutrients (Holden and Firestone, 1997). Intrinsic bioremediation is economically viable and is a major technique for the clean-up of soils contaminated with oil (Hart, 1996). Each method of bioremediation has its own advantages and limitations and these must be considered for each site.

5.1.3. The Importance of Molecular Biology and Microbial Ecology in Bioremediation.

Microbial ecology examines the microbial community with respect to the interactions and relationships between microorganisms and their environment. *In situ* bioremediation techniques, aim to provide optimal conditions in the soil for certain groups of microorganisms, hence an understanding of their ecology and habitat is beneficial to the development of such techniques (Hart, 1996). A limitation of the bioremediation process is the unpredictability of the efficiency of the method. This may be improved to some degree by biological monitoring of the process in addition to the chemical and physical analysis that is usually ongoing at a site. Molecular techniques have been utilised to monitor bioremediation field trials. These techniques have usually focused on catabolic genes (Brockman, 1995). Nucleic acid can be extracted from the samples and screened for the presence of a specific gene, for example *nahA* - naphthalene dioxygenase gene (Herrick *et al.*, 1993), *merA* - mercury reduction gene (Bruce *et al.*, 1992) and *bphC* - 2,3,-dihydroxybiphenyl dioxygenase gene (Erb and Wagner-Dobler, 1993). Catabolic gene detection can be

PCR amplification-based or gene detection can be achieved by hybridisation to a specifically labelled probe (Brockman, 1995). Activity can also be estimated using RNA approaches, with population shifts being observed using fingerprinting techniques (Hart, 1996).

It is essential to monitor the total microbial community in bioremediation studies as many biotic relationship will exist which are involved in the breakdown of contaminating substances. Many contaminating compounds in the soil are broken down by a process of cometabolism (Madsen, 1998) and sequential degradation pathways (Sayler, 1998). Cometabolism can be defined as the process whereby a compound, which does not support the growth of a microorganism, can be modified or degraded by that organism, in the presence of a second growth-supporting substrate (Pritchard *et al.*, 1995). An example of this phenomenon is the growth of *Sphingomonas paucimobilis* on fluoranthene as its sole energy and carbon source. If this strain is then exposed to other PAH compounds (individually), many of these high molecular weight PAH appear to be transformed (cometabolised) in some way. The procedure for this process is unknown, but it is hypothesised that enzymes created for the breakdown of fluoranthene also hydroxylate and possibly open aromatic rings in the other PAH compounds. Degradation of pollutants by mixed communities is highly complicated and not yet fully understood, however, it has been proposed that pollutant breakdown reactions, such as cometabolism, will be dependent on the microbial diversity present.

An understanding of the ecology of the microbes involved in bioremediation is essential for a better understanding of the process and may allow the enhancement of degradation rates (Head, 1988). Increased understanding of *in situ* microbial ecology, improved methodology, and government and public pressures are the likely reasons for the widespread increase in the number of bioremediation projects being undertaken (Sayler, 1991; Hart, 1996). Methods used include sequencing and probing indigenous populations, and the monitoring of community changes by fingerprinting techniques (Brockman, 1995).

5.1.4. Monitoring a Bioremediation Field Trial.

BG plc. (formerly part of British Gas) owns over one thousand gaswork sites. These are potentially contaminated with the by-products of the coal gasification process which was carried out on some sites, for periods in excess of 50 years. The main contaminants present in the soil from the coal gasification process include coal tar containing polyaromatic hydrocarbons (PAH), complex cyanides and heavy metals. Phenols and ammonia are often present in the groundwater. Such sites have often been producing gas for over fifty years. Currently 90% of BG plc.'s pollution is transported to landfill (BG plc., 1998).

Many gas production sites are now redundant due to the introduction of other gas sources (such as North sea gas) which has led to the closure of these plants (Bewley *et al.*, 1989). Now these sites are no longer required, if the contaminated land could be remediated then the land could be used or sold. The traditional method for removal of pollutants is by landfill. Landfill is a finite process, which does not degrade the pollutant compound but removes it to another area. BG plc. are investigating new technologies for pollution removal including bioremediation, soil and solvent washing and vapour extraction (BG plc., 1998). A three year, £2 million programme began in 1997 with the aim of developing new technologies for the assessment, remediation and monitoring of contaminated land (BG plc., 1998).

A test site in Sheffield has been established for the evaluation of bioremediation technology (Fig 5.3). This site was involved in gas production and storage for over 50 years and numerous contaminants are present that are associated with the gas production industry, including PAH compounds. To prepare the site for the study, physical removal of solid waste was necessary to provide a homogeneous soil for bioremediation, with any material larger than a house brick being removed. Once a homogeneous soil was obtained, it was placed into a unused tar tank and tar added and the soil mixed thoroughly to make the PAH distribution as homogeneous as possible. The polluted soil was placed into the bioremediation treatment pits, which were lined with breeze blocks.

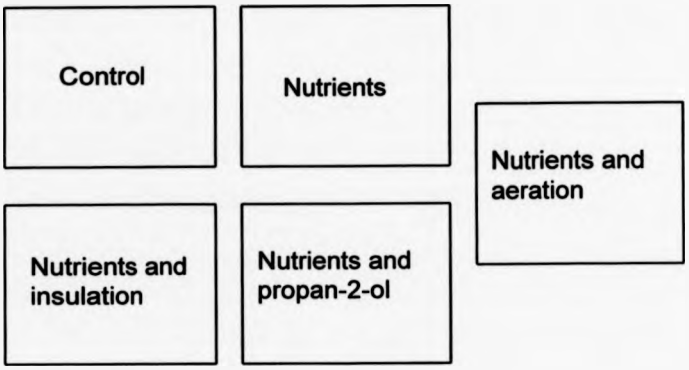
Figure 5.3. An aerial photograph of the BG plc. bioremediation experiment site in Sheffield.

The Bioremediation treatment site in Sheffield, the different treatment pits are indicated by the arrow.

Each pit held approximately 50 tonnes of soil and was subjected to a different treatment regime, (Fig 5.3 and 5.4). The treatment was carried out *ex situ* to allow better process control. The treatments monitored in this study include nutrient addition, nutrient addition and aeration, nutrient addition and insulation, nutrient and propan-2-ol addition, and a control pit (Fig 5.4). Nutrients were added to all treatment pits with the exception of the control and each treatment will be referred to by its additional treatment. Exact details of the treatments cannot yet be released, as this is commercially sensitive information. Most of the treatments are thought to increase rates of biodegradation by optimisation of conditions for the microorganisms present. Propan-2-ol was added to act as a co-solvent to deliver the hydrophobic PAH to the microorganism in the aqueous phase. Samples were taken from the pits throughout the year for analysis. Efforts to obtain homogeneous samples were employed and multiple ten cores were taken from each pit and used for chemical analysis. The ten soil samples were then combined and these combination samples were used for the microbiological analysis. Abiotic factors, such as pH, water content and temperature, were recorded for each of the pits. Specific chemical analyses were performed and the concentrations of the 16 PAH compounds designated by the US Environmental Protection Agency (EPA) as priority pollutants were determined (Section 9.4).

The community analysis techniques validated in Chapters 3 and 4 were applied to monitor the biodegradation process in the BG plc. experiment. These techniques enabled a greater understanding of the microbial community involved in the bioremediation treatment. The BG plc. experiment provided an ideal opportunity to assess the affects of different conditions and time on the microbial community present in the heavily polluted soil.

Figure 5.4. A Schematic Representation of the Bioremediation Treatments.



5.1.5. Aims.

- Using selective plate counts determine if large shifts in selected populations can be observed in the bioremediation treatment pits.
- To apply molecular genetic techniques for the identification of the dominant organisms present and to examine overall changes in diversity in the treatment pits.
- To investigate functional activity within the treatment pits and determine if the metabolic potential alters with the different bioremediation strategies.
- To examine if there is a correlation between potential metabolic activity, genetic diversity and culturable numbers with increasing degradation of pollutant compounds in the treatment pits.

5.2. Results.

5.2.1. Selective Plate Counts.

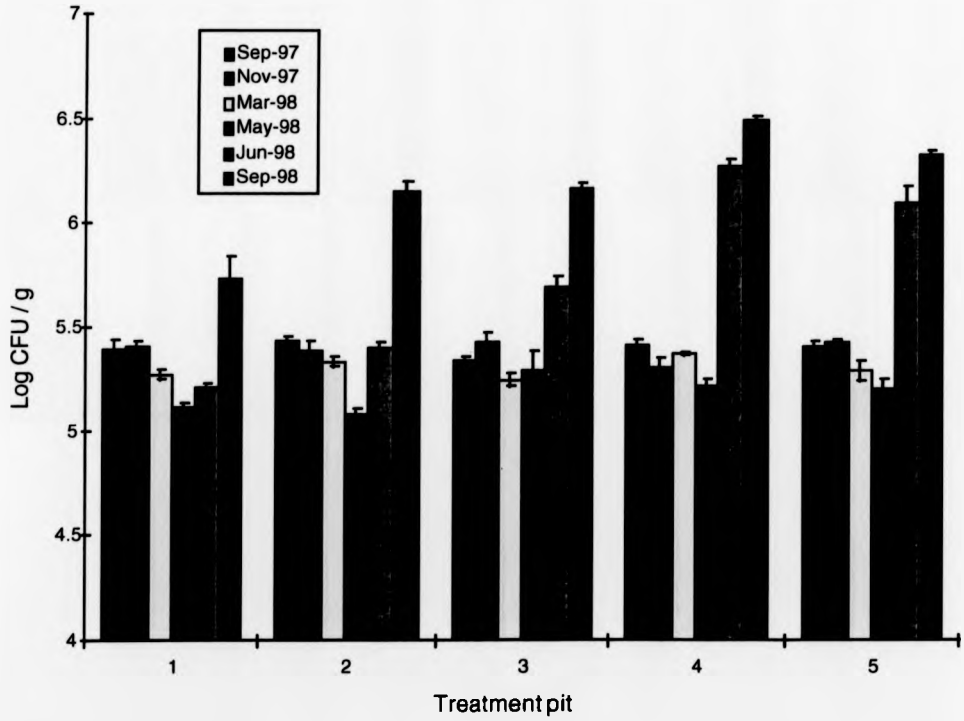
Selective plate counts were performed as in Section 2.4.1 and total, pseudomonad and actinomycete counts enumerated. The actinomycete counts were similar in all treatments over the first four sample dates, however, an increase was observed in the June samples from the aeration and propan-2-ol treatment pits (Fig 5.5). The September 1998 samples all had higher numbers, this increase was larger in the propan-2-ol and insulated pits. There were significant differences ($p<0.05$) in the number of actinomycete propagules in the different treatments over time (Table 5.1). Statistical tests were performed to determine if there were significant differences between the different treatment pits, indicated that samples taken in May, June and September 1998 were significantly different (Table 5.2.).

Table 5.1. One-way ANOVA statistical tests to determine if significant differences exists with each treatment over time at $p<0.05$.

Treatment Pit	Pseudomonad counts	Actinomycete counts	Total culturable counts
Control	234.5	17.54	22.7
Nutrients	73.2	103.8	53.9
Nutrients and aeration	285.4,	39.2	15.37
Nutrients and propan-2-ol	564	299.9	385.6
Nutrients and insulation	14.14	105.6	58.16

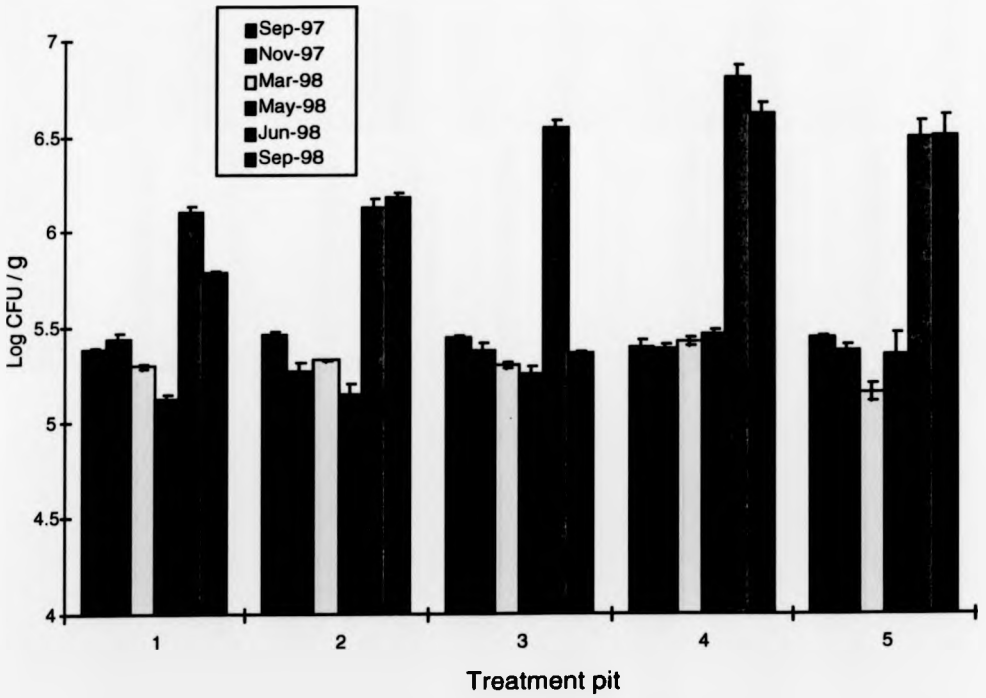
F critical value determined as $F_{5,12} = 3.1$

Figure 5.5. Actinomycete selective plate counts from the different bioremediation treatment pits.



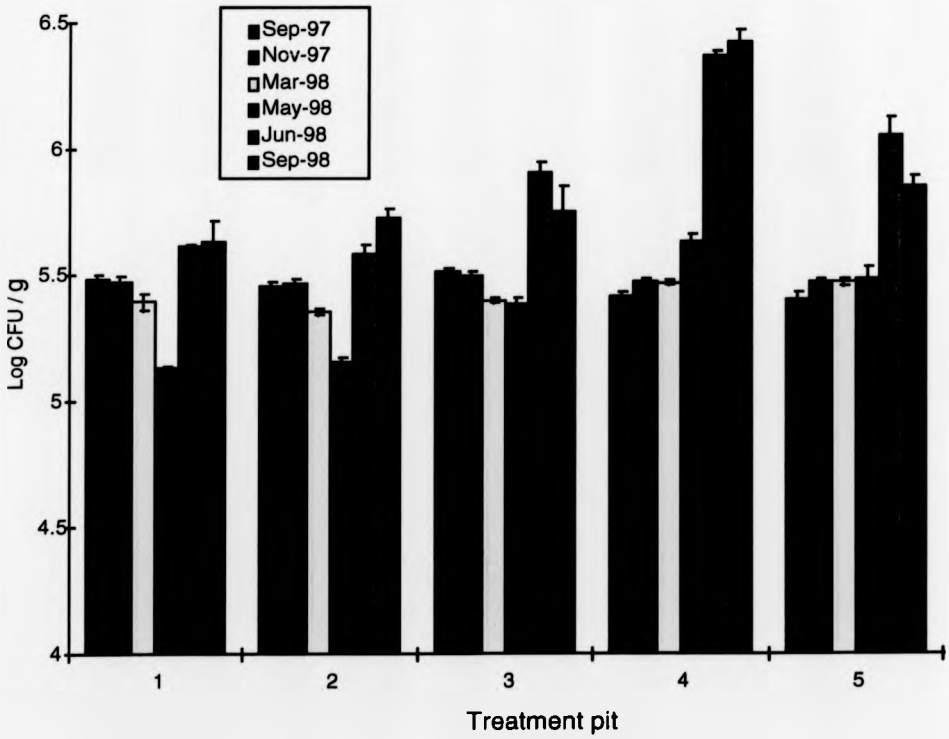
Treatment pits; 1 = control pit, 2 = nutrient addition, 3 = aeration, 4 = propan-2-ol, 5 = insulation. The counts of culturable actinomycetes were determined at time points throughout the year in the different treatment pits. The error bars shown represent the standard error of three replicates.

Figure 5.6. Pseudomonad selective plate counts from the different bioremediation treatment pits.



Treatment pits; 1 = control pit, 2 = nutrient addition, 3 = aeration, 4 = propan-2-ol, 5 = insulation. The counts of culturable pseudomonad were determined at time points throughout the year in the different treatment pits. The error bars shown represent the standard error of three replicates.

Figure 5.7. Total culturable counts on nutrient agar for different bioremediation treatment pits.



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Treatment pits; 1 = control pit, 2 = nutrient addition, 3 = aeration, 4 = propan-2-ol, 5 = insulation. Total culturable counts were determined at time points throughout the year in the different treatment pits. The error bars shown represent the standard error of three replicates.

Pseudomonad counts were similar for all treatments until June 1998 when there was an increase in bacterial numbers in all treatment pits. High counts were also obtained in September, with the exception of the aeration treatment, where pseudomonad numbers decreased. The pseudomonad and actinomycete counts were of similar numbers and showed a similar overall pattern in all treatment pits (Fig 5.6). There were significant differences ($p<0.05$) in pseudomonad numbers in all treatments over time (Table 5.1). Also significant differences were observed between the different treatments at the same time points, except for September, November 1997 and March 1998 (Table 5.2).

Table 5.2. Statistical results from one-way ANOVA tests performed between different treatments at different time points at $p<0.05$

Sample date	Pseudomonad counts	Actinomycete counts	Total culturable counts
Sept. 1997	4.0	***1.3**	6.2
Nov. 1997	***1.3,**	***1.5**	17.2
March 1998	***0.5**	***3.0**	7.9
May 1998	21.1	9.2	76.2
June 1998	90.5	87.2	59.1
Sept. 1998	91.1	25.65	21.53

F critical value determined as $F_{4,10} = 3.5$

* Non-significant results.

Total culturable counts on nutrient agar indicated that bacterial numbers were similar to the pseudomonad and actinomycete counts (Fig 5.7). On the last two sample dates, there was an increase in colony numbers; this increase being considerable in the propan-2-ol and insulated pits. Significant differences were observed in each

treatment over time in total culturable numbers (Table 5.1). Bacterial counts were also significantly different at each time point between treatments (Table 5.2).

5.2.2. Biolog Analysis.

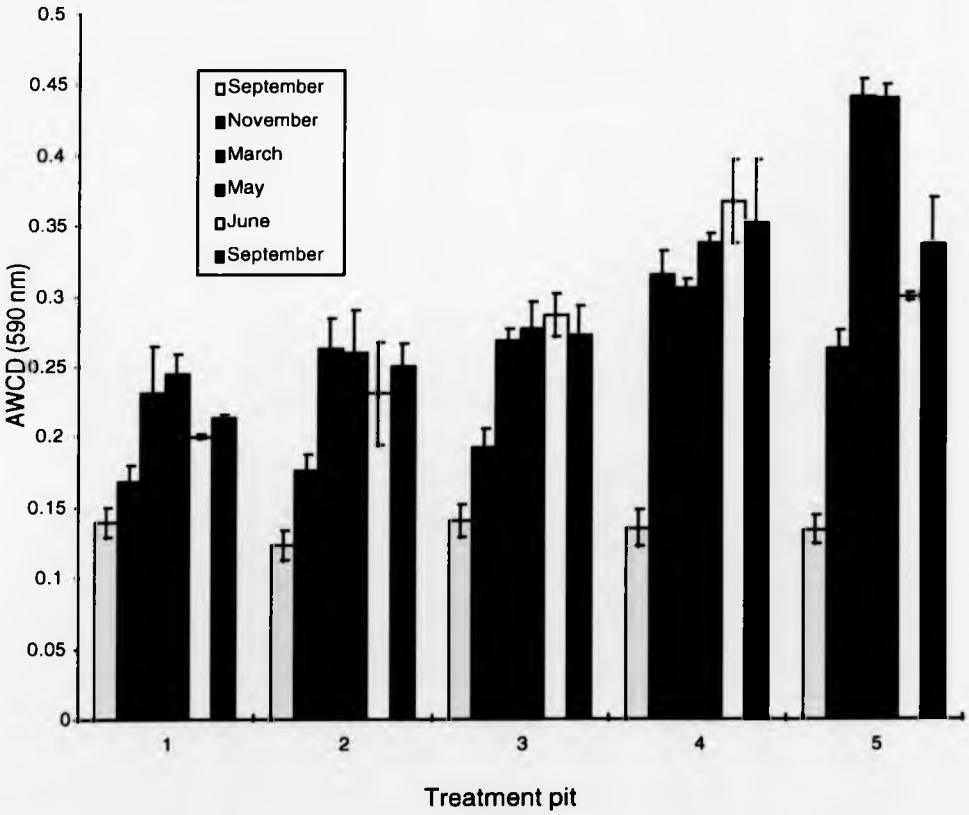
The potential metabolic activity of each treatment pit was analysed using the Biolog system and the average well colour development (AWCD) was determined for each sample (Section 2.8). Metabolic activity increased in the control pit over the year, peaking in March and May (Fig. 5.8). A decrease in activity was observed on the last two sample dates, although the levels remained higher than observed on the first two sample dates. The nutrient treated pit and the control pit showed comparable levels of metabolic activity. The pit treated with aeration had higher levels of potential metabolic activity than the control and nutrient treatment pits. The propan-2-ol and insulated pits showed the largest increases in potential metabolic activity. Statistical tests (one-way ANOVA's) indicated significant differences in each treatment over time (Table 5.3).

Table 5.3. One-way ANOVA statistical analysis to determine if significant differences exists with each treatment over time at $p<0.05$.

Treatment	Test statistic
Control	6.03
Nutrients	6.02
Nutrients and aeration	14.8
Nutrients and propan-2-ol	12.7
Nutrients and insulation	51.4

F critical value determined as $F_{5,12} = 3.1$

Figure 5.8. Comparison of Bioremediation Treatments Using Biolog Metabolic Analysis.



Treatment pits; 1 = control pit, 2 = nutrient addition, 3 = aeration, 4 = propan-2-ol, 5 = insulation. Biolog analysis was performed over the year in the different treatment pits. AWCD was recorded for each treatment at each time point to allow a comparison of metabolic activity. The error bars shown represent the standard error of three replicates.

Table 5.4. One-way ANOVA statistical analysis to determine if significant differences exist at each time point between different treatments at $p<0.05$.

Sample date	Test statistic
September 1997	* 0.4
November 1997	21.9
March 1998	18.8
May 1998	20.8
June 1998	8.7
September 1998	4.6

F critical value determined as $F_{4,10} = 3.5$

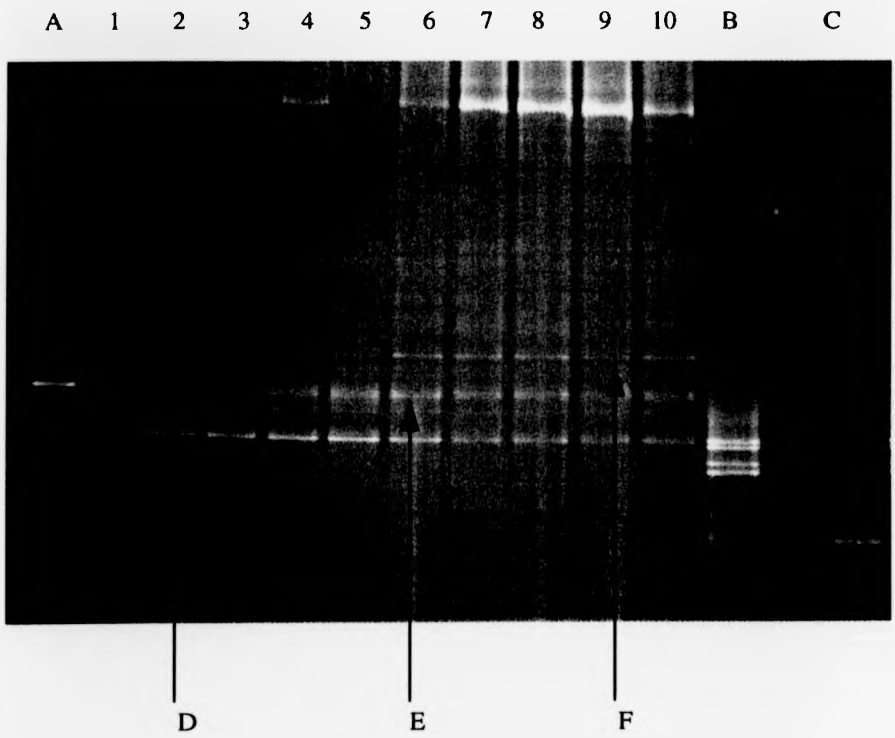
* Non-significant results.

Statistical analysis indicated that significant differences existed between different treatments at each sample time point with the exception of the first date of sampling, (Table 5.4).

5.2.3. Genetic Analysis.

DNA was extracted from each treatment pit and used as a template for universal 16S rRNA PCR amplification. The products were separated on DGGE gels (Section 2.6). DGGE analysis of the September and November (1997) samples, revealed a very defined community with a few dominant species present (Fig 5.9). The September and November communities produced three major bands, although the intensity of the bands varied. In September, a band identified as *Xanthomonas codiaei* was of the highest intensity, while in November analysis the *X. codiaei* band decreased in intensity and a band identified as *Pseudomonas stutzeri* was more intense.

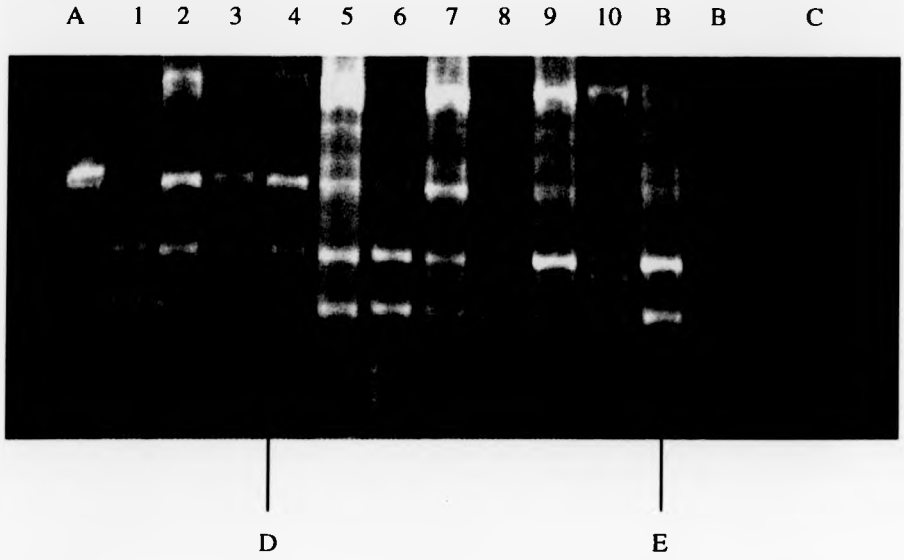
Figure 5.9 DGGE analysis of 16S rRNA amplified using universal primers from BG plc. treatment pits from September and November 1997 samples.



A = *Pseudomonas putida,* B = *Streptomyces lividans*, C = *Rhodococcus rhodochrous.* Bands D, E and F were excised from the gel and sequenced . D = *Xanthomonas codiaei* (93%), E = *Pseudomonas putida* (96%), F = *Pseudomonas stutzeri* (95%). Figures in brackets represent percentage similarity with 16S rRNA for three replicate sequences.

1 = Control, Sept. 1997, 2 = Aeration, Sept. 1997, 3 = Nutrients, Sept. 1997, 4 = Insulated, Sept. 1997, 5 = Propan-2-ol, Sept. 1997, 6 = Control, Nov. 1997, 7 = Aeration, Nov. 1997, 8 = Nutrients, Nov. 1997, 9 = Insulated, Nov. 1997, 10 = Propan-2-ol, Nov. 1997.

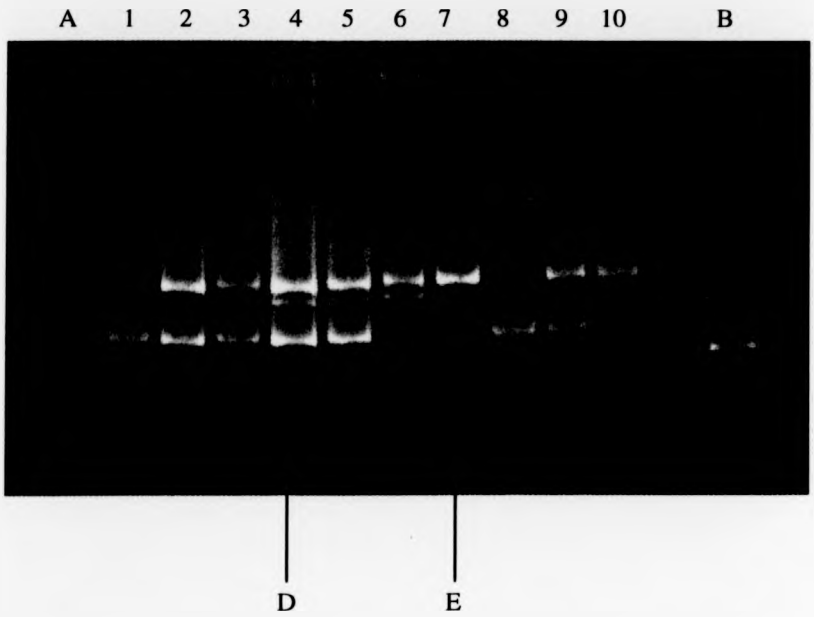
Figure 5.10. DGGE analysis 16S rRNA PCR products amplified using universal primers, from BG plc. Treatment pits from March and May samples.



A = *Pseudomonas putida,* B = Bioreactor samples, C= *Streptomyces lividans.* Bands D and E were excised from the gel and sequenced.
D = *Pseudomonas putida* (94%), E = *Xanthamonas codiaei* (91%). Figures in brackets represent percentage similarity with 16S rRNA for three replicate sequences.

1 = Control, March 1998, 2 = Aeration, March 1998, 3 = Nutrients, March 1998, 4 = Insulated, March 1998, 5 = Propan-2-ol, March 1998, 6 = Control, May 1998, 7 = Aeration, May 1998, 8 = Nutrients, May 1998, 9 = Insulated, May 1998, 10 = Propan-2-ol, May 1998.

Figure 5.11. DGGE Analysis of 16S rRNA PCR products amplified using universal primers, from BG plc. treatment pits from June and September Samples.



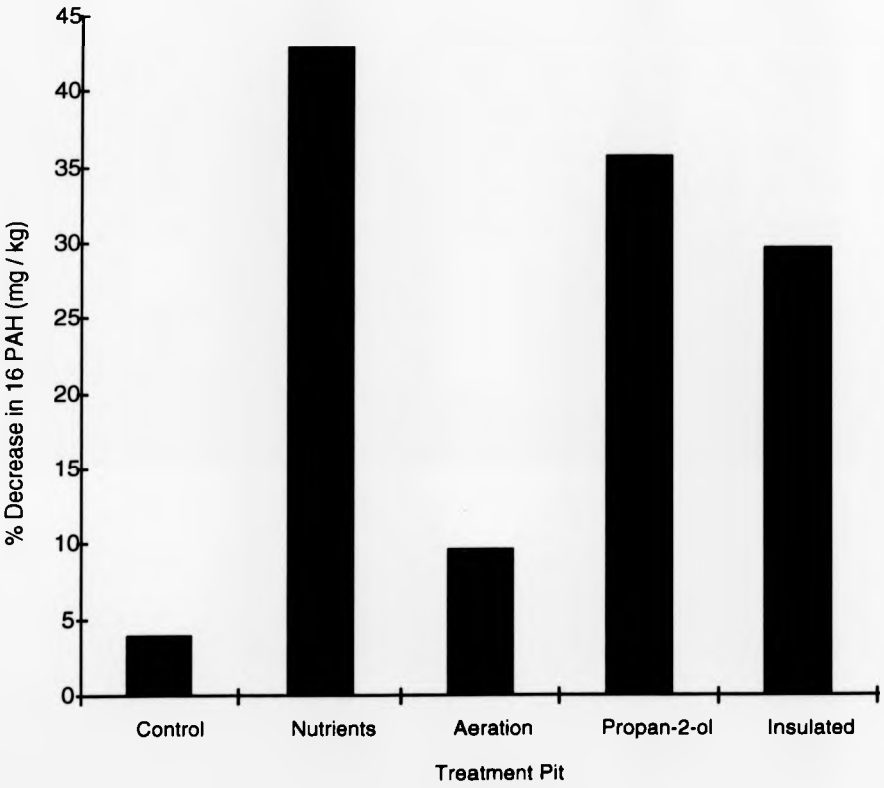
A = *Pseudomonas putida,* B = *Rhodococcus rhodochrous*. Bands D and E were excised from the gel and sequenced. D = *Pseudomonas aeruginosa* (93%), E = *Pseudomonas stutzeri* (91%). Figures in brackets represent percentage similarity with 16S rRNA for three replicate sequences.

1 = Control, June 1998, 2 = Aeration, June 1998, 3 = Nutrients, June 1998, 4 = Insulated, June 1998, 5 = Propan-2-ol, June 1998, 6 = Control, Sept. 1998, 7 = Aeration, Sept. 1998, 8 = Nutrients, Sept. 1998, 9 = Insulated, Sept. 1998, 10 = Propan-2-ol, Sept. 1998.

Samples from the different treatment pits in March (1998) and May (1998) indicated a more complex bacterial community than samples taken in September (1997) or November (Fig 5.9). This may have resulted from different bacterial species proliferating in the different treatment pits. The control pits (lanes 1 and 6) had a less complex banding pattern with three dominant bands as on the first sample dates. The samples from May had more bands present than on the March sample date. The lanes which appeared to have the most genetically diverse communities (i.e. the most bands) were from the propan-2-ol treatment in March and the propan-2-ol and insulated pit in May. Different banding patterns were observed in samples from different pits, attempts to sequence other bands were unsuccessful. However, *Pseudomonas putida* was identified and this was one of the dominant bands in March. In May *X. codiaei* was represented by a band of higher intensity whilst *P. putida* bands were of a lower intensity.

June and September (1998) samples were also examined by DGGE (Fig 5.11). The community banding patterns did not appear to be as diverse as the March and May samples. Two or three bands dominated in most lanes, however, other bands could be detected at low intensity in some lanes. Two bands which could be observed in all treatments (at lower intensity in the controls), were sequenced and identified as *Pseudomonas aeruginosa* and *P. stutzeri* (Fig 5.11). Another band was observed in the propan-2-ol and insulated pits but attempts to sequence this band were unsuccessful. In September the *P. aeruginosa* and *P. stutzeri* bands could still be observed but that of *P. stutzeri* was of a brighter intensity. The additional band in the propan-2-ol and insulated pits in the June samples was also present in the control pit, and in those treated with nutrients and aeration in September. Only *P. aeruginosa* and *P. stutzeri* bands could be detected in the propan-2-ol and insulated treatments of September (1998). Nutrient and aeration treatment pits in June had a faint band at a high position in the gel, with an equivalent band in the nutrient and aeration pit in September (1998).

Figure 5.12. Percentage decrease in 16 US EPA polyaromatic hydrocarbons (PAH) in the treatment pits from September 1997-June 1998 (from BG plc.).



Percentage decreases were calculated from the mean difference of 10 values. Treatments are 1 = control pit, 2 = nutrient addition, 3 = aeration, 4 = propan-2-ol, 5 = insulation. Reproducibility data restricted by BG plc.

5.2.4. Percentage Decrease in 16 PAH Compounds in the Treatment Pits from September 1997 to June 1998.

Analysis of the treatment pits to determine levels of the 16 US EPA PAH compounds was performed by BG plc. (Fig 5.12). Pollutants decreased over time in each treatment, the control pit had the lowest rates of pollutant degradation. Decreases were calculated from the median value of 10 measurements and data for the reproducibility (standard errors) were not provided. However from this analysis, the nutrient, propan-2-ol and insulated pits appear to have the largest decreases in the concentration of the 16 PAH compounds with time.

5.3. Discussion.

Selective plate counts were performed for actinomycetes, pseudomonads and total culturable bacteria. The numbers and overall patterns were similar for each count. Pseudomonad and actinomycete counts were very similar over the year, with pseudomonad counts being slightly higher. All bacterial counts were relatively stable throughout the year, until June and September 1998, when a large increase in propagules was observed. The greatest increases in propagule counts were detected in the propan-2-ol and insulated treatments this may have been due to a reduction in pollutants which facilitated higher rates of bacterial survival. This reduction in polluting compounds may have been due to the warmer weather conditions over the summer months, which could have increased degradation activity and bacterial numbers. A smaller increase in bacterial numbers was also observed in the control pit suggesting that the increase may have been due in part to warmer temperatures of the summer months or ageing of the pollutant, reducing its toxicity to the microbes present (Head, 1998).

The nutrient treatment pit had very similar propagule counts numbers to the control pit indicating that nutrients alone did not have a major effect on the microbial

community. The pit treated with aeration and nutrients had higher bacterial numbers than the control and nutrient pits, although the highest bacterial counts were in the propan-2-ol and insulation treatments. All three selective counts allowed the analysis of large changes in bacterial numbers in the treatment pits. For example, all three counts indicated a large increase in bacterial numbers on the last two sampling dates, especially in the propan-2-ol treated and insulated pits.

Biolog analysis was used to examine the functional activity of the bacterial communities in the different treatment pits, over the year. Potential metabolic activity increased in all treatments, including the control, over the year. This was probably due to the mild winter and the warmer summer months, leading to an increase in the metabolic activity of the indigenous microorganisms. Potential metabolic activities in the pits treated with nutrient addition or aeration were similar to the control, although activity in the aeration pit was always slightly higher. This analysis indicated that the potential metabolic activity was at different levels in the different treatments and that the propan-2-ol and insulated treatment pits had the greatest increases. This large increase in potential metabolic activity may have been due to these treatments providing optimal conditions for bacterial survival and activity. The insulated pit, due to its warmer temperatures, may support higher levels of potential metabolic activity. While in the propan-2-ol treatment pit, the PAH compounds will be present in a more soluble form, enhancing their availability to the microorganisms, as a nutrient source.

Genetic analysis was performed in order to examine the microbial community present in each of the treatment pits, to overcome non-culturability problems and identify bacterial species which may play a key role in the environment. The DGGE analysis allowed comparison of communities via fingerprinting patterns and identification of major species was also possible via sequencing. The results of this genetic analysis indicated the presence of a succession of microorganisms over the course of the year. These changes in the microbial community may have been the result of seasonal fluctuations in the climate and soil conditions. The control pits also revealed these community changes but at a much lower level, maybe indicating

that the different treatments enhanced the community changes. The communities present in the treatment pits in the first four samples appeared to be progressively diverse, however, the last two sample dates decreased in complexity. A reduction in diversity could be due to high competition with highly adapted microorganisms that were capable of surviving and degrading the pollutants, and therefore dominating in the treatments. This would cause the treatment pit to have a reduced microbial diversity through the dominance of a few highly adapted species. Higher resolution sampling may allow a more detailed analysis of changes in the microbial community, in the different treatments over time.

Changes in the bacterial community could be observed with time and treatment, by the genetic analysis. Control pits in each of the sample dates appear to have the same bands but at a much lower intensity indicating that the treatments increased bacterial numbers and diversity. There was a correlation between the results of the metabolic and genetic analysis. The propan-2-ol and insulated treatment pits, which had the highest metabolic potential and bacterial numbers on selective plates, often had the highest number of bands at the highest intensity. The three methods of analysis used in this study provide complementary results in the examination of the bacterial communities present. To further investigate if the dominant microorganisms in the DGGE gels were also found in the selective plate counts, probes could be developed for these species and used in colony blot screening of the isolates obtained from the selective plates. This analysis would allow a comparison of the traditional selective plate techniques and the genetic analysis, to determine if the techniques used analysed different subsections of the microbial community.

The most commonly identified genera in the genetic analysis was the pseudomonad group. The predominance of these microorganisms may be due to their involvement in a wide range of biodegradation reactions in the environment. Faison (1992) stated that pseudomonads appeared to be the most effective degraders of xenobiotic compounds. Actinomycetes were present in similar numbers to pseudomonads on selective plate counts, although in the DGGE analysis the majority of the bands sequenced were pseudomonads with no actinomycetes DNA detected. Many groups

of actinomycetes have resistance and degradation pathways of pollutants, for example rhodococci (Finnerty, 1992). It was surprising when no actinomycete DNA was detected, especially the aeration pit where the moisture content was low, providing favourable conditions for actinomycete growth. This may indicate a bias in the PCR reaction (Heuer *et al.*, 1997). To detect and investigate the diversity of the actinomycete group specific PCR analysis was used (Heuer *et al.*, 1997), however, these primers also amplified other high G+C microorganisms such as pseudomonads, when tested with type strains, in this study (Section 4.2.4).

To investigate this further, probes could be developed for the pseudomonad species which appear to be dominant in the genetic analysis. These probes could then be used to screen total isolates by colony hybridisation, or dot blotting techniques. This would determine if the pseudomonads isolated by the genetic analysis corresponded with the culturable community isolated in this study. To ascertain if these species were active in the environment, analysis of mRNA by RT-PCR techniques, could be performed.

There are limitations to the DGGE method. However, if applied with other data sets such as selective plate counts and further analysis of the gels then this method provides a sensitive tool for community analysis. One of the limitations is that bands representing different species can migrate to the same position in the gel. This can be seen in Fig 5.9 where a band that was sequenced and identified as *X. codiaei* was present at the same position on the gel as a band present from the *S. lividans* type strain. This reinforces the need for positive identification of the band by sequencing or probing, as identification by position on the gel alone is unreliable (Ferris *et al.*, 1996). Another problem with DGGE analysis is the occurrence of multiple bands for type strains, for example in Fig 5.9 the *S. lividans* type strain produces multiple bands. These multiple bands have occurred in many studies using 16S rRNA analysis (M. Girvan and I. Foley pers.comm.) and are probably the result of multiple copies of the 16S gene being present. With this limitation in mind, caution must be applied before relating the number of bands to the number of species present (Heuer and Smalla, 1997b). Although when sequencing bands from communities separated

on DGGE gels this problem did not occur, this has been observed in other studies (K. Smalla, pers.comm.). The genetic analysis is dependent upon the same conditions being applied to each sample as well as the sensitivity of the PCR reaction. A rigorous attempt was made to make the conditions of the analysis as uniform as possible and each PCR reaction was performed in triplicate and combined together prior to analysis. Although the PCR is not quantitative, the same conditions have been applied throughout the experiment to all of the samples, and therefore qualitative comparisons can be made. To allow quantitative comparisons, other systems such as quantitative PCR (Q-PCR) and densitometry (Romanowski *et al.*, 1993; Fuller, *et al.*, 1998) could be employed to enable a quantitative assessment of changes in structure of the bacterial community.

The results in Fig 5.12 for the percentage decrease of PAH compounds do not include the last sample date. This study indicates that there was substantial bacterial activity at this last sample date. It is possible that further high rates of degradation of pollutants may have occurred, which may alter this data set. Degradation of the 16 PAH compounds was observed in all the pits, including the control. This may be the result of volatile compounds leaving the soil and also due to the increased levels of aeration of the soil resulting from the removal of core samples. This could be determined by examining the ratio of heavy-to-light PAH compounds (R.Swannell pers.comm). Pollutant breakdown was observed at a lower rate in the aeration treatment, this may be due to the decreased water content adversely affecting the abiotic conditions for microorganisms. High rates of PAH degradation were observed in the propan-2-ol and insulated treatments. This observation correlated with the high numbers and increased metabolic activity in these pits. Anaerobic processes are thought to play an important role in the degradation of PAH compounds (Bewley, 1990). Future studies will be conducted to investigate the anaerobic component of the microbial community by BG plc.. Biological treatment of PAH-contaminated soil often leads to a reduction in the lighter PAH compounds, leaving the heavier PAH compounds which are often carcinogenic (Madsen, 1998). This was observed in the treatment pits with the lighter PAH being degraded before the heavier PAH compounds (R. Morris pers. comm.).

This field trial has been carried out on the macroscale, allowing a better understanding of the bioremediation process in the environment. Although microcosms experiments do not allow an accurate picture of the environmental system, the whole experiment can be sampled, therefore, reducing the affects of sample location. An inherent problem of mesoscale experiments is that activity will be unevenly distributed in hot spots throughout the site. Care must be taken in the sampling regime to achieve representative samples (van Elsas and Smalla, 1997) as each 50 tonne treatment pit will have hot spots of activity, but mostly the bulk soil will have low rates of degradation. It is likely that gradients of abiotic factors may exist throughout the pit and this could be an interesting point for study in the future.

In a similar study, the biodegradation of oil was examined using DGGE and carbon dioxide evolution rates (Swannell *et al.*, 1998). This study determined that population changes were extremely rapid and that the communities altered considerably over time. The condition that limited the rates of biodegradation appeared to be temperature. This finding was in agreement with the observations of this study, where high rates of metabolic potential and bacterial numbers were found in the insulated pit which had an increased temperature. A bioremediation study which focused on disused gasification sites showed that the use of surfactants increased bioremediation efficiency. Surfactants are thought to make PAH compounds present in a more readily available form (Hughes, *et al.*, 1997). This finding is consistent with this study, as higher levels of activity, bacterial numbers and genetic diversity were detected in the propan-2-ol treatments. It is hypothesised that the PAH compounds were in a more readily available form, which led to enhanced degradation. The propan-2-ol however may have been used as a carbon source where it may have a role in the cometabolism of PAH compounds. Further studies would have to be carried out to analyse if the propan-2-ol was degraded by microorganisms and involved in cometabolism of PAH compounds.

To improve bioremediation efficiency, studies must be performed in the environment with quantitative analytical and molecular tools to monitor and control the population

dynamics of biodegradative microorganisms (Sayler 1991). In this study, molecular techniques were applied to overcome problems of non-culturability of most microorganisms along with the more traditional approach of selective plating. The microorganisms that have been identified in the polluted samples can now be isolated and further analysis performed to examine their optimal conditions in the laboratory. These isolates could also be examined to identify the catabolic genes that they may possess.

Community analysis is essential in bioremediation systems, as it is unlikely that one microorganism will be capable of degrading all pollutants present in a contaminated soil. In order to degrade polluting compounds the microbial community will interact by processes such as cometabolism (Madsen, 1998) and sequential degradation pathways (Sayler, 1998). Therefore, information regarding the microbial consortia present will provide a deeper understanding of the biodegradation process.

Molecular techniques provide essential information for monitoring bioremediation. This type of analysis does have limitations including the cost of sample analysis and the as yet incomplete knowledge of genes controlling biodegradation in the environment. Molecular analysis of bacterial communities is not yet well enough understood to be a stand alone assay (Head, 1998). However, with increasing numbers of bioremediation studies and a better understanding of the process, the monitoring of the bacterial communities may provide a sensitive tool for treatment assessment in the future.

5.4. Future Work.

* Further analysis of the physical and chemical data of the treatment pits could be performed, including heavy to light PAH ratios.
* Microorganisms that have been identified from contaminated soil by genetic analysis could be isolated and further analysis with RNA techniques may determine if these microorganisms are active and dominant in the environment.
* Probes could be made for the pseudomonad species identified in this study and used to determine if these are the dominant microorganisms in the environment, or if their dominance in the genetic analysis is the result of PCR bias.
* Analysis of isolates on the selective plates by pseudomonad probes to determine if the species found to be dominant by the genetic analysis are the same isolates found by culturing studies.
* Actinomycete specific primers could be developed and used to analyse the diversity of the actinomycete group in these treatment pits. Further analysis with Q-PCR techniques could determine if the universal primers do preferentially amplify the pseudomonad species.
* The industrial collaborators BG plc. are performing analysis of the anaerobic community in the different bioremediation treatment pits, by selective enrichment and culturing techniques. It would be of interest to compare the aerobic and anaerobic studies.
* To determine if propan-2-ol is used as a carbon source by the microorganisms labelled propan-2-ol studies could be performed to examine if this is involved in cometabolism.
* The treatment pits could be examined for Archaea and other bacterial genera using specific primers and the determination of specific activities by using functional gene probes and primers. Such as *nahA* specific primers and internal probe for the naphthalene dioxygenase gene (Herrick *et al.*, 1993),

Chapter 6

Assessment of The Biolog Community Analysis Technique.

6.1. Biolog Analysis.

Metabolic activity may be used as an indicator to examine the functional activity of microbial communities in environmental samples. Microorganisms differ in their abilities to degrade substrates and these differences in phenotype have been used to identify microorganisms in pure cultures (Konopka, 1998). It has been proposed if an environmental niche changes due to stress or disturbance, the microbial flora contained within it will alter (Atlas, 1984). These shifts in the microbial community will then lead to changes in the metabolic capabilities of the community. The relationship between functional diversity and genetic diversity remains unclear, although analysis of potential metabolic activity of a sample may give an indication of microbial community function and activity (Zak *et al.*, 1994).

Biolog plates have been previously introduced in Section 1.5.4. These commercially produced plates contain 96 wells, 95 of the wells contain a carbon source and one well (the control well) has none. The carbon sources are representatives from eleven substrate groups including alcohols, amino acids and phosphorylated compounds. Each well also contains a basal medium and a tetrazolium dye which indicates oxidation of the carbon source by virtue of a colour change. Biolog GN (Gram-negative) and GP (Gram-positive) plates were originally produced for the identification of clinically important isolates and therefore have a range of carbon sources commonly utilised by these groups (Bochner, 1989b). GN and GP plates have 62 carbon sources in common. When using GN and GP Biolog plates for community analysis, the wells containing carbon sources are not selective for Gram positive or Gram negative organisms. The differential utilisation of carbon sources in the plates, however, may permit discrimination for different groups of bacteria.

For microbial community analysis the soil samples have to be shaken with various dispersants to remove the bacterial fraction from the soil matrix. Once the bacterial fraction has been obtained, it is inoculated into the Biolog plates. After incubation with the various carbon sources, a characteristic fingerprint for the sample that represents the functional capabilities of the bacterial community is obtained (Garland

and Mills, 1991). These fingerprints can be compared by various methods, such as principle component analysis (PCA), or the average well colour development (AWCD) which measures overall colour development on the plate (Garland and Mills, 1991). AWCD analysis facilitates rapid comparisons of overall potential metabolic activity between soils (Garland, 1996).

Metabolic fingerprints have allowed the discrimination of many different microbial communities in a wide range of environments including the rhizosphere, inoculated rhizosphere and bioreactor monitoring (Garland and Mills, 1994), six different plant-associated bacterial communities (Zak *et al.*, 1994), differences in metabolic activity in different size particles of the same soil (Winding, 1994), comparative analysis of plant phyllospheres (Heuer and Smalla, 1997a), and differences between field and greenhouse soil (Ibekwe and Kennedy, 1998). Biolog community analysis has also determined shifts in microbial communities under hydrocarbon pollution (Wünsche *et al.*, 1995), heavy metal stress (Knight *et al.*, 1997), and amended with genetically-modified plant materials (Donegan *et al.*, 1995). The majority of sole carbon source utilisation studies have focused on the Biolog GN plate for community analysis, with only a few studies analysing GP plates (Wünsche *et al.* 1995, and 1996; Haack *et al.*, 1995).

Garland (1996), suggested that future studies should focus on the development and understanding of the Biolog system rather than the application of the method to new environments. By investigating the effects of incubation time and plate type on Biolog community analysis, further information may be provided on what the Biolog patterns represent in environmental terms. In this study, Biolog GN and GP plates were inoculated with Warwick soil, incubated at 24°C and read after 48, 72 and 96 h. The AWCD was recorded at each time point. To determine how representative Biolog analysis was of the whole microbial community, DNA extractions were performed on selected wells (Section 2.8.4) at the specified times to examine the microbial communities present in the wells.

6.1.2. Aims.

* To examine the effect of Biolog plate type when analysing microbial communities.
* To investigate the effects of incubation time on AWCD and diversity present in the Biolog wells.
* To determine if there is a relationship between optical density in the wells in the Biolog plate and microbial diversity detected by DGGE gels.
* To determine how representative the Biolog analysis was of the total microbial community present.

6.2. Results.

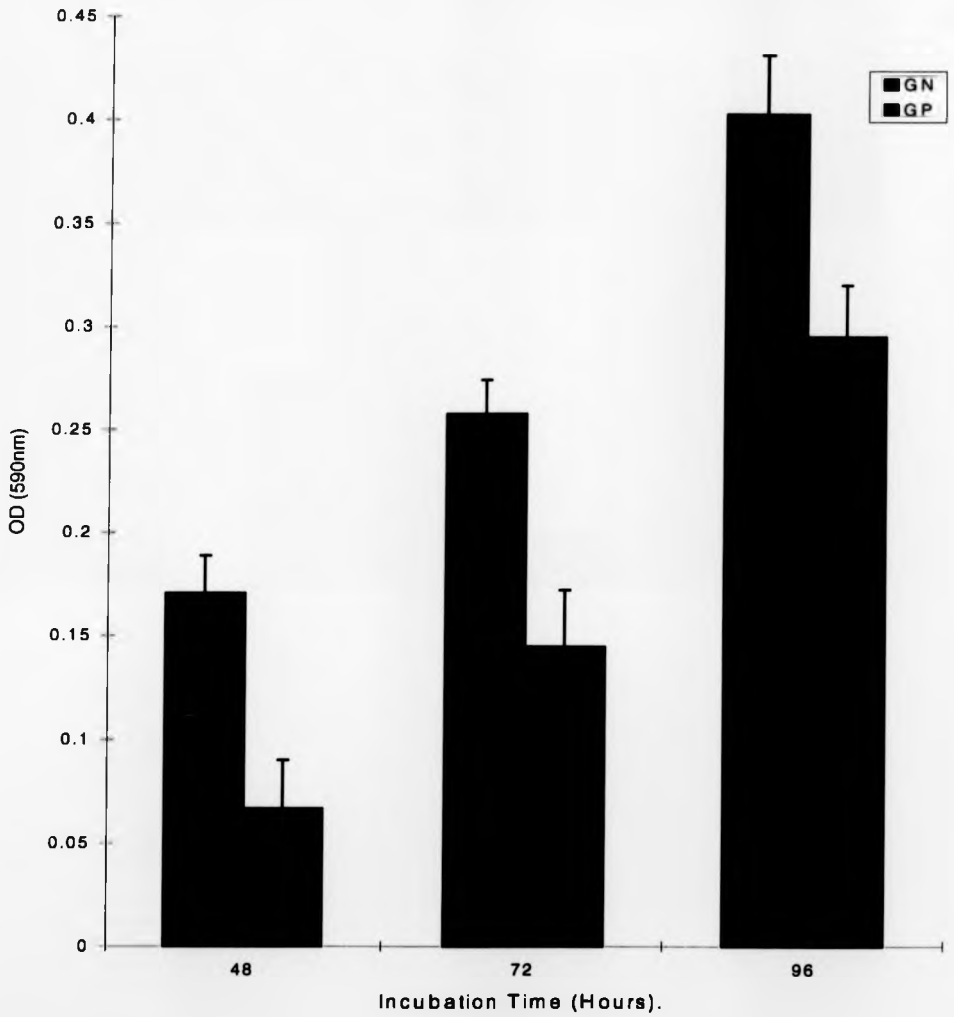
6.2.1 Inoculum Plate Counts.

Before addition of the inoculum to the Biolog plate, an aliquot was plated onto nutrient agar plates. The total culturable count for the inoculum was determined as 1.3×10^6 CFU / ml.

6.2.2. AWCD Using GN and GP Biolog Plates After 48 to 96 h Incubation.

The AWCD increased reproducibly for both the GP and GN plates with time. The AWCD of GN plates increased at a faster rate than that of the GP plates (Fig. 6.1). The difference in colour production may be due to the GP plate containing novel carbon sources which may be more recalcitrant to microbial degradation than those in the GN plate.

Figure 6.1 Average Well Colour Development of GN and GP plates.



Average well colour development for Biolog GP and GN plates at 48, 72 and 96 hours. The error bars shown represent the standard error of three replicates.

AWCD results for the GN plates have smaller standard error indicating higher levels of reproducibility compared with GP plates. This difference in reproducibility may be due to novel carbon sources being present on the GP plate.

A statistical test (one-way ANOVA) was performed to determine if a significant difference existed between the AWCD of GP and GN plates at each time point. There was a significant difference at each time point [48 h ($F_{1,4}$= 13.4, p = 7.7), 72 h ($F_{1,4}$= 18.8, p = 7.7), 96 h ($F_{1,4}$= 8.1, p = 7.7)], for both plates there were also significant differences between AWCD at each time point between the GN and GP plates [GN ($F_{2,6}$ = 34.51, p = 5.1), GP ($F_{2,6}$ = 23.5, p = 5.1)].

6.2.3. DGGE Analysis of GN and GP Biolog Plates.

To examine the bacterial community present in some wells on the Biolog plates, DNA was extracted from 12 predetermined wells (11 carbon sources and the control well) and 16S rRNA PCR performed using universal primers. After amplification the products were separated on DGGE gels to examine the diversity present in comparison to the sample before inoculation into Biolog plates. The number of bands detected by DGGE analysis increased with incubation time on both the gels produced from GN and GP plates (Figures 6.2 and 6.3). From Fig. 6.2 it can be seen that GN gels contain one dominant band in nearly all the lanes, including the control, and after 72 h, this band is still dominant. After 96 h there appears to be six main bands present in the wells, including the control.

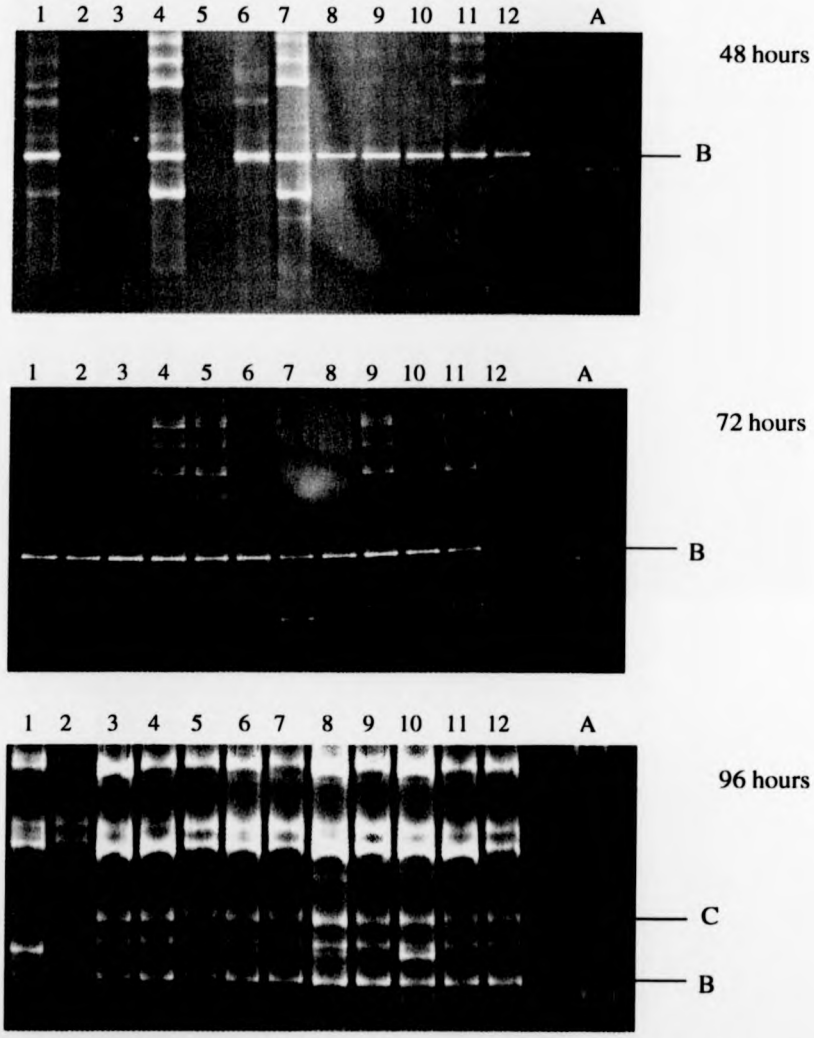
The GN-DGGE gel at 96 h has very similar / identical banding patterns in all lanes but some of the banding patterns are established at an earlier time. For example lanes 4, 5, 9, 10 and 11 have the same banding pattern at 72 h, but at 96 h the bands have an increased intensity. After 48, 72 and 96 h, the control well on the GN plate appears to have the same DGGE pattern but at lower intensity. Although this is not a quantitative method, it may be an indication that there were fewer bacteria in this well.

Analysis of the GP-DGGE showed that after 48 h there is one major band present on the DGGE gel, this being in a similar position to the dominant band on the GN gels (Fig 6.3). After 72 h the GP-DGGE gels have a similar band pattern compared with GN gels. Again there is one dominant band, but in the case of the GP plate it is not present in all of the lanes. After 96 h in the GP-DGGE gel, the majority of lanes with detectable banding patterns are very similar to the GN-DGGE gel banding patterns. In some lanes on the GP-DGGE gel, however, there are no detectable banding patterns.

In the GP plates, bands could not be detected from the PCR products derived from the control well samples although bands were detected in the GN plate controls. This was unexpected as the same soil inoculum and conditions were used for both GN and GP plates and both control wells should contain the same nutrients, levels of dye and have been inoculated and incubated under the same conditions. The basal medium in the GP and GN plates, however, is different (Biolog, pers. comm.). Due to patent issues the exact contents of the well are not published, but Biolog confirmed that the GP medium contains fewer nutrients than the GN plate. The lower concentration of nutrients in the GP plates will support less microbial growth, which in turn could lead to a reduced intensity and number of bands on the DGGE gel.

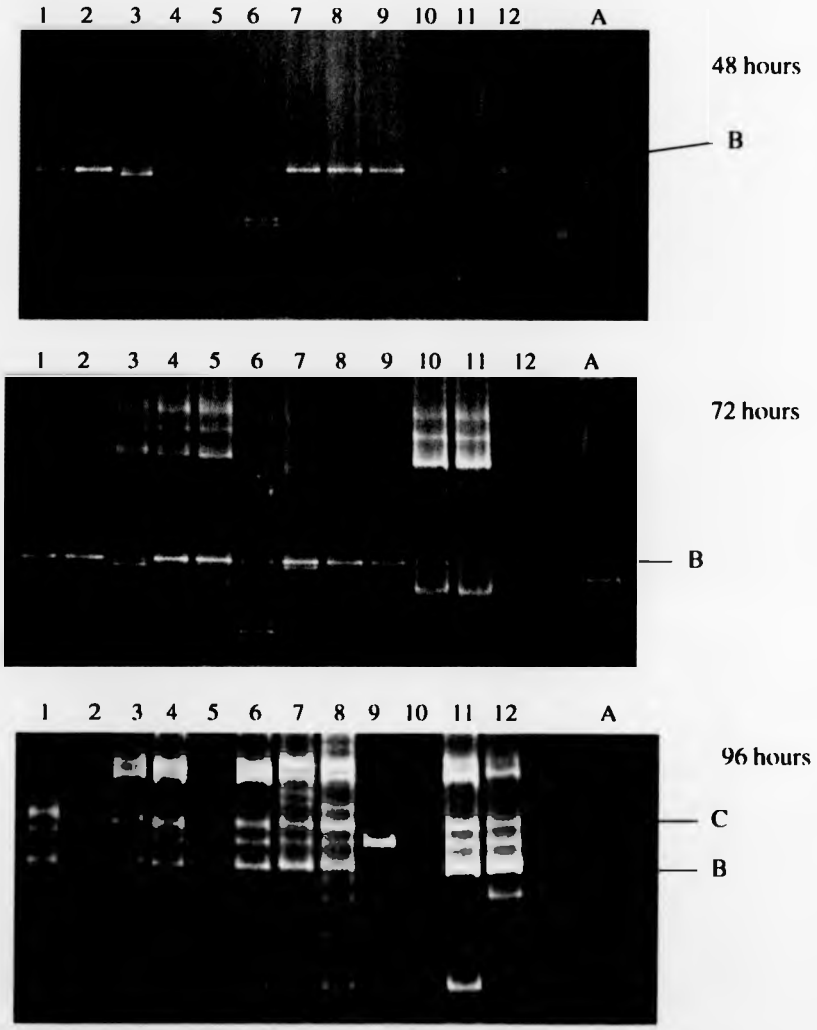
In summary, the GP gel banding patterns are similar to the GN patterns when communities are detected, although in the GP plates a number of carbon sources do not appear to support growth. In the GN-DGGE gels, bands were observed appearing in more carbon sources at an earlier time point. The community present in the Biolog wells is considerably less diverse than the total soil community (Section 3.5). When Warwick soil total community DNA is analysed, a smear of DNA bands is obtained by PCR-DGGE analysis (Baker, 1997), while the Biolog wells appear to support a few species in high numbers.

Figure 6.2 DGGE analysis of selected wells on Biolog GN plates after 48, 72 and 96 h of incubation.



Lanes 1-12 are DNA extracts from various carbon sources on Biolog GN plates. 1= a-D-glucose, 2 = mono-methyl-succinate, 3 = tween 80, 4= p-hydroxyphenylacetic acid, 5 = glycerol, 6 = putrescine, 7 = glucose-6-phosphate, 8 = L-phenylalanine, 9 = urocanic acid, 10 = bromo-succinic acid, 11 = succinamic acid , 12 = control well, A = *Pseudomonas fluorescens*. B and C indicate the bands that were sequenced and identified as *Pseudomonas putida* and *Pseudomonas aeruginosa* respectively.

Figure 6.3 DGGE analysis of selected wells on Biolog GP plates after 48, 72 and 96 h of incubation.



Lanes 1-12 are DNA extracts from various carbon sources on Biolog GP plates. 1= tween 40, 2 = D-gluconic acid, 3 = maltose, 4 = D-alanine, 5 = methyl pyruvate, 6 = thymidine, 7 = putrescine, 8 = D-C-a-glycerol phosphate, 9 = 2, 3, butanediol, 10 = succinamic acid, 11 = a-D-glucose, 12 = control well, A = *Pseudomonas fluorescens*. B and C indicate the bands that were sequenced and identified as *Pseudomonas putida* and *Pseudomonas aeruginosa* respectively.

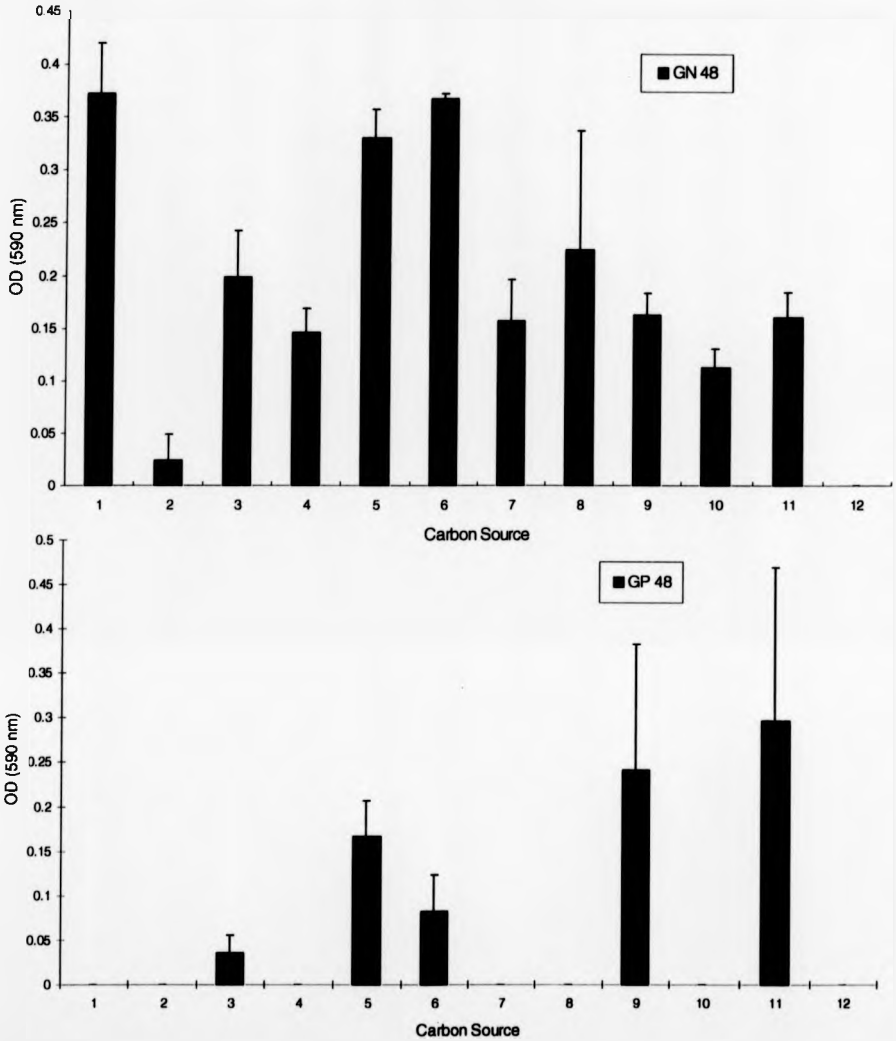
6.2.4. Sequence Analysis of Selected Bands.

Bands from the DGGE gels were excised and sequenced (Section 2.6.6). This allowed the identification of key species in the Biolog wells. *Pseudomonas putida* and *Pseudomonas aeruginosa* (94 and 96% sequence similarity with 16S rRNA, respectively) appear to be dominant organisms growing in the selected wells on the plate. Attempts were made to sequence other bands on the gels but were unsuccessful.

6.2.5. Optical Density Development and the Detection of Bands on DGGE Gels.

The optical densities recorded after 48 h incubation for the 11 selected carbon sources from the GN and GP plates (corrected for colour in control wells) are represented graphically in Fig. 6.4. The optical density of the control well has been subtracted leaving the net difference. There is no correlation between the optical density in the Biolog wells and the number of bands detected on the DGGE gels. For example, at 48 h on the GP plate, carbon sources 7 and 8 show no detectable increase in optical density (Fig. 6.4) although a band is present on the DGGE gel from each lane. Figures 6.5 and 6.6 show that optical density increases over longer periods of incubation. At 96 h, all 11 of the selected wells showed an increase in optical density. On the DGGE gel at this time, a number of wells produced no detectable bands. This may be due to the optical density in the control well being high, leading to no detectable increase in the optical density in the carbon source wells, although it may support a number of microbial groups. With increasing optical density over the 96 h the reproducibility of the optical density in the wells increases, this may be due to differences in bacterial number in the wells after 48 and 72 h.

Figure 6.4 Optical density of selected wells on GN and GP Biolog plates at 48 h.

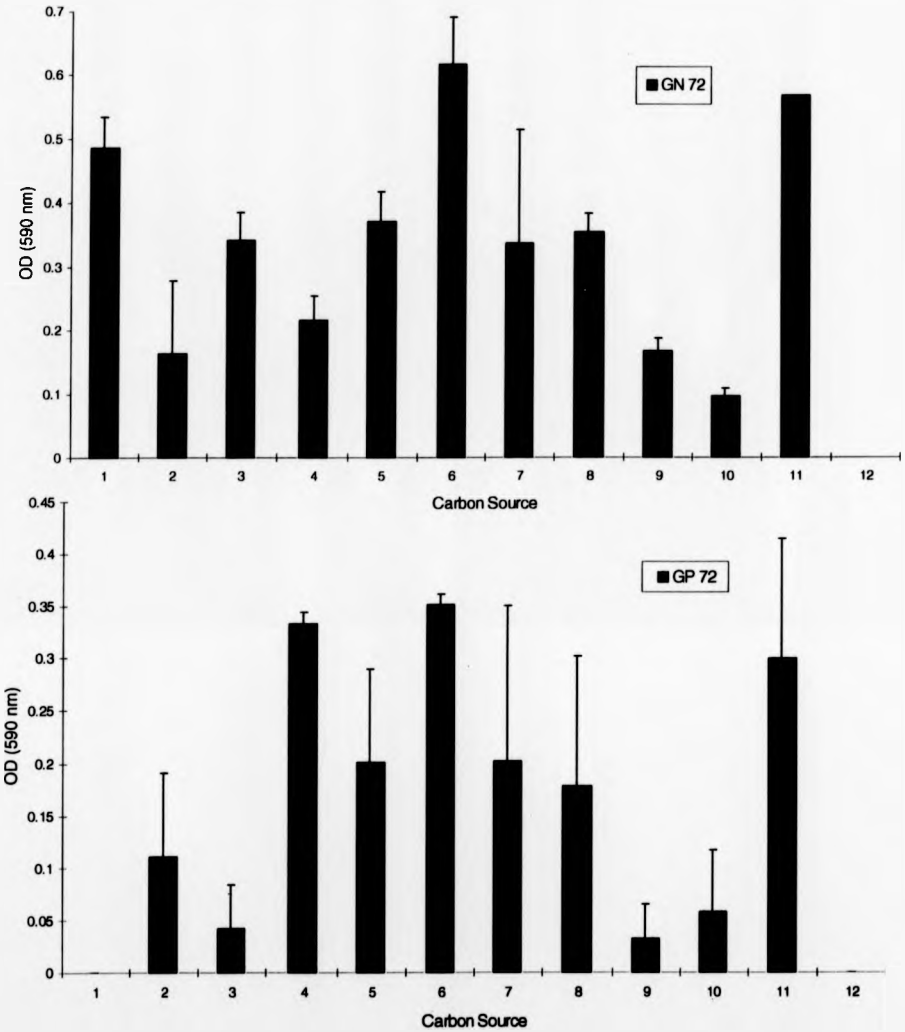


GN 1= a-D-glucose, 2 = mono-methyl-succinate, 3 = tween 80, 4= p-hydroxyphenylacetic acid, 5 = glycerol, 6 = putrescine, 7 = glucose-6-phosphate, 8 = L-phenylalanine, 9 = urocanic acid, 10 = bromo-succinic acid, 11 = succinamic acid , 12 = control well.

GP 1= tween 40, 2 = D-gluconic acid, 3 = maltose, 4 = D-alanine, 5 = methyl pyruvate, 6 = thymidine, 7 = putrescine, 8 = D-C-a-glycerol phosphate, 9 = 2, 3, butanediol, 10 = succinamic acid, 11 = a-D-glucose, 12 = control well.

The error bars shown represent the standard error of three replicates.

Figure 6.5 Optical density of selected wells on GN and GP Biolog plates at 72 h.

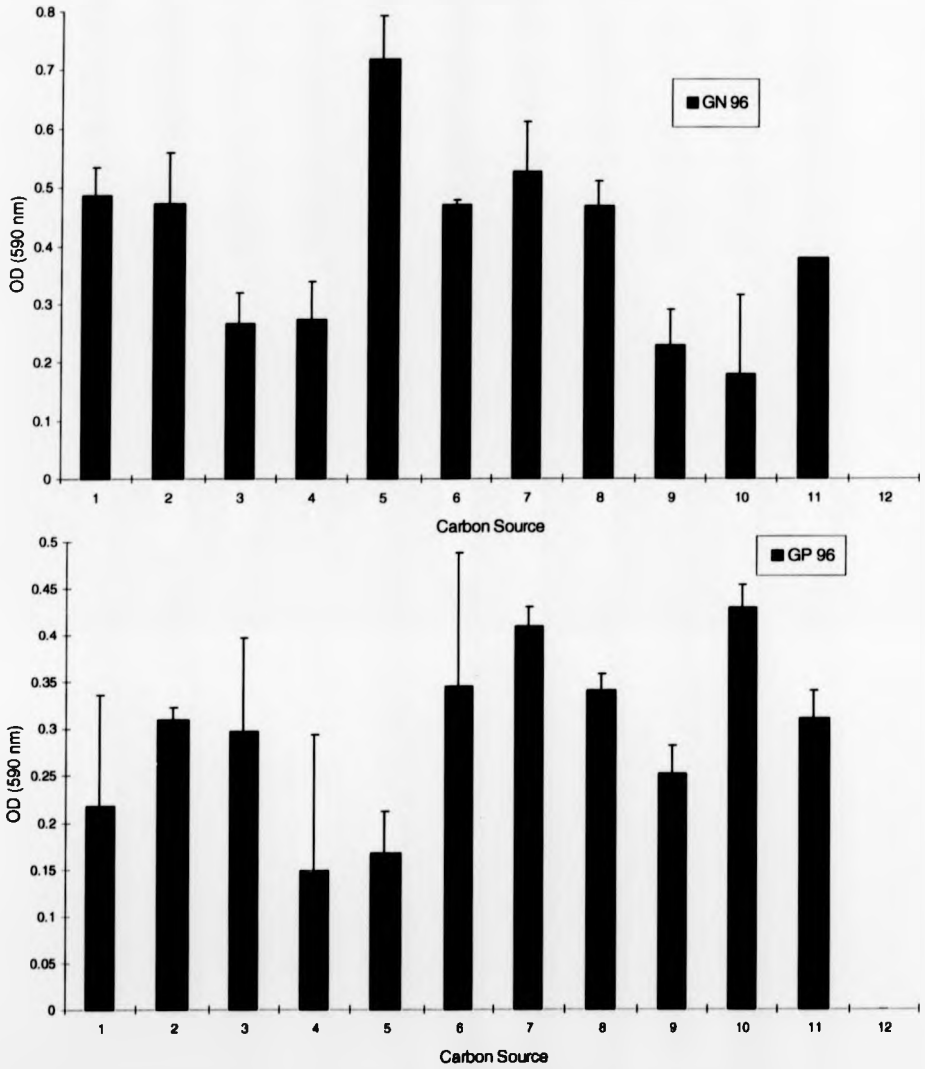


GN 1= a-D-glucose, 2 = mono-methyl-succinate, 3 = tween 80, 4= p-hydroxyphenylacetic acid, 5 = glycerol, 6 = putrescine, 7 = glucose-6-phosphate, 8 = L-phenylalanine, 9 = urocanic acid, 10 = bromo-succinic acid, 11 = succinamic acid , 12 = control well.

GP 1= tween 40, 2 = D-gluconic acid, 3 = maltose, 4 = D-alanine, 5 = methyl pyruvate, 6 = thymidine, 7 = putrescine, 8 = D-C-a-glycerol phosphate, 9 = 2, 3, butanediol, 10 = succinamic acid, 11 = a-D-glucose, 12 = control well.

The error bars shown represent the standard error of three replicates.

Figure 6.6 Optical density of selected wells on GN and GP Biolog plates at 96 h.



GN 1= a-D-glucose, 2 = mono-methyl-succinate, 3 = tween 80, 4= p-hydroxyphenylacetic acid, 5 = glycerol, 6 = putrescine, 7 = glucose-6-phosphate, 8 = L-phenylalanine, 9 = urocanic acid, 10 = bromo-succinic acid, 11 = succinamic acid , 12 = control well.

GP 1= tween 40, 2 = D-gluconic acid, 3 = maltose, 4 = D-alanine, 5 = methyl pyruvate, 6 = thymidine, 7 = putrescine, 8 = D-C-a-glycerol phosphate, 9 = 2, 3, butanediol, 10 = succinamic acid, 11 = a-D-glucose, 12 = control well.

The error bars shown represent the standard error of three replicates.

6.3. Discussion.

The communities that develop within the wells of Biolog GN and GP plates are very similar on the basis of their DGGE profiles. Sequence analysis from the DGGE gels indicated the dominant presence of two *Pseudomonas* species. This indicates that the Biolog plates favour the selection of fast growing copiotrophic microorganisms. These findings are in agreement with a previous study by Smalla *et al.* (1998), who determined that the dominant microorganisms belonged to the γ *Proteobacteria.* Pseudomonads may not be the dominant group in the environment, but in the Biolog well they may out compete the slow growing oligotrophic microorganisms (Heuer and Smalla, 1997a; Smalla *et al.*, 1998). The results in this study indicate that the carbon source utilisation patterns of the Biolog plate are not representative of all the microorganisms present but represent a proportion of the community that can adapt quickly to the conditions of the Biolog plate. Biolog carbon utilisation analysis is capable of discriminating between environmental samples, but caution must be used before relating these patterns to the whole bacterial community (Konopka *et al,* 1998).

In comparison with total soil DGGE analysis, the Biolog wells showed limited diversity, this has also been shown by Smalla *et al.* (1998). This reduction of community diversity could be the result of the Biolog plate providing a specialised environment. This artificial environment is very different to the conditions normally found in the soil and the high levels of nutrient in the well can lead to substrate-induced death (Konopka *et al.*, 1998). The Biolog plate provides a mainly aquatic environment that has no spatial structure which is a major factor regulating microbial activity in soils (Young and Ritz, 1998).

The link between potential metabolic diversity and actual functional activity is not fully elucidated (Zak *et al.*, 1994). Heuer and Smalla (1997a) found that some isolated strains did not give a carbon utilisation pattern when added to the Biolog plates. This may result from the microorganism existing in an extremely specialised

niche, such that its does not degrade any of the carbon sources present on the plate. However, when considering environmental isolates, it is more likely that the microorganisms may have been present in a nutrient-poor habitat and when presented with such a nutrient rich, warmer and, to some extent, aquatic environment, will take a considerable time to adapt to these new conditions before carbon utilisation can begin (Konopka *et al.*, 1998). Verschuere *et al.* (1997) examined the changes in model communities following inoculation into Biolog wells. They determined that the metabolic activity of some strains was masked by others when inoculated together at the same density. Their data suggested that species which are present in low numbers can sometimes have a dominant effect on colour production.

The control wells in the GN plates appear to support the same community as the wells with carbon sources. This suggests that bacteria are able to survive and grow using the basal medium and, unless the carbon source in the well is inhibitory, the community will reach a climax of a few well-adapted species. The microbial community may be able to survive using carbon and other nutrients from the initial inoculum (Konopka *et al.*, 1998). However, no bands were detectable in the GP control wells by DGGE, this was probably due to the basal medium containing lower levels of nutrients than the GN plates and, consequently bacterial numbers being below the detection limits. These results indicate that the community does not survive using nutrients from the inoculum, but probably utilises the basal medium.

Some of the carbon sources on the GN and GP plates are shared but the two plates do not have the same banding patterns present in the DGGE gel. This may be due to the GP plates having lower concentrations of nutrients present in the well in comparison with the GN plate.

There are biases involved in the genotypic examination of the Biolog plates used in this study. For example, only partial lysis of the cells in the well may occur during the DNA extraction procedure. Also, in the PCR reaction, there will be PCR biases which may affect the structure of the community when analysed by DGGE. Presence of bands without a corresponding optical density increase may result from microbes

present in the wells not actively respiring, but still being detectable by molecular methods.

There are a number of additional limitations inherent to the Biolog system including the selectivity of the assay which is based on respiration in an aerobic system. This selectivity means that strictly anaerobic bacteria will be excluded from the community analysis (Winding, 1994). The microtitre wells in the Biolog plate indicate degradation of the carbon compounds by respiration detection and will, therefore, have the same limitations as any other cultivation technique (Konopka *et al.*, 1998). To be representative of the soil, the Biolog assay requires cells to be removed from the soil matrix and any bacteria that are strongly adhered to soil particles will not be included in the assay. The well of the Biolog plate is in reality an aquatic environment. The highest concentration of nutrient sources are probably at the bottom of the well with a biofilm existing at the interface. If a biofilm exists on the interface of the well, high levels of bacteria would be located in this area, but this region may not be extracted for the DNA analysis. There may be anaerobic regions at the bottom of the well, with different bacterial communities than the top aerobic region of the well. Kersters (1997) noted that Biolog production batch number affected metabolic profile development. Any Biolog community analysis studies performed temporally must consider this. Inoculum density has been found to play an important role in the development of carbon utilisation patterns (Garland and Mills, 1994), although, it has been proposed that incubations of over 48 h can overcome this variability (Wünsche *et al.*, 1995). The reproducibility of the optical density on the eleven carbon sources increased at each sample point, this is probably a result of differences in the inoculate.

Other methods to assess metabolic activity *in situ* exist, such as *in situ* catabolic potential (ISCP), whereby different carbon source are added directly to the soil (Degens and Harris, 1997). ISCP may yield a more accurate picture of the activity as the bacterial habitat / soil structure is maintained, which is important in functional diversity (Young and Ritz, 1998). This method, however can be very labour intensive and not facilitate the comparison of a number of soils.

To summarise, DGGE analysis revealed that the Biolog plates do have an enrichment effect on the bacterial community and contain a greatly reduced community diversity when compared to the total sample. The Biolog carbon source utilisation analysis does enable the separation of communities and allows comparisons of metabolic diversity for the dominant species, and is also a rapid and reproducible method (Smalla *et al.*, 1998). Although caution must be used when applying the results to the total community as the dominant species in the Biolog plate may not be the predominant microorganisms in the environment (Garland, 1997).

6.4. Future Work

* Examine other wells on the Biolog plates as the other different carbon sources may have different genetic profiles.
* Determine if distinct bacterial communities exist in different areas of the wells.
* Use different soil samples to analyse if these effects are observed in different soil types.

Chapter 7

General Discussion.

7.1. General Discussion.

Understanding and evaluating soil fertility requires a polyphasic approach in which physical, chemical and biological factors are analysed. Fertility is closely related to soil aggregate structures, aggregates being formed by various chemical, physical and biological interactions. The soil microbial community plays an essential role in the formation and subsequent stability of soil aggregates. Soil fertility is also dependent upon many nutrient cycling and decomposition reactions performed by microorganisms in the soil (Prosser, 1997). It is important to assess the microbial community changes and the extent of damage caused to soil structure by the addition of anthropogenic pollutants when compared with a microbial community of a fertile soil with good structure (Harris, 1994). A understanding of the effect that pollution has on the microbial soil community could lead to the development of more efficacious bioremediation strategies.

An examination of the location of bacterial groups on the microscale to relate microbial interactions with soil structure was performed in this study. A Warwick soil was separated into water-stable aggregates by a physical soil fractionation technique. The use of a combination of methods, including culturable counts, taxonomic 16S rRNA gene analysis and potential metabolic activity analysis using the Biolog system identified actinomycete and pseudomonad populations at different locations in the soil. Actinomycetes were mainly located in the larger soil fractions suggesting that they may be involved in binding the soil microaggregates together. Pseudomonad species were predominantly located in the smaller soil aggregates and may be trapped within these smaller aggregates or, their distribution may result from the bacteria having strong electrostatic attractions with clay particles. To confirm if the proposed distribution of the actinomycete and pseudomonad populations present in the soil are correct then methods such as confocal microscopy or *in situ* hybridisations in soil, combined with soil embedding and sectioning techniques could be used.

The structure of the soil may provide protection to microorganisms in the presence of abiotic stresses such as pollution. This may be a consequence of the soil providing many microniches which may physically separate microbial communities from the impacts of abiotic stress (Coleman *et al.*, 1994). This has been observed with the distribution of heavy metals in soil, much lower levels of heavy metals being detected in the inner regions of aggregates (Wilcke and Kaupenjohann, 1997).

Different sized soil fractions were analysed for potential metabolic activity, the highest levels being detected in the second largest fraction. Elevated potential metabolic activity may result from the presence of increased bacterial numbers within this fraction, although no significant differences were supported by culturable plate counts. The higher metabolic activity may also result from a more metabolically diverse or active collection of bacteria residing in this aggregate size, this may reflect a more nutrient enriched environment.

The location of different bacterial groups in the soil may provide additional tools for the detection of new groups of commercially important microbes. *Streptomyces* species appeared to be the most taxonomically diverse in one soil fraction (Baker, 1997). This soil fraction may provide the source of a more targeted approach for pharmaceutical discovery of novel bioactive compounds as an alternative to screening the total soil. Because new multiply resistant strains are arising more commonly in clinical situations (Levy, 1998) this approach may provide an important step in the detection of more diverse bioactive microorganisms which is central to the discovery of novel chemotherapeutics.

Spatial patterns of microbial diversity are important at the macroscale and microscale level. A taxonomic study by Felske and Akkermans (1998) indicated that soil sampled over a considerable area, with different agricultural and fertiliser histories, supported the same microbial communities. Measuring communities at the macroscale should have produced greater differences in the microbial community if gradients in soil conditions were present. It can be hypothesised that the microbial communities in different habitats in a large site do vary, but these difference in the

communities are small and cannot be detected by taxonomic methods. This lack of variation reported by taxonomic analysis may indicate that only the dominant species in the communities were detected and this may be a limitation of the taxonomic techniques. However, Tiedje (1995) postulated that bacterial diversity on large geographical scale may not reveal an extensive increase in diversity as high levels of diversity are already present at the microscale. In this study high levels of taxonomic diversity were detected in the Warwick and Doncaster non-polluted soils at the microscale level in each of the different soil fractions. This may have interesting consequences with regard to the discovery of novel compounds. Instead of obtaining many samples from a large geographical area, less samples may be taken with subsequent analysis focusing on the microscale level.

The relationship between diversity and functional ability is not yet completely understood (Zak *et al.*, 1994; Degens, 1998a). It is often assumed that microbial communities can adapt to different abiotic stresses if the microbial diversity of the system is high due to the functional redundancy of the community. It was recently reported, however, that a decreases in functional diversity of microbial communities did not result in the reduction of the biotic processes (Degens 1998b). Clearly, further studies are required to establish if diversity in the environment is essential for the functioning of the soil system, or if diversity is a consequence of the existence of many microhabitats in the soil. To evaluate this, comparisons can be made between changes of microbial communities in stressed and non-stressed soil environments.

The understanding of microbial community structure and composition can provide a baseline measurement against which the impact of environmental changes can be measured (Tiedje, 1995). Pritchard *et al.* (1995) discussed the role of microbial diversity in hydrocarbon breakdown. In order to obtain the greatest flexibility for biodegradation, microorganisms must interact closely to allow mutually beneficial interactions for pollutant degradation in the environment (Bianchi and Bianchi, 1995). The mechanisms of breakdown of mixtures of pollutants is very complex, it may be that a unique microbial diversity is required as a consequence of a number of cometabolism and sequential reactions. Knowledge about the composition of

communities involved in the biodegradation process may lead to the development of communities with enhanced degradation abilities (Pritchard *et al.*, 1995). The microbial community can also be used as a tool for monitoring environmental change as a consequence of its ability to adapt rapidly to different abiotic factors. This procedure has been used when monitoring the affects of pollution on microbial communities by a pollution-induced community tolerance (PICT) technique (Pennanen *et al.* 1996).

A comparison of Warwick soil with the heavy metal polluted Doncaster soil was performed using a polyphasic approach involving culturable counts, taxonomic analysis and potential metabolic activity. The polluted soil had lower culturable counts, taxonomic diversity, and potential metabolic activity in comparison with the Warwick and Doncaster non-polluted soil. Low diversity and low bacterial population was closely related to a poor soil aggregate structure in the polluted soil. It is hypothesised that the reduced soil structure is a result of poor nutrient status of the soil, in addition to a lack of microaggregates that would otherwise provide a suitable environment for microbial growth and activity (Young and Ritz, 1998). The reduction in the diversity of microbial communities and their subsequent activities in soil has a detrimental effect on soil fertility and structure. These effects leading to a damaging cycle of soil structure and fertility loss, allowing the soil to be eroded.

In this current study microcosm systems containing different proportions of polluted and non-polluted soil were used to provide an of how the microbial communities are altered with different levels of contaminant. In general culturable counts and taxonomic diversity decreased with increasing proportions of polluted soil. Whilst the potential metabolic activity was stimulated by the presence of small amounts of polluted soil, although, decreases in activity were observed with the addition of higher levels of polluted soil. The observed increase of metabolic potential may be the result of the stimulation of the Warwick soil microbiota by the addition of an inorganic element that may be present in low amounts in the Warwick soil. Microcosm systems allowed the analysis of the soil communities with time, it was found that as time increased, the culturable plate analysis detected similar propagule

numbers but the genetic analysis appeared to decrease over the 80 days. This could be a result of the overall community decreasing, but because the selective plates in this study only focused on two ubiquitous populations it may not detect this overall community decline. Future work could focus on the manipulation of conditions such as nutrient supply and the microbial community response monitored to achieve a greater understanding of the soil microbial community dynamics in the microcosm system.

In a field trial in collaboration with a BG plc the use of a polyphasic approach allowed the bioremediation process to be monitored. The identification of taxonomically dominant microorganisms indicated which microorganisms may be involved in the bioremediation process. These pollution resistant microorganisms can then be cultured in high numbers and introduced again into the treatment pits to enhance biodegradation rates. The isolates could also be studied in greater detail to determine their mechanisms of resistance and pathways by which they break down pollutants. These pathways may provide candidates for the selection of degradative genes and cloned into field application vectors (Lajoie *et al.*, 1993) or studied further to yield probes for further analysis of the environment.

In the BG plc. treatment study, the culturable numbers, genetic diversity and metabolic potential were linked to biodegradation of the PAH pollutants in the treatment pits. This suggests that the polyphasic approach could be used as a basis for monitoring field trials. Many novel bioremediation techniques, such as electrodialytic methods, are currently used to remediate land (Ottosen, 1997). It is important to assess the effects of these techniques on the microbial community and not just on the physical and chemical properties of the soil. A polyphasic approach, as performed in this study, can provide such information about the soil microbial community present.

A number of soil microbial communities were examined using the polyphasic approach. Each method detects a different element of the overall diversity present in

the microbial community. Standage (1998) examined the relationship between molecular detection of gentamicin resistance in soil community DNA and selective isolation of gentamicin-producing micromonosporas and the correlation between these methods was less than 50%. This observation has also been made in 16S rRNA clone libraries and other functional genes including *mer*A, where novel sequences have been obtained that cannot be attributed to isolated bacteria (Ward *et al.*, 1995, Hart *et al.*, 1998). It can be hypothesised that the direct molecular taxonomic methods allow a greater understanding of the whole community without the problems of culturability on selective plates (Liesack *et al.*, 1997). No one method can provide information on the function, genetic constituents and relationships involved in a microbial community. Therefore, ecological studies of an environment must adopt a polyphasic approach. The challenge is to compare and contrast the results from the different methods to achieve a more representative picture of the environment. Tiedje (1995) addressed this issue and determined that each method has different resolution capabilities, that is coarse or fine levels of characterisation. Biolog analysis has a coarse level of resolution as it does not detect individuals, but allows qualitative comparisons of different communities. Traditional culture-based techniques provide a fine level of resolution of the community, allowing the identification of species in the environment and with further analysis may yield information on the function performed in the environment. Genotypic techniques can provide both coarse and fine levels of characterisation. Coarse resolution of the microbial community is gained from overall fingerprinting techniques and can identify microorganisms by sequence analysis.

Selective plate counts are a traditional method of community analysis and serve to provide an overview of culturable population changes in this study. The selective plating technique also enabled the acquisition of isolate collections from the different soils studied. These isolates yield essential information about the environment and their function within it, which cannot be gained by 16S rRNA analysis taxonomic information (White, 1994). Phenotypic methods, such as enrichment cultures allow the discrimination of function and form of the microorganism to be examine (Head *et al.*, 1998). The principle limitation with cultivation is that the majority of

microorganisms in the environment are thought to be unculturable (Torsvik *et al.*, 1990b; Ward *et al.*, 1995). The microorganisms which can be cultured in the laboratory may not necessarily be the dominant populations in the environment (Stotzky, 1997).

Taxonomic analysis using the 16S rRNA has been utilised in many studies to overcome the limitations of the culturable plate counts (Rölleke *et al.*, 1996; Ferris *et al.*, 1997; Felske and Akkermans, 1998). Taxonomic analysis facilitated the monitoring of microbial communities by a fingerprinting technique and allowed the identification of dominant microbes. These genotypic techniques have become the methods of choice for many molecular studies and have been used to detect numerous bacteria in the environment that cannot, as yet, be cultured by traditional methods (Liesack and Stakebrandt, 1992) and to detect microbial activity (Felske *et al.*, 1997). There are limitations inherent in genotypic analysis which must be recognised and these include difficulties with DNA extractions, bias in PCR reactions and in clone libraries (Hansen, *et al.*, 1998; Liesack *et al*, 1997; Wang and Wang, 1996). In this current study precautions were taken against biases in community DNA extraction, for example multiple DNA sample extraction and PCR. Future studies should address the bias in DNA extractions by utilising a range of different DNA extraction techniques and combining the DNA extracts to obtain a more representative sample of DNA from the soil.

The Biolog system may not be representative of the whole microbial community since wells on the Biolog plate provide a specialised enriched environment which have the limitations of any other culture-based technique. To determine if the Biolog method was representative of the whole community, further analysis was performed on selected wells on the plate. The taxonomic analysis revealed a very defined community that was similar in each of the wells, including the control. Growth in the control well indicates that the bacteria may be metabolising using the basal media present in the wells and therefore not necessarily be utilising the carbon source in the test wells. This is an important point that should be taken into consideration when using the Biolog system to indicate changes in functional diversity. Taxonomic

identification of species from the Biolog plates indicated that pseudomonads were the dominant microorganisms in the selected wells, although these may not necessarily be dominant in the environment. This was supported by culturable plate count analysis as pseudomomad and actinomycete populations appeared to be present in equivalent numbers. Taxonomic analysis also indicated that there was a diverse community present before Biolog analysis. This dominance of pseudomonad species in the wells is probably due to the high nutrient levels and short incubation time favouring copiotrophic microorganisms.

Biolog analysis was used to examine and compare the potential metabolic activity of the different soil samples. Studies of the Biolog system indicated that the plates reflected the activity of the major culturable fraction of the soil system. This study indicated that pseudomonad species were a predominant microorganism in some of the Biolog wells. This may indicate that the Biolog system favours fast growing copiotrophic microorganisms rather than the dominant oligotrophic microorganisms in the soil. A limitation of the Biolog system is that it requires the homogenisation of the soil, and hence, removal of all soil structure. This may affect activity in the sample. In future studies *in situ* methods, such as *in situ* catabolic potential (ISCP) could be used and may provide more meaningful information about the activity of the total microbiota (Degens, 1998a). Biolog analysis provided information about the potential metabolic activity of the samples, but to gain a better understanding of the soil environment *in situ,* further studies could use mRNA techniques to examine activity genotypically. These techniques are labour intensive and if a rapid method of screening a large number of samples is required, then Biolog analysis may be used. Biolog analysis allowed a rapid and reproducible view of a subset of the community which gave an overall indication of the potential activity of the soil. Activity measurements can often be effected by the presence of contaminants in the soil, for example, ATP analysis cannot be used reliably in soils polluted with heavy metals (McCarty *et al.*, 1998). This must be taken into consideration when choosing a method of measuring activity.

Molecular techniques provide essential information when monitoring bioremediation. This type of analysis does have limitations including the cost of sample analysis and the incomplete knowledge of genes controlling biodegradation in the environment. Molecular analysis of bacterial communities is not yet well enough understood to be a stand alone assay (Head, 1998). However, with increasing numbers of bioremediation studies and a better understanding of the process, the monitoring of the bacterial communities may provide a sensitive tool for treatment assessment in the future. The microbial community structure and dynamics must be understood to gain a deeper insight into and allow the optimisation of the bioremediation process. The continued use of a polyphasic approaches will play a key role in this strategy.

It is anticipated that in the future there will be an increasing demand for suitable land for both agricultural and development purposes. Commercial and public pressures will therefore demand that polluted land is remediated. Landfill is currently the main method of contaminated land disposal, however, bioremediation strategies will hopefully become the method of choice. It is envisaged that in the future a suite of catabolic gene probes will be available for the rapid screening of the degradative potential of a bacterial community at a bioremediation site. Also, the quantification of these activities by mRNA analysis, may be possible *in situ*. The microbiological analysis of a prospective bioremediation site will be hopefully become as routine as the physical and chemical analysis of a site is currently. In the late eighties and early nineties it was believed that bioremediation would involve the isolation or creation of a 'superbug' that would be capable of degrading a specific chemical this microorganism being inoculated into the soil. This rather simplistic view has been replaced by a more holistic approach to bioremediation, based on the realisation that the biodegradation process is mediated by a consortia of many bacterial populations, with many relationships involved, such as cometabolism. As more sensitive techniques become available these biotic relationships will be better understood.

Chapter 8

References.

8. References

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Chapter 9

Appendices

9. Appendices

9.1. Total metal content determined by inductively coupled mass spectrophotometry in Warwick and Polluted and Non-polluted Doncaster soils.

	Soil		
Total Metal mg/kg	**Polluted Doncaster**	**Non-polluted Doncaster**	**Warwick**
Arsenic	44	28	8.0
Cadmium	<0.5	1.1	<0.5
Chromium	120	14	5.8
Copper	110	71	9.8
Iron	25000	19000	10000
Lead	270	97	19
Mercury	0.71	<0.1	<0.1
Nickel	39	22	13
Selenium	200	62	32
Zinc	170	170	65

Determined by Analytical Environmental Services (Ltd).

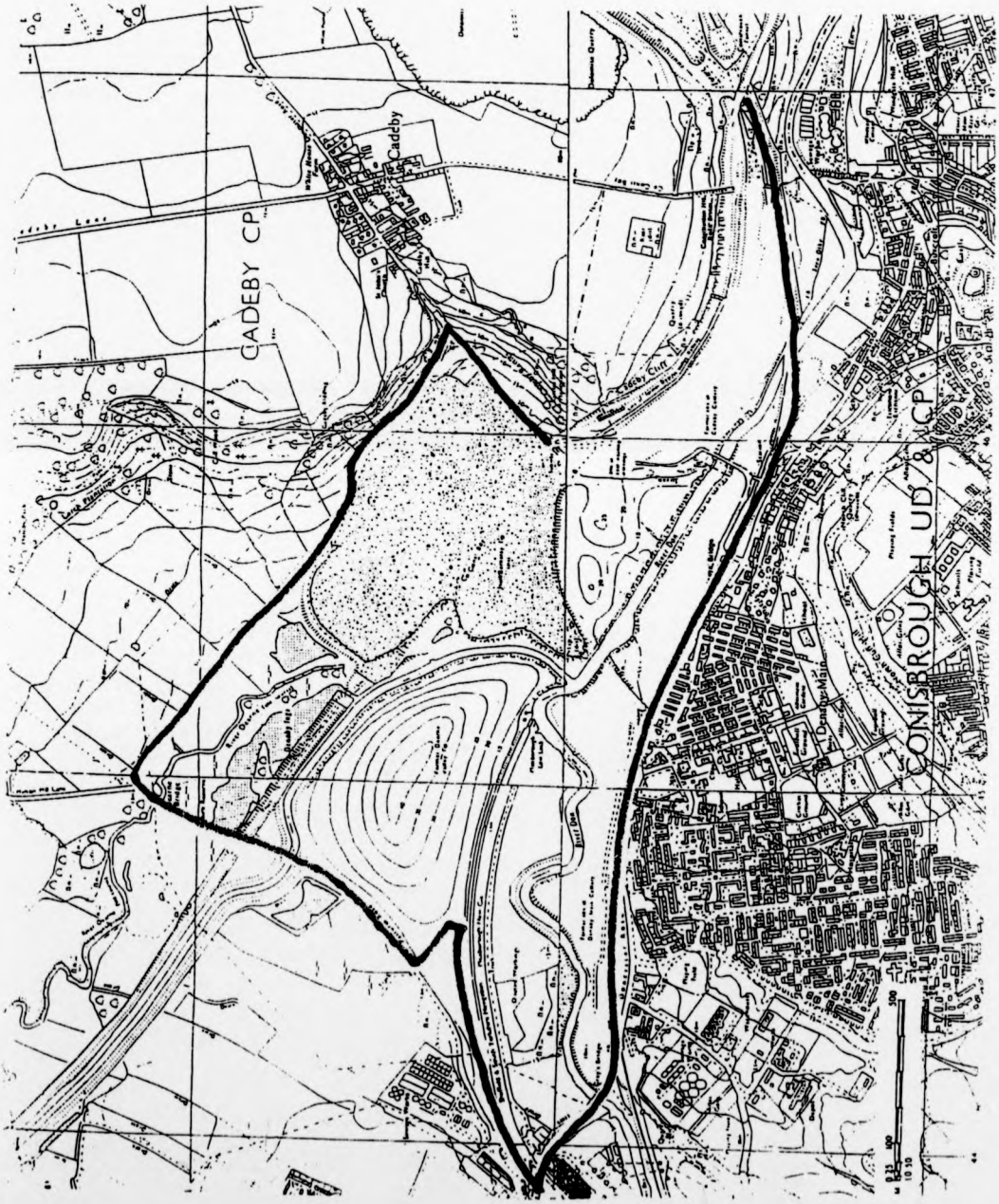
9.2 Site Descriptions.

Warwick: Soil was obtained from a local field site (Cryfield Hall, University of Warwick). Analysis of particle size gave a composition of (percent dry weight); 63.6% sand, 18.4% silt, 11.7% clay and 6.2% loss on ignition. The pH of fresh soil was 6.5-6.8 (Herron and Wellington, 1990) and after drying 6.7-6.9 (1:1).

Doncaster polluted: Soil was collected from the Earth Centre (Red House, Tickhill Square, Denaby Main, South Yorks.). Soil pH was determined as pH 2-4. Samples were taken from the east side of the 'Whaleback' this pit heap is 40 m in height with gentle slopes. In the 70's after the Aberfan disaster the heap was lowered and the dredgings from Mexborough New Cut Canal were placed on to it as top soil. This poor top soil from the canal contained contaminants from the local steel industry and this has increased signs of weathering and erosion (Hobbs, 1996).

Doncaster non-polluted: Samples were taken in the pony field area beside the river Don, soil pH was determined as pH 5-6. This area is regularly flooded by the canal the vegetation includes some wooded areas. Ponies grazed on the field until recently. This site has never been used for mine waste disposal.

9.3. A Map of the Earth Centre Site



9.4. The 16 Polyaromatic hydrocarbons compounds designated as priority pollutants by the US Environmental Protection Agency.

16 US EPA Polycyclic Aromatic Hydrocarbons		
Name	**Chemical Formula**	**Molecular Weight**
Naphthalene	C10H8	128.17
Acenaphthylene	C12H8	152.20
Acenaphthalene	C12H10	154.21
Fluorene	C13H10	166.22
Phenanthrene	C14H10	178.23
Anthracene	C14H10	178.23
Fluoranthene	C16H10	202.26
Pyrene	C16H10	202.26
Benz(a)anthracene	C18H12	228.29
Chrysene	C18H12	228.29
Benz(b)fluoranthene	C18H12	228.29
Benz(k)fluoranthene	C18H12	228.29
Benz(a)pyrene	C20H12	252.32
Indeno(1,2,3-cd)pyrene	C22H14	278.35
Dibenz(ah)anthracene	C22H14	278.35
Benzo(ghi)perylene	C22H12	276.34