An Assessment of Synthetic Landfill Leachate Attenuation in Soil and the Spatial and Temporal Implications of the Leachate on Bacterial Community Diversity

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Declaration

I do hereby declare that the work documented in this dissertation, unless otherwise stated, is the result of my own investigations based on original proposals with my supervisors.

____________________________ ____________________________

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"*Vires per fidem, Modestia in magnitate*"

Dedication

To my family and the spirituality we all share

Thank you Ma!

Abstract

The temporal fate of selected parameters, including redox potential; pH; phenol; nitrates; sulphates; copper and zinc, of a young synthetic acetogenic phase landfill leachate was assessed by perfusing a series of sequential soil (Hutton) microcosms (arrays) at two hydraulic loading rates (HLR). We chose HLRs that were representative of areas in South Africa with typically elevated rainfall (Pietermaritzburg – HLR*h*) and one with relatively low rainfall (Kimberley – HLR*l*). Preliminary phenol, copper, and zinc adsorption investigations on gamma radiation sterilized soil and unsterilized soil revealed superior adsorption rates for each compound in the unsterilized soil. This revealed the importance of the biological component of soil in phenol, copper, and zinc attenuation in soil. The results presented in this thesis suggest that the HLR of leachate into soil arrays contributes to significant differences in the fate of the landfill leachate parameters mentioned earlier. In addition, we assessed the temporal and spatial succession of bacterial community diversity in each of the soil arrays by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). Prior to this, we compared two soil DNA isolation techniques, the modified method of Duarte *et al.* (1998) (Bead Beat) and the commercial Mo-Bio UltraClean™ Soil DNA isolation kit (Kit). The DNA isolated by the Kit method was significantly superior regarding purity and absence of DNA fragmentation. However, the Bead Beat method produced a significantly higher yield per reaction before further purification with Wizard™ Clean-Up columns produced DNA extracts of similar purity at the cost of a significant reduction in DNA yield. The Kit method was chosen for future DNA isolation and PCR-DGGE based on the quality of the PCR amplicons generated from the Kit isolated DNA. PCR-DGGE was further optimized by comparing the efficiency and sensitivity of a silver stain against ethidium bromide. Silver stain generated DGGE gels with greater number of bands (species richness – S) and stronger band signal intensities. Captured DGGE fingerprints generated data that were subjected to the Shannon-Weaver Index (H') and the associated Shannon-Weaver Evenness Index (E_H) to measure the change in spatial and temporal bacterial diversity. There was a significant shift in S and H' for both HLRs but a significant change in E_H was only observed for HLR*h*. Furthermore, a temporal comparison of S and H' between both HLRs revealed significant

differences throughout the investigation. Canonical Correspondence Analysis (CCA) revealed spatial distribution of bacterial community diversity with depth. Effects of phenol concentration, redox potential, and pH of the effluent leachate on bacterial community diversity was tentatively assessed by three-dimensional graphical representation on PlotIT 3.2 software. Bacterial community diversity showed a decrease with elevated pH and phenol concentration along with decreasing redox potentials for both HLRs. While this study reveals the spatial and temporal dynamics of bacterial community diversity *in situ*, it provides important evidence with respect to: (i) the effects of rainfall / leaching rates (HLR) on spatial and temporal bacterial community succession; (ii) the importance of the biological component in natural attenuation; (iii) the ability of soil, previously unexposed to landfill leachate, to initiate natural attenuation of phenol and other leachate constituents; (iv) the capacity of PCR-DGGE to fingerprint successional changes in bacterial community diversity, (v) and the potential to clone and sequence selected members of bacterial associations for future reference in environmental remediation strategies.

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Chapter One

1. Literature Review

1.1 Introduction

One of the oldest and simplest forms of biotechnology is landfilling. Apart from its simplicity, the popularity of this technology as a solid waste disposal option remains unmatched (Sulfita, Gerba, Ham, Palmisano, Rathje and Robinson, 1992; El-Fadel, Findikakis and Leckies, 1997) due largely to the relative low cost in comparison to other solid waste disposal options (Senior and Balba, 1987). Therefore, landfills will continue to be the most attractive route for solid waste disposal. Indeed, research has shown that up to 95 % of solid waste generated worldwide, is currently emplaced within landfills (Cossu, 1989). Alternatives to landfilling are considered as volume reducing processes because they produce waste fractions that ultimately end up in landfills (El-Fadel *et al.*, 1997). Although differences in classification, sampling, and analytical methods make direct comparisons difficult, globally there exists a significant difference in refuse composition. This is not only evident between countries, but also evident within countries between different cultures; socio-economic groups; and socio-economic areas (Senior, 1990; Al-Fadel *et al.*, 1997). Despite the variability in waste composition, total organic content (in particular cellulosic material) constitutes the highest percentage of solid waste (El-Fadel *et al.*, 1997). It is this organic material that is susceptible to aerobic and anaerobic degradation, giving rise to leachate and gas as a consequence of numerous interacting variables (Senior, 1990; El-Fadel *et al.*, 1997).

Simplistically, leachate is water that has percolated through emplaced waste, forming a solution carrying with it suspended and soluble material to the bottom of the landfill (Senior, 1990; Novella, Ekama and Blight, 1996). Initial variables such as refuse composition; moisture content; microbial inoculum; the electron acceptors and donors present in the refuse (Novella *et al.*, 1996), as well as first-tier variables such as, geology; hydrogeology; hydrometereology; refuse emplacement strategies; cover permeability and topography; vegetation cover; site after-use; season and time, all undergo complex interactions with

second-tier variables such as, redox potential; pH; and temperature. These factors will have a direct influence on the quality and quantity of the leachate produced (Senior, 1990).

Landfill leachate present at the bottom of the landfill or at the interface of an impermeable layer within the landfill, either travels laterally to a point where it discharges to the ground's surface as a "seep", or it will move through the face of the landfill and into subsurface formations (Cheremisinoff, Gigliello and O'Neill, 1984; Reinhard, Goodman and Barker, 1984). Depending on the nature of the subsurface formations and the absence of suitable leachate controlling systems, leachate has been reported to be associated with the contamination of aquifers underlying landfills (Novella *et al.*, 1996; El-Fadel *et al.*, 1997). The actual impact that a given landfill has on its surroundings depends on the practice at that landfill and the corresponding quality of the surrounding areas. Emissions originating from the landfill itself are a direct result of the landfill, and these emissions in their broadest sense, can be defined as any kind of matter released to the area surrounding the landfill. Depending on the route of these landfill generated emissions they may cause unacceptable changes to the quality of the surrounding environment (Christensen, 1989). One such emission is landfill leachate, which is by far the most significant threat to the environment, in particular to groundwater resources (Novella *et al.*, 1996; El-Fadel *et al.*, 1997). Therefore, the impact of waste disposal on the environment is a major problem to a country like South Africa, which is facing serious water shortages in the near future. Groundwater resources form a fundamental part of the countries water supplies and this role is expected to increase significantly (Parsons, 1992; DWAF, 2004).

Factors such as the redox capacities and reactivities of reduced and oxidised compounds, in addition to other factors present in the leachate and receiving aquifer, play a major role in the development of sequential redox environments when a strongly reduced leachate enters the subsurface (Albrechtsen and Christensen, 1994; Bjerg, Rugge, Pedersen and Christensen, 1995). Research indicates that redox environments in leachate plumes, contribute significantly to contaminant attenuation in the subsurface (eg. Bjerg *et al.*, 1995; Rugge, Bjerg and Christensen, 1995; Mikac, Cosovic, Ahel, Andreis and Tovcic, 1998).

Biological uptake, a mechanism by which microorganisms either breakdown or adsorb leachate (Bagchi, 1994), is dependant on the establishment of microbial populations in response to the nature of the contaminant loading. To predict the fate of the contaminants, it is essential to study the actual pollution plumes so as to gain knowledgeable insights into the mechanisms occurring within such a complicated framework (Williams and Higgo, 1994). Since the redox conditions, persisting in the plume, are largely governed by a sequence of microbially mediated reactions (Lensing, Vogt and Herrling, 1994), it is only fitting that studies focusing on the distribution and diversity of the microbial populations within the plume will provide information required to elucidate, and ultimately harness, the interactions between microorganisms and contaminants (Ludvigsen, Albrechtsen, Ringelberg, Ekelund and Christensen, 1999; Roling, van Breukelen, Braster, Lin and van Verseveld, 2001). Ultimately this may lead to improved landfill design and construction by aiding the development of tools for predicting and monitoring natural degradation in subsurface environments in the vicinity of a landfill (Roling *et al.*, 2001). With this in mind, this series of investigations were aimed at analysing the attenuation of chemicals and the consequential microbial fauna in soil exposed to a landfill leachate poised at pH 5.5.

The importance of individual processes is highly variable for a variety of contaminants, since most of these reactions depend on the formation of characteristic sequences of redox zones in the subsurface, ranging from methanogenic to aerobic (Mikac *et al.*, 1998). Thus, the presence of microbial populations in the soil below a landfill may be beneficial for the attenuation of organic (Rugge *et al.*, 1995) and inorganic leachate constituents. However, this attenuation is not permanent since microbially fixed contaminants can be released by mineralisation as the biological population dies (Bagchi, 1994) or remobilised as the groundwater levels increase (Ahel, Mikac, Cosovic, Prohic and Soukup, 1998). It is possible to study the decay rate and pollutant release by these microorganisms using bio-kinetic principles (Bagchi, 1994). In so doing, this may aid in ascribing a form of numeric assessment that describes the contribution of microorganisms in attenuating subsurface contaminants.

1.2 The Role of Sanitary Landfilling in Solid Waste Disposal

The disposal of domestic waste into landfills has been in practice for more than 5000 years with the earliest evidence first appearing in Northern European Stone Age communities. These communities deposited waste in kitchen middens by emplacement strategies similar to those employed at present. The products of solid-state fermentations were used for agricultural (1900 B.C) and military (1530 A.D) purposes in Minoan Crete and Tudor England, respectively. However, evidence suggests that by the turn of the 20 th century the purpose of waste exploitation shifted to waste incineration to generate heat and electricity (White-Hunt, 1980; White-Hunt, 1981a; White-Hunt, 1981b).

Presently, refuse continues to be exploited by direct incineration for heat production (Senior, Watson-Craik and Kasali, 1990), while several other possibilities have emerged, including methane generation by anaerobic digestion (Kinman, Nutini, Walsh, Vogt, Stamm and Rickabaugh, 1987; Boeckx, Van Cleemput and Villaralvo, 1996). Although the refuse mass can be used to produce value added chemicals (by low-energy pyrolysis or acid hydrolysis); proteins (by Single Cell Protein or vermiculite technologies), and compost, low cost disposal to landfill remains the favourable option (Senior *et al.*, 1990).

Over the years there has been an exponential increase in the global population and industrial development. Inevitably, this has been accompanied by an increase in the volume and complexity of waste entering landfills (Sinclair, 1994). Therefore, it is perhaps no co-incidence that there has been a subsequent increase in the exploitation of landfills by current communities. Such exploits have been brought to fruition by the recognition of landfills to act as anaerobic filters for the treatment of industrial liquids and sludge effluents (co-disposal) (Senior *et al.*, 1990) in addition to the potential of landfills to act as anaerobic digesters for methane production (Kinman *et al.*, 1987; Senior *et al.*, 1990; Boeckx *et al.*, 1996). Within industrialised nations the major portion (60-70 %) of solid waste is land disposed by landfilling, composting, or land farming (Cheremisinoff, 1990), confirmation that modern methods of refuse management concentrates on the elucidation and harnessing of the potential energy within refuse (Large, 1983).

In the past, waste managers in South Africa have considered a landfill as a hole in the ground into which waste was buried and forgotten (“tomb concept”). Nowadays an engineered approach is employed when disposing of solid wastes (Diaz, 1994) and choosing solid waste disposal sites (Sumathi, Natesan and Sarkar, 2008) with specific governmental organisations responsible for establishing criteria for the selection; investigation; design; permitting; preparation; operation; closure and monitoring of the landfills (Department of Water Affairs and Forestry, 1998a) so as to minimise environmental hazards. The importance of proper landfill management including leachate and biogas management have subsequently gained tremendous recognition (Novella, Ross, Lord, Greenhalgh, Stow and Fawcett, 1996) leading to definitive investigations into the scientific design and operation of a landfill, followed by the effective long term reclamation of sites (Senior *et al.*, 1990).

Solid wastes are disposed into landfills by one of three basic methods: area, ramp, and trench (Cheremisinoff, 1990). Each method involves solid waste disposal in a manner that minimises environmental hazards by spreading and compaction into the smallest practical volume, followed by the application and compaction of cover material (commonly soil) at the end of each disposal day. Any landfill devoid of such operations cannot be termed “sanitary” (Diaz, 1994), and appropriately such a facility is unacceptable in modern society. Expansive populations and growing urbanisation has forced societies to live closer to waste disposal facilities, resulting in increasing levels of awareness to problems resulting from poor waste management. This, in conjunction with several other factors, has necessitated improvements in the quality of waste management (Cossu, 1989) by activating thorough investigations into the scientific and engineering processes involved in sanitary landfilling (Senior *et al.*, 1990; Sinclair, 1994).

1.3 Waste Classification, Sanitary Landfill Site Selection and Design

In designing a sanitary landfill it is of paramount importance to obtain a full characterisation of the waste to be landfilled. The pollution potential of landfilled waste can only be projected if the physical and chemical character of the waste is understood (Cheremisinoff, 1990) and if the environmental impact that such an installation will have on

the immediate site and surroundings is completely assessed. This assessment is concluded by the procedure termed Environmental Impact Assessment (EIA), which evaluates the relationship between the proposed landfill and the environment in which it is to be implemented by taking into consideration technical, legal, economic, social, and environmental aspects with the sole purpose of formulating a judgement (Andreottola, Cossu and Serra, 1989).

The Department of Water Affairs and Forestry (1998b) follows a classification system that distinguishes between wastes that require maximum precaution (extreme hazard wastes) and wastes that require lesser control (limited risk wastes) during disposal, on the assumption that no wastes are entirely non-hazardous. An assessment of the waste quality and quantity, adverse biological susceptibility, and the conditions of handling can be used to determine the hazard rating posed by the waste and as such two broad classes of waste exist: General Wastes and Hazardous Wastes (Department of Water Affairs and Forestry, 1998b). Collectively these classes encompass the following types of wastes:

- Municipal solid waste, the composition of which may change by virtue of separate collection activities;
- Mass waste, an example of which is mine waste;
- Demolition waste or residues from demolition waste recycling plants;
- Soil;
- Wastewater sludges; and
- Bulky waste

(Stegman, 1989).

The selection of a site for waste disposal requires the examination of multiple considerations. These considerations include the physical and chemical characteristics of the waste, the environmental and ecological impacts surrounding the proposed site (Pacey, 1989; Cheremisinoff, 1990), as well as legislation governing the location and design of the sanitary landfill in conjunction with public perception and acceptance (Glysson, 1990; World Bank Technical Paper, 1989; Nathanson, 2000).

Globally, the protections of aquifers are essential if water demands are to be satisfied for the future. Therefore, from an environmental perspective the geo-hydrology and geology are the definitive factors influencing the suitability of the proposed site since these settings will represent essential barriers against pollutant migration into the surrounding environment (Knox, 1989; Lechner, 1989, Pacey, 1989; Stief, 1989; World bank Technical Paper, 1989; Carra and Cossu, 1990; Cheremisinoff, 1990; Jewaskiewitz, 1992; Parsons, 1994; Parsons and Jolly, 1994; Jolly, 1996; Nathanson, 2000). The inherent attenuation potential of the saturated and unsaturated zones directly below the bottom of a landfill and downstream of the proposed site is vital for the preservation of acceptable groundwater quality in and around the proposed location (Stief, 1989). The Environmental Resources Management Inc. (1981) concluded that knowledge of the soil-pH, cation exchange capacity (CEC), and permeability would facilitate educated insights into the capacity of the soil to attenuate leaching chemicals. Generally accepted hydraulic conductivities for soil at a proposed site are in the range of 1 x 10^{-7} $cm.s^{-1}$ or lower. Sites with higher conductivities require the implementation of a liner system with average conductivities ranging between 1 x 10^{-7} $cm.s^{-1}$ and 1 x 10^{-12} $cm.s^{-1}$ (Pacey, 1989).

The World Bank Technical Paper (1989) presents a joint study sponsored by the World Bank, The World Health Organisation (WHO), and UNEP and lists an array of engineering, environmental, and economic criteria as essential for landfill site selection. It is often the case that a proposed site is rejected on political rather than technical grounds, albeit satisfactory compliance with the listed criteria. The general public and the politicians representing the public tend to equate landfills with "dumps" and are reluctant to allow sites in their communities (Nathanson, 2000). In the South African context, DWAF (1998a) "Minimum Requirements" caters for public participation in landfill development. The Department of Environmental Affairs and Tourism (DEAT) integrates this aspect of the DWAF guidelines into the Environmental Impact Assessment Regulations (EIAR) that they govern.

Landfills can be constructed in old quarries (originating from the extraction industry) as "below ground" sites, as "above ground" inclusive of valley fill landfills, and in "below ground" excavations built for the specific purpose of landfilling (Parker, Bateman and Williams, 1993). However, few existing sites are ideal for landfill purposes, but many that are

not can be moulded into suitable landfills by thorough planning and sound engineering (World Bank Technical Paper, 1989; Druyts and Legge, 1996). Traditionally, landfill sites have been classified into three groups:

- Class 1 Landfills (containment sites) contain refuse and leachate within the site by the inclusion of a barrier (synthetic- or natural clay- liner) of extremely low permeability in the design and construction phase of a landfill;
- Class 2 Landfills (attenuation sites) permit slow leachate migration with natural sand and gravel layers facilitating significant attenuation of leachate constituents, and shale barriers that provide protection for deeper aquifers; and
- Class 3 Landfills (rapid migration site) allow rapid leachate migration into the surrounding environment with minimal attenuation of leachate constituents by way of fissured strata (Senior, 1991).

Increasingly, class 1 landfill sites are becoming the norm (Senior, 1990) as industrialised nations opt for fewer but larger operational landfills that have stringent scientific designs (Senior, Watson-Craik, Sinclair and Jones, 1991).

Revision of design technology with respect to experience gained from existing landfills, coupled with the subsequent application of any practical and environmentally acceptable options, is necessary for the secure design of waste disposal sites (Jewaskiewitz, 1992). Consequently, a replacement landfill classification system is in operation in South Africa as stipulated in the "Minimum Requirements" guidelines. The system rejects class 3 sites and introduces a gradation system between class 1 and class 2 sites (Ball and Bredenhann, 1992). General waste landfills are subdivided into four classes (Communal, Small, Medium, and Large) based on the scale of the waste stream and the size of the operation. These classes are further divided on the basis of the site-water balance, where a positive water balance indicates the potential for significant leachate generation. All sites with a positive water balance, with the exception of Communal sites, require leachate management systems. However, all landfills receiving hazardous waste must have leachate management systems in place irrespective of the site-water balance. Landfills receiving hazardous wastes are designed, engineered, and operated under maximum stringency (DWAF, 1998a).

As a consequence of precedents of groundwater pollution, modern landfills are designed to keep adverse environmental impacts to a minimum (Wall and Zeiss, 1995). Modern landfill design must incorporate adequate containment systems, by way of natural- or synthetic- liners; leachate and biogas management facilities (Kennedy, Hamoda and Guiot, 1988; Binder and Bramryd, 2001); and groundwater monitoring facilities (Cheremisinoff, 1990; Blight and Bredenhann, 1992; Parsons, 1994; Nathanson, 2000).

1.4 Environmental Impacts of Sanitary Landfills

The term "environmental impact" specifically describes alterations of the environment resulting from activities associated with the implementation of a scheme. These alterations may evoke both positive and negative impacts (Andreottola *et al.*, 1989) depending on the nature of the installation, as is the case with sanitary landfills. Preceding inappropriate waste management strategies have resulted in numerous incidents of environmental pollution and public health problems (Scott, 1982). DWAF (1998a) deemed sanitary landfilling environmentally acceptable in South Africa on condition that their "Minimum Requirements" guidelines are sufficiently adhered to. Failure to do so can result in adverse short- and long-term impacts on the environment. This review focuses on the long term impacts of leachate generation that is generally associated with incorrect site selection; design; preparation; and operation (Chian and DeWalle, 1976; Venkataramani, Ahlert and Corbo, 1984; Christensen, 1989; DWAF, 1998a). Present trends dictate that even small construction projects must include an environmental impact study before project construction can be approved (Nathanson, 2000).

1.4.1 Landfill leachate

The greatest area of environmental concern with regards to sanitary landfills lies in the uncontrolled infiltration of leachate into the environment and subsequent pollution of surface and groundwater (Canziani and Cossu, 1989). The percolation of rainfall and other sources of water through emplaced waste in landfills eventually result in the production of a liquid, possessing extreme pollution potential. This liquid is termed landfill leachate (Dass, Tamke and Stoffel, 1977; Kennedy et al., 1988; Clement and Thomas, 1995; Frigon, Bissaillon, Paquette and Beaudet, 1997; Cecen and Aktas, 2001). The physico-chemical composition of leachate depends on the composition of emplaced waste, hydro-geologic conditions, site-specific operational parameters (Kennedy et al., 1988; Clement and Thomas, 1995), the landfill age (Chan, Davey and Geering, 1978; Clement and Thomas, 1995), season, climate, and rainfall (Venkataramani *et al.*, 1984) that results in an expansive variation in leachate quality and quantity between sites (Smith and Weber, 1990).

Climatic conditions, including rainfall patterns, are important factors that affect leachate volume and quality (Knox, 1985) and as such the site-water balance of a landfill is an important consideration in determining the volume of leachate likely to be produced (County Surveyors Society, 1984) and treated so as to avert the potential of the leachate to cause environmental damage. Numerous equations have been formulated for the purpose of describing water balance at an existing or potential landfill site (Holmes, 1980; Harrington and Maris, 1986; World Bank Technical Paper, 1989; Nyhan, Hakonson and Drennon, 1990; U.K Department of the Environment, 1994). In the South African context, DWAF (1998a; 1998b) makes use of a simple equation to calculate the climatic water balance. This Climatic Water Balance is defined as:

$$B = R - E \qquad \text{Eq1.1}$$

where B is the climatic water balance in mm; R is the rainfall in mm; and E is the evaporation from a soil surface in mm of water (calculated as the product of a standard factor and the corresponding reference-pan evaporation). This equation is based on the assumption that climate, specifically rainfall and evaporation, is the major contributing factor to leachate

production. DWAF (1998a; 1998b) states that a positive climatic water balance (B^+) for more than one year in five years of testing is indicative of significant leachate generation and warrants a leachate management system.

The equation presented by Nyhan *et al.* (1990) considers runoff and soil water storage as vital interacting variables in determining the expected quantity of leachate. Bagchi (1994) states that the water balance method is only applicable for landfills composed of a highly permeable layer of soil as final cover, since the infiltration of water decreases with the reduction in permeability of the final cover. Measured field data for leachate generation from landfills are few and far between, hence the reliance on indirect methods of prediction that are prone to dramatic errors of estimation during empirical conversions (Nyhan *et al.*, 1990). Ehrig (1983) investigated the relationship between the compaction of waste and leachate production. He concluded: (1) for wastes compacted by crawler tractors, 25 – 50 % of the precipitation emerged as leachate; and (2) for wastes compacted by steel-wheeled compactors, 15 – 25 % of the precipitation materialized as leachate. Leachate production is often observed within a few months of new landfill operations. This occurs when the absorptive capacity of the emplaced waste is exceeded, resulting in saturation of the waste with water (Maris, Harrington, Biol and Chismon, 1984) followed by the release of the excess liquid as leachate.

Leachate compositon varies significantly among landfills (Scott, 1982; Christensen, Kjeldsen, Albrechtsen, Heron, Nielsen, Bjerg and Holm, 1994). However, there exists a general consensus among researchers that a leachate should contain organic and inorganic constituents; heavy metals (Chian and DeWalle, 1976; Scott, 1982; Christensen *et al.*, 1994; Clement and Thomas, 1995); suspended solids (Dass *et al.*, 1977); and microorganisms such as bacteria and viruses (Knoll, Rump and Schneider, 1983; Senior, 1991).

Christensen *et al*. (1994) described the organic content of leachate as a bulk parameter engulfing a variety of organic degradation products ranging from small volatile fatty acids that dominate acid-phase leachate to the more refractory organic matter, characteristic of well stabilized methanogenic-phase leachate. Of particular importance in determining the adverse impacts of leachate on the environment is the age of the landfill generating the leachate,

specifically detailing the change from early acetogenic conditions, where leachates of high organic strength prevail, to late methanogenic conditions, where leachates contain lower proportions of non-oxidized organic compounds (Robison and Gronow, 1992). This phase change in leachate is reflected in the ratio of chemical oxygen demand (COD) to total organic carbon (TOC), which determines any relationship present between the organic content in a leachate and the corresponding age of the landfill in question. The COD:TOC ratio tends to decrease with an increase in the age of the landfill (Cameron and McDonald, 1982; Lo, 1996), thereby indicating the phase shift from acetogenic (COD:TOC = ± 3.3) to methanogenic (COD:TOC = ± 1.16) leachate (Venkataramani *et al.*, 1984). Harmsen (1983) concluded that 95 % of the TOC of acid-phase leachate consisted of volatile fatty acids and that only 1.3 % of the TOC consisted of compounds with a molecular weight (MW) greater than 1000. Conversely, the methanogenic-phase leachate contained a TOC of which 32 % was made up of high molecular weight compounds (MW > 1000). Johansen and Carlson (1976) further stated that 90 % of the TOC in acid-phase leachate consisted of acetic, propionic and butyric acids. Acid-phase leachate contained volatile amines and alcohols as apposed to the methanogenic-phase leachate that was devoid of such compounds (Harmsen, 1983). Apart from the COD:TOC ratio, there exists other ratios of chemical parameters that correlate directly with landfill age. These include the biological oxygen demand (BOD) to TOC; total volatile solids (VS) to total fixed solids (TS); sulphate (SO_4^{-2}) to chloride (Cl^{-1}) (Chian and DeWalle, 1976); and BOD:COD (Ehrig, 1989). In contrast to these ratios, the VS:TS ratio showed direct proportionality to landfill age (Venkataramani *et al.*, 1984; Lo, 1996). The BOD:COD ratio of acetogenic leachate is usually high (≥ 0.4), indicating good biodegradability under conditions of low pH and gas production. This is in stark contrast to methanogenic phase leachate, which is characterized by low BOD:COD ratios (< 0.1) at relatively higher pH and gas production rate (Ehrig, 1989).

The inorganic constituents in leachate include the cations: calcium, magnesium, potassium, sodium, ammonium, iron, and manganese; and the anions: chloride, hydrogen carbonate, and sulphate at concentrations that significantly exceed the drinking-water quality standards (Christensen *et al.*, 1994). Generally, there exists an inverse proportional relationship between landfill age and the concentration of inorganic constituents (Lo, 1996).

The ratio of SO_4^{-2}:Cl^{-1} decreases with an increase in the landfill age. This is due to the inert, non-biodegradable chloride compound maintaining a stable concentration, while there is a decrease in the sulphate concentration as a result of the anaerobic biological reduction of sulphate to sulphide as the landfill ages (Anderson and Dornbush, 1967; Lo, 1996). The sulphide ions can in turn react with metal cations and form insoluble metal sulphide precipitates (Lo, 1996; Reinhart and Al-Yousfi, 1996; Binder and Bramryd, 2000) thereby immobilising the metal ions. Methanogenic phase leachate is characterised by neutral pH (Christensen *et al.*, 1994; Lo, 1996), high concentrations of ammonia (County Surveyors Society, 1984; Knox, 1985; Harper, Manoharan, Mavinic and Randall, 1996), a TOC:NH_3-N (ammoniacal-nitrogen) ratio of 1:1 as apposed to a 13-16:1 ratio observed in acetogenic phase leachate, and low concentrations of iron (County Surveyors Society, 1984). The inorganic components in landfill leachate play an important role in controlling redox environments and attenuating heavy metals (Christensen *et al.*, 1994).

Heavy metals are usually present in the waste and landfill leachate at modest concentrations, where impact on process management and toxicity within the configuration of a landfill (Ahring and Westerman, 1983). The heavy metals in acetogenic phase landfills possess high solubility and mobility because of the lower pH and elevated volatile fatty acid concentrations (Loch, Lagas and Haring, 1981; Harmsen, 1983) and by virtue of this fact young leachate contains high concetrationsof heavy metals. In contrast, the high pH and low fatty acid content warrants the immobilisation of heavy metals as metal sulphide precipitates in stabilised landfills, resulting in depressed concentrations in older leachates (Loch *et al.*, 1981; Harmsen, 1983; Binder and Bramryd, 2001). A typical leachate analysis that illustrates the diversity in parameter values observed between leachates of different ages is presented in Table 1.1. The characterisation of such leachate parameters is of vital importance for the selection and implementation of appropriate treatment and disposal technologies (Smith and Weber, 1990).

The treatment of leachate and gas are both directly related by the processes governed by a range of organisms during the decomposition of landfill waste (Robinson and Luo, 1991). There are three major phases during waste decomposition that contribute to the characteristic biology of a landfill (Robinson and Luo, 1991; Senior, 1991) and the leachate produced.

Table 1.1 Average values for leachate analysis parameters showing differences between acetic and methanogenic phases (adapted from Ehrig, 1989)

Parameter	Acetic Phase	Methanogenic Phase
pH	6.1	8
BOD_5 (mg.l^{-1})	13 000	180
COD (mg.l^{-1})	22 000	3000
BOD_5/COD	0.58	0.06
SO_4 (mg.l^{-1})	500	80
Ca (mg.l^{-1})	1200	60
Mg (mg.l^{-1})	470	180
Fe (mg.l^{-1})	780	15
Mn (mg.l^{-1})	25	0.7
Zn (mg.l^{-1})	5	0.6
Sr (mg.l^{-1})	7	1

The first phase is dominated by the aerobic processes (Robinson and Luo, 1991) involving bacteria, including actinomycetes, fungi, and other higher organisms, including invertebrates. The aerobic metabolism produces a range of chemical intermediates as well as terminal stable products such as humic compounds, carbon dioxide, and water. The exothermic reactions of aerobic metabolism contributes significantly to the elevation of temperatures (maximum = 80 ^{o}C) which together with anti-microbial agents from the refuse results in the inactivation of numerous pathogens and contributes significantly to the site-water balance by virtue of the water produced (Senior, 1991). The second phase is characterised by anaerobic and facultative organisms (acetogenic bacteria) (Robinson and Luo, 1991) that hydrolyse and ferment cellulose and other putrescibles to simpler compounds such as volatile

fatty acids (Barlaz, Schaefer and Ham, 1989). Acetic acid, carbon dioxide and hydrogen are produced during the fermentation of more reduced products by oxidation under anaerobic conditions (Beker, 1987). The final phase of refuse decomposition is characterised by strict anaerobic metabolism of methanogenic bacteria, which consume the simple organic compounds and produce carbon dioxide and methane (Beker, 1987; Robinson and Luo, 1991). The assumption made is that young leachate is characterised by organisms dominating the first two phases of refuse decomposition, while older leachate is dominated by those microorganisms controlling the final phase of decomposition.

There is little doubt that waste disposal by land is a major contributor to the global degradation of aquifers (Schultz and Kjeldsen, 1986; Kjeldsen, 1993; Parsons and Jolly, 1994). This has initiated extensive leachate management practices to afford greater protection of water resources (Thornton, Tellam and Lerner, 2000) from the hazardous sunstances that may be present in landfill leachate (Smith and Weber, 1990).

1.5 Treatment Methods for Landfill Leachate

Prior to the collection and treatment of landfill leachate, strategies to minimise leachate generation must be employed or environmentally friendly leachate disposal options must be investigated (Scott, 1982). Scott (1982) presents an efficient appraisal that details such practices. Ultimately, global legislation demands that leachate be treated before it is discharged into the environment (Frigon *et al.*, 1997) since even the most stringent site management can only reduce leachate quality and quantity but cannot eradicate it (Bull, Evans, Weschler and Cleland, 1983). Researchers recognise that the collection and treatment of landfill leachate is among the major problems associated with the operation of a sanitary landfill (Lema, Mendez and Blazquez, 1988; Britz, Venter and Tracey, 1990). The strength and composition of a landfill leachate is governed by the age of the source landfill (Venkataramani *et al.*, 1984), with the concentration of inorganic and organic compounds (Lo, 1996), BOD and COD (Doeden and Cord-Landwehr, 1989) showing some variability over time (Scott, 1982). Therefore, the selection of an optimum leachate treatment strategy must consider fluctuations in the chemical characteristics of the leachate over time (Boyle and Ham,

1974; Cameron and Koch, 1980; Chu, Cheung and Wong, 1994; Lo, 1996; DWAF, 1998a) and the tolerance of the chosen strategy against the leachate characteristics (Kettunen and Rintala, 1998).

Chian and DeWalle (1976) concluded that aerobic and anaerobic leachate treatment technologies were best suited to the treatment of leachates with a high volatile fatty acid content, typical of leachates collected from recently leaching landfills while the physico-chemical technologies are better suited to the treatment of stabilized leachates. In most instances the chemical complexity of landfill leachate will require multiple treatment processes (Scott, 1982), most effectively involving a combined physico-chemical and biological treatment approach (Palit and Qasim, 1977) before the quality of the effluent satisfies legislative regulations (Scott, 1982). A proposed leachate management system must be simple, economical, and energetically efficient so that maximum operational benefit can be achieved (Cossu, Stegmann, Andreottola and Cannas, 1989).

1.5.1 Biological treatment

Biological treatment processes are simple and economical when compared to the majority of other leachate treatment options. The economic viability of the method is further improved by the inherent ability of an acclimated microbial population to utilize organic carbon and other essential nutrients present in the leachate (Venkataramani and Ahlert, 1984). The BOD:COD ratio provides a solid indication of the degree of biodegradability of the organic content present in the leachate. A ratio of approximately 0.5 (typical of young leachate) is indicative of proficient biodegradation of the organic content in the leachate whereas the opposite holds true for an older leachate with a ratio <0.5, and in the case of the latter biological treatment of the leachate is not recommended (Cossu *et al.*, 1989). Therefore the nature of the leachate being treated plays a central role in determining the mode of biological leachate treatment i.e. aerobic or anaerobic (Kennedy and Lentz, 2000).

Aerobic treatment

Aerobic bio-stabilization of sanitary landfill leachate has been extensively investigated. The principle modes that have been assessed include: activated sludge, aeration lagoons, extended-aeration, and oxidation ditch processes (Venkataramani *et al.*, 1984). Other systems of note include: biological filters, rotating biological contactors (RBC) (Knox, 1985; Cossu *et al.*, 1989), semi-continuous fed batch systems (Cecen and Aktas, 20010, continuous-flow systems (Harper *et al.*, 1996; Cecen and Aktas, 2001), and sequencing batch biofilm reactors (White and Schnabel, 1998; Kennedy and Lentz, 2000).

Bull *et al*. (1983) revealed that aerated processes were capable of treating organic wastes to stringent levels of quality. They concluded that organic and heavy metal constituents of leachate were rapidly removed by aerobic oxidation.

The treatment of high-ammonia landfill leachate has received considerable attention (Knox, 1985; Carley and Mavinic, 1991; Robinson and Luo, 1991; Harper *et al.*, 1996; Cecen and Aktas, 2001). Harper *et al.* (1996) established that a single-sludge, nitrification-denitrification process was capable of removing significant concentrations of ammonia and total nitrogen from landfill leachate under aerobic solid retention times (SRT) ranging from six to ten days. Carley and Mavinic (1991) arrived at a comparable conclusion and further stated that the addition of an external carbon source to carbon deficient methanogenic leachate was imperative for the occurrence of optimum nitrification-denitrification of high-ammonia landfill leachate. Martiensen and Schops (1997) also tackled the problem of high-ammonia landfill leachate by using a novel aerobic/anoxic fixed film reactor and a activated sludge bioreactor.

Studies have reported biological system failures as a consequence of bio-available phosphorus deficiencies (Palit and Qasim, 1977; Robinson, Barber and Maris, 1982; Scott, 1982) and heavy metal toxicities (Harper *et al.*, 1996). Conversely, Cameron and Koch (1980) reported impressive heavy metal removal efficiencies in aerobic treatment systems.

Activated sludge systems do not function efficiently when treating high-strength leachates (young leachates). Aerobic treatment technologies generally have quicker treatment rates (Bull *et al.*, 1983) but have the added disadvantage of heavy sludge production (Venkataramani *et al.*, 1984; Frigon *et al.*, 1997) coupled to the necessity of sludge disposal and greater operational costs (Bull *et al.*, 1983; Frigon *et al.* 1997).

Anaerobic treatment

Anaerobic treatment methods offer an impressive alternative to the aerobic options, by virtue of the technology's immense potential for the production of treated effluents of comparable quality, in addition to associated advantages of low costs, energy production through methane generation (Frigon *et al.*, 1997; Kennedy and Lentz, 2000), production of a solids residue that can be used as a cover material in landfills (Kennedy and Lentz, 2000), and the production of smaller quantities of sludge (Venkataramani *et al.*, 1984; Frigon *et al.*, 1997). However, the inability of the anaerobic system to treat ammonia present in the leachate ranks as a major disadvantage for the system (World Bank Technical Paper, 1989).

Numerous systems have been proposed and evaluated for the anaerobic treatment of landfill leachate. These include: upflow anaerobic sludge blanket (UASB) reactors (Kennedy, Hamoda and Guiot, 1988; Britz *et al.*, 1990; Kennedy and Lentz, 2000), bench-scale anaerobic digesters (Cameron and Koch, 1980; Lin, 1991; Myburg and Britz, 1992; Myburg and Britz, 1993); anaerobic filters (Chian and DeWalle, 1976); and anaerobic lagoons (Cossu *et al.*, 1989). Treatment success rates vary between researchers, with the quality of the treatment achieved often dependant on the character of the leachate being treated (Bull *et al.*, 1983; Lin, 1991; Frigon *et al.*, 1997; Kennedy and Lentz, 2000).

Boyle and Ham (1974) showed a 90 % removal of BOD when the hydraulic retention time in an anaerobic system was greater than ten days with temperatures ranging between 23 °C and 30 °C. Bull et al. (1983) further demonstrated a 95 % BOD removal and 100 % soluble iron removal as a sulphide precipitate. Myburg and Britz (1993) went even further to demonstrate 80 – 95 % COD removal efficiency in a hybrid digester operated at mesophilic

temperatures at a hydraulic retention time of one day. Kennedy and Lentz (2000) published COD removal rates ranging between 71 – 92 % for UASB and sequencing batch reactors with hydraulic retention times ranging between 12 – 24 hours. Anaerobic systems are often sensitive to shock loads and toxic substances (Venkataramani *et al.*, 1984). However, Myburg and Britz (1993) demonstrated the ability of their anaerobic hybrid digester to withstand shock loads within specific limits.

Final discharge of anaerobically treated leachate requires further physico-chemical treatment of the organic- and nitrogenous content (Bull *et al.*, 1983); and sulphide- and chloride content (Kennedy and Lentz, 2000). A combined approach, incorporating biological- and physico-chemical treatment technologies will enable the complete treatment of landfill leachate (Venkataramani and Ahlert, 1984).

1.5.2 Physico-chemical treatment

In contrast to the ineffectiveness of biological treatment processes on stabilised landfill leachate, physico-chemical approaches on such waste streams often produces impressive outcomes. Conversely, poor results have been achieved when physico-chemical options have been used for the treatment of young landfill leachate (Venkataramani *et al.*, 1984). This indicates that the physico-chemical approaches are more effective for the treatment of landfill leachate that is characterised by fulvic components, the concentration of which is very high in biologically treated leachate and leachate radiating from old landfills (Scott, 1982).

There have been numerous physico-chemical treatment options evaluated. These include: chemical precipitation and oxidation (Thornton and Blanc, 1973; Ho, Boyle and Ham, 1974; Sletten, Benjamin, Horng and Ferguson, 1995), adsorption (Ho *et al.*, 1974; Chian and DeWalle, 1976; Mclellan and Rock, 1988), reverse osmosis (Chian and DeWalle, 1976; Scott, 1982; Ehrig, 1989), and vaporization (Ehrig, 1989).

There is no single treatment option that can provide optimum remediation for a heterogenous waste stream such as landfill leachate. A combined physico-chemical treatment

preceded by a biological treatment option is the approach most recommended. This type of approach guarantees harnessing of the advantages inherent in both technologies (Venkataramani and Ahlert, 1984; Ehrig, 1989, Bagchi, 1994). Indeed, Chian and DeWalle (1976) further stated that physico-chemical treatments such as activated carbon and reverse osmosis were best suited as secondary treatment processes following the biological treatment of young leachates, or as a primary treatment option for treating stabilised leachates.

1.5.3 Recirculation

Experimental evidence suggests that recirculation of landfill leachate through a municipal waste landfill increased the rate of organic pollutant stabilisation (Scott, 1982; Bull *et al.*, 1983) and heavy metal removal (Scott, 1982), in some instances by facilitating the rapid development of anaerobic bacterial populations (Bull *et al.*, 1983) thereby promoting methanogenesis. It has been further stated that leachate recirculation may provide advantages that include: a function as a temporary storage facility (Cureton, Groenevelt and McBride, 1991; Pohland and Al-Yousfi, 1994); enhancing the adsorptive attenuation of organic and reduced inorganic leachate constituents in the aerobic soil environment (Tittlebaum, 1982; Cureton *et al.*, 1991; UK DoE, 1994); minimising dry zones within the emplaced refuse thereby maximising refuse degradation throughout the landfill (Senior, 1991); and increasing evaporative losses of leachate by spray irrigation, thereby decreasing the total volume of leachate (Robinson and Maris, 1985; UK DoE, 1994).

Leachate recirculation satisfies the primary criteria that influence the dynamic, microbially mediated process of landfill stabilisation. The primary criteria governing landfill stability are waste characteristics, available nutrients and moisture, and existing operational procedures; all of which are satisfied by controlled leachate management and recirculation through the landfill (Pohland and Al-Yousfi, 1994).

Chapman and Ekama (1992) stated that experimental data, presented by numerous researchers, concerning recirculation were often contradictory. They further proposed that such contradictions were chiefly a consequence of using leachates of different ages. Research

conducted by Novella, Ekama and Blight (1996) supported the conclusions of Chapman and Ekama (1992), thereby emphasising the need to consider aspects such as the age of the emplaced waste and resultant leachate when planning and designing a recirculation regimen.

However, recirculation technology is not without its risks and disadvantages. These include: the added risk of leachate infiltration into the subsurface posed by the additional volume of liquid resulting from continued leachate application, which poses the risk of groundwater pollution (Robinson and Maris, 1985; Doedens and Cord-Landwehr, 1989); lateral discharge of leachate as a consequence of compacted or layers of low permeability within the waste (Robison and Maris, 1985; UK DoE, 1994); concentrating of salts (UK DoE, 1994) and heavy metals (Doedens and Cord-Landwehr, 1989); surface clogging leading to leachate ponding (Robinson and Maris, 1985; UK DoE, 1994); and the exacerbation of odours as a consequence of open irrigation practices (UK DoE, 1994).

1.6 Redox Environments of a Landfill Leachate Pollution Plume

There are numerous reports detailing groundwater contamination as a consequence of inadequate landfill leachate management systems and as a result of primitive landfills devoid of such practices (e.g., Lyngkilde and Christensen, 1992a; Bjerg *et al.*, 1995; Mikac *et al.*, 1998; Roling, van Breukelen, Braster, Lin and van Verseveld, 2001). Therefore, it is inevitable that environmental pollution, with particular reference to groundwater contamination, has been been associated with landfills (Griffin, Shimp, Steele, Ruch, White and Hughes, 1976; Baun, Jensen, Bjerg, Christensen and Nyholm, 2000; Cozzarelli, Suflita, Ulrich, Harris, Scholl, Schlottmann and Christensen, 2000).

The high inorganic (Bjerg *et al.*, 1995) and organic concentration of landfill leachate provides an ideal substrate for microbial processes (Cozzarelli *et al.*, 2000; Roling *et al.*, 2001) within the subsurface environment (Ludvigsen, Albrechtsen, Ringelberg, Ekelund and Christensen, 1999). This coupled with complex chemical reactions can result in significant changes in aquifer geochemistry and microbiology downstream of a landfill, with these changes being mirrored in the sequential development of redox zones in time and space

(Williams and Higgo, 1994; Bjerg *et al.*, 1995; Roling *et al.*, 2001). Methane production, sulphate reduction, iron reduction, nitrate reduction, manganese reduction, and aerobic zones (Lynkgilde and Christensen, 1992a; Lensing, Vogt and Herrling, 1994; Williams and Higgo, 1994; Bjerg *et al.*, 1995; IWACO, 1997; Lovely, 1997; Mikac *et al.*, 1998; Ludvigsen *et al.*, 1999) have been identified as the characteristic redox zones present in a landfill leachate pollution plume, with an overall distribution downgradient from the landfill (Figure 1.1) (Lovely, 1997). In the light of such a statement, emphasis must be placed on the fact that the redox potentials increase away from the landfill thereby reflecting the overall distribution of the individual redox processes stated previously (Bjerg *et al.*, 1995; IWACO, 1997).

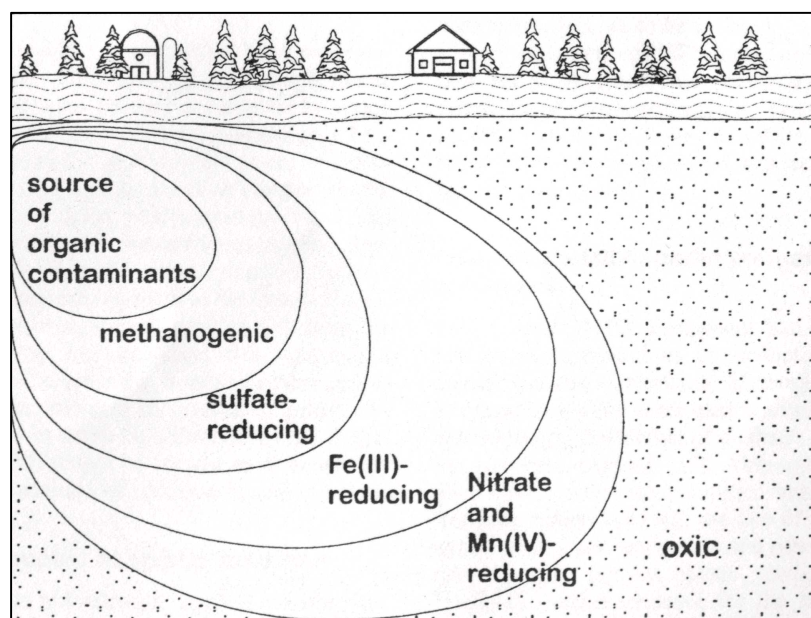

Figure 1.1 **Typical distribution of redox conditions prevalent in an aquifer polluted by an organic contaminant (Lovely, 1997).**

The entrance of a high organic load into the subsurface causes a rapid depletion of oxygen, and as a consequence of this, the kinetics of microbial metabolism becomes dependant on the interactions between the organic carbon present in the migrating leachate, the availability of soluble and insoluble electron acceptors and donors (Lynkgilde and Christensen, 1992a; Cozzarelli *et al.*, 2000), and the kinetics of the actual redox processes (Lynkgilde and Christensen, 1992a). This results in areas expressing dominant terminal electron-accepting

processes (TEAPs) (Lovely, 1997; Cozzarelli *et al.*, 2000) which confer dominant redox zones within the framework of a leachate plume (Lovely, 1997). Lynkgilde and Christensen (1992a) stated that the existence of such a sequence of redox environments is a hypothesis, based on the assumption that significant quantity of: free oxygen, nitrate, sulphate, iron and manganese compounds being present in the subsurface environment. They further concluded that the absence of an electron acceptor would render the corresponding redox environment non-existent. Bjerg *et al.*, (1995) presented a comprehensive description of the prevailing conditions within the individual redox environments of a typical landfill leachate pollution plume.

Natural attenuation is a process by which the concentration of landfill leachate constituents is reduced by natural phenomenon. Based on their definitions, Senior (1990) and Bagchi (1994) identified the following as the possible attenuation mechanisms in the subsurface:

i. Adsorption
ii. Biological uptake
iii. Cation- and Anion-exchange reactions
iv. Filtration, and
v. Dilution reactions

Rugge *et al.* (1995) stated that biological degradation can only be proposed as a possible attenuation mechanism, when there is a failure to associate the disappearance of waste stream constituents in the plume with any of the remaining attenuation processes. Bjerg *et al.* (1995) and Mikac *et al.* (1998) further stated that it is of fundamental importance to associate pollutant attenuation/degradation to the prevailing redox environments in the plume. Such reactions often aid in the development of a specific redox state thereby facilitating similar reactions in such an environment (Bjerg *et al.*, 1995). Bouwer and Edwards (1983a; 1983b) found that numerous organic compounds produced varied responses to biodegradation under differing redox environments. They concluded that the prevailing redox environments played a key role in the biotransformation of these organic compounds, since an essential factor affecting the biotransformation process is the type of electron acceptor available to the microbial systems. In a series of investigations, detailing the distribution and migration of

organic compounds in the subsurface, Williams and Higgo (1994), concluded that prevailing redox conditions and the related microbial populations will play a defining role in determining the degradation fate of the organic compounds. Cozzarelli *et al.* (2000) further stated that the rate at which organics are degraded by the microbiology of the iron reducing, sulphate reducing and methanogenic zones, depends largely on the balance between the reaction rates prevalent in these zones and the rate at which leachate is supplied to the subsurface.

The vast chemical reactions, typical of a leachate plume, are often dominated by the heterotrophic activities of numerous bacterial groups (Lensing *et al.*, 1994; Williams and Higgo, 1994). The abundance of both anaerobic and heterotrophic organisms decreases with increasing depth in the subsurface, which is possibly a result of a decrease in the supply of essential nutrients and electron acceptors as well as an increase in anti-microbial contaminants through the build-up of xenobiotics (Williams and Higgo, 1994). Ludvigsen *et al.* (1999) showed that cell numbers decreased with increasing distance from the landfill by analysing phospholipid fatty acid (PLFA) and adenosine triphosphate (ATP) content in samples traversing the area of a landfill leachate polluted aquifer. Methanogens are restricted to the most polluted and reduced section of the plume corresponding to the section closest to the landfill (Rugge *et al.*, 1995; IWACO, 1997; Ludvigsen *et al.*, 1999), while sulphate reducers were shown to decrease with increasing distance from the landfill (IWACO, 1997). Research has shown that in some cases methanogenesis and sulphate reduction are exclusive of each other (Bjerg *et al.*, 1995), but other studies have provided evidence that suggests the co-existence of methanogens and sulphate reducers (Beeman and Suflita, 1987; 1990; Cozzarelli *et al.*, 2000). Lynkgilde and Christensen (1992) identified the iron reducing zone as the largest zone in the plume. The importance of this zone in the oxidation cycle of organic matter was highlighted by Albrechtsen and Christensen (1994) and Roling *et al.* (2001). Iron-, manganese- and nitrate reducers were identified in pockets throughout the plume in lower concentrations than within the boundaries of the respective active redox zones in the plume (IWACO, 1997).

Researchers agree that the biologically mediated redox environments of a landfill leachate pollution plume plays a central role in the natural attenuation of leachate contaminants, and remains a key factor in determining the ultimate fate of such contaminants in the plume (Lynkgilde and Christensen, 1992a; 1992b; Bjerg *et al.*, 1995; Winderl, Anneser, Griebler, Meckenstock, Lueders, 2008).

1.7 Genotypic Profiling of Microbial Associations

The inevitable use of molecular biological techniques to characterize microorganisms in their natural habitats were borne from the elementary realization that traditional microbiological methods of enrichment and isolation had failed to detect the vast majority of microbes, resulting in gross underestimates of the complexity of innate microbial communities (Picard, Ponsonnet, Paget, Nesme and Simonet, 1992; Muyzer, De Waal and Uitterlinden, 1993; Amann, Ludwig and Schleifer, 1995; Ferris, Muyzer and Ward, 1996; Santegoeds, Nold and Ward, 1996; Watanabe, Teramoto, Futamata and Harayama, 1998; Gelsomino, Keijzer-Wolters, Cacco and van Elsas, 1999; Wise, McArthur and Shimkets, 1999; Jackson, Churchill and Roden, 2001). Culture based identification fails to mimic the overall conditions prevalent in natural habitats thereby imposing selection pressures that proliferate only a small percentage of the natural populations (Muyzer *et al.*, 1993; Ferris *et al.*, 1996; Santegoeds *et al.*, 1996; Felske and Akkermans, 1998a; El Fantroussi, Verschuere, Verstraete and Top, 1999). Conventional microscopy has limited use, since a variety of microbes bind to natural sediments thereby masking their existence (Muyzer *et al.*, 1993). In addition, numerous microorganisms share similar morphologies that make them inseparable by conventional microscopy (Amann *et al.*, 1995; Ferris *et al.*, 1996). Amann and co-authors (1995) presented a comprehensive review detailing the percentage of microorganisms that are culturable from various natural environments by traditional methods of microbiology. Borneman, Skroch, O'Sullivan, Palus, Rumjanek, Jansen, Niehuis and Triplett (1996) supported these conclusions by stating that the majority of microbes (90-99 %) in environmental samples are indeed unculturable.

Recent history has witnessed a dynamic approach, poised at overcoming the drawbacks associated with traditional culture-dependent methods. This approach has a molecular base focused on the analysis of nucleic acid, extracted from environmental samples, aimed at studying the microbial diversity of natural communities (Muyzer *et al.*, 1993; Muyzer and Ramsing, 1995; Borneman *et al.*, 1996; Ferris *et al.*, 1996; Brinkhoff and Muyzer, 1997; Kowalchuk, Stephen, De Boer, Prosser, Embley and Woldendorf, 1997; Felske and Akkermans, 1998a; Felske and Akkermans, 1998b, Lloyd-Jones and Lau, 1998; Watanabe *et al.*, 1998; Dunbar, Takala, Barns, Davis and Kuske, 1999; El Fantroussi *et al.*, 1999; Gelsomino *et al.*, 1999; Macnaughton, Stephen, Chang, Peacock, Flemming, Leung and White, 1999; Duineveld, Kowalchuk, Keijzer, van Elsas and van Veen, 2001). These nucleic acid based protocols are often more stable, less time-consuming, and offer greater detail from which sound phylogenetic conclusions can be drawn (Schneider and De Bruijn, 1996; Boivin-Jahns, Bianchi, Ruimy, Garcin; Daumas and Christen; 1995; Louws, Rademaker and De Briujn, 1999). However, each of these methods provides different levels of taxonomic resolution (Louws et al., 1999) and as such must be applied appropriately by weighting the quantity of information required with the associated disadvantages of the chosen protocol.

The application of these techniques in molecular microbial ecology relies principally on the manipulation of the 16S ribosomal DNA (rDNA) sequences that are extracted from environmental samples (Felske, Wolterink, Van Lis and Akkermans, 1998; Cho and Kim, 2000). The average bacterial 16S rRNA (or its gene) has a length of approximately 1500 nucleotides (nt), which when fully or almost completely sequenced (> 1000 nt), can provide sufficient information for accurate phylogenetic placement (Pace, Stahl, Lane and Olsen, 1986; Amann *et al.*, 1995). The 16S rRNA and its corresponding gene from the bacterial genome functions as an ideal indicator of microbial diversity in community profiling by virtue of the following criteria:

- presence in all species of the population;
- they may be analysed as rDNA or by reverse transcription of the rRNA into copy DNA (cDNA);
- shows variation in its sequence between species; and

- it is universally accessible for all species of a population by a common method (Woese, 1987).

The 16S rRNA consists of numerous domains, with the string of domains of the primary structure being less conserved than the highly conserved secondary structure. The 16S rRNA approach relies profoundly on the use of oligonucleotide primers for the placement of microorganisms into specific taxa. These primers range from universal and domain-specific to group-specific (Amann *et al.*, 1995). Dojka, Hugenholtz, Haack and Pace (1998) directly amplified the 16S rDNA from aquifer sediment by using universally conserved or *Bacteria*- or *Archaea*-specific primers to phylogenetically characterise the dominant microbial populations existent in a series of redox zones present in a hydrocarbon- and chlorinated-solvent pollution plume. Numerous researchers have used universal primers specific to the domain *Bacteria* so as to amplify 16S rDNA or the corresponding fragment, extracted from diverse environments (e.g. Felske, Rheims, Wolterink, Stackebrandt and Akkermans, 1997; Fournier, Lemieux and Couillard, 1998; Eichner, Erb, Timmis and Wagner-Dobler, 1999; Cho and Kim, 2000). Other researchers have used a combination of *Bacteria*- and group-specific primers to identify precise factions of microbes (e.g. sulphate reducing bacteria) present in an environmental sample (Teske, Wawer, Muyzer and Ramsing, 1996; Brinkhoff and Muyzer, 1997). Not satisfied with this degree of specificity, other workers progressed further by utilising group-specific primers corresponding to the 16S rDNA and compared the generated profiles with profiles spawned by the amplification of group-specific gene sequences (e.g. *amoA* genes of the autotrophic ammonia oxidising bacteria) with the corresponding primers (Watanabe *et al.*, 1998; Ivanova, Stephen, Chang, Bruggemann, Long, McKinley, Kowalchuk, White and Macnaughton, 2000).

Generally speaking, nucleic acid based bacterial characterisation protocols can be divided roughly into polymerase chain reaction (PCR) amplification-dependent and PCR amplification-independent approaches (Louws et al., 1999). Torsvik, Daae, Sandaa and Øvreås (1998) reviewed their pioneering work in the field of direct genomic analysis by reassociation kinetics, for the determination of microbial community structure and diversity.

The analysis of plasmid profiles characteristic of microbial isolates was also demonstrated as a partial but direct method of genomic analysis that facilitated microbial diversity studies (Schütt, 1990; Louws *et al.*, 1999). Another direct genomic analysis method involves the digestion of total DNA by specific restriction endonucleases, which generates characteristic profiles on gels (Schneider and de Bruijn, 1996). This method is called Restriction Fragment Length Polymorphisms (RFLPs). A fourth direct fingerprinting method involves the profiling of low molecular weight (LMW) RNA, in the form of the 5S rRNA or transfer RNA (tRNA). The method is based on the electrophoretic separation of LMW on a polyacrylamide gel together with the corresponding data capture of the characteristic profiles for comparative purposes (Höfle, 1990). The technique has been applied to diverse environments (Höfle, 1992; Höfle and Brettar, 1996). Generally, genomic analyses that are independent of PCR-amplification are less specific, rapid, and sensitive than PCR-amplification dependent protocols (Louws *et al.*, 1999). Both methods have the added disadvantages associated with DNA extraction from natural environments (Jackson, Harper, Willoughby, Roden and Churchill, 1997), however, since relatively larger quantities of DNA are required for PCR-independent reactions, the negative aspects are magnified in such instances.

PCR-amplification dependent approaches that focus on the cloning and sequencing of the 16S rDNA of environmental samples have thus far dominated the initial ventures into the molecular age of microbial identification (eg. Bornemann *et al.*, 1996; Godon, Zumstein, Dabert, Habouzit and Moletta, 1997; Kuske, Barns and Busch, 1997; Felske *et al.*, 1998; Lloyd-Jones and Lau, 1998; Widmer, Seidler, Gillevet, Watrud and Giovanni, 1998). However, this approach is time-consuming and laborious and as such incapable of coping with the high sample throughput required to monitor sequential changes in microbial community structure (Muyzer and Smalla, 1998; Eichner *et al.*, 1999; Gelsomino *et al.*, 1999). Since microbial ecology is the study of microbe-microbe interactions and the characteristic interactions with their environment (Muyzer and Smalla, 1998), protracted monitoring of the associated systems are essential to gaining definitive conclusions with regards to community structure and diversity, since it is this concept that is fundamental for analysing phenomena such as succession, colonisation, and response to disturbances (as in the case of this study) (Eichner *et al.*, 1999). For this purpose, genetic fingerprinting techniques are ideally suited

(Muyzer, 1999). Muyzer (1999), further states that genetic fingerprinting techniques provide unique nucleic acid profiles that enable comparisons of genetic diversity over time and between microbial communities from diverse environments.

There are a variety of fingerprinting techniques available for the study of microbial community dynamics and diversity within natural environments (Muyzer and Smalla, 1998; Louws *et al.*, 1999; Muyzer, 1999; Cho and Kim, 2000). Many of these techniques, but not all, rely on the direct amplification of the 16S rDNA or part-thereof, before further analysis by the fingerprinting technique of choice (Cho and Kim, 2000).

These techniques include: single strand conformational polymorphism (SSCP) (Orita, Iwahana, Kanazawa, Hayashi and Sekiya, 1989; Lee, Zo and Kim, 1996; Schweiger and Tebbe, 1998); randomly amplified polymorphic DNA (RAPD - also referred to as DNA amplification fingerprinting – DAF) (Manulis, Valinsky, Lichter and Gabriel, 1994; Breen, Rope, Taylor, Loper and Sferra, 1994; van Rossum, Schuurmans, Gillis, Muyotcha, van Verseveld, Stouthamer and Boogerd, 1995; Pooler, Ritchie and Hartung, 1996; Röling and van Verseveld, 1996; Momol, Lamboy, Norelli, Beer and Allowinckle, 1997; Clerc, Manceau and Nesme, 1998); restriction fragment length polymorphisms (RFLP's) (George, Bustamam, Cruz, Leach and Nelson, 1997; Leeflang and Smit, 1997; Manceau and Horvais, 1997; Cho and Kim, 2000), otherwise known as amplified ribosomal DNA restriction analysis (ARDRA) when the amplification of RFLP's is based only on the 16S rDNA portion of the genome (Schramm and Amann, 1999; Muyzer, 1999); the variant of RFLP's (Schramm and Amann, 1999) that incorporates a fluorescent label on one of the two primers used in the reaction – terminal restriction fragment length polymorphisms (t-RFLP's – Clement, Kehl, DeBord and Kitts, 1998; Liu, Marsh, Cheng and Forney, 1997; van der Maarel, Artz, Hanstra and Forney, 1998), otherwise referred to as fluorescent restriction fragment length polymorphisms (FluRFLP's – Bruce, 1997); bisbenzimide-polyethylene glycol (Bb-PEG) conjugate electrophoretic analysis (Muyzer, 1999; Demkin, Edelstein, Zimin, Edelstein and Suvoron, 2000); denaturing gradient gel electrophoresis (DGGE) (eg. Muyzer *et al.*, 1993; Ferris *et al.*, 1996; Kowalchuk *et al.*, 1997; Duarte, Rosado, Seldin, Keijzer-Wolters and van Elsas, 1998; Head, Saunders and Pickup, 1998; Jackson and Churchill, 1999; Ralebitso, Röling, Braster,

Senior and van Verseveld, 2000; Röling, van Breukelen, Braster, Goeltom, Groen and van Verseveld, 2000; Duarte, Rosado, Seldin, de Araujo and van Elsas, 2001; Jackson, Churchill and Roden, 2001) and the related technique called temperature gradient gel electrophoresis (TGGE) (Felske, Engelen, Nubel and Backhaus, 1996; Felske *et.al.*, 1997; Felske *et.al.*, 1998; Muyzer and Smalla, 1998); amplified fragment length polymorphisms (AFLP's) (Janssen, Coopman, Huys, Swings, Bleeker, Vos, Zabeau and Kersters, 1996; Louws *et al.*, 1999); and the collective protocol termed rep-PCR which is based on the use of PCR with primer sequences analogous to regions of naturally occurring interspersed repetitive sequences. The procedure (rep-PCR) can target all or one of three families of repetitive sequences in a genome, viz. repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequence, and the BOX elementwhich comprises the boxA; boxB; and box C subunits (de Bruijn, 1992; Louws, Fulbright, Stephens and de Bruijn, 1994; Schneider and de Bruijn, 1996; Meintanis, Chalkou, Kormas, Lymperopoulou, Katsifas, Hatzinikolaou, and Karagouni, 2008).

Ideally, microbial community diversity studies involving DNA fingerprinting protocols should engage multiple approaches so as to counteract the negative aspects of each technique while accentuating the benefits of the "positives" innate in each protocol. Muyzer (1999) provides a concise overview of the positive and negative facets of such techniques.

1.7.1 Denaturing gradient gel electrophoresis (DGGE)

Fischer and Lerman (1980; 1983) pioneered the electrophoretic separation of DNA molecules in a specific gradient of denaturant. Their work detailed the behaviour of Phage λ DNA in denaturing gradient gels, thereby setting the bar for the separation of DNA fragments based on nucleotide base-pair variations. The procedure allows the separation of DNA fragments of the same length but with sequence variations that produce partially melted double-stranded DNA molecules when exposed to the linear gradient of DNA denaturants. The melting of DNA fragments proceeds in discrete melting domains (stretches of base-pairs with an identical melting temperature). The migration of the DNA molecule stops once the

domain with the lowest melting temperature (T*m*) reaches its melting point in a specific position in the denaturing gel. Molecules with different sequences will stop migrating at different positions in a gel because sequence variations within domains infer different melting temperatures to characteristic molecules (Myers, Fischer, Lerman and Maniatis, 1985; Muyzer and Smalla, 1998). However, to detect sequence variation in the highest temperature melting domain of a DNA molecule, a guanine-cytosine rich DNA sequence (GC-clamp) (Myers et al., 1985) must be attached to the 5'-end of one primer of a primer pair (Muyzer et al., 1993). The GC-clamp functions as a domain with high melting properties (Myers, Maniatis and Lerman, 1987; Muyzer and Smalla, 1998) that prevents complete strand dissociation of the double-stranded molecule (Myers *et al*., 1985; Muyzer and Smalla, 1998), which would significantly reduce the resolving power of the gel (Myers *et al*., 1985).

Muyzer and company (1993) were the first to apply this PCR-dependent fingerprinting technique to microbial ecology. Today the method has been applied to diverse environments by numerous researchers. These environments include: microbial biofilms from sea sediments (Muyzer et al., 1993) and hot springs (Santegoeds et al., 1996; Ferris et al., 1996), coastal sand dunes (Kowalchuk et al., 1997), and crop producing soils (Gelsomino et al., 1999; Marschner, Crowley and Lieberei, 2001), to mention just a few. The application of DGGE in such environments ranges from comparative studies detailing DNA extraction and purification techniques (Niemi, Heiskanen, Wallenius and Lindström, 2001) to investigating the microbial composition of enrichment cultures under selective pressures (Santegoeds et al., 1996). However, as in the context of this study, DGGE has been extensively used to profile community diversity (e.g. Teske et al., 1996; Duarte et al., 1998; Ralebitso et al., 2000; Röling et.al., 2000) and the monitoring of population dynamics within perturbed environments (El Fantroussi et al., 1999).

Most of the molecular methods that depend on nucleic acid analysis, have one major limitation, it being the quality and to a lesser extent the quantity of nucleic acid recovered from environmental samples (Jackson et al., 1997; Head et al., 1998; Niemi et al., 2001). The separation of humic substances from DNA is an integral part of achieving high quality DNA that is suitable for successive applications such as PCR (Jackson et al., 1997; Niemi et al.,

2001). There are numerous protocols available for extracting DNA from environmental samples (Head et al., 1998), but few address the comparative efficiency of these methods. Niemi and co-workers (2001) addressed this issue by conducting a comparative study of DNA isolation protocols by using PCR-DGGE community fingerprinting as the basis for their comparisons. It is essential that one realises that procedures succeeding DNA extraction and purification are also prone to introducing errors or bias (Farrelly, Rainey and Stackebrandt, 1995). PCR amplification contributes significantly to introducing bias to all PCR-dependent fingerprinting technologies, inevitably affecting measures of community composition (Teske et al., 1996; Head et al., 1998; Eichner et al., 1999). Apart from errors resulting from inappropriate methodology, the genomic properties of the bacterial cell contributes significantly to the introduction of bias. Farrelly and company (1995) addressed such properties *en route* to concluding that it was impossible to quantify the representative populations of a structured community without prior knowledge of the *rrn* gene copy number and the genome size of the individual species.

Although PCR-DGGE does have its limitations, the technology has significant worth in the field of microbial ecology (Jackson, Roden and Churchill, 2000) and as in the case of this study, the focus of which concentrates on the comparative analysis of microbial diversity in perturbed environmental conditions.

1.8 Objectives

Diverse redox environments may develop within a leachate plume as a consequence of strongly reduced leachate migrating through the unsaturated zone of soil before entry into an aquifer. The development of such environments is dependent on the redox capacities and reactivities of leachate compounds, as well as other factors present in the leachate and the receiving subsurface material (Bjerg *et al*., 1995). The capacity for microbial attenuation of leachate contaminants within the leachate plume has received very little attention in the literature. With this in mind, the central aim of this study was to investigate the biological mechanisms of attenuation prevalent within a landfill leachate contaminated soil microcosm, bearing in mind the following principle objectives:

1. To assess the adsorptive capacity and inherent biological influence of a Hutton soil on selected constituents of a "young", synthetic, acetogenic phase landfill leachate;
2. To assess the fate of selected constituents of a the synthetic leachate over time by using a series of laboratory-scale soil microcosms so as to mimic the behaviour of landfill leachate in soil beneath a landfill;
3. To optimize; DNA extraction from the soil microcosms, and DGGE staining techniques.
4. To generate DNA profiles using PCR-DGGE to assess changes in bacterial community diversity occurring within the laboratory-scale soil microcosms; and
5. To identify selected members of the microbial associations involved in leachate bio-degradation/attenuation by cloning and sequencing of the 16SrDNA.

Characterisation of the microbial communities resident in soil beneath a "seeping" landfill could potentially shed light on the biological attenuation potential of these communities, be it qualitative or quantitative. The microbial profiles of primary interest were those occurring in the nitrate reducing, sulphate reducing, and methanogenic phases of the leachate plume.

Chapter Two

2. Materials and Methods

2.1 Soil Material

A Hutton soil (silty loam), rich in iron and aluminum oxides was used throughout the study. The soil was obtained from a field (Apple Gate Farm, Merrivale, Howick, Republic of South Africa) with sparse grass cover and no recent history of cultivation. Soil selection and collection was partially based on the criteria and recommendations of Loch, Lagas and Haring (1981); Rees and King (1981), Artiola-Fortuny and Fuller (1982); Kjeldsen, Kjolholt, Schultz, Christensen and Tjell (1990); Shaw and Burns (1996; 1998); Nay, Snozzi and Zehnder (1999a and 1999b); Wong, Cheung and Wong (2000). The soil was air-dried and passed through 2.0 mm and 0.5 mm sieves, to ensure homogeneity of the soil, in preparation for re-packing into cylindrical glass columns (15 cm long, 4.3 cm internal diameter). The physical and chemical characteristics of the soil were determined by Cedara Agricultural College, Soil Laboratory, Department of Agriculture and Environment, Ceadara, KwaZulu-Natal, Republic of South Africa (Appendix A).

2.1.1 Soil moisture content

The soil was air dried for 10 hours in direct sunlight before 100 g were spread evenly on a glass Petri dish (15 cm internal diameter). The soil was dried in an oven (100 ^{o}C) for 24 hours, and re-weighed. The difference in weight before and after oven drying was taken as the moisture content of the soil. Triplicate determinations were performed.

2.1.2 Total soil pore volume (Total Porosity)

The total pore volume of the soil was calculated by using the following equation:

$$Total\ Porosity = \frac{1 - bulk\ density}{particle\ density} \text{...} Eq2.1$$

Hence, it was necessary to determine first the bulk density and the particle density (*Pd*) of the soil. Triplicate determinations were performed.

2.1.3 Bulk density

Glass columns (15 cm long, 4.3 cm internal diameter) fitted with rubber bungs, were weighed before and after filling with air dried Hutton soil (Tan, 1996). The soil was compacted by hand tapping the bottom of the stoppered columns at least 20 times so as to produce an internal soil column of approximately 10 cm. The bulk density was then calculated as follows:

$$Bulk\ Density = \frac{ovendried\ mass\ of\ soil}{total\ volume\ of\ soil\ sample} \text{..} Eq2.2$$

2.1.4 Particle density (*Pd*)

An average of 160 g of air dried soil was added to a 1000 m*l* graduated measuring cylinder and 300m*l* of distilled water were added. The slurry was stirred thoroughly with a glass rod to displace the air in the soil. After standing for five minutes the volume of the soil plus water was recorded. The volume increase accounted for by the soil was recorded as the volume of the soil solids. The pre-determined soil moisture content (2.2) was accounted for in determining the total volume of water used in the experiment (Tan, 1996). The *Pd* was then calculated as follows:

$$Pd = \frac{ovendried\ mass\ of\ soil}{volume\ of\ water\ displaced\ by\ soil} \text{..} Eq2.3$$

2.2 Construction of Soil Microcosms

Glass columns, with dimensions described in 2.1.3, were packed with 201.0 g of pre-sieved air-dried soil to a height of approximately 10 cm and a standard bulk density of 1.384 $g.cm^{-3}$. Glass wool discs (2 cm thick) were placed at both ends of the soil columns to facilitate even distribution / exit of applied synthetic leachate. Each column was closed with a two-port rubber bung at the top (to measure methane gas release at different depths) and a single port rubber bung at the base. No head-space was present between the upper rubber bung and the top of the soil column. The columns were saturated from the base with one pore volume (75.92 ml) of distilled water and left to equilibrate over a period of 50 days (Shaw and Burns, 1998). Subsurface soil in the unsaturated zone may be, at worst, completely saturated by leachate. Therefore, it would make sense to investigate attenuation mechanisms under such condition (Bagchi, 2004). A total of 64 columns were prepared and were assembled into sequential soil microcosms (SSM) as described in Chapter Four.

2.2.1 Column harvesting and soil sampling

When the respective SSM's had reached the desired redox states each column was removed and sampled (Appendix B, steps 1-7). Rubber bungs were removed (steps 1 - 2) and the soil column within the glass cylinder was gently pushed out using a circular wooden plunger whilst ensuring that an equal distribution of pressure was used (steps 3 - 4). The resulting soil column was dissected into three equal parts (± 50 g wet weight) and labeled (steps 5 - 7) and stored at 4EC until further analyses could be carried out.

2.3 Eluents

2.3.1 Synthetic leachates

Two synthetic leachate concentrates (10x) were prepared; one with nutrient supplements and the other without (Appendix C) (Smith, Senior and Dicks, 1999b). The relevant concentration of each constituent is given in Table 2.1. Leachate concentrates (10x) were filter sterilized through 0.22 Φm filter membrane (Millipore, USA) using a 20 *l* Millipore Corporation filter tank equipped with a 142 mm filter holder and 124 mm pre-filter (Millipore, USA) and attached to a air compressor (Hobbycraft 1.1 kW Air Compressor, South Africa).

Concentrations of phenol, zinc and copper used were above those found in natural landfill leachates, in order to create a situation which represented a high risk for groundwater contamination. The concentrations of the remaining inorganic components were selected to approximate the normal ranges found in natural landfill leachates (Smith *et al.*, 1999b). Phenol was chosen as a representative of phenolic substances found in soil humic material and because it occurs as a common industrial contaminant (Albrechtsen and Winding, 1992; Guerin, 1999). Upon dilution both leachates were acidified with 1M hydrochloric acid (pH5.0) (Smith *et al.*, 1999b) and sparged with oxygen-free nitrogen for a minimum of two hours before use. Both leachates were titrated with a few drops of 0.01 M $Na_2S.9H_2O$ (Saarchem, Merck) to secure a redox (E*h*) environment of 160 - 200 mV (Hrapovic and Rowe, 2001). These chemical alterations ensured a synthetic mixture that modeled a young stable landfill leachate characteristic of landfill leachate entering soil below a “waste tip”.

2.3.2 Nutrient supplements

Micronutrient stock solutions

Two micronutrient stock solutions adapted from the trace element supplement described by Coutts, Senior and Balba (1987) were made up containing the following ($mg l^{-1}$ sterile-distilled water):

A: $FeCl_2.4H_2O$, 1500; $MnCl_2.4H_2O$, 197; $CaCl_2$, 90; $CoCl_2.6H_2O$, 238; $AlCl_3$, 50; H_3BO_4, 62; $NiCl_2.6H_2O$, 24.

B: $NaMoO_4.2H_2O$, 48.4; $NaSeO_3.5H_2O$, 2.55; $Na_2WO_4.H_2$, 3.3.

Vitamins

The vitamin stock solution contained the following (mgl^{-1} sterile-distilled water): pyridoxine-HCl, 20; *p*-aminobenzoic acid, 19; Ca-D-pantothenate, 30; nicotinic acid, 50; riboflavin, 30; thiamine-HCl, 20; biotin, 10; folic acid, 10; cyanocobolamine, 20.

Each of the solutions were filter sterilised (0.22 Φm cellulose acetate, Millipore) and stored at 4EC for no longer than two months.

Five millilitres of each filter sterilized nutrient supplement stock solutions were added to 100 m*l* of the concentrated synthetic leachate and the volume made up to 1000 m*l* with distilled water prior to being fed to the respective columns. The E*h* was verified prior to every feed (2.4.1).

2.3.3 Hydraulic loading rates (HLR's)

Degassed synthetic landfill leachate was fed to the respective columns via. 20 m*l* plastic syringes at a rate of 40 m*l* (HLR*h*) and 20 m*l* (HLR*l*) every ten days, since studies have revealed that discontinuous application of compounds more closley resembles field conditions than continuous application (Shaw and Burns, 1998). These hydraulic loading rates were based on 25 % of the mean annual precipitation (MAP) of a high rainfall region (viz. Pietermaritzburg, KwaZulu-Natal) and a typically arid region (viz. Kimberley, Western Cape) found in South Africa (Ehrig, 1983; Canzianni and Cossu, 1989) (Appendix D).

Leachate migration within a landfill and the surrounding environment are influenced by a range of factors such as the local rainfall; surface runoff; and water retention properties of the surrounding medium (Crawford and Neretnieks, 2001). The resultant hydraulic loading

rates (HLR) were designated HLR*h* and HLR*l*, respectively and these designations were retained throughout the study.

Rainfall maps[1] and data[2] were supplied by The Computing Center for Water Research (CCWR), University of Natal, Pietermaritzburg, Republic of South Africa (Appendix C).

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[1]Mr Mark Horan, University of Kwazulu Natal, BEEH, Pietermaritzburg

[2]Mr Youdeshan Naidoo, University of Kwazulu Natal, CCWR, Pietermaritzburg

Table 2.1 Component concentrations (mg.l^{-1}) of two synthetic leachates.

Leachate Component	Component Concentrations (mg.l^{-1}) in Synthetic Landfill Leachates	
	A	A-mn
phenol	500	500
Zn	100	100
Cu	25	25
NO_3	286	286
NH_4-N	12	12
K	30	30
Na	532	532
Mg	100	100
Ca	100	100
Cl	1523	1523
SO_4	135	135
supplements	absent	present

2.4 Leachate Analyses

Due to economic constraints coupled with the large number of replicated samples collected, analyses was restricted to single determinations for each sample. The following analyses were performed:

2.4.1 Redox potential

Leachate redox potential was determined with a Crison Pt / $AgCl_2$ redox probe attached to a Crison MicropH 2002 meter. A Fe^{2+}/Fe^{3+} standard solution (39.21 g ferrous ammonium sulphate, 48.22 g ferric ammonium sulphate and 56.2 m*l* of 98 % (v/v) sulphuric acid made up to 1 *l* with distilled water and stored at 4EC) was used to calibrate the instrument. The calibration was deemed accurate when the solution gave a redox value between 460 - 470 mV. Measurements were taken immediately upon sampling (10 m*l*) each microcosm using a 10 m*l* gas-tight glass syringe (SGE International, Australia).

2.4.2 pH

Leachate pH determinations were made with a Crison pH probe in conjunction with a Crison MicropH 2001 meter. The instrument was calibrated using pH 4.0 and pH 7.02 standards (Crison).

2.4.3 Phenol

Residual phenol was quantified using gas chromatography (Varian 3600 Gas Chromatograph) equipped with a flame ionization detector. The gas chromatograph was fitted with a glass column, (length, 2.4 m; i.d, 3.0 mm) packed with 5 % OV-101 on 80/100 mesh Chromosorb W. The oven temperature was maintained at 70 ^{0}C for 30 seconds after which temperature was increased at a rate of 50 ^{0}C min^{-1} to 150 ^{0}C. The injection port and detector temperatures were 200 and 250 ^{0}C, respectively. Nitrogen was used as a carrier gas at a flow rate of 30 ml.min^{-1}. Samples were filtered through a 0.22 µm cellulose acetate membrane

before injection of 1 μ*l* into the GC. The phenol concentration was determined by peak area comparison with phenol standards (0 - 600 mg.l^{-1}) using Delta chromatography computer software.

2.4.4 Heavy metals

A Varian SpectrAA-200 Series Atomic Absorption Spectrophotometer (AAS) equipped with a Varian SPS-S auto-sampler was used. Samples were centrifuged in an Eppendorf centrifuge (Model 5410), at 10000 x g for 10 minutes to remove any precipitates or colloids present and were then stored at 4EC. Each sample was diluted ten-fold prior to analysis for Zn^{2+} and Cu^{2+} in solution.

Standards (1, 5 and 10 mgl^{-1}) were made up from ultra pure AAS reagents (Saarchem) and stored at 4EC for not longer than one month. Conditions used for the analysis of each metal are outlined in Table 2.2.

Table 2.2 Conditions used for heavy metal analysis by Atomic Absorption Spectrophotometry.

Metal	Wavelength (nm)	Lamp Current (mA)	Slit Width (nm)	EHT (volts)	Flame	Instrument Mode
Cu^{2+}	324.8	4	0.5	250	air-acetylene	absorbance
Zn^{2+}	213.9	5	1.0	229	air-acetylene	absorbance

2.4.5 Nitrates

Residual nitrate concentrations were determined by direct colorimetry at 410 nm with a Milton Roy Spectronic 301 spectrophotometer.

Reagents

4 M Sodium hydroxide:

NaOH pellets (160 g) were dissolved in 600 m*l* of distilled water and the resulting solution diluted to 1000 m*l* in a volumetric flask.

5% salicyclic acid:

Salicyclic acid (5 g) was dissolved in 95 m*l* of 98 % (v/v) sulphuric acid.

1000 mg.l^{-1} NO_3-N stock solution:

Dry potassium nitrate (7.223 g) was dissolved in distilled water and the resulting solution diluted to 1000 m*l* in a volumetric flask.

The three reagents were stored at room temperature.

A range of standards (0 - 200 mg.l^{-1}) were made up from the 1000 mg.l^{-} NO_3-N stock solution. Salicyclic acid (1 m*l*) was added to 0.5 m*l* sample or standard and the mixture was allowed to stand for 30 minutes. Ten millitres of 4 M NaOH was then added to each reaction and the resulting solution was allowed to stand for a further hour for colour development. A water blank was also prepared in this manner by substituting distilled water in place of the sample. A second set of blanks were prepared for each NO_3-N concentration by adding 0.5 m*l* of each NO_3-N standard to 1 m*l* sulphuric acid (98 %) instead of the salicyclic acid solution. The second blank attempted to minimize the effects of pigmentation in the coloured leachate extracts. A standard curve was constructed from which residual nitrate concentrations were determined.

2.4.6 Sulphates

Residual leachate sulphate concentrations were quantified with the Spectroquant® analysis system (Merck Laboratory Supplies, Germany). Briefly, 2.5 m*l* of undiluted sample was mixed with specified amounts of two reagents in a screw-cap test tube and incubated in a shaker water bath at 40EC for five minutes. Thereafter, a third reagent was added to the mix and thoroughly shaken before the mixture was filtered (Whatman® N° 1, England). The filtrate was collected in a screw-cap test tube and combined with a fourth reagent before being placed in a water bath at 40EC for seven minutes. The test tube was then placed in the Spectroquant® Photometer SQ 200 (Merck Laboratory Supplies, Germany) set on filter position 3 to measure the concentration of residual sulphate in the sample.

2.4.7 Methane

Gas samples (100 μ*l*) were collected with a 250 μ*l* gas-tight glass syringe (Hamilton, Switzerland) from the gas outlet (Figures 4.1) of each of the columns and injected into a Varian 3600 gas chromatograph equipped with a flame ionization detector and universal injector. A glass column (length 1.45 m, i.d. 3.0 mm) packed with Propak T (80/100 mesh) was used. The injector, detector and column temperatures were maintained at 110EC, 200EC, and 35EC, respectively. Methane standards (5, 15, 25, 50, and 100 % v/v) were prepared from pure methane gas (Fedgas, South Africa). The methane concentration of each sample was calculated by comparison of peak areas with the standards.

2.5 Genotypic Profiling of Microbial Communities Associated with Soil Microcosms

2.5.1 Deoxyribonucleic acid (DNA) isolation from soil

2.5.1.1 "Bead Beat" method

A modified version of the protocol described by Duarte *et al* (1998) was used to extract DNA from soil (Appendix E). Briefly, 1.0 g (wet weight) of soil, 100 μ*l* 20 % SDS, 800 μ*l* 120 mM sodium phosphate buffer (pH 8.0) and 500 μ*l* phosphate saturated liquid phenol (pH 5.5) were added to a sterile bead-beater tube containing 0.6 g of glass beads (0.1 mm diameter). The tube was placed in a Mini Bead-Beater 1000 (Braun Cell Homogenizer, Melsungen, Germany) for one minute at a speed of 4200 rpm. The resultant slurry was placed in a water bath at 60EC for 10 minutes before bead-beating for a further one minute. The sample was then centrifuged (Eppendorf Centrifuge 5410) at 10000 x g for three minutes. The aqueous upper phase was extracted and placed in a sterile eppendorf tube containing 600 μ*l* phosphate saturated liquid phenol (pH 5.5). The mixture was centrifuged at 10000 x g for three minutes, afterwhich, the aqueous upper phase was extracted once more and placed in a sterile Eppendorf tube, to which 600 μ*l* of phenol:chloroform:isoamylalcohol (25:24:1) was added. This mixture was centrifuged at 10000xg for three minutes, followed by a repeat extraction with phenol: chloroform: isoamylalcohol addition and centrifugation. The resultant aqueous upper phase was transferred to a new eppendorf tube and the volume was determined before the DNA was precipitated with 0.1 volume 3 M sodium acetate (pH 5.5) and 0.6 volume isopropanol , followed by incubation on ice for at least 30 minutes. The sample was then centrifuged at 10000 x g for 20 minutes afterwhich the supernatant was discarded. The remaining pellet was washed once with 200 μ*l* 70 % ethanol and centrifuged for a few seconds at 10000 x g before the resultant supernatant was carefully removed and discarded. The pellet was allowed to air dry for a minimum of 15 minutes before resuspending in 50 μ*l* TE buffer. The extracted DNA was stored at -20EC. DNA extraction was carried out in duplicate for all samples and the products of each duplicate were pooled for further analysis (Felske and Akkermans, 1998b).

2.5.1.2 DNA isolation kit

The isolation of total genomic DNA from the soil samples were made with an UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) following the recommended protocol (Appendix F). To standardize the extraction one gram of soil sample was used. DNA extraction was carried out in duplicate for all samples and the products of each duplicate were pooled for further analysis (Felske and Akkermans, 1998b).

2.5.2 Detection and quantification of DNA

Detection

A 1.2 % (m/v) agarose gel was prepared by combining 0.3 g of electrophoresis grade agarose (Whitehead Scientific Ltd.) with 25 m*l* of 1* TAE buffer (Appendix G). The mixture was heated in a microwave (Tedelex) for 20 seconds, and then gently swirled before heating for a further 20 seconds untill the agarose had dissolved. After cooling to ± 50°C, 1.5 μ*l* ethidium bromide stock solution (10mg.ml^{-1}) was added and the solution was poured into a gel chamber (Hoefer Scientific Instruments). A comb (8 toothed) was placed at the upper end of the chamber and left to set at ambient temperature. Once solidified, the gel was placed in an electrophoresis chamber (Hoefer Scientific Instruments) which contained ± 200 m*l* of running buffer (1*TAE).

Extracted DNA sample (7 μ*l*) was mixed with loading buffer (2 μ*l*) and loaded into the gel wells. A molecular weight marker (1 kB marker, Boehringer Mannheim) and a positive control (DNA of *Escherichia coli*) were loaded into lanes 1 and 8, respectively. The gel was run at 100 volts (BioRad Power Pac 300) for 40 minutes. The bands were visualized with an UV-transilluminator (Chromo-Vue TM-36, San Gabriel USA) and the image captured with Imagestore 5000 software (Ultra Violet Products).

Quantification

The extracted DNA was quantified with a Gene Quant Pro (Pharmacia Biotech). The DNA was blanked against 63 μ*l* of sterile Milli-Q water before 7 μ*l* of sample was added (10^{-1} dilution). The absorbance was measured at (nm) 260, 280, and 230 by using a 70 μ*l* quartz cuvette with one millimeter path length. Quantification facilitated a direct comparison between the two DNA extraction methods used.

2.5.3 16S rDNA amplification by the Polymerase Chain Reaction (PCR)

Step 1: Optimization

PCR conditions for the amplification of environmental DNA samples and a DGGE marker (Lin Bin marker from H.W van Verseveld, personal communication) were based on conditions previously optimized for at Vrije Universiteit van Amsterdam, Netherlands (H.W van Verseveld, personal communication) (Table 2.3 and 2.5). The annealing temperature was adjusted from 54ºC to 55 ºC to increase the annealing specificity of the primers and DNA template. The number of cycles was also increased from 30 to 32 so as to increase the quantity of amplicon.

Table 2.3 Reagents used for the PCR amplification of 16S rDNA.

Reagent per reaction tube	Volume (μl)
Forward primer (f357gc) (0.01 M)	1.0
Reverse primer (R518) (0.01 M)	1.0
dNTPs (10 mM)	1.0
Bovine serum albumin (10 mg.ml^{-1})	1.0
Taq polymerase buffer (10 x concentrate)	2.5
Taq polymerase (5µ.µl^{-1})	0.5
Sterile Milli-Q water	17.0
Target DNA	1.0
Total volume	**25**

Step 2: PCR Amplification

DNA was amplified for application to denaturing gradient gels under electrophoresis (DGGE). The variable V3 region of 16S rDNA (Saiki, Scharf, Faloona, Mullis, Horn, Erlich and Arnheim, 1985), which corresponds to positions 341 and 534 in *E. coli* (Muyzer *et al.*, 1993), was amplified with universal prokaryote primers corresponding to conserved regions of the 16S rDNA genes (Medlin, Elwood, Stickel and Sogin, 1988) (Table 2.4). The guanine-cytosine clamp (GC clamp) was incorporated into the forward primer by the addition of a 40-nucleotide GC-rich sequence (GC clamp) at its 5' end (Muyzer *et al.*, 1993).

Table 2.4 Characteristic properties of the primers used for PCR amplification of 16S rDNA.

#Forward Primer (f357gc)	
Property	Character
Sequence	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'
Length	57-mer
T_m	> 75 ^{0}C
GC content	91.2 %
Molecular weight	17253 g.mol^{-1}
#Reverse Primer (R518)	
Sequence	5'-ATT ACC GCG GCT GCT GG-3'
Length	17-mer
T_m	57.6 ^{0}C
GC content	64.7 %
Molecular weight	5202 g.mol^{-1}

= Supplied by Whitehead Scientific, Cape Town and Isogen, Vrije Universiteit, Netherlands

Polymerase chain reaction amplifications were made in an automated thermal cycler containing a refrigerant system (Applied Biosystems GeneAmp PCR System 2400, Singapore). The programme used is shown in Table 2.5 (H.W van Verseveld, personal communication). Two control tubes were included in each PCR run: a positive control, which contained DNA from a reference culture of *E. coli*; and a negative control with sterile Milli-Q water replacing template DNA. PCR products were stored at -20 ^{0}C.

Table 2.5 Polymerase Chain Reaction Programme adapted from Vrije Universiteit, Netherlands and applied on a thermo-cycler.

File	Effect on DNA	Temperature (^0C)	Time (minutes)
Time Delay	Initial denaturation	92	4
Step Cycle (32 cycles)	Denaturation	92	0.5
	Annealing	55	1
	Extension	68	1
Time Delay	Final extension	72	5
Soak	Cooling	4	user defined

Step 3: Visualization of PCR Products

PCR amplicons were visualized by agarose gel electrophoresis following the method described in 2.5.2. A 100bp molecular weight marker (Boehringer Mannheim) was used to determine amplicon size.

2.5.4 Parallel denaturing-gradient gel electrophoresis (DGGE)

DGGE was carried out using the DCode^{TM} Universal Mutation Detection System (Bio-Rad). A description of reagent preparation, assembly, casting, and running of denaturing gradient gels is presented in Appendix H.

2.5.5 Staining of DGGE gels

2.5.5.1 Silver staining method

Denaturing gradient gels were transferred from the electrophoretic chamber on an electrophoresis glass plate to a clean glass tray (50 cm x 30 cm). Each gel was fixed twice, for a minimum of 10 minutes, with fresh Fixation solution (250 m*l*) (Appendix I) and gentle agitation on a flat surface rotary shaker (Hoefer Red Rotor). Thereafter, the fixation solution was removed and the gel was rinsed with MilliQ water (Millipore Corporation) before 250 m*l* of freshly prepared 0.1 % $AgNO_3$ solution was poured onto the gel. The gel was gently agitated in darkness for a further 20 minutes. Afterwhich the $AgNO_3$ solution was removed and the gel rinsed with MilliQ water. The gel was then transferred to a second glass tray and allowed to develop by gentle agitation in 250 m*l* Developing solution for 25 - 30 minutes in darkness. Development of stained bands was monitored carefully to ensure optimum results. The developing solution was removed and the gel rinsed with MilliQ water. Finally, 250 m*l* Stop Mix was poured onto the gel to prevent further development. The gel was gently agitated in this solution for a minimum of 10 minutes before air drying and capturing of the image using a VersaDoc™ Imaging System coupled with Quantity One® 1-D Image Analysis Software (Bio-Rad).

2.5.5.2 Ethidium bromide

Denaturing gradient gels were transferred from the electrophoretic chamber on an electrophoresis glass plate and placed in a glass tray (50 cm x 30 cm). Running buffer (400 m*l*) from the electrophoretic chamber was poured onto the gel and 6 μ*l* of ethidium

bromide (10 mg.ml^{-1}) (Appendix H) was added. The gel was gently agitated for 1-2 hours on a flat surface rotary shaker (Hoefer Red Rotor) in darkness. The bands on the gel were visualised and photographed as stated in 2.5.2.

2.5.6 Analysis of DGGE banding patterns

The captured digital images were analyzed with Quantity One® 1-D Image Analysis Software (Bio-Rad) using uniform detection criteria standardized for the detection of bands for all gels.

2.5.6.1 Assessment of bacterial community structure by species diversity indices

The structural dynamics of the bacterial species occurring in each sample was assessed by applying the following species diversity indices:

i *Species Richness (S)* - where the number of bands appearing on a gel lane represents the number of species in that sample (Muyzer *et al.,* 1993; Jackson *et al.*, 2001; McCaig, Glover and Prosser, 2001).

ii *Shannon-Weaver Index (H')* - where the peak intensity (Pi = peak intensity of the ith band expressed as a proportion of the total peak intensity of a lane) of each band appearing on a gel lane was used to measure microbial diversity per sample (McCaig *et al.*, 2001; Girvan, Bullimore, Pretty, Osborn and Ball, 2003; Koizumi, Kojima and Fukui, 2003; Camargo, Okeke, Bento and Frankenberger, 2005) in order to factor both species richness and evenness into a single measure (Hill, Walsh, Harris and Moffett, 2003).. The following equation was used (Begon, Harper and Townsend, 1986):

$$H' = -\sum Pi \log Pi \text{.........................} Eq2.4$$

iii *Shannon-Weaver Evenness Index (E_H)* - this is an index that assigns a numerical grading that describes the equivalent abundance of all species occurring in a sample (Haack, Fogarty, West, Alm, McGuire, Long, Hyndman and Forney, 2004). The index

describes the evenness of the species in the sample by comparison of the observed species abundance (H') and the theoretical species abundance (H_{max}), assuming each band in a sample lane has equal peak intensities (Camargo *et.al.*, 2005). The index is calculated from the following equation (Hill *et.al.*, 2003; Ramírez-Saad, Sessitsch, and Akkermans, 2003)

$$E_H = \frac{H'}{H_{\max}} \text{.......................} Eq2.5$$

where $H_{max} = ln\ S$.

iv *Simpson's Index (D)* - Like the Shannon-Weaver Index, this index measures the diversity by considering the prevalent abundance and species richness of a community by calculating the proportionate (Pi) peak intensity contribution of each species (S) in a sample and applying these values in the following equation (Edwards, Lilley, Timms-Wilson, Thompson and Cooper, 2001; Hill *et.al.*, 2003):

$$D = \sum Pi^2 \text{........................} Eq2.6$$

Begon and co-workers (1986) referred to the Simpson's Index of Diversity as the reciprocal of the above equation.

i *Simpson's Equitability Index (E_D)* - the index describes the distribution of abundance within the sample by expressing the reciprocal of the observed diversity ($1/D$) as a proportion of the maximum diversity (D_{max}) that would be possible in the same sample if the abundance (Pi) was equally distributed among the contributing species (S), thus (Begon *et al.*, 1986):

$$E_D = \frac{D}{D_{\max}}^{-1} \text{......................} Eq2.7$$

where $D_{max} = S$.

2.5.7 Clone bank

The genomic DNA extracted from two columns (sample 12 = array A*h*, harvested after 12 days and sample C1 = untreated soil) was used to establish a clone bank according to the method described by Felske, Wolterink, van Lis and Akkermans (1998). Thus, polymerase chain reaction primers 8f (5'-CAC GGA TCC AGA CTT TGA T(CT) (AC) TGG CTC AG-3') and 1512r (5'-GTG AAG CTT ACG G(CT)T AGC TTG TTA CGA CTT-3') were used to amplify 16S rDNA sequences. The products were then cleaned with the Qiaquick Rep Purification Kit (Qiagen, Hilden, Germany) and cloned in pGEM-T linear plasmid vector and *Escherichia coli* JM109 competent cells as specified by the manufacturer (Promega, Madison, Wisconsin, USA). Randomly-selected recombinants/clones were reamplified with the primer set F357-GC/R518 (Isogen Bioscience BV, Maarsen, Netherlands) and the products were compared on a DGGE gel with the initial association profiles. Some of the recombinants were then selected for sequencing.

2.5.8 Sequencing of randomly-selected clones

To obtain the partial sequences of the 16S rDNA, reamplification of selected 8f-1512r clones was made with the T7/sP6 primer set (Isogen Bioscience BV, Maarsen, Netherlands). Sequencing PCR was performed with an ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit (Perkin-Elmer) and the purified products were run in SEQUAGEL-6 sequence gel (National Diagnostics, USA). Both strands of the 16SrDNA gene fragments were sequenced. Basic Logical Alignment Search Tool (BLAST) Network Service was used to compare the sequences with available databases to determine their approximate phylogenetic affiliations.

Chapter Three

3. An Assessment of the Natural Content and Adsorptive Capacity of the Soil Matrix for Zinc, Copper, and Phenol

3.1 Experimental

3.1.1 Phenol and heavy metal extraction from soil

For each of the extraction methods, all glassware was thoroughly washed and oven-dried at 105°C prior to use.

3.1.1.1 Phenol extraction by Soxhlet

Positive controls were created by spiking air-dried soil with a known concentration of phenol to achieve soil phenol concentration of 1000 $mg.kg^{-1}$ (Khan, 2002). An aqueous phenol preparation was used to mimic, as close as possible, the natural entry of the compound into the soil. Briefly, non-sterile air-dried soil (5 g) was weighed into a glass vial and 1 m*l* of phenol solution was added to the soil. The soil was mixed thoroughly and allowed to equilibrate at ambient temperature overnight.

The extraction method employed was modified from EPA Method 3540 (USEPA, 1986) and Khan (2002). Dichloromethane (100 m*l*) (Saarchem) and a few boiling chips were placed in a round-bottom flask seated in a thermal heater. This was attached to a Soxhlet extractor, containing a nitro-cellulose thimble with 5 g of soil (spiked or unspiked), and attached to a water-cooled condenser. The extraction was sustained for 8 hours at 10 refluxes per hour. The extract was then passed through a plug of anhydrous $MgSO_4$ (oven-dried at 400 ºC prior to use).The extract was cooled and filtered through Watman No. 1 (Merck) filter paper and the total volume was reduced, with a rotovaporator (Heidolph), to 10 m*l* and 2 m*l* for the spiked and non-spiked samples, respectively. Residual dichloromethane was evaporated under a low flow of nitrogen gas. Duplicate extractions were carried out for each spiked and non-spiked sample.

3.1.1.2 Heavy metal extraction from soil

Analyses courtesy of Mr. V. Dorasamy[3] and Cedara Agricultural College, Cedara, Republic of South Africa (Handbook of Standard Soil Testing Methods for Advisory Purposes, 1990).

3.2 Adsorptive Behavior of Phenol, Copper and Zinc in a Sterile and Non-sterile Hutton Soil

**see Appendix J for reagent preparation*

3.2.1 Phenol

Stock solutions of phenol were prepared by dissolving phenol (Sarchem) in full strength synthetic leachate (Appendix A) to achieve concentrations of (mg.l^{-}): 50, 100, 200, 300, 400, 500 and 600 respectively. Ten grams of sieved, pre-gamma radiated soil [Gamwave Pty (Ltd)] or non-sterile soil were mixed with 50 ml of each of the respective phenol stock solutions in a 100 m*l* centrifuge tube. Duplicate tubes were setup for each concentration of phenol.

The sealed tubes were allowed to shake on an end-over-end shaker at a speed of 24 rpm for 48 hours. Following a period of equilibration a sample (2 m*l*) from each tube was centrifuged (Eppendorf Centrifuge 5410) at 14000 rpm (8 minutes). Phenol concentrations were then determined (2.4.3).

[3] Mr. V. Dorasamy, University of Natal, Department of Soil Science, Pietermaritzburg

3.2.2 Copper and zinc

Stock solutions of copper and zinc were prepared separately by dissolving $Cu(NO_3)_2.3H_2O$ and $Zn(NO_3)_2.6H_2O$ in full strength synthetic leachate (Appendix A) to achieve concentrations of (mg.l^{-1}): 25, 50, 75, 100, 125 and 150 respectively for each metal. Ten grams of sieved, pre-gamma radiated soil [Gamwave Pty (Ltd)] or non-sterile soil were mixed with 50 ml of each of the respective stock solutions. Duplicate tubes were setup for each concentration of metal.

The sealed tubes were allowed to shake on an end-over-end shaker at a speed of 24 rpm for 48 hours. Following a period of equilibration a sample (2 m*l*) from each tube was centrifuged (Eppendorf Centrifuge 5410) at 14000 rpm (8 minutes). Copper and zinc concentrations were then determined (2.4.4).

3.2.3 Adsorption of copper and zinc from mixed solutions

To determine the adsorptive behaviour of zinc and copper mixtures, stock solutions (ratio 1:1) were prepared by dissolving $Zn(NO_3)_2.6H_2O$ and $Cu(NO_3)_2.3H_2O$ in half strength synthetic leachate (Appendix A) to achieve a concentration range of 25 - 150 mg.l^{-1}. Adsorption experiments were carried out in the same fashion as described above (3.2.2).

3.2.4 Analysis of data

The amount of sorbed metal was calculated according to the following equation (Kan, Fu,and Tomson, 1994; Swalaha, Datadin and Choonawala, 2002):

$$qtot = \frac{(Ci - Cres)V}{W} \text{..} Eq3.1$$

where q*tot* is the quantity of metal or phenol adsorbed per unit mass of soil (mg.kg^{-1}); C*i* is the initial concentration of adsorbate in solution (mg.l^{-1}); C*res* is the equilibrium concentration of the adsorbate left in solution after contact with the soil (mg.l^{-1}); *V* is the volume of solution used for each reaction (*l*); and *W* is the dry weight of the soil used in each reaction (kg).

The results were presented in a graph of C*res* against q*tot* and the data was fitted to the Langmuir (Murali and Aylmore, 1983) (*Eq*3.2) and Freundlich (Campbell and Davies, 1995; Novella, Ballard, Stow, Ross, Blight and Vorster, 1999) (*Eq*3.3) equations:

$$qtot = \frac{QCresK_L}{(1 + KCres)} \text{...} Eq3.2$$

$$qtot = KfCres^{1/N} \text{...} Eq3.3$$

where Q is the maximum quantity of solute adsorbed per unit mass of adsorbant (mg.kg^{-1}); K_L is the Langmuir bonding energy coefficient or equilibrium distribution coefficient and is related to the energy of adsorption (Arias *et al.*, 2005b); K_f is the Freundlich equilibrium distribution coefficient or adsorption constant and represents a measure of metal or phenol adsorption (Agbenin and Olojo, 2004) where the K_f value is directly proportional to the adsorption capacity (Ghiaci, Abbaspur, Kia and Seyedayn-Azad, 2004) and strength of binding (Khan and Anjaneyulu, 2005) to the soil and; N is the Freundlich power coefficient or intensity coefficient, where $1/N = 1$ corresponding to linear adsorption (Ghiaci *et al.*, 2004).

Fitting of the adsorption isotherms to Eq. 3.2 and 3.3, and the calculation of the governing parameters was established by simple linear regression (Gentstat Release 8.1) for the investigations involving single compounds in isolated systems (3.2.1 and 3.2.2). General linear regression, by way of stepwise regression of two explanatory variables on a single response variable, was used to describe investigations involving the mixed solutions of copper and zinc (3.2.3). The goodness of fit to each equation was evaluated in terms of the linear correlation coefficient, r.

3.3 Results and Discussion

3.3.1 Phenol and heavy metal extraction from soil

3.3.1.1 Phenol extraction by Soxhlet

Phenol extraction from Hutton soil was performed to test the hypothesis that the incidence of phenol would be symptomatic of the presence of phenol metabolising microorganisms within the soil. The degradation of numerous hazardous compounds, including phenol, by indigenous soil borne microbial communities has been widely documented (Scott, Wolf and Lavy, 1982; Willems, Lewis, Dyson and Lewis, 1996; Romantschuk, Sarand, Petanen, Peltola, Jonsson-Vihanne, Koivula, Yrjala and Haahtela, 2000; Schie and Young, 2000; Khan and Anjaneyulu, 2005). An average Soxhlet extraction efficiency of 67.85 % was obtained from soil samples spiked with phenol. These results confirmed the success of the extraction protocol and recovery rates documented by Khan *et.al.* (2002). The quantity of phenol extracted from the unspiked soil was 2.33 $mg.kg^{-1}$. This intrinsic presence of phenol was thought to be an important factor for the establishment and subsequent proliferation of indigenous microbial populations capable of degrading phenol (within limits) present in the synthetic leachate. The presence of phenol could be a result of the solubilization of phenolic compounds, contained in terrestrial vegetation, during the natural degradation (Dobbins, Thornton-Manning, Jones and Federle, 1987) and animal digestion of plant material (van Schie and Young, 2000). Hrapovic and Rowe (2002) hypothesised that under difficult conditions for natural degradation of volatile fatty acids a favourable environment for metabolism of compounds can be achieved given adequate time. Guerin (1999) found evidence to support the view that in soils containing organic contaminants, such as phenols and polycyclic aromatic hydrocarbons (PAH), there are innate microbial communities that possess metabolic capacities that enable them to survive, and when given ample stimulation, thrive.

3.3.1.2 Heavy metal extraction from soil

The Hutton soil contained 6.6 $mg.kg^{-1}$ and 2.0 $mg.kg^{-1}$ of Cu and Zn respectively. For a suitable assessment of retention and/or removal of Cu and Zn from the leachate it was necessary to qualify the pre-existence of Cu and Zn in the Hutton soil, since this history would impact to an extent on the physico-chemical capacity of the soil. In addition, pre-exposure of the indigenous microbial community to the metals would enhance their survival and growth capacity in the face of increasing heavy metal concentrations which would facilitate greater bioaccumulation of Cu and Zn (Gadd, 1992; Costley and Wallis, 2001). Copper and zinc exhibit divergent behaviours in soil. Copper has a low mobility, whereas zinc tends to be more mobile in soil. Furthermore, the type of soil plays a significant role in influencing the behaviour of these metals and other components of the landfill leachate percolating through the soil. Although physical and chemical interactions between the heavy metals, the soil matrix (Loch *et al.*, 1981), and various other fractions of the landfill leachate (Calace, Liberatori, Petronio and Pietroletti, 2001) account for the retention of a large proportion of the heavy metals from leachate, biogeochemical processes significantly enhances this retentive capacity (Cozzarelli *et al.*, 2000; Mori, Hatsu, Kimura and Takamizawa, 2000).

3.3.2 Adsorptive behavior of phenol, copper and zinc in a sterile and non-sterile Hutton soil

These experiments were aimed at assessing the behavior of a synthetic landfill leachate containing copper, zinc and phenol on soil containing indigenous microbial communities and comparing this to a gamma-radiated soil devoid of viable microorganisms. These preliminary investigations potentially provide a measure of biological attenuation of the compounds in question against a back-drop of physical and chemical attenuation within the soil. Findings could provide valuable information on the retentive abilities of the biological component of the soil, if indeed any exists. Furthermore, the biological component of attenuation can later be characterized and possibly be used as a gauge for biological attenuation potential in other natural environments. The adsorptive behavior of the compounds could also provide insight in to the migratory potential of these compounds into groundwater and surrounding resources.

3.3.2.1 Phenol

Adsorption of phenol onto untreated soil followed a pattern typical of an S-type isotherm (Figure 3.1) which suggests that phenol adsorption is influenced and assisted by the phenol molecules previously adsorbed on the soil colloids (Isaacson and Frink, 1984; Khan and Anjaneyulu, 2005). This shape was not as pronounced for the sterilized soil as indicated by the poorer fit to the Langmuir model (Table 3.1) (Isaacson and Frink, 1984). Alloway, 1995 (cited by Agbenin and Olojo, 2004) recommended the use of the distribution quotient (K_d) to compare sorptive capacities of soils for metals under controlled conditions. The K_d, calculated by dividing the concentration of adsorbed compound by the equilibrium concentration of the compound in solution (Agbenin and Olojo, 2004; Arias, Pérez-Novo, Lopez and Soto, 2005a) indicated that the total amount of phenol adsorbed by the gamma-radiated soil (K_{dg} = 1.43 l.kg^{-1}) was 1.5 times less than that adsorbed by the untreated soil (K_{du} = 2.17 l.kg^{-1}). This means that for every milligram of phenol adsorbed per kilogram of gamma-radiated soil, 1.5 mg of phenol is adsorbed to the same amount of untreated soil. This suggested that the biological component of the soil plays a role in the phenol uptake, be it by metabolic degradation or adsorption. Scott *et al.* (1982 and 1983) conducted similar experiments to determine the adsorption and degradation of phenol in autoclaved and non-sterile soil. They were able to demonstrate that microbial biomass played a significant role in the adsorption / degradation of phenol, and those differences in adsorption / degradation rates were more apparent at low concentrations of phenol.

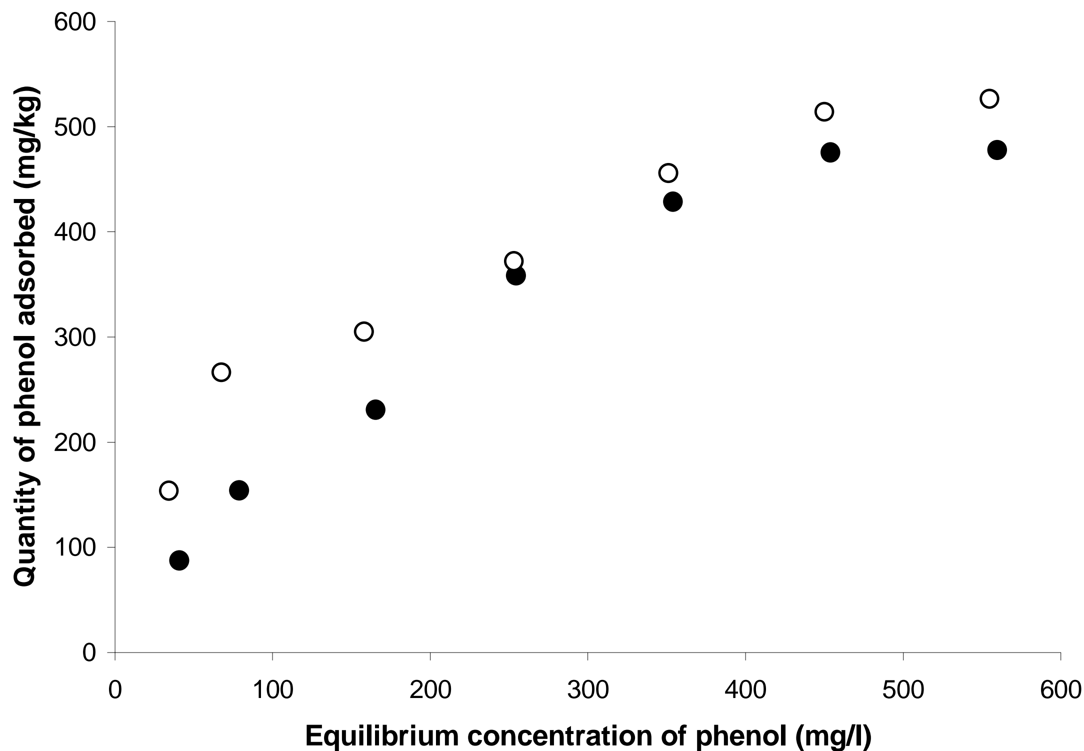


Figure 3.1 **Adsorption isotherms of phenol derived from gamma-radiated and untreated Hutton soil. Open symbols represent untreated and closed symbols represent gamma-radiated soil.**

The Langmuir model provided a satisfactory fit for both soil treatments, with r-values of 0.92 (untreated soil) and 0.85 (gamma-radiated soil) (Table 3.1). Langmuir constants (K_L and Q) indicated greater adsorption of phenol in the gamma-radiated soil with the lower K_L corresponding to a higher Q value. However, this finding contradicts the previous one which indicated that phenol adsorption (as expressed by Kd – values) was greater in the untreated soil. This discrepancy could be ascribed to the differences in the linear relationship expressed in the Langmuir equation for each treatment. In the case of the untreated soil 83.8% of the variance of total phenol adsorbed could be explained by the equilibrium concentration of phenol whereas only 71.8% of this variance was explained in the model given for the gamma-radiated soil. The opposite is true for the Freundlich model, where data corresponding to the gamma-radiated soil demonstrated a better fit ($r = 0.95$) than the untreated soil ($r = 0.80$), with the corresponding N-values confirming the greater linear relationship of the gamma-radiated soil data. In both cases the value of N was not less than 1.0, indicating favourable adsorption

of phenol to both soil treatments. The greater amount of phenol adsorbed by the untreated soil was confirmed by the higher K_f for the untreated soil (Ghiaci *et al.*, 2004).

Table 3.1 Langmuir and Freundlich adsorption model parameters estimated from the linear regression of data accumulated from phenol adsorption investigations involving gamma-radiated and untreated soil.

Soil Treatment	Langmuir Equation			Freundlich Equation		
	r	K_L	Q	r	K_f	N
Gamma-radiated	0.85	0.003	782.47	0.95	7.70	1.49
Untreated	0.92	0.006	673.90	0.80	37.34	2.38

Enhanced adsorption and degradation of phenolic compounds in soils rich in organic carbon and clay content (i.e. similar to the Hutton soil used in this study) has been demonstrated by Khan and Anjanyulu (2005). Lo, Mak and Lee (1997) demonstrated the need for modified clay liners as secondary defense to landfill leachate migration when geomembranes below landfills were breached. Bhandari, Novak, Burgos and Berry (1997) further demonstrated that mixtures of phenols could be attenuated within a surface soil rich in organic matter. They further showed that selected phenolic compounds from the initial group were biodegraded in the soil following inorganic attenuation, providing sufficient bio-stimulation was made available. If this is indeed the case with this study, then the subsequent investigations (Chapter 4) should highlight the biological attenuation of phenol from the synthetic landfill leachate, and its influence on the community structure of indigenous microorganisms.

3.3.2.2 Copper and zinc

The Hutton soil adsorbed, on average, more copper than zinc per unit weight of soil over the concentration range investigated (Figure 3.2). Copper and zinc adsorption by gamma-radiated and untreated soil were found to follow the shape of Type III and Type II isotherm patterns, respectively, which was indicative of adsorbents containing a large pore size distribution (Swalaha *et al.*, 2002). This indicated that the heterogeneity of the soil mineralogy had a major bearing on the behaviour of the adsorbates copper and zinc (Antilėn, Főrster, Del Confetto, Rodier, Fudym, Venezia, Deganello, and Escude, 2004). However, the zinc adsorption isotherm profile for both soil treatments, exhibited a definite saturation limit. This saturation limit was attained much sooner in soil that has been exposed to gamma-radiation (Figure 3.2).

Copper adsorption data for both soil treatments could not be fitted to the Langmuir equation. In contrast, the zinc adsorption data fitted both the Langmuir and Freundlich equations equally ($r = 0.96$) for the gamma-radiated soil and there was a pronounced improvement in fit to the Langmuir equation ($r = 0.99$) when the soil was left untreated (Table 3.2). Within the literature, varying trends have been reported. Arias *et al.*, (2005a) found that adsorption data of copper and zinc with a range of soils was better fitted to the Freundlich equation as apposed to the Langmuir model. Subsequently, in a related study Arias, Pérez-Novo, Osorio, López and Soto (2005b) found that adsorption data for zinc could not be fitted to the Freundlich equation. Meanwhile, Mesquita and Vieira e Silva (2002) found that their adsorption data for copper and zinc had a superior fit to the Langmuir equation. These differences in isotherm equation fitness were attributed to numerous factors which include, nature of adsorption medium (van Hullebusch, Peerbolte, Zandvoort and Lens, 2005; Swalaha *et.al.*, 2002), pH of the adsorption medium (Arias *et al.*, 2005) and metal solutions, concentration and composition of background (viz. electrolytes, organic matter) (Khan and Anjaneyulu, 2005), and possible modifications to the soils physical, chemical, and biological character brought about by gamma-radiation (Shaw, Beaton, Glover, Killham and Meharg, 1999).

Table 3.2 **Langmuir and Freundlich parameters extrapolated from the linear regression of data from copper and zinc adsorption investigations on gamma-radiated and untreated soil.**

Soil Treatment	Metal	Langmuir Equation			Freundlich Equation		
		r	K_L	Q	r	K_f	N
Gamma-radiated	Cu	na	na	na	0.90	80.24	0.774
	Zn	0.96	0.588	716.85	0.96	236.74	1.637
Untreated	Cu	na	na	na	0.95	51.06	0.720
	Zn	0.99	1.506	581.95	0.89	236.51	2.469

Contrary to expectations, the Hutton soil adsorbed more zinc than copper over the concentration range (25 mg.l^{-1} – 150 mg.l^{-1}) for both soil treatments. The average distribution quotients (K_d), indicated that the total amount of metal adsorbed by the gamma-radiated soil (K_{dg} = 382.36 l.kg^{-1}) was double that adsorbed by the untreated soil (K_{du} = 173.34 l.kg^{-1}) (Agbenin and Olojo, 2004). The adsorption isotherms (Figure 3.2) showed that zinc and copper require lower equilibrium concentrations of metal for greater adsorption from solution when the soil is treated by gamma-radiation. The average distribution quotients for zinc (K_{dgzn} = 264.68 l.kg^{-1}) was 3.6 times greater than that observed for zinc adsorbed on the untreated soil and 2.2 times greater than the amount of copper adsorbed by the gamma-radiated soil (K_{dgcu} = 117.68 l.kg^{-1}). The gamma-radiated soil adsorbed 1.2 times more copper than the untreated soil. Interestingly, the untreated soil adsorbed 1.4 times more copper (K_{ducu} = 100.14 l.kg^{-1}) than zinc (K_{duzn} = 73.20 l.kg^{-1}). The higher K_f values for zinc than copper, highlights the greater retention and strength of binding of zinc to the soil, be it gamma-radiated or untreated. The values of *N* are greater for zinc than for copper which indicates a greater linear relationship between zinc and its equilibrium concentration. The preference of the gamma-radiated soil for zinc retention ($K_{dzn} > K_{dcu}$) is in disagreement with observations made by Agbenin and Olojo (2004) and Arias *et al.* (2005a). Their findings were however, in agreement with the adsorption findings observed on the untreated soil ($K_{dcu} > K_{dzn}$).

The findings presented in this study clearly demonstrated that gamma-radiation significantly altered the adsorptive behaviour of the copper and zinc, by inverting the natural retentive behaviour of the cations albeit to a minimum extent. Gamma-radiation is recommended as a method for soil sterilization because of the nominal disturbance it is perceived to cause natural soils (Trevors, 1996; and McNamara, Black, Beresford and Parekh, 2003). The implication of these findings is that the overall impact of biological organisms on metal retention in the Hutton soil could not be reached unambiguously. However, these findings are noteworthy since gamma-radiation is commonly used as a tool in investigations that assess the impact of biology in soils.

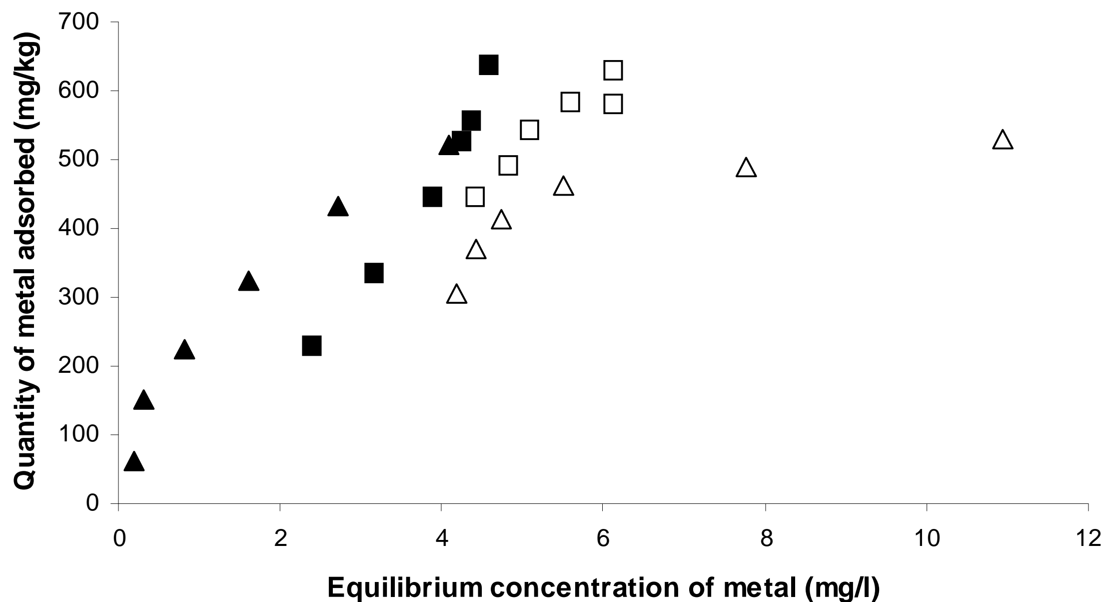


Figure 3.2 **Adsorption isotherms of copper (■) and zinc (▲) from mono-component systems. Open and closed symbols represent untreated and gamma-radiated soils respectively.**

3.3.2.3 Adsorption of copper and zinc from mixed solutions

In general, the adsorption isotherms determined for both heavy metals was similar to that observed in each of the mono-component investigations (Figure 3.3). The H-type isotherm evident for copper and the linear isotherm for zinc, for both soil treatments, imply that the Hutton soil has a stronger affinity for copper than for zinc (Agbenin and Olojo, 2004). This was clearly noticeable in the untreated soil. The amount of zinc adsorbed ($K_{dgzn} = 42.76$ *l*.kg^{-1}) by the gamma-radiated soil remained greater than that observed for copper ($K_{dgcu} = 38.22$ *l*.kg^{-1}). However, this difference was 50% less than that observed for the corresponding mono-component systems of the two metals. Again, more copper ($K_{ducu} = 59.77$ *l*.kg^{-1}) was adsorbed than zinc ($K_{duzn} = 31.32$ *l*.kg^{-1}) by the untreated soil, and this was a 50% increase in the difference observed for the two metals in the mono-component systems. The total amount of metal adsorbed by the gamma-radiated soil ($K_{dg} = 80.98$ *l*.kg^{-1}) was less than that adsorbed by the untreated soil ($K_{du} = 91.09$ *l*.kg^{-1}), this was in stark contrast to the mono-component systems. These findings are in line with other studies that highlight the competitive relationship of copper and zinc for binding sites on natural soils (Mesquita and Vieria e Silva, 2002; Agbenin and Olojo, 2004; Arias *et al.*, 2005b).

Fitting the adsorption data to the Freundlich equation conferred suitable r-values (0.99) for both metals and soil treatments. The equilibrium concentrations of copper ($p < 0.05$) and, to a greater extent, zinc ($p < 0.001$) played significant roles in determining the magnitude of copper and zinc adsorption in the untreated soil. The equilibrium concentration of zinc in the gamma-radiated soil was the only statistically significant factor ($p < 0.001$) in determining the zinc and copper adsorptive response. The adsorption data did not fit the Langmuir model as well as the Freundlich model.

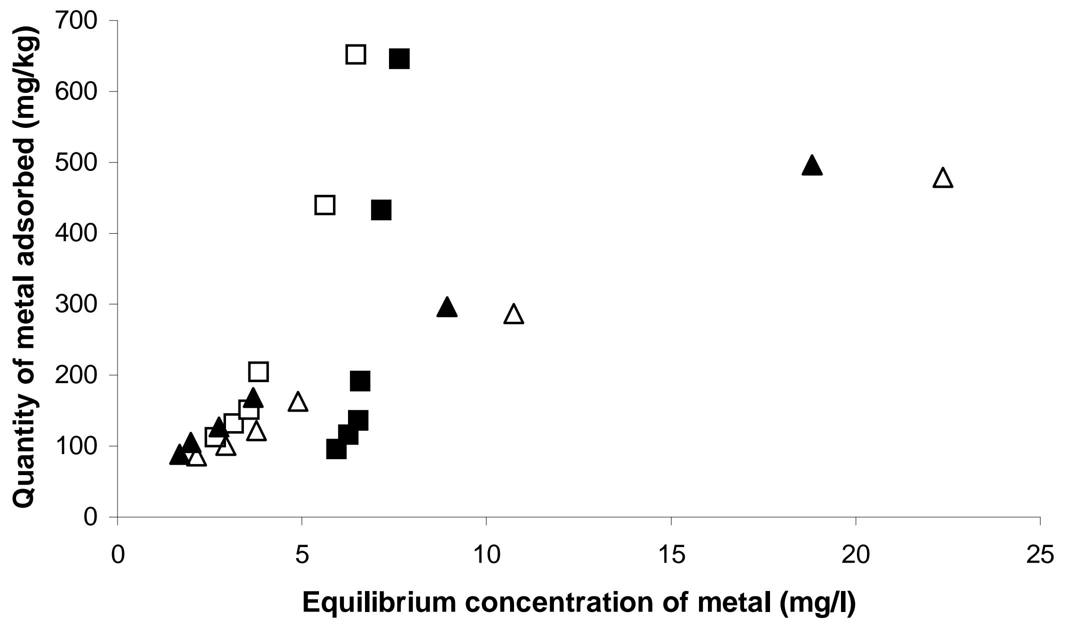


Figure 3.3 **Adsorption isotherms of copper (■) and zinc (▲) from mixed metal solutions maintained at a 1:1 ratio over a defined range of concentrations. Open symbols and closed symbols represent untreated and gamma-radiation treated soils respectively.**

The dual presence of copper and zinc in a landfill leachate reflects a competitive relationship for adsorption sites in the soil since the nett concentrations of copper and zinc adsorbed to the soil (both treatments) in the mono-component systems are far greater than that observed in the dual-metal adsorption systems. This is in agreement with Arias *et.al*. (2005b) and Markiewicz-Patkowska, Hurthouse and Przybyla-Kij, (2005).The preference for copper adsorption over zinc in the untreated Hutton soil is in contrast to that observed in the sterilized soil. Such behaviour could be attributed to the biological component present in the untreated soil. However, one must be cautious when making such a statement since, according to Langmuir (1996) (cited by Markiewicz-Patkowska, *et al.,* 2005); the adsorptive behaviour of metals in soil is dependent on numerous factors. In this instance all of these factors were controlled within specific limits. Knowledge of the adsorptive behaviour of single and multi-metal leachates in soil devoid of microbial communities could provide useful information geared towards assessing the effect biological components have on attenuation / migration of these compounds into groundwater as well as other natural resources.

3.4 Conclusion

The primary objectives in this study were to assess the existing concentrations of phenol, copper, and zinc present in Hutton soil and to evaluate the adsorptive capacity of this soil for the same compounds. With this in mind the experimental evidence suggests that the following conclusions can be drawn:

- The presence of phenol, copper, and zinc in the undisturbed Hutton soil was indicative of the fact that the resident autochthonous microorganisms would have had prior exposure to such compounds. Although, the compounds were present in relatively low concentrations in comparison to those encountered in polluted soils, previous authors have emphasised the importance of the pre-exposure of microorganisms to organic compounds (Guerin, 1999), and heavy metals (Gadd, 1992; Costly and Wallis, 2001) prior to the successful attenuation of these toxic compounds.

- The average distribution quotients (K_d), indicated that the total amount of phenol adsorbed by the gamma-radiated soil ($K_{dg} = 1.43\ l.kg^{-1}$) was 1.5 times less than that adsorbed by the untreated soil ($K_{du} = 2.17\ l.kg^{-1}$) suggesting that the biological component of the soil plays an important role in phenol attenuation.

- In single metal adsorption systems, the gamma-radiated soil displayed a preference for the adsorption of zinc over copper. However, the association was reversed when the soil was left untreated, meaning that the adsorption of copper was favoured over zinc. Here again, the role of the biological components of the soil in determining the behaviour of the heavy metals in soil is highlighted.

- In dual-metal adsorption systems, the metal-soil interactions were the same as that observed for the single-metal systems. However, the dual-metal system highlighted the competitive adsorption of copper over zinc in the untreated soil, and to an extent in the gamma-radiated soil treatment. The gamma-radiated soil still showed a preference for zinc adsorption in the dual-metal system, but the difference between copper and

zinc adsorption was 50 % less than that observed in the single-metal systems. This suggests that the biological component of soil plays a significant role in determining the competitive adsorptive behaviour of copper and zinc in the Hutton soil. The favoured attenuation of copper over zinc in the untreated soil suggests that there would be a greater potential for zinc pollution of receiving groundwater over copper in cases of zinc and copper co-contamination of the subsurface.

Chapter Four

4. Investigating the Fate of a Synthetic Landfill Leachate Perfused Through Sequential Soil Microcosms at Two Hydraulic Loading Rates (HLRs)

4.1 Experimental

4.1.1 Construction and operation of sequential soil microcosms

Four glass columns were arranged in series with gas outlets linked to a common gas trap containing 0.1 % (m/v) zinc acetate (Figure 4.1). Hydrogen sulphide production was detected as an insoluble zinc sulphide precipitate. A total of sixteen arrays were assembled in two categories, each consisting of eight replicate arrays. The average effective pore volume for each array was 303.68 m*l* per net soil column length of 40 cm. Over a 32 week period arrays were perfused with synthetic leachate A at either a high hydraulic loading rate (HLR*h* - arrays A*h*; B*h*; C*h*; D*h*) of 20 m*l* or a low hydraulic loading rate (HLR*l* - arrays A*l*; B*l*; C*l*; D*l*) of 10 m*l* every five days. Selected arrays were destructively sampled, in duplicate for each HLR, at week 12 (A*h* and A*l*) and at week 32 (B*h* and B*l*). From weeks 36 – 80 the remaining arrays were perfused with leachate A-mn. Destructive samplings of arrays C*h* and C*l* took place at week 52 followed by the final sampling at week 80 (D*h* and D*l*). The total Bacterial populations of all the soil samples of the respective destructively sampled arrays were profiled by DNA isolation in association with PCR-DGGE. These results appear in Chapter Six.

A temperature range of 17 ºC to 22 ºC was maintained throughout the experiment (VanGulck and Rowe, 2004). The microcosms were pre-flushed with oxygen-free nitrogen (OFN) (Fedgas, South Africa) before sealing with industrial strength marine silicone sealant (Bostik®, Bostik Ltd, England) so as to minimize atmospheric interference with the internal environment of the microcosms. Prior to sampling, with a 10 m*l* gas-tight glass syringe (SGE International, Australia), all the collection vessels were over-gassed with oxygen-free nitrogen (OFN) (Fedgas, South Africa). Initially, leachate samples were taken fortnightly for the first month (weeks 2 and 4), thereafter sampling was undertaken on a monthly basis. Redox

measurements were made immediately after sampling prior to leachate samples being stored at 4EC for further analysis.

4.1.2 Statistical analysis

Regression analyses were carried out using GenStat Release 8.1 statistical software. Polynomial and Gompertz regression functions were used to model the fate of leachate constituents; phenol, copper, zinc, nitrate, and sulphate along with the chemical parameters; redox and pH, over time at the two different hydraulic loading rates. In addition, the regression functions facilitated the statistical differentiation of the two hydraulic loading rates.

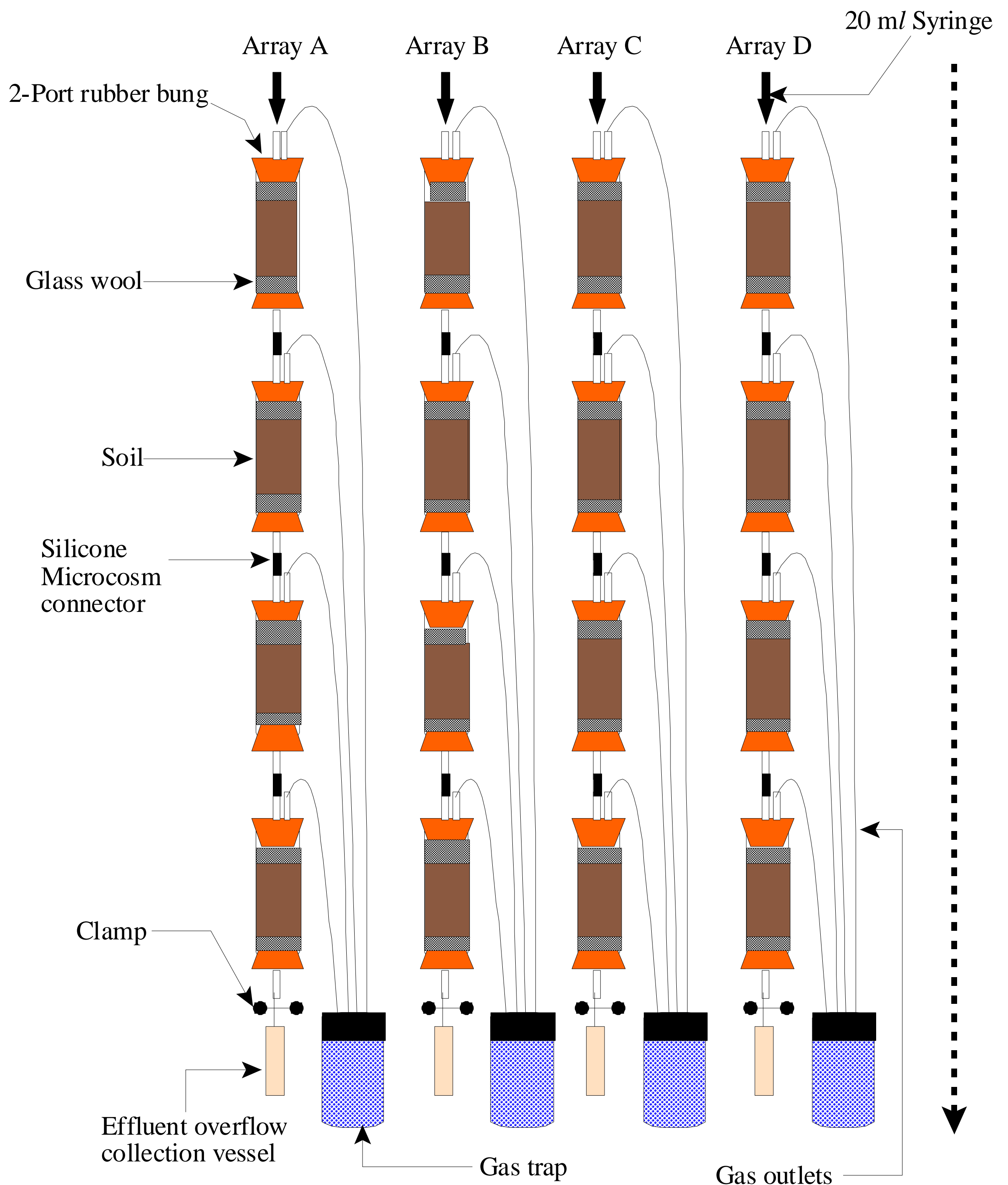


Figure 4.1 **Schematic diagram representing arrays of soil microcosms perfused with a synthetic landfill leachate at a High (HLR*h*) or Low (HLR*l*) hydraulic loading rate to investigate the fate of chemical constituents of the leachate.**

4.2 Results and Discussion

Laboratory-scale soil microcosms were used to evaluate the fate of selected constituents and chemical parameters of a young, synthetic, acetogenic phase landfill leachate perfused at two HLRs so as to mimic the associated leachate / soil interactions occurring beneath a landfill. The results from duplicate arrays for each HLR, are presented as mean values of duplicate arrays in all Figures. Furthermore, only data from duplicate arrays of D*h* and D*l* are presented since leaching in these arrays continued over the full 80 weeks. The data from arrays A, B and C were used to decide when to destructively sample an array over the course of the investigation period. These arrays were used to measure and assess the timeline of microbial succession with respect to microbial diversity, numerical dominance, and evenness of distribution (Chapter Six). The results for leachate constituents' phenol, copper, zinc, nitrates, and sulphates are expressed as relative concentration ratios (c/c_o), where c and c_o are the residual (outflow) and initial (inflow) concentrations (mg.l^{-1}), respectively.

4.2.1 pH

The pH of the effluent leachate for both HLRs showed a gradual increase from the initial pH 5.0 throughout the investigation (Figure 4.2). The pH never dropped below 5.9 and by the end of the investigation had increased to 7.4 and 6.9 for arrays D*h* and D*l*, respectively.

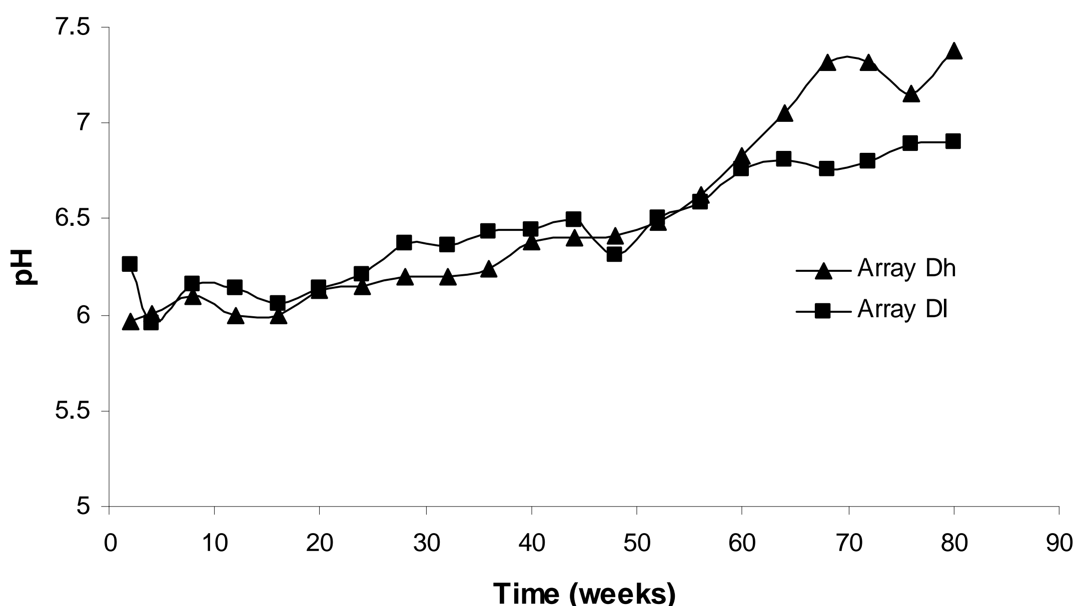


Figure 4.2 **Transformation of the pH of landfill leachate after leaching through soil microcosms at two different hydraulic loading rates (HLRs) over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days.**

At the destructive sampling times 12, 32, and 52 weeks the pH of effluent leachate from array D*l* was consistently higher but similar to that of array D*h*. From week 60 pH of effluent leachate from array D*h* increased above that recorded for array D*l* where a marked increase in pH, from 6.7 to 7.3 during week 60 to 80, was recorded. Over the same period array D*l* reflected a minimal pH increase from 6.7 to 6.8. The greater hydraulic loading of array D*h* may have contributed to a more rapid onset of anaerobic conditions within the microcosm and effectively facilitate an increase in the pH. The elevated pH could be a consequence of the degradation of phenol to organic carbon by microorganisms leading to an increase in environmental pH (Cozzarelli *et al.*, 2000; Vanbroekhoven, Van Roy, Gielen, Maesen, Ryngaert, Diels, Seuntjens, 2007). Moreover, the presence of sulphate coupled with reduced redox conditions and increasing pH can lead to the formation of sulphides (evident in the zinc acetate gas traps as a white precipitate) which would result in the scavenging of H^+ and heavy metals such as zinc and copper.

Differences in the rate of change in pH between the two treatments are more evident towards the latter stages of the investigation (weeks 60 – 80). This was attributed to the higher

concentrations of phenol (within non-toxic concentration limits) available for microbial metabolism in array D*h* that could have promoted increased oxygen consumption. This could have lead to an earlier onset of anaerobiosis and the subsequent increase in pH as a consequence of the removal of H^+ from solution during the reduction reactions in the absence of free oxygen (Smith *at. al.*, 1999b) coupled with the release of OH^{-1} during phenol degradation. Consequently, when one considers the redox (4.2.2) relationship over time for both treatments it is clear that the redox state of array D*h* stabilized at 60 weeks whereas the redox potential in array D*l* increased. Furthermore, the redox state of array D*h* showed a constant decline over time as apposed to the fluctuating pattern evident in array D*l*. Smith and her colleagues (1999b) concluded that the limited supply of organics required for microbial metabolism can contribute to changes in the redox state and a consequent delay in pH increase of a micro-environment.

Although there were relatively small differences between the pH recorded for both treatments, the regression analysis of pH against time reflected two significantly different equations for both treatments (Table 4.2). The data reflected an adequate fit to the Gompertz response function for both treatments after grouping (R^2 = 95.7 %). The equations revealed a superior rate of change in pH (0.06685) for array D*h* for the duration of the investigation. The equations further revealed that the major change in pH occurred towards the latter stages of the experiment i.e. week 56 (56.6) and 60 (61.39) for arrays D*l* and D*h*, respectively. The timing of these pH changes coincided with the lowest redox states recorded for both treatments. Although Hoeks and Borst (1982) concluded that the optimum pH for methanogenesis was between 6.5 and 7.0 in soil below landfills, methane production was never detected.

Table 4.1 Regression response functions of pH (*y*) on time (*x*) after grouping of treatments

$$y_{Dh} = 7.3894 - 1.389 \exp[-\exp(0.06685(x - 61.39))]$$

$$y_{Dl} = 7.1130 - 1.327 \exp[-\exp(0.02750(x - 56.6))]$$

4.2.2 Redox potential (E_h)

The redox potential of effluent leachate for both treatments over the course of the experiment is shown in Figure 4.4. At the first sampling interval (week 2) an average E_h of 672 mV was recorded for array D*h* whereas for array D*l* an E_h of 23 mV was recorded. The Eh of effluent leachate from array D*h* decreased over the course of the study, reaching negative E_h conditions at week 26, stabilizing at approximately E_h -240mV from week 52 onward. In contrast, the E_h of the effluent leachate from array D*l* increased for the first 24 weeks, reaching a maximum E_h of +220 mV before dropping down to negative E_h values. From weeks 44 to 68 the redox states of both treatments were comparable. Thereafter, divergent paths were observed for arrays D*l* and D*h* with the former becoming less anaerobic while the latter stabilized between -210 mV and -240 mV, this occurs largely as a consequence of microbial metabolism which influences the redox cascade. This cascade generally begins with microorganisms utilizing aerobic respiration followed by those microorganisms that employ denitrification (nitrate reduction) as part of their metabolic pathways; this in turn leads to microbes using manganese and iron as terminal electron acceptors respectively. Depletion of these heavy metals facilitates the use of sulphate as the next terminal electron acceptor in the redox cascade. In other words, all microbes capable of using sulphate as a terminal electron acceptor during metabolism would thrive and out-compete non-sulphate reducing microorganisms (Lensing *et al.*, 1994). The decrease in E_h occurs as a consequence of the availability of the different electron acceptors over time and space. Depletion of oxygen necessitates a switch to the next available terminal electron acceptor (nitrates), and this sequence of reactions continues until methanogenesis persists. Different electron acceptors yield different amounts of energy during microbial metabolism. The energy yield generally decreases as metabolically able microbes' progress from oxygen through to sulphate as the terminal electron acceptor. The continuous acceptance of electrons by these terminal electron acceptors along the redox cascade ensures a continuous decrease in electrical charge along the redox cascade i.e. from positive E_h, associated with oxygen as the terminal electron acceptor, to negative E_h associated with sulphate acting as the terminal acceptor. Sulphate reduction activity was confirmed in each array by the deposition of metal sulphide in the gas traps connected to the soil arrays. The values never decreased below this and perhaps this explains

the failure to mimic complete methanogenic conditions, indicated by the recorded redox states of the emerging effluent and the lack of methane production. Among other factors such as pH and temperature, sulphate is a significant factor that influences redox states in subsurface environments (Beeman and Sulfita, 1990). Sulphate levels recorded for the soil arrays of both treatments remained elevated for the majority of the investigation (4.2.6), and only begin to recede after weeks 40 (array D*h*) and 48 (array D*l*), coinciding with the establishment of sulphate reducing potentials, particularly in array D*h* (Figure 4.4). The composition of landfill leachate frequently includes sulphates (Christensen *et al.*, 1994), and to this end Lovely and Klug (1983) concluded that this constituent contributes significantly to halting progression to methanogenesis. Ehlers (1999) achieved sulphate reducing conditions during his assessment of dual co-disposal of activated sewage sludge plus phenol with refuse but failed to mimic methanogenesis. He concluded that the continued presence of sulphate as an available electron acceptor, in the presence of a hypothetically common substrate, prevented effective competition from methanogens by virtue of metabolic energy yield-available substrate dynamics. Christensen *et al.* (2001) concluded that the limited time associated with laboratory experiments of this nature made it difficult for the development of an undisturebed and stable redox environment.

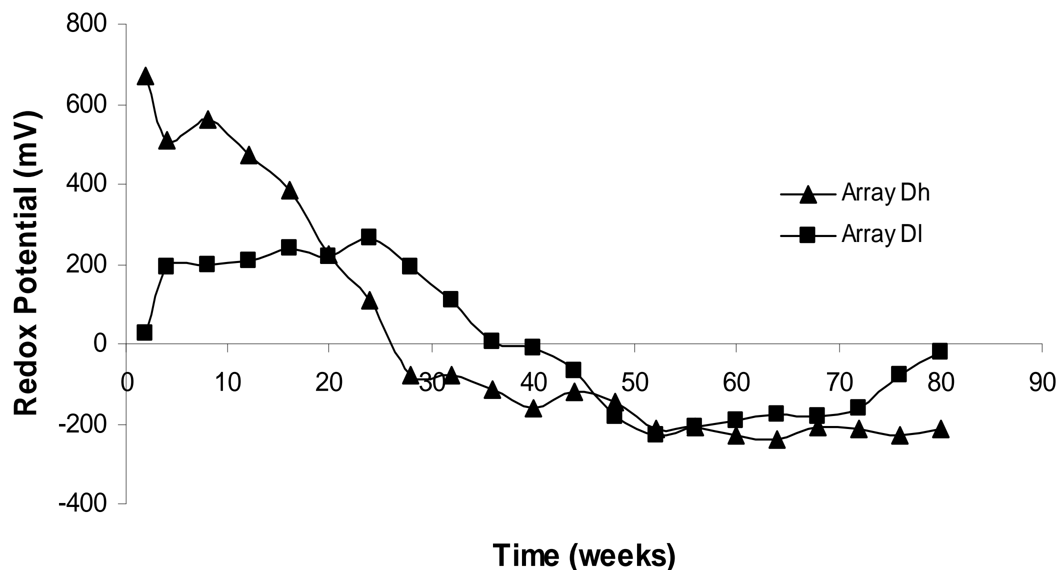


Figure 4.3 **Transformation of the redox potential of effluent synthetic landfill leachate after leaching through soil microcosms at two different hydraulic loading rates (HLRs) over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days.**

Table 4.2 Regression response functions of Redox Potential (*y*) on time (*x*) after grouping of treatments

$$y_{Dh} = 595.1 - 808.9 \exp[-\exp(-0.1190(x - 17.713))]$$

$$y_{Dl} = 192.0 - 348.6 \exp[-\exp(-0.1982(x - 34.40))]$$

Regression analysis accounted for 95.2 % of the variation when the redox data for both treatments were grouped and regressed against time. Table 4.3 contains two significantly different regression equations generated by the Gompertz model. The rate of change in E_h for array D*l* (0.1982) was higher than that observed for array D*h* (0.1190). However one has to consider that there was a steady drop in E_h recorded for array D*h* throughout the investigation until stabilization from week 52 while the E_h state of array D*l* was punctuated by periods of fluctuations (Figure 4.4). The Gompertz model further illustrates that the chief sequence of reduction was triggered at week 17 (17.713) and week 34 (34.40) for arrays D*h* and D*l*, respectively (Table 4.3). This indicated a lag of 17 weeks before reduction was effectively triggered in array D*l*.

The actual redox conditions prevalent in each soil microcosm will play a significant role in determining the microbial populations present (Williams and Higgo, 1994). The survival and proliferation of these populations will in turn depend on their ability to utilize the organic and inorganic constituents introduced to the microenvironment. The governing redox processes play a pivotal role in determining the level of toxicity posed by the organic and heavy metal constituents of the landfill leachate on the microbial populations (Lensing *et al.*, 1994). Hence, this "one-dimensional" approach attempts to define the prevalent Bacterial populations at various redox states achieved in the soil microcosms perfused with leachate at the two HLR investigated.

4.2.3 Phenol

The results for the attenuation of the phenol component of the landfill leachate are presented in Figure 4.4. Over the first twelve weeks of the investigation all the arrays of the two treatments, HLR*l* and HLR*h*, reflected a 95 – 99 % and 67 – 82 % reduction in phenol, respectively. At 32 weeks the arrays perfused at HLR*l* continued to reflect a higher capacity for phenol attenuation with 67 – 79 % as apposed to 52 – 65 % by the arrays perfused at HLR*h*. However, the subsequent supplementation of vitamin and trace minerals to the synthetic leachate at week 36 coincided with there being a similar capacity for phenol attenuation at week 44 for each of the HLRs under investigation. From week 68 the rate of phenol attenuation leveled off for both treatments (45 – 48 %). The lag in phenol attenuation demonstrated between the two treatments after the first 40 weeks could be a consequence of the different redox potentials (4.2.2) evident in the respective soil arrays which were in turn a consequence of the difference in hydraulic loading. Arrays perfused at HLR*h* often resulted in water-logging, rendering them anoxic or anaerobic sooner than arrays perfused at HLR*l*. This would facilitate a more rapid degradation of phenol in aerobic conditions as apposed to anoxic or anaerobic conditions. Various reports in the literature indicate that naturally occurring soil microbes are capable of degrading phenol over a vast concentration range. However, the rate of phenol degradation depends on the redox state of a system, with estimated half-life phenol degradation ranging over a few days under aerobic conditions to several weeks under anaerobic conditions (Lerner, Thornton, Spence, Banwart, Botrell, Higgo, Mallinson, Pickup and Williams, 2000; Shibata, Inoue, and Katayama, 2006). Phenol degradation can proceed without bio-augmentation, however, suitable stimulation of metabolic conditions have been shown to increase degradation rates (Guerin, 1999).

In the phenol sorption investigation previously described (3.3.2.1), results showed that the phenol attenuation by the Hutton soil was low [12 – 17 % (m/m)]. Non-sterile soil was found to have a greater phenol attenuation capacity [16.95 % (m/m)] than sterile soil [12.39 % (m/m)] which supports the assertion that naturally occurring microorganisms play a role in phenol attenuation/degradation. The results from this investigation reflect a greater capacity for phenol attenuation under both HLR with the relative phenol concentrations never

reaching unity. Failure to achieve breakthrough at the two HLR investigated is not uncommon. Kjeldson *et al.* (1990) found that a range of chlorophenols and nitrophenols were firmly retarded by two subsurface soils and showed no breakthrough during the investigation period. Research into a landfill leachate plume in Vejen, Denmark found that phenol disappearance from the leaching waters was due to biological degradation (Baun, Reitzel, Ledin, Christensen, Bjerg, 2003). This assumption is further supported by Smith and Novak (1987) who concluded that the attenuation of phenol in subsurface soils was attributed entirely to biodegradation.

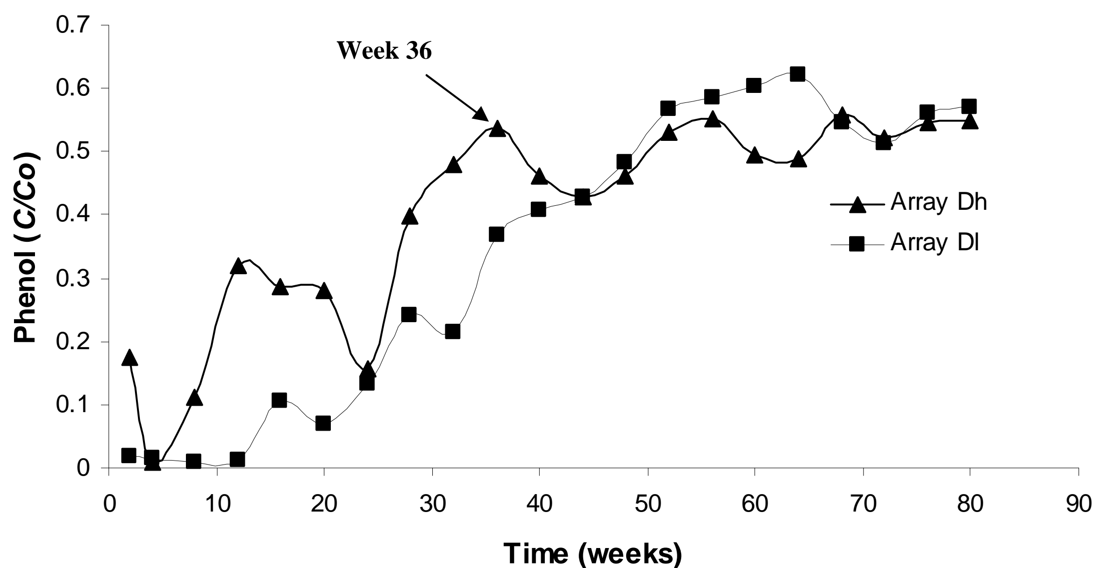


Figure 4.4 **Relative concentration ratios of phenol (*c/c_o*) in landfill leachate after leaching through soil microcosms at two different hydraulic loading rates (HLRs) over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days. Where, *c* and *c_o* are the outflow and inflow concentrations (mg.l^{-1}) in the leachate, respectively.**

Regression analysis of relative phenol concentration against time reflected an adequate fit of the data to cubic response functions for both treatments after grouping (R^2 = 95.8 %). The two response functions (Table 4.1) were significantly different at the linear, quadratic and cubic level but there was no significant difference evident between the intercepts.

Table 4.3 **Regression response functions of relative phenol (*y*) concentration on time (*x*) after grouping of treatments**

$$y_{Dh} = -0.0746 + 0.03011x - 0.0005297\,x^2 + 0.000003162x^3$$

$$y_{Dl} = 0.0099 - 0.00299x + 0.0004993\,x^2 - 0.000004806x^3$$

The regressions response functions reflect a difference in the behavior of the phenol component of leachate when the soil microcosms are leached at the two HLR under investigation. The initial rate of phenol attenuation in array D*h* was lower than that in array D*l* over the first 40 weeks, thereafter array D*h* consistently attenuated more phenol up until 68 weeks. The initial difference in attenuation could be a consequence of the adaptive response of resident microbial populations to different HLRs and hence phenol concentrations in the respective arrays. The different HLRs, and therefore different phenol concentrations, influence the time for acclimation of the micro-biota to the leachate components in addition to altering the internal chemistry of the arrays thereby influencing the rates of phenol attenuation/degradation. The results indicate that the higher HLR delivers a greater phenol shock load to the arrays and this in turn triggers a greater time lag before phenol metabolism can resemble those removal efficiencies produced by arrays receiving a lower concentration of phenol (HLR*l*). When populations of heterogeneous microorganisms, acclimated to phenol in a system of anaerobic-anoxic-aerobic reactors, were exposed to phenol shock loads they initially lost their phenol removal capacities before establishing new phenol removal efficiencies (Chakraborty and Veeramani, 2005). After all, the development of different redox conditions in leachate plumes depends largely on the degradation of organic compounds, in this case phenol, entering that plume (van Breukelan, 2003); similarly, the prevalent redox zonation will determine the rate of pollutant attenuation by the soil (Lønborg, Engesgaard, Bjerg and Rosbjerg, 2006). Furthermore, the rates of organic compound degradation in the leachate plume is not only dependant on the presence/absence of specific electron acceptors/donors but is also dependant on the diversity of microorganisms present (van Breukelan, 2003). Shibata *et al.* (2006) found that aerobic microbial degradation of phenols and associated derivatives was much faster than its anaerobic counterparts. Therefore it is not surprising that the rate of phenol degradation for both treatments decreases as the

microenvironment of the soil arrays becomes more anaerobic (4.2.2). This is mirrored by the decrease in the concentrations of preferred electron acceptors, starting with oxygen through to nitrates, and followed by sulphates.

4.2.4 Copper and zinc

The cation exchange capacity of the Hutton soil used in the arrays was high enough to account for the attenuation of all the copper and zinc leached through the soil for both HLRs. The total charge of exchange sites available for cation exchange amounted to 19.616 $mmol_c$ per soil array. When one considers that the total ionic charge of the copper and zinc in the synthetic leachate was 0.239 $mmol_c$ per pore volume and 0.9288 $mmol_c$ per pore volume, respectively; the assumption made was that the CEC of the soil was sufficient to attenuate all of the copper and zinc contained in the leachate. Furthermore, if copper was the only constituent of the leachate and the CEC was the exclusive mechanism of copper attenuation, then each soil array would become saturated with copper after 82 pore volumes (or 889 weeks) for array D*h* and 164 pore volumes (or 1778 weeks) for array D*l*. Similarly, breakthrough for zinc would occur only after 21 (or 227 weeks) and 42 (or 454 weeks) pore volumes for arrays D*h* and D*l*, respectively. When one considers the dual-metal system, used in the synthetic leachate, saturation was calculated to occur after 16 (or 172 weeks) and 32 (or 346 weeks) pore volumes for arrays D*h* and D*l*, respectively. It must be emphasized that these calculations and the assumptions drawn from them are based entirely on single-metal and multi-metal solutions, whereas the synthetic leachate was a multi-constituent system. The results presented here are consistent with the trends documented in the metal adsorption study discussed in Chapter 3 (3.2.2) which shows the competitive nature of metal adsorption.

Analysis of the leaching of copper as a function of time (Figure 4.5a) showed that the relative concentration of copper present in the effluent for both treatments was extremely low. The assumption is that the majority of the copper that was introduced via the leachate was attenuated by the chemical and biological character of the soil. The inability to achieve breakthrough over 80 weeks at the investigated HLRs lends a degree of credence to the earlier calculations regarding the CEC of the soil relative to the total ionic charge of the metal.

However, there is a difference in the behavior of copper over the two HLRs that is evident in Figure 4.5a. After 54 weeks there was a spike in the relative concentration of copper for array D*h* followed by a decrease. There was little change evident in array D*l*. At 56 weeks the pH of the effluent recorded for array D*h* was 6.481 and continued to increase to 7.375 (Figure 4.3). The initial increase could be attributed to the adsorptive behavior of the soil since the relative concentration of copper would increase as more copper was introduced to the system until saturation of all the adsorptive sites on the soil occurred (C/Co = 1), which in this case was not achieved.

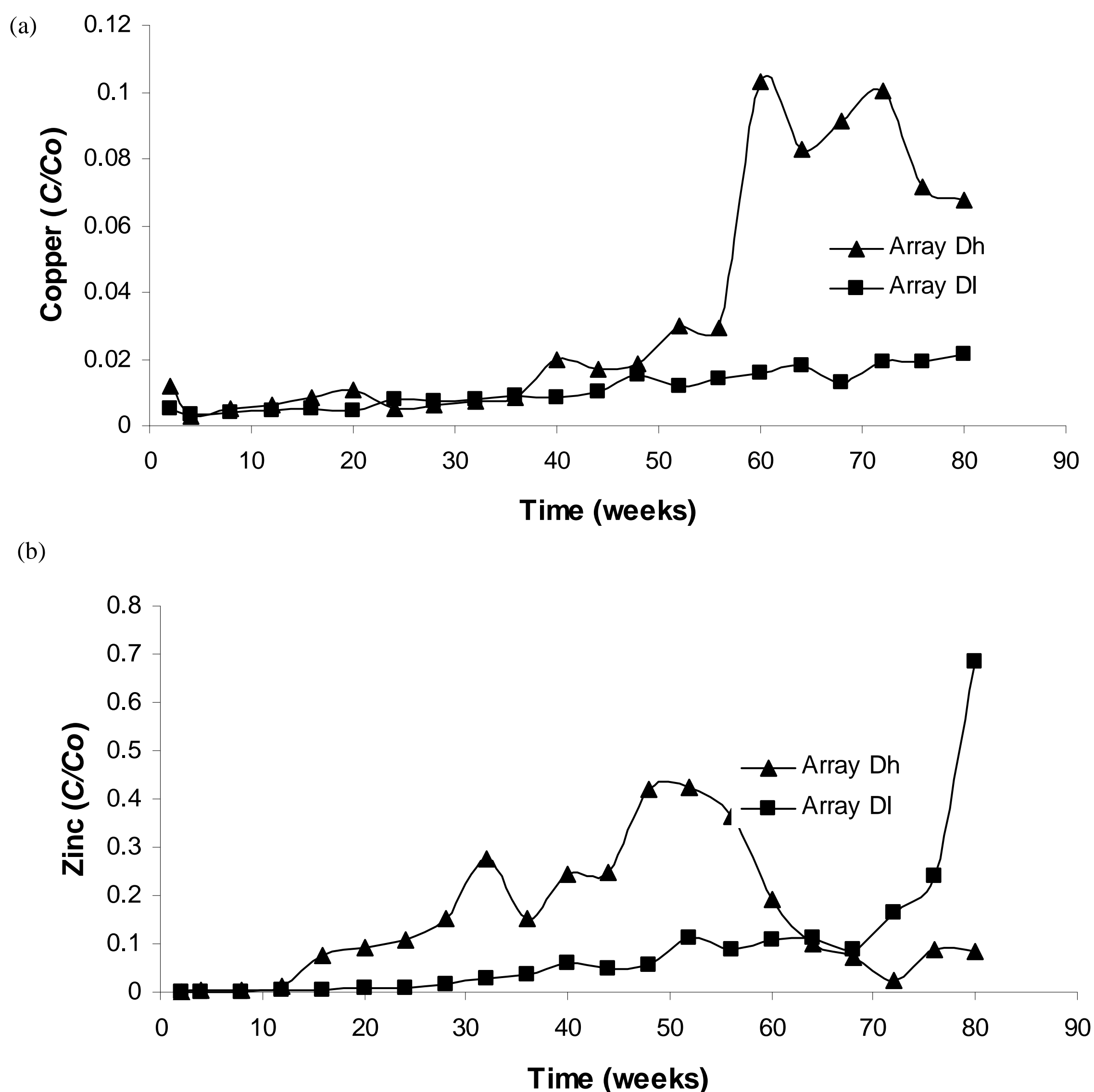


Figure 4.5 **The relative concentrations (*C/Co*) of (a) copper and (b) zinc in the synthetic leachate after leaching through soil microcosms at two different hydraulic loading rates (HLRs) over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days. Where, *c* and c_o are the outflow and inflow concentrations (mg.l^{-1}) in the leachate, respectively.**

The pH of the leachate plays a significant role in determining the mobility of copper. Markiewicz-Patkowska, Hursthouse and Przybyla-Kij (2005) discovered that at near neutral pH the solubility of most heavy metals was severely restricted by numerous physico-chemical interactions. The subsequent decrease in *C/Co* of copper could be a result of precipitation

which in turn could lead to an overestimation of the adsorptive capacity of the soil. Conditions in both soil microcosms become progressively anaerobic as indicated by the drop in redox potential of the leachate (Figure 4.3); this in itself can contribute to increased retardation of the heavy metals in the leachate by way of sorption and precipitation (Williams and Higgo, 1994) which is a common occurrence during the biological reduction of sulphate to sulphide and the subsequent reaction of metal ions to form metal sulphide precipitates (Hoa, Liamleam and Annachhatre, 2007).

The first 12 weeks demonstrated maximum absorption of zinc by the soil over both HLRs (Figure 4.5b). Thereafter (weeks 20 to 60), there was a noticeable decrease in zinc adsorption observed for array D*h* relative to array D*l*. Premature release of zinc from the soil binding sites could be attributed to the sharp decrease in the redox state observed for array D*h* (Figure 4.3). Under partially anaerobic conditions zinc tends to form oxides more readily with Fe and Mn and can subsequently become electron acceptors that have increased mobility. Copper forms stable complexes with organic matter more freely than reduced copper oxides under similar conditions (Ramos, Hernandez and Gonzalez, 1994). However, between weeks 52 and 72 the adsorption of zinc in array D*h* showed a marked increase (42 % overall) such that 98 % of the zinc from the leachate was adsorbed. This increase in adsorption coincided with an effluent pH of 7.0. The assumption made is that the increase in effluent pH resulted in the immobilization of the zinc by precipitation (Smith *et al.*, 1999b; Ming-Kiu, Zhen-Li, Calvert, and Stoffella, 2005). In contrast, array D*l* reflected a 59 % decrease in adsorption from weeks 68-80. The pH of array D*l* never exceeded 7.0, the threshold pH required for the precipitation of zinc from the effluent (Smith *et al.*, 1999b). This, in conjunction with phenol concentrations that were higher in the effluent of array D*l* relative to array D*h*, could have effected the formation of zinc-phenol complexes thereby increasing the mobility of the metal (Smith, Sacks and Senior, 1999a).

The difference between the two HLR treatments was further emphasized by regressing the *C/Co* of each metal against time. The data displayed an adequate fit to two significantly different cubic and quadratic response functions for copper ($R^2 = 90.9$ %) and zinc ($R^2 = 83.5$ %), respectively (Table 4.4). The regressions response functions reflect a

difference in the behavior of each metal when the soil microcosms are leached at the two HLR under investigation. This was expected since the adsorption of metals by soil is governed, amongst numerous other factors, by the amount of metal applied to the soil in relation to the CEC of that soil. As the amount of metal applied to the soil increases, the total amount of metal adsorbed increases but the percentage of metal adsorbed over time decreases as the adsorption sites on the soil becomes saturated (Markiewicz-Patkowska *et al.*, 2005).

The soil microcosms reflected a higher capacity for the attenuation of copper than zinc at the investigated concentrations and HLRs. This implies that should landfill leachate containing zinc and copper penetrate the bottom of a landfill, there would be a greater potential for attenuating copper than zinc. The result was consistent with earlier investigations (Chapter 3) detailing the attenuation of metals by "sterile" and "non-sterile" soils as well as research carried out by Agbenin and Olojo (2004). Over the course of the investigation the accumulation of heavy metals in the soil can have a toxic effect on the resident microorganisms in the soil thereby affecting the diversity and evenness of microbial distribution in the soil.

Table 4.4 Distinct regression response functions of relative metal concentration (y) on time (x) after grouping of treatments according to hydraulic loading

Metal	Regression Equation
Copper	$y_{Dh} = 0.1121 - 0.00616x + 0.0002214\, x^2 - 0.000001439x^3$
	$y_{Dl} = 0.0628 + 0.00026x + 0.000022\, x^2 - 0.000000158x^3$
Zinc	$y_{Dh} = -0.1032 + 0.02824x - 0.0003090\, x^2$
	$y_{Dl} = -0.0074 + 0.00453x + 0.0000191\, x^2$

4.2.5 Nitrate

Over the first 52 weeks the relative concentration ratio of nitrate for each treatment reflected significantly divergent patterns (Figure 4.6). The c/c_o of nitrate in array D*h* was close to zero for the duration of the investigation whereas, array D*l* exhibited elevated c/c_o nitrate, reaching a peak at week 20. This suggests that nitrate removal in array D*l* between weeks 4 and 28 was at its lowest and this coincided with the elevated redox states recorded for array D*l* over the same time scale (Figure 4.4). The gradual decrease in the nitrate concentration in the effluent is indicative of continued nitrate reduction in array D*l* and is comparable with the gradual decrease in the redox state documented for the array over this period i.e. a gradual shift from nitrate reduction to other redox states downstream in the redox cascade. In contrast nitrate levels for array D*h* registered close to zero after 20 weeks. The redox state of the array constantly decreased, with the redox state dropping below the nitrate reducing potentials (below 200 mV) after 20 weeks and below zero after 24 weeks. The lower rate of nitrate depletion coupled with the higher rate of phenol degradation recorded for array D*l* point towards there being a greater period of aerobic and nitrate reducing activity over the first 40 weeks, when compared to array D*h*. When Pedersen, Bjerg and Christensen (1991) correlated nitrate profiles with groundwater and sediment characteristics in a sandy aquifer, they observed significant removal of nitrates in the unsaturated zones above the aquifer water table as a consequence of dentrification in anaerobic microenvironments. Nitrate is a mobile ion in solution and its fate in the soil arrays can primarily be attributed to conversion to other nitrogenous forms, such as microbial biomass, or attenuation by the soil (Ding, Zhang and Cheng, 2001).

The low levels of nitrates available for electron transfer during microbial processes coupled with the decreasing redox potential of the environment point towards the shift in the redox state of the array from a nitrate > iron/manganese > sulphate reducing conditions. The inference made by the previous statement is one of sequential redox activity generating genuinely bordered redox zones within a plume, however, such reactions occur simultaneously and the different redox zones often overlap each other as the plume expands such that areas of denitrification often incorporate iron and sulphate reduction (Scholl, Cozzarelli, and

Christenson, 2006). These redox zones incorporate diverse groups of bacteria in the same area some of which are active while others remain dormant until conditions become suitable for active metabolism (Christenson et al., 2001).

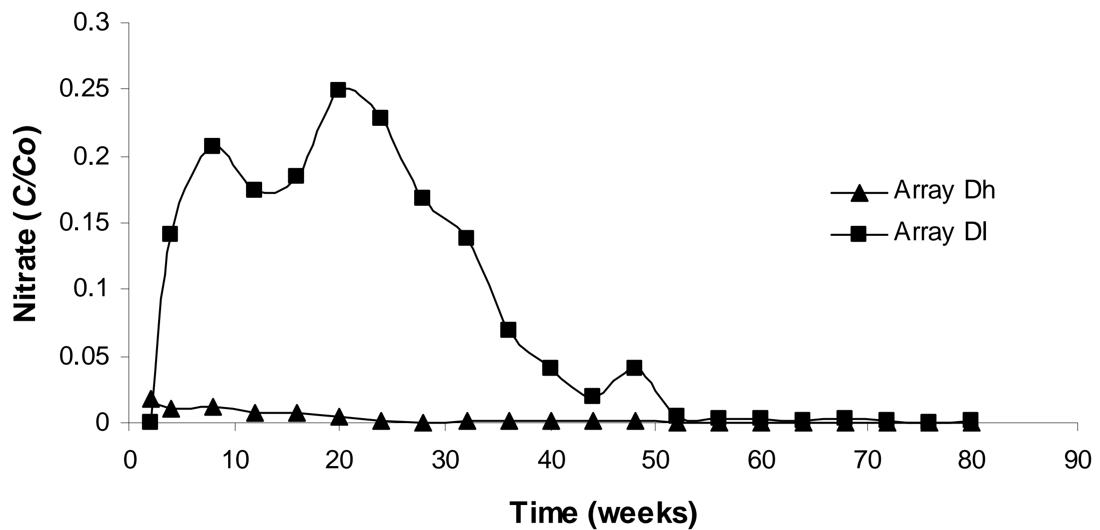


Figure 4.6 **Relative concentration of nitrate (c/c_o) in landfill leachate after leaching through soil microcosms at two different hydraulic loading rates (HLRs) over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days. Where, *c* and c_o are the outflow and inflow concentrations ($mg.l^{-1}$) in the leachate, respectively.**

Regression of c/c_o inorganic nitrate on time produced an adequate fit of the data to the Gompertz model ($R^2 = 97.4$ %). The two significantly different equations generated by the model after grouping of the two treatments are presented in Table 4.5. The superior rate of change parameter derived from equation y_{Dl} for array D*l* (0.1270) points to the accentuated change of inorganic nitrate over time brought on by an initial reserve of nitrate followed by a rapid reduction over a shorter time. The rate of change in nitrate observed in array D*h* (0.1232) is a product of instant nitrate depletion characterized by minimal changes from zero over the duration of the investigation, irrespective of further nitrate addition to the system.

Table 4.5 **Regression response functions of realative inorganic nitrate concentration (*y*) on time (*x*) after grouping of treatments**

$$y_{Dh} = 0.1258 - 0.1040 \exp[-\exp(-0.1232(x - 12.94))]$$

$$y_{Dl} = 0.4514 - 0.4150 \exp[-\exp(-0.1270(x - 35.612))]$$

4.2.6 Sulphate

The fate of sulphate within the sequential soil microcosms is shown in Figure 4.7. Over the first 36 weeks both treatments displayed similar trends of attenuation / release of sulphate from the synthetic leachate (Figure 4.7). Interestingly both treatments reached unity at 24 (array D*l*) and 28 weeks (array D*h*), with the concentration of sulphate being released from array D*h* doubling after 40 weeks. Pedersen *et al.*(1991) reported leaching sulphate concentrations that were in excess of estimated potential sulphate concentrations entering a shallow sandy aquifer via the unsaturated sediment zone above the water table. They concluded that oxidation of reduced sulfur compounds can take place in microenvironments conducive to oxidation resulting in the release of added sulphate into the aquifer. However, there was a steady decrease in c/c_o sulphate recorded for array D*h* from 40 to 80 weeks. This could be a consequence of the stable reduced redox state of the array over the same period (Figure 4.4), and brought about in part by the depletion of the nitrates as electron acceptors, resulting in the consumption of the next available electron acceptor i.e SO_4. Interestingly, zinc adsorption (Figure 4.5b) over the same period increased for array D*h*, possibly reflecting the precipitation of zinc as a sulfide from the effluent leachate during the predominant sulphate reducing environment. Erses and Onay (2003) reported significant decreases in the heavy metal content of a synthetically prepared leachate when heavy metal sulphides formed in their landfill simulating reactors as a result of sulfate reduction. Research into the effects of redox potential on the biogeochemistry of another divalent transition metal ion (arsenic) revealed that the metals solubility was considerably reduced under sulphate reducing potentials of -250 mV (Signes-Pastor, Burló, Mitra, and Carbonell-Barrachina, 2006). Conversely, the c/c_o sulphate arising from array D*l* leveled off at an average c/c_o of 1.3 for the remainder of the investigation after peaking at 1.6 at 24 weeks. This could be a consequence of the oscillating

redox state of array D*l* over time (Figure 4.4) brought about in part by the delayed consumption of the nitrates as electron acceptors, resulting in the accumulation of sulphate prior to gradual consumption.

The occurrence of sulphate in the effluent, coupled with the reduced E_h and depleted nitrate concentrations recorded for both treatments at the termination point of the investigation, indicates that sulphate reduction was the predominant process occurring at the time in array D*h*. The higher E_h and c/c_o of sulphate recorded for array D*l* points to the possible predominance of iron reducing conditions within the array.

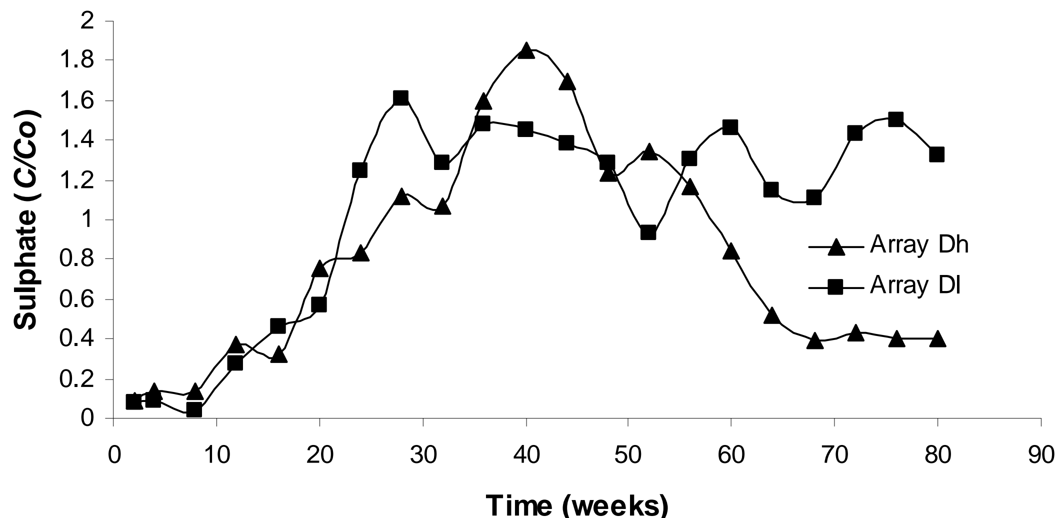


Figure 4.7 **Relative concentration of sulphate (c/c_o) in landfill leachate after leaching through soil microcosms over time. (▲) High HLR at 20 m*l* every 5 days, and (■) Low HLR at 10 m*l* every 5 days. Where, *c* and c_o are the outflow and inflow concentrations ($mg.l^{-1}$) in the leachate, respectively.**

Regression analysis of the c/c_o of sulphate on time presented a cautious fit to the quadratic model. Only 77.4 % of the variance about the sulphate data could be explained by the passage of time after grouping the treatments for comparative purposes. The model predicted significant differences between the two equations (Table 4.6) at the quadratic level but non-significant differences between the respective intercepts. The analysis confirms the assumption that the sulphate attenuation in the microcosms varies with changing HLRs. The

higher HLR produces an environment that is more reduced resulting in conditions conducive to the partial or complete reduction of sulphate.

Table 4.6 Regression response functions of relative sulphate (*y*) concentration on time (*x*) after grouping of treatments

$$y_{Dh} = -0.293 + 0.07596x - 0.0008944\, x^2$$

$$y_{Dl} = -0.137 + 0.05598x - 0.0004974\, x^2$$

4.3 Conclusion

The principle objective of this Chapter was to assess the fate of selected constituents of a young, synthetic, acetogenic phase landfill leachate over time by using a series of laboratory-scale soil microcosms so as to mimic the behavior of landfill leachate and associated interactions in soil beneath a landfill. With this in mind the experimental evidence suggests that the following conclusions can be drawn:

- The hydraulic rate at which landfill leachate seeps into the soil below a landfill contributes significantly to the fate of the different chemical constituents of the landfill leachate.

- In conjunction with the results of phenol adsorption on sterile and non-sterile soil in Chapter Three, the data provided by this study suggests that there was clear evidence of biological attenuation of phenol in the subsurface. As the internal environment of the sequential soil microcosms became more anoxic the relative concentration of phenol leaving the microcosms increased, confirming slower phenol attenuation rates with increased anoxic conditions.

- The persistence of sulfate in the landfill leachate prevents the development of methanogenic conditions and therefore the development of a complete redox pollution plume in the soil microcosms.

- The attenuation of copper over zinc by the soil microcosms was superior, implying a greater capacity for the pollution of the receiving groundwater by zinc in the presence of copper.

- The synthetic landfill leachate showed clear evidence of temporal changes with respect to chemical characteristics (pH and redox potential) and composition (phenol, nitrate, sulphate, copper and zinc) after perfusion through the sequential soil microcosms. These changes could possibly be related to the diverse physiological activity of different microbial groups.

- The successful achievement of a model system which was able to mimic microbial successional events which are relevant to subsoil environments likely to be found / occur beneath a landfill.

Chapter Five

5. Optimization of DNA Extraction and DGGE Staining Techniques

5.1 Experimental

5.1.1 Evaluation of DNA isolation methods for PCR-DGGE analysis of bacterial communities present in a soil

This experiment involved a comparison of two physico-chemical DNA isolation methods detailed in Chapter Two (2.9.1). They were the Mo-Bio™ Ultra-Clean Soil DNA Isolation Kit (hereafter refered to as the Kit method) and a modified version of the DNA isolation protocol documented by Duarte *et al.*(1998) (hereafter refered to as the Bead Beat method) which included the use of Wizard™ Clean-Up columns as a final step during DNA purification. Random samples (sixteen) of a soil exposed to conditions detailed in Chapter 4 were subjected to both methods and evaluated accordingly. Purity and yield of the extracted soil microbial DNA was the chief criteria used to evaluate the efficacy of each isolating technique. The purity was determined spectrophotometrically (GeneQuant Pro, Applied Biosystems) by computing the absorbance ratios at 260:280 nm (A260/A280) and 260:230 nm (A260/A230) so as to assess the extent of protein and humic acid contamination, respectively. The yield was calculated by spectrophotometer analysis at A260. Agarose gel [(1.2 % (m/v)] electrophoresis integrating a 1 kb and 100 bp marker (Promega) was used to identify the size of the extracted DNA fragments and the evaluation of the associated PCR reactions, respectively.

The economic viability (time and cost per sample) of each technique was also considered. Furthermore, DGGE was used as a tool to assess the contribution of each extraction method on the diversity of the extracted DNA. The isolation techniques detailed in Chapter Two (2.9.2; 2.9.3; and 2.9.4) was used as a foundation for the comparisons.

5.1.2 Evaluation of denaturing gradient gel staining methods

Three random samples of DNA (isolated from a soil subjected to the investigation detailed in Chapter Four) and a soil borne *Bacillus* isolate (Mr. C.H. Hunter from The University of KwaZulu-Natal, personal communication) were treated by PCR-DGGE (2.9.3 and 2.9.4). Sample one comprised a mixture of DNA isolated by the Kit from three control columns that were leached with water. The second and third DNA samples originated from soil sample 9 from array A*h* that was destructively sampled at week 12 (Chapter Four) using the Kit and the Bead Beat method, respectively. Each sample was subjected to duplicate PCR reactions that were visually qualified on an agarose gel (2.9.2) and loaded onto a denaturing gradient gel. The resultant gel was divided into two separate gels, with each gel containing all the amplified samples. One gel was silver stained (2.9.5.3) whilst the other was stained with ethidium bromide (2.9.5.4). The basis for the comparison of the staining methods was the clarity of the banding profile, and the number of bands present.

5.1.3 Statistical analysis

A general linear model (GLM) was used to run a pairwise t-test to compare the purity, yield, and humic content of the DNA isolated by the Bead Beat and Kit methods (MoBio Laboratory, USA). In addition, the Bead Beat isolated DNA was further purified using a Wizard™ Clean-up Kit (Promega) and the resultant DNA was also subject to the pairwise t-test comparison. If the t-test was significant, ($P < 0.05$), the means were separated using the Student Neuman Keuls test using SAS (version 6.12). The pairwise t-test was also used to compare the overall means for Species Richness (S), Shannon-Weaver Index (H'), Simpson's Index (D), Shannon-Weaver Evenness Index (E_H), and the Simpson's Equitability Index (E_D) achieved for the two DNA isolation techniques.

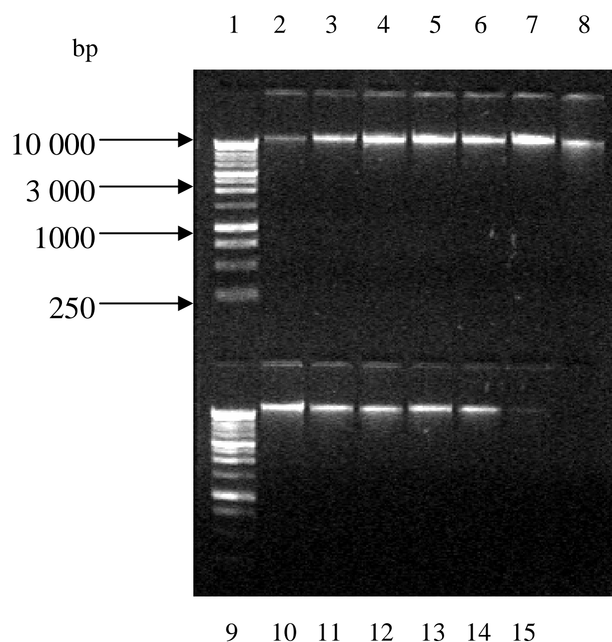
5.2 Results and Discussion

5.2.1 Evaluation of DNA isolation methods for PCR-DGGE analysis of bacterial communities present in a soil

5.2.1.1 Detection by agarose gel electrophoresis

The DNA fragments isolated by the commercial kit were visibly larger, and more distinct than that yielded by the Bead Beat method (Plates 5.1a and b). The Bead Beat isolated DNA revealed DNA fragments of variable sizes across all samples as evidenced by the DNA smear effect on the agrose gel (Plate 5.1b) (Bertrand, Poly, Van, Lombard, Nalin, Vogel and Simonet, 2005). The DNA concentration was not estimated from the agarose gel because of the absence of a quantitative marker. The Kit method incorporates chemical and homogenisation with specialised beads of an undisclosed nature while the Bead Beat method makes use of glass beads. Furthermore the vigorous agitation associated with the Bead Beat method was replaced by shaking on a vortex in the Kit method thereby decreasing the shearing forces of the treatment. This factor in addition to others; such as bead beating time and speed, buffer volume and temperature, and the amount and type of beads used in the extractions contribute significantly to the quality and quantity of DNA extracted (Bürgmann, Pesaro, Widmar and Zeyer, 2001). Krsek and Wellington (1999), Lloyd-Jones and Hunter (2001), and Niemi *et al.* (2001) has all reported similar findings whereby the quality and quantity of DNA is dependent on the time and speed of bead beating. There exists an inverse relationship between the speed and frequency of bead beating in relation to the fragmentation of DNA extracted while the opposite holds true for DNA yield (Bürgmann *et al.*, 2001). It is interesting to note that DNA of a larger molecular weight was trapped in the wells of the agarose gel containing DNA isolated by the kit while only five wells containing the bead beat isolated DNA revealed such an occurrence. The implications of this is unknown, however, this could affect the diversity of the isolated DNA. Both methods recommended the use of one gram of soil per reaction; this was ideal for the purpose of comparison in addition to the finding of Felske and Akkermans (1998b), who demonstrated that one gram of soil, contained a bacterial representation prevalent over several hundred square meters of soil.

(a)



(b)

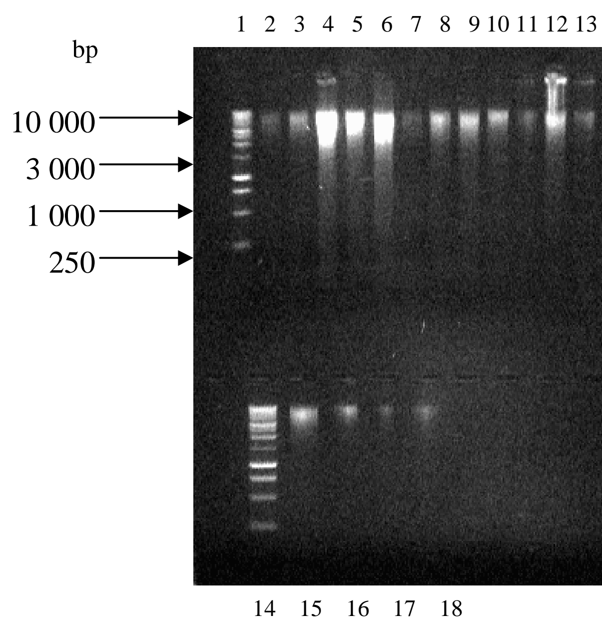


Plate 5.1 **Agarose gel electrophoresis of DNA (5 µ*l*) isolated from soil samples taken from soil array A*h* that was perfused with synthetic leachate. (a) Mo-Bio Ultra Clean™ isolated DNA, lanes: 1 kb DNA ladder (Promega) (1 and 9); samples 1 to 12 (samples from soil array A*h*) (2 – 8 and 10 – 14); untreated dry soil (Cd) (15). (b) Bead Beat isolated DNA, lanes : 1 kb DNA ladder (Promega) (1 and 14); samples 1 to 12 (samples from array A*h*) (2 – 13); untreated soil perfused with water (15 – 17); untreated dry soil (Cd) (18).**

5.2.1.2 Quantification by spectrophotometry and PCR

Pair-wise t-tests showed that there were significant differences in DNA purity, yield and humic content between the two DNA isolation methods and the consequent Wizard™ Clean-Up (Promega) treatment of the Bead Beat isolated DNA (Table 5.1). The DNA yield attained attained using the Kit was significantly lower ($P < 0.001$) (average yield = 36.23 ng.μl^{-1}) than that achieved with the Bead Beat protocol (average yield = 349.70 ng.μl^{-1}). Conversely, the purity achieved with the Kit isolation (average A260/A280 = 1.63) was significantly superior ($P = 0.0026$) to the Bead Beat protocol (average A260/A280 = 1.23). A260/280 ratios close to 1.8 indicates DNA of higher purity whereas lower ratios are indicative of protein contamination. A260/A230 measurements indicated that humic contamination was greater for the Bead Beat protocol (0.29) than the Kit (0.63) (Figure 5.1), with the pair-wise t-test confirming a significant difference ($P = 0.0007$) in humic contamination between both treatments. Low A260/A230 ratios are indicative of humic acid contamination (Yeates, Gillings, Davison, Altavilla and Veal, 1998). Cullen and Hirsch (1998) reported that a heat treatment step (70 ºC – as in the case of this study) used in conjunction with bead beat protocols resulted in an increase in brown humic residue and lowered DNA yield. Similar findings were true for this study, with the exception that there was no increase in yield for the protocol devoid of heat treatment (Kit).

In this investigation, Bead Beat DNA extracts ranged from dark brown to beige in colour suggesting the presence of humic and fulvic acids, in addition to an organic soluble PCR inhibitor which is known to adversely affect DNA polymerase during PCR (Španová, Rittich, Štyriak, Štyriaková and Horák, 2006). The presence of large amounts of humic acid in the isolated DNA can also give rise to errors during the calculation of DNA yield (Steffan, Goksøyr, Bej, and Atlas, 1988). DNA yield estimations by A260 measurement can account for as much as a 10 fold overestimation in the presence of elevated humic acid contamination (Cullen and Hirch, 1998; Lloyd-Jones and Hunter, 2001). Bearing this in mind, treatment of the bead-beat isolated DNA with the Wizard™ Clean-Up columns was found to significantly increase ($P = 0.0048$) the purity of the isolated DNA such that there was no longer a significant difference ($P = 0.1736$) in DNA purity as compared to the DNA isolated by the Kit.

However, there was a significant decrease (P = < 0.0001), as much as 25 times, in DNA yield after treatment with the Wizard™ columns (Figure 5.1).

Table 5.1 Pair-wise t-test comparison of purity (A260/A280), yield, and humic content (A260/A230) of DNA isolated using two different isolation methods and a DNA clean-up step.

Factors Compared	**DNA Purity**	
	P-value	Significance Rating
Bead Beat vs Kit	0.0026	***
Bead Beat vs Wizard	0.0048	***
Kit vs Wizard	0.1736	ns
	DNA Yield	
Bead Beat vs Kit	<0.0001	***
Bead Beat vs Wizard	<0.0001	***
Kit vs Wizard	0.0496	*
	Humic Content	
Bead Beat vs Kit	0.0007	***
Bead Beat vs Wizard	0.0018	***
Kit vs Wizard	0.0251	*

***significant at P = 0.01; ns not significant (based on duplicate extractions)
Bead Beat = modified method of Duarte et al. (1998)
Kit = MoBio Ultra Clean™ DNA Isolation Kit
Wizard = modified method of Duarte et al. (1998) plus the use of Wizard™ Clean-up columns

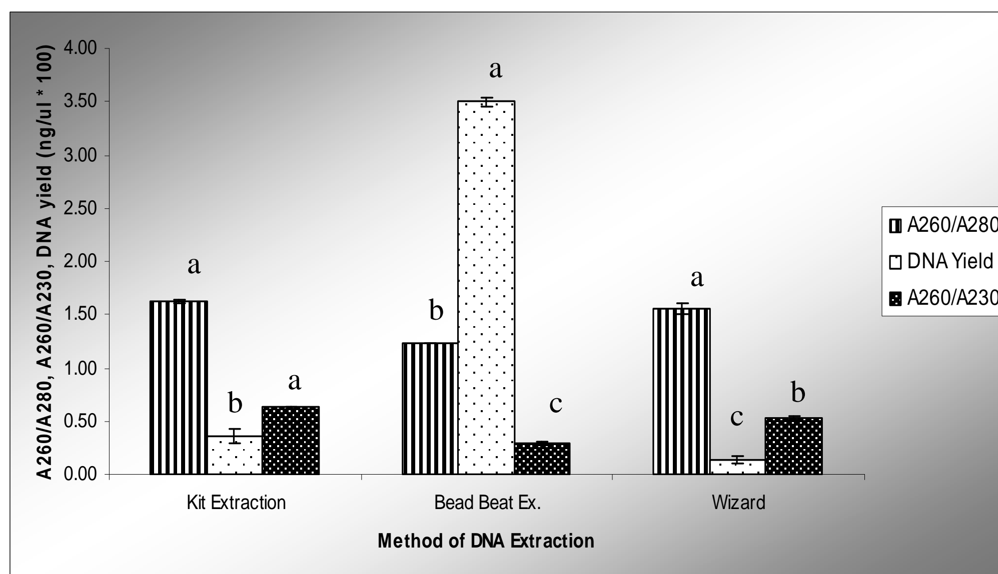


Figure 5.1 **Purity (A260/A280 and A260/A230) and yield (A260) measurements of DNA isolated from soil arrays using two DNA isolation methods [MoBio Ultra Clean™, Modified Bead Beat version of Duarte *et al.* (1998)], and Wizard™ Clean-up columns as a clean-up step.**

The difference in yield was also less significant (P = 0.0496) between the Wizard™ treated DNA and the Kit isolated DNA. Robe *et al.* (2003) also noted that DNA yields from soil isolations were significantly reduced after purification and they found that this had a negative impact on the amplification of DNA sequences that were present in low numbers.

The purity and yield of the isolated DNA are fundamental to achieving success with subsequent downstream molecular manipulations, in this case PCR and DGGE (Picard *et al.*, 1992; Cullen and Hirsch, 1998; Krsek and Wellington, 1999). DNA purity has a major bearing on the successful amplification of the DNA template during PCR (Cullen and Hirsch, 1998; Krsek and Wellington, 1999). Attempts to amplify the DNA isolated by the bead beat protocol proved unsatisfactory even after successive dilutions (1:10 to 1:1000) of the isolated DNA (result not shown). Negligible product was achieved for only a few samples at these dilutions and these were found to be unsuitable to generate DGGE profiles. Similar amplification constraints have been reported by Erb and Wagnerdöbler (1993).

High levels of humic material present in isolated DNA extracts have been shown to contribute significantly to the failure of subsequent PCR amplifications (Cullen and Hirsch,

1998; Lloyd-Jones and Hunter, 2001; He, Xu and Hughes, 2005). Moreover, the presence of phenolic compounds and heavy metals (which are common constituents of landfill leachate) in soil contribute to the inhibition and / or decrease in the sensitivity of subsequent PCR reactions (Robe, Nalin, Capellano, Vogel and Simonet, 2003; de Lipthay, Ezinger, Johnsen, Aamand and Sørensen, 2004). Protein impurities in soil-DNA extracts also have a negative impact on PCR amplification (Krsek and Wellington, 1999).

Purification of the Bead Beat DNA extract with Wizard™ Clean-up columns was considered an essential pre-treatment to render DNA purity suitable for satisfactory PCR amplification. This approach is well documented, although the exact commercial clean-up columns may differ (Duarte *et al.*, 1998; Niemi *et.al.*, 2001). Wizard™ column pretreated DNA was successfully amplified indicating that a clean-up step was essential.

A PCR product of approximately 200 bp (theoretically 180 bp) was clearly visible for reactions incorporating DNA from both isolation methods as template (Plate 5.2). The kit isolated DNA produced clearly defined products with no evidence of unused primer (Plate 5.2a). After PCR amplification using Bead Beat DNA as template for the reaction, the resulting product contained a larger amplified artefact (approximately 300 bp) in addition to the target segment (Plate 5.2b). However the product generated by the target sequence was visibly more substantial than this artefact. Furthermore there was a larger amount of unused primer.

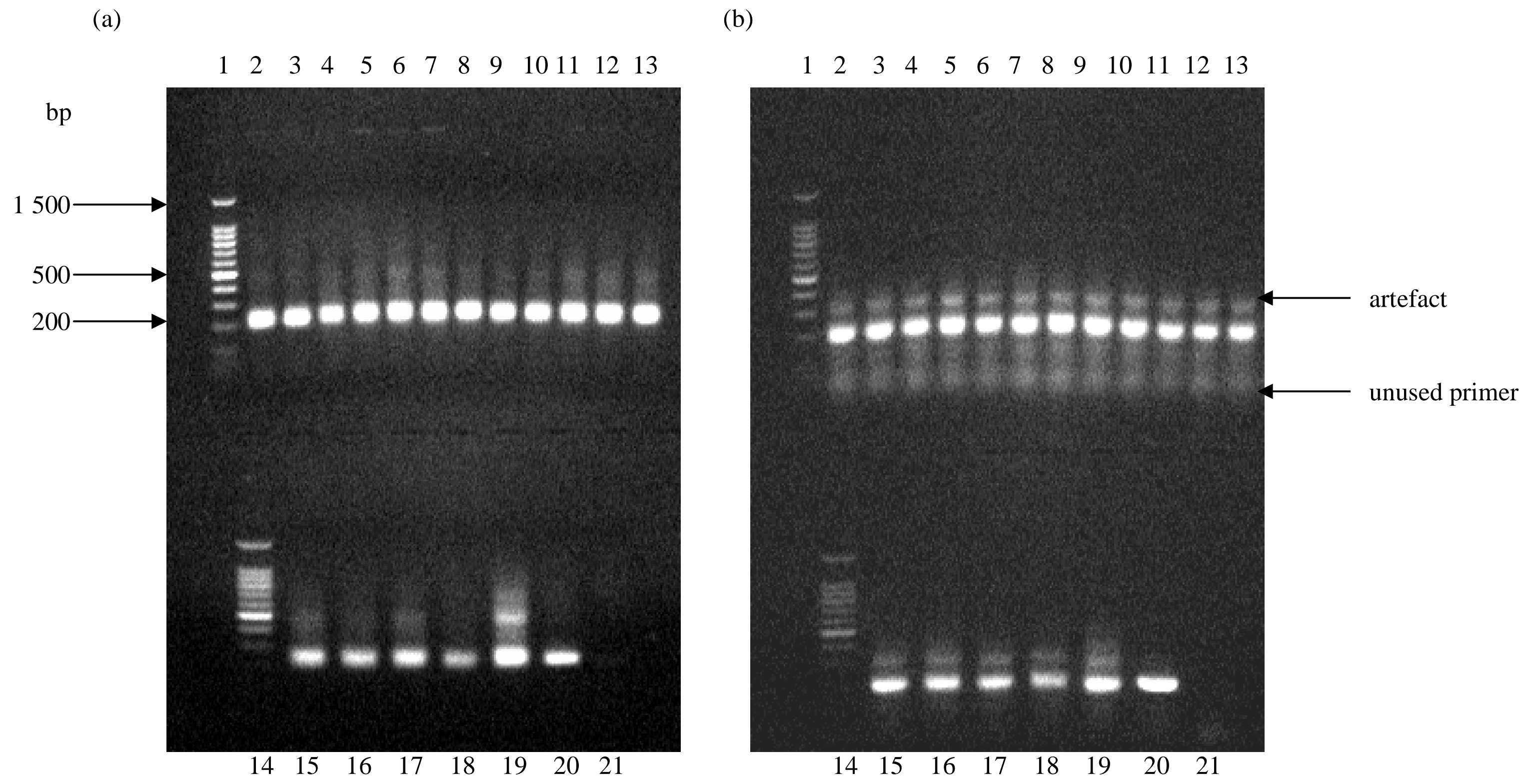


Plate 5.2 **Amplification of 16S rDNA fragments from soil samples of array A*h* demonstrating the effect of DNA method on the quality of PCR product. (a) Mo-Bio Ultra Clean DNA isolation kit, lanes: 100 bp DNA ladder (Promega) (1 and 14); samples 1 to 12 (samples of array A*h*) (2 – 13); wet controls (C1; C2; C3) (15 - 17); dry control (Cd) (18); DGGE marker (19); positive control (Bacillus isolate) (20); and negative control (21) . (b) Bead beat isolation, lanes correspond to those described in (a).**

5.2.1.3 Influence of DNA extraction method on bacterial community structure and relatedness determined by DGGE

PCR products derived from the two extraction methods were run in two separate denaturing gradient gels to assess the community profiles generated for identical soil samples. Differences in band number and band distribution by PCR-DGGE are shown in Table 5.2 and Plate 5.3, respectively. The occurrence of such differences in the diversity profiles is not unique to this study. Krsek and Wellington (1999) found that for each manipulation of a DNA isolation technique different microbial diversity profiles were achieved. Lipthay *et al.* (2004) have also demonstrated similar findings; DNA isolated from three soils by employing three separate DNA isolation techniques (viz. sonication, freeze-thawing-grinding, and bead-beating) where each produced distinct community diversity profiles for each soil type.

The average number of DNA bands visible was 17 and 15 per gel for DNA isolation by the Kit and Bead Beat method respectively. These differences were found to be significant ($P = 0.018$). On average fewer bands were visible for the control soils (samples 13-16, Table 5.2). This was unexpected since a lower *S*, which indicates a lower bacterial diversity, is often associated with pollution perturbation (Camargo *et al.*, 2005). This is a view shared by Rasmusen and Sørensen (2001), who found that mercury contamination of an agricultural soil, reduced the genetic diversity of the soil bacterial community. It is speculated that the synthetic leachate treatment contributed additional carbon sources and electron acceptors thereby promoting increased levels of soil bacterial community diversity. The overall reduction in *S* for both treatments over time, as a consequence of the accumulation effects of the leachate, will be discussed in Chapter Six.

Table 5.2 **Influence of DNA isolation method on the numbr of bands visualized by Denaturing Gradient Gel Electrophoresis generated from identical soil samples.**

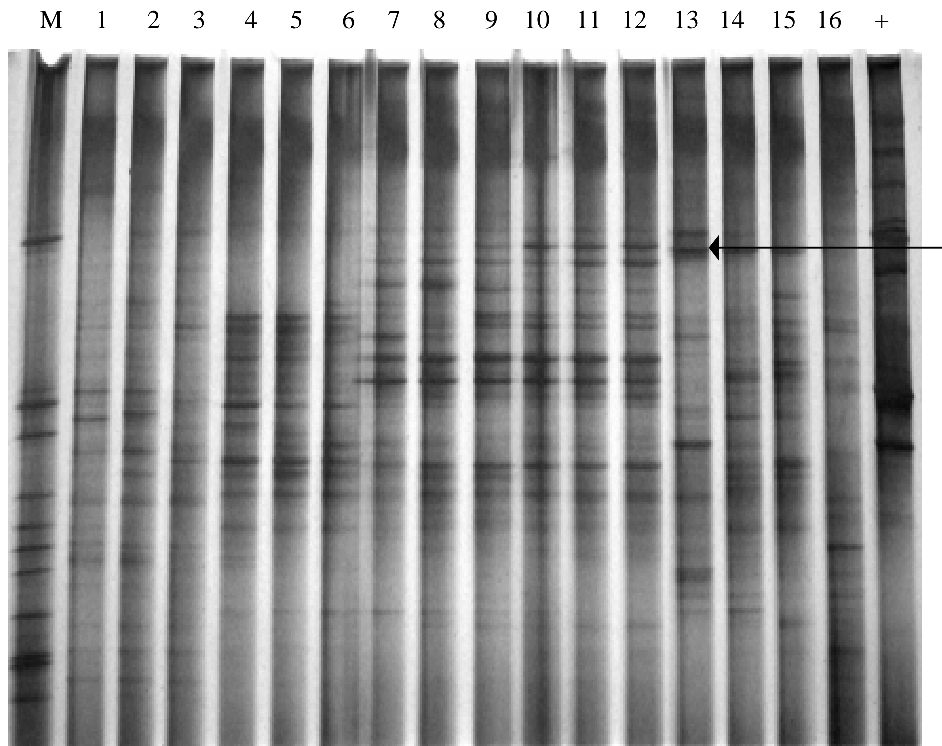
DNA Extraction Method	Number of DNA Bands per Sample on Denaturing Gradient Gels																	
	M	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	+
Mo-Bio Kit	12	21	22	17	18	17	16	19	21	22	18	19	19	20	16	15	11	11
Bead Beat Method	11	12	14	11	20	19	16	14	16	17	18	23	16	21	13	12	10	10

*Samples 1-12 originated from soil array A*h* that was destructively sampled after 12 weeks of leaching with the synthetic leachate
*Samples 13-15 originated from soil perfused with water only
*Sample 16 was untreated soil
*M was the DGGE marker (Vrije Universiteit van Amsterdam, H.W. van Verseveld)
*+ *Bacillus* soil isolate

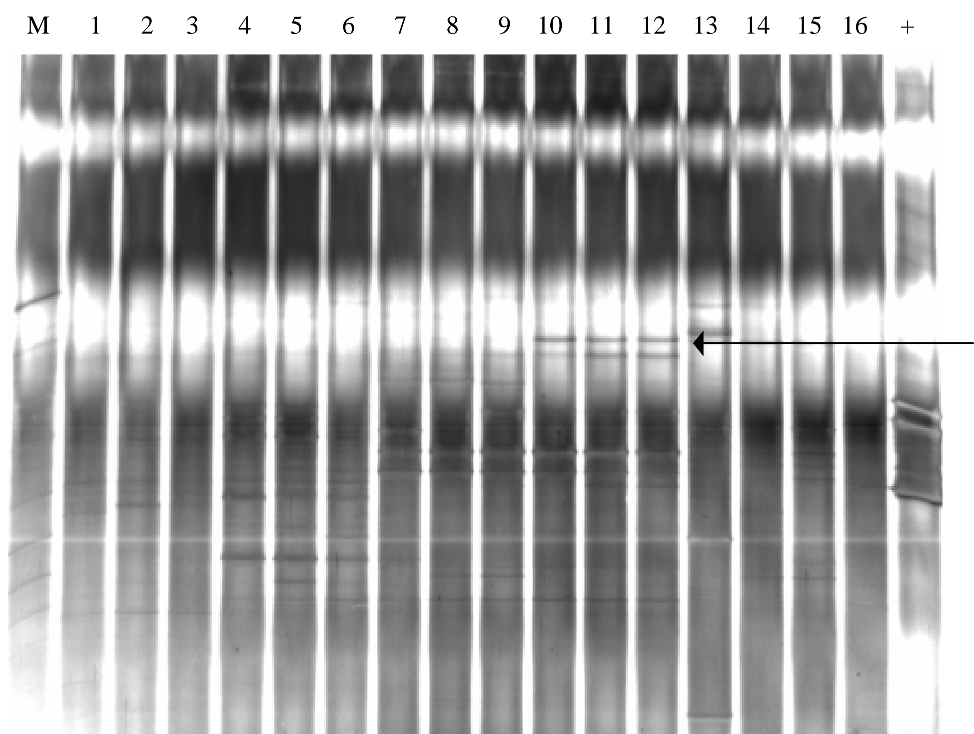
The DGGE marker and *Bacillus* soil isolate (positive control) produced similar patterns in both gels but were more difficult to visualise on the gel containing the PCR products originating from Bead Beat isolated DNA due to dark background stain of the gradient gel. This contributed significantly to the inability to capture some of the bands in lanes with relatively lighter background staining as a consequence of contrast limitations on the gel documentation system. Conversely, the gel containing the PCR products originating from Kit isolated DNA developed with a uniform background stain making the capture of the image relatively uncomplicated (Plate 5.3). There was evidence of smearing for all the samples depicted on Plate 5.3 (b) and in some instances this made it difficult to distinguish between individual bands. On investigating the spatial homogeneity of bacteria in grassland soils, Felske and Akkermans (1998a) generated community profiles consisting of a mixture of high; medium; and low intensity bands. They noticed that the bands of lower intensity sometimes resulted in smears in the profiles generated and this impacted on the inability to separate individual bands of this intensity. The prominent bands for all of the samples are more evident in Plate 5.3 (a) than on Plate 5.3 (b). The darker bands appear to be more distinct on both gels, whereas the lighter bands are either absent or more difficult to visualise on Plate 5.3 (b). PCR amplified products for both gels were prepared, electrophoresed, and stained simultaneously so one can cautiously assume that variations in the quality of the gel images may be attributed to the quality and quantity of the PCR products which were the result of DNA isolated by two different methods. Gelsomino *et al.* (1999) noticed a similar trend when they compared direct and indirect DNA isolation methods on a Flevo silt loam soil from Waginingen in the Netherlands.

Plate 5.3 **Silver stained Denaturing Gradient Gels illustrating the community profiles generated for common soil samples (Lanes 2 – 16) exposed to; (a) Mo-Bio Ultra Clean Soil DNA Isolation Kit and (b) Modified version of Duarte *et al.*(1998) Bead Beat DNA Isolation method. Lanes M and + correspond to the DGGE Marker and a *Bacillus* isolate from soil. The arrow (←) represents distinct bands visible on both gels.**

(a)



(b)



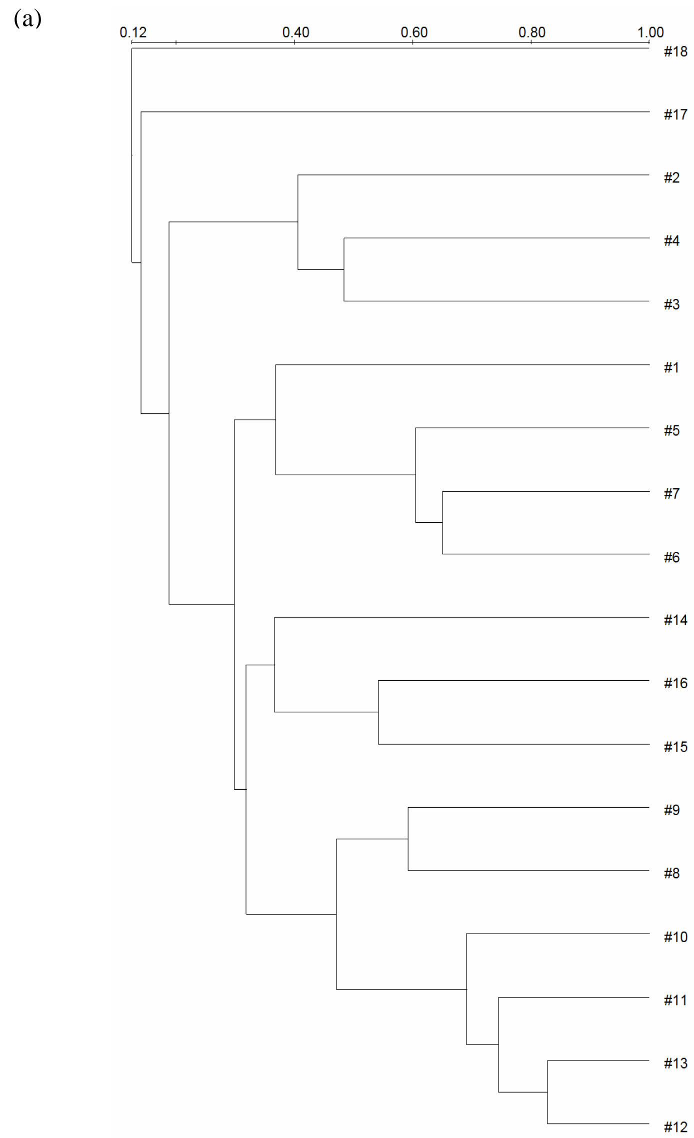
Cluster analysis performed on the DGGE profiles using the unweighted pairwise grouping method with mathematical averages (UPGMA, Dice co-efficient of similarity) showed dissimilar cluster relationships between samples for each DNA isolation method (Figure 5.2). For example, PCR-DGGE of samples isolated using the Kit method showed clustering for samples 3 and 4 at 50% similarity with sample 2 joining the cluster directly at 40%. PCR-DGGE of the bead-beat isolated DNA showed that samples 3 and 4 also clustered at 50% but sample 2 only clustered indirectly at 10% similarity. Samples 5/6/7 formed a discrete cluster at 60% for the Kit isolated DNA products, with samples 5 and 6 clustering at 60% for the Bead Beat method and only indirectly pairing with sample 7 at 10% correspondence. Samples 8 and 9 for the Kit isolated products clustered at 60% while samples 10/11/12/13 paired as a discrete cluster at 65%. Both of these distinct clusters paired at 40%. The Bead Beat method paired samples 8 and 9 at 50%, with 10/11/13 pairing at 40% as a discrete cluster. However sample 12 joined both clusters indirectly at only 15% similarity. The controls leached with water both formed discrete clusters at 35% and 30% for the products of Kit and Bead Beat isolation respectively. However, the phylogenetic tree for the Kit isolated DNA products was rooted by the original soil sample (17) which paired indirectly with cluster 14/15/16 at 15% whereas pairing for the Bead Beat method occurred at double the similarity to samples 15 and 16 only. For this method sample 17 served as a link between samples 15/16 and with sample 14 thereby linking cluster 15/16/17 and rooting this tree as the out-group for all the samples. This original soil sample was linked to all the samples of the investigation at 15% for the Kit isolated PCR products while pairing for the Bead Beat method occurred below 10% similarity.

The divergent phylogenetic relationships between the samples for each method relates to the presence/absence of DNA bands on the gels. The results suggest that some degree of similarity exists between the DNA isolation methods by virtue of similar clustering relationships between samples. However there are undoubtedly major differences in the number of bands depicted by PCR-DGGE as well as the prominence of specific bands isolated by each method. In this case the two DNA isolation techniques produced different phylogenetic relationships for identical soil samples. Other researchers have reported similar conclusions (Niemi *et al.*, 2001; Lipthay *et al.*, 2004). Lipthay *et al.* (2004) further concluded

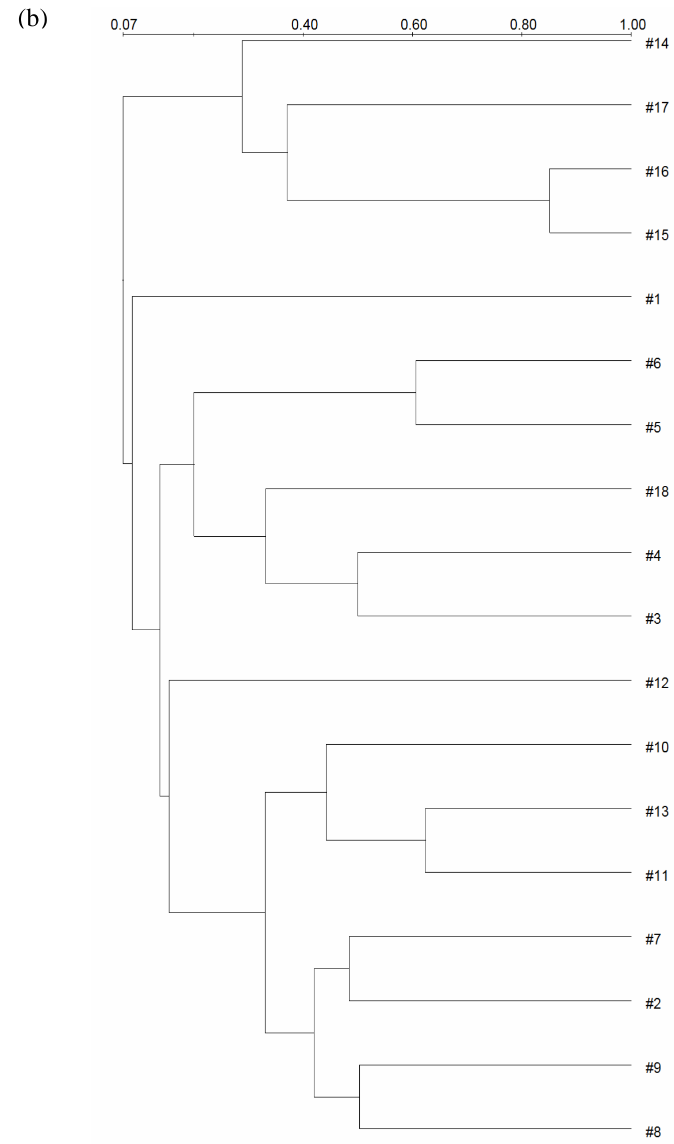
that the choice of DNA isolation protocol must also impact on the functional diversity of the profiled community.

Figure 5.2 **Cluster analysis of soil samples by the unweighted pairwise grouping method of mathematical averages (UPGMA, Dice co-efficient of similarity). (a) PCR-DGGE of DNA isolated by the Mo-Bio Ultra Clean Soil DNA Isolation Kit and (b)) PCR-DGGE of DNA isolated by the Modified version of Duarte *et al.*(1998) (Bead Beat method). Sample numbers: Soil samples (2-13) originate from the landfill leaching experiment detailed in Chapter 4; with soil samples (14-16) the control samples that were leached with water only; soil sample (17) the original untreated soil; and samples (1 and 18) the DGGE marker and *Bacillus* soil isolate respectively.**

(a)



(b)



5.2.1.4 Assessment of bacterial community diversity

In describing the structure of any given community it would be reckless to assume that *S* alone gives a complete summation of rare and common bacterial species. Hence, we turn to diversity indices that attempt to describe the distribution of species within ecological communities by incorporating measures of *S* and evenness into a single value (Townsend, Begon and Harper, 2003). The various measures of general ecological diversity that were traditionally reserved for higher organisms have recently been adapted to describe species diversity in microbial communities (Atlas and Bartha, 1998). Initially these indices were applied to microbial communities only after the analysis of morphological and physiological traits, these procedures often requiring the culturing of the microbial communities. It was thought that the application of these mathematical diversity measures required identification to a definite species or genus level by cultivation (Borneman *et al.*, 1996). However, the advent of numerous genetic/molecular approaches has reduced the dependence of microbial community profiling on culture dependant methods. This has paved the way for the application of diversity equations without the need for complete identification and culturing of microbial communities (Jackson *et al.*, 2001; McCaig *et al.*, 2001; Koizumi *et al.*, 2003; Haack *et al.*, 2004). The basis of the application lies in the defining of an operational taxonomic unit (OTU), which is a single nucleic acid band in the case of DGGE (Jackson *et al.*, 2001; Kocherginskaya, Aminov and White, 2001; Haack *et al.*, 2004).

This investigation included the use of the Shannon-Weaver Index (*H'*), Simpson's Index (*D*), Shannon-Weaver Evenness Index (E_H), and the Simpson's Equitability Index (E_D) to confirm that the observed differences in *S* resulting from the isolation of DNA using two different methods translates into variations in the numerical distribution of the bacterial species isolated. We compared diversity measures over a range of sampling depths for identical samples subjected to the two isolation methods and found that the community profiles generated not only differed in terms of species richness (*S*) and phylogenetic relatedness, but also in terms of the relative abundance of the composite species of each sample (diversity indices). The average *H'* calculated for the PCR-DGGE amplicons of DNA isolated by the Kit was significantly different ($P = <0.001$) from that recorded for the Bead

Beat method. Overall, sample for sample, the PCR-DGGE amplicons of DNA isolated by the Kit showed greater diversity than that generated by the Bead Beat method (Figure 5.3a). This superior diversity was confirmed by lower *D* indices recorded for the kit isolated DNA amplicons (Figure 5.3b) which effectively reveals that there is a lower probability that two species (bands) identified in a community (sample) will be the same species, thereby reflecting a higher diversity (Krebs, 1985; Edwards *et.al*., 2001). The difference identified between the means of *H'* for both treatments were confirmed by significantly different mean *D* values (P = <0.001). This study made use of two diversity measures (Shannon-Weaver Diversity Index and Simpson's Index) and their related equitability equations so as to increase the validity of the conclusion drawn from this study (Kocherginskaya *et al.*, 2001).

Each diversity index distributes a different weighting in each equation to composite species. The Shannon-Weaver Index gives more weight to bands with relatively low signal intensities while the Simpson's Index tends to apply additional weight to dominant bands with relatively high signal intensities (Hill *et al.*, 2003). Essentially, Simpson's index measures the diversity of the most numerically predominant amplicons which are the direct products of the selected DNA isolation methods (Kocherginskaya *et al.*, 2001). Thus, by calculating these indices both possible extremes, which may be encountered in any sample, can be taken into account.

The lower evenness indices measured for the PCR-DGGE amplicons of DNA isolated by the Bead Beat method indicate the presence of a greater number of numerically dominant species (bands) isolated by this method (Figures 5.4a and b). This method favoured the extraction of bacterial DNA from certain species over others. This was supported by average E_H and E_D values of 0.92 and 0.69 respectively. Conversely, the Kit method showed evenness indices closer to one (average E_H=0.95 and E_D=0.78), indicative of the isolation of a more equitable or even distribution of all extracted bacterial DNA per soil sample. Consequently, significant differences were recorded between the mean E_H (P = 0.002) and E_D (P = 0.002) of both treatments. The E_H and E_D ranged from 0.97 to 0.93 and 0.85 to 0.67 respectively for the DNA isolated by the Kit method and 0.96 to 0.87 and 0.80 to 0.49 respectively for the Bead Beat method. The broader range recorded for both evenness indices in relation to the Bead

Beat method implies a greater variability experienced in reproducing the isolation of similar proportions of DNA across the samples. An evenness index of one indicates a proportionate distribution of microorganisms (Camargo *et al.*, 2005).

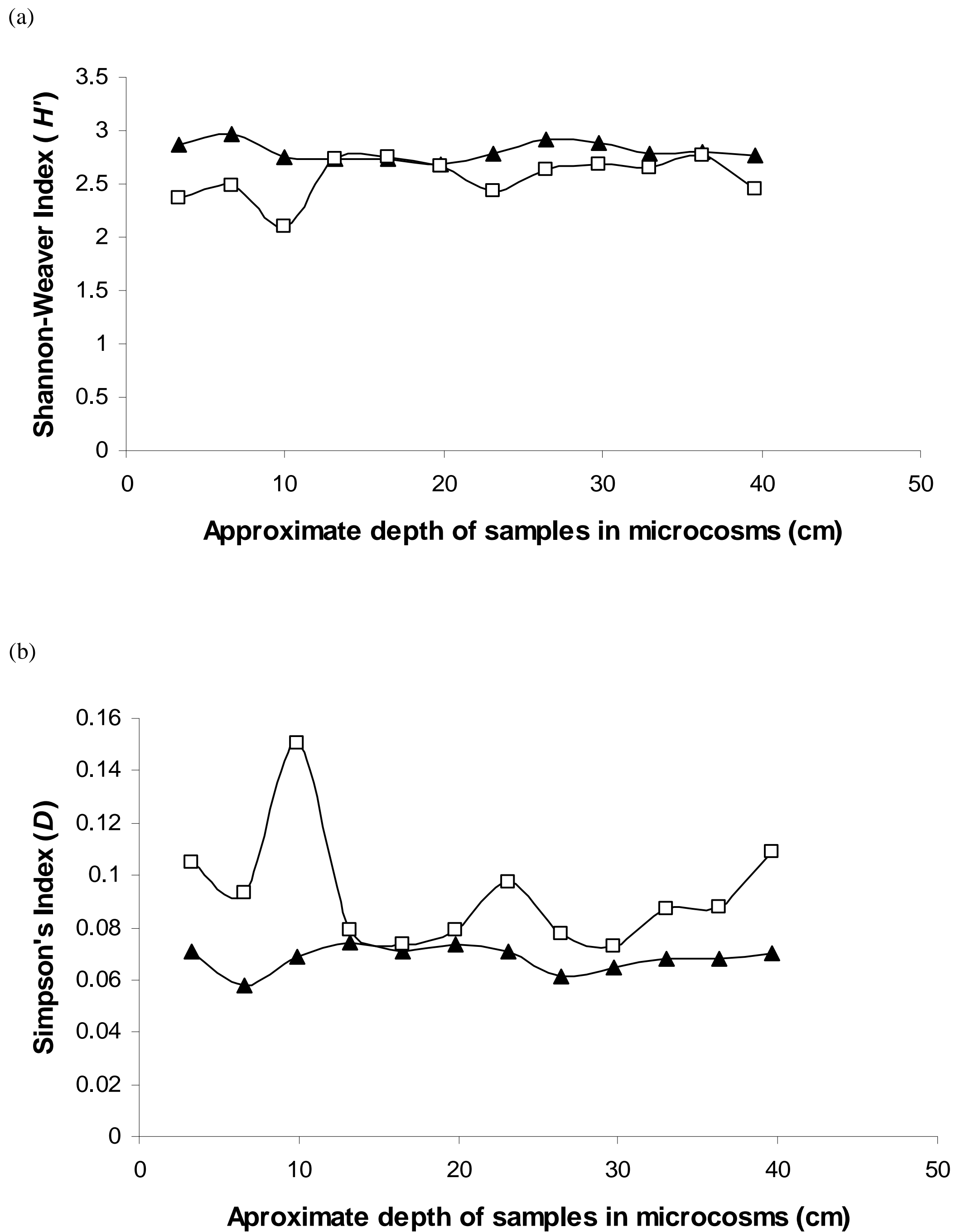


Figure 5.3 **Diversity analyses of data generated by PCR-DGGE of DNA isolated using a Mo-Bio Ultra Clean Soil DNA Isolation Kit (Kit method) (▲) and DNA isolated using a Modified version of Duarte *et al.*(1998) (Bead Beat method) (□). Soil samples were taken from soil array A*h* perfused with synthetic landfill leachate over 12 weeks (Chapter Four). The generated DGGE data was applied to (a) Shannon-Weaver Diversity Index and (b) Simpson's Diversity Index.**

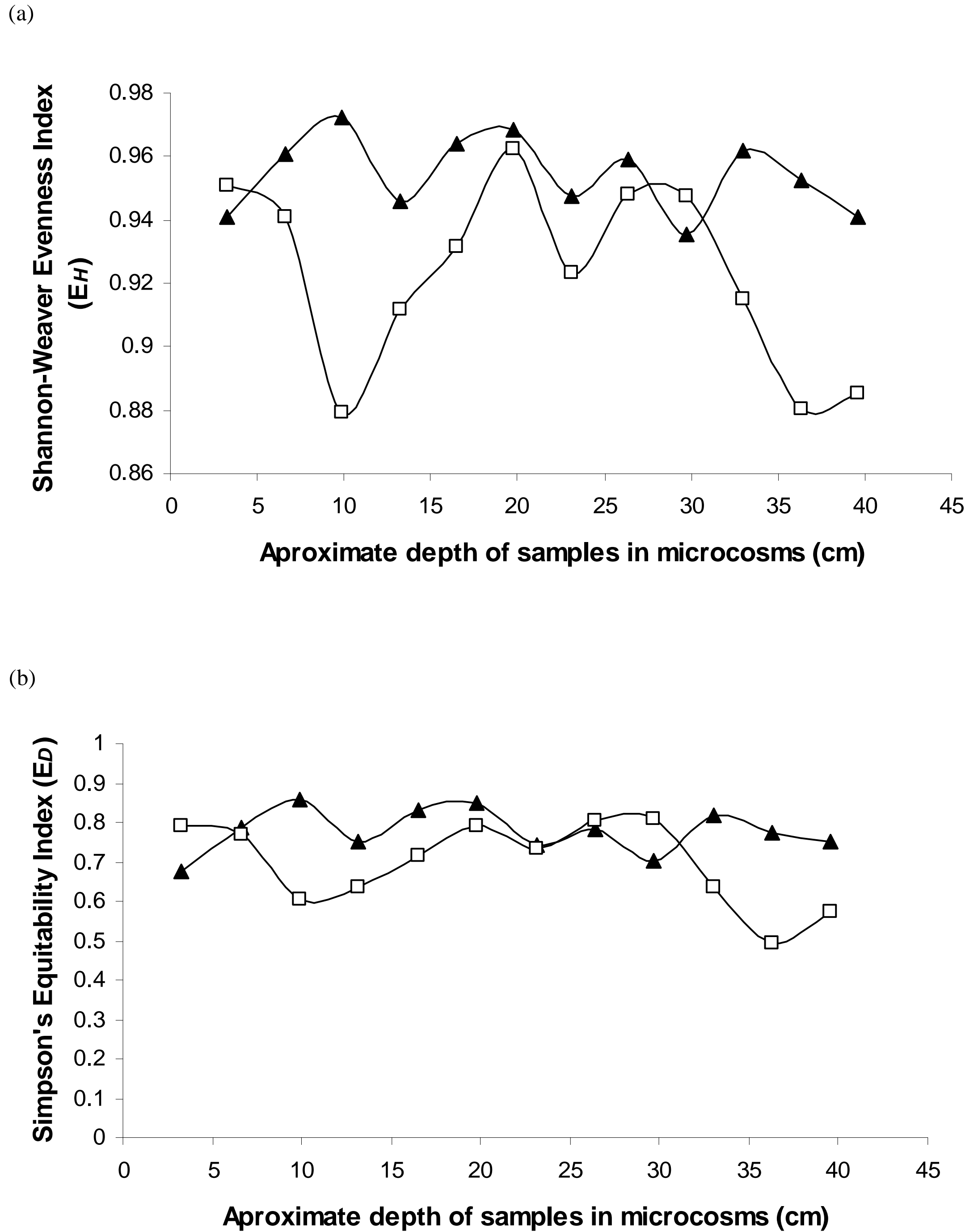


Figure 5.4 **Diversity analyses of data generated by PCR-DGGE of DNA isolated using a Mo-Bio Ultra Clean Soil DNA Isolation Kit (Kit method) (▲) and DNA isolated using a Modified version of Duarte *et al.*(1998) (Bead Beat method) (□). Soil samples were taken from soil array A*h* perfused with synthetic landfill leachate over 12 weeks (Chapter Four). The generated DGGE data was applied to (a) Shannon-Weaver Evenness Index and (b) Simpson's Evenness Index.**

Commercial DNA isolation kits, exclusively designed for the isolation of microbial DNA from soil and sediments, offer a convenient method that is quick, simple, routinely reproducible, and appropriate for successive DNA isolation reactions. However, caution must be exercised when relying exclusively on single isolation methods since there are bound to be recalcitrant bacteria present from which DNA is not readily isolated. Any given protocol will favour the isolation of DNA from bacteria most susceptible to the physico-chemical methods employed thereby contributing significantly to the composition and diversity of the prevalent microbial community (Roose-Almsaleg, Garnier-Sillam, and Harry 2001). The choice of DNA isolation method revolves around numerous factors; these include the efficiency of isolation and purification, the quality of the isolated DNA for downstream reactions, and the representative constitution of the isolated DNA in any given sample. Roose-Almsaleg *et al.* (2001) stated that the choice of a DNA isolation protocol centres on a compromise between quality, representative composition, and destined applications of the isolated DNA while taking into account that each isolation technique suffers some form of bias or limitation. It is imperative that each isolation technique is adapted for the type of sample in question (Zhou, Bruns and Tiedje, 1996). Since it is not only the parent sample that will influence the extraction efficiency of the technique but also the subsequent treatments that the sample is exposed to will have a significant bearing on the efficiency of the DNA isolation technique. Dubey, Tripathy, and Upadhyay (2005) stated that an ideal DNA isolation technique would; process all samples uniformly, isolate DNA from all the members of a soil microbial community, process multiple samples over a satisfactory time scale, and produce DNA of satisfactory yield and purity. Additionally, the quantity of DNA isolated will determine, in some measure, the numerical diversity and numerical dominance of autochthonous bacteria in the soil sample but not necessarily the functional dominance in the community (Krsek and Wellington, 1999).

5.3 Evaluation of Denaturing Gradient Gel Staining Methods

There have been numerous studies evaluating the influence of DNA extraction methods on the quantity and quality of DNA yields (Mumy and Findlay, 2004; Lloyd-Jones and Hunter, 2001; Jackson, Harper, Willoughby, Roden, and Churchill, 1997), as well as on the PCR-DGGE profiles generated (de Lipthay *et al.*, 2004; Niemi *et al.*, 2001; Gelsomino *et al.*, 1999). However, there seems to be little or no documentation on the effects of DNA staining procedures on the diversity profiles generated by PCR-DGGE. With this in mind we assessed the influence of a silver stain and ethidium bromide in visualizing DGGE bands derived from identical samples that were subjected to electrophoresis on the same gel (Table 5.3 and Plate 5.4). The silver stain resolved significantly more bands per sample than the ethidium bromide stained gel (P = 0.003). Sample for sample, the community diversity reflected by the silver stained gel was significantly greater than that observed for the ethidium bromide stained gel ($P_{h'}$ = 0.002 and P_D = 0.013) (Figure 5.5). However, there were no significant differences recorded for the evenness distribution of the data captured from both staining methods.

The *H'* index is dependent on the *S* and *E* of any sample in question and as such for any given numerical composition and distribution of the bacteria among the composite species, there will be an increase in *H'* with an increase in *S* (Townsend *et al.*, 2003). The *H'* index is more sensitive to changes that occur in the number of individuals found in less common species (Hill *et.al.*, 2003) therefore the increased sensitivity of the silver stain enables the detection of species (bands) and quantities thereof that would not be possible when using an ethidium bromide stain. A similar argument can be offered for the *D* index of diversity which gives greater weighting to numerically abundant species (bands) (Krebs, 1985; Edwards *et al.*, 2001).

Table 5.3 **The number of DNA bands per sample that were visible on two Denaturing Gradient Gels after silver and ethidium bromide staining.**

Staining Method	Number of DNA Bands per duplicated Sample on Denaturing Gradient Gels				
	1	2	3	4	5
Silver Stain	24	10	20	25	24
Ethidium Bromide	13	6	11	13	16

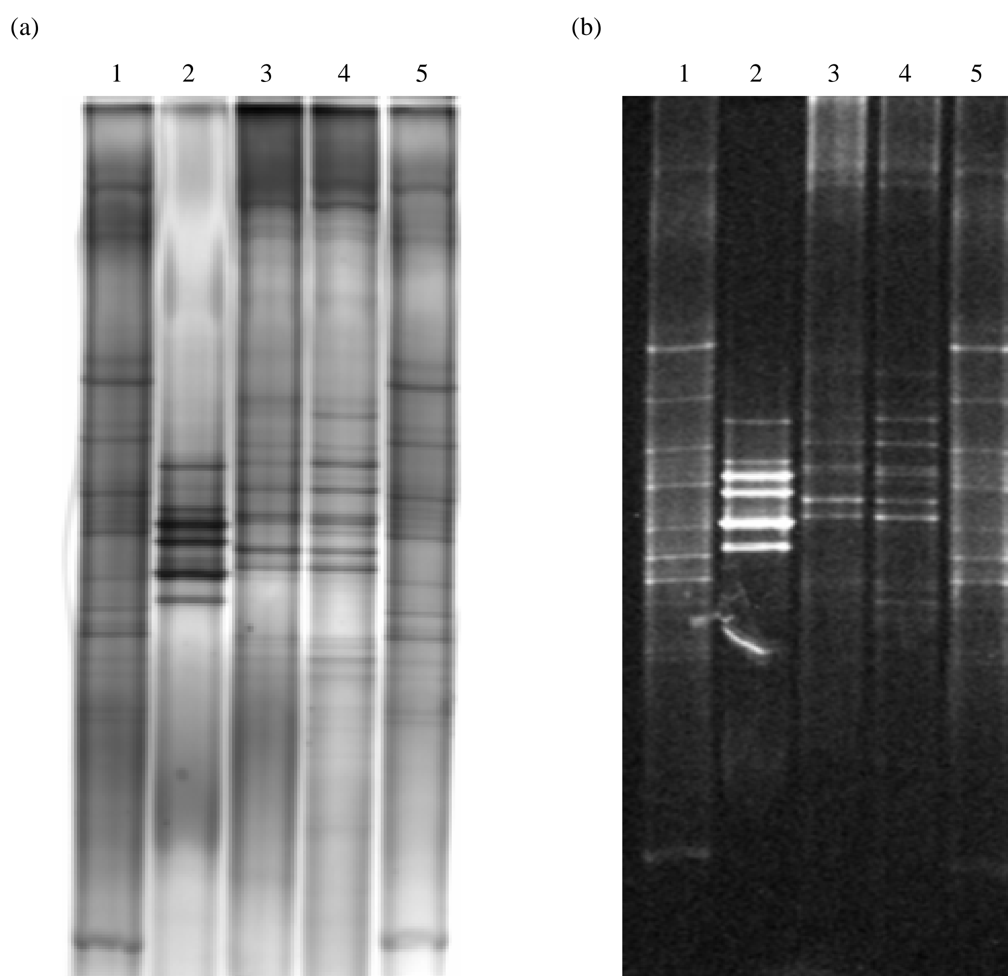


Plate 5.4 **Denaturing Gradient Gels illustrating the differences in banding profiles achieved with two DNA gel staining techniques. (a) Silver stained gel; and (b) Ethidium bromide stained gel. Lanes: combined control soils (1 and 5); *Bacillus* soil isolate (2); soil sample 9 with DNA isolation by Modified version of Duarte *et al.*(1998) (3); and soil sample 9 with DNA isolation by Mo-Bio Ultra Clean Soil DNA Isolation Kit (4).**

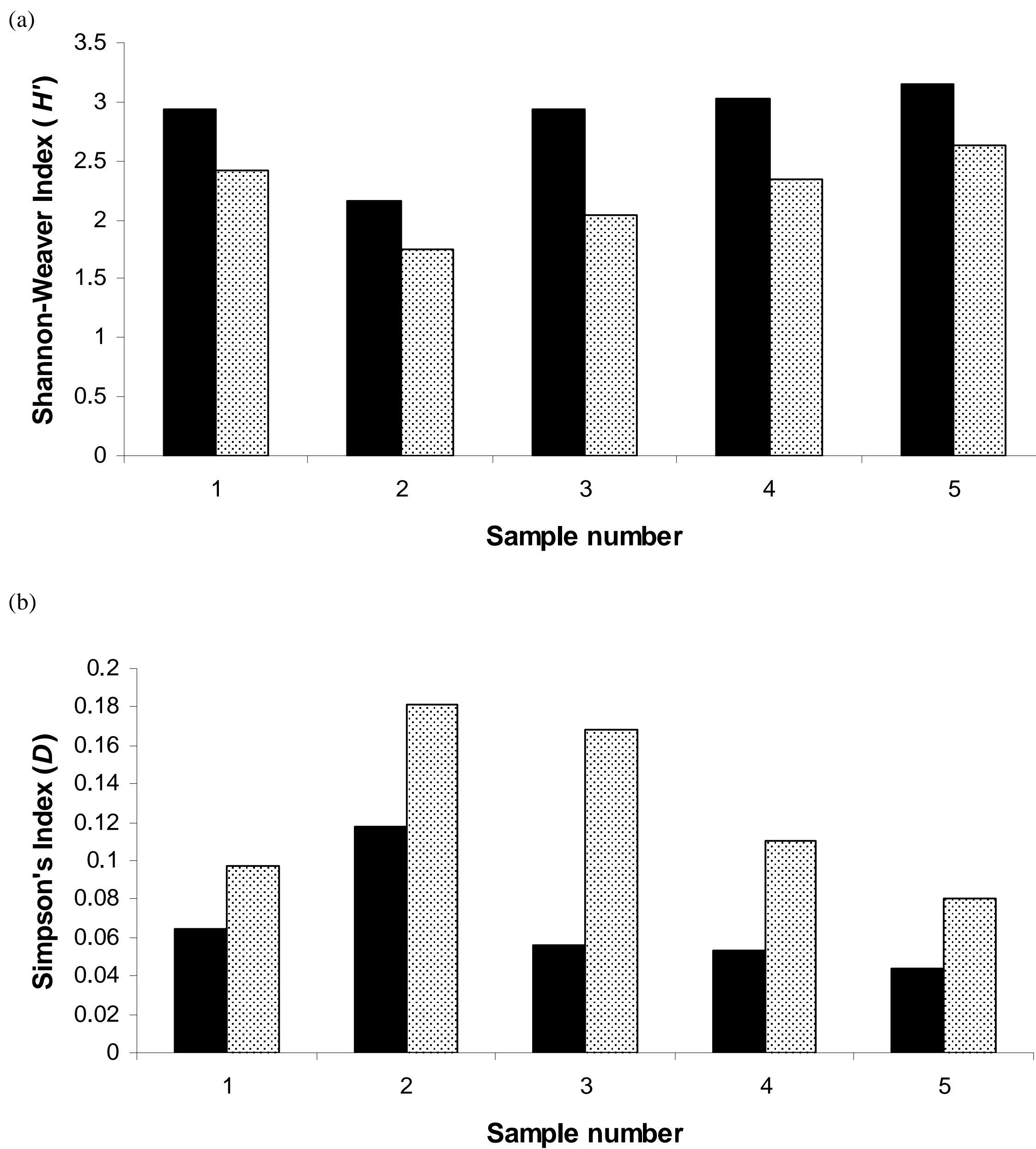
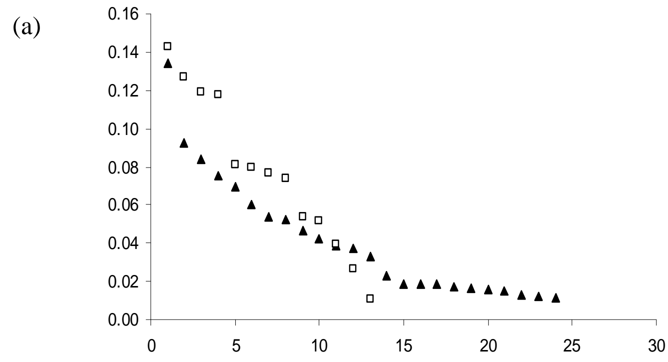


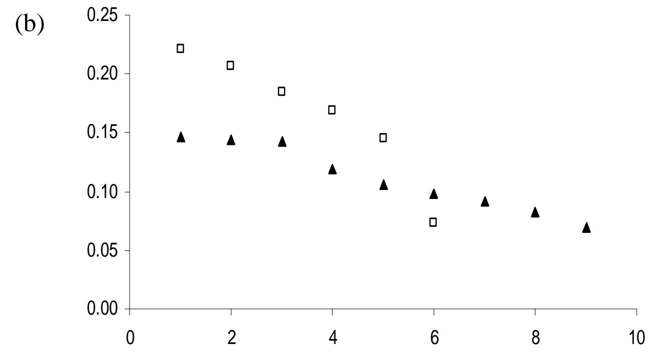
Figure 5.5 **Diversity analyses of data generated by PCR-DGGE of DNA isolated from soil and the subsequent staining of identical gels by (■) Silver stain and (□) Ethidium bromide stain. Soil samples: (1 and 5) combined control soils originating from three control columns that were leached with water only; (2) *Bacillus sp.*soil isolate; (3) soil sample 9 with DNA isolation by Modified version of Duarte *et al.*(1998); (4) and soil sample 9 with DNA isolation by Mo-Bio Ultra Clean Soil DNA Isolation Kit. Soil sample 9 originated from the landfill leaching experiment detailed in Chapter 4. The data generated follows application to (a) Shannon-Weaver Index and (b) Simpson's Index.**

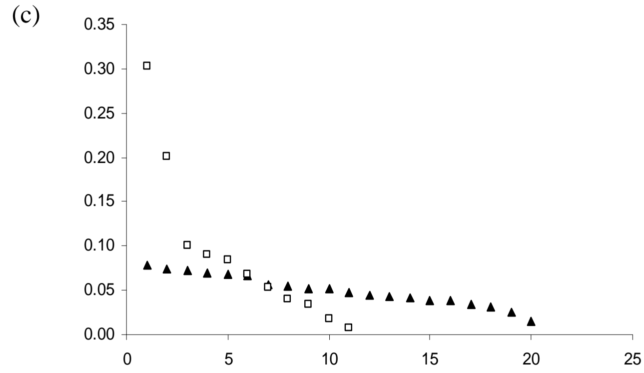
The information derived from analysing the captured data into diversity measures illustrates clear differences in community diversity between replicate samples when the DGGE gels in question were stained by either silver or ethidium bromide stains. These differences were demonstrated by the capture of different *S* and peak signal intensities which in turn have a profound effect on the calculated community diversity indices and dominance relationships of the species (bands) present in each sample. These differences are further exemplified when the data was depicted as rank-abundance graphs (Figure 5.6) which plot the relative proportion of the composite species in a sample against the corresponding rank of a species in that sample i.e. a band that contributes the highest proportion of peak signal intensity (highest quantity) to a given sample would be assigned a corresponding rank of one (Townsend *et.al.*, 2003). The steeper the gradient of the rank-abundance plot the greater the dominance of the more commonly occurring species (bands with high peak signals) over the other species in that sample with lower peak signals (Townsend *et al.*, 2003). All the rank-abundance plots (Figure 5.6a-e) confirmed the findings of the diversity measures. The ethidium bromide stained samples were dominated by a fewer number of species punctuated by relatively large changes in abundance from one cluster of species (bands) to the next while the silver stained samples revealed a less dominant existence of composite species. An increase in slope is indicative of a reduced numerical equitability between species of the population i.e. an uneven numerical distribution of the species comprising the population (Jackson *et al*., 2001). The ideal would be a situation where rank-abundance diagrams are plotted for all samples of a given investigation since these diagrams take into account the full array of peak signal intensities in a given sample and displays them as an individual data point as apposed to assimilation into various equations (Townsend *et al.*, 2003). However, the quantity of data generated in this investigation would make such an approach unfeasible. The divergent dominance relationships between identical samples exposed to either of the two staining protocols was a consequence of the increased sensitivity of silver staining which enabled the capture of bands that were not detected on the ethidium bromide stained gel. The diagrams further illustrate differences in *S* between identical samples in addition to differences in the relative numerical distribution of the composite species of a sample.

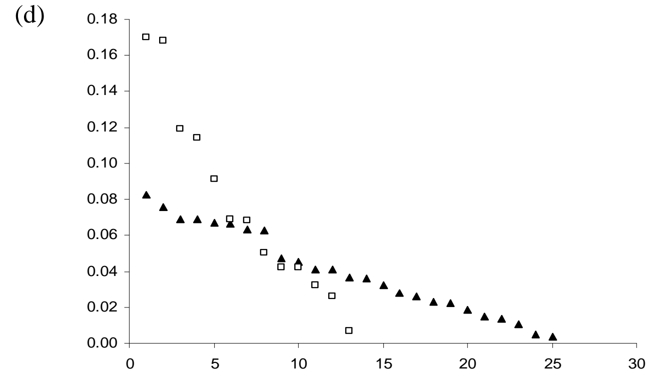
Ultimately, the choice of DGGE staining protocol will have a profound effect on the type and amount of data captured by image capture software. As a result the information accumulated from manipulating the captured images does contain differences in relation to *S*, diversity of bacterial communities, numerical distribution of composite species (evenness of distribution), and dominance relationships between composite species of a sample. It is therefore crucial to standardize methods and be cogniscent of the limitations associated with each method starting with isolating DNA, through to PCR-DGGE, gel staining, image capture and data analysis, and finally application of the data to relevant expanatory equations.

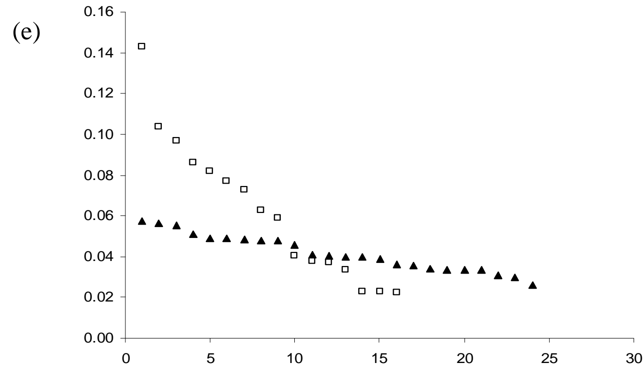
Figure 5.6 **Comparison of rank-abundance plots of silver (▲) and ethidium bromide (□) stained DGGE gels. Plots per soil sample: (a and e) combined control soils originate from three control columns that were leached with water only; (b) *Bacillus* soil isolate; (c) soil sample 9 with DNA isolation by Modified version of Duarte *et al.*(1998); (d) and soil sample 9 with DNA isolation by Mo-Bio Ultra Clean Soil DNA Isolation Kit. Soil sample 9 originated from the landfill leaching experiment detailed in Chapter Four.**











Relative Abundance by Peak Intensity (*Pi*)

Species Rank per Sample

5.4 Conclusion

The objectives of this Chapter were three fold. The first objective was to optimize the isolation of DNA from soil samples originating from the microcosms detailed in Chapter Four. The second was to optimize conditions for DGGE and evaluate efficacy of the associated silver and ethidium bromide gel staining techniques. Lastly, the data generated from the captured DGGE images were subjected to a range of ecological diversity indices analyses to determine their suitability in measuring the Bacterial diversity of the soil samples. With this in mind, the experimental evidence suggests that the following conclusions can be drawn:

- The Kit method produced DNA of a lower yield but of superior quality with respect to purity and humic acid content when compared to the DNA isolated by the Bead Beat method. The DNA extracted by the Bead Beat method required further purification by Wizard™ Clean-Up columns which significantly reduced the yield. This increased the purity such that there was no longer a significant difference between the DNA purity of the two extraction methods. In comparison to the amplicons produced from PCR of the Bead Beat DNA, the amplicons achieved by using DNA extracted by the Kit method was of superior quality. Furthermore, these amplicons produced DGGE images of better clarity in addition to significantly different banding patterns. Based on this evidence, the Kit method was selected as the preferred method for use in future investigations (Chapter Six).

- DNA staining procedures were shown to have a significant influence on the banding profiles generated by PCR-DGGE. Identical samples that were subjected to electrophoresis on the same gel, prior to staining with either silver or ethidium bromide stains, generated DGGE images that differed with respect to S and band signal intensities. The silver stained gels generated images that had greater S and stronger band signal intensities This resulted in differences in downstream analysis that were dependent on such data extrapolated from the captured primary image e.g. diversity indices. With this in mind the silver stain was maintained for future investigations so as to maximize output data.

- The use of *H'* and *D* to assess Bacterial community diversity in the treated soil supported the findings and conclusions drawn by previous researchers. When the peak densities of more abundant bands were altered, the consequence was a greater shift in *D* as apposed to that observed for *H'* (data not shown). Alternately, *H'* represents an index that is less affected by changes in the composition of rare and dominant bands, thereby accommodating a broader weighting of these components (Hill, 1973) and representing an intermediate range between *S* and *D* (Hill *et al.*, 2003). Furthermore, values of *H'* allows for the application of t-tests for statistical comparisons by virtue of their normal distribution (Hill et al., 2003). With this in mind *H'* was subsequently applied as the defining diversity index used in subsequent experiments.

Chapter Six

6. PCR-DGGE Characterization of Bacterial Associations from Soil Microcosms Arrays perfused with a Synthetic Landfill Leachate at two Hydraulic Loading Rates (HLRs)

6.1 Experimental

The experimental set-up of the soil microcosm arrays and their operating parameters have been previously described (Chapter Four).

6.1.1 Microcosm harvesting and soil sampling

When the respective arrays of microcosms had reached a redox state deemed to represent the redox potentials expected of nitrate reducing, sulphate reducing, and methanogenic environments, each column constituting an array was removed and destructively sampled (weeks 12, 32, 52, 80) (Appendix B). Firstly, silicone seals were broken and the rubber bungs removed from both ends of each column. The soil within the glass column was gently pushed out using a circular wooden plunger to ensure an equal distribution of pressure. Each soil column was dissected into three equal sections of ± 50 grams (wet weight). A total of twelve samples were taken from four columns making up a single soil microcosm array at the following depths (cm): 1.5, 4.5, 7.5, 10.5, 13.5, 16.5, 19.5, 22.5, 25.5, 28.5, 31.5, and 34.5. The duplicate soil samples were pooled and thoroughly mixed before storage at 2-4 ºC prior to performing DNA extraction (2.5.1.2) and PCR-DGGE (2.5.3 and 2.5.4).

6.1.2 Data analysis of DGGE images

Captured DGGE images were analyzed with QuantityOne® 1-D Analysis Software (Bio-Rad) to determine band numbers and average signal intensities of each band across the gels. Cluster analysis and dendrogram construction was performed using the unweighted pair-group method using arithmetic averages to illustrate lane similarities within gels.

Canonical correspondence analysis (CCA) (CANOCO 4.5 - Ter Braak and Šmilauer, 1997) was used to establish the influence of depth on the Bacterial composition of each microcosm. Microbial community profiles were analyzed at various depths in each microcosm based on band position and the relative intensity of each band for all samples. The statistical significance of the depth-community composition relationship was assessed by the Monte Carlo permutation test using 499 random permutations for each gel.

The Shannon-Weaver (*H'*) Index was used to assess the microbial diversity prevalent in the treated soil microcosms. The related Shannon-Weaver Evenness Index (E_H) was used to measure the distribution of the species (bands) comprising a community (sample) (2.5.6.1).

General Analysis of Variance (GenStat Release 8.1) was used to compare mean Species Richness (*S*), Shannon-Weaver Index (*H'*), and Shannon-Weaver Evenness Index (E_H) over the four sampling times for both hydraulic loading rates (HLR).

The two sample pair-wise t-test (GenStat Release 8.1) was used to determine the significance of differences between the mean Species Richness (*S*), Shannon-Weaver Index (*H'*), and the Shannon-Weaver Evenness Index (E_H) recorded for the two different hydraulic loading rates (HLR) at each sampling time.

6.1.3 Comparative analysis of redox, pH, and phenol landfill leachate data with bacterial diversity data

Redox, pH, and phenol leachate data generated from the respective soil microcosm arrays (Chapter Four) were subjected to area under the curve (AUC) calculations (GenStat Release 8.1). In total, four values corresponding to the four sampling times were generated for each HLR treatment. Mean Bacterial diversity data (*S*, *H'*, and E_H) was generated by calculating the mean of each diversity measure recorded for each soil array by PCR-DGGE at the four sampling times. This mean diversity data, together with the AUC data generated for redox, pH, and phenol was subjected to analysis on PlotIT 3.2 to generate 3-D graphs to illustrate the relationship between redox, pH, and phenol characteristics of the effluent landfill leachate on the Bacterial diversity of the leached soil arrays. Further to this, Spearman Rank Correlation coefficients (ρ) (GenStat Release 8.1) were calculated to

determine the relationship between effluent leachate redox potential, pH, and phenol concentration.

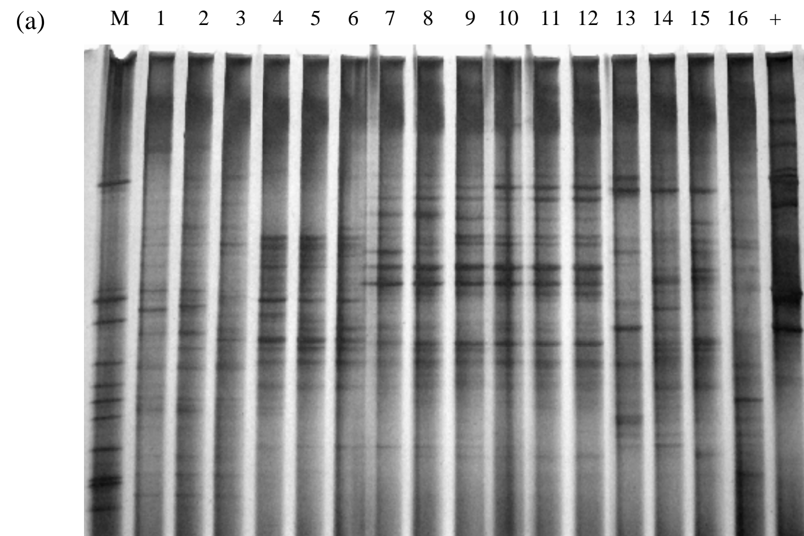
6.2 Results and Discussion

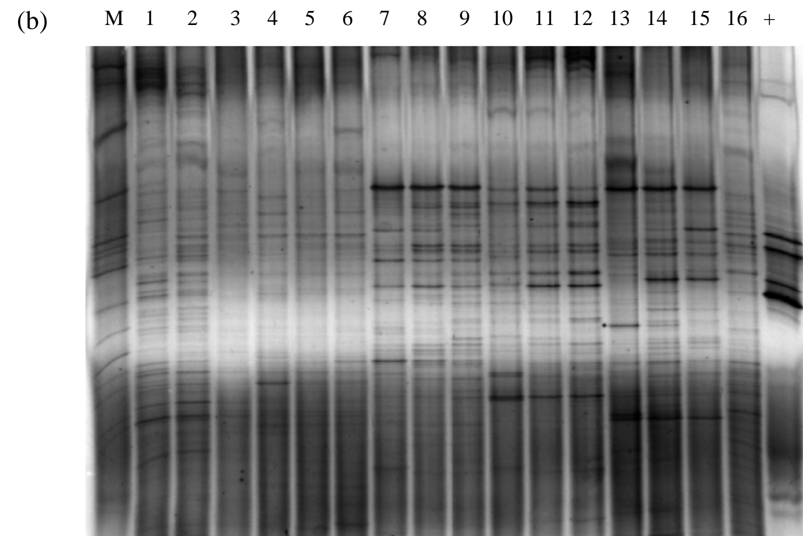
6.2.1 Analysis of bacterial community profiles from soil microcosm arrays at two hydraulic loading rates (HLRs) over time by DGGE

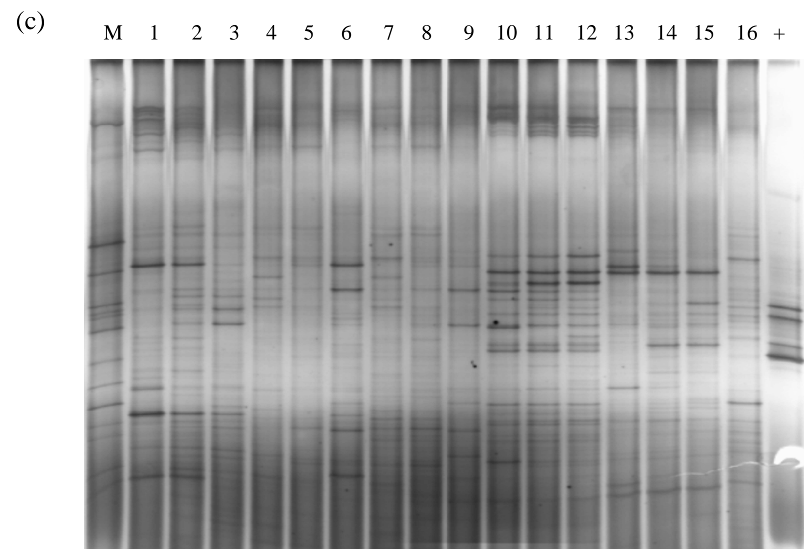
6.2.1.1 Analysis of temporal and spatial changes in bacterial community structure

The DGGE community profiles generated from soil microcosm arrays for each HLR over the experimental period are shown in Plates 6.1 and 6.2. The reproducibility of PCR and DGGE profiles for the DGGE marker (M) and *Bacillus* (+) soil isolate was found to be qualitatively satisfactory.

Plate 6.1 **Denaturing gradient gels showing PCR-amplified 16S rDNA amplicons from four soil microcosm arrays perfused with synthetic landfill leachate at an elevated hydraulic loading rate (HLR*h*). Microcosms were destructively sampled at week 12 (a), 32 (b), 52 (c), and 80 (d) and was sectioned into twelve portions ranging from 1.5 cm to 34.5 cm(Lanes 1 – 12). Lane: (M) DGGE Marker consisting of 13 clones (H.W van Verseveld, Vrije Universiteit van Amsterdam, Netherlands); (13-15) control microcosm perfused with de-ionized water and sampled at depths of 1.5, 4.5, and 7.5 cm; (16) sample of original, untreated soil; and (+) *Bacillus* isolate from soil.**


(a)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 +


(b)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 +


(c)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 +

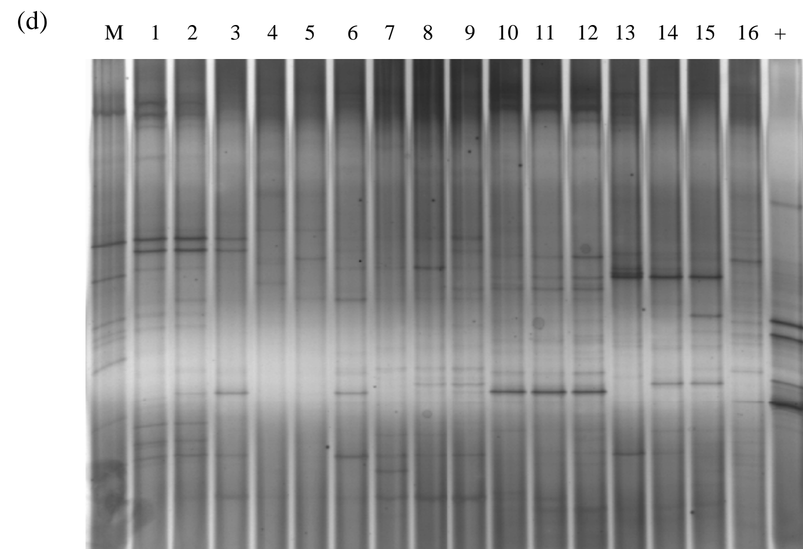
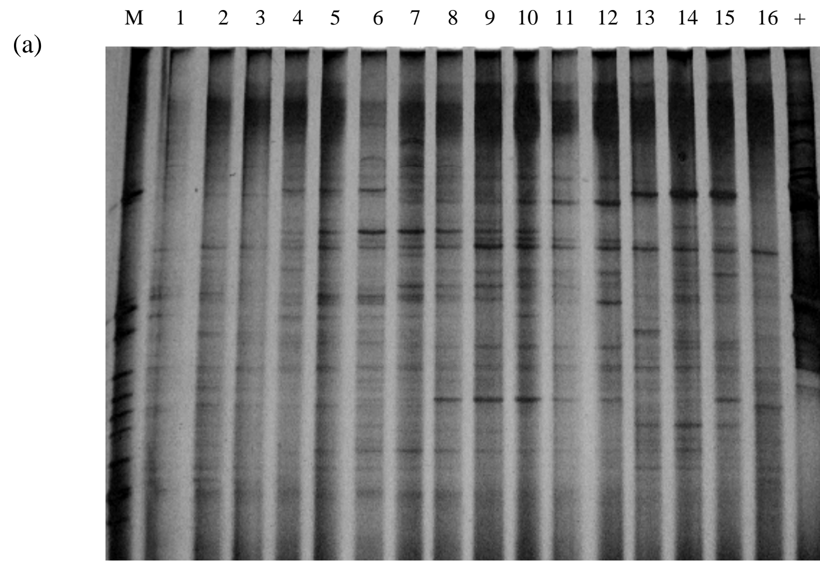
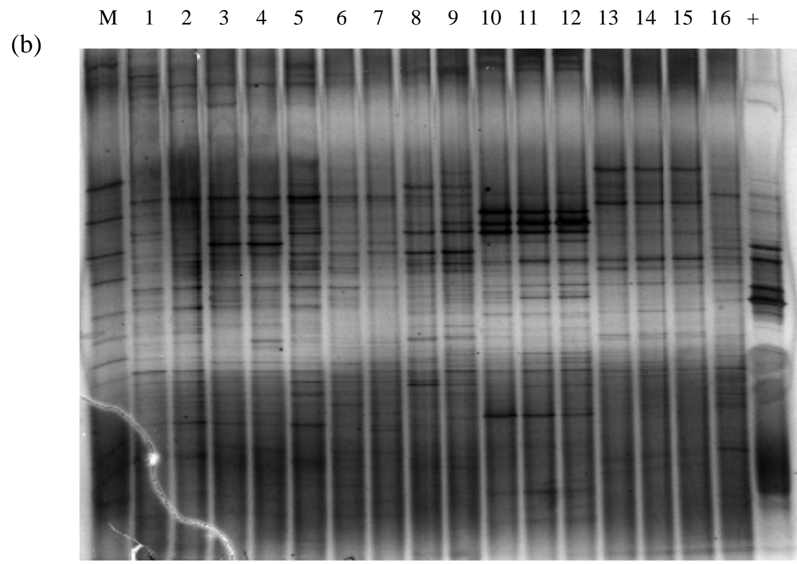

(d)
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 +

Plate 6.2 **Denaturing gradient gels showing PCR-amplified 16S rDNA amplicons from four soil microcosm arrays perfused with synthetic landfill leachate at a lower hydraulic loading rate (HLR*l*). Microcosms were destructively sampled at week 12 (a), 32 (b), 52 (c), and 80 (d) and was sectioned into twelve portions ranging from 1.5 cm to 34.5 cm(Lanes 1 – 12). Lane: (M) DGGE Marker consisting of 13 clones (H.W van Verseveld, Vrije Universiteit van Amsterdam, Netherlands); (13-15) control microcosm perfused with de-ionized water and sampled at depths of 1.5, 4.5, and 7.5 cm; (16) sample of original, untreated soil; and (+) *Bacillus* isolate from soil.**

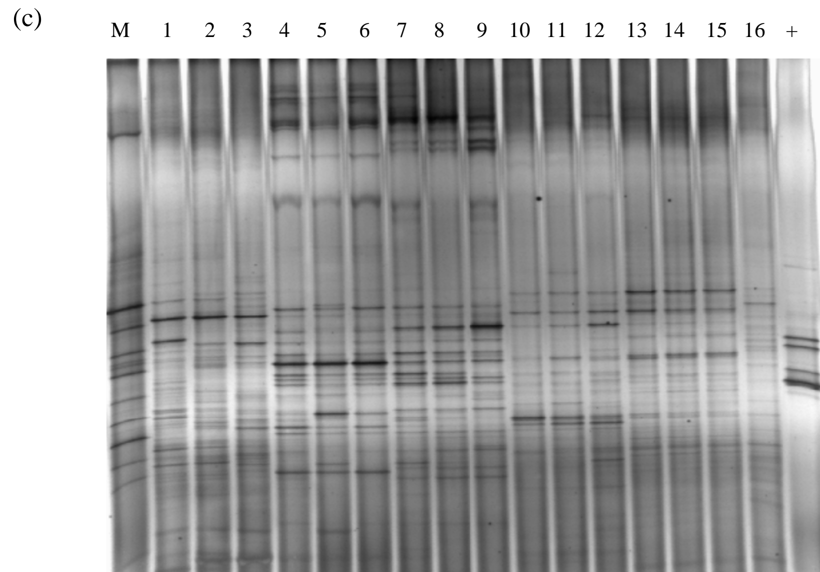
(a)



(b)



(c)



(d)

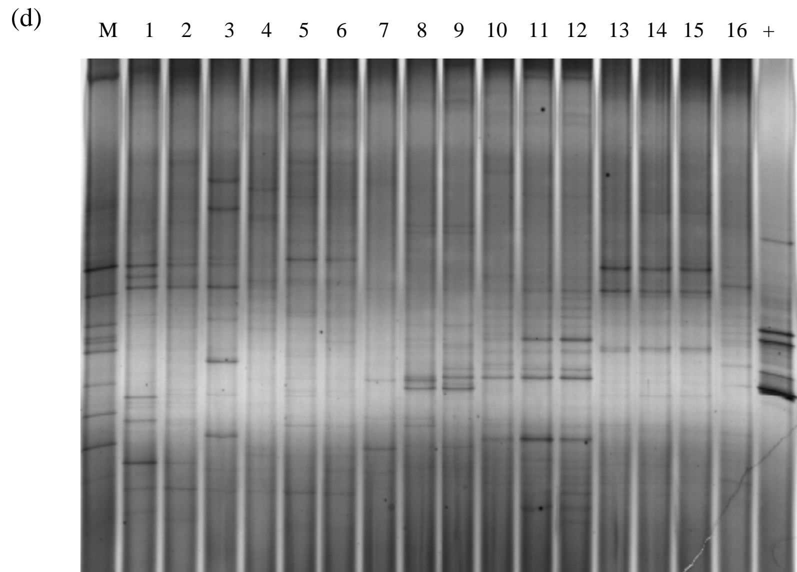
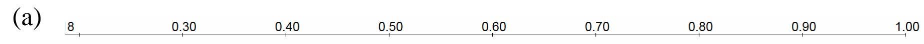
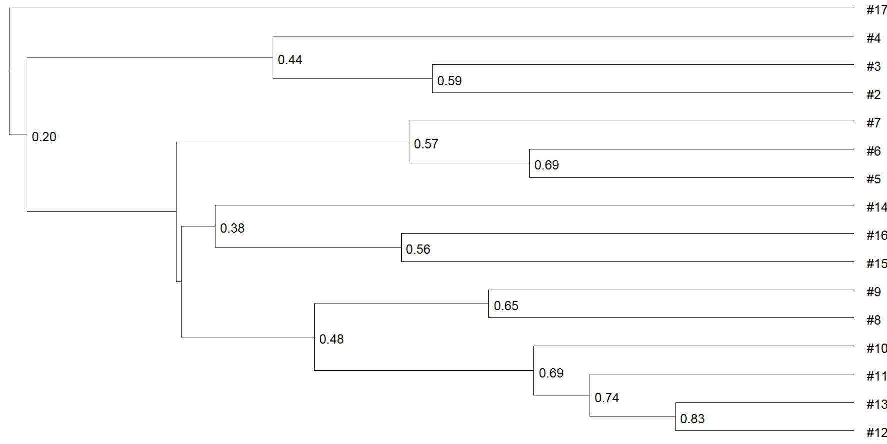


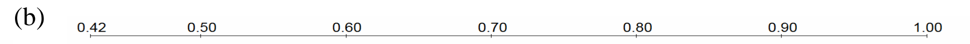
Figure 6.1 **Dendrogram analysis (UPGMA) of DGGE bacterial community profiles of the four soil microcosm arrays perfused with synthetic landfill leachate at an elevated hydraulic loading rate (HLR*h*). Microcosms were destructively sampled at weeks 12 (a), 32 (b) 52 (c), and 80 (d) and then sectioned into twelve portions (# 2 – #13) each representing the following depths in the soil profile: 1.5, 4.5, 7.5, 10.5, 13.5, 16.5, 19.5, 22.5, 25.5, 28.5, 31.5, and 34.5 (cm). Control microcosm perfused with de-ionized water (#14-16) was sampled at depths 1.5, 4.5, and 7.5 cm. The original, untreated soil was represented by #17.**

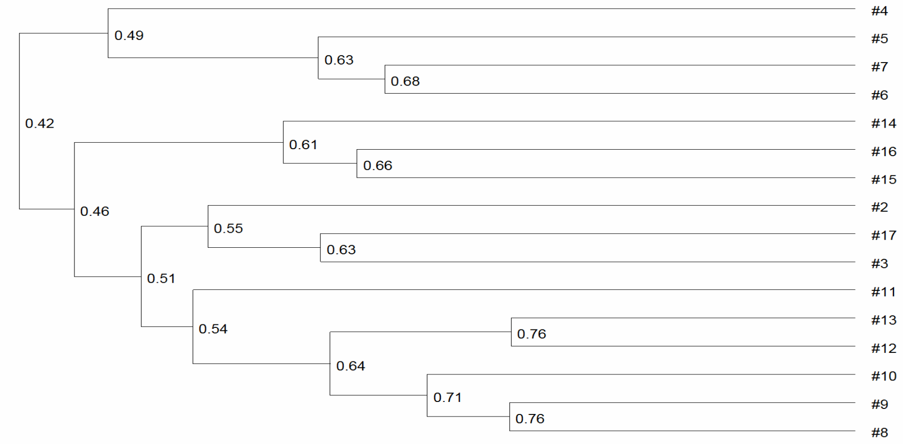
(a)



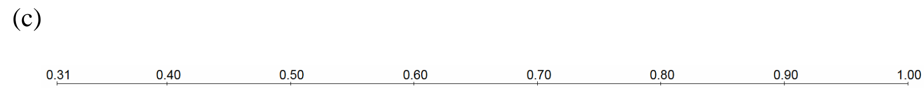


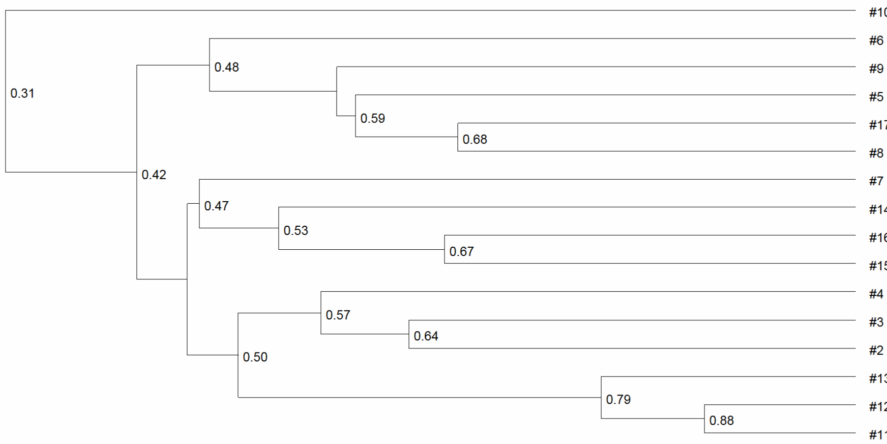
(b)



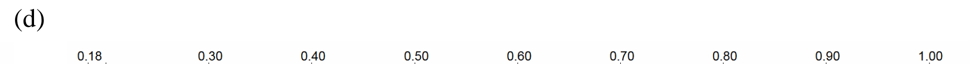


(c)





(d)



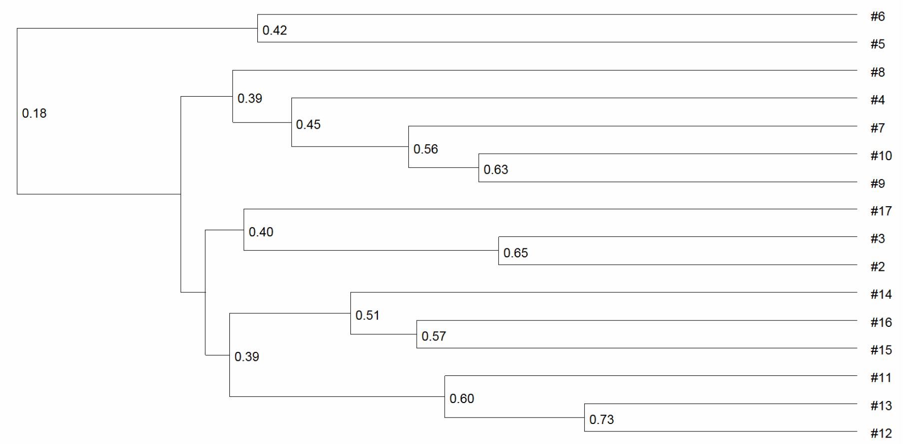
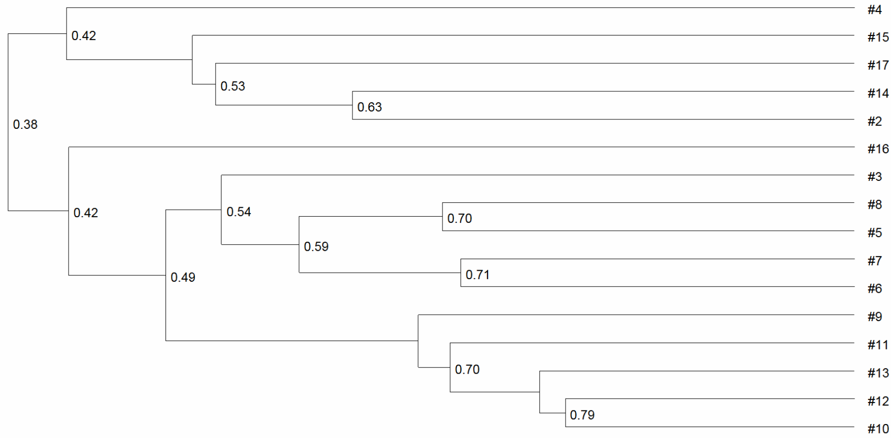
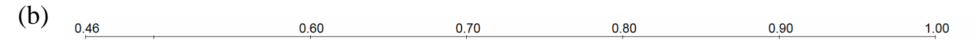


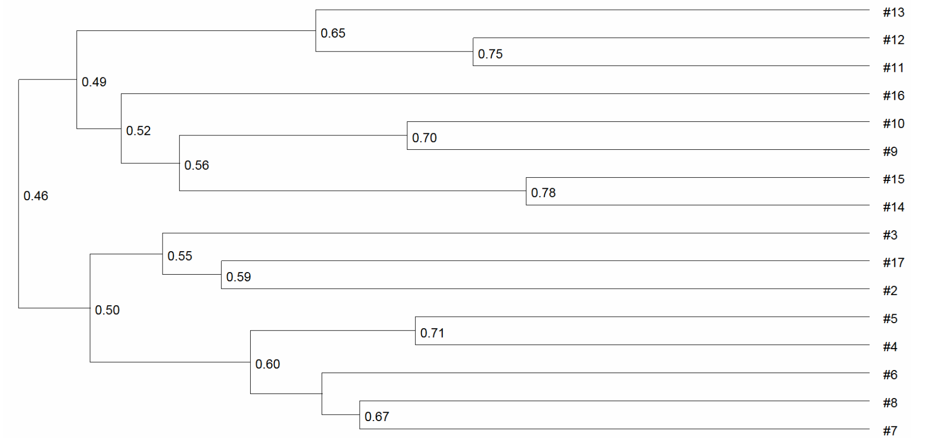
Figure 6.2 **Dendrogram analysis (UPGMA) of DGGE bacterial community profiles of the four soil microcosm arrays perfused with synthetic landfill leachate at a lower hydraulic loading rate (HLR*l*). Microcosms were destructively sampled at weeks 12 (a), 32 (b) 52 (c), and 80 (d) and then sectioned into twelve portions (# 2 – #13) each representing the following depths in the soil profile: 1.5, 4.5, 7.5, 10.5, 13.5, 16.5, 19.5, 22.5, 25.5, 28.5, 31.5, and 34.5 (cm). Control microcosm perfused with de-ionized water (#14-16) was sampled at depths 1.5, 4.5, and 7.5 cm. The original, untreated soil was represented by #17.**

(a)

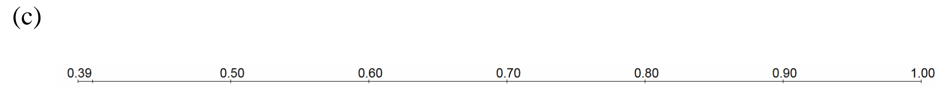


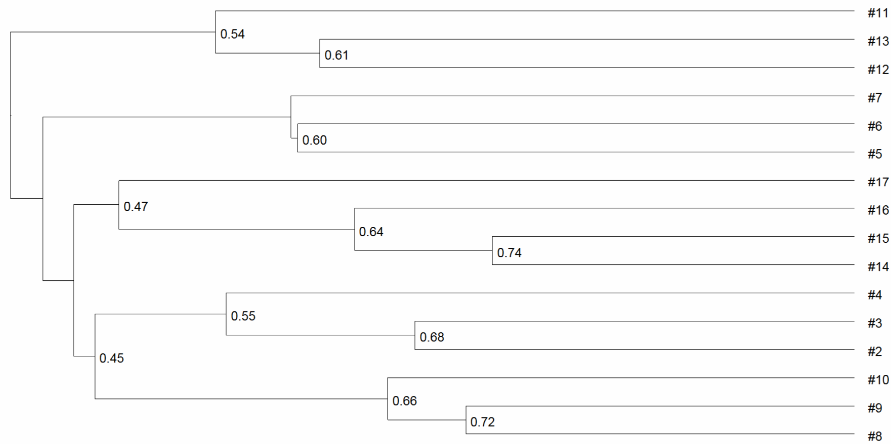
(b)



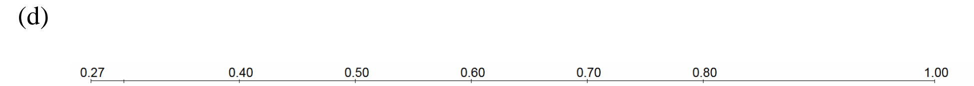


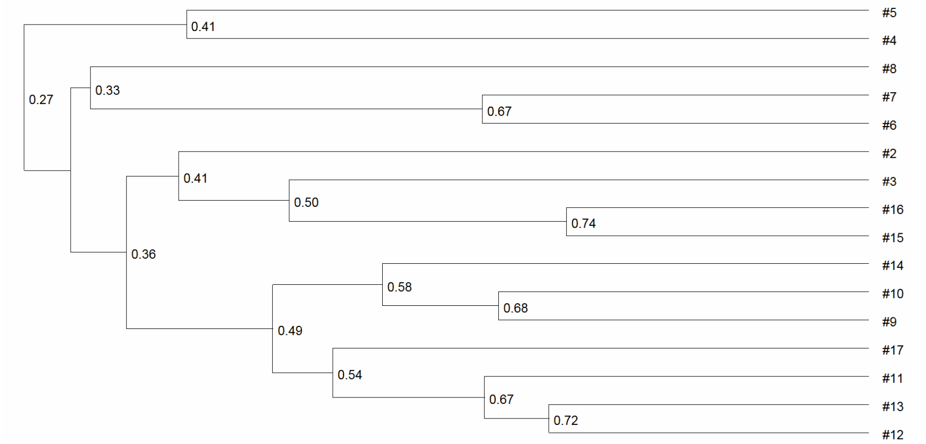
(c)





(d)





Cluster analysis dendrograms of these DGGE profiles are shown in Figure 6.1 and 6.2. The samples of both treatments generally clustered with depth at each sampling interval, indicating the localized successional changes occurring within each array over time. The influence of depth on bacterial succession was also demonstrated on a diesel contaminated site, where an increase in soil depth produced a decrease in hydrocarbon degrading bacteria (Maila, Randima, Surridge, Drønen and Thomas, 2005).

However, the effect of depth was less evident in arrays A*l* and B*l* sampled at weeks 12 (Figure 6.2a) and 32 (Figure 6.2b), respectively. The relatedness observed for these two arrays was relatively erratic compared to the clustering observed for arrays C*l* (Figure 6.2c) and D*l* (Figure 6.2d). It was assumed that the effects of a lower HLR of landfill leachate on the soil bacterial composition would become evident at a slower rate as a consequence of the slower supply of organic and inorganic compounds to the developing succession of bacterial communities. For example, treatment HLR*h* and HLR*l* showed initial rooting of the dendrograms at 18 % and 38 %, respectively (Figures 6.1a and 6.2a). The overall relatedness increased to 42 % (HLR*h*) (Figure 6.1b) and 46 % (HLR*l*) (Figure 6.2b) followed by a decrease to 31 % (Figure 6.1c) and 39 % (Figure 6.2c) after 52 weeks. Figures 6.1d and 6.2d show a further decrease to 16 % (HLR*h*) and 27 % (HLR*l*) in overall sample relatedness for both treatments at the final week of sampling. These results suggest that the bacterial community structures initially become less distinct over time, before diverging in their overall relatedness. Moreover, the commuities in the arrays perfused at HLR*l* show less of a change in cluster rooting than that observed for arrays perfused at HLR*h*. Indeed, Wünsche, Bruggemann, and Babel (1995) showed that changes in the concentration of available carbon result in modifications to substrate utilization patterns by prevalent microorganisms and this in addition to existing selection pressures, associated with increasing depth, plays a major role in determining the surviving microbial complement (Maila *et al.*, 2005).

Overall cluster analysis revealed that the number and type of bacteria occurring at the selected depths of the soil subsurface varies. The duration of and rate at which landfill leachate is perfused through the soil microcosms appeared to have an effect on the rate at which bacterial compositions stabilize. The differences observed for the two HLRs were attributed to differences in nutrient and terminal electron acceptor availability at various depths in the soil profile. As a result, distinct physico-chemical microenvironments could

have established leading to differences in microbial composition at different times. Indeed, researchers investigating the microbial diversity of soil under varied conditions concluded that the prevaling conditions as well as the resources available in a soil environment has a major influence on the functional and numerical diversity of the existing microbial populations (Zhou, Xia, Treves, Wu, Marsh, O'Neil, Palumbo, and Tiedje, 2002).

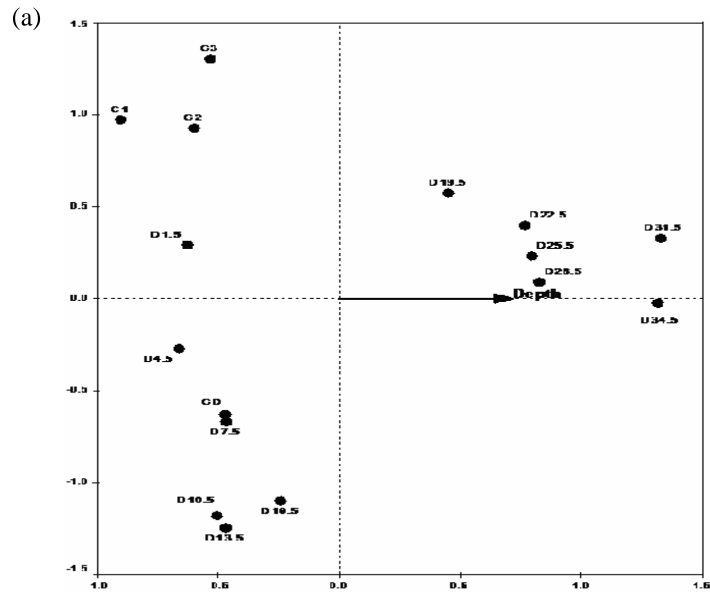
Unlike UPGMA cluster analysis, which only relates samples with similar banding patterns on DGGE profiles, multivariate analyses such as canonical correspondence analyses (CCA) takes into consideration individual band intensities while correlating the resultant banding patterns with the environmental variables affecting the banding profiles (Salles, van Veen, and van Elsas, 2004). CCA of the DGGE profiles across all sampling times and both treatments revealed a significant relationship between soil depth and Bacterial composition (Figure 6.3 and 6.4). Environmental variables such as redox potential, pH, concentration of anions and available carbon where not taken into account because of limitations in the experimental design. The relationship between depth and Bacterial composition of the treated arrays was not random and this was reflected in the correlations and levels of statistical significance achieved with the CCA (Table 6.1). CCA confirmed the cluster relationship recognized by UPGMA analysis. The samples tended to cluster relative to depth for both treatments and displayed a progressively linear change in Bacterial composition from one sample to the next with increasing depth (data displaying the change in composition with depth measured along a CCA-axis is not displayed).

Table 6.1 **Correlation coefficients and levels of significance achieved after subjecting DGGE band density data to CCA and the Monte Carlo Permutation Test. The test assessed the significance of depth on the Bacterial composition in the respective soil arrays over time and at two HLRs.**

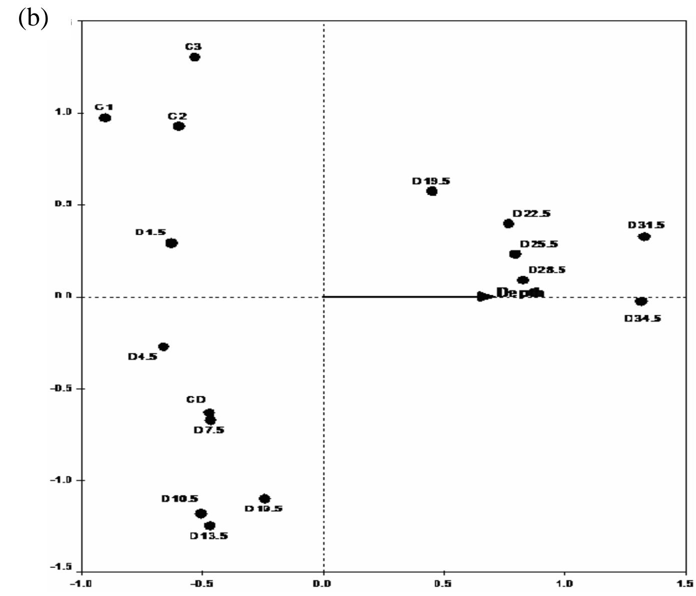
Treatment	Sampling Time (weeks)	Correlation	p-value
HLR*h*	12	0.955	0.006
	32	0.955	0.006
	52	0.910	0.0740
	80	0.927	0.010
HLR*l*	12	0.964	0.002
	32	0.945	0.004
	52	0.948	0.010
	80	0.948	0.004

Figure 6.3 **First two axes of a canonical correspondence analysis (CCA) revealing the effects of soil depth (→) on Bacterial composition of soil samples (•) treated at loading rate HLR*h*. D1.5-D34.5 represents the depth of samples in an array. C1, C2, and C3 represent the control microcosms perfused with de-ionized water. CD was the original, untreated soil. Plots represent destructive sampling of arrays at different times (weeks): (a) 12; (b) 32; (c) 52; and (d) 80.**

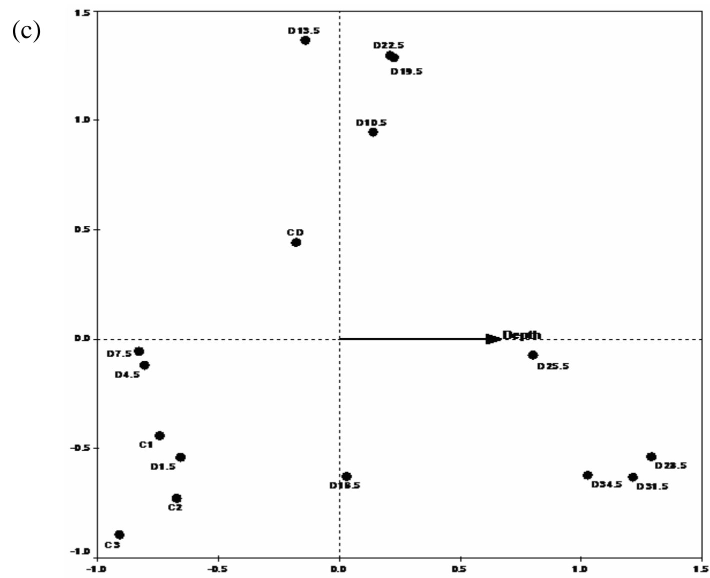
(a)



(b)



(c)



(d)

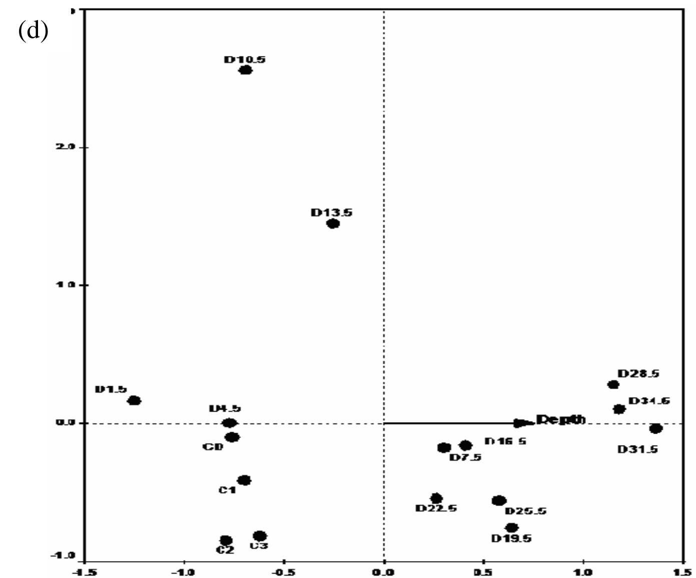
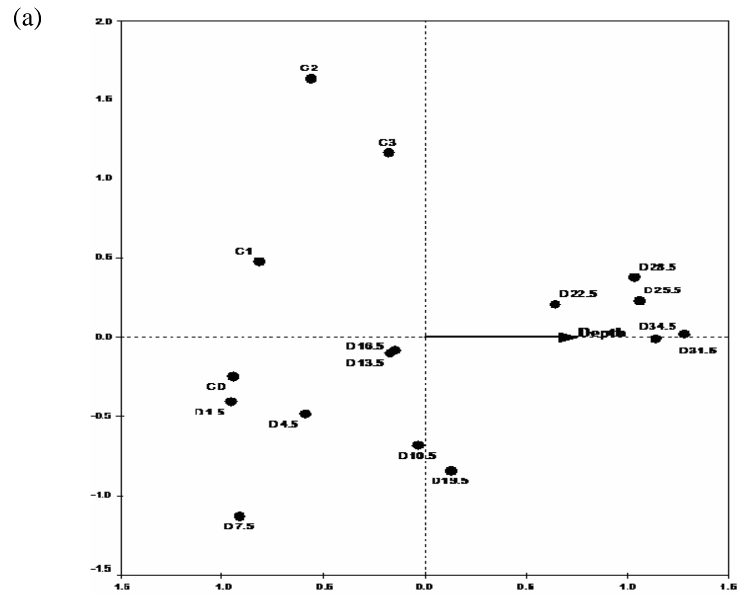
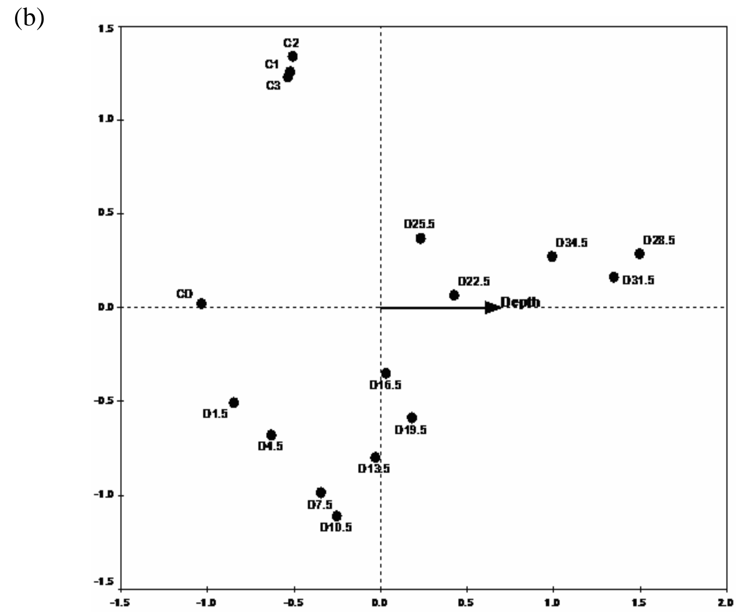
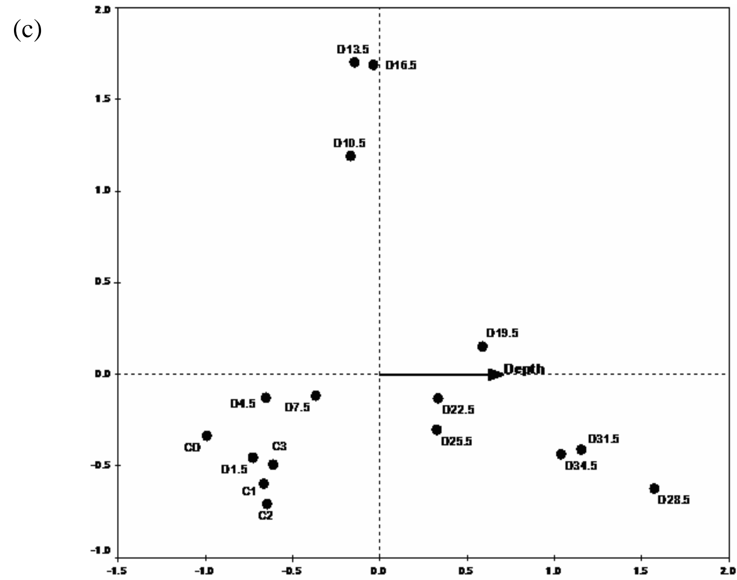
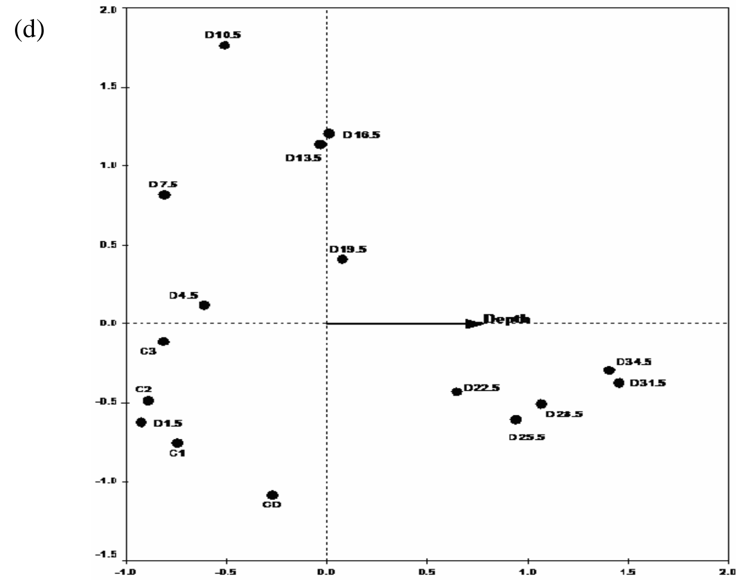


Figure 6.4 **First two axes of a canonical correspondence analysis (CCA) revealing the effects of soil depth (→) on Bacterial composition of soil samples (•) treated at loading rate HLR*l*. D1.5-D34.5 represents the depth of samples in an array. C1, C2, and C3 represent the control microcosms perfused with de-ionized water. CD was the original, untreated soil. Plots represent destructive sampling of arrays at different times (weeks): (a) 12; (b) 32; (c) 52; and (d) 80.**


(a)
C2
C3
C1
D28.5
D22.5
D25.5
D34.5
Depth
D16.5
D13.5
D31.5
CD
D1.5
D4.5
D10.5
D19.5
D7.5


(b)
C2
C1
C3
D25.5
D34.5
D28.5
D22.5
D31.5
CD
Depth
D16.5
D1.5
D19.5
D4.5
D13.5
D7.5
D10.5


(c)
D13.5
D16.5
D10.5
D19.5
Depth
D4.5
D7.5
D22.5
CD
C3
D25.5
D31.5
D1.5
D34.5
C1
C2
D28.5


(d)
D10.5
D16.5
D13.5
D7.5
D19.5
D4.5
Depth
C3
D34.5
D31.5
C2
D22.5
D1.5
D28.5
C1
D25.5
CD

6.2.1.2 Bacterial community diversity

There are several ecological diversity equations available for comparing bacterial diversity within and between communities. Hill *et al.* (2003) presents a detailed review on a range of ecological indices previously used to describe bacterial community diversity. For reasons stipulated in Chapter Five (5.2.1.4, Page 113, paragraph 2), this section of the study focuses on the Shannon-Weaver Index (*H'*) and its derivation; the Shannon-Weaver Evenness Index (E_H).

6.2.1.2.1 Species richness (*S*)

All four of the soil microcosms belonging to the two leachate treatments (HLR*h* and HLR*l*) showed similar trends in *S* throughout the investigation (Figure 6.5). The general *S*-Depth (Figure 6.5) profiles generated for each microcosm (A; B; C; and D) at the designated sampling times showed an overall increase in band number from week 12 (T1) to week 32 (T2) followed by a decrease measured at week 52 (T3) and a further decline in overall *S* with depth calculated at week 80 (T4). This trend was evident in both leachate treatments. The initial increase in *S* extended from the surface of all arrays of treatment HLR*h*, culminating in the peak *S* measured at a depth of 4.5 cm (Figure 6.5a). The same pattern was evident for arrays A*l* and D*l*, sampled at T1 and T4, respectively. Peak *S* for the arrays B*l* and C*l* were recorded at a depth (cm) of 13.5 and 7.5, respectively (Figure 6.5b). *S* for both treatments reflected an initial increase with depth, thereafter *S* decreased with depth at sampling times T2 and T3, followed by a further decrease in *S* recorded at T4, however overall *S* never decreased below the initial *S* recorded at T1. Williams and Higgo (1994) found that anaerobic and heterotrophic organisms decreased with depth in an aquifer contaminated by industrial effluents, and concluded that the high concentrations of pollutants had become increasingly bacteriocidal, and this coupled to the reduction in electron acceptors and vital nutrients with depth had caused the depletion of bacterial populations with depth. Ludvigsen *et al.* (1999) discovered that there was a greater proportion of active microbial biomass closest to the landfill, thriving in response to the entering pollutants, for the purpose of this study the top end of the soil arrays were seen as the area closest to the landfill and as such mean *S* was highest at this end of the arrays.

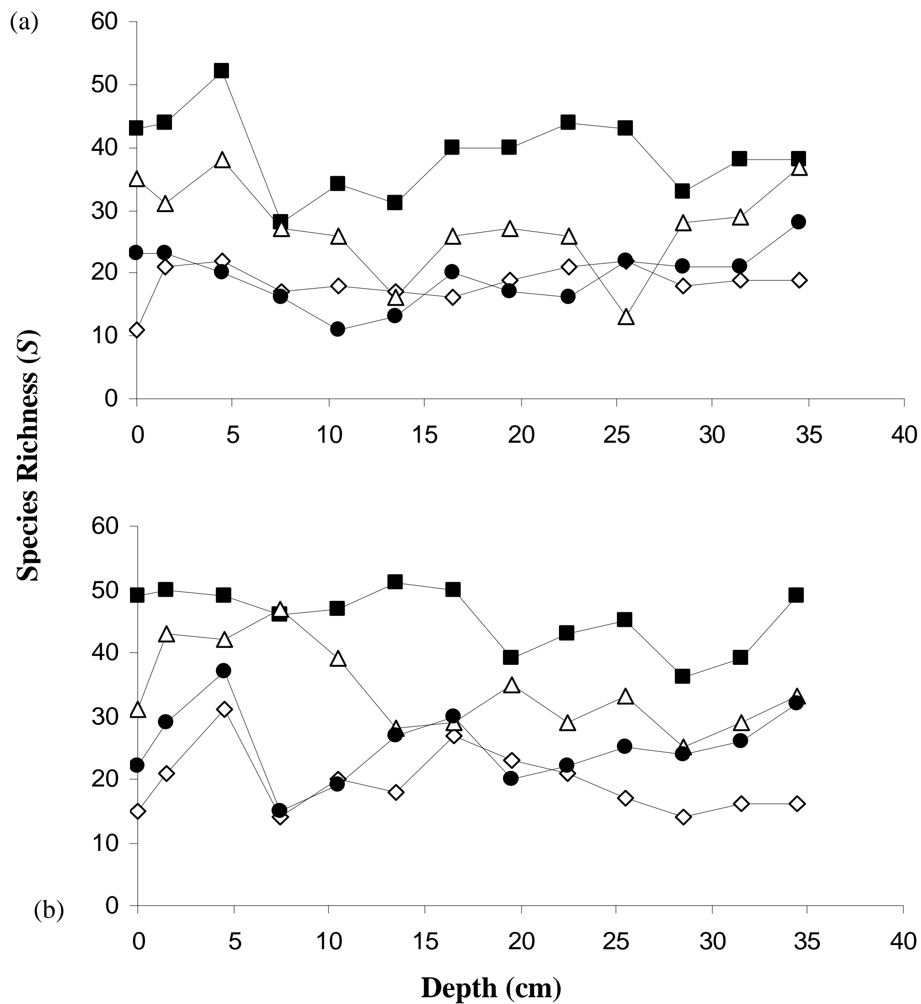


Figure 6.5 Changes in the Bacterial species richness (*S*) over depth and time. Symbols correspond to destructively sampled soil arrays A (◊), B (■), C (∆), and D (●) at times (weeks): 12 (T1); 32 (T2); 52 (T3); and 80 (T4), respectively. (a) Treatment HLR*h* and (b) Treatment HLR*l*.

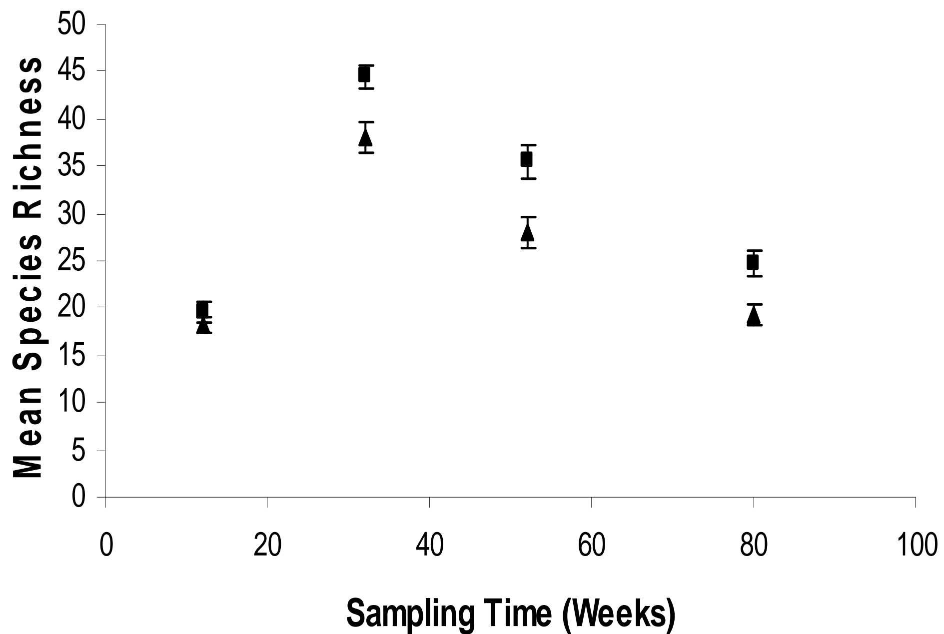


Figure 6.6 Changes in the mean species richness measured at weeks 12 (T1), 32 (T2), 52 (T3), and 80 (T4) in soil arrays A, B, C, and D, respectively. Symbols correspond to treatments HLR*h* (▲) and HLR*l* (■).

The mean *S* recorded for treatment HLR*h* at T1 was significantly different from that recorded at T2 and T3 (F=<0.001) but not significantly different from that recorded at T4 (Table 6.2). In contrast, mean *S* recorded for treatment HLR*l* was significantly different (F=<0.001) at all sampling times (Table 6.2). Mean *S* was highest in array B, followed by arrays C, D, and A for both treatments.

Bacterial growth and incidence of organic contamination share a close correlation (Arora, Linde, Revil, and Castermant, 2007). The periodic supply of leachate to each set of arrays ensured a supply of substrates required for bacterial growth. This then appeared to lead to an increase in the overall *S* for both treatments (T1-T3). However it is feasible that eventually, the supply of phenol containing leachate became toxic leading to a decrease in *S* as observed in treatment HLR*h*. The non significant change in *S* observed at T1 and T4 supports this explanation. The difference in *S* observed for treatment HLR*l* at T1 and T4 remained significant by virtue of a larger difference in *S*, lending further support to the theory of toxicity under higher leachate loading rates.

Table 6.2 **General Analysis of Variance comparing the mean Species Richness (*S*), Shannon-Weaver Index (*H'*), and the Shannon-Weaver Evenness Index (*E*) over time, and for two different hydraulic loading rates (HLR).**

Treatment	**Sampling Times**	**Mean Measures of Diversity**		
		S	*H*	*E*
HLR*h*	Time 1	18.19^{a}	2.750^{a}	0.9502^{a}
	Time 2	38.06^{c}	3.407^{c}	0.9402^{ab}
	Time 3	27.94^{b}	3.105^{b}	0.9417^{ab}
	Time 4	19.25^{a}	2.683^{a}	0.9157^{b}
F-value		<0.001	<0.001	0.067
l.s.d		3.750	0.1669	0.02649
s.e.d		1.875	0.0835	0.01325
HLR*l*	Time 1	19.56^{a}	2.779^{a}	0.9409^{a}
	Time 2	44.50^{d}	3.540^{d}	0.9341^{a}
	Time 3	35.50^{c}	3.344^{c}	0.9410^{a}
	Time 4	24.81^{b}	2.968^{b}	0.9306^{a}
F-value		<0.001	<0.001	0.856
l.s.d		3.930	0.1688	0.02887
s.e.d		1.964	0.0844	0.01444

The mean *S* recorded along the vertical profile of the soil arrays were compared for both leachate treatments at the four sampling intervals (Figure 6.6). Treatment HLR*l* showed consistently higher mean *S* over the four sampling times. Both treatments followed the same trend regarding *S*, an initial increase in mean *S*, peaking at T2, followed by a steady decrease at T4. The difference in mean *S* recorded for both treatments at T1 was not significant (P=0.283), however significant differences between the treatments were found at T2 (P=0.002), T3 (P=0.002), and T4 (P=<0.001) by virtue of the pairwise-t-test. This adds to the body of evidence supporting the conclusion that pollutants supplied at a higher HLR become increasingly toxic to the existent Bacterial communities, thereby affecting the number of surviving/thriving species (*S*). A band (species) initially detected using PCR-DGGE may not be adequately amplified to generate an intensity significant enough to warrant detection in a sample later on in the investigation, even though that band may still be present (Jackson *et al.*, 2001). In this investigation the number of samples made it impractical for comparison of replicated samples over time since samples were analyzed on different DGGE gels over the course of the investigation.

6.2.1.2.2 Shannon-Weaver index of diversity (H')

The influence of soil depth on *H'* for both leachate loading rates is shown in Figure 6.7. All soil arrays of both treatments follow similar trends with depth, with the major difference being the change in *H'* at each depth over the four sampling times. The highest *H'* values at each depth were produced by array B (week 32) of both treatments, subsequently followed by arrays C (week 52), A (week 12), and D (week 80) for treatment HLR*h* and arrays C, D, and A for treatment HLR*l*.

Redox conditions within each soil array were found to be dynamic, constantly changing over time. This was attributed to change in chemical and microbial compliments. This constant state of flux could therefore be expected to affect the metabolic response of the resident microorganisms. Indeed, Løngborg *et.al.* (2006) concluded that the rate of xenobiotic compound degradation varied depending on prevailing redox conditions. Therefore, it stands to reason that the redox changes recorded for the soil arrays over time could have triggered a change in the metabolic capabilities and composition of predominant microorganisms leading to changes in the *H'* over time and depth. The general decrease in *H'* with depth over the period of investigation was more pronounced in

arrays B and C; presumably the result of nutrient and electron acceptor supply and demand through the vertical profiles of the arrays. The migration of leachate pollutants through the soil profile can further contribute to lower *H'* values indicative of reduced bacterial diversity. Maila, *et al.* (2005) came to a similar conclusion when studying the microbial diversity of different soil layers at a site polluted by hydrocarbons.

The mean *H'* recorded for treatment HLR*h* at T1 was significantly different from that recorded at T2 and T3 (F=<0.001) but not significantly different from that recorded at T4 (Table 6.2). In contrast mean *H'* recorded for treatment HLR*l* was significantly different (F=<0.001) at all sampling times (Table 6.2). As with mean *S*, the mean *H'* was highest in array B, followed by arrays C, D, and A for both treatments. This is not surprising, since *S* and *H'* are positively correlated (Hill *et al.*, 2003).

A comparison of the mean *H'* recorded for each soil array of both treatments at the comparative sampling times revealed similar trends but different levels of diversity (Figure 6.7). At each of the four sampling intervals treatment HLR*l* showed higher mean *H'*. The trend was similar to that plotted by mean *S* over time, i.e. an initial increase in mean *H'*, peaking at T2, followed by a steady decrease to T4. If *H'* is expressed as $e^{H'}$ (Hill *et al.*, 2003), essentially, this implies that at T1; T2; T3; and T4 the samples reflected a mean *H'* corresponding to 15; 30; 22; and 14 equally abundant bands, respectively for treatment HLR*h*. By comparison, the mean *H'* reflected for treatment HLR*l* at the respective times was indicative of *H'*-values representing 16; 35; 28; and 19 equally abundant bands (*S*). Clearly, there are differences between these values and those recorded for *S* (Table 6.2). The difference between these calculated values and observed values was due to unevenness in the Bacterial populations comprising the samples and can be attributed to the difference in HLR observed between the two treatments.

The difference in mean *H'* recorded for both treatments at T1 was not significant (P=0.629), however, significant differences between the treatments were found at T2 (P=0.002), T3 (P=0.013), and T4 (P=<0.001) using the pairwise-t-test. A consequence of these findings is that a doubling of landfill leachate supply to the soil beneath a landfill could result in a significant decrease in *H'* over time. This would mean that the soil profile would become more characteristic of a pollutant perturbed system, made up of fewer, yet more pronounced, bacterial communities.

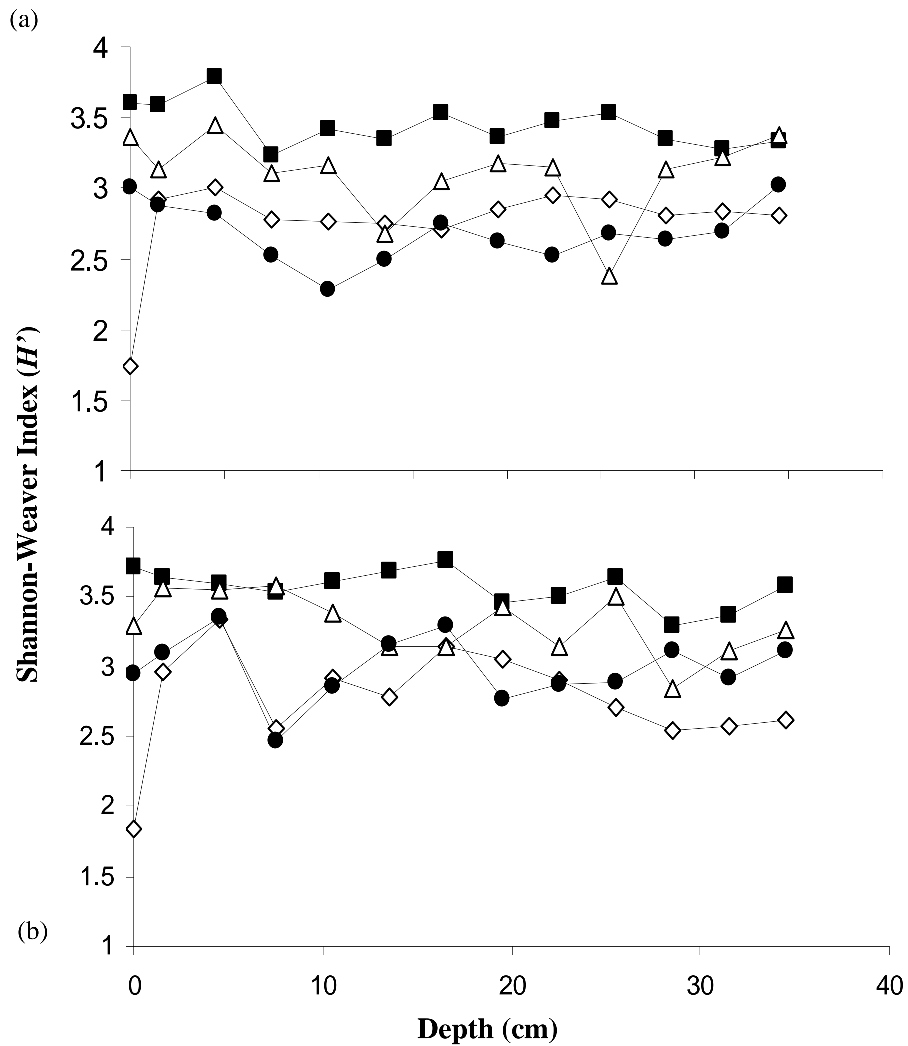


Figure 6.7 Changes in the Bacterial species diversity reflected by the Shannon-Weaver Index (*H'*) over depth and time. Symbols correspond to soil arrays A (◊), B (■), C (∆), and D (●) that were destructively sampled at times (weeks): 12 (T1); 32 (T2); 52 (T3); and 80 (T4), respectively. (a) Treatment HLR*h* and (b) Treatment HLR*l*.

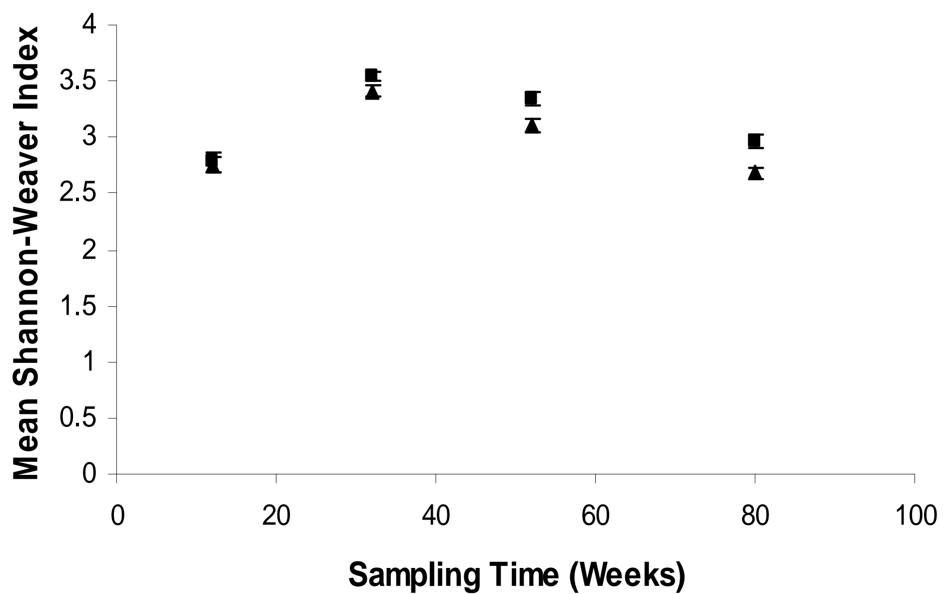


Figure 6.8 Changes in the mean Shannon-Weaver Index measured at weeks 12 (T1), 32 (T2), 52 (T3), and 80 (T4) in soil arrays A, B, C, and D, respectively. Symbols correspond to treatments HLR*h* (▲) and HLR*l* (■).

6.2.1.2.3 Shannon-Weaver Evenness index of diversity (E_H)

The relationship between depth and E_H over time for both HLR treatments are shown in Figure 6.9. The assumption with respect to E_H is that the most numerically equitable community must contain equal numbers of all species comprising the community, resulting in a E_H equal to one (Camargo *et al.*, 2005). In the context of this investigation this means that the most numerically equitable sample must contain DNA bands (species), each with equal signal intensities. The changes in E_H with depth revealed a stable trend for array A (T1) for both treatments. As time progresses the trend became more erratic along the soil profile depth for both treatments, indicative of changes in the microenvironment from one depth to another that contributes significantly to the changes in community evenness at each depth over time. At T1 there was a general increase in E_H with depth for both treatments, however, at the remaining sampling intervals E_H reflected a general decrease with depth for both treatments with this pattern being most prominent at T4. The general decrease in E_H from T1 to T4 for both treatments, suggests that there was an overall decrease in Bacterial diversity and that the conditions became favorable for the dominance of specific species.

The mean E_H recorded for both treatments over all sampling times showed no overall significant differences (Table 6.2). However, one could state that the overall level of significance excluding the effect of time on community evenness as being random was much smaller for treatment HLR*h* (F=0.067) in comparison to treatment HLR*l* (F=0.856). Evidence of this was observed in the difference between *E* at T1 and T4 for treatment HLR*h* (Table 6.2). Therefore, one could speculate that, given sufficient time, the effect of time on community evenness could become significant under the two leachate HLR treatments investigated.

A comparison of the mean E_H recorded for each soil array of both treatments at the comparative sampling times revealed similar trends but different levels of community evenness (Figure 6.10). Treatment HLR*h* showed greater community evenness over T1 and T2. At T3 the mean E_H recorded was the same for both treatments, followed by a more pronounced decrease in mean E_H for treatment HLR*h* in comparison to HLR*l*. This represented a deviation from the trajectories plotted for the previous measures of community diversity (mean *S* and *H'*) where treatment HLR*l* reflected constantly higher

mean S and H' over all sampling times. E_H can be expressed as $e^{H'}/S$ (Hill *et al.*, 2003), from this ratios of 0.86; 0.79; 0.80; and 0.76 were derived for T1, T2, T3 and T4 of the HLR*h* treatment. This means that of the S recorded for T1; T2; T3; and T4 (Table 6.2) of treatment HLR*h*, the unevenness of the mean species abundance (band density) gave each sampling time a mean value of 86 %; 79 %; 80 %; and 76 % of the expected mean E_H if all the species (bands) had an equal abundance at the respective sampling times. One can arrive at a similar conclusion for treatment HLR*l*, where unevenness accounted for 18 %; 23 %; 20 %; and 22 % of the mean species abundance at T1; T2; T3; and T4, respectively. There are numerous factors that contribute to the unevenness exhibited in each of the soil arrays. An investigation examining bacterial diversity by amplified ribosomal DNA restriction analysis (ARDRA) in zinc contaminated agricultural soils found decreasing bacterial diversity and associated evenness with increasing zinc pollution (Moffett, Nicholson, Uwakwe, Chambers, Harris, and Hill, 2003).

The difference in mean E_H recorded for both treatments at all sampling times was not significant by virtue of the pairwise-t-test. Mean E_H (Table 6.2) recorded at all sampling times and for both treatments was close to unity, indicating an even yet numerically dynamic distribution of members of the Bacterial community over time.

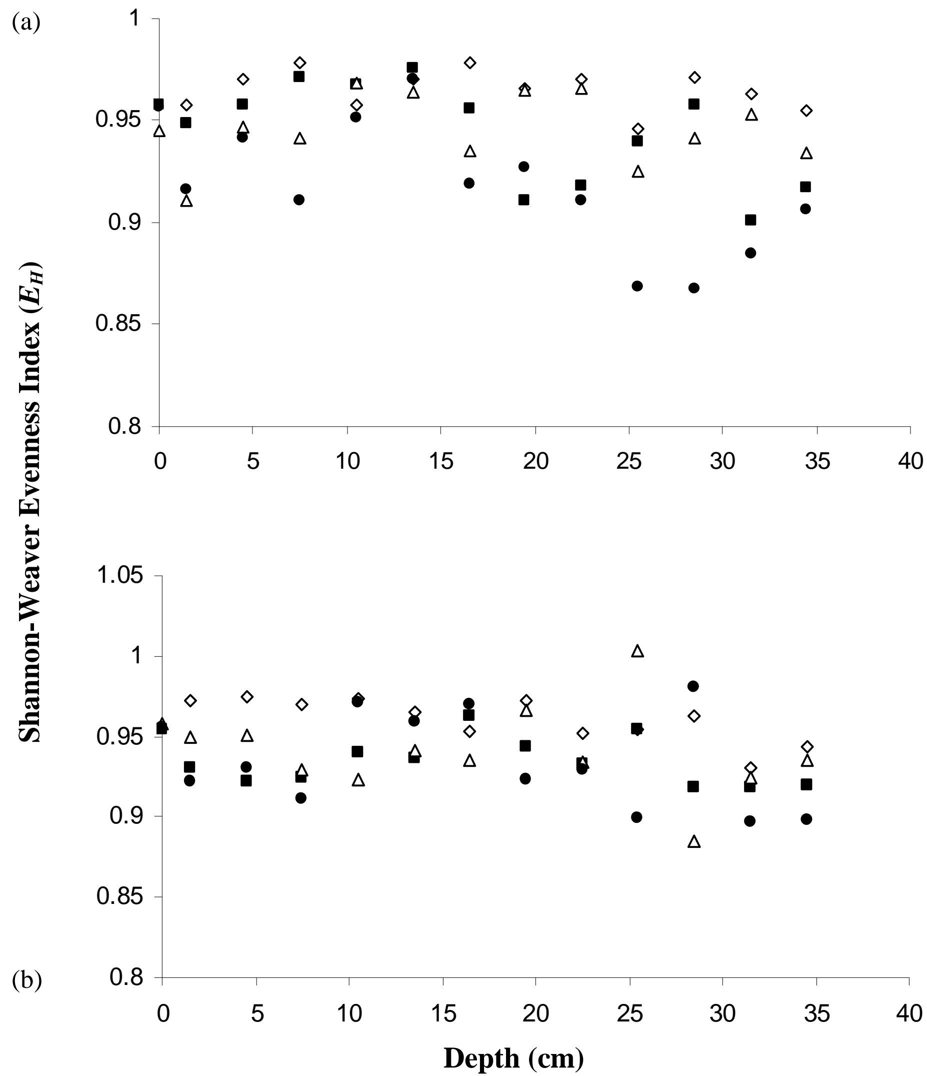


Figure 6.9 **Changes in the Bacterial species evenness reflected by the Shannon-Weaver Evenness Index (E_H') over depth and time. Symbols correspond to soil arrays A (◊), B (■), C (Δ), and D (●) destructively sampled at times (weeks): 12 (T1); 32 (T2); 52 (T3); and 80 (T4), respectively. (a) Treatment HLR*h* and (b) Treatment HLR*l*.**

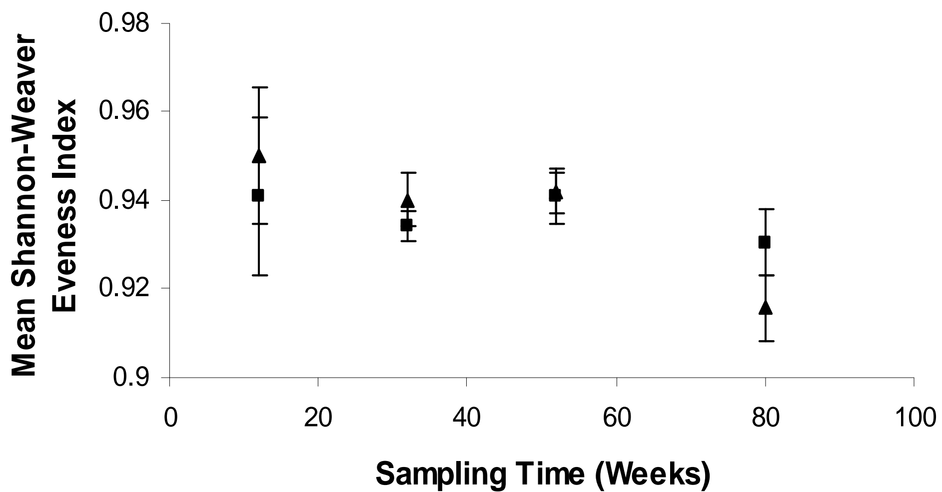


Figure 6.10 **Changes in the mean Shannon-Weaver Index measured at weeks 12 (T1), 32 (T2), 52 (T3), and 80 (T4) in soil arrays A, B, C, and D, respectively. Symbols correspond to treatments HLR*h* (▲) and HLR*l* (■).**

Table 6.3 **The significance of P-values of the two sample pair-wise t-test for comparing the Species Richness (*S*), Shannon-Weaver Index (*H'*), and the Shannon-Weaver Evenness Index (*E*) for two different hydraulic loading rates (HLR) over time**

Factors Compared	P-value			
	Time 1	Time 2	Time 3	Time 4
S_H vs S_L	0.283	0.002	0.002	<0.001
H'_H vs H'_L	0.629	0.002	0.013	<0.001
E_H vs E_L	0.055	0.356	0.926	0.116

6.2.1.3 The effects of the redox, pH, and phenol concentration of landfill leachate on the bacterial community diversity

6.2.1.3.1 Redox and pH

The influence of redox and pH changes on *S*, *H*, and E_H are represented in Figure 6.11. The three-dimensional plots relating changing redox and pH conditions with the average *S* over time reflect similar trajectories for treatments HLR*h* (Figure 6.11a) and HLR*l* (Figure 6.11b). However, the trends along the trajectories are more prominent in the latter treatment as apposed to the former. It is possible that the more rapid change in redox over a given time contributed to a faster decrease in *S* by virtue of greater leachate toxicity and waterlogging of arrays as a consequence of a higher HLR. This toxicity further selects only those Bacterial species capable of surviving under such redox environments, which are not only brought on by prevailing physico-chemical conditions but also by the metabolic capabilities of the microorganisms themselves. Likewise, the lower HLR reflected smaller changes in the redox-time relationship permitting greater adaptability over the same course of time, therefore a higher average *S* is evident over a more gradual redox-time gradient. A decrease in *S* with decreasing redox conditions over time is more evident for treatment HLR*h* than treatment HLR*l*. In other words, as the average redox state over a given time becames more anoxic, the number of bands (Bacterial species) decreased. Whether the decrease in *S* is redox related remains to be seen, since Ludvigsen *et al.* (1999) found no significant correlation between quantitative redox processes and redox-specific bacteria.

The selection pressure posed by changing pH over time appears to have less of an impact on *S* over time when compared to the contributions of the redox environment over both treatments.

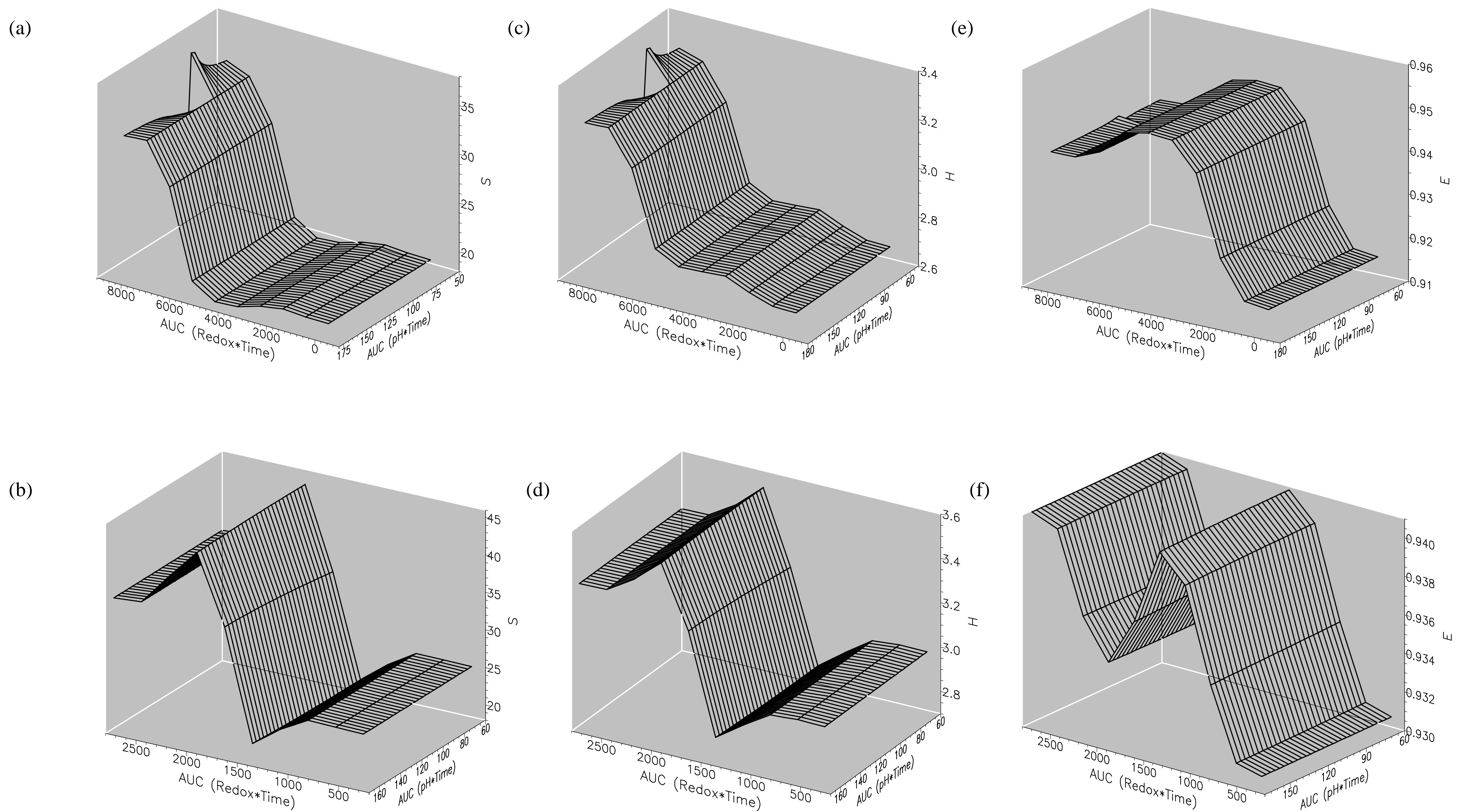


Figure 6.11 **Three Dimensional (3-D) surface representations of "area under the curve" (AUC) data for Redox*Time (x-axis) and pH*Time (y-axis) plotted against Bacterial measures of diversity (z-axis) as follows: (a) and (b) Species Richness (*S*), (c) and (d) Shannon-Weaver Index (*H*), (e) and (f) Shannon-Weaver Evenness Index (*E*). Treatment HLR*h* is represented by (a); (c); and (e) while treatment HLR*l* is represented by (b); (d); and (f), for the respective measures of diversity.**

A similar relationship is evident for both leachate treatments when AUC redox*time and AUC pH*time are plotted against average *H'* over time (Figures 6.11c and d). However, the pH appears to have more of an effect on average *H'* than on average *S*, as the redox conditions become more anoxic for treatment HLR*h* (Figure 6.11c). There is a decrease in *H'* with increasing pH*time. There is the emergence of a stronger influence of redox*time on *H'*, as apposed to *S*, for treatment HLR*h* specifically since conditions become increasingly anoxic. Therefore, the effects of increasing pH in reduced redox environments manifests by changing the numerical composition (intensity of bands over time) of different Bacterial species (bands) over time. Indeed, Singh (2001) showed that methanogenesis was successfully initiated in conditions of increasing soil pH with the corresponding decreasing Eh. This was evident for both treatments in this investigation, since redox and the corresponding pH were shown to share a strong negative correlation ($\rho_{HLRh} = -0.919$; $p < 0.001$ and $\rho_{HLRl} = -0.744$; $p < 0.001$), implying that any change in one characteristic would instigate a significant but reverse change in the remaining characteristic.

With respect to treatment HLR*h*, there is an initial increase and stabilization in *E* as the AUC redox*time changes from 8000 to 3000 (Figure 6.11e). Thereafter, there is a noticeable, decline in *E* from 0.95 to 0.91 (Table 6.2) as conditions become more anoxic. Comparatively, the change in *E* for treatment HLR*l* is less perceptible, only showing non-significant changes (6.2.1.2.3) at the third decimal place, from 0.940 to 0.930 (Figure 6.11f). The relatively steady state of *E* for treatment HLR*l* over time can be explained by the lower rate of landfill leaching, making it possible for a greater proportion of the Bacterial species to adapt and survive. In Chapter Four (4.2.2) we discussed the lag of 17 weeks between both leaching treatments before the major sequence of reduction was triggered first with treatment HLR*h*; this time lag would provide sufficient time to account for differences in *E* between the two treatments, thereby influencing the numerical composition (intensity of bands) and distribution (presence/absence of bands) of different Bacterial species over time. As with *S* and *H'*, AUC pH*time has an inverse relationship with *E* for both treatments.

6.2.1.3.2 Redox and phenol

The interpolated effects of changing redox and relative phenol consumption on *S*, *H*, and *E* are represented in Figure 6.12. The positive relationship between redox*time and *S*, *H'*, and *E* was reiterated i.e. a general decrease in the redox state in the soil arrays resulted in an initial increase followed by a decrease in the investigated diversity measures for both treatments. In comparison to the AUC redox*time effects, phenol*time effects are less noticeable at the identical points of comparison on the graphs. The effects yield a similar plot pattern to the effects discussed in 6.2.1.3.1 in this Chapter. During the initial stages of leaching when conditions appear to be more oxidized than reduced, there is a definite elevation in *S* when the relative phenol concentration is low, followed by a decrease in *S* as the uptake is reduced (Figure 6.12a). This pattern is evident only for treatment HLR*h*. The lower rate of leaching saw no change in *S* during the initial stages of redox monitoring, however there was an increase in *S* (35 – 45 bands) as the redox*time state shifted from an AUC of 2500 to 2000, proceeded by a steep decline to 20 species at a AUC redox*time state of 1500 (Figure 6.12b). Both systems showed stability at different AUC redox*times, with treatment HLR*h* stabilizing earlier from 2000 and treatment HLR*l* from 900. The bioavailability of phenol and the prevalent redox environments play a key role in determining *S* since it is these two factors that will ultimately determine the rate of phenol degradation (Guerin, 1999).

The effects of redox and phenol on *H'* are represented in Figure 6.12c and d. They reflect a similar trend as that projected for their effects on *S*. However, from an AUC redox*time of 2000 *H'* continues to decrease for treatment HLR*h* and at the final sampling time the projected *H'* is 2.7 whereas for treatment HLR*l* the projected *H'* is 3.0 having stabilized at an AUC redox*time of 900. One can assume that the major contributor to the differences in diversity is the different rates of leaching with the synthetic leachate. Treatment HLR*h* supplies more carbon, nutrients and water to generate more rapid changes in the redox state of the soil arrays, thereby leading to changes in the type and number of Bacterial species. The effects of greater phenol loading are evident in Figure 6.12c, where at the termination of the experiment, there appears to be a more noticeable reduction in *H'* as phenol accumulates in the soil arrays. This trend is less noticeable in soil arrays perfused at HLR*l* (Figure 6.12d).

The effects of redox*time and phenol*time on *E* follow a similar trajectory to that projected for the effects of redox*time and pH*time on *E* (Figure 6.12e and f). There is a general decrease in *E* with an increase in phenol concentration over time. This is highlighted by a comparison of the two treatments, with a greater change in *E* documented for treatment HLR*h* (0.95 – 0.91) as apposed to that recorded for HLR*l* (0.940 – 0.930) over the investigation.

Here again, redox potential and phenol degradation share a relationship that is negatively correlated for both treatments ($\rho_{HLRh} = -0.811$; $p < 0.001$ and $\rho_{HLRl} = -0.857$; $p < 0.001$) (4.2.2 and 4.2.3). The correlation between organic carbon and redox potential in soil environments was shown to be non-linear in nature (Singh, 2001). However, the rates of phenol degradation decrease under aerobic and anaerobic conditions, and can be distinguished further under nitrate-and sulphate reducing and methanogenic conditions (van Schie and Young, 2000). It is true that our knowledge of aerobic phenol degrading bacteria is more advanced than phenol degrading pathways involving anerobic bacteria. The aerobic phenol degrading bacteria employ metabolic pathways that make use of oxygen dependent enzymes that enable quicker degradation of phenolics. The range of anaerobic bacteria, including methanogenic, sulphate, iron and nitrate reducing bacteria, make use of multiple anaerobic degradation pathways often containing oxygen sensitive carboxylase enzymes (van Schie and Young, 2000).

However with respect to the two cases discussed thus far (6.2.1.3.1 and 6.2.1.3.2), the change in redox state appears to be the dominating factor in determining the response of Bacterial diversity for both treatments.

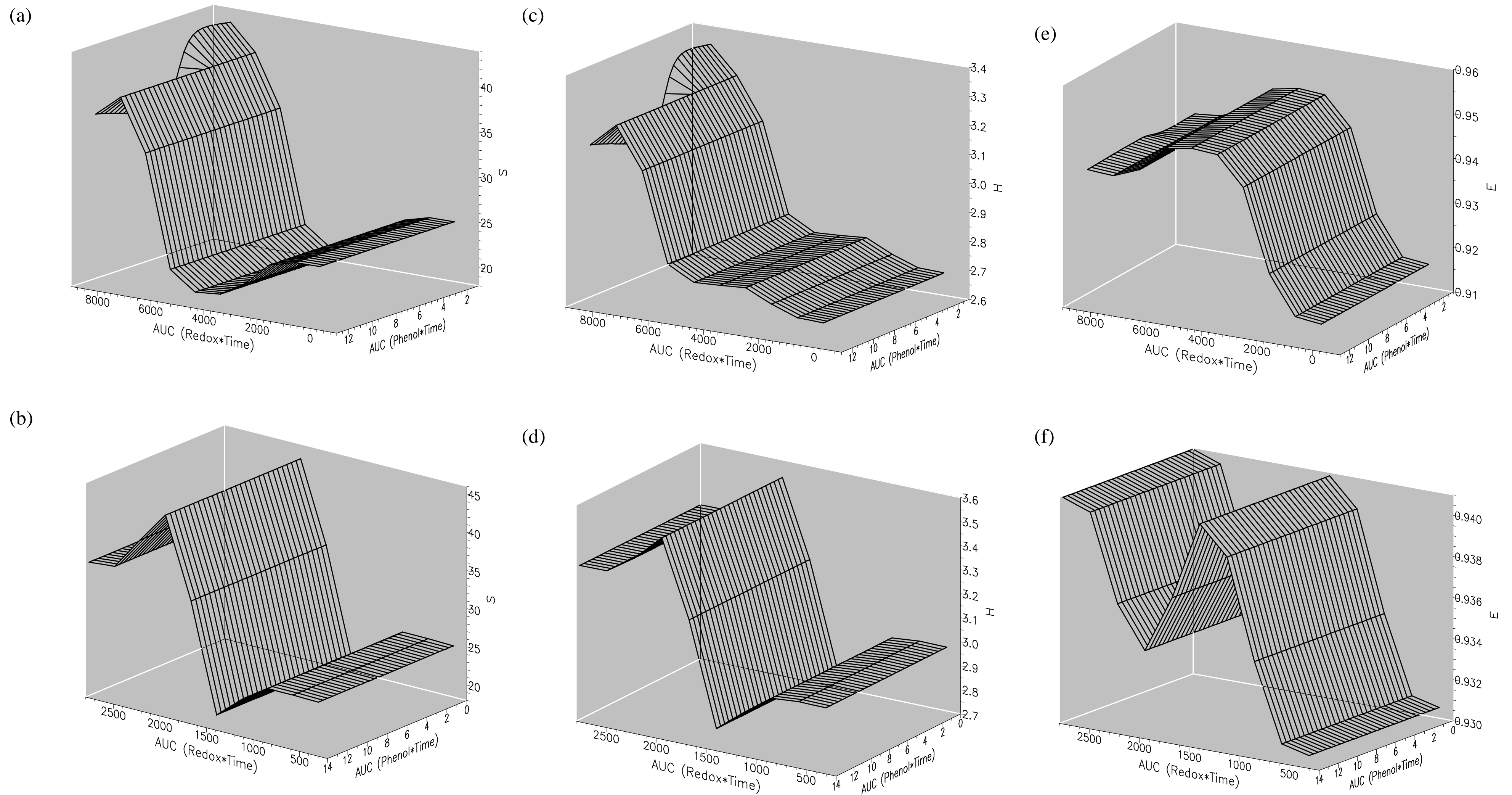


Figure 6.12 **Three Dimensional (3-D) surface representations of "area under the curve" (AUC) data for Redox*Time (x-axis) and Phenol*Time (y-axis) plotted against Bacterial measures of diversity (z-axis) as follows: (a) and (b) Species Richness (*S*), (c) and (d) Shannon-Weaver Index (*H*), (e) and (f) Shannon-Weaver Evenness Index (*E*). Treatment HLR*h* is represented by (a); (c); and (e) while treatment HLR*l* is represented by (b); (d); and (f), for the respective measures of diversity.**

6.2.1.3.3 pH and phenol

The projected effects of phenol and pH on Bacterial diversity are more visible on the three-dimensional plots that obviate the effects of redox potential on the soil arrays described in Chapter Four (Figure 6.13). With respect to *S* and *H'*, a general increase in pH over time results in an increase in diversity up to a threshold, after which any further increase in AUC pH*time forces a decrease in *S* and *H'* for both treatments. The start-to-threshold range of *S* projected for treatment HLR*h* and HLR*l* under the effects of AUC pH*time were 18-37 and 19-44 species, respectively (Figures 6.13a and b). The start-to-threshold range for *H'* was 2.75-3.40 and 2.77-3.54, respectively (Figures 6.13c and d). Regarding the effects of phenol*time, the start-to-threshold ranges projected for *S* and *H'*, when pH effects were optimal for the acquisition of maximum diversity, were 34-37 and 39-44 species for treatments HLR*h* and HLR*l*, respectively . In terms of *H'*, these values were 3.25-3.40 (HLR*h*) (Figures 6.13a and b) and 3.35-3.54 (HLR*l*) (Figures 6.13c and d). In Chapter Four, regression analysis of phenol (4.2.3) and pH (4.2.1) revealed significantly different responses between the two leaching treatments which in turn have contributed, accordingly, to significantly different overall *S* (6.2.1.2.1)and *H'* (6.2.1.2.2) between both treatments.

An altogether different trajectory was projected for the effect of pH*time on *E* for both leaching treatments (Figures 6.13e and f). The trajectory for treatment HLR*h* implies a constant decrease in Bacterial community evenness as the pH increased over time, punctuated by a temporary plateau measuring between 90 and 120 AUC pH*time (Figure 6.13e). Much the same pattern is evident for treatment HLR*l* but with two apparent differences; the first being the prominent increase in *E* evident between 90 and 120 AUC pH*time and the second being the relatively non-significant ($F=0.856$, Table 6.2) change in *E* (change projected only over the third decimal place) (Figure 6.13f). By comparison, the change in *E* over time for treatment HLR*h* was deemed non-significant ($F=0.067$, Table 6.2) so one can conclude that the average change in *E* over time for treatment HLR*h* was relatively non-random when compared to the change in average *E* detected for treatment HLR*l*.

Phenol concentration and the corresponding pH were shown to share a strong positive correlation ($\rho_{HLRh} = 0.852$; $p < 0.001$ and $\rho_{HLRl} = 0.880$; $p < 0.001$), implying that

any change in one characteristic would instigate a significant change, in the same direction, in the remaining characteristic.

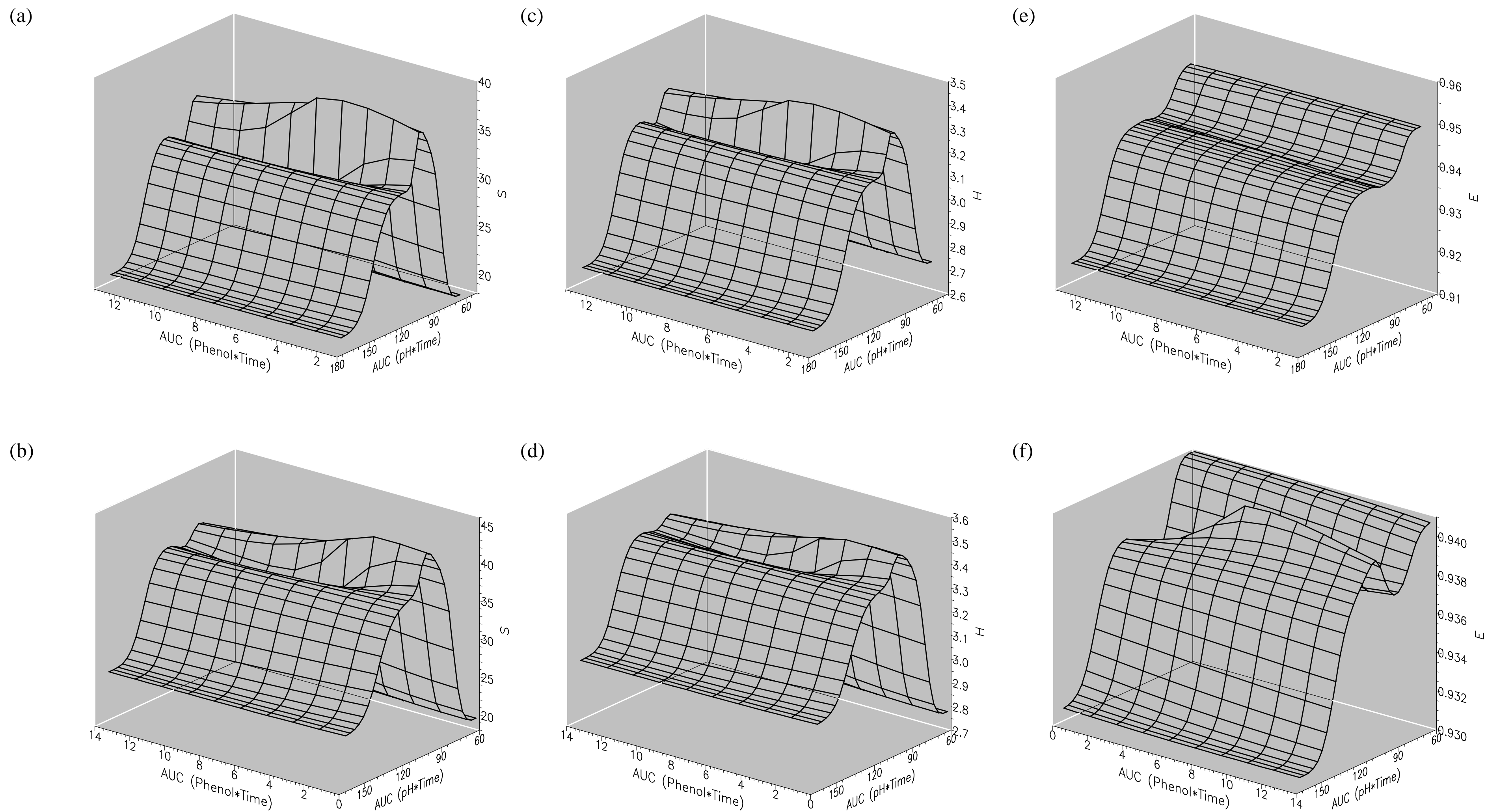


Figure 6.13 **Three Dimensional (3-D) surface representations of "area under the curve" (AUC) data for Phenol*Time (x-axis) and pH*Time (y-axis) plotted against Bacterial measures of diversity (z-axis) as follows: (a) and (b) Species Richness (*S*), (c) and (d) Shannon-Weaver Index (*H*), (e) and (f) Shannon-Weaver Evenness Index (*E*). Treatment HLR*h* is represented by (a); (c); and (e) while treatment HLR*l* is represented by (b); (d); and (f), for the respective measures of diversity.**

6.3 Conclusion

The principal objective of this study was to assess the diversity of the Bacterial associations that develop from naturally occurring communities in soil, under changing physico-chemical conditions, by PCR-DGGE when exposed to a synthetic landfill leachate. Molecular profiling of the Bacterial communities revealed the following information:

- This investigation revealed a significant shift in Bacterial community diversity with respect to *S* and *H'* for both HLRs over time. However, a significant shift in *E* was only observed for microcosms leached at HLR*h*. These results suggest that the quantity of rainfall received by a landfill indirectly plays a significant role in determining the rate at which Bacterial community diversity changes in the subsurface below a landfill. This in turn directly affects the rate of subsurface bio-attenuation of leachate components and the subsequent rate of groundwater pollution. The correlation between quantitative redox processes and redox-specific Bacteria requires further investigation. A combined, redox-position-specific field and laboratory approach utilizing PCR-DGGE (using 16S rDNA primers specific for different redox-specific Bacteria), cloning and sequencing can address this issue.

- The effects of a single environmental factor on the Bacteria composition of the soil microcosms were difficult to assess since many, if not all, the factors confound each other. However, determining the three dimensional association of redox, phenol, and pH on Bacteria diversity sheds some light on the relative impact of each of these factors on Bacteria diversity. Depressed redox potentials coupled with exposure to increasing concentrations of phenol and elevated pH generally lead to a decrease in Bacteria diversity (*S*, *H'*, and *E*). Bacterial community changes tended to manifest earlier when exposed to HLR*h* treatment. The changing redox potentials, pH, and phenol concentrations appeared to have a more pronounced effect on *E* followed by *H'* and *S*. This was anticipated since *H'* and *E* are dependant on the density (numbers) of the surviving Bacterial species (bands).

- UPGMA cluster analysis and CCA revealed Bacteria communities that clustered with depth at each of the four sampling intervals. Moreover, CCA described a progressively linear change in Bacteria composition from one sample to the next that corresponded with an increasing depth at all sampling intervals. The establishment of different Bacteria associations at different depths within the microcosms suggests a dynamic relationship between the attenuation of specific leachate components and the depth at which this attenuation transpires by virtue of the changing metabolic abilities inherent amongst the diverse microbial associations and the changing physico-chemical environment. To take this study further, the different associations could be cloned and sequenced in an attempt to match the resultant Bacteria identities with specific metabolic capabilities that enable the attenuation of specific leachate components. These associations can be useful in leachate treatment facilities.

- The assessment of community diversity revealed significant differences between mean *S* and *H'* for both treatments over all four sampling times. However, a comparison of the mean *S* and *H'* between treatments revealed significant differences at sampling times T2, T3, and T4 but not at T1. In fact the statistical differences, for *S* and *H'*, between the two treatments increased with time thereby highlighting the greater mean *S* and *H'* recorded for treatment HLR*l* in comparison to treatment HLR*h*. Conversely, there were no significant differences established for the mean E_H at all sampling times for both treatments, although one could argue that the overall level of significance determined when comparing mean E_H over time for treatment HLR*h* was close to significant ($P = 0.067$) and as a consequence one could argue that the effect of the higher leachate loading on the Bacteria community E_{H} was not random when compared to treatment HLR*l*. The rate of hydraulic loading of soil below landfills can affect the Bacteria diversity of the soil and indirectly affect the bio-attenuation of landfill leachate by virtue of the numbers, types, and distribution of the surviving Bacteria species.

Chapter Seven

7. General Conclusion and Future Prospects

Over the years, microbiologists have strived to characterize and identify individual microorganisms. The understanding that microbial communities, as apposed to the actions of individual populations, posess far greater impacts on natural processes brought with it new possibilities with respect to microbial characterization, identification, and manipulation. Increasingly, molecular approaches to microbial ecology have surpassed that which was provided by conventional culture-dependent methods of microbial isolation and characterization, providing fast and reliable evidence for manipulation in a variety of disciplines, including wastewater treatment strategies, environmental impact assessments, and contaminant transport and degradation within a range of natural and synthetic waste streams.

The effective treatment of waste streams, including landfill leachate, still presents a worldwide concern. The ever increasing demands of the world's population on industrial output, inevitably leads to the generation of higher levels of domestic and industrial waste which legislation demands be treated to constituent levels deemed fit for release into natural surroundings. However, there seems to be no universal agreement detailing the techniques and strategies employed during the treatment of landfill leachate due, in particular, to the dynamic and varied nature of this wastewater. Conventional leachate treatment strategies are often expensive to employ or posess limitations with respect to performance over time. Alternatively, passive natural attenuation, of which microorganisms play a major role, is a resource that can be employed to, at the very least; slow down pollutant migration (Swett and Rapaport, 1998). Therefore, it is of fundamental importance that each landfill leachate be treated on its own merits taking into account its unique composition in relation to the treatment strategies on offer, bearing in mind the potential of the indigenous microbial potential for natural attenuation of pollutants (Primo, Rivero and Ortiz, 2007).

Carbon, inorganic components, heavy metals, and xenobiotic compounds that enter the soil below a landfill are subjected to a variety of bio-geochemical processes. These

processes have a significant effect on the surrounding environment with respect to redox zonation, groundwater pollution, microbiological community structure and diversity, pollutant migration and attenuation rates (Christensen *et al.*, 2001). Attenuation of leachate constituents in soil beneath a landfill are dependent on the formation of characteristic redox zones that range from strongly reduced environments that are dominated by methanogenic activity through a sequence of cascading reactions that culminate in aerobic conditions. The microbial community structure and composition is in constant flux in each of these redox zones varying in response to the composition of the flowing leachate and the changing redox conditions as the leachate plume expands. Within each of these redox zones, there are specific bacterial communities present that associate with each other and the prevaling chemical environment. Within the soil microcosms decreasing redox potentials coupled with increasing pH and phenol concentration was associated with an overall decrease in Bacteria community diversity over time. Essentially, this relationship translates into temporal and spatial changes in the rate of natural attenuation of contaminants in the subsurface.

There are reports detailing microbial profiling of subsurface environments that have been polluted by various waste streams (Röling *et al.*, 2000; Röling *et al.*, 2001). However, these deal with specific landfill sites that produce leachate with corresponding pollution plumes and microbial community structure and composition that are indigenous to those landfills. Other laboratory investigations involved the use of leachate collected from existing landfills before perfusion of soil microcosms or inoculation of soil microcosms with specific microbial associations prior to leaching with a synthetic leachate (Kjeldson *et al.*, 1990; Smit *et al.*, 1999b). In this study we attempted to create redox conditions similar to those expected to be encountered in a subsurface. This was achieved by controlled perfusion of sequential soil microcosms with a synthetic leachate so that we could explore the temporal and spatial change in microbial community structure and diversity under changing redox environments without the introduction of external inoculum. Moreover, we attempted to identify the dynamic temporal relationship, if any, between the soil bacterial community and the physico-chemical environment in the soil microcosms. The resident bacterial community survived and adapted to exposure to the syntethic leachate, first increasing before decreasing in overall diversity at both HLRs that

were investigated. This highlighted the emergence of greater diversity between the initial surviving indigenous communities before specific physico-chemical conditions within the soil microcosms brought about the adaptation and proliferation of specific types of Bacteria capable of surviving in the prevailing conditions. The pattern of succession was tenuously linked to changes in the redox state, pH, and concentration of phenol of the effluent leachate which in turn influences the temporal and spatial distribution of bacterial communities and physico-chemical environments within the soil microcosms. The development of successive redox zones in the path of the leachate flow would enable the successive degradation of pollutants by different microbial associations present in the leachate plume (Williams and Higgo, 1994), until the majority of the leachate plume is dominated by sulphate and methanogenic conditions which would limit the range of contaminant degradation by these microrganisms (Van Breukelan, 2003). In a study tracing the degradation of xenobiotic compounds in a leachate plume in Denmark, benzene and the herbicide Mecoprop were not degraded in the anaerobic section of the plume, whereas phenols were degraded throughout the plume (Baun *et al.*, 2003). Nielsen, Albrechtsen, Heron and Christensen (1995) found that microbial degradation of specific phenolic compounds was position specific within the developing anaerobic leachate plume. Apart from the redox chemistry of the soil microcosms, other confounding factors such as pH and contaminant toxicity play an important role in determining microbial metabolism and therefore contaminant migration in the subsurface (Lerner *et al.*, 2000).

Traditional culture-dependent methods rely on the provision of suitable substrates and nutrients necessary for the growth of microorganisms of interest. However, this approach suffers several limitations some of which are discussed in Chapter One (1.7). On the other hand, microbial molecular ecology is significantly dependent on the quality of the isolated DNA. PCR or cloning techniques require template nucleic acid that has minimum fragmentation and high purity (Bürgman *et al.*, 2001). Subsequent downstream reactions employing the amplified products of the isolated nucleic acid, such as gel electrophoresis, gel staining and data capture, are also indirectly dependent on the purity, yield and quality of the isolated nucleic acid (Chapter Five). The heterogenous soil matrix presents a unique environment from which nucleic acids are isolated (Bürgman *et al.*, 2001). A common problem during the isolation of nucleic acids from soil is the simultaneous extraction of

humic acids and protein complexing organic soluble PCR inhibitors (Španová *et al.*, 2006). Different DNA isolation protocols possess significantly different potentials for the removal of these PCR inhibitors, thereby influencing the efficiency of PCR-DGGE and the bacterial community fingerprints generated for replicate soil samples (Chapter Five). This in turn affects the captured gel data and subsequent mathematical extrapolation of the data when using ecological indices. In essence, the choice of DNA isolation technique has a major bearing on the type, quantity, and quality of data retrieved for bacterial fingerprinting. Therefore, it is recomended that a multiple DNA isolation approach is taken to maximize information retrieval. Future work must focus on standardizing and maximizing nucleic acid isolation from soil so that maximum information can be obtained for the harnessing of global microbial fingerprinting databases (*see later* Subsurface Specimen Banking Concept).

This study showed that the choice of DGGE gel staining technique had a major bearing on the information extrapolated from captured gel images (Chapter Five). The differences in sensitivity of the stains made a significant contribution to the quantity and quality of fingerprint data retrieved which in turn translated to differences in the ecological diversity indices that were used to describe the bacterial community fingerprints. Although silver staining is physically and economically demanding, the information retrived from the gels was superior to that supplied by ethidium bromide stained gels (Chapter Five).

In the past, little or no attention was given to the potential contribution of the microbiological aspects involved in waste treatment and minimisation strategies. However, there is ever increasing evidence advocating the invaluable contributions that microbiology can make to the growing concern of waste disposal. Microbially mediated natural attenuation of contaminants in the subsurface has been demonstrated elsewhere under existing landfills (Baun *et al.*, 2003; Van Breukelan, 2003). This study proves that microbial attenuation can occur in a soil perfused with a representative synthetic landfill leachate, without prior prolonged exposure to the leachate or inoculation with specific microbial associations. This adds to the growing body of evidence increasing the confidence of all parties involved in waste management. The identification of microbial

associations specific to redox potential, contaminant concentration and other confounding factors by the ever expanding techniques of molecular ecology can lead to the generation of a microbial contaminant attenuation database (Subsurface Specimen Banking - SSB) from which newly discovered microbial associations involved in natural attenuation can be uploaded together with data describing their physico-chemical environment, biochemistry, and genetics (Röling and van Verseveld, 2002). In this study alone, just two samples indicated the presence of three uncultured and an unidentified Bacteria from a total of eight clones that were sequenced and compared on the Basic Logical Alignment Search Tool (BLAST) Network Service (data not shown) (2.5.7 and 2.5.8). Of the remaining four clones, two were identified as *Anaeromyxobacter dehalogenans* (accession nr. AF382400); one as *Azospirillum brasilense* (accession .nr. AB16srrn2); and the other as an uncultured *Proteobacterium* Sva0812b (accession nr. UPR241045).

Knowledge of this nature could aid in the future prediction of contaminant migration and degradation rates below landfills that share common aspects of microbiology and physico-chemical environments. For instance, the microbial attenuation of a specific group of contaminants can be determined by investigating the genetic constitution of a given microbial community by molecular techniques, including isotope probing, nucleoside labelling, and fingerprinting protocols. The information gained can then be cross-referenced with information present on the SSB to assess the potential contaminant attenuation capacity of the latest microbial associations (Röling and van Verseveld, 2002). For instance, the presence of *Anaeromyxobacter dehalogenans* points to the presence of a facultative anaerobic bacterium within a community that is capable of using nitrate, iron and chlorinated phenolic compounds as terminal electron acceptors (Sanford, Cole and Tiedje, 2002; Truede, Rosencrantz, Liesack and Schnell, 2003). Dissimilatory iron reducing bacteria, like *Anaeromyxobacter*, are common in soils and aquifers and couple the oxidation of organic matter with a suitable electron acceptor like iron. Furthermore, *Anaeromyxobacter* is an ideal candidate for bioremediation of contaminated sites because of its tolerance to environmentally relevant changes in redox and pH conditions in addition to its tolerance to phenolic compounds (He and Sanford, 2003).

It is in the opinion of this author that any natural soil has the potential to naturally attenuate a range of organic and inorganic contaminants. Given sufficient time, suitable redox environments can develop as a consequence of dynamic microbial metabolism and physico-chemical interactions which in turn can provide remediation strategies with an advantage when dealing with contaminated subsurface environments.

References

Adams, V.D. (1990). *Water and Wastewater Examination Manual.* Lewis Publishers, Michigan, USA, 153-154.

Agbenin, J.O. and Olojo, L.A. (2004). Competitive adsorption of copper and zinc by a Bt horizon of savanna Alfisol as affected by pH and selective removal of hydrous oxides and organic matter. *Geoderma* **119**, 85-95.

Ahel, M., Mikac, N., Cosovic, B., Prohic, E. and Soukup, V. (1998). The impact of contamination from a municipal solid waste landfill (Zagreb, Croatia) on underlying soil. *Water, Science, and Technology* **37**, 203-210.

Ahring, B.K. and Westerman, P. (1983). Toxicity of heavy metals to thermophilic anaerobic digestion. *European Journal of Applied Microbiology and Biotechnology* **17**, 365-370.

Albrechtsen, H.J. and Winding, A. (1992). Microbial biomass and activity in subsurface sediments from Vejen, Denmark. *Microbial Ecology* **23**, 303-317.

Albrechtsen, H.J. and Christensen, T.H. (1994). Evidence of microbial iron reduction in a landfill leachate-polluted aquifer (Vejen, Denmark). *Applied and Environmental Microbiology* **60**, 3920-3925.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**, 143-169.

Anderson, J.R. and Dornbush, J.N. (1967). Influence of sanitary landfills on groundwater quality. *Journal of the American Water Works Association* **59**, 457-470.

Andreottola, G., Cossu, R. and Serra, R. (1989). A Method for the assessment of environmental impact of sanitary landfill. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 367-389.

Antilėn, M., Főrster, J.E., Del Confetto, S., Rodier, E., Fudym, O., Venezia, A.M., Deganello, G., and Escude, M. (2004). Characterisation of the porous structure of Chilean volcanic soils by nitrogen adsorption and mercury porosimetry. *Journal of the Chile Chemistry Society* **49**, 313-318.

Arias, M., Pérez-Novo, C., Lopez, E. and Soto, B. (2005a). Competitive adsorption and desorption of copper and zinc in acid soils. *Geoderma* **133**, 151-159.

Arias, M., Pérez-Novo, C., Osorio, F., Lopez, E. and Soto, B. (2005b). Adsorption and desorption of copper and zinc in the surface layer of acid soils. *Journal of Colloid and Interface Science* **288**, 21-29.

Arora, T., Linde, N., Revil, A., and Castermant, J. (2007). Non-intrusive characterisation of the redox potential of landfill leachate plumes from self potential data. *Journal of Contaminant Hydrology* **92**, 274-292.

Artiola-Fortuny, J., and Fuller, W.H. (1982). Phenols in municipal solid waste leachates and their attenuation by clay soils. *Soil Science* **133**, 218-226.

Atlas, R.M. and Bartha, R. (1998). Development of microbial communities. In *Microbial Ecology: Fundamentals and Applications, fourth edition*. Benjamin/Cummings Science Publishing, California, USA, 174-217.

Bagchi, A. (1994). *Design, Construction, and Monitoring of Landfills*. John Wiley and Sons, New York, USA.

Bagchi, A. (2004). *Design of Landfills and Integrated Solid Waste Management*. John Wiley and Sons, New York, USA.

Ball, J.M. and Bredenhann, L. (1992). Minimum requirements for waste disposal facilities. In *Proceedings of Waste Management in a Changing Society, WasteCon. '92*, Johannesburg, South Africa, 113-128.

Barlaz, M.A., Schaefer, D.M. and Ham, R.K. (1989). Bacterial population development and chemical characteristics of refuse decomposition in a simulated sanitary landfill. *Applied and Environmental Microbiology* **55**, 55-65.

Baun, A., Jensen, S.D., Bjerg, P.L., Christensen, T.H. and Nyholm, N. (2000). Toxicity of organic chemical pollution in groundwater downgradient of a landfill (Grindsted, Denmark). *Environmental Science and Technology* **34**, 1647-1652.

Baun, A., Reitzel, L.A., Ledin, A., Christensen, T.H., Bjerg, P.L. (2003). Natural attenuation of xenobiotic organic compounds in a landfill leachate plume. *Journal of Contaminant Hydrology* **65**, 269-291.

Beeman, R.E. and Suflita, J.M. (1987). Microbial activity of a shallow unconfined groundwater aquifer polluted by municipal landfill leachate. *Microbial Ecology* **14**, 39-54.

Beeman, R.E. and Suflita, J.M. (1990). Environmental factors influencing methanogenesis in a shallow anoxic aquifer: A field and laboratory study. *Journal of Industrial Microbiology* **5**, 45-58.

Begon, M., Harper, J.L. and Townsend, C.R. (1986). The nature of the community. In *Ecology: Individuals, Populations and Communities*. Blackwell Scientific Publications, Oxford, 591-628.

Beker, D. (1987). Control of acid-phase degradation. In *Proceedings ISWA International Symposium on Process Technology and Environmental Impact of Sanitary Landfill*, Cagliari, Italy, 1-22.

Bertrand, H., Poly, F., Van, V.T., Lombard, N., Nalin, R., Vogel, T.M. and Simonet, P. (2005). High molecular weight DNA recovery from soils prerequisite for biotechnological metagenomic library construction. *Journal of Microbiological Methods* **62**, 1-11.

Bhandari, A., Novak, J.T., Burgos, W.D. and Berry, D.F. (1997). Irreversible binding of chlorophenols to soil and its impact on bioavailability. *Journal of Environmental Engineering* **123**, 506-513.

Binder, M. and Bramryd, T. (2001). Environmental impacts of landfill bioreactors in comparison to former landfill techniques. *Water, Air, and Soil Pollution* **129**, 289-303.

Bjerg, P.L., Rugge, K., Pedersen, J.K. and Christensen, T.H. (1995). Distribution of redox sensitive groundwater quality parameters downgradient of a landfill (Grinsted, Denmark). *Environmental Science and Technology* **29**, 1387-1394.

Blight, G.E. and Bredenhann, L. (1992). Engineering aspects of landfill design. In *Proceedings of the Third Southern Africa Anaerobic Digestion Symposium 1992*, Pietermaritzburg, South Africa, 211-219.

Boeckx, P., Van Cleemput, O. and Villaralvo, I. (1996). Methane emission from a landfill and the methane oxidising capacity of its covering soil. *Soil Biology and Biochemistry* **28**, 1397-1405.

Boivin-Jahns, V., Bianchi, A., Ruimy, R., Garcin, J., Daumas, S. and Christen, R.S. (1995). Comparisons of phenotypical and molecular methods for the identification of bacterial strains isolated from a deep subsurface environment. *Applied and Environmental Microbiology* **61**, 3400-3406.

Borneman, J., Skroch, P.W., O'Sullivan, K., Palls, J.A., Rumjanek, N.G., Jansen, J.L., Nienhuis, J. and Triplett, E.W. (1996). Molecular microbial diversity of an agricultural soil in Wisconsin. *Applied and Environmental Microbiology* **62**, 1935-1943.

Bouwer, E.J. and McCarty, P.L. (1983a). Transformation of 1- and 2- carbon halogenated aliphatic organic compounds under methanogenic conditions. *Applied and Environmental Microbiology* **45**, 1268-1294.

Bouwer, E.J. and McCarty, P.L. (1983b). Transformation of halogenated organic compounds under denitrification conditions. *Applied and Environmental Microbiology* **45**, 1295-1299.

Boyle, W.C. and Ham, R.K. (1974). Biological treatment of landfill leachate. *Journal of the Water Pollution Control Federation* **46**, 860-872.

Breen, A., Rope, A.F., Taylor, D., Loper, J.C. and Sferra, P.R. (1995). Application of DNA amplification fingerprinting (DAF) to mixed culture bioreactors. *Journal of Industrial Microbiology* **14**, 10-16.

Brinkhoff, T. and Muyzer, G. (1997). Increased species diversity and extended habitat range of sulphur-oxidizing *Thiomicrospira* spp. *Applied and Environmental Microbiology* **63**, 3789-3796.

Britz, T.J., Venter, C.A. and Tracey, R.P. (1990). Anaerobic treatment of municipal landfill leachate using an anaerobic hybrid digester. *Biological Wastes* **32**, 181-191.

Bruce, K.D. (1998). Analysis of *mer* gene sub-classes within bacterial communities in soils and sediments resolved by fluorescent-PCR-restriction fragment length polymorphism profiling. *Applied and Environmental Microbiology* **63**, 4914-4919.

Bull, P.S., Evans, J.V., Wechler, R.M. and Cleland, K.J. (1983). Biological technology for the treatment of leachate from sanitary landfills. *Water Research* **17**, 1473-1481.

Bürgmann, H., Pesaro, M., Widmar, F. and Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods* **45**, 7-20.

Calace, N., Liberatori, A., Petronio, B.M. and Pietroletti, M. (2001). Characteristics of different molecular weight fractions of organic matter in landfill leachate and their role in soil sorption of heavy metals. *Environmental Pollution* **113**, 331-339.

Camargo, F.A.O., Okeke, B.C., Bento, F.M. and Frankenberger, W.T. (2005). Diversity of chromium-resistant bacteria isolated from soils contaminated with dichromate. *Applied Soil Ecology* **29**, 193-202.

Cameron, R.D. and Koch, F.A. (1980). Trace metals and anaerobic digestion of leachate. *Journal of the Water Pollution Control Federation* **52**, 283-293.

Cameron, R.D. and McDonald, E.C. (1996). Toxicity of landfill leachate. *Journal of the Water Control Federation* **52**, 760-769.

Campbell, L.S. and Davies, B.E. (1995). Soil sorption of caesium modelled by the Langmuir and Freundlich isotherm equations. *Applied Geochemistry* **10**, 715-723.

Canziani, R. and Cossu, R. (1989). Landfill hydrology and leachate production. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 185-211.

Carley, B.N. and Mavinic, D.S. (1991). The effects of external carbon loading on nitrification and denitrification of a high ammonia landfill leachate. *Journal of the Water Pollution Control Federation* **63**, 51-59.

Carra, J.S. and Cossu, R. (1990). Introduction. In International Perspectives on Municipal Solid Wastes and Sanitary Landfilling, ed. Carra, J.S. and Cossu, R. Academic Press,United Kingdom, 1-14.

Cecen, F. and Aktas, O. (2001). Effect of PAC addition in combined treatment of landfill leachate and domestic wastewater in semi-continuously fed batch and continuous-flow reactors. *Water SA* **27**, 177-188.

Chakraborty, S. and Veeramani, H. (2005). Response of pulse phenol injection on an anaerobic-anoxic-aerobic system. *Bioresource Technology* **96**, 761-767.

Chan, K.Y., Davey, B.G. and Geering, H.R. (1978). Interaction of treated sanitary leachate with soil. *Journal of Environmental Quality* **7**, 306-310.

Chapman, G. and Ekama, G. (1992). The effect of sewage sludge co-disposal and leachate recycling on refuse stabilisation. In *Proceedings of Waste Management in a Changing Society, WasteCon. '92*, Johannesburg, South Africa, 477-487.

Cheremisinoff, P.N., Giglielo, K.A. and O'Neill, T.K. (1984). *Groundwater Leachate: Modelling, Monitoring and Sampling*. Technomic Publishing, Pennsylvania, USA.

Cheremisinoff, P.N. (1990). Landfill lining: means for leachate control. In *Encyclopedia of Environmental Control Technology: Hazardous Waste Containment and Treatment, volume 4*, ed. Cheremisinoff, P.N. Gulf Publishing, Houston, 41-64.

Chian, E.S.K. and DeWalle, F.B. (1976). Sanitary landfill leachates and their treatment. *ASCE Journal of Environmental Engineering Division* **102**, 411-431.

Cho, J.C. and Kim, S.J. (2000). Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. *Applied and Environmental Microbiology* **66**, 956-965.

Christensen, T.H. (1989). Environmental aspects of sanitary landfilling. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 19-25.

Christensen, T.H., Kjeldsen, P., Albrechtsen, H.J., Heron, G., Nielsen, P.H., Bjerg, P.L., and Holm, P.E. (1994). Attenuation of landfill leachate pollutants in aquifers. *Critical Reviews in Environmental Science and Technology* **24**, 119-202.

Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Djensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H.J., and Heron, G. (2001). Biogeochemistry of landfill leachate plumes. *Applied Geochemistry* **16**, 659-718.

Chu, L.M., Cheung, K.C. andWong, M.H. (1994). Variations in the chemical properties of landfill leachate. *Environmental Management* **18**, 105-117.

Clement, B. and Thomas, O. (1995). Application of ultra-violet spectrophotometry and gel permeation chromatography to the characterisation of landfill leachates. *Environmental Technology* **16**, 367-377.

Clement, B.G., Kehl, L.E., DeBord, K.L. and Kitts, C.L. (1998). Terminal restriction fragment patterns (TRFPs), a rapid PCR-based method for the comparison of complex bacterial communities. *Journal of Microbiological Methods* **31**, 135-142.

Clerc, A., Manceau, C. and Nesme, X. (1998). Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **64**, 1180-1187.

Cossu, R. (1989). Role of landfilling in solid waste management. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 3-9.

Cossu, R., Stegmann, R., Andreottola, G. and Cannas, P. (1989). Biological treatment. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 251-283.

Costley, S.C. and Wallis, F.M. (2001). Bioremediation of heavy metals in a synthetic wastewater using a rotating biological contactor. *Water Research* **35**, 3715-3723.

County Surveyors Society (1984). *The Treatment of Leachates from Landfill Sites*. Report Number 41, English County Councils, United Kingdom.

Cozzarelli, I.M., Suflita, J.M., Ulrich, G.A., Harris, S.H., Scholl, M.A., Schlottmann, J.L. and Christenson, S. (2000). Geochemical and microbiological methods for evaluating anaerobic processes in an aquifer contaminated by landfill leachate. *Environmental Science and Technology* **34**, 4025-4033.

Crawford, J. and Neretnieks, I. (2001). Simulation of the redox buffer depletion rate in landfills of combustion residue waste materials. *Water, Air, and Soil Pollution* **128**, 223-242.

Cullen, D.W., and Hirsch, P.R. (1998). Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biology and Biochemistry* **30**, 983-993.

Cureton, P.M., Groenevelt, P.H. and McBride, R.A. (1991). Landfill leachate recirculation: effects on vegetation vigor and clay surface cover infiltration. *Journal of Environmental Quality* **20**, 17-24.

Dass, P., Tamke, G.R. and Stoffel, C.M. (1977). Leachate production at sanitary landfill sites. *ASCE Journal of Environmental Engineering Division* 103, 981-988.

de Bruijn, F.J. (1992). Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. . *Applied and Environmental Microbiology* **58**, 2180-2187.

Demkin, V.V., Edelstein, M.V., Zimin, A.L., Edelstein, I.A. and Suvorov, M.M. (2000). Detection of sequence variation in PCR-amplified fragments of *omp2* gene fromthree species of the family Chlamydiaceae using agarose gel electrophoresis containing bisbenzimide-PEG. *FEMS Microbiology Letters* **184**, 215-218.

Department of Water Affairs and Forestry (1998a). Waste Management Series. *Minimum Requirements for Waste Disposal by Landfill, Second Edition*.

Department of Water Affairs and Forestry (1998b). Waste Management Series. *Minimum Requirements for the Handling, Classification and Disposal of Hazardous Waste, Second Edition.*

Department of Water Affairs and Forestry (2004). National Water Resource Strategy – "Our Blue Print for Survival", first edition.

Diaz, L.F. (1994). The use of site characteristics in the design of sanitary landfills. In: *Proceedings of WasteCon '94*, Somerset West, South Africa, 38-47.

Ding, A., Zhang, Z., Fu, J. and Cheng, L. (2001). Biological control of leachate from municipal landfills. *Chemosphere* **44**, 1-8.

Dobbins, D.C., Thornton-Manning, J.R., Jones, D.D. and Federle, T.W. (1987). Mineralization potential for phenol in subsurface soils. *Journal of Environmental Quality* **16**, 54-58.

Doedens, H. and Cord-Landwehr, K. (1989). Leachate recirculation. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 231-249.

Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. (1998). Microbial diversity in a hydrocarbon-chlorinated-solvent-contaminated aquifer undergoing intrinsinc bioremediation. *Applied and Environmental Microbiology* **64**, 3869-3877.

Druyts, F.H.W.M., Legge, K.R. (1996). Landfill design in crisis: A reviewers perspective. In *Proceedings of Waste Management – In Crisis, WasteCon. '96*, Durban, South Africa, 89-97.

Duarte, G.B., Rosado, A.S., Seldin, L., Keijzer-Wolters, A.C. and van Elsas, J.D. (1998). Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *Journal of Microbiological Methods* **32**, 21-29.

Duarte, G.F., Rosado, A.S., Seldin, L., de Araujo, W. and van Elsas, J.D. (2001). Analysis of bacterial community structure in sulphurous-oil-containing soils and detection of species carrying dibenzothiophene desulfurization (*dsz*) genes. *Applied and Environmental Microbiology* **67**, 1052-1062.

Dubey, S.K., Tripathy, A.K. and Upadhyay, S.N. (2005). Exploration of soil bacterial communities for their potential as bioresource. *Bioresource Technology* , article in press.

Duineveld, B.M., Kowalchuk, G.A., Keijzer, A., van Elsas, J.D. and van Veen, J.A. (2001(. Analysis of bacterial communities in rhizosphere of Chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology* **67**, 172-178.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A. and Kuske, C.R. (1999). Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**, 1662-1669.

Edwards, M.L., Lilley, A.K., Timms-Wilson, T.H., Thompson, I.P. and Cooper, I. (2001). Characterisation of the culturable heterotrophic bacterial community in a small eutrophic lake (Priest Pot). *FEMS Microbiology Ecology* **35**, 295-304.

Ehrig, H.J. (1983). Quality and quantity of sanitary landfill leachate. *Waste Management and Research* **1**, 53-68.

Ehlers, G.A.C. (1999). Preliminary Investigation of Nutrient Supplementation of, and Heavy Metal Mobilization by, Dual (Phenol/Activated Sewage Sludge) Co-Disposal with Refuse. MSc. Thesis, University of Natal.

Ehrig, H.J. (1989). Leachate quality. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 213-229.

Eichner, C.A., Erb, R.W., Timmis, K.N. and Wagner-Döbler, I. (1999). Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge pollutant community. *Applied and Environmental Microbiology* **65**, 102-109.

El-Fadel, M., Findikakis, N.A. and Leckies, J.O. (1977). Environmental impacts of solid waste landfilling. *Journal of Environmental Management* **50**, 1-25.

El Fantroussi, S., Verschuere, L., Verstraete, W. and Top, E.M. (1999). Effects of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiling. *Applied and Environmental Microbiology* **65**, 982-988.

Environmental Resources Management, Inc. (1981). *Technical Criteria for Identification and Screening of Sites for Hazardous Waste Facilities.* Environmental Resources Management, Inc., West Chester, Pennsylvania.

Erb, R.W. and Wagnerdöbler, I. (1993). Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Applied Environmental Microbiology* **59**, 4065-4073.

Erses, A.S. and Onay, T.T. (2003). In situ heavy metal attenuation in landfills under methanogenic conditions. *Journal of Hazardous Materials* **B99**, 159-175.

Farrelly, V., Rainey, F.A. and Stackebrandt, E. (1995). Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* **61**, 2798-2801.

Felske, A., Engelen, B., Nubel, U. and Backhaus, H. (1996). Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Applied and Environmental Microbiology* **62**, 4162-4167.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. and Akkermans, A.D.L. (1997). Ribosome analysis receives prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology* **143**, 2983-2989.

Felske, A. and Akkermans, A.D.L. (1998a). Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils. *Microbial Ecology* **36**, 31-36.

Felske, A. and Akkermans, A.D.L. (1998b). Prominent occurrence of ribosomes from an uncultured bacterium of the Verrucomicrobiales cluster in grassland soils. *Letters in Applied Microbiology* **26**, 219-223.

Felske, A., Wolterink, A., Van Lis, R. and Akkermans, A.D.L. (1998). Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). *Applied and Environmental Microbiology* **64**, 871-879.

Ferris, M.J., Muyzer, G. and Ward, D.M. (1996). Denaturing gradient electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* **62**, 340-346.

Fischer, S.G. and Lerman, L.S. (1980). Separation of random fragments of DNA according to properties of their sequences. *Proceedings of the National Academy of Sciences* USA **77**, 4420-4424.

Fischer, S.G. and Lerman, L.S. (1983). DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with meting theory. *Proceedings of the National Academy of Sciences* USA **80**, 1579-1583.

Fournier, D., Lemieux, R. and Couillard, D. (1998). Genetic evidence for highly diversified bacterial populations in wastewater sludge during biological leaching of metals. *Biotechnology Letters* **20**, 27-31.

Frigon, J.C., Bisaillon, J.G., Paquette, G. and Beaudet, R. (1997). Anaerobic treatment of landfill leachate. *Canadian Journal of Microbiology* **43**, 937-944.

Gadd, G.M. (1992). Microbial control of heavy metal pollution. *In Microbial Control of Pollution*, eds. J.C. Fry, G.M. Gadd, C.W. Jones and I. Watson-Craik. Cambridge University Press, Cambridge, 59-88.

Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G. and van Elsas, J.D. (1999). Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient electrophoresis. *Journal of Microbiological Methods* **38**, 1-15.

George, M.L.C., Bustamam, M., Cruz., W.T., Leach, J.E. and Nelson, R.J. (1997). Movement of *Xanthomonas oryzae* pv. Oryzae in Southeast Asia detected using PCR-based DNA fingerprinting. *Phytopathology* **87**, 302-309.

Ghiaci, M., Abbaspur, A., Kia, R. and Seyedayn-Azad, F. (2004). Equilibrium isotherm studies for the sorption ofbenzene, toluene, and phenol onto organo-zeolites and as-synthesized MCM-41. *Separation and Purification Technology* **40**, 217-229.

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M. and Ball, A.S. (2003). Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Applied and Environmental Microbiology* **69**, 1800-1809.

Glysson, E.A. (1990). Solid waste. In *Standard Handbook of Environmental Engineering*, ed. Corbitt, R.A. McGraw-Hill, USA, 8.1-8.193.

Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F. and Moletta, R. (1997). Microbial 16S rDNA diversity in an anaerobic digester. *Water Science and Technology* **36**, 49-55.

Griffon, R.A., Shimp, N.F., Steele, J.D., Ruch, R.R., White, W.A. and Hughes, G.M. (1976). Attenuation of pollutants in municipal landfill leachate by passage through clay. *Environmental Science and Technology* **10**, 1262-1268.

Guerin, T.F. (1999). Bioremediation of phenols and polycyclic aromatic hydrocarbons and creosote contaminated soil using ex-situ landtreatment. *Journal of Hazardous Materials* **B65**, 305-315.

Haack, S.K., Fogarty, L.R., West, T.G., Alm, E.W., McGuire, J.T., Long, D.T., Hyndman, D.W. and Forney, L.J. (2004). Spatial and temporal changes in microbial community structure associated with recharge-influenced chemical gradients in a contaminated aquifer. *Environmental Microbiology* **6**, 438-448.

Handbook of Standard Soil Testing Methods for Advisory Purposes. (1990). Soil Science Society of South Africa. 19/1.

Harper, S.C., Manoharran, R., Mavinic, D.S. and Randall, C.W. (1996). Chromium and nickel toxicity during the biotreatment of high ammonia landfill leachate. *Water Environment Research* **68**, 19-24.

Harrington, D.W. and Maris, P.J. (1986). The treatment of leachate. A UK perspective. *Water Pollution Control* **85**, 45-56.

He, Q. and Sanford, R.A. (2003). Characterization of Fe(III) reduction by chlorespiring *Anaeromyxobacter dehalogenans*. *Applied and Environmental Microbiology* **69**, 2712-2718.

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* **35**, 1-21.

Hill, M.O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology* **54**, 427-431.

Hill, T.C.J., Walsh, K.A., Harris, J.A. and Moffett, B.F. (2003). Using ecological diversity measures with bacterial communities. *FEMS Microbiology Ecology* **43**, 1-11.

Ho, S., Boyle, W.C. and Ham, R.K. (1974). Chemical treatment of leachates from sanitary landfills. *Journal of the Water Pollution Control Federation* **46**, 1776-1791.

Hoa, T.T.H., Liamleam, W. and Annachhatre, A.P. (2007). Lead removal through biological sulfate reduction process. *Bioresource Technology* **98**, 2538-2548.

Hoeks, J. and Borst, R.J. (1982). Anaerobic digestion of free volatile fatty acids in soils below waste tips. *Water, Air, and Soil Pollution* **17**, 165-173.

Höfle, M.G. (1990). Transfer RNAs as genotypic fingerprints of Bacteria. *Archives of Microbiology* **153**, 299-304.

Höfle, M.G. (1992). Bacterioplankton community structure and dynamics after large-scale release of non-indigenous bacteria as revealed by low molecular weight RNA analysis. *Applied and Environmental Microbiology* **58**, 3387-3394.

Höfle, M.G. and Brettar, I. (1996). Genotyping of heterotrophic bacteria from the central Baltic Sea by use of low-molecular-weight RNA profiles. *Applied and Environmental Microbiology* **62**, 1383-1390.

Holmes, R. (1980). The water balance method of estimating leachate production from landfill sites. *Solid Wastes* **70**, 20-31.

Hrapovic, L. and Rowe, R.K. (2001). Intrinsic degradation of volatile fatty acids in laboratory-compacted clayey soil. *Journal of Contaminant Hydrology* **58**, 221-242.

Isaacson, P.J. and Frink, C.R. (1984). Nonreversible sorption of phenolic compounds by sediment fractions: The role of sediment organic matter. *Environmental Science and Technology* **18**, 43-48.

Ivanova, I.A., Stephen, J.R., Chang, Y.J., Brüggemann, J., Long, P.E., McKinley, J.P., Kowalchuk, G.A., White, D.C. and Macnaughton, S.J. (2000). A survey of 16S rRNA and *amoA* genes related to autotrophic ammonia-oxidizing bacteria of the β-subdivision of the Class Proteobacteria in contaminated groundwater. *Canadian Journal of Microbiology* **46**, 1012-1020.

IWACO (1997). *Intrinsic Capacity of Aquifers to Degrade Pollution from (Old) Landfills*. Report Number 1073200, Dienst Water en Milieu, Provincie Zuid-Holland, Netherlands.

Jackson, C.R., Harper, J.P., Willoughby, D., Roden, E.E. and Churchill, P.F. (1997). A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Applied and Environmental Microbiology* **63**, 4993-4995.

Jackson, C.R., Harper, J.P., Willoughby, D., Roden, E.E. and Churchill, P.F (1997). A simple, efficient method for the separation of humic substances and DNA from environmental samples. *Applied and Environmental Microbiology* **63**, 4993-4995.

Jackson, C.R. and Churchill, P.F. (1999). Analysis of microbial communities by denaturing gradient gel electrophoresis: applications and limitations. *Recent Research Developments in Microbiology* **3**, 81-91.

Jackson, C.R., Roden, E.E. and Churchill, P.F. (2000). Denaturing gradient gel electrophoresis can fail to separate 16S rDNA fragments with multiple base differences. *Molecular Biology Today* **1**, 49-51.

Jackson, C.R., Churchill, P.F. and Roden, E.E. (2001). Successional changes in bacterial assemblage structure during epilithic biofilm development. *Ecology* **82**, 555-566.

Jansen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeua, M. and Kersters, K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* **142**, 1881-1893.

Jewaskiewitz, S.M. (1992). A review of current trends and new developments in the design and operation of landfill sites. In *Proceedings of Waste Management in a Changing Society, WasteCon. '92*, Johannesburg, South Africa, 279-296.

Johansen, O.J. and Carlson, D.A. (1976). Characterisation of sanitary landfill leachates. *Water Research* **10**, 1129-1134.

Jolly, J.L. (1996). Geohydrological investigations at waste disposal sites – when does groundwater constitute an aquifer? In *Proceedings of Waste Management – In Crisis, WasteCon. '96*, Durban, South Africa, 249-257.

Kan, A.T., Fu, G. and Tomson, M.B. (1994). Adsorption / desorption hysteresis in organic pollutant and soil / sediment interaction. *Environmental Science and Technology* **28**, 859-867.

Kennedy, K.J., Hamoda, M.F. and Guiot, S.G. (1988). Anaerobic treatment of leachate using fixed film and sludge bed systems. *Journal of the Water Pollution Control Federation* **60**, 1675-1683.

Kennedy, J.M. and Lentz, E.M. (2000). Treatment of landfill leachate using sequencing batch and continuous flow upflow anaerobic sludge blanket (UASB) reactors. *Water Research* **34**, 3640-3656.

Kettunen, R.H. and Rintala, J.A. (1998). Performance of an on-site UASB reactor treating leachate at low temperature. *Water Research* **32**, 537-546.

Khan, F. (2002). Bioremediation of chemically contaminated soil: Extraction / analysis methodology development. University of Natal, PhD Thesis.

Khan, Z. and Anjaneyulu, Y. (2005). Influence of soil components on adsorption-desorption of hazardous organics-development of low cost technology for reclamation of hazardous waste dumpsites. *Journal of Hazardous Materials* **B118**, 161-169.

Kinman, R.N., Nutini, D.L., Walsh, J.J., Vogt, W.G., Stamm, J. and Rickabaugh, J. (1987). Gas enhancement techniques in landfill simulators. *Waste Management and Research* **5**, 13-25.

Kjeldsen, P., Kjolholt, J., Schultz, B., Christensen, T.H., and Tjell, J.C. (1990). Sorption and degradation of chlorophenols, nitrophenols and organophosphorus pesticides in the subsoil under landfills – laboratory studies. *Journal of Contaminant Hydrology* **6**, 165-184.

Kjeldsen, P. (1993). Groundwater pollution source characterisation of an old landfill. *Journal of Hydrology* **142**, 349-371.

Knoll, K.H., Rump, H.H. and Schneider, W. (1983). Effects of toxic pollutants on indicator germs in large-scale solid-waste ecosystems. *Ecotoxicology and Environmental Safety* **7**, 463-474.

Knox, K. (1985). Leachate treatment with nitrification of ammonia. *Water Research* **19**, 895-904.

Knox, K. (1989). Practice and trends in landfilling in the UK. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 533-547.

Kocherginskaya, S.A., Aminov, R.I. and White, B.A. (2001). Analysis of rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecological approaches. *Anaerobe* **7**, 119-134.

Koizumi, Y., Kojima, H. and Fukui, M. (2003). Characterization of depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments. *FEMS Microbiology Ecology* **46**, 147-157.

Kowalchuk, G.A., Stephen, J.R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorf, J.W. (1997). Analysis of ammonia-oxidising bacteria of the β-subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified16S ribosomal DNA fragments. *Applied and Environmental Microbiology* **63**, 1480-1497.

Krebs, C.J. (1985). Species diversity I: theory. In *Ecology:The Experimental Analysis of Distribution and Abundance*, third edition. Harper and Row Publishers, New York, 513-542.

Krsek, M., and Wellington, E.M.H. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *Journal of Microbiological Methods* **39**, 1-16.

Kuske, C.R., Barns, S.M. and Busch, J.D. (1997). Diverse uncultured bacterial groups from soils of the arid southwestern United States that are present in many geographical regions. *Applied and Environmental Microbiology* **63**, 3614-3621.

Large, P.J. (1983). Methylotrophy and methanogenesis. In *Aspects of Microbiology*, ed. Cole, J.A., Knowles, C.J. and Schlessinger, D. Van Nostrand Reinhold Co. Ltd., United Kingdom, 1-69.

Lechner, P. (1989). The Austrian guidelines for sanitary landfilling. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 523-531.

Lee, D.H., Zo., Y.G. and Kim, S.Y. (1996). Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Applied and Environmental Microbiology* **62**, 3112-3120.

Leeflang, P. and Smit, E. (1997). Use of the Expand™ PCR system to amplify the 16S ribosomal genes for the characterization of bacterial communities in soil. *Biochemica* **1**, 16-18.

Lema, J.M., Mendez, R. and Blazquez, R. (1988). Characteristics of landfill leachate and alternatives for their treatment: A review. *Water, Air, and Soil Pollution* **40**, 223-250.

Lensing, H.J., Vogt, M. and Herrling, B. (1994). Modelling of biologically mediated redox processes in the subsurface. *Journal of Hydrology* **159**, 125-143.

Lerner, D.N., Thornton, S.F., Spence, M.J., Banwart, S.A., Botrell, S.H., Higgo, J.J., Mallinson, H.E.H, Pickup, R.W. and Williams, G.M. (2000). Ineffective natural attenuation of degradable organic compounds in a phenol contaminated aquifer. *Ground Water* **38**, 922-928.

Lin, C.Y. (1991). Anaerobic digestion of landfill leachate. *Water SA* **17**, 301-306.

Lloyd-Jones, G. and Lau, P.C.K. (1998). A molecular view of microbial diversity in a dynamic landfill in Quebec. *FEMS Microbiology Letters* **162**, 219-226.

Lo, I.M.C. (1996). Characteristics and treatment of leachates from domestic landfills. *Environment International* **22**, 433-442.

Lo, I.M.C., Mak, K.M. and Lee, S.C.H. (1997). Modified clays for waste containment and pollutant attenuation. *Journal of Environmental Engineering* **123**, 25-32.

Loch, J.P.G., Lagas, P. and Haring, B.J.A.M. (1981). Behaviour of heavy metals in soil beneath a landfill; results of model experiments. *The Science of the Total Environment* **21**, 203-213.

Lønborg, M.J., Engesgaard, P., Bjerg, P.L. and Rosbjerg, D. (2006). A steady state redox zone approach for modelling the transport and degradation of xenobiotic organic compounds from a landfill site. *Journal of Contaminant Hydrology* **87**, 191-210.

Louws, F.J., Fullbright, D.W., Stephens, C.T. and de Bruijn, F.J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. . *Applied and Environmental Microbiology* **60**, 2286-2295.

Louws, F.J., Rademaker, J.L.W. and de Bruijn, F.J. (1999). The three Ds of PCR-based genomic analysis of Phytobacteria: Diversity, Detection, and Disease Diagnosis. *Annual Review of Phytopathology* **37**, 81-125.

Lovely, D. and Klug, M. (1983). Sulphate reducers can outcompete methanogens at freshwater sulfate concentrations. *Applied and Environmental Microbiology* **45**, 187-192.

Lovey, D.R. (1997). Potential for anaerobic bioremediation of BTEX in petroleum contaminated aquifers. *Journal of Industrial Microbiology and Biotechnology* **18**, 75-81.

Ludvigsen, L., Albrechtsen, H.J., Ringelberg, D.B., Ekelund, F. and Christensen, T.H. (1999). Distribution and composition of microbial populations in a landfill leachate contaminated aquifer (Grinsted, Denmark). *Microbial Ecology* **37**, 197-207.

Lui, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**, 4516-4522.

Lyngkilde, J. and Christensen, T.H. (1992). Redox zones of a landfill leachate pollution plume (Vejen, Denmark). *Journal of Contaminant Hydrology* **10**, 273-289.

Lyngkilde, J. and Christensen, T.H. (1992b). Fate of organic contaminants in the redox zones of a landfill leachate pollution plume (Vejen, Denmark). *Journal of Contaminant Hydrology* **10**, 291-307.

Macnaughton, S., Stephen, J.R., Chang, Y.J., Peacock, A., Flemming, C.A., Leung, K.T. and White, D.C. (1999). Characterization of metal-resistant soil Eubacteria by polymerase chain reaction – denaturing gradient gel electrophoresis with isolation of resistant strains. *Canadian Journal of Microbiology* **45**, 116-124.

Maila, M.P.,Randima,P., Surridge,K., Drønen, K., Thomas, E.C. (2005). Evaluation of microbial diversity of different soil layers at a contaminated diesel site. *International Biodeterioration and Biodegradation* **55**, 39-44.

Manceau, C. and Horvais, A. (1997). Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. tomato. *Applied and Environmental Microbiology* **63**, 498-505.

Manulis, S., Valinsky, L., Lichter, A. and Gabriel, D.W. (1994). Sensitive and specific detection of *Xanthomonas campestris* pv. Pelargonii with DNA primers and probes identified by random amplified polymorphic DNA analysis. *Applied and Environmental Microbiology* **60**, 4094-4099.

Markiewicz-Patkowska, J., Hurthouse, A. and Przybyla-Kij, H. (2005). The interaction of heavy metals with urban soils: sorption behaviour of Cd, Cu, Cr, Pb and Zn with a typical mixed brownfield deposit. *Environment International* **31**, 513-521.

Maris, P.J., Harrington, D.W., Biol, A.I. and Chismon, G.L. (1984). Leachate treatment with particular reference to aerated lagoons. *Water Pollution Control* **84**, 521-536.

Markiewicz-Patkowska, J., Hursthouse, A. and Przybyla-Kij, H. (2005). The interaction of heavy metals with urban soils: sorption behaviour of Cd, Cu, Cr, Pb and Zn with a typical mixed brownfield deposit. *Environmental International* **31**, 513-521.

Marschner, P., Crowley, D.E. and Lieberei, R. (2001). Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. *Mycorrhiza* **11**, 297-302.

Martienssen, M. and Schops, R. (1997). Biological treatment of leachate from solid waste landfill sites – Alterations in the bacterial community during the denitrification process. *Water Research* **31**, 1164-1170.

McCaig, A.E., Glover, L.A. and Prosser, J.I. (2001). Numeriacal analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Applied and Environmental Microbiology* **67**, 4554-4559.

McLellan, J.K. and Rock, C.A. (1988). Pretreating landfill leachate with peat to remove metals. *Water, Air, and Soil Pollution* **37**, 203-215.

McNamara, N.P., Black, H.I.J., Beresford, N.A. and Parekh, R. (2003). Effects of acute chemical irradiation on chemical, physical and biological properties of soil. *Applied Soil Ecology* **24**, 117-132.

Meintanis, C., Chalkou, K.I., Kormas, K.A., Lymperopoulou, D.S., Katsifas, E.A., Hatzinikolaou, D.G., and Karagouni, A.D. (2008). Application of rpoB sequence similarity analysis, REP-PCR and BOX-PCR for the differentiation of species within the genus Geobacillus. *Letters in Applied Microbiology* **46**, 395-401.

Mesquita, M.E. and Vieira e Silva, J.M. (2002). Preliminary study of pH effect in the application of Langmuir and Freundlich isotherms to Cu-Zn competitive adsorption. *Geoderma* **106**, 219-234.

Mikac, N., Cosovic, B., Ahel, M., Andreis, S. and Tovcic, Z. (1998). Assessment of groundwater contamination in the vicinity of a solid waste landfill. *Water, Science, and Technology* **37**, 37-44.

Ming-Kiu, Z., Zhen-Li, H.E., Calvert, D.V. and Stoffella, P.J. (2006). Extractability and mobility of copper and zinc accumulated in sandy soils. *Pedosphere* **16**, 43-49.

Moffett, B.F., Nicholson, F.A., Uwakwe, N.C., Chambers, B.J., Harris, J.A., and Hill, T.C.J. (2003). Zinc contamination decreases the bacterial diversity of agricultural soil. *FEMS Microbiology Ecology* **43**, 13-19.

Momol, M.T., Momol, E.A., Lamboy, W.F., Norelli, J.L., Beer, S.V. and Alowinckle, H.S. (1997). Characterization of *Erwinia amylovora* strains using random amplified polymorphic DNA fragments (RAPDs). *Journal of Applied Microbiology* **82**, 389-398.

Mori, K., Hatsu, M., Kimura, R. and Takamizawa, K. (2000). Effect of heavy metals on the growth of a methanogen in pure culture and coculture with a sulphate-reducing bacterium. *Journal of Bioscience and Bioengineering* **90**, 260-265.

Mumy, K.L and Findlay, R.H. (2004). Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. *Journal of Microbiological Methods* **57**, 259-268.

Murali, V. and Aylmore, L.A.G. (1983). Competitive adsorption during solute transport in soils: 1. Mathematical models. *Soil Science* **135**, 143-150.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**, 695-700.

Muyzer, G. and Ramsing, N.B. (1995). Molecular methods to study the organisation of microbial communities. *Water Science and Technology* **32**, 1-9.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**, 127-141.

Muyzer, G. (1999). Genetic fingerprinting of microbial communities – Present status and future perspectives. In *Microbial Biosystems: New Frontiers. Proceedings of the 8th Symposium on Microbial Ecology*, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Myburg, C. and Britz, T.J. (1992). Anaerobic treatment of landfill leachate with a COD concentration > 20 kg/m^3.d, using an anaerobic hybrid digester. In *Proceedings of the Third Southern Africa Anaerobic Digestion Sysposium, 1992*, Pietermaritzburg, South Africa, 220-236.

Myburg, C. and Britz, T.J. (1993). Influence of higher organic loading rates on the efficiency of an anaerobic hybrid digester while treating landfill leachate. *Water SA* **19**, 319-324.

Myers, R.M., Fischer, S.G., Lerman, L.S. and Maniatis, T. (1985). Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research* **13**, 3131-3145.

Myers, R.M., Maniatis, T. and Lerman, L.S. (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology* **155**, 501-527.

Nathanson, J.A. (2000). Municipal solid waste. In *Basic Environmental Technology: Water Supply, Waste Management, and Pollution Control*, third edition. Prentice Hall, New Jersey, 309-346.

Nay, M., Snozzi, M., Snozzi, M., and Zehnder, J.B. (1999a). Fate and behavior of organic compounds in an artificial saturated subsoil under controlled redox conditions: The sequential soil column system. *Biodegradation* **10**, 75-82.

Nay, M., Snozzi, M., Snozzi, M., and Zehnder, J.B. (1999b). Behavior of chemical contaminants under controlled redox conditions in an artificial sequential soil column system and in batch cultures. *Biodegradation* **10**, 405-414.

Nielsen, P.H., Albrechtsen, H.J., Heron, G. and Christensen, T.H. (1995). In situ and laboratory studies on the fate of specific organic compounds in an anaerobic leachate plume, 1. Experimental conditions and fate of phenolic compounds. *Journal of Contaminant Hydrolog* **20**, 27-50.

Niemi, R.M., Heiskanen, I., Wallenius, K. and Lindström, K. (2001). Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *Journal of Microbiological Methods* **45**, 155-165.

Novella, P.H., Ekama, G.A. and Blight, G.E. (1996). The effects of leachate recycling and recirculation on leachate and refuse stabilisation. In *Proceedings of Waste Management – In Crisis, WasteCon. '96*, Durban, South Africa.

Novella, P.H., Ross, W.R., Lord, G.E., Greenhalgh, M.A., Stow, J.G. and Fawcett, K.S. (1996). *The Co-disposal of Wastewater Sludge with Refuse in Sanitary Landfills*, Water Research Commission Report, Cape Town City Council Engineer's Department.

Novella, P.H., Ballard, R.H., Stow, J.G., Ross, W.R., Blight, G.E. and Vorster, K. (1999). *Practical application of special waste co-disposal with municipal refuse at the Coastal Park Landfill Bioreactor*. Report to the Water Research Commission, WRC Report No. 606/1/99.

Nyhan, J.W., Hakonson, T.E. and Drennon, B.J. (1990). A water balance study of two landfill cover designs for semi-arid regions. *Journal of Environmental Quality* **19**, 281-288.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989). Detections of polymorphisms of human DNA by gel electrophoresis as single strand conformational polymorhisms. *Proceedings of the National Academy of Sciences* USA **86**, 2766-2770.

Pace, N.R., Stahl, D.A., Lane, D.J. and Olsen, G.J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. *Advances in Microbial Ecology* **9**, 1-55.

Pacey, J. (1989). Landfill design concepts in the United States. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 559-576.

Palit, T. and Qasim, S.R. (1977). Biological treatment kinetics of landfill leachate. *ASCE Journal of Environmental Engineering* **103**, 353-366.

Parker, R.J., Bateman, S. and Williams, D. (1993). Design and management of landfills. In *Geotechnical Management of Waste and Contamination: Proceedings of the Conference on Geotechnical Management of Waste and Contamination*, Sydney, ed. Fell, R., Phillips, T. and Gerrard, C. AA Balkema, Rotterdam, 209-252.

Parsons, R. (1992). Preventing groundwater contamination by waste disposal activities: are we doing enough? In *Proceedings of Waste Management – In Crisis, WasteCon. '96*, Durban, South Africa.

Parsons, R. (1994). Groundwater monitoring at waste disposal sites: more than just sinking a borehole and grabbing a sample. In: *Proceedings of WasteCon '94*, Somerset West, South Africa.

Parsons, R. and Jolly, J.L. (1994). The Development of a Systematic Method for Evaluating Site Suitability for Waste Disposal Based on Geohydrological Criteria. WRC Report Number 485/1/94, Water Research Commission, Pretoria, South Africa.

Pedersen, J.K., Bjerg, P.L. and Christensen, T.H. (1991). Correlation of nitrate profiles with groundwater and sediment characteristics in a shallow sandy aquifer. *Journal of Hydrology* **124**, 263-277.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X. and Simonet, P. (1992). Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology* **58**, 2717-2722.

Pohland, F.G. and Al-Yousfi, B. (1994). Designs and operations of landfills for optimum stabilisation and biogas production. *Water, Science, and Technology* **30**, 117-124.

Pooler, M.R., Ritchie, D.F. and Hartung, J.S. (1996). Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic concensus PCR data and and generation of multiplexed PCR primers useful for the identification of this phytopathogen. *Applied and Environmental Microbiology* **62**, 3121-3127.

Primo, O., Rivero, M.J., and Ortiz, I. (2007). Photo-Fenton process as an efficient alternative to the treatment of landfill leachates. *Journal of Hazardous Materials* **153**, 834-842.

Ralebitso, T.K., Röling, W.F.M., Braster, M., Senior, E. and van Verseveld, H.W. (2000). 16S rDNA-based characterization of BTX-catabolizing microbial associations isolated from a South African sandy soil. *Biogedradation* **11**, 351-357.

Ramírez-Saad, H.C., Sessitsch, A. and Akkermans, A.D.L. (2003). Microbial diversity in the bacterial community and the fluorescent pseudomonads group in natural and chlorobenzoate-stressed and peat-forest soil. *Microbiology Research* **158**, 47-54.

Ramos, L., Hernandez, L.M. and Gonzalez, M.J. (1994). Sequential fractionation of copper, lead, cadmium and zinc in soils from or near Doñana National Park. *Journal of Environmental Quality* **23**, 50-57.

Rasmusen, L.D. and Sørensen, S.J. (2001). Effects of mercury contamination on the cultural heterotrophic, functional and genetic diversity of the bacterial community of soil. *FEMS Microbiology Ecology* **36**, 1-9.

Rees, J.F., and King, J.W. (1981). The dynamics of anaerobic phenol biodegradation in Lower Greensand. *Journal of Chemical Technology and Biotechnology* **31**, 306-310.

Reinhard, M., Goodman, N.L. and Barker, J.F. (1984). Occurrence and sistribution of organic chemicals in landfill leachate plumes. *Environmental Science and Technology* **18**, 953-961.

Reinhart, D.R. and Al-Yousfi, A.B. (1996). The impact of leachate recirculation on municipal solid waste landfill operating characteristics. *Waste Management and Research* **14**, 337-346.

Robe, P., Nalin, R., Capellano, C., Vogel, T.M., and Simonet, P. (2003). Extraction of DNA from soil. *European Journal of Soil Biology* **39**, 183-190.

Robinson, H.D., Barber, C. and Maris, P.J. (1982). Generation and treatment of leachate from domestic wastes in landfills. *Water Pollution Control* **81**, 465-478.

Robinson, H.D. and Maris, P.J. (1985). The treatment of leachates from domestic waste in landfill sites. *Journal of the Water Pollution Control Federation* **57**, 30-38.

Robinson, H.D. and Luo, M.M.H. (1991). Characterisation and treatment of leachates from Hong Kong landfill sites. *Journal of the Institution of Water and Environmental Management* **5**, 326-335.

Robinson, H. and Gronow, J. (1992). Groundwater protection in the UK: Assessment of the landfill leachate source-term. *Journal of the Institution of Water and Environmental Management* **6**, 229-236.

Röling, W.F.M. and van Verseveld, H.W. (1996). Characterization of *Tetragenococcus halophila* poulations in Indonesian soy mash (kecap) fermentation. *Applied and Environmental Microbiology* **62**, 1203-1207.

Röling, W.F.M., van Breukelen, B.M., Braster, M., Goeltom, M.T., Groen, J. and van Verseveld, H.W. (2000). Analysis of microbial communities in a landfill leachate polluted aquifer using a new method for anaerobic physiological profiling and 16S rDNA based fingerprinting. *Microbial Ecology* **40**, 177-188.

Röling, F.M., van Breukelen, B.M., Braster, M., Lin, B. and van Verseveld, H.W. (2001). Relationship between microbial community structure and hydrochemistry in a landfill leachate polluted aquifer. *Applied and Environmental Microbiology* **67**, 4619-4629.

Röling, F.M. and van Verseveld, H.W. (2002). Natural attenuation: What does the subsurface have in store? *Biodegradation* **13**, 53-64.

Romantschuk, M., Sarand, I., Petanen, T., Peltola, R., Jonsson-Vihanne, M., Koivula, T., Yrjala, K. and Haahtela, K. (2000). Means to improve the effect of insitu bioremediation of contaminated soil: an overview of novel approaches. *Environmental Pollution* **2**, 179-185.

Roose-Almsaleg, C.L., Garnier-Sillam, E. and Harry, M. (2001). Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology* **18**, 47-60.

Rugge, K., Bjerg, P.L. and Christensen, T.H. (1995). Distribution of organic compounds from municipal solid waste in the groundwater downgradient of a landfill (Grinsted, Denmark). *Environmental Science and Technology* **29**, 1395-1400.

Salles, J.F., van Veen, J.A., and van Elsas, J.D. (2004). Multivariate analyses of *Burkholderia* species in soil: Effect of crop and land use history. *Applied and Environmental Microbiology* **70**, 4012-4020.

Sanford, R.A., Cole, J.R. and Tiedje, J.M. (2002). Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic Myxobacterium. *Applied and Environmental Microbiology* **68**, 893-900.

Santegoeds, C.M., Nold, S.C. and Ward, D.M. (1996). Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. *Applied and Environmental Microbiology* **62**, 3922-3928.

Schie, P.M. and Young, L.Y. (2000). Biodegradation of phenol: Mechanisms and applications. *Bioremediation Journal* **4**, 1-18.

Schneider, M. and De Bruijn, F.J. (1996). Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic pattern analysis. *World Journal of Microbiology and Biotechnology* **12**, 163-174.

Scholl, M.A., Cozzarelli, I.M. and Christenson, S.C. (2006). Recharge processes drive sulfate reduction in an alluvial aquifer contaminated with landfill leachate. *Journal of Contaminant Hydrology* **86**, 239-261.

Schramm, A. and Amann, R. (1999). Nucleic acid based techniques for analysing the diversity, structure, and dynamics of microbial communities in wastewater treatment. In *Environmental Processes – Wastewater and Waste Treatment*, vol. IIa of the series: Biotechnology, 2nd edition, eds. Rehm, H.J., Reed, G., Pühler, A. and Stadler, P. Wiley-VCH, Weinheim, Germany, 85-108.

Schultz, B. and Kjeldsen, P. (1986). Screening of organic matter in leachates from sanitary landfills using gas chromatography combined with mass spectrometry. *Water Research* **20**, 965-970.

Schütt, C. (1990). Plasmids and their role in natural aquatic bacterial communities. In *Aquatic Microbial Ecology: Biochemical and Molecular Approaches*, eds. Overbeck, J. and Chróst, R.J. Springer-Verlag, New York, USA. 160-183.

Schweiger, F. and Tebbe, C.C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology* **64**, 4870-4876.

Scott, M.P. (1982). Options for the treatment of municipal and chemical waste leachate. *Public Health Engineer* **10**, 119-126.

Scott, H.D., Wolf D.C. and Lavy, T.L. (1982). Apparent adsorption and microbial degradation of phenol by soil. *Journal of Environmental Quality* **11**, 107-112.

Scott, H.D., Wolf D.C. and Lavy, T.L. (1982). Adsorption and degradation of phenol at low concentrations in soil. *Journal of Environmental Quality* **12**, 91-95.

Senior, E. and Balba, M.T.M. (1987). Landfill biotechnology. In *Bioenvironmental Systems*, volume II, ed. Wise, D.L. CRC Press, Boca Raton, Florida, 17-65.

Senior, E., Watson-Craik, I.A. and Kasali, G.B. (1990). Control/promotion of the refuse methanogenic fermentation. *Critical Reviews in Biotechnology* **10**, 93-118.

Senior, E., Watson-Craik, I.A., Sinclair, K.J. and Jones, R. (1991). Refuse exploitation: A role for the applied microbiologist? In *Proceedings of the Ninth International Conference on Global Impacts of Applied Microbiology and Biotechnology*, Valletta, Malta.

Senior, E. (1991). *Lay Wasted Waste*. Inaugural Lecture, University of Natal, Pietermaritzburg, South Africa.

Shaw, L.J., and Burns, R.G. (1996). Construction and equilibration of a repacked soil column microcosm. *Soil Biology and Biochemistry* **28**, 1117-1120.

Shaw, L.J., and Burns, R.G. (1998). Biodegradation-transport interactions of pulse applied 2,4-D in repacked soil column microcosms. *Journal of Environmental Quality* **27**, 1472-1478.

Shaw, L.J., Beaton, Y., Glover, L.A., Killham, K. and Meharg, A.A. (1999). Re-inoculation of autoclaved soil as a non-sterile treatment for xenobiotic sorption and biodegradation studies. *Applied Soil Ecology* **11**, 217-226.

Shibata, A., Inoue, Y., and Katayama, A. (2006). Aerobic and anaerobic degradation of phenol derivatives in various paddy soils. *Science of the Total Environment* **367**, 979-987.

Signes-Pastor, A., Burló, F., Mitra, K., and Carbonell-Barrachina, A.A. (2007). Arsenic biogeochemistry as affected by phosphorus fertilizer addition, redox potential and pH in a west Bengal (India) soil. *Geoderma* **137**, 504-510.

Sinclair, K.J. (1994). The Co-disposal of Sewage Sludge with Domestic Refuse and the Potential Importance of Landfill Nitrogen Transformations. PhD Thesis, University of Strathclyde.

Singh, S.N. (2001). Exploring correlation between redox potential and other edaphic factors in field and laboratory conditions in relation to methane efflux. *Environment International* **27**, 265-274.

Sletten, R.S., Benjamin, M.M., Horng, J.J. and Ferguson, J.F. (1995). Physical-Chemical treatment of landfill leachate for metals removal. *Water Research* **29**, 2376-2386.

Smith, J.A., and Novak, J.T. (1987). Biodegradation of phenols in subsurface soils. *Water, Air, and Soil Pollution* **33**, 29-42.

Smith, E.H. and Weber Jr., W.R. (1990). Comparative assessment of the chemical and adsorptive characteristics of leachates from a municipal and an industrial landfill. *Water, Air, and Soil Pollution* **53**, 279-295.

Smith, D.C., Sacks, J. and Senior, E. (1999a). Irrigation of soil with synthetic landfill leachate – speciation and distribution of selected pollutants. *Environmental Pollution* **6**, 429-441.

Smith, D.C., Senior, E. and Dicks, H.M. (1999b). Irrigation of land with synthetic landfill leachate – breakthrough behaviour of selected pollutanta. *Water, Air, and Soil Pollution* **109**, 327-342.

Španová, A., Rittich, B., Štyriak, I., Štyriaková, I. and Horák, D. (2006). Isolation of polymerase chain reaction-raedy bacterial DNA from Lake Baikal sediments by carboxyl-functionalised magnetic polymer microspheres. *Journal of Chromatography A* **1130**, 115-121.

Stegman, R. (1989). Principles of landfilling – The current approach. In In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 11-17.

Stief, K. (1989). Multi-barrier concept in West Germany. In *Sanitary Landfilling: Process, Technology, and Environmental Impact*, ed. Christensen, T.H., Cossu, R. and Stegman, R. Academic Press, United Kingdom, 549-557.

Suflita, J.M., Gerra, C.B., Ham, R.K., Palmisano, A.C., Rathje, W.L. and Robison, J.A. (1992). The world's largest landfill. A multidisciplinary investigation. *Environmental Science and Technology* **26,** 1486-1495.

Sumathi, V.R., Natesan, U. and Sarkar, C. (2008). GIS-based approach for optimized siting of municipal solid waste landfill. *Waste Management* **28**, 2146-2160.

Swalaha, M.F., Datadin, S. and Choonawala, B.B. (2002). *Assessment and application of imported biomass for the bioremediation of heavy metal effluents*. Report to the Water Research Commission, WRC Report No. 1083/1/02, 26.

Swett, G.H. and Rapaport, D. (1998). Natural attenuation: Is the fight right? *Chemical Engineering Progress* **94**, 37-43.

Tan, K.H. (1996). *Soil Sampling Preparation and Analysis*. Marcel Dekker Inc., New York, USA, 86-95.

Ter Braak C.J.F. and Smilauer P. (1997). Canoco for Windows Version 4.51. Biometrics – Plant Research International, Wageningen, The Netherlands.

Teske, A., Wawer, C., Muyzer, G. and Ramsing, N.B. (1996). Distribution of sulphate-reducing bacteria in a stratified Fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoeresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology* **62**, 1405-1415.

Thornton, R.J. and Blanc, F.C. (1973). Leachate treatment by coagulation and precipitation. *ASCE Journal of Environmental Engineering Division* **99**, 535-544.

Thornton, S.F., Tellam, J.H. and Lerner, D.N. (2000). Attenuation of landfill leachate by UK Triassic sandstone aquifer materials: (1) Fate of inorganic pollutants in laboratory columns. *Journal of Contaminant Hydrology* **43**, 327-354.

Tittlebaum, M.E. (1982). Organic carbon content stabilisation through landfill leachate recirculation. *Journal of the Water Pollution Control Federation* **54**, 428-487.

Townsend, C.R., Begon, M. and Harper, J.L. (2003). Patterns in species richness. In *Essentials of Ecology*, second edition. Blackwell Scientific Publications, Oxford, 330-365.

Trevors, J.T. (1996). Sterilization and inhibition of microbial activity in soil. *Journal of Microbiological Methods* **26**, 53-59.

Truede, N., Rosencrantz, D., Liesack, W. and Schnell, S. (2003). Strain FAc12, a dissimilatory iron reducing member of the *Anaeromyxobacter* subgroup of Myxococcales. *FEMS Microbiology Ecology* **44**, 261-269.

UK Department of the Environment (1994). Waste Management Paper 26F: Landfill Co-disposal (Draft Consultation).

Van Breukelan, B.M. (2003). Natural Attenuation of Landfill Leachate: A Combined Biogeochemical Process Analysis and Microbial Ecology Approach. PhD Thesis, Vrije Universiteit van Amsterdam.

van der Maarel, M.J.E.C., Artz, R.R.E., Hanstra, R. and Forney, L.J. (1998). Association of marine Archaea with the digestive tracts of two marine fish species. *Applied and Environmental Microbiology* **64**, 2894-2898.

Van Gulck, J.F. and Rowe, R.K. (2004). Evolution of clog formation with time in columns permeated with synthetic landfill leachate. *Journal of Contaminant Hydrology* **75**, 115-139.

van Hullebusch, E.D., Peerbolte, A., Zandvoort, M.H. and Lens, P.N.L., (2005). Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction. *Chemosphere* **58**, 493-505.

van Rossum, D., Schuurmans, F.P., Gillis, M., Muyotcha, A., van Verseveld, H.W., Stouthamer, A.H. and Boogerd, F.C. (1995). Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L) roots. *Applied and Environmental Microbiology* **61**, 1599-1609.

van Schie, P.M. and Young, L.Y. (2000). Biodegradation of phenol: mechanisms and applications. *Bioremediation Journal* **4**, 1-18.

Vanbroekhoven, K., Van Roy, S., Gielen, C., Maesen, M., Ryngaert, A. Diels, L. and Seuntjens, P. (2007). Microbial processes as key drivers for metal (im)mobilization along a redox gradient in a saturated zone. *Environmental Pollution* **148**, 759-769.

Venkataramani, E.S. and Ahlert, R.C. (1984). Rapid aerobic biostabilisation of high strength industrial landfill leachate. *Journal of the Water Pollution Control Federation* **56**, 1178-1184.

Venkataramani, E.S., Ahlert, R.C. and Corbo, P. (1984). Biological treatment of landfill leachates. *CRC Critical Reviews in Environmental Control* **14**, 333-377.

Wall, D.K. and Zeiss, C. (1995). Municipal landfill biodegradation and settlement. *ASCE Journal of Environmental Engineering* **121**, 214-224.

Watanabe, K., Teramoto, M., Futamata, H. and Harayama, S. (1998). Molecular detection, isolation, and physiological characterisation of functionally dominant phenol degrading bacteria in activated sludge. *Applied and Environmental Microbiology* **64**, 4396-4402.

White-Hunt, K. (1980). Domestic refuse: a brief history (Part 1). *Solid Wastes* **70**, 609-615.

White-Hunt, K. (1981a). Domestic refuse: a brief history (Part 2). *Solid Wastes* **71**, 159-169.

White-Hunt, K. (1981b). Domestic refuse: a brief history (Part 3). *Solid Wastes* **71**, 285-292.

White, D.M. and Schnabel, W. (1998). Treatment of cyanide waste in a sequencing batch biofilm reactor. *Water Research* **32**, 254-257.

Widmar, F., Seidler, R.J., Gillevet, P.M., Watrud, L.S. and Di Giovanni, G.D. (1998). A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (sensu stricto) in environmental samples. *Applied and Environmental Microbiology* **64**, 2545-2553.

Willems, H.P.L., Lewis, K.J., Dyson, J.S. and Lewis, F.J. (1996). Mineralization of 2,4-D and atrazine in the unsaturated zone of a sandy loam soil. *Soil Biology and Biochemistry* **28**, 989-996.

Williams, G.M. and Higgo, J.J.W. (1994). *In situ* and laboratory investigations into contaminant migration. *Journal of Hydrology* **159**, 1-25.

Winderl, C. Anneser, B., Griebler, C., Meckenstock, R.U. and Lueders, T. (2008). Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar-oil contaminant plume. *Applied and Environmental Microbiology* **74**, 792-801.

Wise, M.G., McArthur, J.V. and Shimkets, L.J. (1999). Methanotroph diversity in landfill soil: Isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Applied and Environmental Microbiology* **65**, 4887-4897.

Woese, C.R. (1987). Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Wong, J.W.C., Cheung, K.C. and Wong, M.H. (2000). Environmental implications of soils amended with anaerobically digested sewage sludge in Hong Kong. *Water, Air, and Soil Pollution* **124**, 23-36.

World Bank Technical Paper (1989). *The Safe Disposal of Hazardous Wastes: The Special Needs and Problems of Developing Countries, volume II.* Report Number 93, ed. Batstone, R., Smith, J.E. and Wilson, D. The World bank Publications Division, Washington.

Wünsche, L., Bruggemann, L. and Babel, W. (1995). Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution. *FEMS Microbiology Ecology* **17**, 295-306.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., Veal, D.A. (1998). Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online* (*www.biological procdures.com*) **1**, 40-47.

Zhou, J., Bruns, M.A. and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62**, 316-322.

Zhou, J., Xia, B., Treves, D.S., Wu, L.Y., Marsh, T.L., O'Neill, R.V., Palumbo, A.V., and Tiedje, J.M. (2002). Spatial and resource factors influencing high microbial diversity in soil. *Applied and Environmental Microbiology* **68**, 326-334.

Appendix A

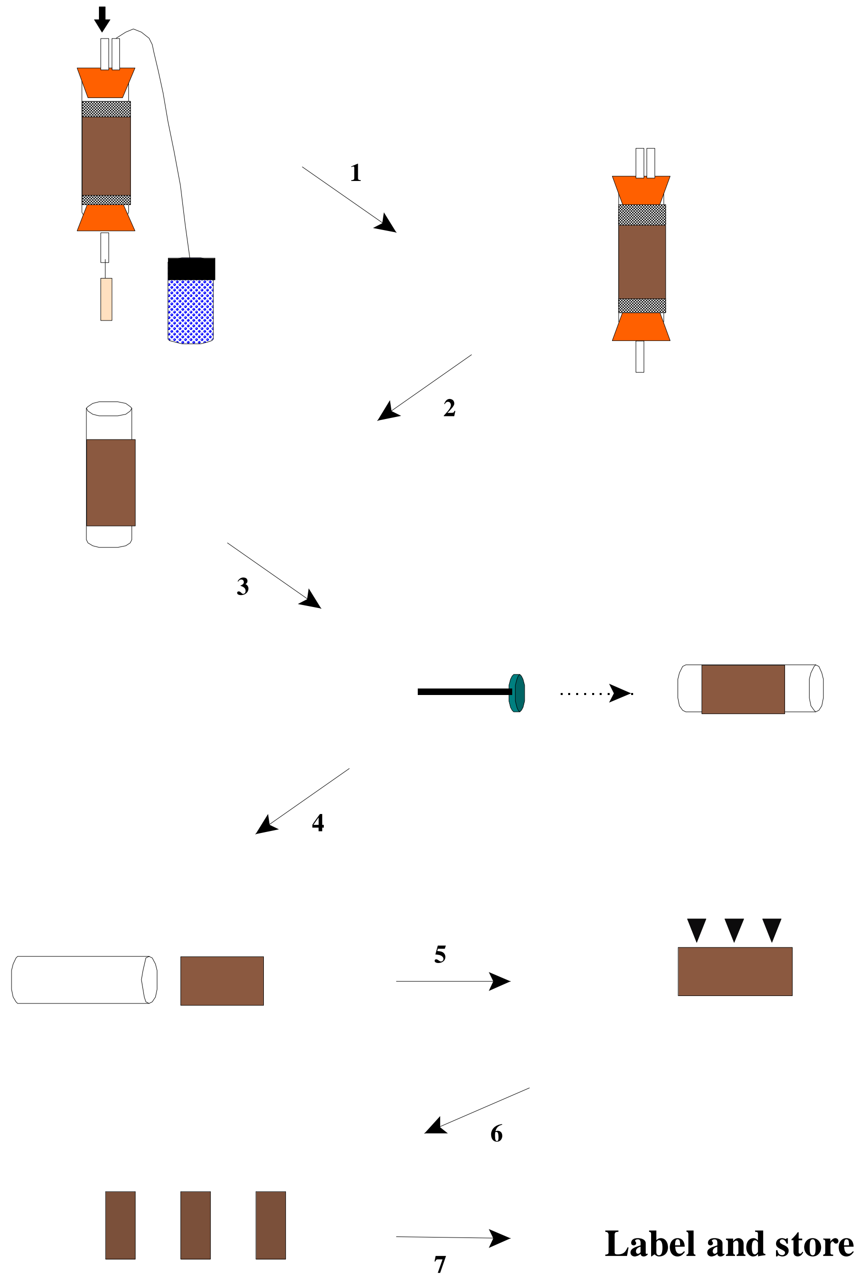
A. Characteristics of Soil Material

Table A.1 Summary of the physical and chemical characteristics of a Hutton soil.

Particle Size Distribution	Percentage (w/w)
Coarse Sand 2 - 0.5 mm	1.34
Medium Sand 0.5 - 0.25 mm	1.57
Fine Sand 0.25 - 0.1 mm	6.38
Silt	22.97
Clay <0.002 mm	67.57
Mineralogy of Clay Fraction (%) (w/w)	
Kaolinite	40 - 60
Vermiculite	40 - 60
Exchangeable Cations ($cmol_c$ kg^{-1})	
Ca	10.0
Mg	3.42
Na	0.74
K	0.6
Fe	0.75
General Characteristics	
Organic Carbon (%) (w/w)	3.31
P ($mg.l^{-1}$)	5.0
pH (H_2O)	6.36
pH (KCl)	5.05
CEC ($cmol_c$ kg^{-1})	2.44

Appendix B

B. Column Harvesting and Soil Sampling



Key:

▼ = Knife

= Applied force

= Plunger

Appendix C

C. Eluents

C.1 10 x's Concentrated Leachate

The leachate contained the following (g.l^{-1} distilled water):

$Cu(NO_3)_2.3H_2O$, 0.951; $Zn(NO_3)_2.6H_2O$, 4.549; NH_4Cl, 0.451; KNO_3, 0.791; NaCl, 11.897; $MgCl_2.6H_2O$, 8.365; $CaCl_2.2H_2O$, 3.668; Na_2SO_4, 2.0; C_6H_6O, 5.0.

C.2 Leachate A

10 x's concentrated leachate (100 m*l*) was diluted with distilled water to 1 *l*.

C.3 Leachate A-mn

10 x's concentrated leachate (100 m*l*), 5 m*l* each of the trace element, trace mineral and vitamin solutions (2.4.2) were combined and diluted to 1 *l* with distilled water.

C.4 Sodium Sulphide Solution (0.01 M)

Sodium sulphide ($Na_2S.9H_2O$) (0.12 g) (Saarchem) was dissolved and diluted with distilled water to 50 m*l*.

Appendix D

D. Rainfall data used to determine Hydraulic Loading Rates (HLR's)

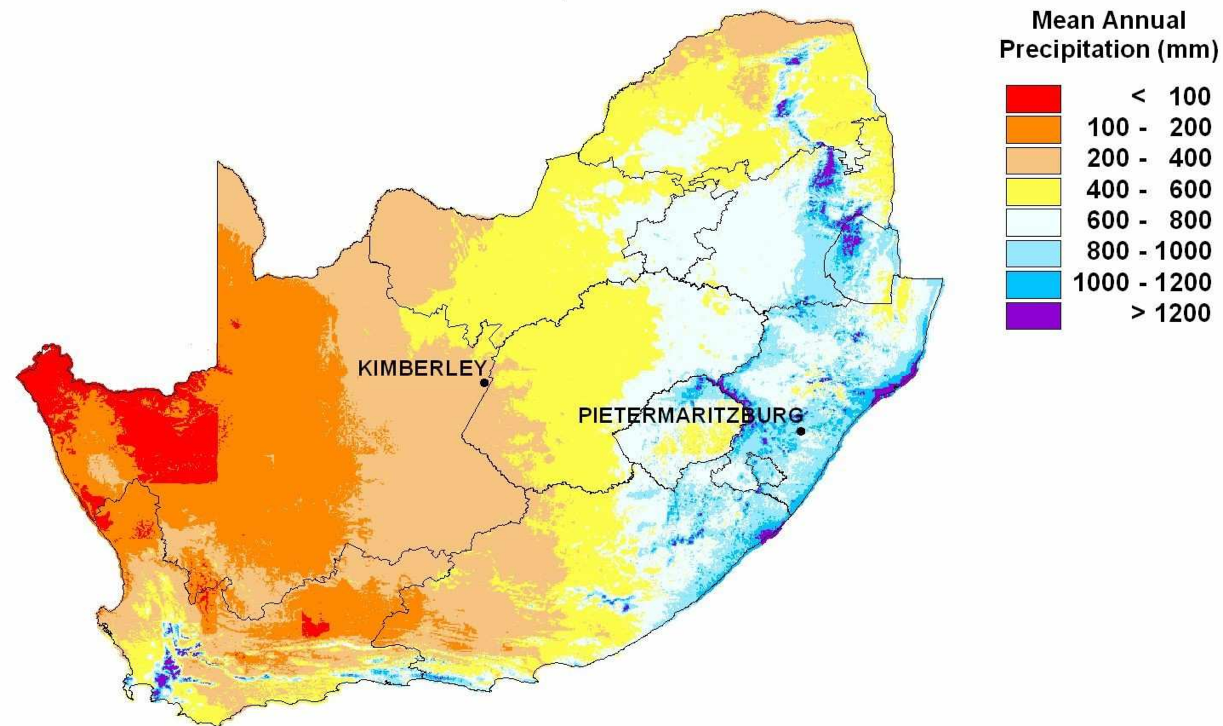


Figure D.1 Mean Annual Precipitation of South Africa after Schulze, Lynch and Dent (1989).

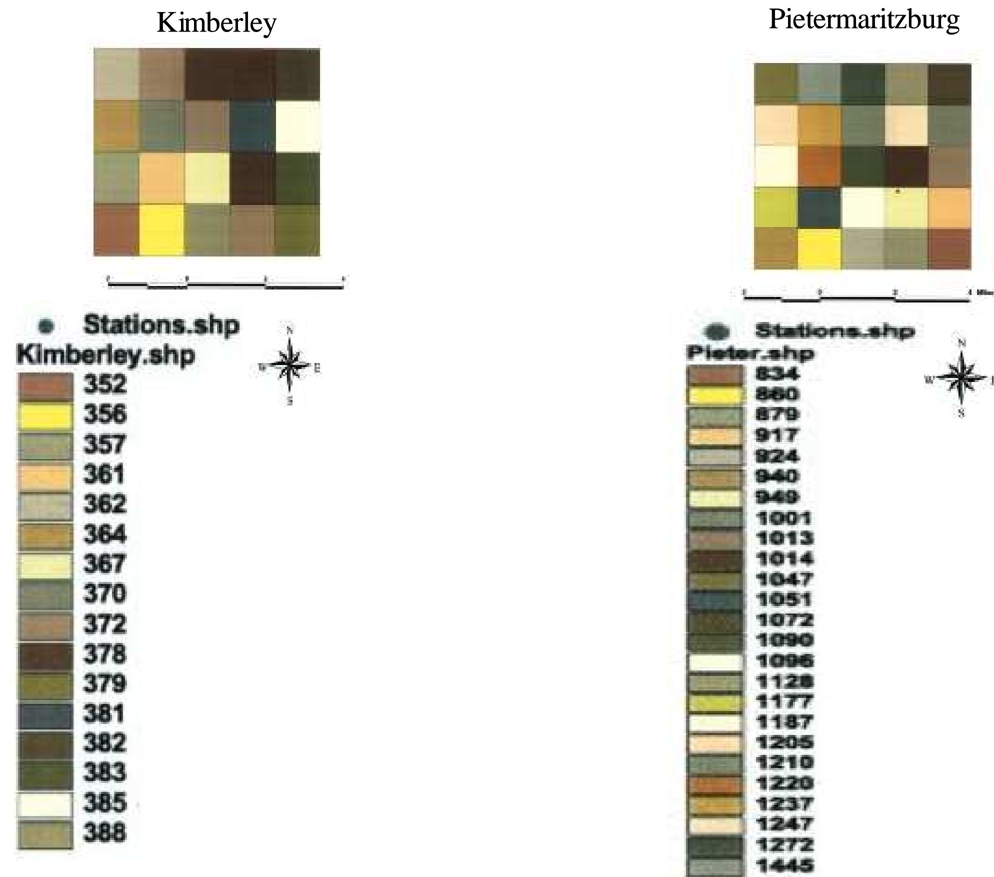


Figure D.2 **Rainfall records of Pietermaritzburg and Kimberley at specific stations (positions specified in the data to follow) over a maximum period of 25 years within the Republic of South Africa (CCWR). Each grid represents the recorded mean annual precipitation over a given year (Kimberley has missing data).**

Table D.1 **Actual Mean Annual Precipitation (MAP) Data recorded for Pietermaritzburg over 25 years at specific geographical positions around the city, represented by the mapped grid detailed in Figure D.2.**

lat	long	MAP	sum 25yr	□MAP	25%MAP (mm)
29.56	30.28	1047	27015	1080.6	270.15
29.56	30.30	1445			
29.56	30.31	1272			
29.56	30.33	1128			
29.56	30.35	1072			
29.58	30.28	1205			
29.58	30.30	1237			
29.58	30.31	1210			
29.58	30.33	1247			
29.58	30.35	1001			
29.60	30.28	1187			
29.60	30.30	1220			
29.60	30.31	1090			
29.60	30.33	1014			
29.60	30.35	1013			
29.61	30.28	1177			
29.61	30.30	1051			
29.61	30.31	1096			
29.61	30.33	949			
29.61	30.35	917			
29.63	30.28	940			
29.63	30.30	860			
29.63	30.31	924			
29.63	30.33	879			
29.63	30.35	834			

Diameter of glass column = 4.3 cm

Surface Area of glass column (SA) = ☺ r^2

Therefore SA = @PI*(4.3/2)^2
= 14.52201 cm^2

Mean Monthly PPt = 270.15/12 = 22.5125 mm

Mean Daily PPt = 22.5125/30 = 0.7504 mm

Mean PPt per hour = 0.7504/24 = 0.0313 mm

Thus total amount of rain needed to give a HLR of 25 % MAP adjusted for column area is :

HLR*h* = Mean Daily PPt * SA
HLR*h* = (0.7504/10)*14.52201 = **1.090 m*l* per day**
For Series of four columns HLR*l* = 1.090*4 = **4.360 m*l* per day**
Over ten days the:
HLR*h* = 10.90 m*l* for a single column ☞10 m*l*
HLR*h* = 43.60 m*l* for each sequential array of columns ☞40 m*l*

Note :1. Arrays were loaded every 10 days at increments of 20 m*l* every five days.

2. *h* - Denotes high Hydrraulic Loading Rate.

Key:

Lat - latitude of rainfall station
Long - longitude of rainfall station
MAP - mean annual precipitation
sum 25yr - sum of 25 years of rainfall over the area of interest
□MAP - average mean annual precipitation over the area spanning 25 years
25%MAP (mm) - 25 % of the MAP that would theoretically contribute to HLR of a landfill in that area

Table D.2 **Actual Mean Annual Precipitation (MAP) Data recorded for Kimberley over 25 years at specific geographical positions around the city, represented by the mapped grid detailed in Figure D.2.**

lat	long	MAP	sum 12yr	MAP	25%MAP (mm)
28.700	24.667	362.0	4399	366.583	91.646
28.700	24.683	372.0			
28.700	24.700	378.0			
28.717	24.667	364.0			
28.717	24.683	370.0			
28.717	24.700	372.0			
28.733	24.667	357.0			
28.733	24.683	361.0			
28.733	24.700	367.0			
28.750	24.667	352.0			
28.750	24.683	356.0			
28.750	24.700	388.0			

Diameter of glass column = 4.3 cm

Surface Area of glass column (SA) = ☺ r^2

Therefore SA = @PI*(4.3/2)^2
= 14.52201 cm^2

Mean Monthly PPt = 91.646/12 = 7.637 mm

Mean Daily PPt = 7.637/30 = 0.2546 mm

Mean PPt per hour = 0.2546/24 = 0.0106 mm

Thus total amount of rain needed to give a HLR of 25 % MAP adjusted for column area is :

HLR*l* = Mean Daily PPt * SA
HLR*l* = (0.2546/10)*14.52201 = **0.3697 m*l* per day**
For Series of four columns HLR*l* = 0.3697*4 = **1.4788 m*l* per day**
Over ten days the:
HLR*l* = 3.70 m*l* for a single column 4 m*l*
HLR*l* = 14.79 m*l* for each sequential array of columns 20 m*l*

Note :1. Arrays were loaded every 10 days at increments of 10 m *l* every five days.

2. *l* - Denotes Low Hydraulic Loading Rate.

3. The HLR *l* was approximated to 20 m*l* for experimental expediency.

4. 12 years of recorded rainfall was used due to missing data.

Key:

Lat - latitude of rainfall station
Long - longitude of rainfall station
MAP - mean annual precipitation
sum 12yr - sum of 12 years of rainfall over the area of interest
MAP - average mean annual precipitation over the area spanning 25 years
25%MAP (mm) - 25 % of the MAP that would theoretically contribute to HLR of a landfill in that area

Appendix E

E. DNA Extraction by "Bead Beat" method

All samples and reagents were handled with latex or vinyl gloves. Gloves were changed frequently and all tubes were closed when not in use. All glassware was cleaned with detergent and thoroughly rinsed before sterilising by autoclaving at 121EC for 15 minutes. The glassware was then oven-baked at 180EC for at least 6 hours prior to use. The distilled water used in the preparation of all solutions was sterilised by autoclaving at 121EC for 15 minutes. None of the solutions were sterilised after preparation. All solutions were stored at 4EC unless stated otherwise.

E.1 120 mM Primary Sodium Phosphate Solution:

NaH_2PO_4 (MW = 141.96, Saarchem) (1.44 g) was dissolved in distilled water and the final volume was made up to 100 m*l*.

E.2 120 mM Secondary Sodium Phosphate Solution:

$Na_2HPO_4.2H_2O$ (MW = 119.98, Saarchem) (2.14 g) was dissolved in distilled water and the final volume was made up to 100 m*l*.

E.3 120 mM Sodium Phosphate Buffer (pH 8.0):

Secondary sodium phosphate solution (120 mM, 95 m*l*) was mixed with primary sodium phosphate solution (120 mM, 5 m*l*) to give a sodium phosphate buffer (120 mM, pH 8.0) with a ratio of 19:1 (secondary sodium phosphate to primary sodium phosphate).

E.4 Glass Beads:

Glass beads (0.1 - 0.11 mm, 0.6 g) (B. Braun, catalogue # 854 140/0) were placed in screw cap eppendorf tubes (2 m*l*) (Whitehead Scientific, Pty) and sterilised by autoclaving for 15 minutes at 121EC.

E.5 20 % (m/v) Sodium Dodecyl Sulphate (SDS):

SDS (Saarchem) (20 g) was dissolved in 80 m*l* distilled water. The mixture was placed in a water bath maintained at 68EC to aid in the solubilisation of the SDS. The final volume was made up to 100 m*l* with distilled water and the solution was stored at room temperature.

E.6 Phosphate Saturated Liquid Phenol (pH 5.5):

Sodium phosphate buffer (120 mM) (pH 8.0) was decanted over liquid phenol (JT Baker, Sigma) and allowed to stand overnight prior to use. The solution was stored in a dark bottle.

E.7 Phenol:Chloroform:Isoamyl alcohol (25:24:1):

Liquid phenol (JT Baker, Sigma) (25 m*l*), chloroform (Saarchem) (24 m*l*), and isoamyl alcohol (Saarchem) (1.0 m*l*) were combined in a dark bottle and sodium phosphate buffer (pH8.0) (120 mM) was decanted over the mixture. The solution was allowed to stand overnight prior to use.

E.8 3 M Sodium Acetate (pH 5.5):

Sodium acetate (Saarchem) (12.3 g) was added to 40 m*l* distilled water and the pH adjusted with glacial acetic acid (aids solubility). The final volume was made up to 50 m*l* with distilled water.

E.9 1 mM EDTA:

Ethylenediaminetetracetic acid di-sodium salt (Saarchem, $Na_2EDTA.2H_2O$) (0.0186 g) was dissolved in 40 m*l* distilled water, the pH was adjusted to 8.0 with 1 N NaOH and the volume was made up to 50 m*l* with distilled water. The solution was autoclaved at 121EC for 15 minutes and stored at room temperature.

E.10 0.01 M Tris-HCl:

Tris (hydroxymethyl) aminomethane (Sigma) (0.606 g) was dissolved in 40 m*l* distilled water, the pH was adjusted to 8.0 with 1 N HCl and the volume was made up to 50 m*l* with distilled water. The solution was autoclaved at 121EC for 15 minutes and stored at room temperature.

E.11 TE Buffer:

EDTA (1 mM) was mixed with Tris-HCl (0.01 M) (1:1) and the solution was stored at room temperature.

E.12 70 % (v/v) Ethanol:

Ethanol (96 %) (v/v) (Saarchem) (72.92 m*l*) was added to a 100 m*l* volumetric flask and the volume was made up with distilled water. The solution was stored at -20EC.

E.13 Isopropanol:

Commercial grade isopropanol (Saarchem) was used.

Appendix F

F. Mo-Bio UltraClean^{TM} Soil DNA Isolation Kit

Kit Contents

Description	*Amount*
2 ml Bead Solution tubes (contains 550 µl solution)	50
Solution S1	3 ml
IRS solution	10 ml
Solution S2	12.5 ml
Solution S3	45 ml
Solution S4	15 ml
Solution S5	2.5 ml
Spin filter units in 1.9 ml tubes	50
Collection tubes (1.9 ml)	150

Protocol

1. To the 2 m*l* Bead Solution tubes provided, add 0.25 – 1.0 g of soil sample.
2. Gently vortex to mix.
3. **NB Check Solution S1.** If precipitated, heat to dissolve.
4. Add 60 ul of Solution S1 and invert once to mix.
5. Add 200 ul of Solution IRS (Inhibitor Removal Solution). Only required if the DNA is to be used for PCR.
6. Secure bead tubes horizontally on a flat bed vortex pad with "sticky tape" and vortex at maximum speed for 10 minutes. (See alternative lysis method for less DNA shearing).
7. Make sure that the 2 m*l* tubes rotate freely in the centrifuge without rubbing. Centrifuge tubes at 10 000 x *g* for 30 seconds. **Caution**: Be sure not to exceed 10 000 x *g* or the tubes may break.

8. Transfer the supernatant to a clean microcentrifuge tube (provided). **Note:** With 0.25 g of soil and depending upon soil type, expect 400 to 450 µl of supernatant. Supernatant may still contain some particles.
9. Add 250 µl of Solution S2, Vortex 5 seconds. Incubate at 4°C for 5 minutes.
10. Centrifuge the tubes for 1 minute at 10 000 x g.
11. Avoiding the pellet, transfer 450 µl of supernatant to a clean microcentrifuge tube (provided).
12. Add 900 µl of Solution S3 to the supernatant and vortex 5 seconds.
13. Load about 700 µl into a spin filter and centrifuge at 10 000 x g for 1 minute. Discard the flow through and add remaining supernatant to the spin filter and centrifuge at 10 000 x g for 1 minute. **Note:** A total of two loads for each sample processed are required.
14. Add 300 µl of Solution S4 and centrifuge for 30 seconds at 10 000 x g.
15. Discard the flow through.
16. Centrifuge again for 1 minute.
17. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution S4 onto the spin filter.
18. Add 50 µl of Solution S5 to the center of the white filter membrane.
19. Centrifuge 30 seconds.
20. Discard spin filter. DNA in the tube is now application ready. No further steps are required.

We recommend storing DNA frozen (-20℃). Solution S5 contains no EDTA.

Appendix G

G. Detection of isolated DNA by Agarose Gel Electrophoresis

All solutions were stored at 4 EC unless stated otherwise.

G.1 0.1 M EDTA:

Ethylenediaminetetracetic acid di-sodium salt ($Na_2EDTA.2H_2O$) (186.1 g) was dissolved in 700 m*l* distilled water, the pH was adjusted to 8.0 with 10 M NaOH and the volume was made up to 1 *l* with distilled water. The solution was autoclaved at 121 EC for 15 minutes and stored at room temperature.

G.2 50x TAE Buffer:

Tris (hydroxymethyl) aminoethane (242.0 g) (Tris-HCl, Aldrich) and 37.2 g $Na_2EDTA.2H_2O$ (Saarchem) / 200 m*l* 0.5 M EDTA were dissolved in 57.1 m*l* glacial acetic acid and 400 m*l* distilled water. The pH (8.0) was checked and the volume was made up to 1 *l* with distilled water. The solution was autoclaved at 121 EC for 15 minutes and stored at room temperature.

G.3 1x TAE (Running Buffer):

TAE buffer (50x) (20 m*l*) was diluted with 980 m*l* distilled

G.4 Ethidium Bromide Stock Solution:

Ethidium bromide (0.01 g) (Merck) was dissolved in 1 m*l* distilled water to give a working solution of 10 $mg.ml^{-1}$.

G.5 Sample Loading Buffer:

Bromophenol blue (0.05 g), sucrose (40 g) and sodium dodecyl sulphate (SDS) (0.5 g) were dissolved in 20 m*l* of 0.5 M EDTA and 30 m*l* of distilled water. The final volume was made up to 100 m*l* with distilled water.

Appendix H

H. Parallel denaturing-gradient gel electrophoresis (DGGE)

All solutions were stored at 4 EC unless stated otherwise.

H.1 40 % (m/v) Acrylamide/Bis (37.5:1):

Pre-ordered solution (Sigma).

H.2 0 % Denaturing Solution for 8 % Gel:

Acrylamide/bis (20 m*l* of a 40 % (m/v) solution) and 2 m*l* of 50*TAE buffer were added to 78 m*l* sterile Milli-Q water to give a final volume of 100 m*l*.

H.3 100 % Denaturing Solution for 8 % Gel:

Urea crystals (42 g) (Associated Chemical Enterprises cc.) were dissolved in 20 m*l* of 40 % (m/v) acrylamide/bis solution, 2 m*l* of 50*TAE buffer and 40 m*l* of formamide (Sigma) and the volume was carefully made up to 100 m*l* with sterile Milli-Q water, following gentle heating in a water bath (temperature not exceeding 37 EC).

Both the 0 % and 100 % denaturant solutions, for a 8 % polyacrylamide gel, were either degassed under a vacuum for 15 minutes or allowed to stand overnight at 4 EC prior to use. The 100 % denaturant solution was placed in a water bath (temperature not exceeding 37 EC) so as to dissolve any urea crystals that may have formed during storage. Both solutions were stored at 4 EC away from light. A 100 % denaturant solution older than two weeks was never used to produce gels.

H.4 10 % (m/v) Ammonium Persulphate (APS):

Ammonium persulphate (1 g) (Sigma, ultra-pure grade) was dissolved and diluted to 10 m*l* sterile distilled water and stored in 0.5 m*l* aliquots at -20 EC.

H.5 10 M Sodium hydroxide (NaOH):

NaOH (40 g) (Saarchem) was dissolved and diluted to 100 m*l* with sterile distilled water.

H.6 0.5 M EDTA:

Ethylenediaminetetracetic acid di-sodium salt ($Na_2EDTA.2H_2O$) (186.1 g) was dissolved in 700 m*l* distilled water, the pH was adjusted to 8.0 with 10 M NaOH and the volume was made up to 1 *l* with distilled water. The solution was autoclaved at 121 EC for 15 minutes and stored at room temperature.

H.7 1x TAE (Running Buffer):

TAE buffer (50x) (20 m*l*) was diluted with 980 m*l* distilled water.

H.8 Stacking Gel Dye:

Bromophenol blue (hexadecyl trimethylammonium bromide, Sigma) (0.05 g) was dissolved and diluted to 10 m*l* with 1*TAE buffer.

H.9 6*Loading Buffer:

Bromophenol blue (0.05 g), sucrose (40 g) and sodium dodecyl sulphate (SDS) (0.5 g) were dissolved in 50 m*l* of distilled water and 20 m*l* of 0.5 M EDTA and the final volume was made up to 100 m*l* with distilled water.

H.10 Ethidium Bromide Stock Solution:

Ethidium bromide (0.01 g) (Merck) was dissolved in 1 m*l* distilled water to give a working solution of 10 $mg.ml^{-1}$.

H.11 Running Buffer for Electrophoresis Tank (1*TAE):

TAE buffer (20 m*l* of 50*) was diluted with 6860 m*l* of distilled water

The following four steps were applied to the assembly, casting, and running of all the denaturing-gradient gels unless stated otherwise.

Step 1: Initial Set-up

The electrophoresis tank was filled with 7 *l* of fresh running buffer . The temperature control module was placed on top of the tank and the pump and heater were switched on at least 1.5 hours before the gel was loaded to allow the set temperature to be reached. The temperature controller was set to 60 ^{0}C with a ramp rate of 200 ^{0}C.h^{-1}.

Step 2: Gel sandwich assembly

Initially the glass plates were cleaned with soap and water, rinsed with 100/70 % ethanol, then finally with acetone. Subsequent cleaning of the plates were done with soap and water only. “Dust-free” tissue paper was used to wipe the plates dry. The large plate was placed down first, and then the 1.0 mm spacers were placed on top of the large plate followed by the smaller plate on top. The two sandwich clamps were placed on the appropriate sides of the plate-spacer assembly (arrows facing up and towards the glass plates). The sandwich assembly was placed in the alignment slot (slot without cams) of the casting stand and an alignment card was inserted into the sandwich to keep the spacers parallel to each other and in-line with both glass plates at the bottom. The sandwich clamps were then tightened concurrently until they were finger-tight. The sandwich was placed on a sponge in the casting stand and the cams were turned down to lock the sandwich in place (Plate 2.1).

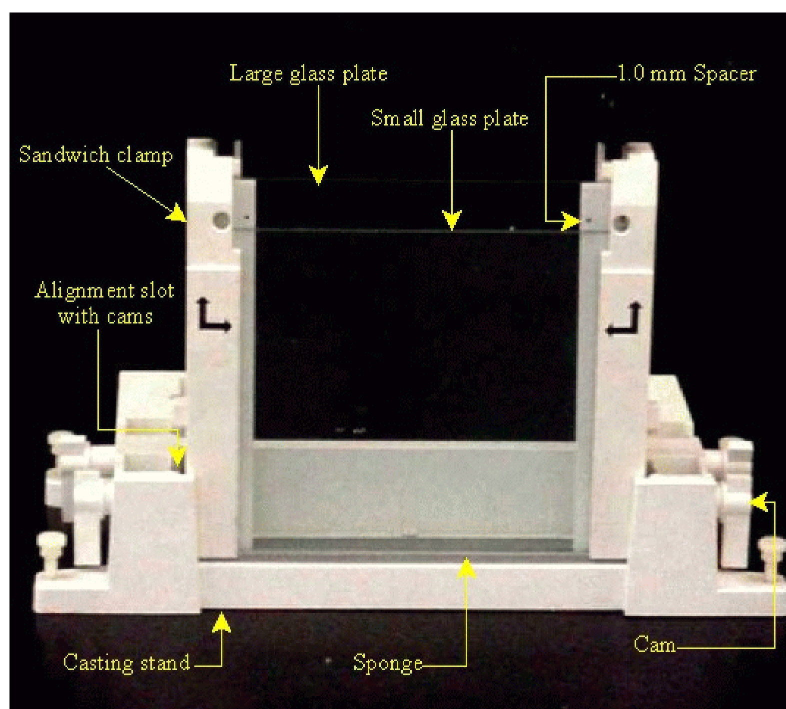


Plate H.1 **Component parts of the gel sandwich assembly.**

Step 3: Casting the DGGE gel

The peristaltic pump (Watson Marlow) that was connected to the gradient mixer (H.W van Verseveld, Vrije Universiteit) and the magnetic stirrer beneath the gradient mixer (Plate 2.2) were turned on 15 minutes before gel casting and checked for satisfactory functioning.

To prepare a variety of gradients 0 % and 100 % stock denaturant solutions for a 8 % (m/v) polyacrylamide gel were prepared and stored at 4 ^{0}C for no longer than a week. A bottom gel [1 m*l* 100 % denaturant solution, 7 μ*l* ammonium persulphate (APS) , and 1 μ*l* N, N, N', N'-tetramethylethylenediamine (TEMED - Sigma, electrophoresis grade)] was pipetted rapidly between the gel plates so as to avoid or detect any leakage.

To prepare denaturant solutions of 40 and 65 %, the desired volumes of 0 and 100 % denaturant solutions (6.6 and 4.4 m*l*, respectively to prepare a 40 % gradient, and 4.4 and 6.6 m*l*, respectively to prepare a 65 % gradient) were pipetted into beakers. A stacking gel of 5 m*l* of 0 % denaturant solution and 20 μ*l* of gel dye was also prepared. The gradient mixer (Plate 2.3) was emptied of its distilled water and the peristaltic pump was stopped. The valve (Plate 2.3) between the two chambers of the gradient mixer was closed, thereby closing the

channel that connected the two chambers after which the needle connected by a tube to the gradient mixer was placed in the middle of the sandwich assembly (Plate 2.2). APS (50 μ*l)* and TEMED (10 μ*l*) were added to the 40 and 65 % denaturant solutions and the solutions mixed thoroughly. The solutions were rapidly decanted into the respective chambers (containing stirrer bars) (Plate 2.4) of the gradient mixer, with the 65 % denaturant solution occupying the chamber closest to the outlet, connected directly to the tubing going to the pump. The pump (pumping at 6.2 m*l*.min^{-1}) was turned on and the valve between the chambers was opened. When the pumping of the solutions neared completion, 35 μ*l* APS and 5 μ*l* TEMED were added to the stacking gel solution and the contents was mixed thoroughly. The valve on the gradient mixer was closed and the stacking gel solution was poured into the chamber connected directly to the tube leading to the pump. After pouring the needle was removed from the gel sandwich and the comb was inserted. The gel was allowed to polymerize for 1.5 - 2 hours.

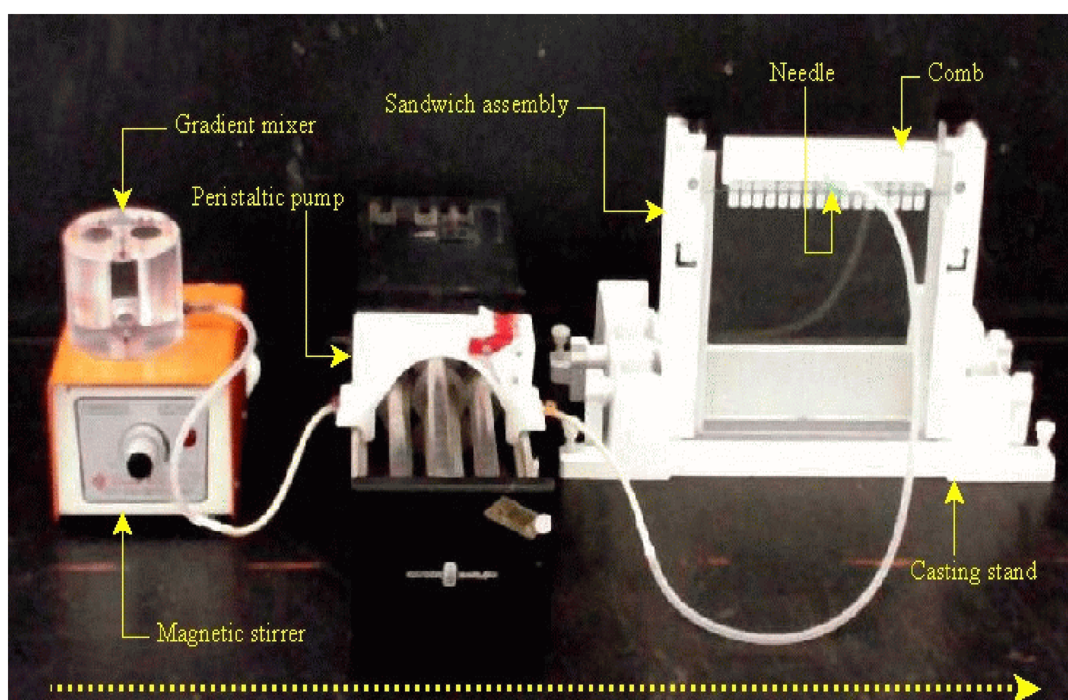


Plate H.2 The Denaturing Gradient Gel pouring system.

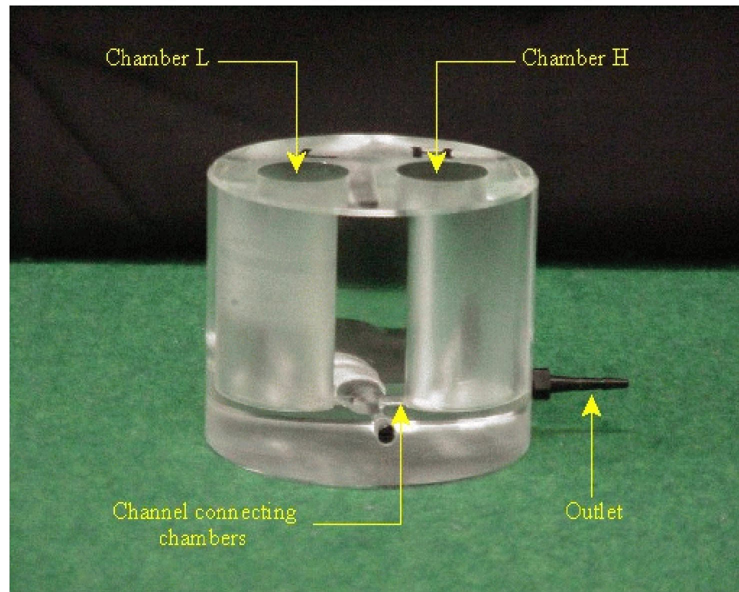


Plate H.3 **The gradient mixing apparatus for DGGE showing the twin mixing chambers.**

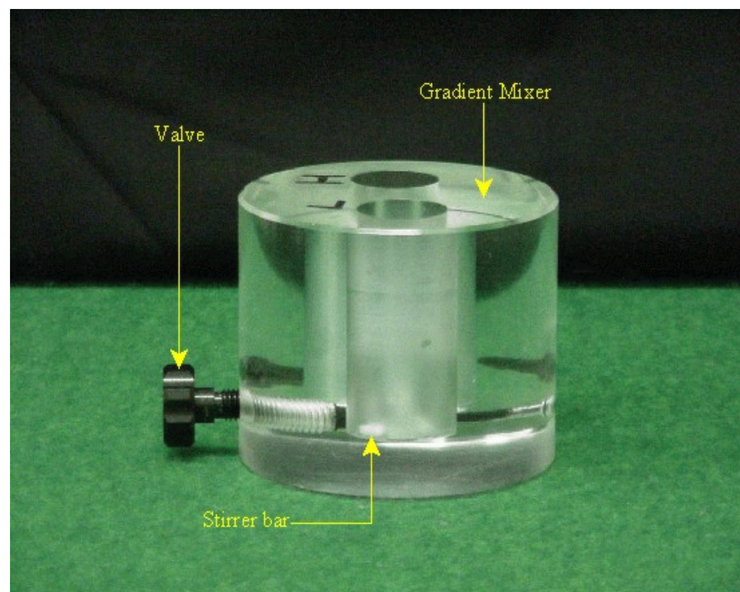


Plate H.4 **The gradient mixer showing the stirrer bars and the valve separating the two chambers.**

Step 4: Running DGGE Gels

After polymerization the comb was removed by pulling it straight up slowly and gently. Each gel was released from the casting stand and the wells were cleared from non-polymerized acrylamide by rinsing (with a 50 m*l* syringe) them with pre-heated running buffer from the electrophoresis tank. Each sandwich assembly, with the short plate facing the core, was inserted into the core apparatus. The control module was turned off and removed. Running buffer (1 *l*) was removed from the tank and the core, together with the attached gel assemblies, was placed in the electrophoresis tank. The upper chamber of the core was filled with 300 m*l* of the running buffer that was removed and the control module was then placed on the top of the tank and the unit was switched on. The system was allowed to reach the desired temperature of 60 ^{0}C before the gel was pre-electrophoresed for 5 minutes at 70 volts. Gelsaver tips (Whitehead Scientific Ltd) were used to load 4 μ*l* loading buffer mixed with 18 μ*l* of sample into the wells of the 8 % (m/v) polyacrylamide gel. All except the outer two wells of the gel were used. The system was run for 16 h at 70 volts (H.W van Verseveld, personal communication). After electrophoresis the power was turned off and the core apparatus was removed from the tank. The gel sandwich was removed from the core with the subsequent removal of the clamps and the shorter glass plate from the gel sandwich. The resultant gels were then stained using the silver staining method or the ethidium bromide method.

Appendix I

Silver staining of Denaturing Gradient Gels

Apart from DNA the protocol also stains other organic compounds, including lipids and proteins, therefore clean gloves were used at all times during the procedure. The DGGE gels were extremely fragile and susceptible to breakage during the numerous stages of the protocol. The solutions were stored at ambient temperature for a maximum of two weeks, unless stated otherwise.

I.1 Fixation Solution:

Ethanol (Saarchem) [100 m*l* of a 100 %(m/v) solution] and acetic acid (Saarchem) (5 m*l*) were added to distilled water to give a final volume of 1000 m*l*.

I.2 1.5 % (m/v) Sodium Hydroxide (NaOH):

NaOH (15 g) (Saarchem) was dissolved and diluted to 1000 m*l* with distilled water.

I.3 0.1 % (m/v) Silver Nitrate ($AgNO_3$):

$AgNO_3$ (0.25 g) (Saarchem) was dissolved and diluted to 250 m*l* with distilled water. The solution was freshly prepared a few minutes prior to use.

I.4 Developing Solution:

Sodium Borohydride ($NaBH_4$) (0.025 g) (Saarchem) and formaldehyde (1 m*l*) (Sigma) were added to 250 m*l* 1.5 %NaOH (m/v). The solution was freshly prepared a few minutes prior to use.

I.5 Stop Mix:

Sodium Bicarbonate (Na_2CO_3) (7.5 g) (Saarchem) was dissolved and diluted to 1000 m*l* with distilled water.

Appendix J

Adsorption Studies

J 1. Adsorption studies of mixed metals at a 1:1 ratio of copper to zinc

! Stock solutions of zinc and copper were prepared by dissolving $Zn(NO_3)_2.6H_2O$ (MW = 297.39) and $Cu(NO_3)_2.3H_2O$ (MW = 241.60) (Saarchem) in half strength synthetic landfill leachate A (Appendix C) (without copper and zinc) to achieve the desired volumes (Table J 1).

Table J.1 **Stock solutions of copper and zinc prepared by dissolving nitrate salts of copper and zinc.**

Premix Concentration (mg.l^{-1})	Postmix Concentration (mg.l^{-1})	Mass of Cu (g)	Mass of Zn (g)	Final Volume (m*l*)
300	150	0.228	0.273	200
150	75	0.114	0.137	200
100	50	0.076	0.091	200
75	37.5	0.057	0.068	200
60	30	0.046	0.054	200
50	25	0.039	0.045	200

! Equal volumes of the respective premix copper and zinc stock solutions were combined to achieve the desired 1:1 postmix metal concentrations of copper:zinc. The final volume of the combined solutions for each concentration was 50 m*l*.

J 2. Individual copper and zinc adsorption studies

! Stock solutions of zinc and copper were prepared by dissolving $Zn(NO_3)_2.6H_2O$ (MW = 297.39) and $Cu(NO_3)_2.3H_2O$ (MW = 241.60) (Saarchem) in full strength synthetic landfill leachate A (Appendix C) (without copper and zinc) to achieve the desired volumes (Table J 2).

Table J.2 Stock solutions of copper and zinc prepared by dissolving nitrate salts of copper and zinc.

Metal Concentration (mg.l^{-1})	Mass of Cu (g)	Mass of Zn (g)	Final Volume (m*l*)
150	0.114	0.137	200
125	0.095	0.114	200
100	0.076	0.091	200
75	0.057	0.068	200
50	0.039	0.045	200
25	0.020	0.023	200

! Each concentration of metal was mixed (50 m*l*) in separate tubes with either sterile or non-sterile Hutton soil for the investigation.

J 3. Phenol adsorption studies

! Stock solution of phenol was prepared by dissolving C_6H_6OH (Saarchem, MW = 94.11) in full strength synthetic landfill leachate A (Appendix C) (without phenol) to achieve the desired volumes (Table J 3).

Table J.3 **Stock solution of phenol prepared by dissolving crystals of phenol in sterile distilled water.**

Desired Phenol Concentration (mg.l^{-1})	Mass of Phenol (g)	Final Volume (m*l*)
600	0.12	200
500	0.10	200
400	0.08	200
300	0.06	200
200	0.04	200
100	0.02	200
50	0.01	200

! Each concentration of phenol was mixed (50 m*l*) with either sterile or non-sterile Hutton soil for the investigation.