
# The Geochemical Controls on Anaerobic Microbial Ecology in a Phenol-Contaminated Sandstone Aquifer

A thesis submitted by

Kieran M. Baker

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Dept. of Chemical and Biological Engineering
Sir Robert Hadfield Building
University of Sheffield
Mappin Street
S1 3JD
United Kingdom

For My Brother, Kristen.

We All Go Down to the Dust, and Weeping Make Our Song.
Alleluia.

# Abstract

The Permo-Triassic sandstone aquifer is one of the most important drinking water resources in the UK. Beneath the Four Ashes site, the aquifer is contaminated with toxic levels of phenolic compounds and inorganic species. Although the hydrochemistry of the groundwater has been fully characterised, no research has assessed contaminant plume development with time using full groundwater profiles. Also, studies have investigated the aerobic microbial ecology at the site, but no research has assessed the anaerobic communities. Anaerobes are important for the remediation of the contamination, as the majority of the contaminated groundwater is anoxic. Furthermore, research has demonstrated that the structure of planktonic (free-living) eubacterial communities at 30m below ground level (mbgl) differ significantly from the community attached to quartz sand suspended in the aquifer at the same depth. However, the diverse mineralogical matrices in sandstones may influence microbial attachment, and therefore the communities attached to quartz may not be representative of the true indigenous attached communities. The aim of this thesis was to update the knowledge on the groundwater hydrochemistry, and to investigate the effects of contaminant load and substratum properties on microbial attachment and anaerobic microbial ecology.

Groundwater samples were taken from multiple depths using 2 depth-discrete boreholes. Concentrations of phenolic compounds in the groundwater were measured using high performance liquid chromatography, and inorganic species were measured by ion chromatography. Chemical profiles show that the groundwater hydrochemistry at borehole 59 (130m from source) remained stable between 1998 and July 2009. However, the data reveals that the distribution of the contaminants changed in August 2009, due to the onset of the pump and treat remediation strategy implemented at the site. Data from borehole 60 (350m from source) shows that the concentration of total phenolics increased markedly at 42-45mbgl between 1998 and 2009, due to variations in contaminant spillage on site, potentially resulting in the plume extending further than the deepest sampling point (45mbgl).

Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA, *napA* (nitrate-reductase) and *dsrB* (dissimilatory sulphate reductase) reveal that the structure of eubacterial and anaerobic communities varied with depth down the groundwater profile, and were therefore influenced by contaminant load. The DGGE profiles of planktonic communities were compared to the communities attached to 3 different geological substrata suspended in the aquifer under the same hydrochemical conditions (i.e. same depth). Profiles reveal that the planktonic communities were highly dissimilar to the attachment communities on all surfaces, and at all samplings. Furthermore, DGGE profiles of the attached communities were compared. Results reveal that the communities attached to Shap granite and sandstone cluster separately to the community attached to quartz, suggesting that substratum properties are an important control governing microbial attachment. This was investigated by quantifying microbial attachment to 6 different geological coupons (apatite, Shap granite, hematite, orthoclase, quartz and shale) suspended in the aquifer. Each coupon provided different physiochemical conditions (hydrophobicity, surface charge and nutrient chemistry) to the system. Results indicate that the most extensive attachment was on coupons that contained at least one microbial growth nutrient (Ca, P, K, Na, Fe, Mn); hematite, apatite, orthoclase and particularly Shap granite, which contains the most nutrients. The least attachment was witnessed on the quartz surface, which contains no microbial nutrients. Quartz has similar surface charge and roughness to orthoclase, and a similar hydrophobicity to the Shap granite. The only difference between the surfaces is nutrient availability. This confirms that substratum chemistry, in particular nutrient content, is a major control on microbial attachment.

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# Abbreviations

| | |
|---|---|
| bp | base pair |
| DGGE | denaturing gradient gel electrophoresis |
| DNAPL | dense non-aqueous phase liquid |
| DsrB | dissimilatory sulphate reductase |
| GR | grid reference (universal transverse Mercator) |
| Mbgl | metres below ground level |
| MNA | monitored natural attenuation |
| NA | natural attenuation |
| NapA | periplasmic nitrate reductase |
| NAPL | non-aqueous phase liquid |
| PCR | polymerase chain reaction |
| rDNA | ribosomal deoxyribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| TPC | total phenolic compounds |
| T-RFLP | terminal restriction fragment length polymorphism |

# Contents

# 1

# General Introduction

## 1.1 Introduction

*"Today, we fear that our groundwater may contain exotic chemicals in levels of parts per trillion or even parts per quadrillion. I note this to accentuate a point. We do not live in a risk-free environment."* (US EPA, 1985).

The above statement highlights the fact that groundwater contamination is an area of utmost concern in today's environmentally-oriented zeitgeist. However, indigenous microbial communities in contaminated groundwater have the capability to degrade toxic levels of organic contaminants. This capability has been utilized as a cheap and non-invasive contaminant remediation method, and is referred to as natural attenuation. To ensure that such a strategy is successful, much research is required to assess the physiochemical controls on microbial growth, community structure and contaminant biodegradation. This is not only important for aerobic communities, but also for anaerobic microbes, as the contaminant load is often located in anoxic environments, due to the rapid utilization of oxygen coupled to organic substrate oxidation.

The aim of this thesis is to investigate the physical and chemical controls on anaerobic microbial ecology within a phenol-contaminated aquifer. The following introduction defines aquifers and groundwater systems, and describes the variety of groundwater contaminants. Then, a description of microbial communities and bioremediation methods is presented before a discussion of the aerobic and anaerobic degradation of phenolic compounds. Finally, an introduction to the field site (Four Ashes) is presented, and the scope of this thesis is described.

## 1.2 Groundwater, Aquifers and Hydrogeology

Groundwater is a subsurface water source stored within pores and fissures of soils and geological formations, and is integral to the hydrological cycle (Brassington, 1999; Waltham, 2009). Such water-bearing rocks are termed aquifers, and are usually formed from arenaceous (sandstones) or karstic (limestone or chalk units) sedimentary rocks (Duff, 2002). Groundwater can also be found in some metamorphic and igneous rocks. Aquifer systems do not only store groundwater, but permit its transmission between areas of recharge to flood plains, surface water

systems and extraction wells (Williams *et al.,* 2001; Duff, 2002). In the UK, the most important aquifer systems are chalk and the Permo-Triassic sandstones of the Midlands (Price, 1996; Duff, 2002).

Aquifers can be unconfined (Figure 1-1), meaning they have an upper surface that is in contact with overlying soils and have a variable water table, allowing water to freely flow in the direction of falling water level elevation (hydraulic gradient). Aquifers can also be confined by layers of aquicludes or aquitards (formations that permit extremely slow or no transmission of water), preventing water from freely rising and falling seasonally (Brassington, 1999; Waltham, 2009). Groundwater in confined aquifers can be under pressure, and the development of artesian groundwater can occur, where the groundwater naturally flows out of the aquifer if a suitable outlet is present (e.g. borehole).

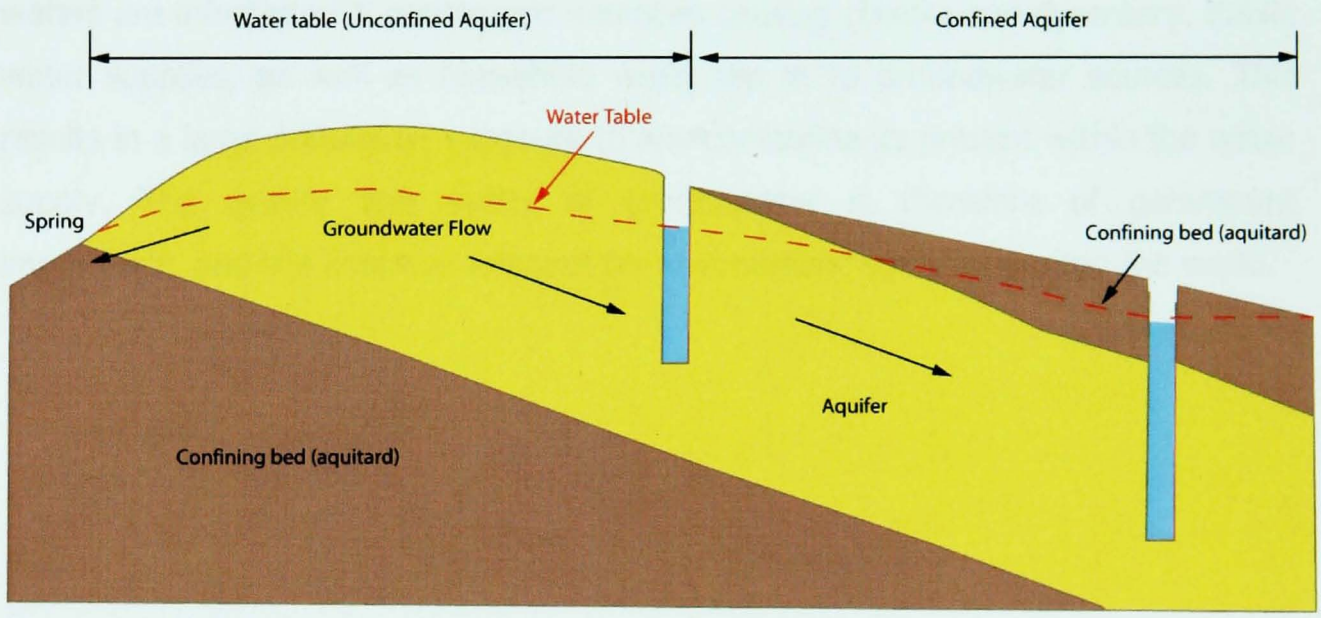



**Figure 1-1** Types of aquifer systems, including an unconfined (water table) and a confined aquifer overlaid by aquitards (modified from Duff, 2002).

Porosity and permeability (hydraulic conductivity) are the main characteristics that define the properties of an aquifer. Porosity is defined as the proportion of the rock that is void space, which can be filled with fluid (Waltham, 2009). There are several types of porosity; primary porosity, which is the main intergranular porosity, secondary porosity derived from fault and joint openness, and vuggy porosity found within limestone rocks where the dissolution of minerals has resulted in caverns or vugs (Duff, 2002). Coarse-grained rocks usually have lower porosities (up to 36% void space) than fine-grained rocks (up to 60% void space).

Permeability is the ease of which a fluid can flow through a porous rock (Domenico and Schwartz, 1998), and hydraulic conductivity (*K*) is the quantitative measure of permeability. This describes the volume of water flowing through a rock unit under the influence of a hydraulic gradient at a given temperature over a given time (Allaby and Allaby, 1999). *K* depends on the nature of the rock (pore size, connectivity, rock fractures and secondary porosity e.g. caverns), the viscosity of the fluid flowing through the rock and the surface tensions and molecular forces associated with the fluid-rock interaction. For these reasons, some high porosity rocks (e.g. clays) store a large volume of water but have an extremely low permeability and hydraulic conductivity (Duff, 2002).

Groundwater supplies contribute more than 90% of the world's fresh water for drinking (Wu, 2002; Alvarez and Illman, 2006), and are especially important in areas of heavy surface water contamination e.g. West Bengal, where the surface waters are infested with pathogenic microbes causing cholera and dysentery. Public water supplies, as well as household wells, tap in to groundwater sources. This results in a large population exposure to any contaminants present within the water supply. The quality and safety of groundwater is therefore of paramount importance, and is a common leitmotif for environment agencies around the world.

## 1.3 Groundwater Contamination

The UK currently has 50,000 contaminated groundwater sites, and there are 400,000 sites throughout the U.S, with cleanup costs topping an estimated $500b (Lerner *et al.*, 2000). Groundwater contaminants include radioactive wastes (Saling and Fentiman, 2001), inorganic effluents (Doble and Kumar, 2005), and more importantly for the scope of this thesis, organic contaminants (Morrison and Boyd, 1992). Petroleum hydrocarbons and chlorinated solvents are the two most commonly encountered toxic contaminants (Alvarez and Illman, 2006). These can result in harmful effects to the receptor, depending upon the degree of exposure. Figure 1-2 highlights important anthropgenic sources of surface and ground water contamination from residential, residential and agricultural sources.

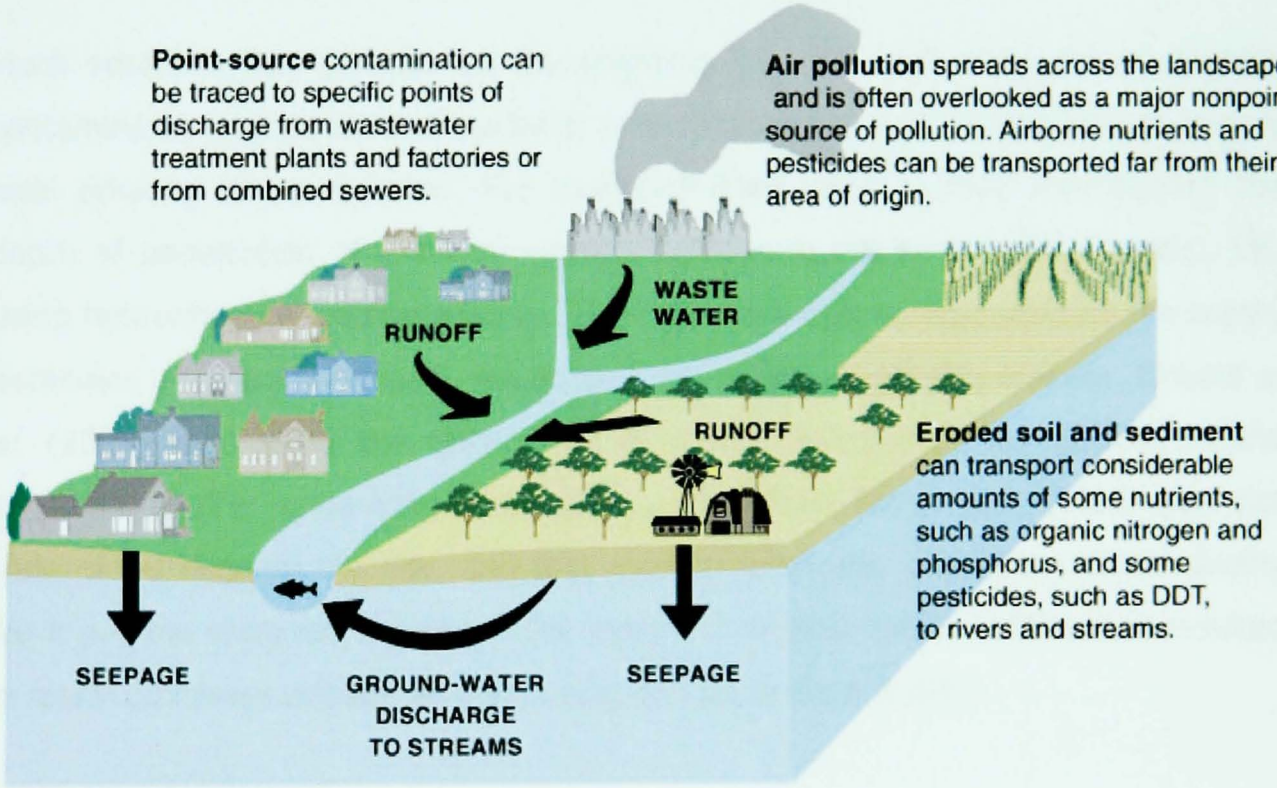



**Figure 1-2** Sources of groundwater contamination (USGS, 2008)

### 1.3.1 Organic Groundwater Contaminants

Aromatic hydrocarbon compounds are the second most common organic compound in nature (Boll *et al.*, 2002). Like many contaminants, organics are derived from a wide variety sources, including air, soil and groundwater (Doble and Kumar, 2005). There are a variety of point-source and non-point source discharges that can result in contamination of ground and surface water. In the UK, one of the major sources

of organic contaminants are sewage treatment works (Harrison *et al.,* 2001), and organic contaminants have a major prevalence in areas of previous industrial occupation. The use of hydrocarbons was initially confined to combustion reactions in the generation of heat and power (Walker, 2001). However, during the 20$^{th}$ century, industrial developments resulted in the production of insecticides, resins, pharmaceuticals, dyestuffs and others (Fetter, 1999), causing the formation of xenobiotics, compounds that have structures different to those produced naturally (Schwarzenbach *et al.,* 1993). There are a variety of organic xenobiotic contaminants that can be found within groundwater systems (Fetter, 2006). These compounds may be released in large quantities, and can either be relatively un-reactive in the environment, or be extremely harmful (Schwarzenbach *et al.*, 1993), some of which are potent carcinogens, such as polyaromatic hydrocarbons (Doble and Kumar, 2005).

Much research has centred on investigating the fate and transport of organic contaminants in groundwater systems, especially urban aquifers that directly supply local drinking water supplies. For example, Rivet *et al.* (1990) investigated the depth of penetration of a tricholorethane spillage in the Birmingham aquifer, UK, using hydrochemical measurements. Their results indicated that 78% of the supply boreholes were contaminated, resulting in an impact on potable sources. Chisala *et al.* (2004) invstigated the distribution of methyl tert-butyl ether (MTBE) in the sanstone aquifer underlying Nottingham, UK. They found that MTBE was not widespread beneath the city, and that most groundwater was of a suitable quality for a potable resource. However, the legacy of organic contamination has resulted in many otherwise potable sources being of substandard quality.

### 1.3.2 Phenolic Compounds

Phenolic compounds are a branch of aliphatic organic compounds that have the general formula ArOH, where Ar denotes the phenyl group (aromatic ring), and OH refers to the hydroxyl radical bonded to the aromatic ring (Fetter, 1999). All phenolic compounds (Figure 1-3) possess this simple structure, plus other functional groups, such as carboxyl and methyl groups, and chlorine (Morrison and Boyd, 1992).

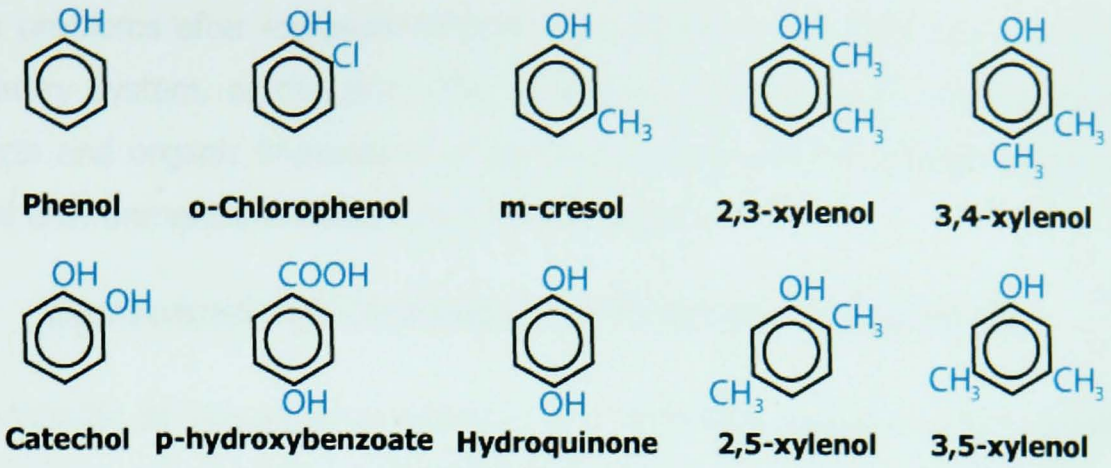



**Figure 1-3** Chemical structures of common phenolic compounds
(Adapted from Fetter, 1999)

The aromatic benzene ring skeleton of phenolic compounds represents around 25% of the Earth's biomass, and results in high resonance energy (~160kJ $mol^{-1}$) causing high stability (Hernandez, 2007). This accounts for the difficulty of ring cleavage during anaerobic degradation. This emphasises the scale at which phenolic compounds are present within our environment. This stability can, however, be reduced by carboxylation, decarboxylation and other chemical rearrangements, which is undertaken by bacteria during biodegradation in both aerobic and anaerobic environments (Hernandez, 2007).

### 1.3.3 Sources of Phenolic Compounds

There are a variety of sources of phenolics, mainly from raw materials such as organic biomass (e.g. lignin, Kleinert and Barth, 2008), to coal carbonization and industrial production as wastes during coal-tar refining (Williams *et al.*, 2001; Semple and Cain, 1996). Phenol is one of the most important chemicals in industry, as it can be used for plastics and resin production (Hernandez, 2007; Chapman, 1972). Trichlorophenol is used as a wood and leather preservative, anti-mildew agent and biocide. It has been noted that industrial effluents released from refineries can produce phenolic groundwater plumes with concentrations reaching 12.5 g $L^{-1}$ (Thornton *et al.*, 2001). Phenolic compounds are therefore prevalent due to current and previous industrial activity. It has been estimated that the global production of phenol surpassed 6.6m tons in 2000 (Hernandez, 2007).

Due to its antiseptic properties, phenol is found within everyday consumables such as mouthwash, medical ointments and gels. The exposure to phenolics can result in

health problems after exposure to toxic concentrations via the alimentary system, respiratory system, or the skin. The increasing production of phenolic-containing products and organic bi-products in industry results in more contamination of the natural environment and public groundwater supplies.

### 1.3.4 Environmental Chemistry of Phenolic Compounds

The solubility of phenolics in water is an important property when considering groundwater contamination and solute transport. This is fundamental to the effects of hydraulic transport of the contaminant through a watercourse. Phenol and cresols readily dissolve in water, due to a high solubility (Fetter, 1988). It results from the hydrogen bonding between the hydroxyl group and the water molecules when in solution. However, if intra-molecular bonding between the functional groups of e.g. nitrophenol occurs, this decreases the solubility as the capacity to bond with $H_2O$ is reduced. Often, the intramolecular bonding (chelation) can actually take place on the intermolecular scale, resulting in the formation phenolic polymers, further reducing solubility (Morrison and Boyd, 1992). Longer chain phenolic compounds (e.g. xylenols) have lower solubility. They can therefore remain as free-product as non-aqueous phase liquids (NAPL) for a considerable period before dissolving, or become sorbed on to grain surfaces. This affects the transport of xylenols in groundwater, and can increase the residence time of the compounds due to poor mixing with groundwater, which can decrease the potential of biodegradation.

Phenolic compounds also possess acidity constants greater than those of alcohols, due to their ability to deprotonate $H^+$ from the structure into the aqueous phase. However, they have varying acidity when different substituents are incorporated into the structure, as the affinity of the compound to lose or gain electrons changes. Other properties of phenol include its colourless appearance, and a sweet smell that is detectable by the human nose when in concentrations of greater than 40ppb (US EPA, 2002).

### 1.3.5 Toxicity of Phenolics

Many national and worldwide guidelines have been set to ensure that exposure to phenolics remains at a minimum. Many health problems occur after the minimum

recommended exposure to phenolics has been reached. From animal studies, Bukowska and Kowalska (2003) noted that phenolics and their derivatives have been found to accumulate in the kidneys or urinary tract and result in gene mutation, potentially leading to cancer. It has also been found that phenol can have deleterious effects on the human immune system, resulting in suppressed response ability (US EPA, 2002a).

Due to the inherent problems associated with toxic exposure to phenolic compounds, drinking water regulations have been implemented. This ensures that the concentrations of phenolic compounds remain as low as possible. Currently, the maximum phenol to be encountered within drinking water should not exceed 0.1mg $L^{-1}$ (HPA, 2007). US EPA (2002) state that the $LD_{50}$ values for phenol associated with mammals is 300-600mg phenol/kg body weight.

## 1.4 Microorganisms and Microbial Biofilms

Microorganisms play an important role in many processes on Earth, and constitute over half of the Earth's total living material (Perry and Stayley, 1997). They have the ability to grow in most environments, from high temperatures and pressures at deep-sea hydrothermal vents (Takaki *et al.*, 2010), to cold environments within glacial settings on Mount Everest (Liu *et al.*, 2009). The various conditions under which microorganisms can exist (Table 1-1) emphasises the wide range of habitats within which they can colonise. This results in the potential for microbial communities to proliferate within contaminated groundwater. It is widely accepted that microorganisms have to ability to degrade contamination *in situ,* and are therefore considered to be useful in the remediation of contaminated land. They are the most ubiquitous agents in the transformation and detoxification of organic chemicals in the environment (Schwarzenbach *et al.*, 1993).

**Table 1-1** Examples of the ranges of habitats that can support microbial life (modified from Flemming, 1990)

| **Environments of Microbial Growth** | |
|---|---|
| Temperature | <-12°C to >110°C |
| pH Range | 0 to >13 |
| Redox Potential | All ranges |
| Salinity | 0 to saturated |

Singular species of microorganisms do exist in nature, but mixed communities are more prevalent due to the advantageous effects of living as part of a cooperative and syntrophic community, such as metabolic exchange and gene transfer. Also, microorganisms can exist primarily in two phases, free-living in solution (planktonic), or attached to, or associated with, a surface (attached). Interest in attached microbial communities has increased over the last 3 decades since the findings that 99.9% of all aquatic organisms grow attached to a surface (Costerton and Wilson, 2004). Techniques, such as confocal scanning laser microscopy, developed in the 1980's, have provided evidence that attaching microorganisms are not just random cell accretions, but organised and complex micro-colonies, known as biofilms (Costerton and Wilson, 2004).

Biofilms play an important role in both the natural environment and the engineering and medical sciences (Wilderer and Characklis, 1989). They can be found attached to metals, minerals, and human tissues such as teeth and the inside of cystic fibrosis lungs (Davey and O'Toole, 2000). This shows that biofilms have a resistance to the levels of chemicals that would usually prevent the proliferation of planktonic microbes. An example is the antibiotic resistance of biofilm-forming *P. aeruginosa* and *B. cepacia* in the lungs of a cystic fibrosis sufferer (Drenkard and Ausubel, 2002; Dales *et al.,* 2009). From an environmental perspective, biofilms exist in response to hostile environmental conditions. These can be complex consortia possessing a number of characteristics that may result in the capability to degrade toxic contaminants. The following provides a brief summary of the attachment of microbial cells to inorganic surfaces.

### 1.4.1 Partitioning in to the Attached Phase

The attachment of microorganisms is dependant on the prevailing environmental conditions. In many groundwater systems, conditions are oligotrophic, and are therefore scarce in terms of soluble macronutrients that are required for microbial growth (Perry and Stayley, 1997). This can become a limiting factor in terms of microbial proliferation, and results in the partitioning of planktonic microbes to the attached phase. Other environmental factors that may initiate microbial attachment are pH, osmolarity, iron availability, temperature, and the build up of toxic contaminants (Davey and O'Toole, 2000; Chapelle, 2001). Nutritive surfaces are

present within geological settings, such as an aquifer, where mineral coatings provide essential growth nutrients, such as nitrogen, phosphorous, potassium and magnesium (Bennett *et al.,* 2001). Surfaces can also provide a suitable carbon source to be used in microbial respiration (O'Toole *et al.*, 2000).

The physical properties of the substratum, such as hydrophobicity, surface topography and charge, also affect the attachment of microorganisms (Hermansson, 1999).

### 1.4.2 Initial Microbial Attachment

Biofilm structures are built via the accretion of single or multiple layers of bacterial or fungal cells upon a substratum. Prior to this, microorganisms have to overcome the chemical and physical properties of the surface. This first phase of attachment involves the interaction of the planktonic cell in the aqueous phase with the substratum (Hermansson, 1999). After a planktonic cell approaches a surface, via sedimentation, convection or other mass transport processes, the cells interact chemically with the surface (Skvaria, 1993). Surface functional groups on the microbial cell and substratum have been found to be important in the determination of the type of chemical bonding that will emerge between the cell and surface (Parikh and Chorover, 2006; Ojeda *et al.,* 2008). Bonding associated with one type of microbe and one surface will undoubtedly differ to those found between other cells and surfaces. Such electrochemical interactions are influenced by the local environment, such as pH, which can alter the surface charge of the substratum. Inorganic substances also effect the initial interaction between the cell and surface (Yelloji Rao *et al.,* 1993). Therefore, much environmental variation within geological ecosystems results in a high variation of microbial attachment (Goldscheider *et al.*, 2006).

After initial chemical bonding, more extensive attachment occurs. This depends on the production of exopolymeric substances (EPS), consisting of polysaccharides, nucleic acids and proteins, which cement surface colonisers both together and to the substratum (Marshall *et al.,* 1971; Sutherland, 2001). An example of the formation of a *Pseudomonas chlorae* biofilm is presented (Figure 1-4). The initial attachment of the cells is dependant on the production and operation of flagella on the cell surface. Surface translocation, including twitching motility and swarming, is

also important during initial attachment. These permit the surface movement of the cells to create a monolayer in preparation for the secondary colonisers. Surface colonisation then continues with the release of outer membrane proteins (OMP) and exopolymeric substances (VPS) to a biofilm matrix (Davey and O'Toole, 2000).

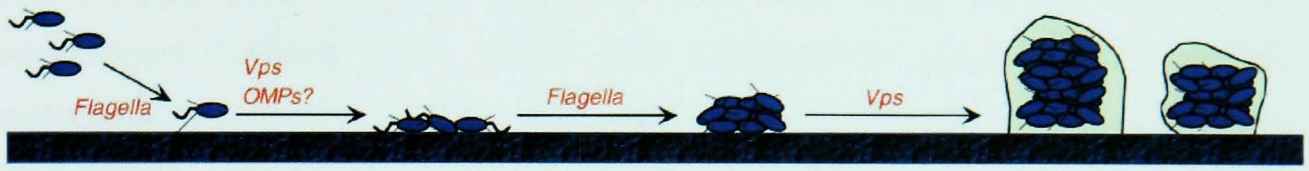



**Figure 1-4** Biofilm development of *V. cholerae* (modified from Davey and O'Toole, 2000).

### 1.4.3 Biofilm Maturation

After initial attachment, secondary colonisers attach to the surface of the primary colonisers, thus commencing cell-cell interaction. The attachment of secondary colonisers is usually due to the production of micro-niches within the biofilm as a result of the environmental conditions. For example, planktonic organisms that can reduce toxic levels of metabolic respiration products attach to the surface, resulting in a cooperative, mixed-culture consortium. In the case of phenol, primary degraders would degrade phenol to substituted catechols, which is degradable by secondary degraders, the products of which would serve to provide nutrients to tertiary degraders, and so on. This is an example of interspecies metabolic product exchange (Schwarzenbach *et al.*, 1993; Davey and O'Toole, 2000). This is where metabolically distinct organisms rely on each other for energy production, and is an example of the previously discussed syntrophism (Davey and O'Toole, 2000).

If a pathway of biodegradation does not exist within a biofilm, quorum sensing can initiate. This is a complex cell-cell interaction mechanism where organisms release signalling molecules (autoinducers) into the biofilm matrix (Miller and Bassler, 2001). This results in communication between organisms leading to an alteration in gene expression and physiological characteristics, including the production of 'inducible enzymes' (Schwarzenbach *et al.*, 1993). This generates an enhanced capability for metabolic activity within the contaminated environment. This interaction, occurring both intra- and interspecies, allows the organisation of biofilm consortia and the efficient degradation of complex chemicals that would not be

wholly degraded by one single organism (McDougald *et al.*, 2006; Miller and Bassler, 2001).

During maturation, cell-cell adhesion also occurs, mediated by further production of EPS (Flemming, 1990). This results in the development of protective layering (an EPS matrix), which provides resistance from toxic levels of chemicals in the environment (O'Toole *et al.*, 2000). EPS increases the absorption of metabolic molecules, which increases the concentration of growth nutrients, making them more readily available to the organisms within the biofilm, increasing the chances of microbial growth (Wolfaardt *et al.*, 1998; Ransom *et al.*, 1999). EPS can provide a diffusion system, mediating the input of certain molecules into the biofilm matrix, and thus protecting the organisms therein. Ransom *et al.* (1999) noted that direct cell-mineral contact is prevented by cross-linked EPS fibrils around the microbes, and that all chemical and physical reactions between cells and the surface to which they are attached are mediated by reaction and transport through EPS.

It is evident that attached growth can occur in heavily contaminated environments (Hiebert and Bennett, 1992; Bennett *et al.,* 2001). Attached communities may have a greater ability to degrade pollutants in hostile environments, and are therefore more advantageous than the planktonic phase. Complex consortia within the biofilm can withstand the harsh environmental conditions and form an integral part of the contaminant degradation within a contaminated site. Moreover, in complex geological systems such as aquifers, environments are seldom uniform due to preferential transport of contaminants related to contaminant and aquifer properties. This can result in steep chemical gradients within the groundwater, and biofilms therefore ensure that the organisms remain within a stable environment (Goldscheider *et al.,* 2006).

## 1.5 Natural Attenuation and Bioremediation of Contaminated Groundwater

70% of all phenolics production is due to the actions of the Western world (Hernandez, 2007), and therefore the responsibility of reducing phenolic contamination within the environment lies with the developed community. Due to the high costs associated with the engineering-based treatments (e.g. pump and treat, filtration, excavation) of heavily contaminated groundwater, new and improved techniques are being developed to decrease the contamination with minimal disruption to the environment. The utilization of natural biological, physical and chemical processes can serve to reduce contaminant load within a carefully monitored site (U.S EPA, 1998). This procedure is defined as Natural Attenuation (NA), and encompasses contaminant advection, sorption, chemical transformations and the use of indigenous micro-organisms that have the metabolic capability to degrade contaminants *in situ* (Barker *et al.,* 1987). Microbial populations can be integral to controlling the fate of contaminants within natural settings (Wu, 2002), and thus, the interest in NA for remediation of contaminated sites has increased significantly over the past decade.

The main contaminant biodegradation processes are aerobic respiration, anaerobic respiration, methanogenesis and fermentation (Williams *et al.*, 2001). Microorganisms gain energy from the proton motive force, where electrons are transferred from an organic compound to an inorganic electron acceptor (Bennett *et al.,* 2001). This system is known as a terminal electron accepting process (TEAP), with electron acceptors including oxygen, nitrate, iron, manganese and sulphate. Thermodynamically, there are differences in ATP (the energy of the cell) generation between the different TEAPs. Aerobic respiration (oxygen) yields the most ATP, and is therefore the favoured TEAP, followed by anaerobic processes and fermentation, which yields the lowest energy (Figure 1-5, Zehnder, 1988; Thornton *et al.*, 2001). During NA, the bioavailability of electron acceptors and organic substrates are important in determining which TEAP will be utilized. If the contaminated site had an abundance of dissolved oxygen throughout the contaminant plume, then aerobic respiration would be utilized throughout. However, in a non-mixed system where there is an oxygen concentration gradient, or if the rate of consumption of oxygen is greater than supply, then the utilization of anaerobic TEAPs would dominate, with

preference being for the most thermodynamically-favourable process (Yolcubal *et al.*, 2001; Londry and Fedorak, 1991). The organisms utilizing the most energetically favourable process would then have a competitive advantage (Londry and Fedorak, 1991). The utilisation of various TEAs can lead to the formation of microbial redox zonation within a contaminated aquifer. Mixing of contaminated and uncontaminated water and hydrodynamic dispersion can result in an aerobic/microaerophillic environment, where oxygen and nitrate would be utilised first. With increasing contaminant concentration, high-energy TEAs would be quickly consumed, followed by other TEAs, moving towards more thermodynamically unfavourable anaerobic respiration processes ($Mn^{IV}$, $Fe^{III}$, $SO_4^{2-}$) and finally methanogenesis and fermentation (Chapelle, 2001; Williams *et al.,* 2001; Rizoulis, 2008). This can result in areas where different TEAPs are in operation. However, concomitant utilisation of anaerobic TEAs has been seen to occur (Chapelle, 2001; Thornton *et al.,* 2001). For example, although fermentation is thermodynamically unfavourable, it occurs independently of other degradation processes. The onset of fermentation can therefore occur before the exhaustion of other respiration processes, as it is controlled only by environmental factors such as pH (Lee *et al.*, 2008). Anaerobic microbial degradation of organic contaminants was found to be extremely important as the chemical degradation of contaminants is insignificant in anoxic environments (Sutton and Barker, 1985).

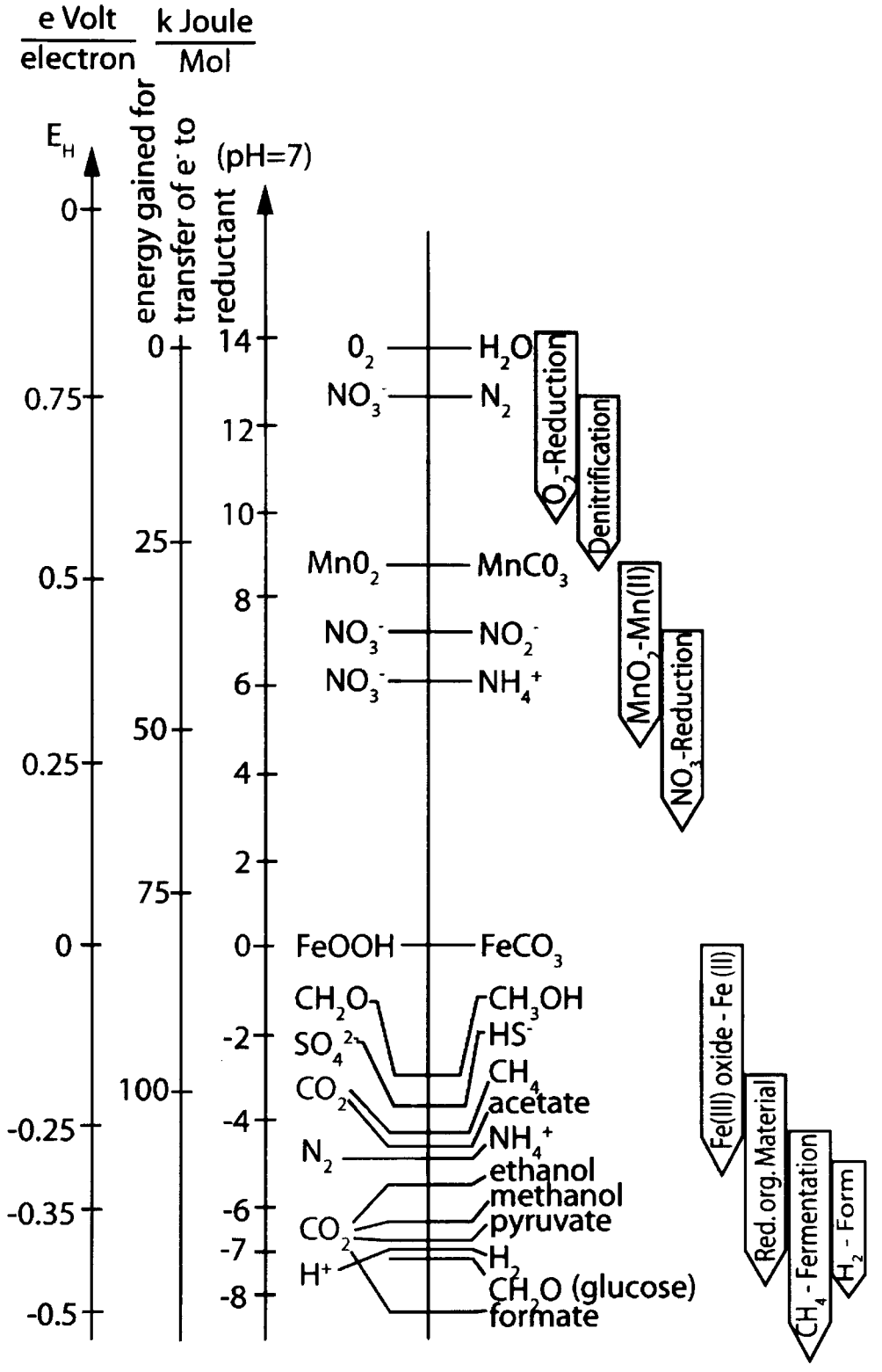



**Figure 1-5** Electron energy diagram showing the sequence of oxidation and reduction reactions in terms of thermodynamics (modified from Zehnder, 1988).

In 1997, 46% of decontamination processes in the U.S involved NA, particularly at sites where underground storage tanks had leaked (EPA, 1998; Tulis *et al.,* 1998). This will inevitably increase, as NA is a cost-effective and practical method of remediation for contaminated groundwater, where engineering-based techniques (e.g. *ex situ* bioreactors) are not suitable and are extremely disruptive to the environment (Loh *et al.,* 2000; Erhan *et al.,* 2004). NA processes have been found to prevent the spread of contaminants since the 1980s. After a significant oil

spillage into groundwater systems in Minnesota, USA, a plume of BTEX (benzene, toluene, ethylene and xylene) compounds was found to be stationary within the aquifer system, despite groundwater flow. This was later attributed to natural attenuation by aerobic/anaerobic microorganisms, which were degrading the contaminants at a similar rate to the contamination infiltration into the groundwater system, preventing the plume from migrating (Chapelle, 2001). Sutton and Barker (1985) found butyric acid to be rapidly attenuated by the actions of anaerobic organisms within a contaminated sandy aquifer. Phenol was also released in to the same sandstone aquifer, and was found to be degraded moderately rapidly by anaerobic degradation. As BTEX and phenol are common contaminants in the environment, microbes often have the metabolic capability to degrade the compounds. This results in NA often being successful.

NA is not always successful however. For example, trichloroethene (TCE) is a degreasing agent that is commonly found as a contaminant in subsurface environments (Lowe *et al.*, 2002). However, microbial degradation processes *in situ* results in the formation of vinyl chloride. This compound is more toxic than TCE, showing that NA has only served to biotransform the contaminant. For NA to be successful, the contaminants must be fully degraded to non-toxic compounds (e.g. ethane, ethane, $CO_2$ and $H_2O$). Also, NA is only effective when nutrient supply and the prevailing environmental conditions permit, and when the indigenous microorganisms have the metabolic capability, as biodegradation is often the most significant mass reduction process (Wilson *et al.*, 2004; Lowe *et al.*, 2002). This results in varying success. Understanding the effect of site-specific variables, such as contaminant stability, physiochemical conditions, and microbial population dynamics, are paramount to assess the true NA potential of a contaminated site (EPA, 1998; Schirmer and Butler, 2004). Monitoring such conditions and processes is known as monitored natural attenuation (MNA). MNA involves assessing the mass loss by biodegradation by monitoring the changes in contaminant concentration and redox-sensitive species over time and with distance from the source (Wilson *et al.*, 2004). This gives an indication of whether NA is successful at a site.

If NA is not a viable method for the clean-up of contaminated sites, it is possible to use bioremediation as an alternative technique. Biologically-mediated remediation by microorganisms can be accomplished using a variety of different methods,

including *ex situ*/above ground treatment of contaminated waters in bioreactors. Other methods involve *in situ* biostimulation or bioaugmentation, which are also known as enhanced bioremediation (Alvarez and Illman, 2006).

*In situ* biostimulation involves stimulating the indigenous microbial population to overcome environmental limitations in order to degrade the contaminants (Figure 1-6). This is best used when conditions within the natural environment are not suitable for efficient biodegradation, and therefore require additions to generate adequate conditions for the most efficient biodegradation to occur. An example would include sparging the groundwater with a terminal electron acceptor (e.g. oxygen, sulphate), macronutrients (P, N), or altering specific conditions such as pH or reducing potential. This can stimulate microbial growth by creating a shift in the microbial population dynamics (Alvarez and Illman, 2006; Iwamoto *et al.,* 2000). The requirement for biostimulation is common in oligotrophic environments, where nutrients are scarce. Biostimulation of the indigenous microbial population using oxygen sparging was successful for the degradation of groundwater contaminated with methyl tertiary butyl ether (Smith *et al.,* 2005) and diesel (Hunkeler *et al.,* 2002), and the addition of acetate was successful at stimulating the anaerobic ($Fe^{III}$ and sulphate-reducing bacteria) microbial immobilization of uranium (Yabusaki *et al.,* 2007).

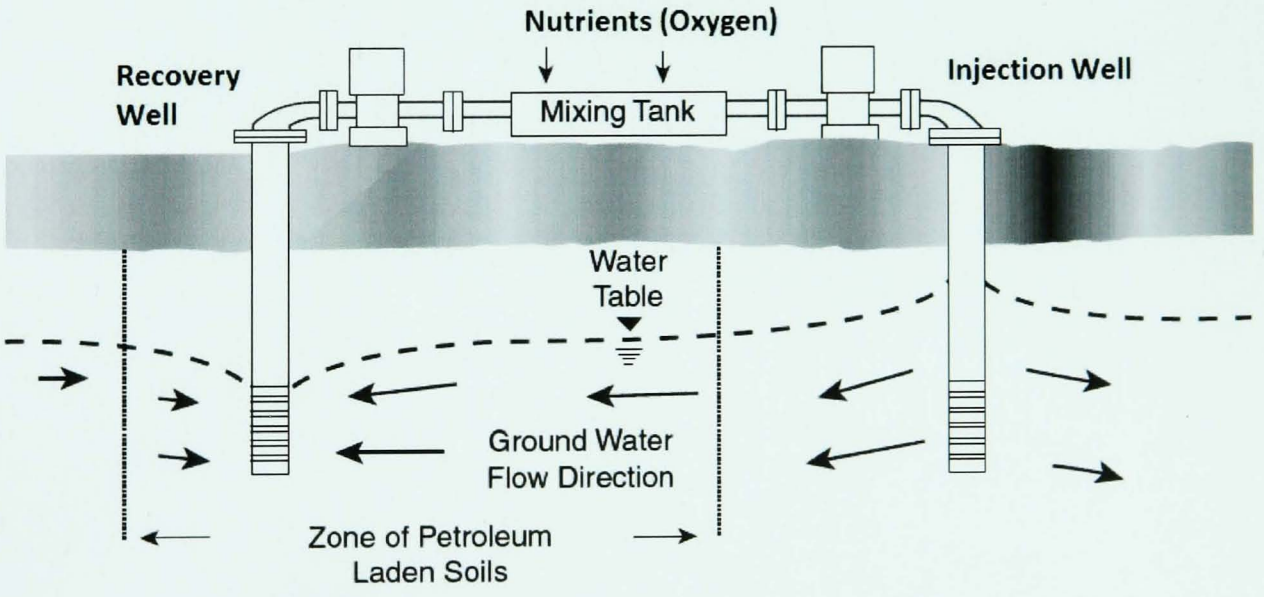



**Figure 1-6** *In situ* aerobic biostimulation strategy where anaerobic waters are removed from the groundwater system (recovery well), and returned via the injection well after mixing with oxygen (modified from Alvarez and Illman, 2006).

If it is found from microcosm or molecular microbiology studies that the indigenous population within the contaminated groundwater does not contain abundant

hydrocarbon degraders, it is possible to perform *in situ* bioaugmentation. This bioremediation technique involves adding exogenous organisms to the system with the capability to degrade the contaminants (Alvarez and Illman, 2006). Bioaugmentation has been successfully used to degrade phenol in a batch bioreactor (Jiang *et al.*, 2006) and for the remediation of chlorinated solvents (Steffan *et al.*, 1999).

Bioaugmentation is not always successful, as the alochtonous communities added to the system are often not well adapted to the environmental stresses that can be caused by a complex contaminant matrix. Also, xenobiotic contaminants are not ubiquitous in the environment, and therefore it can be difficult to find organisms with the suitable catabolic/genetic capabilities to degrade the contaminants (Thompson *et al.,* 2005). It has also been found that hydrogeological properties can prove problematic to bioaugmentation attempts. The attachment of microbes to geological surfaces can result in poor microbial degradation as microbial populations may remain within one area of the aquifer, whereas the contamination can be moving through the system with the groundwater flow (Sutton and Barker 1985; Thomas and Ward, 1994). Indigenous microbial populations can also compete with microorganisms injected in to the system for TEAs and other nutrients, thus reducing the effectiveness of the biodegradation (Chapelle, 2001).

## 1.6 Biodegradation of Phenolic Compounds

### 1.6.1 Aerobic Respiration of Phenolic Compounds

As phenols are biogenic intermediates within the carbon cycle, many micro-organisms possess the metabolic capability to degrade such compounds, enhancing the potential for NA. Under aerobic conditions, phenol is rapidly degraded usually with no lag time (Aronson *et al.,* 1999). The aerobic biodegradation of phenolic compounds utilizes molecular oxygen to cause hydroxylation and cleavage of the aromatic ring, using mono- and di-oxygenase enzymes (Harwood *et al.*, 1999). This results in the production of a non-aromatic intermediate, an unsaturated carboxylic acid chain that can be readily degraded to common metabolites such as acetyl-CoA via central degradation pathways (Figure 1-7). After initial degradation of phenol to catachol using phenol-2-monoxygenase, ring cleavage can occur *ortho* or *meta* to a hydroxyl group on the benzene ring, resulting in different metabolic products (Heider and Fuchs, 1997). Also, the *meta* pathway can utilize nitrate instead of oxygen in the second stage of the degradation.

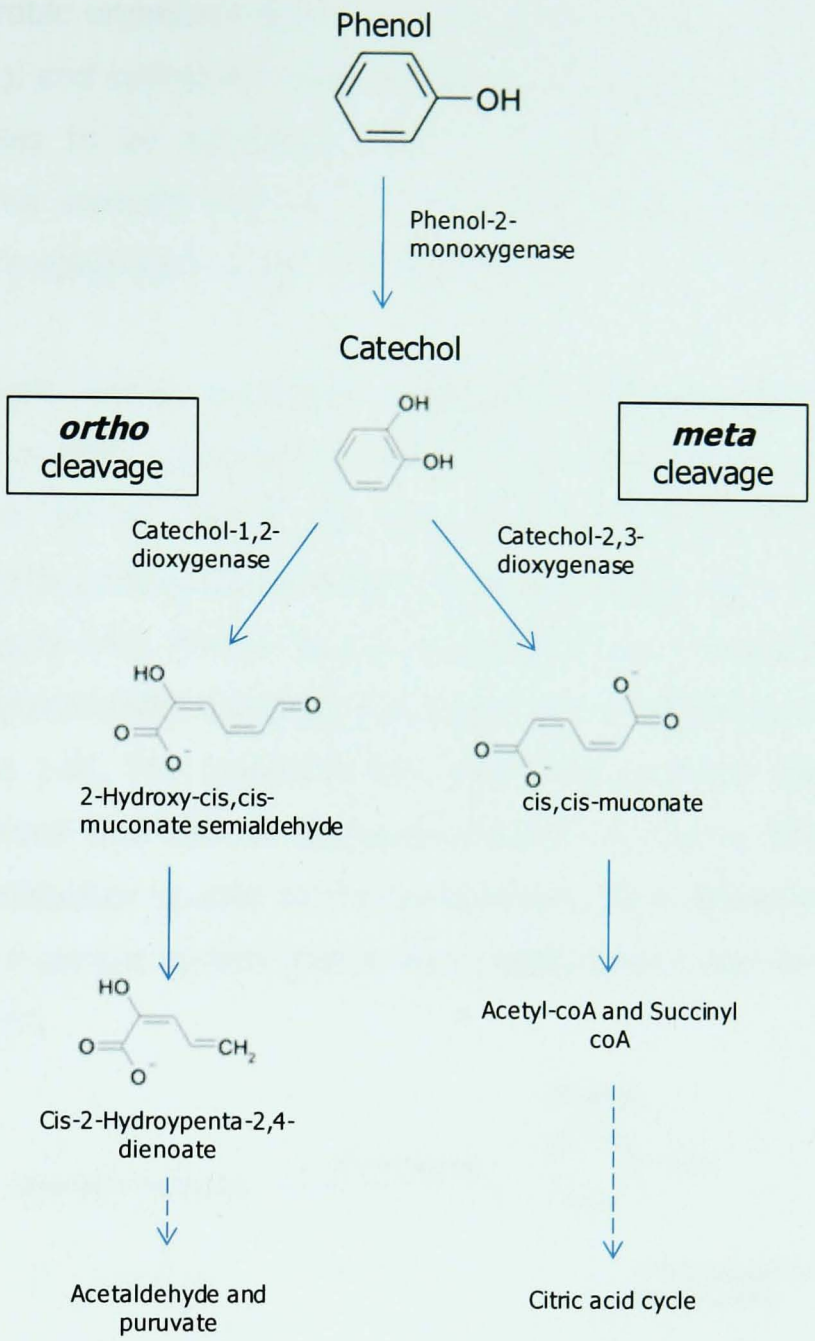



**Figure 1-7** Aerobic degradation pathways for the degradation of phenol (modified from Heider and Fuchs, 1997; Cinar, 2004)

## 1.6.2 Anaerobic Degradation of Phenolic Compounds

There are a number of studies that have discussed the anaerobic degradation of phenolics, and other aromatics. Carboxylation of the aromatic ring by bacterial sulphate- and nitrate-reduction (Bakker, 1977; Evans and Fuchs; 1988; Heider and Fuchs, 1997; Tschech and Fuchs, 1989), and dissimilatory iron-/manganese-reduction coupled to the oxidation of phenolic contaminants have been reported (Lovely and Lonergan, 1990; Broholm *et al.*, 2000; Tor and Lovely, 2001).

However, anaerobic organisms cannot always wholly degrade aromatic substrates (Chapelle, 2001), and syntrophic relationships with fermentors are required in order for NA processes to be successful. This is due to the requirement of initial catabolism of the aromatic ring by the fermenting bacteria, releasing metabolic products that are scavenged by respiring bacteria.

There have been many suggested metabolic pathways for the anaerobic degradation of aromatic compounds, notably the widely accepted 'benzoyl CoA' pathway (Schink *et al.*, 2000). For phenol, this involves degradation by 4-hydroxybenzoate decarboxylase forming 4-hydroxybenzoate, prior to degradation to benzoyl-CoA (Figure 1-8). Phenol can also be degraded to 4-hydroxybenzoate using the phenylphosphate route, using phenol kinase and phenylphosphate carboxylase enzymes (Figure 1-8). This highlights the notion that aromatic degradation takes place via 'peripheral' and 'central' pathways (Heider and Fuchs, 1997). The former representing metabolism specific to the contaminant, be it phenol or benzene, and the latter being a generic system that is more readily undertaken by a wider variety of microorganisms.

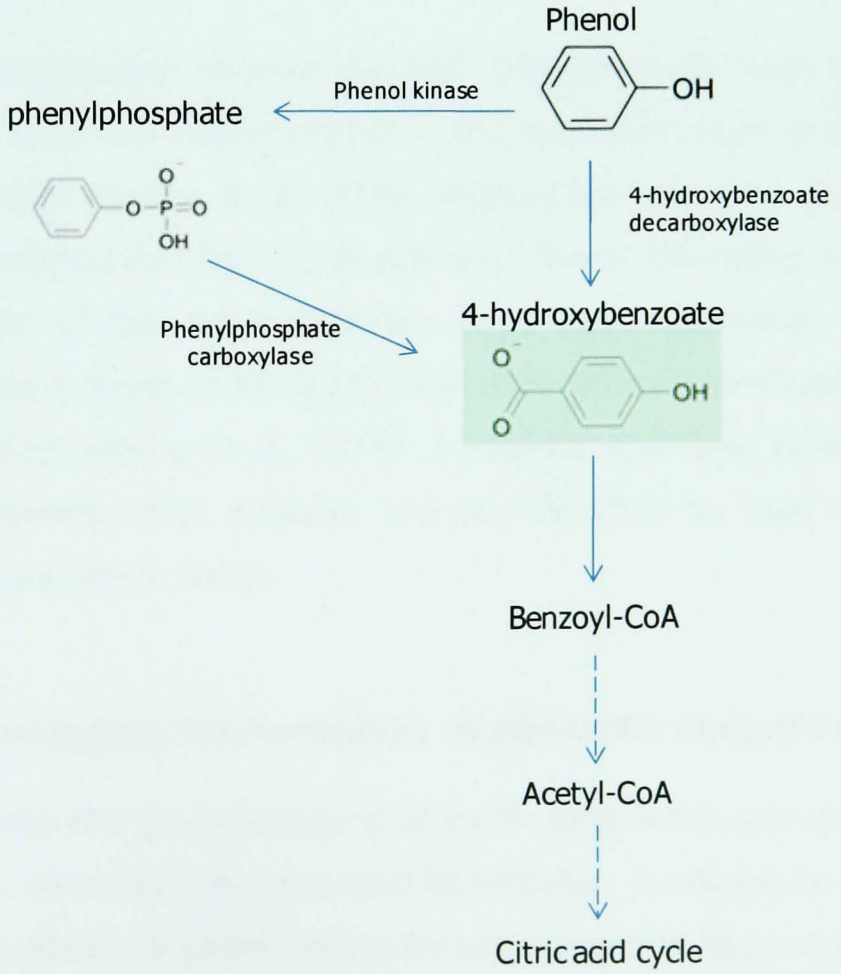



**Figure 1-8** Peripheral anaerobic degradation of phenol to the central benzoyl-CoA (adapted from Heider and Fuchs, 1997)

After initial catabolism during the peripheral pathway, benzoyl-CoA is oxidised to acetyl-CoA, which enters the citric cycle to generate ATP and $CO_2$ (Heider and Fuchs, 1997). Speculation still surrounds the usage of this pathway, and indeed other pathways of phenol degradation, such as the resorcinol, phloroglucinol, catechol, and the more recently discovered hydroxyquinone pathways for phenol degradation (Philipp and Schink, 2000; Schwarzenbach, 1991). It has been suggested that anaerobic organisms, such as sulphate-reducers, methanogens and fermentors may use the same pathway, but more investigation is required (Schink *et al.*, 2000).

The potential number of different anaerobic metabolic pathways of phenol degradation is vast, even when only a number of pure cultures, such as *T. aromatica* (Tschech and Fuchs, 1989; Heider and Fuchs, 1996) and *D. phenolicum* (Bak and Widdel, 1986), are used in the investigations. When considering concomitant mixed-cultures from natural environments, the possibilities increase even further.

The anaerobic degradation of other phenolic compounds has been demonstrated. Cresols are low molecular weight phenolics and have been seen to degrade under anaerobic conditions (Schink *et al.,* 2000). *p*-cresol has also been seen to degrade via the 4-hydroxybenzoate (Figure 1-8) pathway. Due to the higher complexity and molecular weight of the dimethylphenols (xylenols), degradation by anaerobic organisms has been found to be significantly more difficult (Smolenski and Sulfita, 1987; Faber, 1979; Haines *et al.*, 1974). 2,6-xylenol has been reported as being recalcitrant in environmental systems, and can therefore be used as an internal tracer (Broholm and Arvin, 2000).

### 1.6.3 Methanogenic fermentation of phenolic compounds

Fermentation yields the lowest energy of all the biodegradation processes (Zehnder, 1988). However, this does not mean that fermentation is unimportant. It has been noted that fermentation is extremely significant in areas of toxic concentrations of phenolic compounds, resulting from the suppression of respiratory processes by high contaminant loads (Thornton *et al.*, 2001).

Fermentation is regarded as the usage of an organic compound as both electron donor and acceptor, and has been considered as a rather inexpensive technique for the *in situ* degradation of complex organic contaminants (Schink, 1988). Fermentation results in the biodegradation of a substrate to products that are either more, or less, oxidised than the reactant molecule, be it phenol or glucose, via substrate-level phosphorylation (Zehnder and Stumm, 1988; Evans, 1977). This process usually occurs syntrophically with the thermodynamically-poor respiratory processes ($SO_4^{2-}$reduction, $CO_2$; methanogenesis). An example is the fermentation of an organic substrate to hydrogen, which would then be respired using inorganic electron acceptors to complete the degradation process (Figure 1-9).

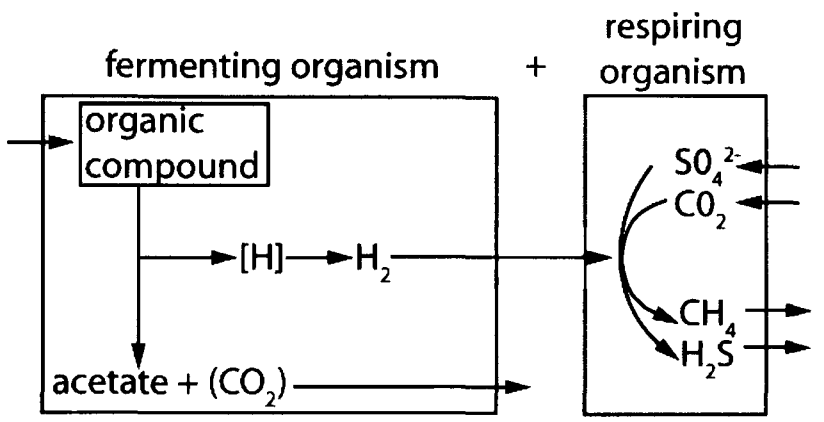



**Figure 1-9** Interspecies degradation pathway for organic compounds, showing initial degradation by fermenting organisms, followed by interspecies transfer of $H_2$ to respiring organisms (Modified from Zehnder, 1988)

Degradation of aromatic compounds by fermentation, with syntrophic degradation by e.g. methanogens, has been extensively studied (e.g. Dwyer *et al.*, 1986). Evans and Fuchs (1988) present an example of the degradation of phenol by fermenting bacteria, followed by a phase of methanogenesis before the degradation process is complete (Figure 1-10). This syntrophic relationship is imperative to ensure that the reaction process can proceed and obey Chatelier's Principle of equilibrium (Hernandez, 2007).

In the absence of inorganic TEAs and/or respiring organisms, fermenting organisms can wholly degrade aromatic organics to non-aromatic products (Figure 1-10). With respect to phenol, fermentation has been known to produce acetate, butyrate and benzoate, with a heavy dependence on $H_2$ and $CO_2$ concentration (Karlsson *et al.*,

2000). Previous studies have noted instances of phenol fermentation to other aromatic intermediates, but to the knowledge of Karlsson *et al.* (2000), their study is the first report of fermentation to non-aromatic products, using phenol as the sole carbon and energy source.

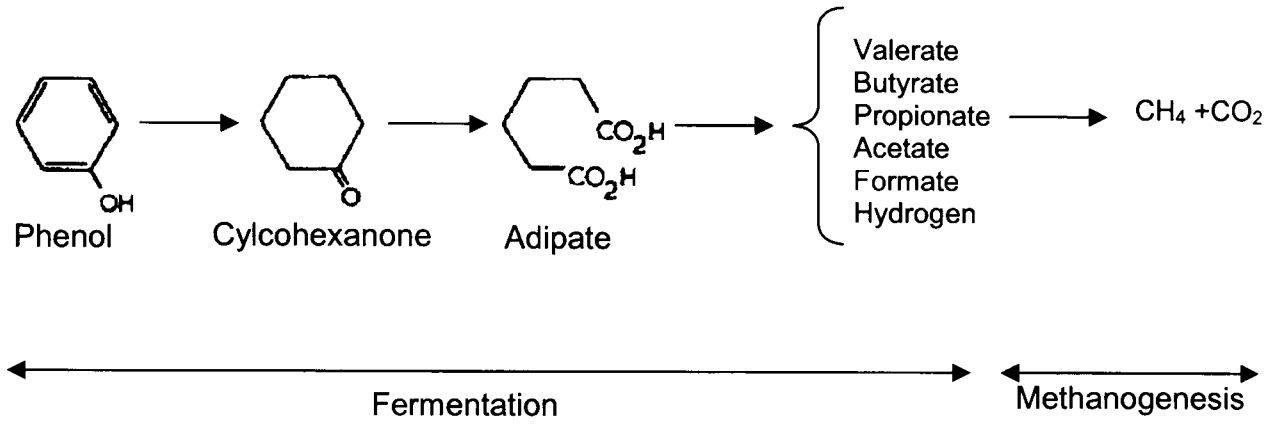



**Figure 1-10** Methanogenic fermentation of phenol to methane and carbon dioxide (adapted from Evans and Fuchs, 1988)

Biodegradation mechanisms for phenolic compounds are complex and extremely variable, depending on the organisms responsible and the conditions of the environmental system. Much research involving both laboratory microcosm and *in situ* investigations is required to provide a complete understanding of the degradation processes that operate in each environment. Studying the degradation pathways requires a separate study that will not be covered in this work.

## 1.7 Experimental Site (Four Ashes, UK)

### 1.7.1 Site Location and History

The Four Ashes site is located 10 miles north of Wolverhampton in the West Midlands, UK (Figure 1-11), and was home to a variety of industries throughout the 20th century. These included the fractionation and distillation of coal tar acids by Midland Tar Distillers (Guanghe *et al.*, 1999, unpublished data), caustic soda lime kilns and feedstocks (Thornton *et al.*, 2001). Both the tar distillery and soda lime kilns were operational from the mid-20th century until closure in the 1980's (Williams *et al.*, 2001).

The site lies on drift deposits of clays, silts, sands, gravels, and glacial alluvium, extending to ~5m below ground level (mbgl) (Williams *et al.*, 2001). The aquifer beneath these deposits is composed of Permo-Triassic conglomeratic and deltaic sandstones, inter-bedded with fresh and brackish water deposits of marls (Morgan, 1973). These rocks form the limb of a NNE trending syncline, and are slightly offset by regional faulting events, and have a westerly direction of dip (approximately 2°) (Guanghe *et al.*, 1999).

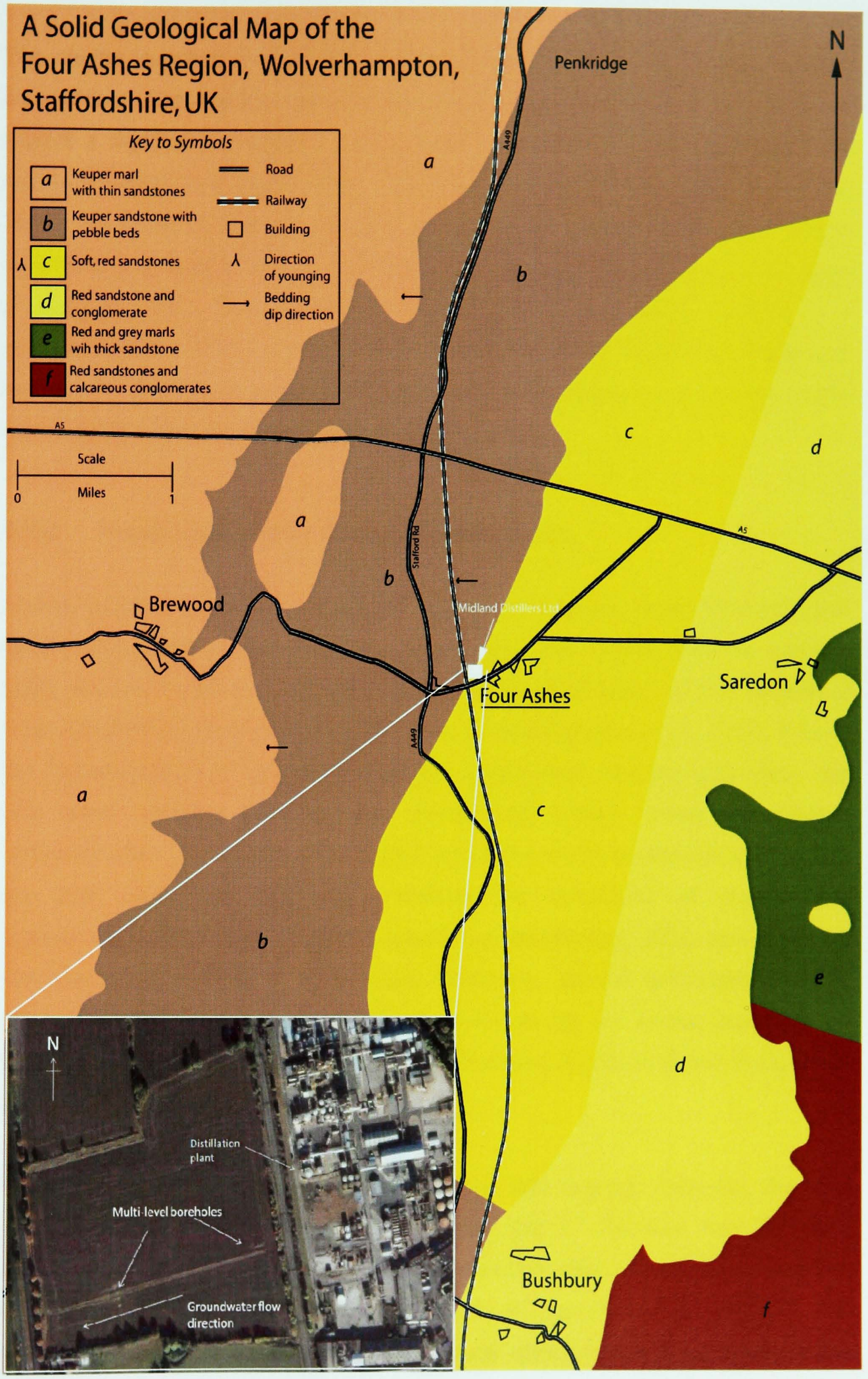



**Figure 1-11** Solid geological map of the area surrounding Four Ashes. Inset: Satellite image of the Four Ashes site.

The aquifer supplies a local groundwater extraction well approximately 1500m downstream. Also, the local surface waterways, the River Penk and Saredon Brook, receive a significant amount of E-W hydraulic baseflow from the aquifer at Four Ashes, resulting in a major output from the groundwater flow (Guanghe *et al.*, 1999). In terms of aquifer hydraulics, most authors agree that the water-table is located around 4mbgl, and that the lateral hydraulic conductivities are in the range of 0.2-1.3m $day^{-1}$. The porosity of the subsurface is 20-30%, resulting in a groundwater flow velocity of 0.7-17m $yr^{-1}$ (Williams *et al.*, 2001). The plume was found to be growing at a rate of 10m $yr^{-1}$, which would result in the contaminants reaching the public supply borehole within 150 years.

### 1.7.2 Properties of the Contaminant Plume

During industrial operation, contaminant spillages of organics, acids, feedstocks and deicing compounds occurred on site. There were no barriers, such as concrete bases and bund walls that would have prevented the infiltration and percolation of these contaminants into the ground beneath the site (Williams *et al.*, 2001). Prior to the implementation of legislation regulating waste input into the subsurface, the only safety measure used to treat contaminant spillage onsite was calcium carbonate chips. These chips were spread across the site in an attempt to neturalise any acid spills, but they still permitted the percolation of organic and othercontaminants into the ground beneath the site (Bottrell, 2010, pers. Comm.). This inadequate method of contaminant treatment, allowed carcinogenic organic contaminants to seep into the aquifer system beneath the works, producing an anchored contaminant plume (Banwart and Thornton, 2003), as shown in Figure 1-13.

The major contaminants include phenol, cresols and xylenols. Currently, the total concentration of organic compounds is around 24g $L^{-1}$ (Banwart and Thornton, 2003), approximately 12.5g $L^{-1}$ of which is phenol (Thornton *et al.*, 2001). A number of modelling studies have been conducted at Four Ashes to quantify the contaminant concentrations, distribution, natural attenuation and migration of the pollutants. The plume is believed to extend 500m in the direction of the groundwater flow, and extend 50-60m deep within the aquifer (Thornton *et al.*,

2001). This has resulted in concern over the site due to the potential of the contaminants to be transported to the local water extraction well.

### 1.7.3 Groundwater Hydrochemistry

Research has been conducted using of a number of pre-existing boreholes at the site to assess the groundwater hydrochemistry (Figure 1-12). Williams *et al.* (2001) collected depth-specific samples using a micro-purge technique from the observation boreholes to analyse a variety of chemical components. Plots of dissolved organic carbon (DOC) and major plume components such as phenol and *o*-cresol were created, displaying evidence as to the concentration gradients within the plume.

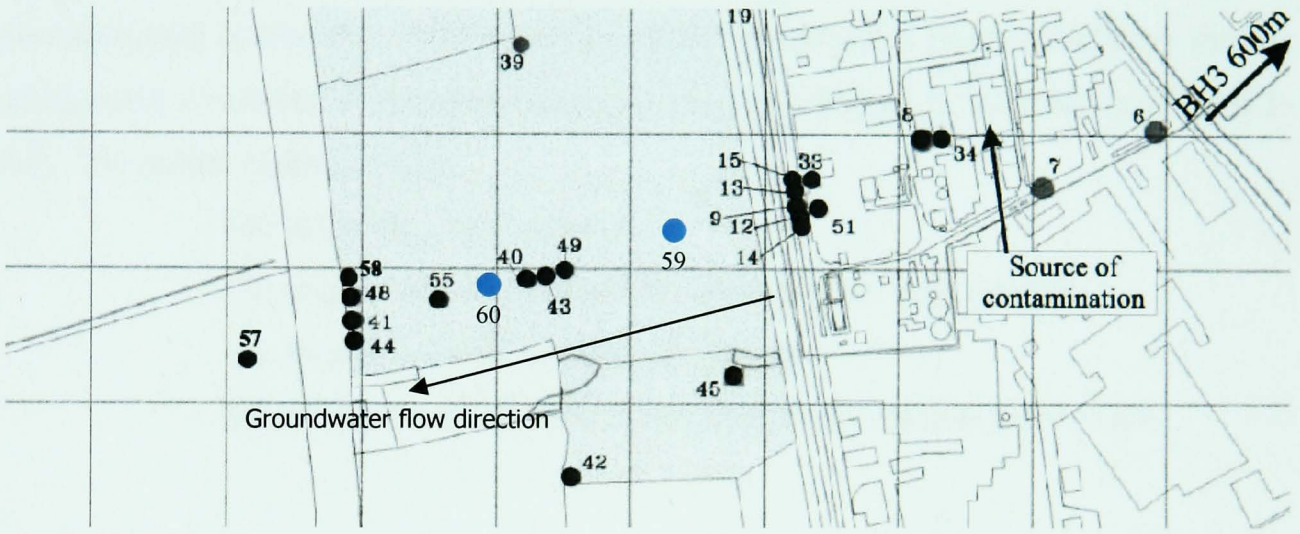



**Figure 1-12** Borehole plan at Four Ashes including location of multi-level sampling (MLS) boreholes 59 and 60 (Thornton *et al.,* 2001; and Williams *et al.,* 2001). Image not to scale.

The upper and lower boundaries of the plume are sharp, and possess the lowest concentration of DOC, phenol (Figure 1-13) and o-cresol, with the maximum concentrations occurring near to the point source and within the anaerobic centre of the plume. The remaining organic species (*p*/*m*-cresol and xylenol) are all believed to be similarly distributed.

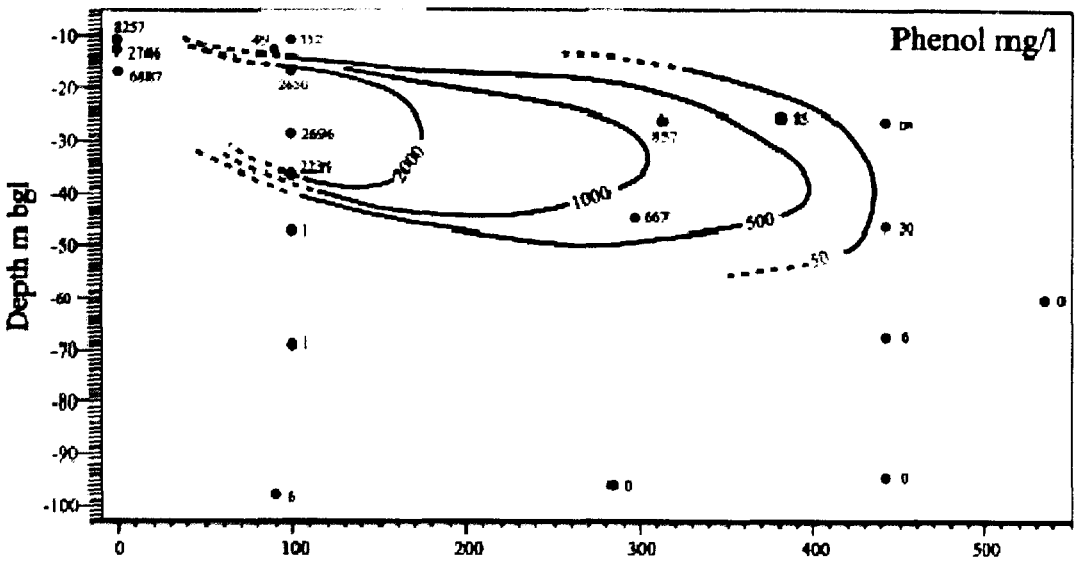



**Figure 1-13** Plume of phenol concentration and distribution in groundwater beneath Four Ashes in 1998 (Williams *et al.*, 2001).

From the DOC data, and data on the spatial distribution of the inorganic contaminants at the site, Williams *et al.* (2001) proposed a plume-chemistry model, suggesting a number of chemical suites in the groundwater at Four Ashes (Figure 1-14). The suites outlined were:

(a) unpolluted groundwater

(b) organically contaminated groundwater, with low pH

(c) As (b) but with significant sodium

(d) Alkaline sodium bicarbonate/sulphate waters, pH = <9.9

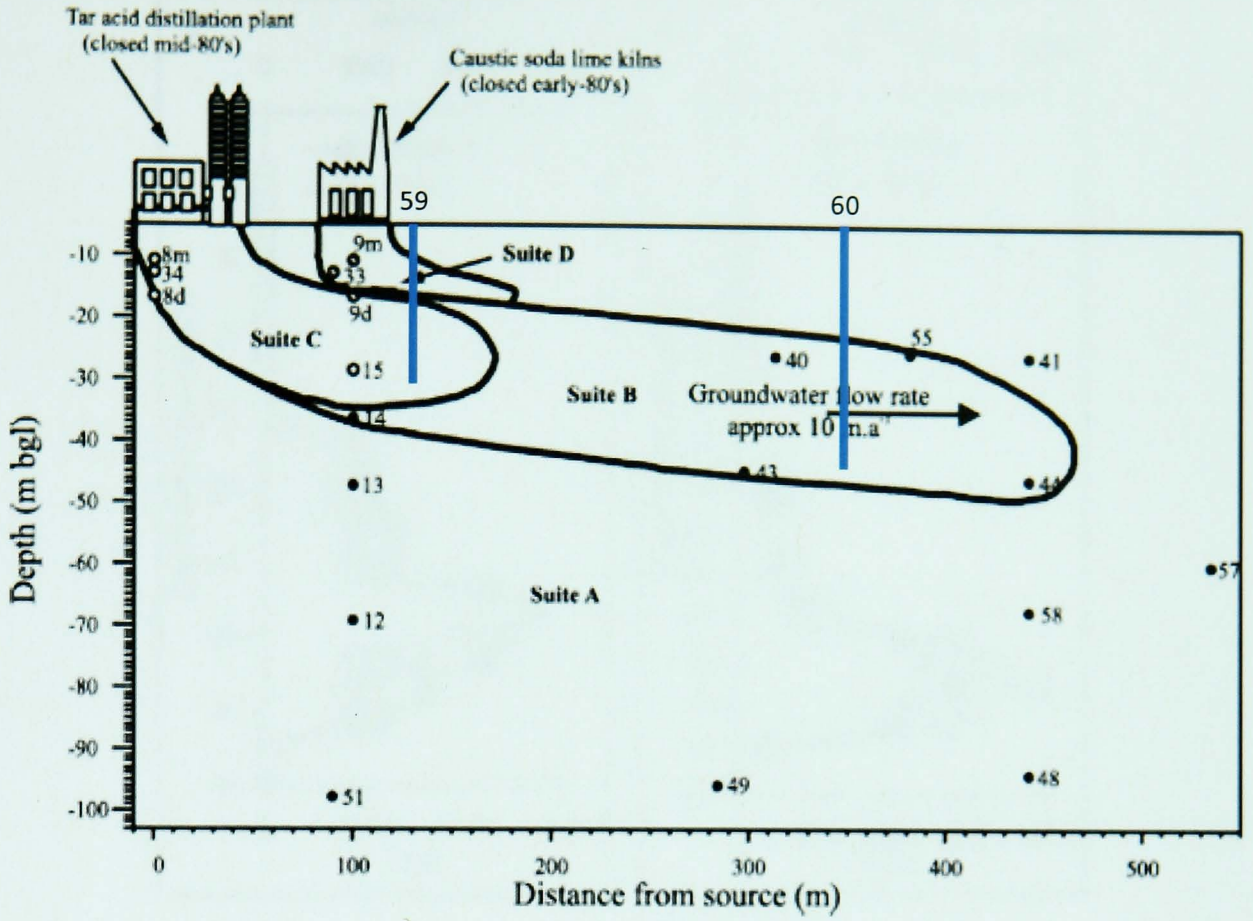



**Figure 1-14** Plume cross-section showing different suites of water composition (Williams *et al.*, 2001). Also shown are the two multi-level sampling boreholes used by Thornton *et al* (2001) to characterise the groundwater hydrochemistry (59 and 60).

Thornton *et al.* (2001) also assessed the chemistry of the plume using two multi-level sampling boreholes (MLS). BH59 is 130m along the flow path sampling 30mbgl and BH60 is 350m along the flow path sampling 45mbgl (Figure 1-12). These provided "high-resolution, level discrete samples of the groundwater" (Thornton *et al.*, 2001). Several profiles were formulated using the MLS to assess the variation of Mn, Fe, organics, pH, $CO_2$, $CH_4$ and others with depth. They found, like Williams *et al.* (2001), that the concentrations of most species vary with depth, with maximum concentrations at 20-30mbgl, corresponding to the centre of the plume. Thornton *et al.* (2001) also found lateral variations in the concentrations of organic compounds in the plume, as higher concentrations of phenolics were encountered downstream from the distillation plant (Figure 1-15), contradicting the conclusions made by Williams *et al.* (2001). However, the method of sample collection and the boreholes used in each investigation differed and will therefore be prone to discrepancy.

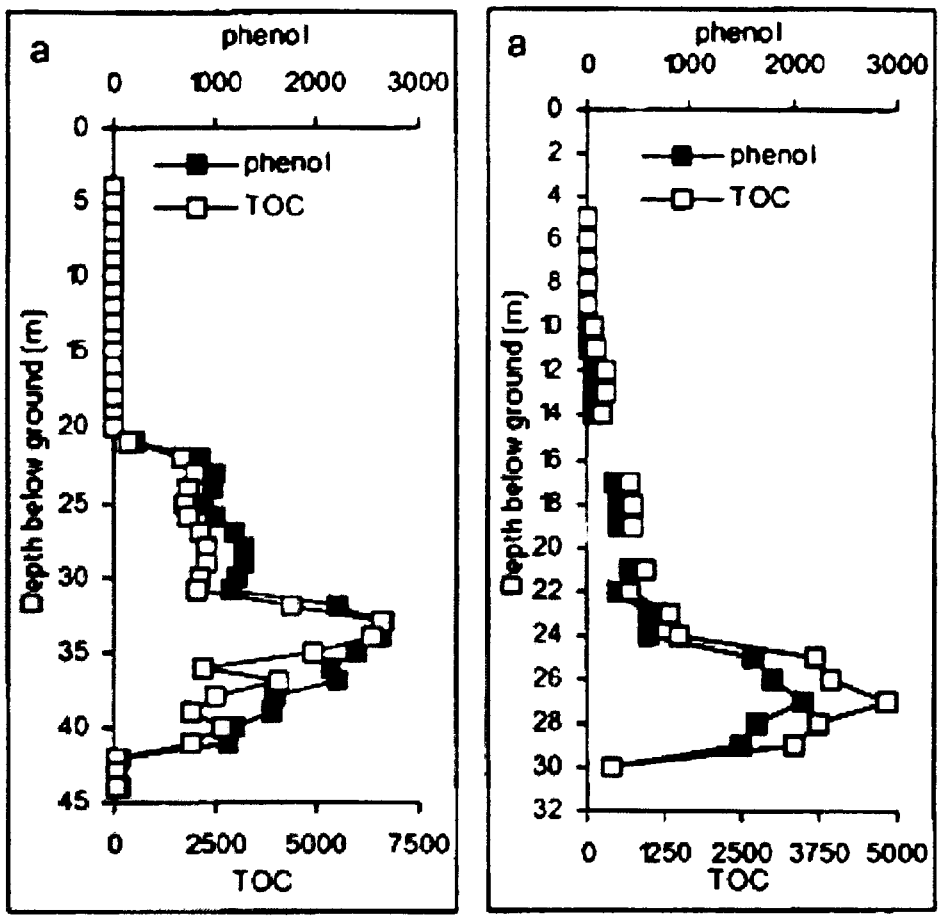



**Figure 1-15** Phenol concentrations as derived from MLS of BH60 (left) and BH59. (Thornton *et al.*, 2001)

Other studies (Spence *et al.*, 2001) also noted that the plume is more concentrated with phenolics further downstream after sampling using the same multi-level sampling boreholes as Thornton *et al.* (2001). This suggests that the input of organic contaminants into the aquifer was stronger during early plume development (Spence *et al.*, 2001).

### 1.7.4 Microbial Assessment of the Contaminant Plume

Pickup *et al.* (2001) assessed microbial presence using the MLS boreholes. After completing total cell counts, it was found that there was a maximum of $7x10^6$ cells $ml^{-1}$ at areas of low phenolics (upstream at borehole 59). In samples containing high levels of phenolics (20-35mbgl), bacterial counts were zero. This provides evidence that a high concentration of phenolic compounds affects the growth of microbial communities.

From molecular (DNA) studies, Pickup *et al.* (2001) found that there is a diverse population of micro-organisms within the plume, and that this was not affected by concentration gradients, as there is a homogenous diversity throughout. The

metabolic activity of the organisms was measured within the same study using API 20NE strips containing a range of test substrates. Once again, contaminant concentration had a profound affect on microbial activity, as at the depths where phenolics concentrations were highest (BH60), no activity was detected. This was surrounded by a zone of suppressed activity, and then high activity around this region, suggesting an activity gradient from aerobic (high) to anaerobic (low to none), coinciding with the phenolics concentration gradient. Activity was found in BH59 at all depths, albeit remarkably lower between 15-25mbgl, once again coinciding with higher phenol concentrations.

The results from the study by Pickup *et al.* (2001) were wholly based on groundwater samples, and therefore only assessed the planktonic biomass within the plume. Rizoulis (2008) compared the diversity of the attached community at 30mbgl to the planktonic microbial community at the same depth using denaturing gradient gel electrophoresis (DGGE). Results revealed that there was a marked difference between the two communities, even though they experienced the same geochemical conditions.

Microbial assessment was also conducted by Williams *et al.* (2001), who found cell counts and diversity similar to that of Pickup *et al.* (2001). Williams *et al.* (2001) also found a distinct lack of culturability from contaminated groundwater, especially where the contaminant concentrations were high and cell counts were low. Williams *et al.* (2001) noted that grossly contaminated areas within the plume did not select for any cell morphotype, or cause the growth of any particular 'monocultures'. It was found that there is a homogenous diversity of cell morphology throughout the plume. However, no investigation of the culturability and cell morphology was performed on the attached biomass within the plume.

### 1.7.5 Natural Attenuation at Four Ashes

Much investigation has been focused on assessing the potential for natural attenuation (NA) at Four Ashes. NA would be a valuable process in ensuring that the contamination is degraded before reaching the local groundwater extraction well.

Lerner *et al.* (2000) noted that NA processes at Four Ashes are occurring, but are extremely slow, especially in anaerobic conditions within the centre of the plume. The aerobic fringe surrounding the plume possesses rapid biodegradation potential. However, anaerobic processes constitute a large proportion of the overall NA process, as only the fringe of the contaminant plume is aerobic. Thornton *et al.* (2001) noted that fermentation is the most important degradation mechanism within the plume core, accounting for 21% of degradation products present, as derived from field experiments on $H_2$ levels and laboratory microcosm studies (Thornton *et al.*, 2001; Wu, 2002).

Williams *et al.* (2001) modelled the different redox processes occurring at Four Ashes from the distributions of redox sensitive species, notably nitrate/nitrite/ammonia, $Fe^{3/2+}$, sulphate/HS and methane (Figure 1-16). Direct geochemical evidence was found for methanogenesis, sulphate-reduction and denitrification on the leading edge of the plume, with the potential to degrade phenolic compounds. However, the model proposed was shown to be too simplistic, as Thornton *et al.* (2001) found that although aerobic respiration and nitrate-reduction is confined only to the plume fringe, other processes, such as metal-oxide- and sulphate-reduction, methanogenesis and fermentation are occurring concomitantly throughout the plume core.

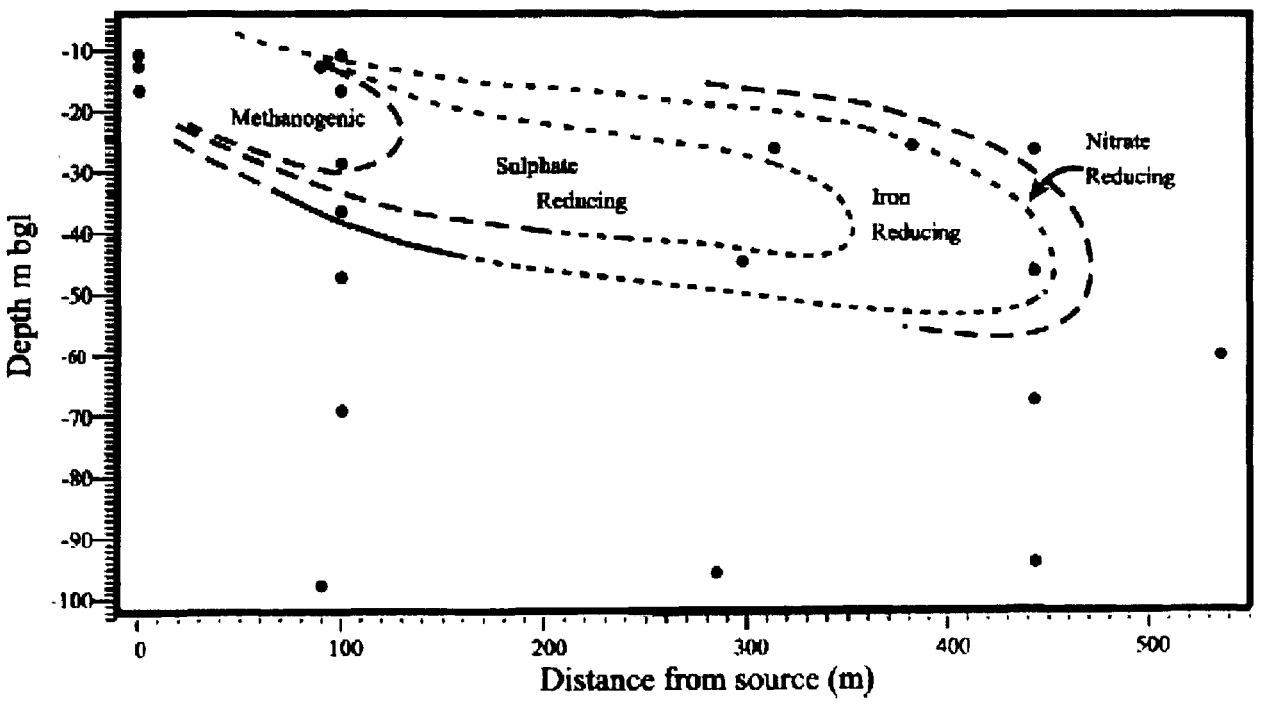



**Figure 1-16** Redox Zones defined from geochemical studies (Williams *et al.*, 2001).

From *in vitro* microcosm studies, Shah (2005) found that in the more heavily contaminated downstream borehole (borehole 60), the onset of methanogenic and fermentative degradation occurred after the exhaustion of sulphate, supporting the notion that there are no common redox zones, as the potential for different redox processes to degrade phenol is present throughout the plume.

Harrison *et al.* (2001) also assessed the potential for NA at Four Ashes using a series of microcosm studies, using anoxic groundwater samples from the boreholes used by Williams *et al.* (2001). The microcosms were used to assess the effect of phenol concentration on the suppression of degradation. Results show that, for aerobic microcosms, rapid degradation occurs at dilute phenol concentrations (<80mg $L^{-1}$), but not where the concentration of phenolic compounds was 5g $L^{-1}$. In moderately dilute microcosms (~200mg $L^{-1}$) biodegradation was occurring at very slow rates. In anaerobic microcosms, nitrate-reduction was occurring, as sulphate levels remained constant throughout, and no $Fe^{2+}$ was produced. However, degradation only occurred within these microcosms up to 660mg $L^{-1}$ (phenol). This suggests that phenol concentration affects the metabolic activity of planktonic organisms within the contaminant plume. Harrison *et al.* (2001) also found that *p*-cresol was degraded only when the total concentration of phenols was below 200mg $L^{-1}$. Elliott *et al.* (2010) assessed the degradation of phenol using novel flow-through column microcosms inoculated with Four Ashes groundwater. Results show that phenol (600µM) was degraded faster as the microbial community became more complex with time, suggesting that microbial community structure, and living as part of complex community, enhances NA potential.

Other studies have assessed particular redox processes in detail. Spence *et al.* (2001a) investigated the processes of denitrification using microcosm experiments, simulating the nitrate-rich plume fringe at Four Ashes. Four Ashes sediment was included within the microcosms to ensure a good approximation of the true environment, and *p*-cresol was the only phenolic compound investigated. It was found that nitrate-reducing bacteria can degrade *p*-cresol up to 200mg $L^{-1}$, agreeing with Harrison *et al.* (2001), whilst simultaneously degrading trace amounts of phenol. Within the plume at Four Ashes, this would only occur on the plume fringe, where hydrodynamic dispersion and mixing can provide nitrate to the system.

Inside the plume, nitrate is undetectable, preventing the onset of nitrate-reduction processes.

Spence *et al.* (2001) modelled the significance of sulphate-reduction for phenol attenuation by analysing the isotopic fractionation of $^{32}S$ and $^{34}S$ within metabolic reactants and products in the Four Ashes groundwater. This allowed the assessment of contaminant concentration thresholds for phenol degradation. It was found that above 2g $L^{-1}$, phenolic compounds are recalcitrant. Lack of an isotopic fractionation within the heavily contaminated downstream area of the plume (borehole 60) suggested that bacterial sulphate-reduction never began due to the high phenolics concentrations. Where bacterial sulphate-reduction has occurred however, it seems that exhaustion of sulphate results in a decline in degradation, causing only 0.05% of the total contaminant load to be degraded.

Shah (2005) also noted that concentrations of >995mg $L^{-1}$ were inhibitory to bacterial sulphate reduction, correlating with the work of Spence *et al.* (2001). This work shows that bacterial sulphate-reduction can degrade high concentrations of phenolic compounds, which has important implications for the potential of NA by sulphate-reducing bacteria. This high threshold is believed to be due to acclimatisation of the organisms within the plume (Wu, 2002).

### 1.7.6 Controls on Natural Attenuation

From previous studies conducted at Four Ashes, it is evident that multiple processes control the evolution of the plume, the contaminant matrix and the NA processes. Firstly, source details are integral to understanding the plume behaviour. The timing, mass and location of initial spillages are important for estimating initial contaminant concentrations, and thus the total degradation of contaminants since infiltration into the aquifer system (Banwart and Thornton, 2003). Quantification of the source strength, biodegradation rates, and hydrogeological parameters, such as groundwater flow velocity and porosity, are important in understanding the nature and evolution of the plume.

Dispersive mixing at the plume fringe is important in controlling the rates of aerobic and microaerophilic degradation. Increased dispersive mixing provides soluble

electron acceptors from the uncontaminated groundwater, allowing rapid aerobic activity and denitrification to occur (Banwart and Thornton, 2003). However, rates of dispersive mixing are slow, suggesting that nutrient transport systems have great control on the NA of the plume. Conversely, biofilms forming on the solid-liquid interface in response to the hostile conditions may not require non-equilibrium mixing as the substrata may contain bioavailable electron acceptors such as $Fe^{3+}$ and $Mn^{4+}$ (Banwart and Thornton, 2003). This negates the problems associated with slow dispersive mixing. Research has yet to assess the geochemical influences on microbial attachment at the site, to quantify microbial attachment and to assess the attached microbial community structure.

Microbial population dynamics are also integral to an accurate assessment of NA potential at the site. As yet, no research has been conducted to provide information on the community diversity and structure of the anaerobic parts of the plume. Rizoulis (2008) found that contaminant levels influence the diversity of eubacterial communities on the plume fringes, but no assessment of the anaerobic community structure (e.g. nitrate/sulphate-reducers and methanogens) has been performed. As the majority of the contaminant load is anoxic, anaerobic communities are integral to the overall NA processes at the site.

### 1.7.7 Pump and Treat Remediation at the Site

In August 2009, the Four Ashes site owners began remediating the contaminant plume. Accordingly, a pump and treat system is being used to purge contaminated water from borehole 60 (350m from the source) and pump it into an on-site treatment system (Thornton, 2009, pers.Comm). Unfortunately, due to the commercially sensitive nature of the operation, the site owners and consultants are unwilling to divulge any information regarding the pumping depths and rates. If available, such data would be valuable in modelling the influence of the pumping on groundwater flow and contaminant transport.

However, qualitative speculation can be made with respect to the main effects of the pumpin on the groundwater system. Whatever the flow regime, groundwater will be drawn from the source downstream towards borehole 59 (130m from

source) and 60. This would potentially change the groundwater contaminant distribution, as purging will remove groundwater at a faster rate than natural flow, thus alterting the groundwater hydrochemistry to reflect source input variation later in plant operation. If high flow rates are being used, it could be expected that the flow of groundwater that had already passed borehole 60 would be reversed, to maximise the remediation efficiency of the system. This would ultimately create a cone of depression, moving the water table to a deeper level in the aquifer. This will strongly influence the distribution of contaminants in the aquifer and modify the natural attenuation processes.

Another variant that may influence contaminant distribution is the depth at which the purging is occurring. Pumping from one depth at high rates may draw groundwater vertically through the aquifer, as recharge will be slower than removal. This will shift contaminants vertically within the aquifer, thus altering the depths at which the maximum concentrations of contaminant were found by Thornton *et al.* (2001).

Microbial degradation and natural attenuation patterns may also be affected by the pump and treat system, as changes in contaminant load may influence community structure. High, turbulent flow rates may result in the enhanced mixing of contaminated and uncontaminated water, thus introducing fresh sources of high-energy electron acceptors into the system, which could enhance natural attenuation. However, vertical draw-down due to high pumping rates will result in parts of the aquifer becoming unsaturated, which will affect the microbial community attached to the aquifer sandstones due to a lack of soluble nutrients, which would be supplied from the groundwater.

As the pumping regime is not known, these possibilities are speculative. However, the possibility of the remediation system influencing groundwater hydrochemistry must be borne in mind when considering the results of any hydrogeological research.

## 1.8 Hypotheses, Aims and Objectives

### 1.8.1 Hypotheses

From the literature survey, extensive aerobic studies have been undertaken, but not anaerobic ones. Investigating the anaerobic microbial community within the groundwater, together with the evolution of the plume and the geochemical influences on microbial attachment would provide a better understanding of the NA at the site. A number of hypotheses can be formulated:

1. The groundwater hydrochemistry at Four Ashes has remained stable since investigations began;
2. Contaminant load affects the structure of anaerobic planktonic and attached microbial communities;
3. Mineral surface properties influence microbial attachment and microbial community structure *in situ*.

### 1.8.2 Aims and Objectives

The main aim of this project is to investigate the effect of phenolic contamination on *in situ* anaerobic microbial ecology in the groundwater at boreholes 59 and 60. Secondly, the geochemical effects on the attachment of microorganisms within the plume will be investigated by suspending surrogate substrata *in situ*. The specific study objectives are:

1. Update the groundwater hydrochemistry data to discern the physicochemical properties of the plume, any changes over time, and assess the distribution of anaerobic terminal electron acceptors in order to highlight zones of potential anaerobic microbial activity;
2. Assess the impact of contaminant load on the distribution and community structure of aerobic and anaerobic microbial communities (attached and planktonic) within the groundwater;
3. Investigate the geochemical controls on microbial attachment *in situ* using surrogate geological substrata.

# 2

# Groundwater Hydrochemistry and Microbial Abundance in the Four Ashes Aquifer

## 2.1 Introduction

Many studies have assessed the microbial abundance and groundwater hydrochemistry in contaminated aquifers (Harvey *et al.,* 1984; Martino *et al.,* 1998; Ludvigsen *et al.,* 1999; Broholm and Arvin, 2000). However, due to time-constraints and site access, it is rare that researchers are able perform a time-series analysis of a site. At Four Ashes, Wolverhampton, UK, the unprotected distillation of coal tars led to the infiltration of phenolic compounds into the underlying Permo-Triassic sandstone aquifer during the 20$^{th}$ century. This produced a 500m-long plume of phenolic contamination (Thornton *et al.,* 2001), which has been the subject of research since 1998. Complete transects of the groundwater hydrochemistry profiles have not been collected since 1998 (Thornton *et al.,* 2001; Spence *et al.,* 2001; Pickup *et al.,* 2001). The study described in this chapter aims to update the knowledge of the groundwater hydrochemistry to assess the development of the contaminant plume with time, and the variation of the different contaminant suites within the groundwater. Such data will provide information regarding the strength and variability of the contaminant source during early plume development in the 1950s. These data could also provide further information on the source-term (timing, mass and locations of spillage) and redox zones in the groundwater (Banwart and Thornton, 2003).

In 2005 and 2006, Rizoulis (2008) investigated the groundwater hydrochemistry, microbial abundance and diversity at the plume fringes within two multi-level, depth-discrete boreholes; borehole 59 (130m from point-source) and borehole 60 (350m from point-source). The aim was to assess how the phenolic contamination influences the aerobic microbial community within the aquifer. However, no research since 1998 has been undertaken to update the knowledge of the microbial abundance within the most grossly contaminated areas of the groundwater, where conditions are anaerobic (Thornton *et al.,* 2001).

It has been estimated that 96% of the contaminant load at Four Ashes is anaerobic, and therefore assessing whether suitable hydrochemical conditions are present for anaerobic communities to thrive is important for understanding the potential for natural attenuation. Thornton *et al.* (2001) found that the anaerobic processes operating at the site include nitrate-, manganese-, iron- and sulphate-reducers,

methanogens and fermenters. All of the respiration processes are slow due to the contaminant matrix and the limited supply of the terminal electron acceptors (TEA). Although sulphate-reduction is thermodynamically unfavourable in comparison to other terminal electron accepting processes (TEAP - see Section 1.5), previous research has found that sulphate levels are high throughout the plume (Thornton *et al.,* 2001; Spence *et al.,* 2001). This would enable sulphate-reduction within the anaerobic areas of the plume, where the influx of more thermodynamically favourable TEA (e.g. nitrate) by transverse mixing of uncontaminated and contaminated water is not possible. Spence *et al.* (2001) and Shah (2005) found, however, that high contaminant loads suppresses microbial sulphate-reduction, but any recent changes in contaminant levels and/or distribution may have resulted in an increase in the activity of sulphate-reducing bacteria (SRB). Sulphate-reduction has been found to be the major TEAP at over 42 sites contaminated with hydrocarbons and therefore could be important at Four Ashes (Weidemeier, 1999). The contaminant matrix does not only affect certain microbial groups, but directly affects total microbial abundance at the site, and therefore updating the information on microbial abundance within the groundwater is central to understanding the effect of any changes in contaminant levels on natural attenuation (Pickup *et al.,* 2001).

The aims of this study are to (i) investigate the groundwater hydrochemistry profiles at borehole 59 and 60 to assess the development of the contaminant plume, (ii) compare the sources and distribution of contaminants with previous research, (iii) assess the distribution of terminal electron acceptors within groundwater profiles that may be suitable for anaerobic microbial processes, especially SRB, (iv) assess the influence of contaminant load on microbial abundance within the aerobic and anaerobic parts of the plume.

## 2.2 Methods

### 2.2.1 Groundwater Sampling using a Multi-Level Sampler

Groundwater samples were collected using two depth-discrete multi-level sampler systems (Figures 2-1 and 2-2) in 2008 and 2009. The two boreholes (59 and 60) were installed by EPSRC and the Environment Agency using water-flushed rotary drilling. These boreholes were used by Thornton *et al.* (2001) and a number of other investigators (Pickup *et al.,* 2001; Spence *et al.,* 2001). The two boreholes are approximately 130m (borehole 59) and 350m (borehole 60) from the point-source of contamination (Figure 1-12). The depths sampled and the dates of sampling are shown in Table 2-1.

**Table 2-1** Depths of sampling within the groundwater profile at the Four Ashes site.

| Borehole | Date | Depth Sampled (mbgl*) |
|---|---|---|
| 59 | February 2008 | 8, 12, 21, 26, 30 |
| | January 2009 | 30 |
| | July 2009 | 6-30+ |
| | August 2009 | 12, 14, 21, 26, 30 |
| 60 | March 2007 | 43, 44, 45 |
| | August 2007 | 45 |
| | July 2009 | 6-45+ |

***mbgl = metres below ground level**

**+samples were taken for groundwater hydrochemistry measurements only**

The method of sampling followed three steps:

1. Equipment set-up: consisting of generator, vacuum pump and bell-jar reservoir, and 4-channel peristaltic pump to remove the sample;
2. Initial purging of sample tubing (~2L) using vacuum pump/bell-jar to remove any stagnant water left in the tubing since the last site visit;
3. Removal of sample using the peristaltic pump (up to 5L).

The amount of water removed from the sample tubing during the initial purge is a function of the volume of water that the tubing can hold (515ml). This is based on the tubing having an internal radius of 2mm, and being up to 45m in length (to

reach the bottom sampling point at 45mbgl within borehole 60). However, due to the water table being 4mbgl, the length of the water column within the tubing is 41m. Therefore, removing 1 to 2L comfortably removes any stagnant water and flushes the sample tubing ready for the removal of a fresh sample.

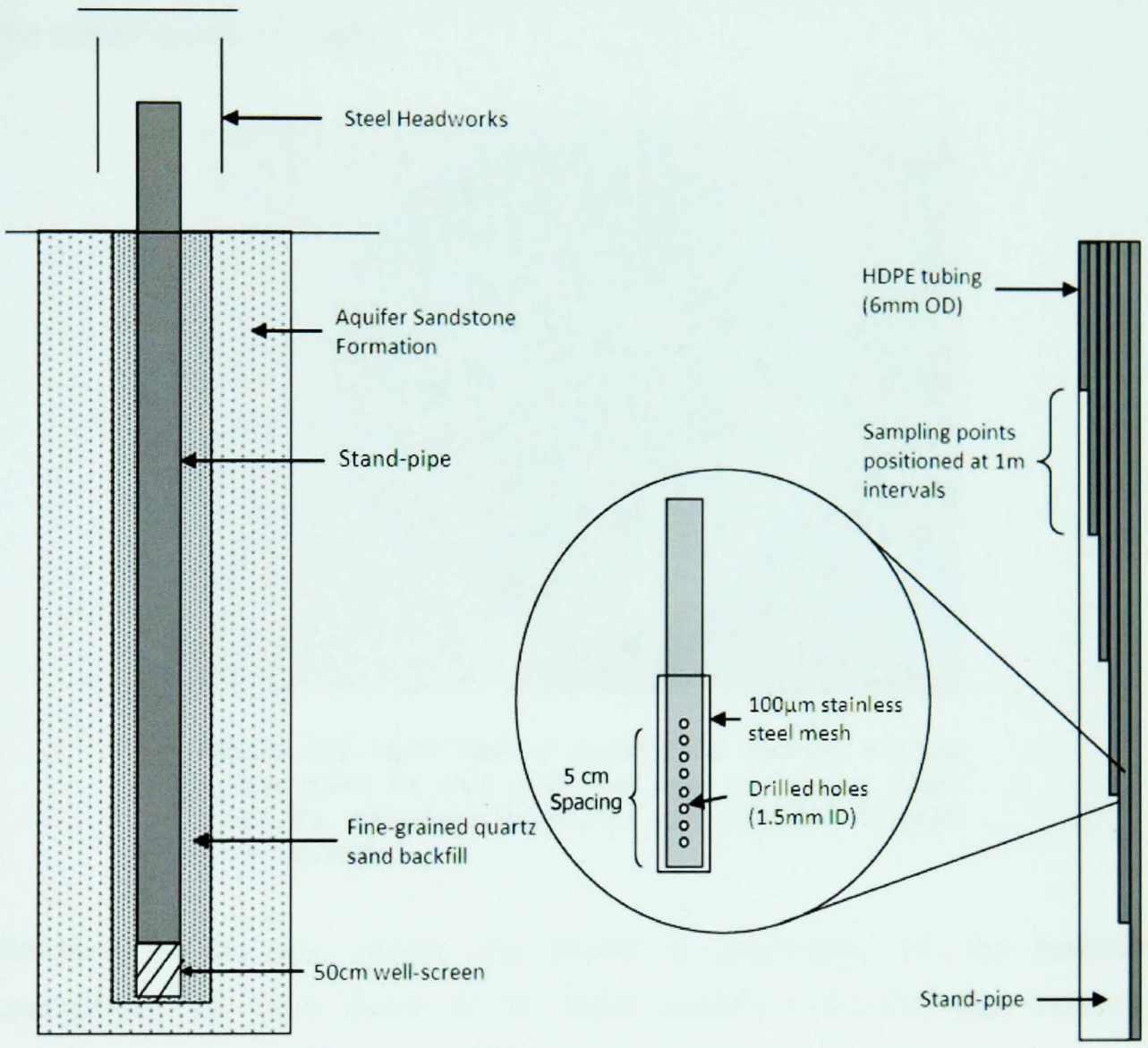



**Figure 2-1** Multi-level sampler system installed at two boreholes at Four Ashes, Wolverhampton (Adapted from Thornton, 2001, unpublished). Left: macroscale structure of the borehole, showing the borehole stand-pipe, well-screen and quartz sand backfill that minimizes the permeability contrast between the formation and the annular spacing. Right: schematic of the port distribution attached to the outside of the stand-pipe.

The method of sample removal was designed to account for the hydraulic conductivities ($K$) of the aquifer (both laterally and vertically) and the groundwater flow rate, to ensure that no cross-contamination between depths occurred during purging. The best and worst case scenarios of sample removal (Figure 2-3) show that, in both cases, no cross contamination of samples from different depths is

achieved during the removal of up to 104.7L of sample. In Figure 2-3a, a uniform spherical removal of sample occurs when the vertical and lateral hydraulic conductivities of the aquifer are assumed to be equal. The removal of 104.7L produces a sampling radius around the port of 0.5m, taking into account an aquifer porosity of ~20% (Thornton *et al.*, 2001). This just reaches the catchment area for the sampling port 1m above.

**Figure 2-2** Aerial view of borehole 59 showing multiple sample tubes for each level, and inner well-casing, down which the well-screen is located and surrogate minerals were suspended.

However, in the real system, the aquifer is anisotropic, i.e. the hydraulic conductivity has been shown to be higher laterally (~0.5m/d) than vertically (~0.1m/d), via the use of pumping tests (Aspinwall & Co., 1992). This is due to inter-bed differences in porosity and permeability. This is demonstrated in Figure 2-3b, where an elongated catchment is assumed. Again, sample cross-contamination between depths does not occur when sampling up to 104.7L.

If any intereference between groundwater samples removed from different depths occurs, a lack of disrete variability would be detected on the plots of contaminant concentration. Conversly, a stepped contaminant profile would suggest that no cross-contamination of samples occurred. The latter was demonstrated by both chemical and biological studies, showing level-discrete variability down the groundwater profiles at both multi-level boreholes (Thornton *et al.*, 2001; Pickup *et al.*, 2001).

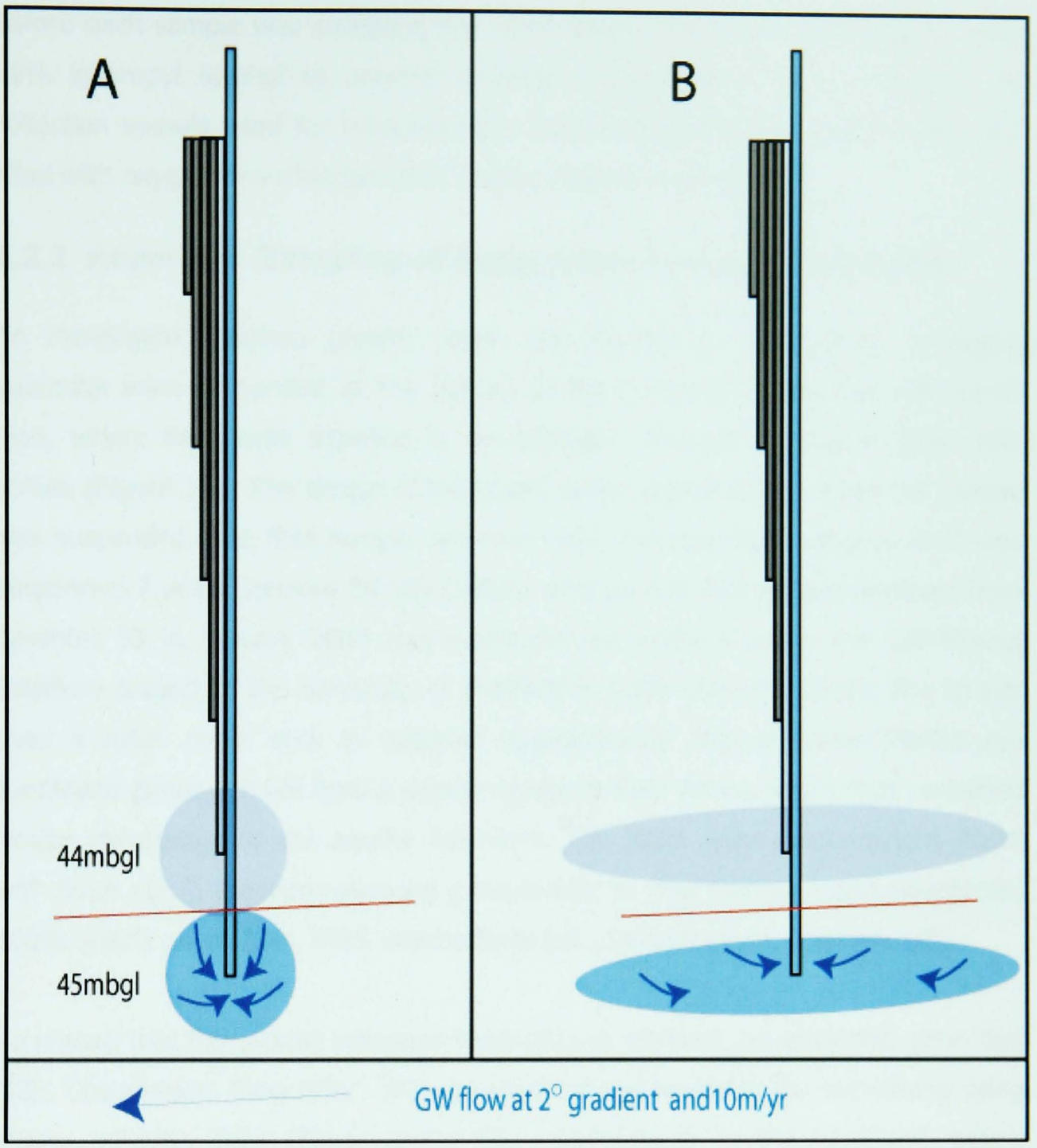



**Figure 2-3** A: Worst case of sample extraction of 104.7L showing radius of sample catchment. B: Actual case of extraction considering lateral and vertical hydraulic conductivity differences. The red line indicates the boundary where sample cross-contamination between depths begins to occur. Images not to scale (Adapted from S. F. Thornton).

After sample collection, groundwater was filtered (Whatman® 0.45µm nylon) and aliquoted on site into glass McCartney vials for analysis of total phenolic compounds, a 20ml scintillation vial for analysis of dissolved ions, and a 15ml polypropylene vial containing nitric acid (5% v/v final concentration) for metal analysis (Thornton *et al.*, 2001). Samples for culture-dependant or molecular (DNA/RNA) assessments were collected unfiltered into 1L sterile Duran bottles (Camlab Ltd., Cambridge, UK) without leaving any headspace.

Before each sample was collected, the HDPE tubing was cleaned thoroughly using 60% isopropyl alcohol to prevent microbial contamination of the samples. All collection vessels used for microbiological assessments were autoclaved and pre-filled with oxygen-free nitrogen (BOC Gases, Guildford, Surrey, UK).

### 2.2.2 Anaerobic Sampling of Suspended Surrogate Substrata

To investigate attached growth within the aquifer at Four Ashes, surrogate substrata were suspended at the bottom of the boreholes within the well stand-pipe, where they were exposed to groundwater through a stainless steel well-screen (Figure 2-2). The design of the vessel varied depending on when the sample was suspended. The first sample retrieved from borehole 60 in August 2007 was suspended 7 years previous by Wu (2002), whereas the first sample retrieved from borehole 59 in January 2009 was suspended by workers within the Cell-Mineral Interface project at the University of Sheffield in 2006 (Rizoulis, 2008). The former used a nylon mesh sock to suspend approximately 5kg of Permo-Triassic red sandstone grains derived from a quarry nearby to Four Ashes, which thus contained similar mineralogy as the aquifer host-rock. The latter used polypropylene flasks with holes cut in the sides allowing groundwater to flow through clean commercial 100% quartz sand (BDH, VWR International Ltd., Leicestershire, England, UK).

To ensure that the sample remained anaerobic on retrieval, an anaerobic glove bag (I2R, Cheltenham, Gloucester, UK) was attached and sealed to the well casing using strong adhesive tape (Figure 2-4). The water-column in the inner-well casing (stand-pipe) was sparged from the base of the borehole with oxygen-free $N_2$ gas (BOC Gases, Guildford, Surrey, UK) using a high-pressure hose (Hozelock Ltd, Birmingham, UK) and gas regulator. This ensured that all dissolved $O_2$ was removed from the water column. This also created a positive pressure and anaerobic atmosphere within the attached glove bag. The sample was slowly removed from the borehole and brought into to the glove bag, whilst still sparging with $N_2$, and placed in a $N_2$-filled anaerobic jar containing Anaerogen™ anaerobic atmosphere generating sachets (Oxoid, Basingstoke, Hampshire, UK), before placing on ice for transport to the laboratory.

On return to the laboratory, sand samples were homogenised in a sterile agar plate using a sterile scalpel, and aliquots were stored in 4% (v/v) formaldehyde for

imaging of bacteria on grains (Section 3.2.1). For molecular analysis, samples were aliquoted into sterile 1.5ml tubes and frozen at -80°C.

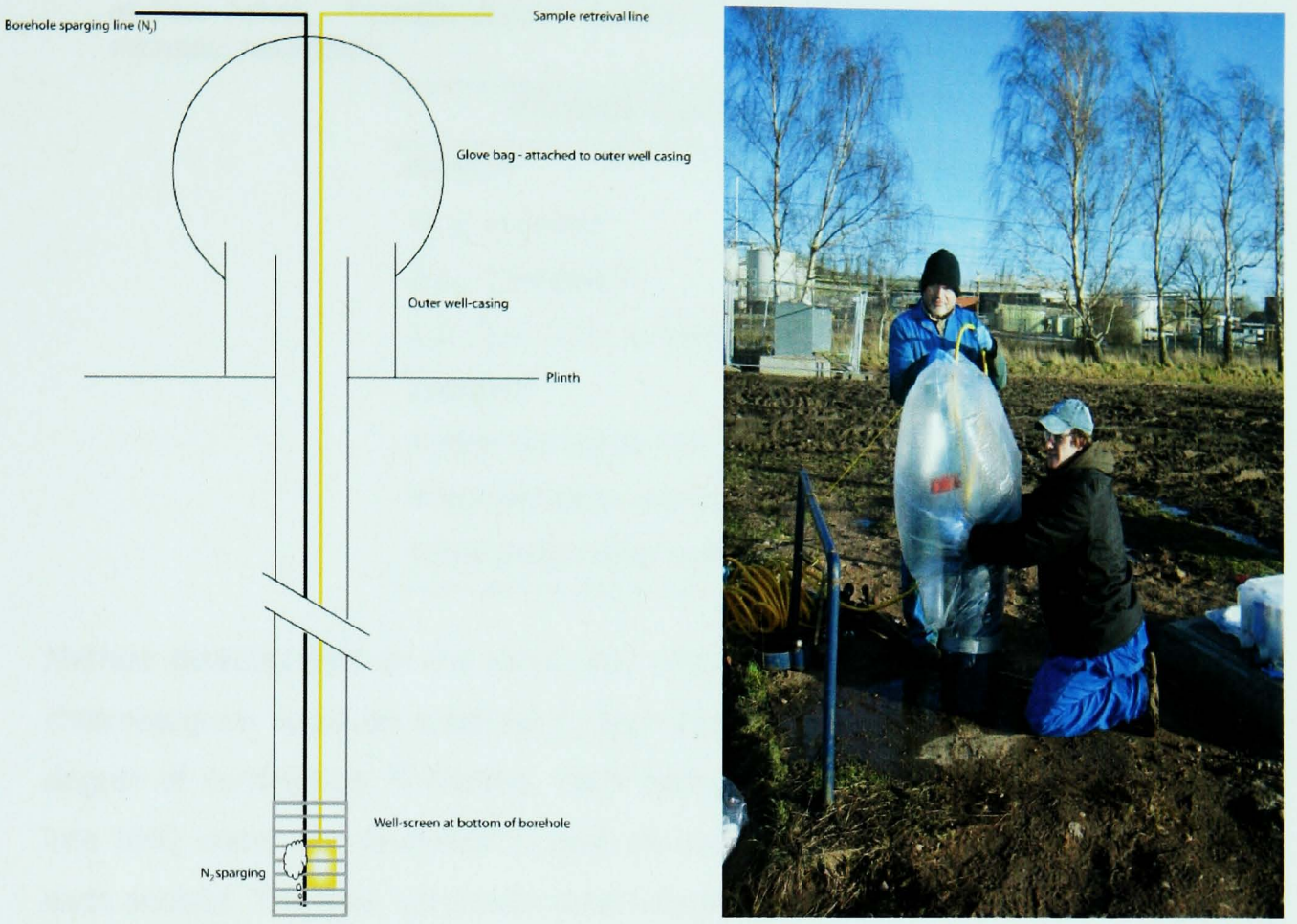



**Figure 2-4** Schematic of anaerobic sample removal strategy (left) used when retrieving suspended surrogate substrata from MLS observation borehole, and actual removal of anaerobic sample from borehole 59.

## 2.2.3 Chemical Analysis of Groundwater

The groundwater was analysed for phenolic compounds (Table 2-2) using a Perkin-Elmer Series 200 high performance liquid chromatograph (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA) with UV/VIS detector. The mobile phase was 40% acetonitrile (pure) and 60% acetic acid solution (0.5% acetic acid v/v in ultra-high quality water (Elga Purelab Ultra, Elga Process Water, Marlow, UK)), with the stationary phase an Alltec® Allspere™ ODS-2 5µm C18 4.6mm x 250mm column (Alltec Associates Inc., IL, USA). 1 ml of filtered sample was required for the analysis, and was run against instrument calibration standards (1, 5, 25, 50, 100, 250, 500mg $L^{-1}$). As no systematic analysis of the detection limit of

the instrument was performed, the detection limit referred to hereafter is the lowest calibration standard (1mg $L^{-1}$).

**Table 2-2** Organic compounds measured in the subsurface contaminant plume at Four Ashes. *=major organic pollutants, $^{+}$= major metabolic products of microbial respiration

| Phenolic Compounds |
|---|
| Phenol* |
| *m, p, o*-cresol* |
| 3,4-, 3,5-xylenol* |
| 2,3-, 2,4-, 2,5-, 2,6-xylenol* |
| Catechol$^{+}$ |
| 4-Hydroxybenzoic Acid$^{+}$ |
| 4-hydroxybenzyl alcohol$^{+}$ |
| 4-hydroxybenzaldehyde$^{+}$ |

Method development on the HPLC was required to ensure that each peak on the chromatogram could be assigned to each phenolic compound (analyte) with a high degree of confidence. Therefore, each phenolic compound was spiked individually into UHQ water and analysed to give an understanding of the retention times for each analyte. The final composite chromatogram showing the retention times for all the analytes is shown in Figure 2-5.

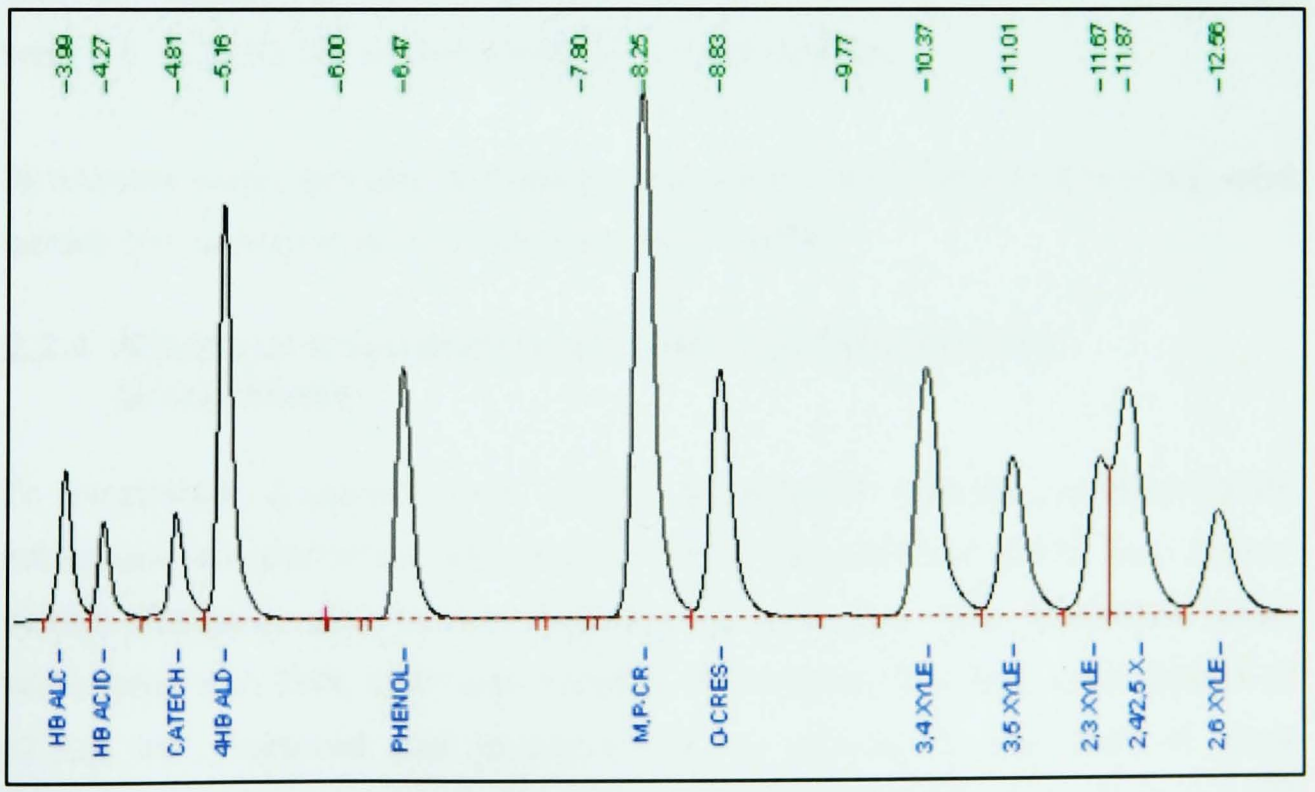



**Figure 2-5** Chromatogram of phenolic compounds and organic metabolites within a calibration standard used in the HPLC analysis of Four Ashes groundwater.

Finally, a certified 5000 mg $L^{-1}$ phenol ampoule (Supelco, Bellefont, PA, USA), diluted to a concentration different to those of the standards (347.1mg $L^{-1}$), was used as an analytical quality control to validate instrument performance. Using a concentration different to any standard ensured that the calibration curve generated from the standards analysis was accurate.

The concentrations of dissolved total Fe and Mn were assessed by atomic absorption spectrometry (AAS) using a Perkin-Elmer AAnalyst 200 (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA). 5ml of acidified sample was analysed alongside calibration standards for Fe (Spectrosol Iron Standard Solution, available from VWR International Ltd., Lutterworth, Leicestershire, UK) and Mn (1000ppm solution, available from Fisher Scientific Ltd., Loughborough, Leicestershire, UK). The calibration levels used were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10mg $L^{-1}$. Samples were assessed for concentrations of dissolved ions (sodium, calcium, nitrate, potassium, phosphorous, sulphate, nitrite, ammonium, bromide, magnesium, fluoride and chloride) using a Dionex DX-120 ion chromatography system (Dionex Ltd, Camberley, UK) fitted with a conductivity cell detection system. The mobile phase was 20mM methane/sulfonic acid (cations) and 1mM sodium hydrogen carbonate (anions). 0.7ml of filtered sample was loaded into a sampling rack and capped with an air-tight plug until analysis. The calibration levels used were 0.5, 1, 5, 10, 25, 50 and 100mg $L^{-1}$ for each species.

All analyses were replicated and diluted where necessary when concentrations were outside the calibration limits of the instrument method.

### 2.2.4 Microbial Enumeration of Total Cell Numbers in Groundwater

To enumerate planktonic cells within groundwater samples, epifluorescence microscopy was performed using 4',6-diamidino-2-phenylindole (DAPI, from Sigma-Aldrich Company Ltd, Dorset, England), a stain that only fluoresces when intercalated with DNA. DAPI was added to the samples to a final concentration of 12.5µg $ml^{-1}$, vortexed and incubated for 15 minutes in the dark at room temperature. Each stained sample was vacuum filtered through a Nucleopore™ 0.22µm black polycarbonate filter (Hobbie *et al.*, 1977) using a Whatman® filter apparatus (Whatman UK Ltd., Maidstone, Kent, UK). The sample was mounted onto

a glass slide and a drop of Baclight™ mounting oil (Invitrogen Ltd, Paisley, UK) was placed in the middle of the filter before covering with a cover-slip. 15 fields of view were imaged using a Zeiss Upright Fluorescence microscope and the 'DAPI' filter cube (365nm excitation).

Samples were viewed and imaged under 63x and 100x oil immersion and counted according to Francisco *et al.* (1973) using ImageJ, an open-source image processing program with available 'cell counter' Java plug-ins. Sample fields of view were opened in ImageJ, and initialised with the 'cell counter' plug-in. Cells were then counted by marking each individual cell, and the total number of cells was calculated by ImageJ. A composite image (Figure 2-6) of the markers and microscope field of view show the marked location of each counted cell. All samples were replicated.

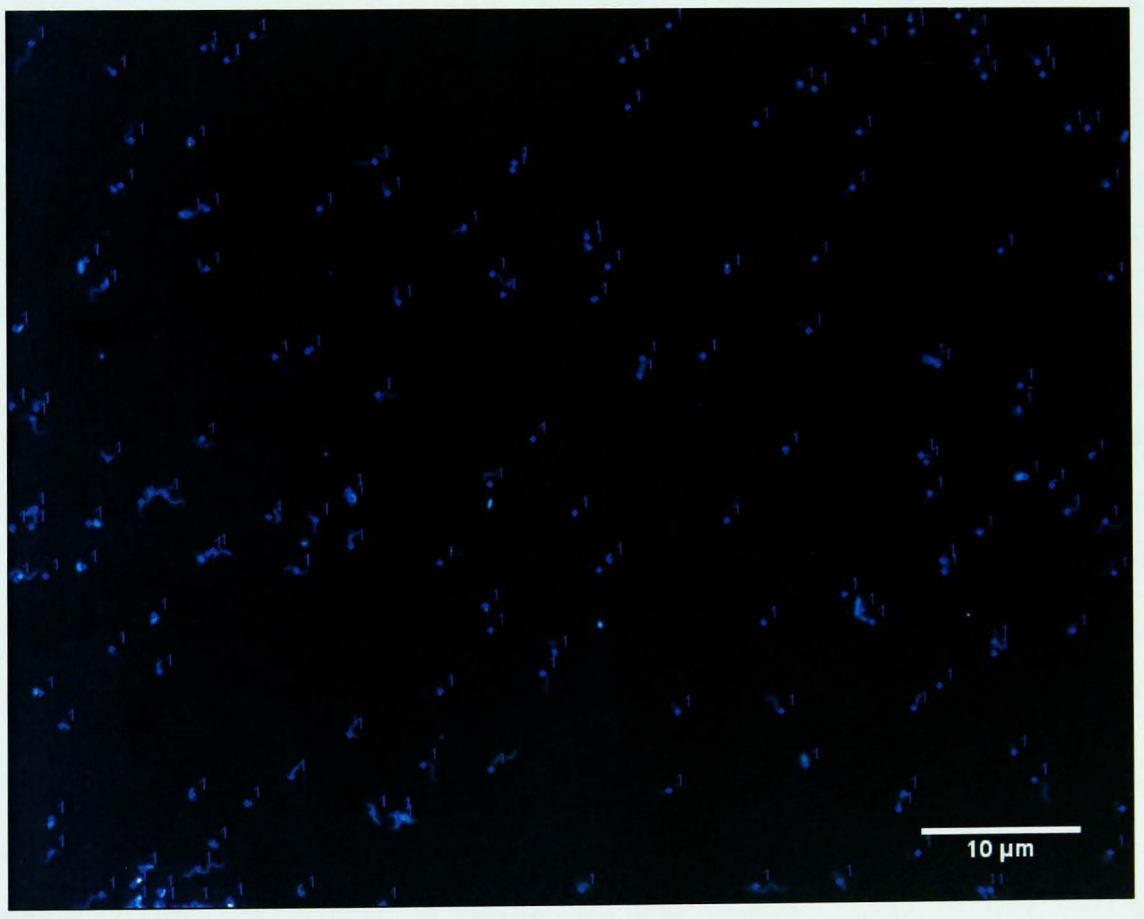



**Figure 2-6** Field of view with marked and counted microbial cells (labelled •[1]), performed using ImageJ.

## 2.3 Results and Discussion

### 2.3.1 Groundwater Hydrochemistry

#### 2.3.1.1 The Spatial Distribution of Organic Contaminants

Groundwater was purged and analysed for a range of organic contaminants as described in Sections 2.2.1 and 2.2.3. The analytical error of the HPLC used to quantify the phenolics in the groundwater was assessed using a certified analytical quality control (Section 2.2.3). Data show that the maximum relative percentage difference between the expected concentration of the control and the actual measured concentration found from all runs was 1.44%. This error is therefore too small to plot on the graphs. Furthermore, the sampling error of 3 standard deviations (99.7% of the data) is also too small to be visible on the graph, showing that sampling was highly reproducible.

Borehole 59

The analysis of groundwater purged from BH59 (Figure 2-7) reveal that TPC (total phenolics concentration) was undetectable down to 10mbgl, where it was found to be 3.2mg $L^{-1}$. Below this, over a 2-3m interval, there was a sharp increase in TPC to 399-648mg $L^{-1}$ at 12mbgl.TPC then gradually increased from 12mbgl down to 24mbgl, where the concentration reached 1200mg $L^{-1}$ in July 2009. TPC sharply increases below this depth to its peak at the plume core (25-28mbgl), where the maximum concentration was 4945 mg $L^{-1}$ at 26mbgl in February 2008. Below the plume core, the TPC sharply decreases at 29mbgl (1633mg $L^{-1}$) and further at 30mbgl where the lower fringe of the contaminant plume is located. At this depth, the TPC ranged between 419mg $L^{-1}$ in February 2008 and 120mg $L^{-1}$ in July 2009.

The concentrations of phenolic compounds measured at Four Ashes are much higher than at other sites in the UK where similar coal tar distillation processes were performed. Lerner *et al.* (1997) found that the Permo-Triassic sandstone aquifer beneath a former Rexco plant in Mansfield, East Midlands was contaminated with only tens of mg $L^{-1}$ of phenolic compounds. Broholm and Arvin (2000) found that the phenolics at this site had either been successfully attenuated or had moved out of the source area to the north east through advection, as they were only detecable in µg $L^{-1}$ at the furthest sampling point from the source. The Rexco plant in the Mansfield ceased to be operational at the same time as Midland Tar Distillers at

Four Ashes. This comparison highlights the extremely high level of contamination that was released from Four Ashes.

Of the phenolic compounds measured at Four Ashes, phenol was the most prominent single contaminant, contributing up to 43% of the total contaminant load. Xylenol isomers contributed up to 32% and *m/p*- and *o*-cresol contributed up to 45% of the total contaminant load. The concentration of phenol in the groundwater (Figure 2-7) was stable until August 2009, when it increased markedly at 12, 14, 21 and 26mbgl. At 30mbgl, the phenol concentration decreased from 177mg $L^{-1}$ in February 2008 to 46mg $L^{-1}$ in August 2009. A similar trend was found for the cresols (Figure 2-7). The concentration of xylenols (Figure 2-7) was inconsistent, with higher concentrations found in February 2008 and August 2009 than were found in January 2009.

In comparison to data from previous studies (Thornton *et al.,* 2001), plume evolution at borehole 59 was evident. The gradient between uncontaminated groundwater and the upper plume fringe was found to be steeper in 2009 than in 1998 (Figure 2-7) due to an increase in TPC at 11-14mbgl. This was also observed in 2005 and 2006 by Rizoulis (2008). A slight decrease in TPC was found between 1998 and July 2009 at 17-19mbgl. At 21-24mbgl, the TPC remained stable throughout samplings until July 2009. The core of the plume (25-28mbgl) also remained stable until July 2009, although the peak concentration in 1998 (5823mg $L^{-1}$) was significantly higher than in 2008 (4935mg $L^{-1}$) and July 2009 (4692mg$L^{-1}$). The TPC at the lower plume fringe (30mbgl) increased from 594mg $L^{-1}$ in 1998 to 1221mg$L^{-1}$ in 2006 (Rizoulis, 2008), before decreasing between 2006 and July 2009 to 120mg $L^{-1}$, suggesting variations in contaminant spillage.

The above trend was found for all phenolic compounds and all samplings between February 2008 and July 2009. However, in August 2009, TPC was found to increase more sharply between the plume fringe at 12mbgl and the plume core at 26mbgl than in previous samplings. Also, the TPC concentration at 26mbgl in August 2009 was lower than found in previous samplings (3576 mg $L^{-1}$). The distribution of organic contaminants in the groundwater in August 2009 differed from all previous samplings (Figure 2-7). TPC on the upper edge of the contaminant plume increased more sharply between 12 and 26mbgl than at previous samplings. The peak

concentration in August 2009 was 3580mg $L^{-1}$, which was lower than at all previous samplings. The change in contaminant distribution suggests that a greater proportion of the contaminant load is located in the upper part of the plume than previously. This could be attributed to variations in contaminant spillage later in plant operation. However, the major change in hydrochemistry occurred within 1 month. This suggests that the onset of the pump and treat system implemented at the start of August 2009 by the site owners, SI Group, is the reason for the marked change. The system operates from borehole 60, withdrawing water downstream and pumping it into an on-site treatment works. Unfortunately, due to the commercially sensitive nature of this operation, no information or data (pumping methods and rates) was available to generate a model to make predictions of future changes to the distribution of the contaminant matrix. The pump and treat system does show, however, that groundwater contaminated later in plant operation was drawn towards borehole 59 from the point source of contamination due to the pumping regime. Fundamentally, the pumping increased the flow of groundwater downstream to borehole 59. This is because the groundwater will have been preferentially drawn through areas of highest hydraulic conductivity (*K* - the ease at which water can pass through a system) in the aquifer. As this aquifer is anisotropic, i.e. has a higher *K* parallel to the dip of the sandstone strata than vertically down the succession, the groundwater will be drawn downstream parallel to the dip of the sandstone beds and groundwater flow. The pumping will have therefore drawn groundwater that was further upstream of borehole 59, downstream. However, if the withdrawal of water by the pump is at one depth only and at a rate greater than groundwater flow, it is possible that some groundwater will have shifted vertically.

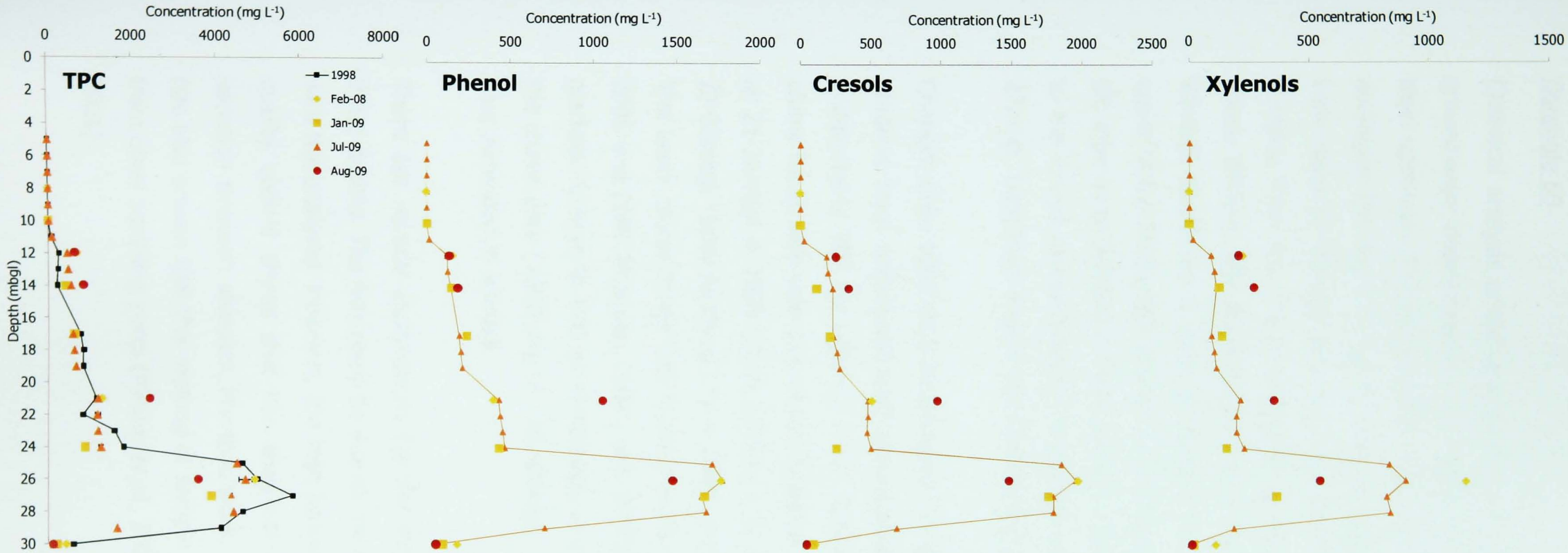



**Figure 2-7** Concentrations of phenolic compounds measured in the Four Ashes groundwater purged from borehole 59 in 2008 and 2009. Figure legend is applicable to all graphs. 2001 The data representing 1998 are from Spence *et al.* (2001).

Borehole 60

Chemical analysis of the groundwater from borehole 60 (Figure 2-8) reveals that groundwater downstream of the contaminant source is more grossly contaminated than upstream. The groundwater TPC measurements at 43-45mbgl in March 2007 averaged 7495mg $L^{-1}$ (Figure 2-8). In August 2007, when measurements of TPC were taken at 45mbgl only, the TPC had increased to 10,099mg $L^{-1}$. In July 2009, samples from the full groundwater profile were analysed. Results showed that above 20mbgl, the groundwater was uncontaminated, but over a 1m interval to 21mbgl, there was a steep increase from undetectable TPC to 1100mg $L^{-1}$. The upper plume fringe was located at 21-27mbgl, below which a sharp increase over a 3m interval to 5480mg $L^{-1}$ was present. The plume core was located below 30mbgl to the lowest sampling depth of 45mbgl, where the peak TPC was 7000mg $L^{-1}$ at 27mbgl. Data from August 2009 followed the trend found in July 2009.

Changes since 1998 were also evident. On the upper plume fringe (21-27mbgl), the gradient from uncontaminated groundwater was steeper in 2009, jumping from undetectable TPC to 1221mg $L^{-1}$ at 21mbgl. Also, the increase from the upper plume fringe was seen to be more 'stepped' than in 1998, with TPC being 2x higher at 21-26mbgl in 1998 (2116-2178mg $L^{-1}$). A steeper gradient was also present at 27-30mbgl (1600-5615mg $L^{-1}$) in 2009, below which the plume core was located. The lower plume fringe was located at 42-45mbgl in 1998. However, results from 2005 and 2006 (Rizoulis, 2008), and 2007/8/9 (this study) show that there was a marked increase in TPC at 42-45mbgl, to concentrations similar to those found in the plume core (>5500mg $L^{-1}$, 30-40mbgl). The plume core was therefore found to have extended to 45mbgl.

There are several explanations for the increase in contaminant concentration at these depths. The first could be due to the sampling and analytical error associated with the analyses. However, the high accuracy and reproducibility of the analytical quality control shows that the analytical error is not able to account for any variation between samples. Previous work, using this multi-level borehole system, has also proven that this method of sample purging does not suffer from interence from other sampling ports (Pickup *et al.*, 2001; Spence *et al.*, 2001; Thornton *et al.*, 2001)

Secondly, variations could be explained by the physical hydrogeology of the site. Vertical hydrodynamic dispersion could have a role in contaminant transport processes at borehole 60 due to rock fracture or high vertical hydraulic conductivity, resulting in preferential vertical flow. However, the hydrogeology of the Four Ashes aquifer is not complex. Hydraulic tests and core analysis by Aspinwall & Co. (1992) found no significant changes in the aquifer hydrogeology down the groundwater profile. Therefore, the Four Ashes aquifer contains neither major fracture networks nor secondary/tertiary porosity, has homogenous hydraulic conductivity throughout and has no major changes in lithology down the succesion. This suggests that the changes are not related to vertical heterogeneities in aquifer geology and therefore differential transport (Thornton *et al.,* 2001). This makes the site suitable for the study of microbial ecology and to assess the source term history, as geological factors do not play an important role in contaminant fate and transport at the site.

This contrasts aquifers that contain, for example, discrete low permeability mud lenses that can result in hydraulic anisotropy (variation down the sucession). This would retard groundwater (and contaminant) flow through the aquifer by forcing the groundwater to take a route around the low permeability region. Such diversions in flow can create flow channels causing a contaminant plume to split creating discrete loci within an aquifer that are more or less contaminated than other regions. An example includes heterogeneities and uncertainties in the Wolston Formation of sandstones and clays at Villa Farm, UK. This site is a former unlined landfill that formed a chloride and phenolics leachate plume in the aquifer beneath the site. Hydrogeological uncertainties led to inaccurate modelling of the migration of the plume through the groundwater system (Mackay *et al.,* 2000). Also, hydrochemical measurements led to conclusions that half of the contamination had been attenuated, as the mass flux of contamination through the aquifer was half of the expected concentration. However, subsequent on-site drilling at Villa Farm revealed that the contaminant plume split in two to flow around the low permeability clay layer, and that the intial hydrochemical measurements missed the deeper plume (Bottrell, 2010, pers. Comm.).

An understanding of these phenomena is especially important prior to the design, construction and implementation of sampling boreholes and *in situ* remediation methods, as the contaminated groundwater may diverge from the expected flow

path. The transport of contaminants in karst (carbonate) aquifers is also very different to that in isotropic sandstone units. The presence of voids can result in distinctly higher and more turbulent flow than in other regions of a system, resulting in complex flow regimes and variations in contaminant transport. An example of a karst system is the chalk aquifer of St. Albans, UK, that contains a contaminant plume derived from an unleaded petroleum spill. Tracer tests at the site using sodium fluoresceine indicated that the aquifer is strongly anistropic due to the development of fissures transverse to the natural hydraulic gradient (Bottrell *et al.*, 2010). Also, hydrochemical data at the site revealed that nitrate and sulphate concentrations within a zone of biodegradation were constantly replenished in the main groundwater system by diffusive equilibration between the fissure network and the pore waters in the chalk matrix (Spence *et al.*, 2005). This study again highlights the simplicity of the Four Ashes hydrogeology.

A lack of vertical hydrodynamic dispersion was also reflected by the 'stepped' increase in contamination found at shallower depths at borehole 60, showing that discrete zones of varying contamination are not affected by vertical dispersion (Dominico and Schwartz, 1998). However, although the horizontal hydraulic conductivity of the aquifer was found to be similar down the groundwater profile, variation in the connection of the void space, or the pore throat, could have an impact on local groundwater flow, resulting in the preferential flow in areas of high pore throat connectivity, causing the variation in concentration with depth.

It could be that the plume was gravity driven due to the high contaminant load (Thornton *et al.,* 2001). Calculations suggested that this was a possibility in areas of intense concentration, but as $K$ is greater laterally than vertically, high vertical dispersion is unlikely, causing the contaminant load to be driven downstream with groundwater flow, parallel to the substrata. Finally, as the contaminant concentrations are still high at 30-40mbgl, this is not consistent with the idea that the contaminant mass is sinking.

A third possibility is the changes in the input source during plume formation in the 1950s. A highly contaminated input source, behind the input corresponding to the plume core seen in 1998 (30-40mgl), would produce a contaminated zone deeper than 40mbgl. This suggests another area of spillage on site, resulting in separate

TPC plumes, as suggested by Thornton *et al.* (2001). As sampling levels do not go deeper than 30mbgl (borehole 59) and 45mbgl (borehole 60), it was not possible to investigate the extent of this source of contamination or to assess whether the plume extends any deeper (Thornton *et al.,* 2001; Williams *et al.,* 2001).

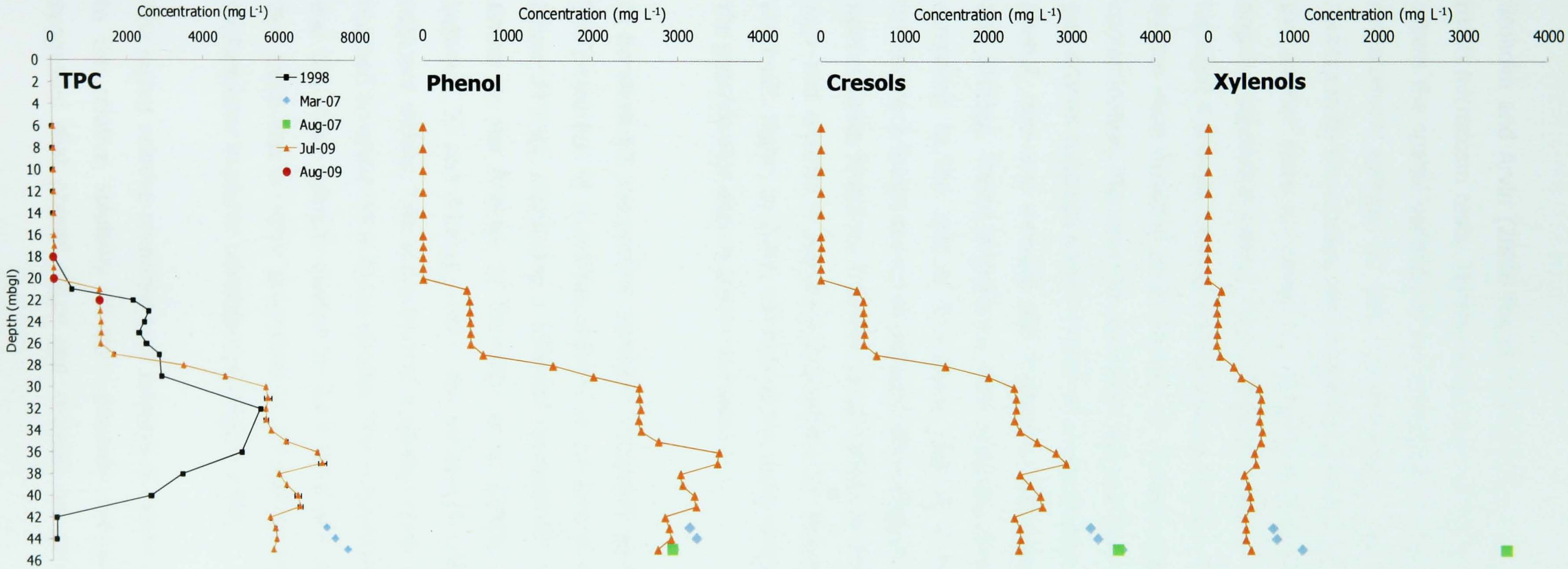



**Figure 2-8** Concentrations of phenolic compounds measured in the Four Ashes groundwater purged from borehole 60 in 2007 and 2008. Figure legend is applicable to all graphs. The data representing 1998 are from Spence *et al.* (2001).

Broholm and Arvin (2000) found 2,6-xylenol to be recalcitrant to microbes during batch microcosm tests. Therefore, 2,6-xylenol can be used as an internal tracer to assess the spatial variation in the proportions of each contaminant and relate it to contaminant spillage on site. To investigate the number of contaminant input sources in to the aquifer, the proportions of each major organic contaminant within the aquifer were compared to 2,6-xylenol (Thornton *et al.*, 2001). Profiles of organic solute/2,6-xylenol ratios (Figure 2-9a) at borehole 59 from July 2009 highlight 4 distinct zones of phenolic contamination. Different proportions of organic solutes were detected at 11-14 and 17-25mbgl, suggesting 2 separate sources of contamination. Moreover, on the lower plume fringe at 30mbgl, another variation in proportions suggests a third source of contamination. Finally, similar proportions of phenol, *m,p*- and *o*-cresol with respect to 2,6-xylenol were detected between 25 and 27mbgl. These proportions were relatively lower with respect to 2,6-xylenol compared to the rest of the plume due to a higher proportion of xylenols, confirming a fourth source of contamination (Figure 2-9b). The organic solute/2,6-xylenol ratios found by Thornton *et al.* (2001) in 1998 are comparable for phenol, *m,p*- and *o*-cresol in July 2009 at borehole 59. However, the ratios for the xylenols are much higher in 2009, confirming the more concentrated input of xylenols into the groundwater later in plume formation.

At borehole 60, the profiles highlight 3 separate sources of organic contamination. The proportion of *m,p*-cresol relative to 2,6-xylenol is higher down to 30mbgl than below 35mbgl, suggesting 2 different contaminant mixtures (Figure 2-9c). This is similar to the findings of Thornton *et al.* (2001). The third source of input is between 30 and 35mbgl, where the solute/2,6-xylenol ratios are lower than the adjacent depths. This suggests that a spillage more concentrated with 2,6-xylenols reached borehole 60 at these depths. The proportions of phenol, *m,p*- and *o*-cresol and the other xylenols relative to 2,6-xylenol are lower than in 1998, confirming that there was a higher proportion of 2,6-xylenol released within the contaminant mixture later in plume development (Figure 2-9d).

The higher relative proportions of xylenols at depth in both boreholes could be due to the relative solubility of each phenolic compound. Thornton *et al.* (2001) suggested that phenol, *m,p*- and *o*-cresol are preferentially dissolved over the

xylenols, which are dense non-aqueous phase liquids (DNAPL). DNAPLs are substances that do not mix with water, but move though pore spaces as free-product. DNAPLs form residual phases that take long periods to dissolve, forming a contaminant source that migrates deeper into the saturated zone due to a specific gravity higher than water, resulting in deep concentrated plumes (Domenico and Schwarz, 1998). This could explain why a relatively high proportion of xylenols is located deeper in the aquifer. Also, during vertical migration of DNAPL, lateral groundwater movement can result in the formation of discrete lenses distributed down the groundwater profile. However, this would only be visible on a small scale, and would not be detected at the sampling resolution used within this study, or account for the marked increase in concentration at depth in borehole 60.

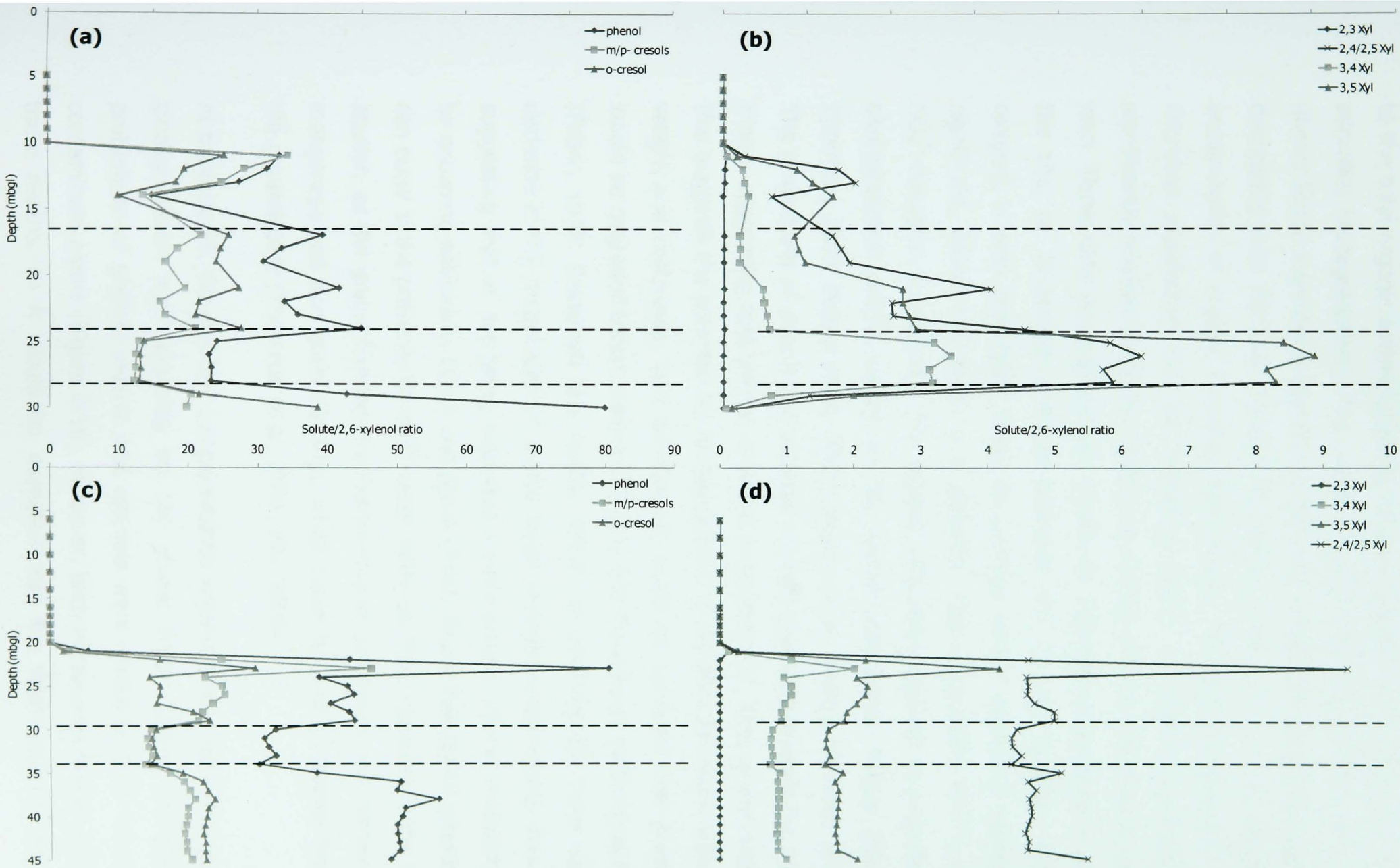



**Figure 2-9** Profiles of organic solute/2,6-xylenol ratios within groundwater at borehole 59 (a and b) and 60 (c and d) in July 2009. Dashed lines mark different contaminant mixtures.

The data from the phenolics analyses can also be used to assess whether microbial respiration may be operating is by comparing the ratios of each major contaminant to the total organic matrix (phenols, cresols, xylenols and organic intermediates of microbial biodegradation). The profile (Figure 2-10) for borehole 59 shows that phenol forms a smaller proportion of the total organic matrix on the plume fringes, decreasing from 38% at 24mbgl to 19% at 11mbgl, due to an increase in the accumulation of organic microbial metabolites. This suggests that the indigenous microbial populations may be degrading phenol to organic intermediates more significantly towards shallower depths, coinciding with the decrease in contaminant load. These data also suggest that anaerobic microbial degradation is occurring at the site, as 11-24mbgl contains abundant Mn, Fe and sulphate. Nitrate (and oxygen) is only detectable down to 12mbgl, where transverse mixing is more significant. Below this depth, it is expected that concomitant $Mn^{(IV)}$-, $Fe^{(III)}$- and $SO_4^{2-}$-reduction is occurring. This agrees with other research sources that suggest biodegradation occurs mainly on the contaminant plume fringes (Banwart and Thornton, 2003; Pickup *et al.,* 2001; Spence *et al.,* 2001a; Thornton *et al.,* 2001). The proportions of cresols and xylenols increase gradually towards the upper plume fringe, suggesting that phenol is being degraded first. This agrees with research that suggests the potential for biodegradation of organics decreases with molecular weight and complexity, and therefore it would be expected that phenol (94.11) would be degraded before cresols (108.14) and the xylenols (122.16) at Four Ashes (Faber, 1979; Smolenski and Sulfita, 1987). At borehole 60, there was a sharp decrease in the proportions of all the major phenolic contaminants above 21mbgl, suggesting that all are being degraded simultaneously. Phenol oxidation may also be occurring abiotically. Ulrich and Stone (1989) found that abiotic phenol oxidation can occur in the presence of $Mn^{(IV)}$-oxide particles. This is possible in the Four Ashes aquifer, as the grains forming the Permo-Triassic sandstone unit contain abundant manganese and iron grain coatings, which could be used to oxidise the phenol in the groundwater (Thornton *et al.,* 2001; Wu, 2002).

At borehole 60, the phenolic compound/total organic matrix ratios also suggest that biodegradation was occurring on the plume fringe (above 20mbgl), as the proportions of phenol, cresols and xylenols were markedly lower than inside the contaminant plume (Figure 2-10). However, both nitrate and oxygen are present at these depths, so it would be expected that the thermodynamically-favourable

aerobic degradation would be occurring, not anaerobic processes. The proportions of phenolic compounds within the plume (below 20mbgl) were high at all depths, suggesting that the high contaminant load is suppressing any microbial degradation.

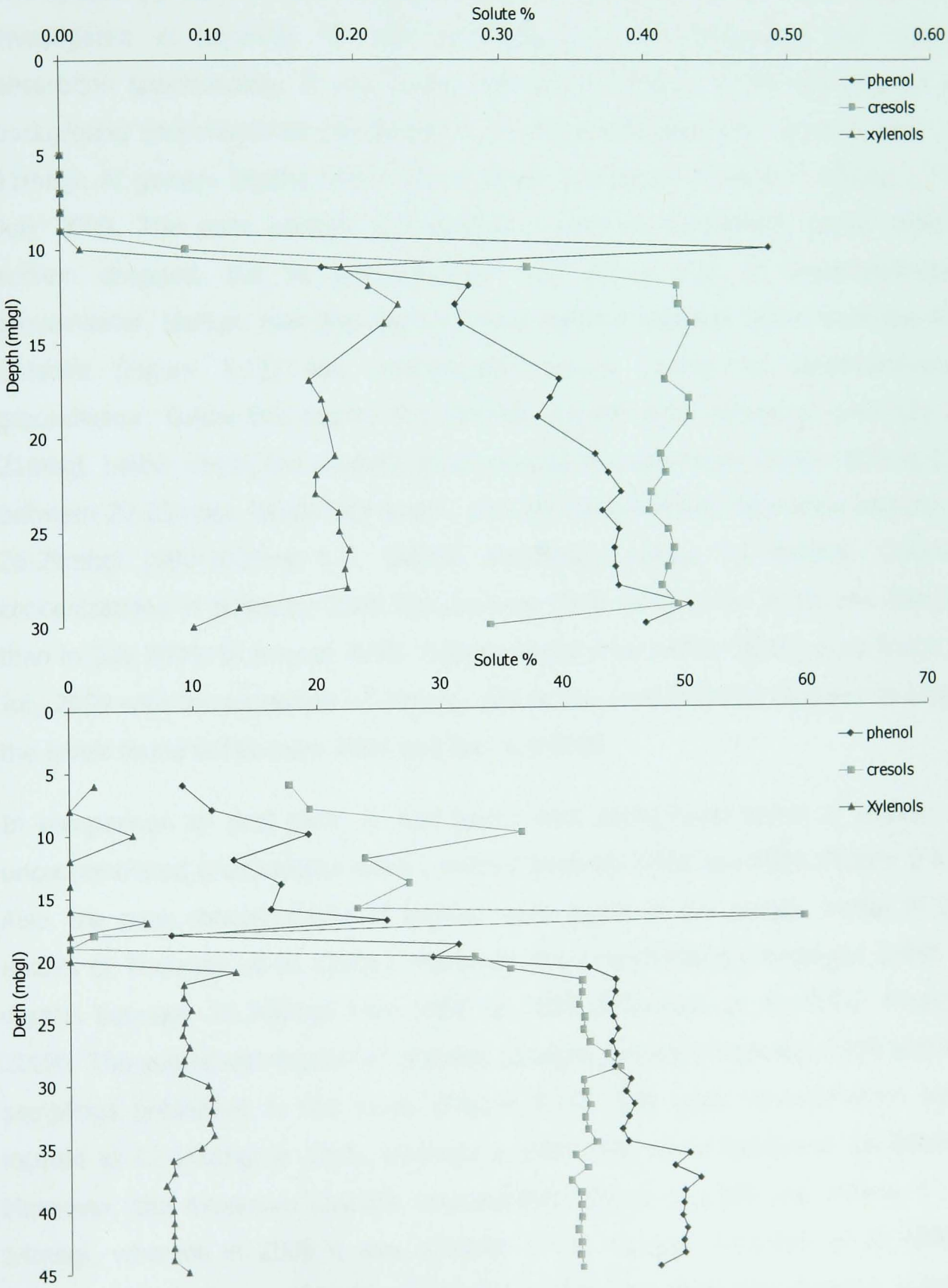



**Figure 2-10** Profiles of percentages that each major phenolic contaminant forms of the total organic matrix within the groundwater at borehole 59 (top) and borehole 60 in July 2009.

### 2.3.1.2 The Spatial Distribution of Inorganic Contaminants

Borehole 59

The spatial variation of the inorganic contaminants in the groundwater was also investigated at borehole 59 and 60 using ion chromatography and atomic absorption spectroscopy. It was found that sodium (Figure 2-11) was present at background concentrations (20-32mg $L^{-1}$) in uncontaminated groundwater down to 11mbgl. At greater depths, there was a sharp increase at 12mbgl to 630mg $L^{-1}$ in July 2009. The peak sodium concentrations were at 13-23mbgl, below which, sodium dropped, but to concentrations still above that of uncontaminated groundwater. Sodium was also the dominant cation measured within borehole 59. Chloride (Figure 2-11) was undetectable above 11mbgl in uncontaminated groundwater. Below this depth, the chloride concentration increased gradually to 21mbgl, below which, the concentration jumped to peak levels (1350-1550mg $L^{-1}$) between 22-25mbgl. Below this depth, chloride concentration decreased sharply at 26-29mbgl (890-1030mg $L^{-1}$), before decreasing further to 30mbgl. Chloride concentrations in February 2008 and January 2009 were lower at 26 and 30mbgl than in July 2009. In August 2009, chloride levels were similar to the ones found in July 2009 with the exception of 30mbgl, where the concentration dropped to below the levels found in February 2008 and January 2009.

In comparison to past data, it was found that background levels of sodium in uncontaminated groundwater were constant between 1998 and 2009 (Figure 2-11). Also, the peak concentrations of sodium were found at 12-15mbgl, similar to the results of Thornton *et al.* (2001). However, the concentrations increased 2-fold at depths between 13-30mbgl from 1998 to 2009 (Thornton *et al.*, 2001; Rizoulis, 2008). The spatial distribution of chloride changed markedly between 1998 and the samplings presented in this study (Figure 2-11). The peak concentrations were located at 12-14mbgl in 1998, whereas in 2009 they were located at 22-25mbgl. Moreover, the maximum chloride concentration found in 1998 was 290mg $L^{-1}$ at 14mbgl, whereas in 2009 it was 1730mg $L^{-1}$ at 21mbgl. Thornton *et al.* (2001) suggested that plumes of NaCl and NaOH, caused due to on-site de-icing, overlap the organic contaminant plume at Four Ashes. The sodium and chloride data confirms this, as a more intense release later in plume development resulted in

more concentrated sodium and chloride profiles at borehole 59 in 2009. A separate source area input of chloride is suggested from the data, due to a change of location of the maximum concentrations found in borehole 59.

Borehole 60

At borehole 60, the spatial distribution of sodium down the groundwater profile in 2007, 2008 and 2009 was similar to 1998 (Figure 2-11), with the peak concentrations located at 21-27mbgl. However, the peak concentration of sodium in the plume (356mg $L^{-1}$) at 22mbgl in 2009 was 5 times higher than the peak concentration in 1998 (70mg $L^{-1}$), suggesting more concentrated releases of sodium later in plume development (Thornton *et al.,* 2001; Williams *et al.,* 2001). The spatial distribution of chloride in 2009 followed a similar trend to 1998 at borehole 60. The peak concentration was found to be 225mg $L^{-1}$ at 33mbgl in 1998 by Thornton *et al.* (2001), and the peak found in 2009 was 275mg $L^{-1}$ at 36mbgl. As chloride concentrations at shallow depths within borehole 60 have not changed markedly since 1998, this suggests a more consistent influx of chloride towards borehole 60 at these depths over the 11 years between samplings. However, as sodium levels have increased, without a marked increase in chloride, this suggests a second source of sodium spillage. Thornton *et al.* (2001) reported that the NaOH plume discovered at borehole 59 was localised around the distillation plant and no evidence suggested that it extended to borehole 60. However, the increase in sodium concentration at shallow depths in borehole 60, without an increase in chloride since 1998, suggests that the NaOH plume has now migrated downstream. Chloride concentration has increased at 35-45mbgl between 1998 and 2009. This highlights that a more concentrated influx of chloride at lower depths, which can be attributed to a separate input source of NaCl. Tracing the concentration contours of the chloride concentrations at depth highlights that the chloride levels seen at the bottom of borehole 60 reflect the increase seen at 17-30mbgl in borehole 59. This shows that the increase at both boreholes is probably the result of the same input source of NaCl, which occurred in pulses resulting in different concentrations in 1998 and 2009.

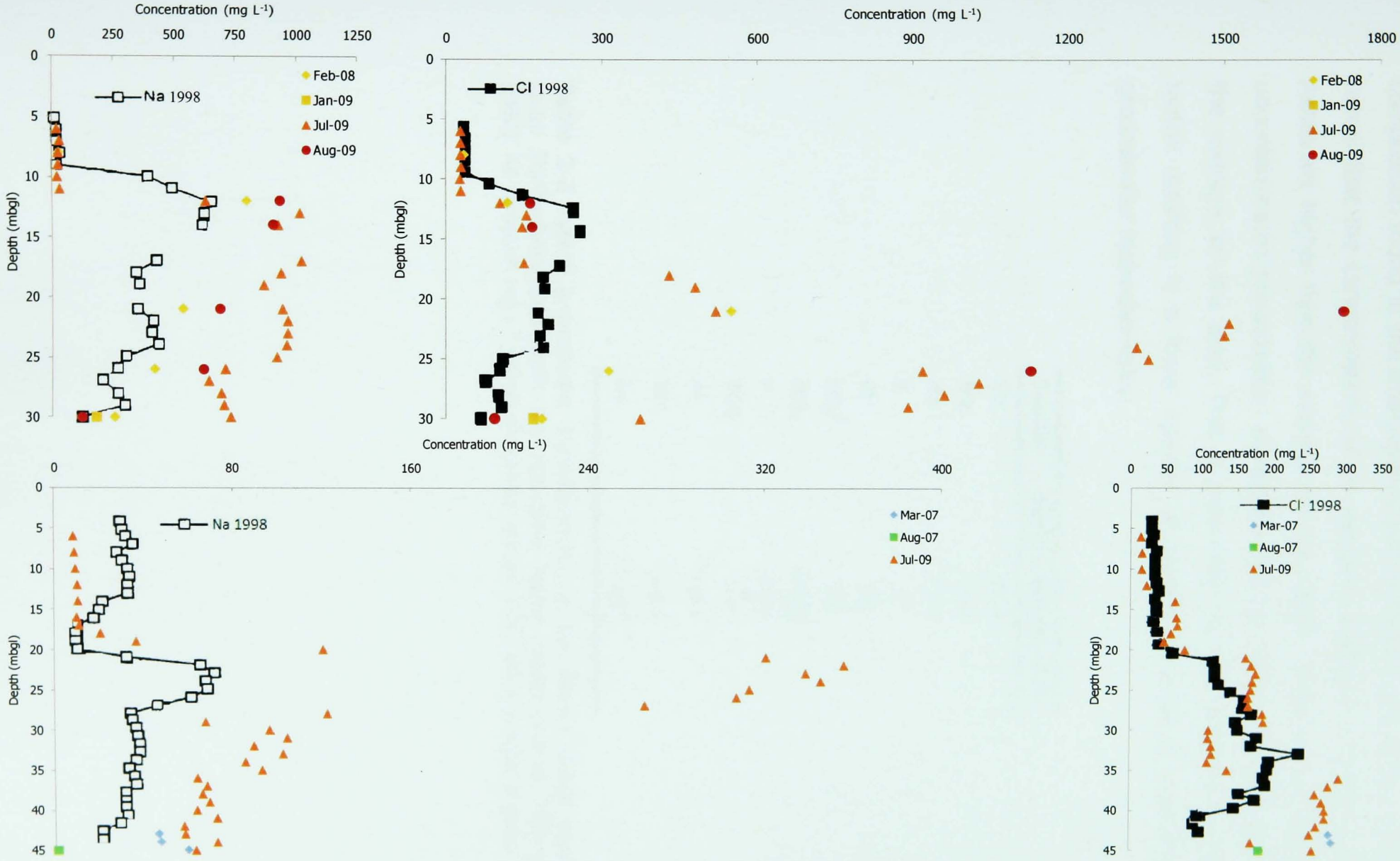



**Figure 2-11** Comparison of groundwater sodium and chloride profiles in 1998, 2007, 2008 and 2009 at borehole 59 (top) and borehole 60 (modified from Thornton *et al.,* 2001)

The data from Four Ashes can also be compared to the average groundwater composition found in the Staffordshire Permo-Triassic sandstone (Table 2-3). Data reveal that the concentrations of all dissolves species in the plume core at Four Ashes are higher than the average for the region. However, outside the plume in uncontaminated groundwater, all species (except nitrate and calcium) were below the average for the area. This is potentially due to variations in the sandstone matrix resulting in a lower number of soluble minerals in equilibrium with the groundwater hydrochemistry.

| Species | Mean Concentration |
|---|---|
| **Ca** | 83.8 |
| **Mg** | 13.3 |
| **Na** | 21.5 |
| **K** | 6 |
| **Cl** | 35 |
| **$SO_4^{2-}$** | 79 |
| **$NO_2^-$** | 0.013 |
| **P** | 0.11 |
| **TOC** | 1.4 |
| **Al** | 5 μg $L^{-1}$ |
| **Mn** | 5 μg $L^{-1}$ |
| **Fe** | 10 μg $L^{-1}$ |

**Table 2-3** Typical groundwater hydrochemistry in the Permo-Triassic sandstone of the south Staffordshire and north Worcestershire region (adapted from Tyler-Whittle *et al.,* 2002). All values in mg $L^{-1}$ unless otherwise stated. TOC refers to total organic carbon.

### 2.3.1.3 Terminal Electron Acceptors and Redox Zones

Nitrate, sulphate, total iron and manganese levels were measured down the groundwater profiles at boreholes 59 and 60 to assess the distribution of anaerobic microbial redox zones. The anaerobic respiration of these TEAs coupled to the oxidation of phenolic compounds has been documented by many researchers (Broholm and Arvin, 2000; Lovely *et al.,* 1989; Nielsen *et al.,* 1995: Smolenkski and Suflita, 1987). Nitrate-reduction is the most thermodynamically-favoured, followed by Mn(IV), Fe(III) and sulphate-reduction (Table 2-4).

**Table 2-4** Gibbs free-energy (ΔG) for the oxidation of phenol by anaerobic terminal electron accepting processes (modified from Thornton *et al.,* 2001).

| Reaction Stoichiometry | TEAP | ΔG (kcal/ equiv) |
|---|---|---|
| $5C_6H_6O + 28NO_3 + 28H^+ \rightarrow 6CO_2 + 3H_20$ | Nitrate-reduction | -26.18 |
| $C_6H_6O_6 + 14MnO_2 + 28H^+ \rightarrow 14Mn^{(II)} + 6CO_2 + 17H_2O$ | $Mn^{(IV)}$-reduction | -25.84 |
| $C_6H_6O_6 + 28FeOOH + 5H^+ \rightarrow 28Fe^{(II)} + 6CO_2 + 45H_2O$ | $Fe^{(III)}$-reduction | -12.93 |
| $2C_6H_6O_6 + 7S0_4 \rightarrow 7S^{(-II)} + 12CO_2 + 6H_2O$ | Sulphate-reduction | -1.03 |

At borehole 59, background concentrations of nitrate (Figure 2-12) in uncontaminated groundwater were found down to 11mbgl, where the concentration was 66mg $L^{-1}$ in July 2009. There was a sharp decrease in nitrate concentration below this depth to 13mbgl where it was undetectable, and remained undetectable until 30mbgl, where it was 40mg $L^{-1}$ in February 2008 and 24mg $L^{-1}$ in January 2009. However, in July and August 2009, nitrate concentration fell to 0.7 and 0.3mg $L^{-1}$, respectively.

It was found that the spatial distribution of nitrate within the groundwater profiles at both boreholes was the inverse of TPC profiles. Background levels of nitrate were found at depths shallower than the TPC plume, and undetectable levels were present inside the TPC plume, suggesting that microbial nitrate-reduction was confined to the plume fringes and uncontaminated groundwater. Williams *et al.* (2001) stipulated that the background levels of nitrate can be attributed to diffuse flow in to the groundwater of nitrate-based fertilisers used on the agricultural fields

that overlie the aquifer in the recharge area. The trend in nitrate concentration with depth was also observed by Thornton *et al.* (2001) and Rizoulis (2008), suggesting the complete depletion of nitrate within the contaminant plume. This also suggests that the more thermodynamically-favourable oxygen was depleted prior to nitrate and therefore a change from aerobic to anaerobic microbial processes occurred, followed by the exhaustion of nitrate as a microbial TEA. Transverse mixing and vertical dispersion of uncontaminated and contaminated groundwater only occurs on the plume fringes, and is therefore not significant enough to increase nitrate levels inside the TPC plume

Sulphate concentrations at borehole 59 (Figure 2-12) in uncontaminated groundwater (down to 10mbgl) were between 25mg $L^{-1}$ and 39mg $L^{-1}$. Below this depth, there was a sharp increase in concentration at 12-17mbgl, where the highest sulphate concentration was found to be 186mg $L^{-1}$ at 12mbgl in February 2008. Sulphate levels then sharply decreased at 18mbgl to 72mg $L^{-1}$ in July 2009. Sulphate levels were similar from 18mbgl to 25mbgl, below which the concentration increased sharply to 97mg $L^{-1}$ until 30mbgl, where the concentration decreased to levels similar to 18-26mbgl. However in January 2009 and August 2009, sulphate levels at 30mbgl were similar to the concentrations found in uncontaminated water above 11mbgl.

This study and Thornton *et al.* (2001) found that sulphate levels were higher than background inside the phenolics plume (Figure 2-7). In 1998, background sulphate levels at borehole 59 (Figure 2-12) were found to be 64-70mg $L^{-1}$ (Spence *et al.,* 2001a), but this decreased to 39mg $L^{-1}$ in February 2008, and further to 25-30mg $L^{-1}$ in July 2009. Sulphate levels at the upper plume fringe (12-14mbgl) were 17-22mg $L^{-1}$ in 1998 (Spence *et al.,* 2001a), but this increased to over 109mg $L^{-1}$ in July 2009, suggesting a discrete zone of increased sulphate concentration, generated by a different area of on-site spillage of mineral acids. Similar concentrations of sulphate were found at 18-25mbgl between 1998 and August 2009. However, much lower levels (2x) were found at 25-28mbgl in 2009. This could be the result of microbial sulphate-reduction. The TPC at these depths decreased between 1998 and 2009 (Figure 2-7), which may have resulted in the onset of bacterial sulphate reduction. However, the TPC was above 2000mg $L^{-1}$ at all samplings between 25-28mbgl at borehole 59. The recalcitrance of phenolic compounds to SRB occurs when the TPC is above 2,000mg $L^{-1}$. Below this, bacterial

sulphate-reduction is active (Spence *et al.*, 2001a; Shah, 2005). Therefore, it is unlikely that the decrease in sulphate levels between 25-28mbgl at borehole 59 is due to bacterial sulphate-reduction, and more likely that it is due to a change in the input of mineral acid spillage later in the plume development. Also, Thornton *et al.* (2001) found from mass balances that contaminant degradation is not significant enough to account for any differences in contaminant concentration. The lower fringe of the plume (30mbgl) had similar levels of sulphate in 1998, February 2008 and July 2009 (approximately 75mg $L^{-1}$). However, in January and August 2009, concentrations were lower (2x). The levels of sulphate and TPC throughout the plume suggest that conditions suitable for SRB activity are present only at the plume fringes where TPC is below 2000mg $L^{-1}$. This agrees with findings of Thornton *et al.* (2001), who found from dissolved $H_2$ concentrations that BSR is confined to the plume fringes, as the centre of the plume is dominated by methanogenesis due to the high contaminant load. The oxidation capacity of sulphate and the reducing capacity of phenol can be calculated to assess whether natural attenuation of phenol by SRB on the plume fringe would theoretically be successful. Using the half reaction for sulphate reduction, the oxidation capacity can be estimated:

$$SO_4^{2-} + 8e^- + 8H^+ \longrightarrow S^{2-} + 4H_2O$$

$$\underline{\text{Oxidation Capacity}} = (C_{\text{dissolved TEA}} \cdot \theta \cdot e^-_{\text{transferred}})/MW_{TEA}$$

Where C is the concentration (g $L^{-1}$), $\theta$ is the aquifer porosity, $e^-_{\text{transferred}}$ is the number of electrons transferred per mole in the redox half reaction, and $MW_{TEA}$ is the molecular weight of the TEA. Results of the calculation show that sulphate has the capacity to oxidise 17.16 moles $m^{-3}$ of phenol on the plume fringe. This was calculated using an average sulphate concentration of 128.8mg $L^{-1}$ (from 13-17mbgl), an aquifer porosity of 0.2, 8 transferred electrons and 96.06g $mol^{-1}$ as the MW. Calculating the reducing capacity of phenol using the same method (9.17 moles $m^{-3}$) showed that the oxidation capacity of sulphate is higher than the reducing capacity of phenol, and therefore natural attenuation using sulphate on the plume fringe should be successful. However, this is a theoretical approximation, so actual phenol consumption by SRB may differ.

Background levels of manganese (Figure 2-12) were found in uncontaminated groundwater down to 11mbgl, below which a sharp increase in concentration was present down to 14mbgl. Below this depth, another sharp increase in concentration was found, down to 19mbgl where the peak concentrations were 21mg $L^{-1}$ in July 2009. A gradual decrease in manganese then occurred down to 30mbgl. Concentrations in February 2008 were lower than other samplings. Manganese was present in much higher levels in borehole 59 in 2009 than in 1998, even though the spatial distributions were similar. The increase was significant by approximately 5x between 1998 and 2009, suggesting a more concentrated release from the source later in plume development and plant operation.

Iron levels (Figure 2-12) were variable throughout sampling. In July 2009, iron concentrations gradually increased from undetectable levels in uncontaminated groundwater to 2.2mg $L^{-1}$ at 28mbgl, before a sharp decrease at 29mbgl to undetectable levels. However, in February 2008, January 2009 and August 2009, concentrations higher than in uncontaminated groundwater were found at all depths sampled, with a peak of 25mg $L^{-1}$ in August 2009.

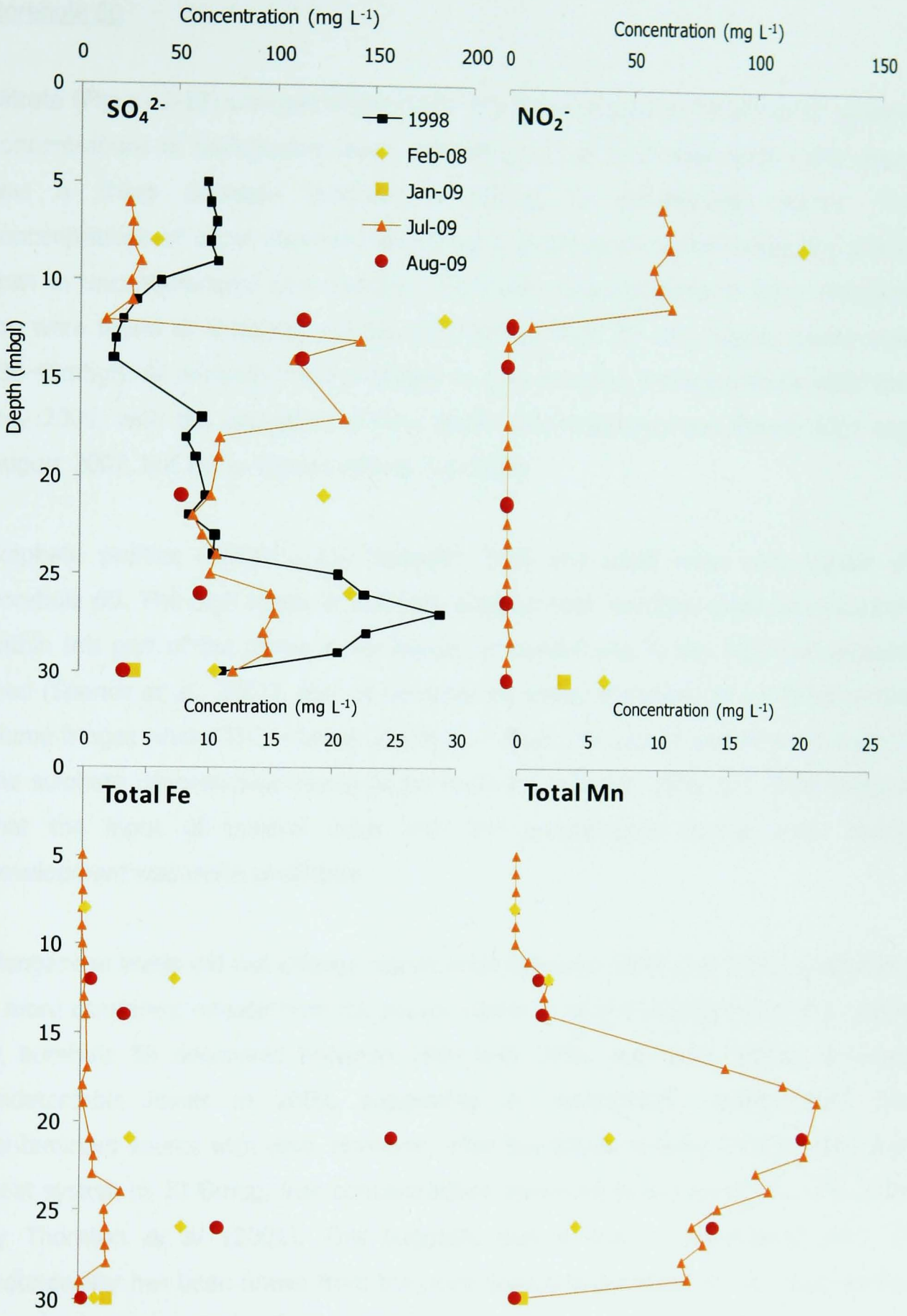



**Figure 2-12** Concentrations of the anaerobic TEA measured in the Four Ashes groundwater purged from borehole 59 in 2008 and 2009. Figure legend is applicable to all graphs. 1998 data from Spence *et al.* (2001).

Borehole 60

Nitrate (Figure 2-13) concentrations within the groundwater at borehole 60 showed concentrations at background levels (50-70mg $L^{-1}$) at 6-17mbgl. Below this, there was a sharp decrease between 17-19mbgl to undetectable levels. The concentrations of other dissolved ions (Figure 2-13) were higher inside the plume than in uncontaminated groundwater. The peak concentrations of each dissolved ion were found at shallower depths (20-29mbgl) than for the organic plume core (30-45mbgl). All anaerobic TEA increased in concentration between March 2007 and July 2009, with the exception of iron, which increased between March 2007 and August 2007, but fell to lower levels in July 2009.

Sulphate profiles (Figure 2-13) between 1998 and 2009 were very similar at borehole 60. The high levels of sulphate suggest that microbial sulphate-reduction within this part of the plume never began, or ceased due to the high contaminant load (Spence *et al.,* 2001). BSR at borehole 60 would therefore be confined to the plume fringes where TPC is below 2000mg $L^{-1}$. Also, the lack of significant change in the sulphate concentration found at borehole 60 between 1998 and 2009 suggest that the input of mineral acids into the groundwater during early plume development was more consistent.

Manganese levels did not change significantly between 1998 and 2009, suggesting a more consistent release from the source earlier in plume development. Iron levels at borehole 59 decreased between 1998 and 2008, and even further to near-undetectable levels in 2009, suggesting an inconsistent release from the contaminant source with time. However, after the implementation of the pump and treat system by SI Group, iron concentrations increased to the levels found in 1998 by Thornton *et al.* (2001). This suggests that a more contaminated zone of groundwater has been drawn from the point source to borehole 59. At borehole 60, iron levels decreased significantly at 21-30mbgl until July 2009, before increasing to levels similar to those found in 1998 by Thornton *et al.* (2001), once again suggesting an influence of the pump and treat system. No metal speciation analysis was performed for iron and manganese. It is therefore not possible to infer any metal microbial respiration trends. It is also not possible to confidently suggest that

conditions are suitable for microbial Mn and Fe-reduction to occur, as the proportions of $Mn^{IV}$ and $Fe^{(III)}$ in the groundwater are unknown.

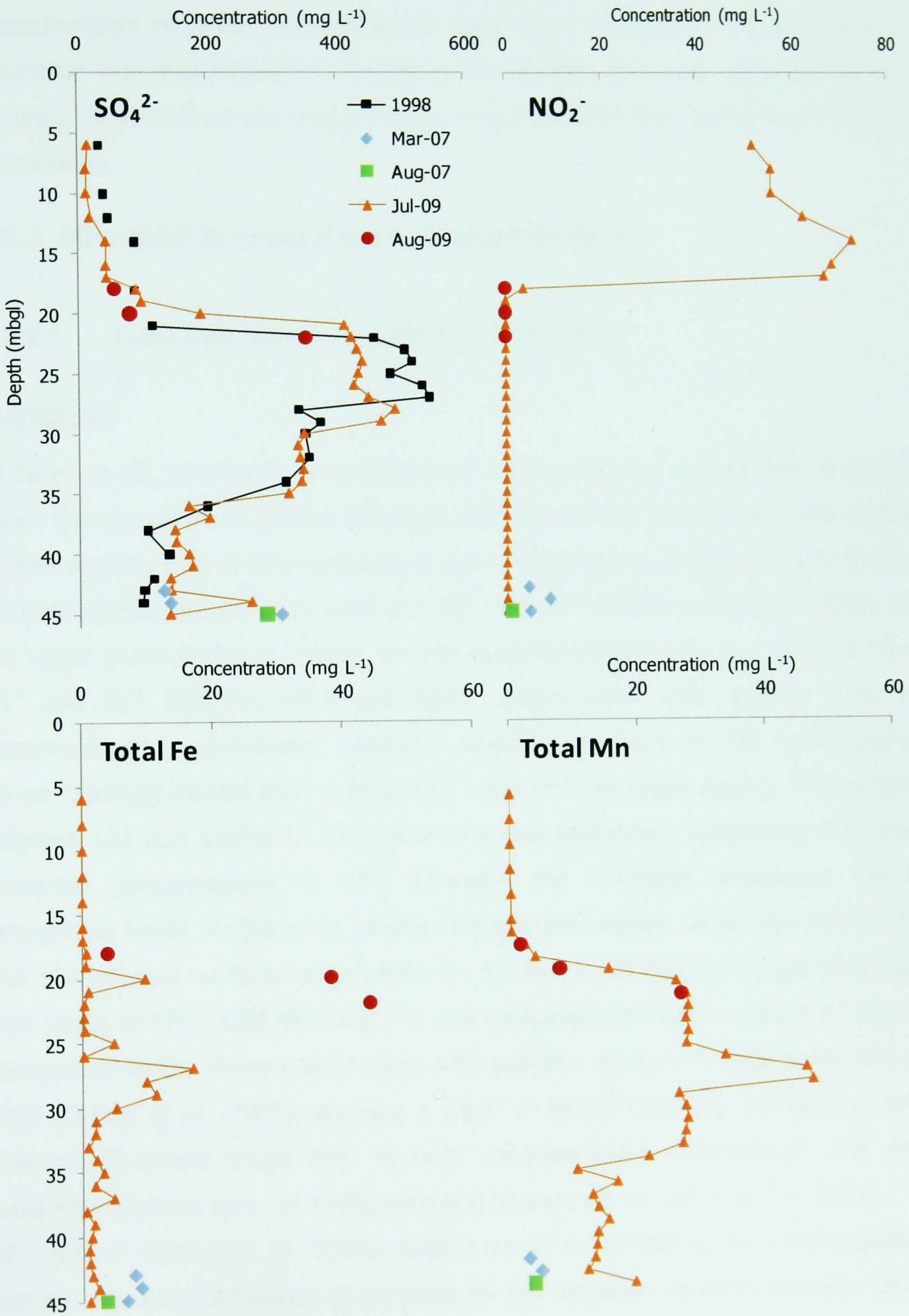



**Figure 2-13** Concentrations of the anaerobic TEA measured in the Four Ashes groundwater purged from borehole 60 in 2007 and 2008.

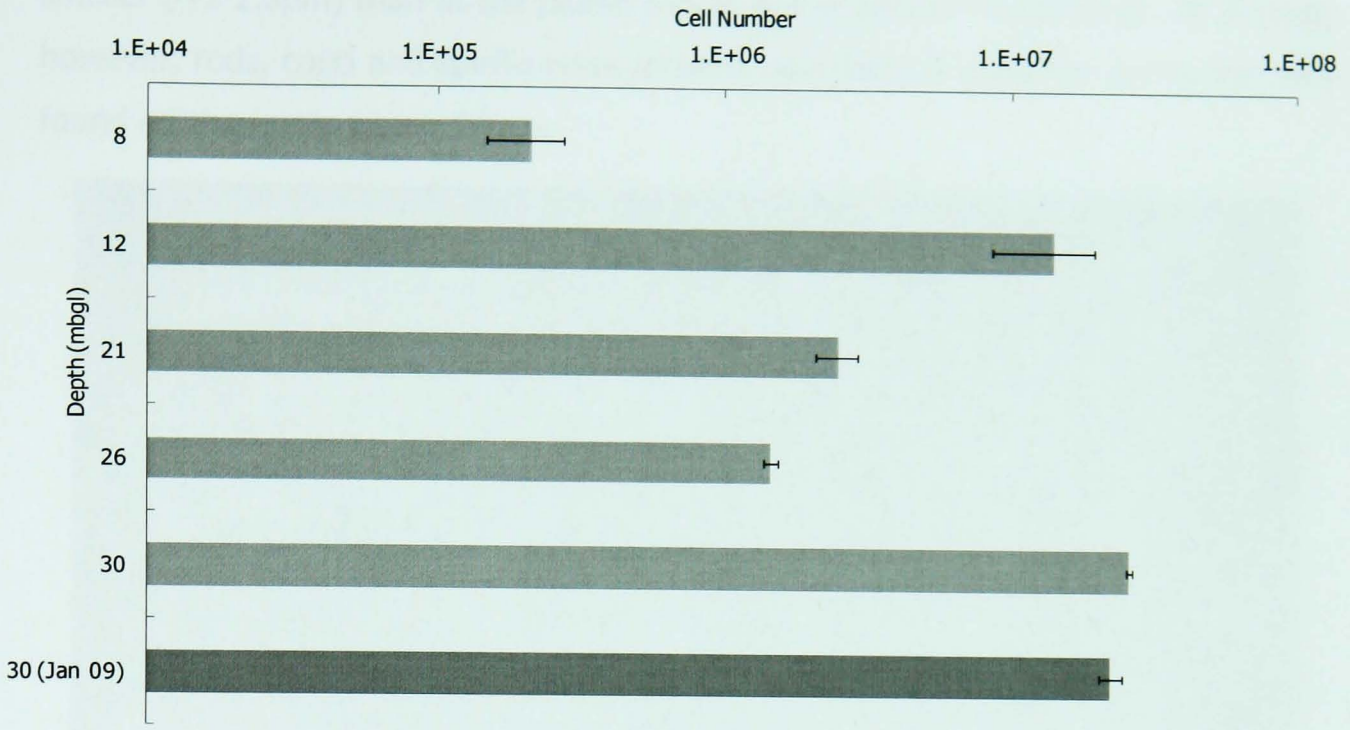
Although measuring the concentrations of TEA in the groundwater provides inference into the the potential microbial redox mechanisms, data from groundwater measurements of redox potential would have been valuable in the prediction of anaerobic microbial respiration trends in the aquifer. However, no practical and accurate method of on-site analysis (e.g. using flow cells) was available at the time of sampling.

## 2.3.2 Microbial Enumeration and Identification

### 2.3.2.1 Total Cell Numbers within Groundwater

Borehole 59

At borehole 59, cell counts were performed on groundwater purged from 6 depths down the groundwater profile, sampling uncontaminated groundwater, the plume fringes and the core of the contaminant plume. Results reveal (Figure 2-14) that in uncontaminated groundwater were 2 x $10^5$ cells $ml^{-1}$ (measured only in 2008). At the upper plume fringe (12mbgl), the cell numbers ranged from 1 x $10^7$ to 1.32 x $10^{-7}$ cell $ml^{-1}$ between 2008 and 2009, which were 100x greater than in uncontaminated groundwater. Similarly, microbial numbers on the lower plume fringe (30mbgl) ranged from 2 to 3 x $10^7$ cells $ml^{-1}$. At these depths, TPC ranged between 122 and 688mg $L^{-1}$ (Figure 2-7) across samplings, suggesting that low-moderate concentrations of TPC increased the microbial abundance above background levels. At the most grossly contaminated depths within the plume (21 and 26mbgl), cell numbers ranged from 7 x $10^5$ to 1 x $10^6$ cells $ml^{-1}$, suggesting that high levels of TPC (1285-4927mg $L^{-1}$) only increased cell numbers by 2-5x above background levels. These results agree with previous work performed at the site in 1998 (Pickup *et al.*, 2001), showing a trend of higher microbial numbers on the contaminant plume fringe than in both uncontaminated groundwater and the contaminant plume core. In 1998, microbial abundance ranged from 2 x $10^5$ to 7 x $10^6$ cells $ml^{-1}$ (Pickup *et al.*, 2001). Aside from minor variations, the data suggests that the microbial abundance at borehole 59 has remained constant between 1998 and 2009.



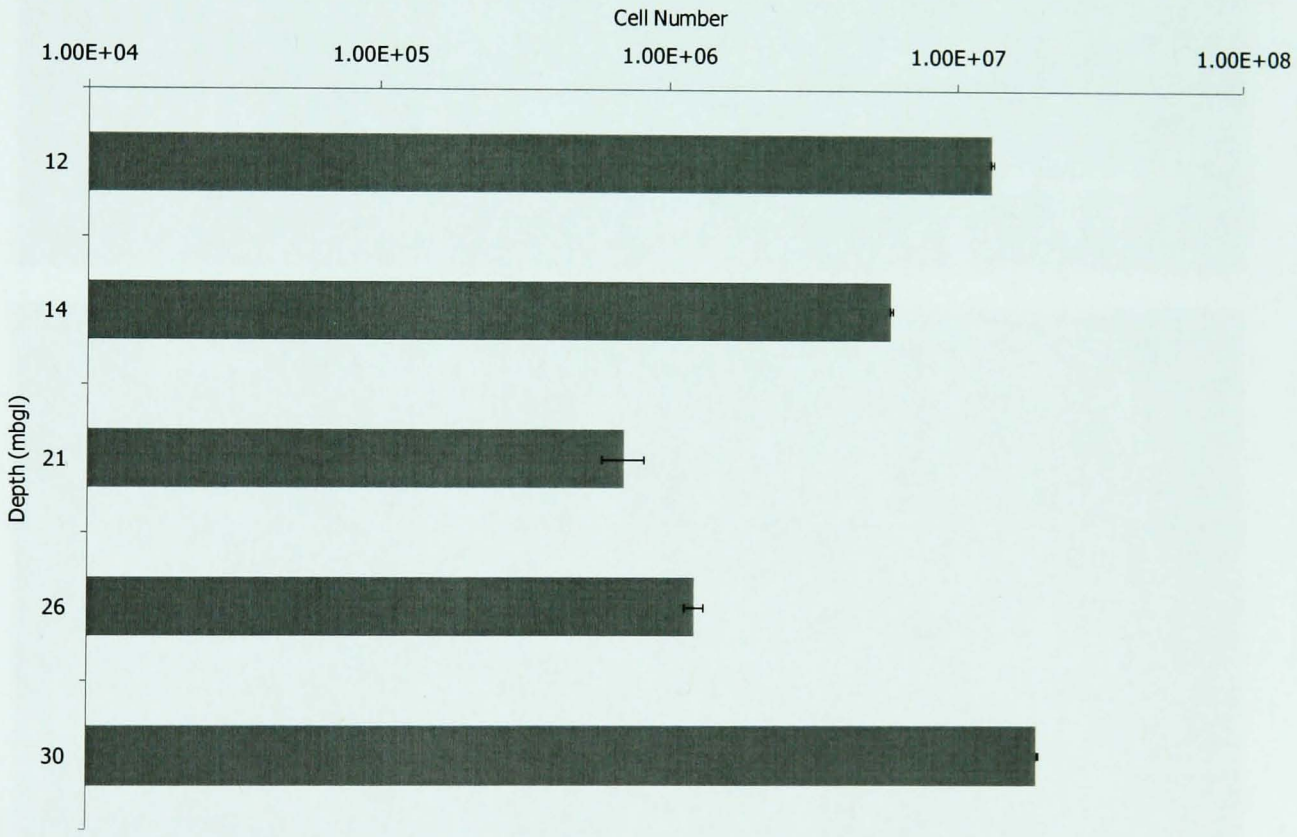




**Figure 2-14** Variation in total cell number with depth at borehole 59. Top: Cell numbers in February 2008 and January 2009, bottom: cell numbers in August 2009.

Images (Figure 2-15) of the stained cells show that there was spatial variation in cell morphology within the groundwater profile. At 8mbgl, outside the contaminant plume, the groundwater was dominated by small (~1µm) rods and cocci. At 12, 14 and 21mbgl, the groundwater contained larger (1.5-32µm) rods. At 26mbgl, the depth where the highest concentrations of contaminants were found, the cells were

smaller (~1-1.5µm) than at the plume fringe, but of similar morphology. At 30mbgl, however, rods, cocci and spirilia were present, and were of a similar size to the cells found on the upper plume fringe.

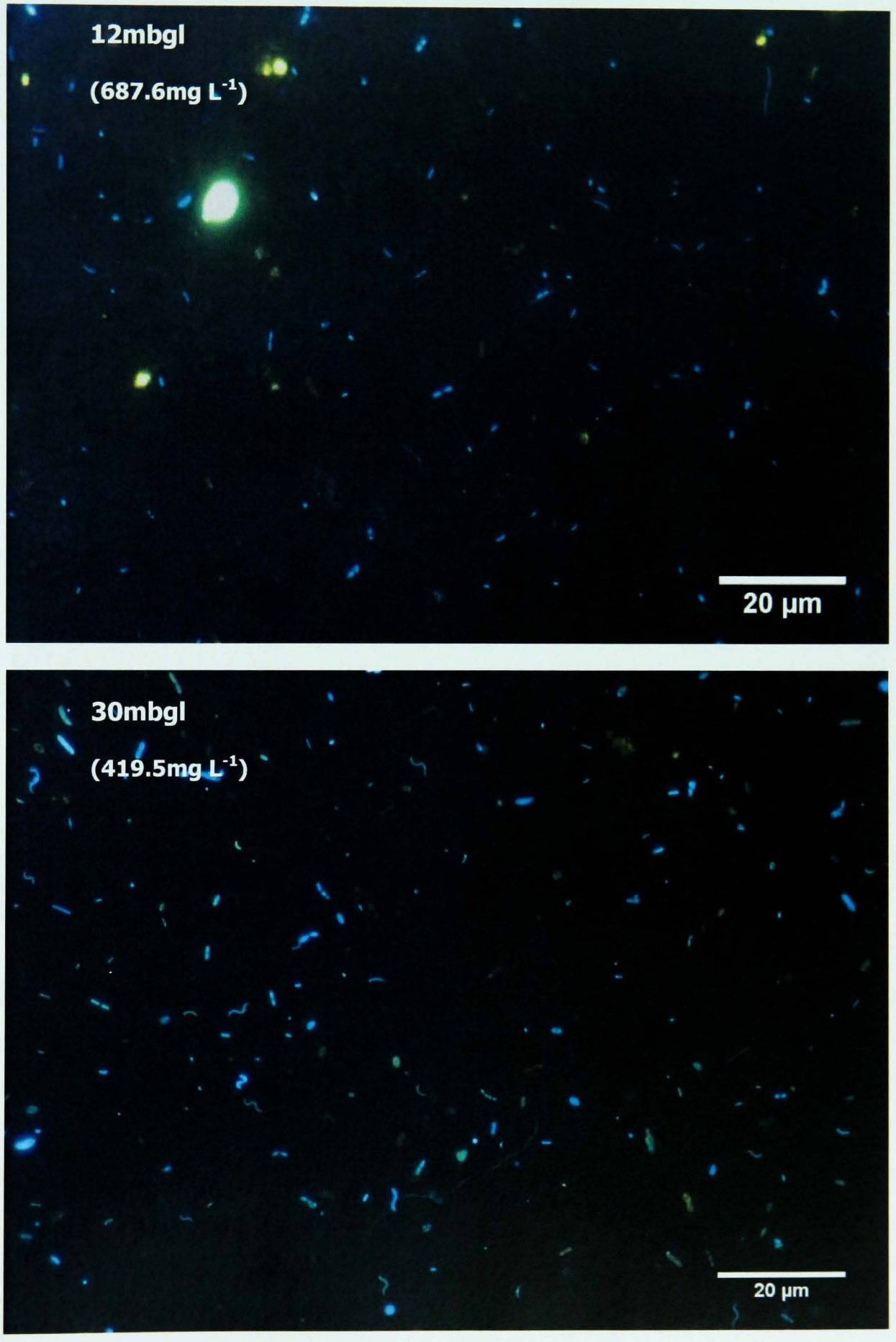



**Figure 2-15** Fluorescence images of planktonic microbial cells stained with DAPI from 12mbgl (top) and 30mbgl at borehole 59 (both from February 2008).

Borehole 60

Total cell counts were unobtainable at 43, 44 and 45mbgl in borehole 60. This was due to a lack of cells and the clustering of suspended particulates (Figure 2-16), which didn't allow for the filtering of more than 5ml of groundwater. The lack of cells coincides with the extremely high concentrations of contaminants found within the groundwater profile (6536-7751mg $L^{-1}$ TPC).

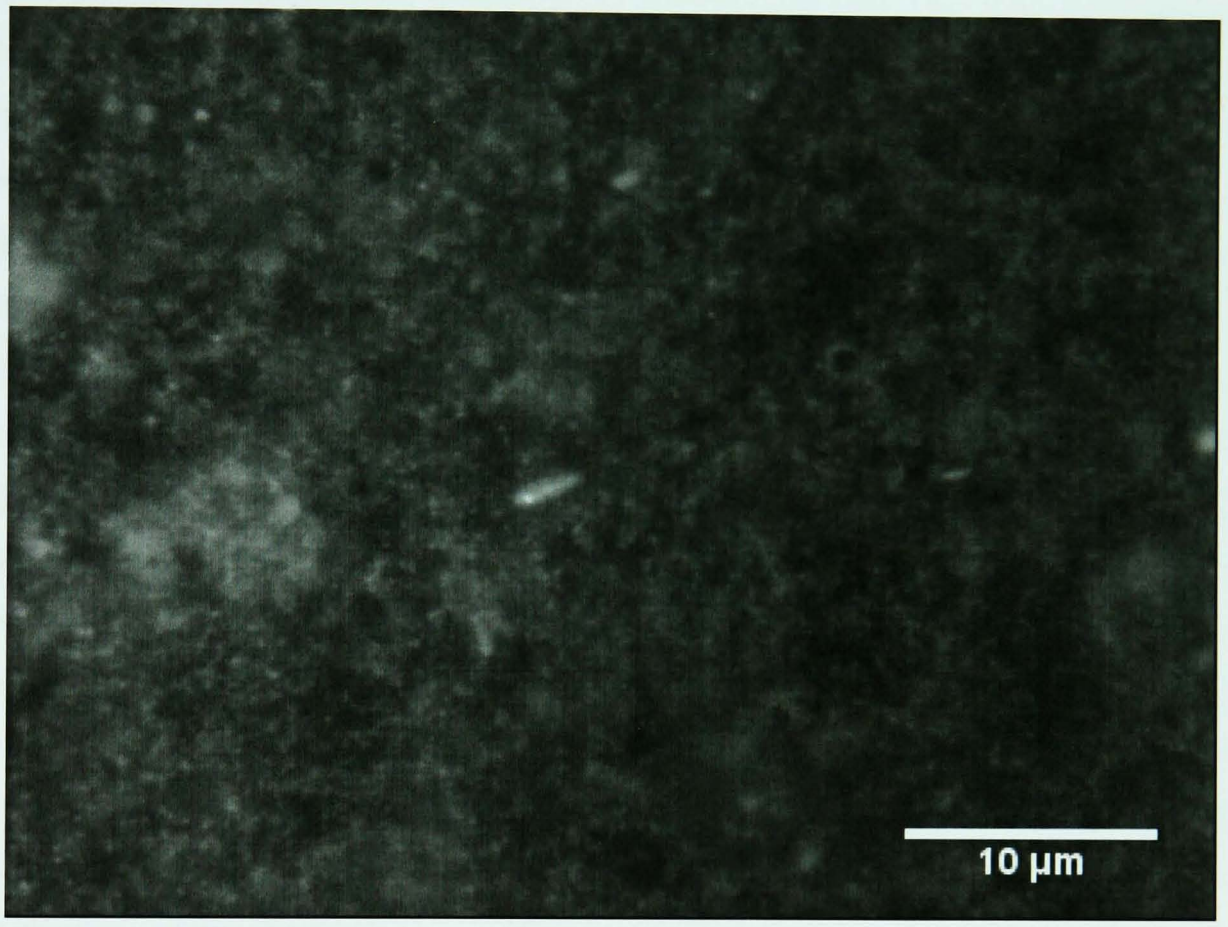



**Figure 2-16** Epifluorescence image of groundwater from borehole 60 at 45mbgl stained with DAPI. Background fluorescence of particulates is evident when 5ml of groundwater is filtered.

Also, there was no visible cell attachment to sand grains suspended in 2003 by Wu (2002) at 45mbgl. Pickup *et al.* (2001) found that cell numbers were too low to count statistically within the most grossly contaminated areas (23, 26 and 33mbgl) of the plume at borehole 60 in 1998. This supports the data found in 2007, as the TPC (up to 5482mg $L^{-1}$) at 23, 26 and 33mbgl in 1998 was lower than at 43-45mbgl in 2007. As the samples were retrieved down to 45mbgl at borehole 60, the change in pressure (from 4.4atm at 45mbgl to 1atm at ground level) may have destroyed the cells present in the samples. Removing samples from 45mbgl and subjecting them to lower pressures may have deleterious effects, as the decrease in pressure during purging may result in cell expansion and bursting.

However, many authors have successfully retrieved and counted microbes from within shallow aquifers (~80mbgl), both at Four Ashes and other sites, to deep oceans at around 11,000m below sea level (Pickup *et al.*, 2001; Williams *et al.*, 2001; Schippers *et al.*, 2005; Kato *et al.*, 1998). Although this is possible, the cultivation and thriving of bacteria is dependent on the pressure they experience within their natural habitat. Madigan, Martinko and Parker (2000) noted that bacteria have thresholds at which they are 'barotolerant', i.e. can exist but not thrive, and 'barophilic', i.e. only thrive at high pressures. The defining threshold where bacteria become barophilic is approximately 300 atm, up to this point bacteria remain barotolerant, resulting in them thriving more at atmospheric pressure. Therefore, there is no reason why the pressure changes resulting from pumping micro-organisms to the surface at Four Ashes would affect their ability to exist, as these organisms are only expected to be barotolerant. They will not possess complicated enzyme systems that a high pressure (obligate) barophile would have to prevent the effects of pressure damaging protein structure causing a loss of functionality. Another adaptive feature is that of the OmpH porin, a specialised diffusion channel in the outer membrane, which is only present in barophilic bacteria as normal porins loose functionality at high pressures. These traits are not transcribed in microbes that are successfully cultivated at 1 atm (Madigan, Martinko and Parker, 2000), and as Four Ashes is nowhere near the high pressures required for such change to occur, the microorganisms recovered will not be physiologically different to the ones seen at atmospheric pressure. It has also been shown that *E. coli* can withstand pressures of up to 450 MPa (Gervilla *et al.*, 1997). Pickup *et al.* (2001), who used the multi-level sampler system at Four Ashes, also obtained total and cultivable bacterial counts from depths up to 45mbgl. Cell counts of $\sim 1 \times 10^6$ cells $ml^{-1}$ were also found by Williams *et al.* (2001) who used a screened borehole system to sample 100mbgl. Therefore, there is ample evidence that the microorganisms within this aquifer system should not be affected by the sampling procedure or the change in environmental conditions on removal from their natural environment.

## 2.4 Conclusion

The spatial variation in contaminant hydrochemistry, anaerobic microbial terminal electron accepters and microbial abundance was investigated down two groundwater profiles at borehole 59 and 60 in the Four Ashes aquifer, Wolverhampton, UK. Results indicate that the spatial variation in phenolic compounds within the groundwater was stable between 1998 and July 2009 at borehole 59, 130m from point source. However, due to the onset of a pump and treat system in August 2009, marked changes in the distribution of phenolic compounds were visible at borehole 59. Changes in groundwater chemistry were also evident at borehole 60, as the TPC plume extended to the lowest sampling point (45mbgl). This was not affected by the pump and treat system, but instead suggests a second, more concentrated and deeper phenolics plume. Organic solute/2,6-xylenol ratios at both boreholes suggest that up to four sources of phenolic compounds were released from the distillation plant. Inorganic contaminants sodium and chloride were also measured at both boreholes. Results suggest that there were areas of high Na concentration within the groundwater, resulting from spillage pulses during the plants operation. Also, data from borehole 60 suggests that the NaOH plume, which was found to be localised near the site by Thornton *et al.* (2001), may have now reached borehole 60, as an increase in Na was detected at shallow depths. If the Na source was from NaCl spillage due to onsite de-icing activities, an increase in $Cl^-$ would have been visible at the same depths. Instead, both $Na^+$ and $Cl^-$ were elevated (in comparison to 1998) below 20mbgl at both boreholes, suggesting an increase in NaCl input into the aquifer later in plume formation from a source separate to the one seen in 1998 at shallow depths.

The range and distribution of anaerobic terminal electron acceptors suggest that conditions are suitable for anaerobic activity. These data, in conjunction with the phenolic compound/total organic matrix ratios suggest that sulphate- and metal-reduction coupled to phenol/cresol oxidation could have been occurring at shallow depths (above 24mbgl) at borehole 59. This was evident due to a decrease in the proportion of phenol/cresol in the total organic matrix towards the upper plume fringe, at which depth nitrate and oxygen are absent. This was

not visible at borehole 60, where aerobic degradation is expected to be occurring only in uncontaminated groundwater (above 20mbgl). The lack of visible degradation within the plume was due to suppression from the high contaminant load.

Total counts of planktonic cells revealed that the presence of TPC on the upper and lower plume fringes (12 and 30mbgl) at borehole 59 caused a 100x increase in cell numbers above background levels in uncontaminated groundwater. However, at higher concentrations of TPC (1285-4927mg $L^{-1}$), cell numbers were only 2-5x greater than background levels. At borehole 60, statistically innumerable cell numbers were found at 42-45mbgl in 2007. This was due to the intense contaminant load.

The data presented in this chapter give an insight in to the potential operation of anaerobic organisms in the groundwater. However, information is required to assess the spatial distribution of anaerobic organisms and to investigate the impact of contaminant load on the indigenous anaerobic communities. As 99% of organisms live attached to, or associated with, a surface, the profiling of attached anaerobic communities is also important to gain a full understanding of the anaerobic microbial ecology in the aquifer (Costerton and Wilson, 2000). This is invesitgated further in Chapters 3 and 4.

# 3

# Anaerobic Microbial Community Structure in the Four Ashes Aquifer

## 3.1 Introduction

Profiling *in situ* planktonic (free-living) microbial communities within a contaminated environment can provide valuable information on the influence of contaminant load on community structure. As microbial habitats are seldom uniform, it is possible that microbial niches would form at different areas within the contaminated environment in response to the geochemical conditions (Chapelle, 2001). A fundamental niche would be that occupied by sulphate-reducing bacteria (SRB). Although a thermodynamically unfavourable terminal electron accepting process (TEAP), bacterial sulphate-reduction has been shown to be a valuable process in the remediation of organic contamination (Shah, 2005). SRB have been used in the reduction of metal-contaminated waters, acid mine drainage and radioactive contamination (Costa and Duarte, 2005; Lovely and Phillips, 1992). Also, SRB have been shown to degrade toxic levels (up to 2000mg $L^{-1}$) of phenol and phenol-derivatives (Shah, 2005; Spence *et al.*, 2001). This revealed that SRB have the ability to provide a useful contribution to the remediation of phenolic contamination. Moreover, other groups of anaerobic organisms (e.g. nitrate-reducing bacteria, archaea) are important when considering the *in situ* natural attenuation of organic contaminants. In aquifers, contaminants can form plumes due to the flowing groundwater. Aerobic and microaerophillic microbial degradation is usually confined to the plume fringes (the boundary between contaminated and uncontaminated groundwater), due to a constant influx of oxygen from uncontaminated waters. Often, this only constitutes a low percentage of the total contaminant degradation, as the majority of the contaminant load can be located in anaerobic waters where oxygen levels are depleted (Banwart and Thornton, 2003). Understanding the role of anaerobic communities within contaminated environments is therefore critical to understanding natural attenuation processes.

Previous research has also highlighted the importance of investigating the microbial community attached to, or associated with, surfaces. Such complex communities are often referred to as sessile communities or microbial biofilms (Section 1.4). In marine environments, 99.9% of the total microbial biomass is present in biofilm communities (Costerton and Wilson, 2004). This is due to the advantages of forming a biofilm, such as protection from high levels of contamination, metabolic exchange between species and genera, and gene

transfer resulting in the induction of catabolic enzymes that are not coded for in the host DNA (Schwarzenbach *et al.*, 1993). This suggests that the attached microbial community can be valuable in natural attenuation processes and the development of an *in situ* bioremediation method, as they have the ability to immobilize and degrade contaminants that are recalcitrant and toxic to planktonic communities (Singh *et al.*, 2006).

Within the Permo-Triassic sandstone aquifer beneath the Four Ashes site, there is prominent organic groundwater contamination, most notably by phenolic compounds (discussed in Chapter 2). It has been demonstrated that this contamination influences microbial abundance within the anaerobic parts of the contaminant plume. Previous research at the site found that eubacterial community diversity (by 16S rRNA fingerprinting) varies with depth on the phenolic plume fringes (Pickup *et al.,* 2001; Rizoulis, 2008). However, research has not been undertaken to profile the anaerobic communities. Also, no investigation into the influence of phenolic contamination on sulphate-reducing (SRB), nitrate-reducing (NRB) and archaeal communities has been performed. Finally, no comparison of the attached and planktonic anaerobic communities has been undertaken.

The aim of this study was to investigate the influence of the groundwater hydrochemistry on the diversity of the indigenous anaerobic communities. Planktonic and attached eubacterial and anaerobic communities (SRB, NRB, archaea) are profiled using denaturing gradient gel electrophoresis (DGGE). The specific objectives of this study are:

1. To profile the eubacterial and anaerobic communities in the groundwater at borehole 59;
    a. To compare community structure at different depths
    b. To assess the influence of groundwater hydrochemistry on community structure
2. To compare both attached and planktonic communities under the same hydrochemical conditions at one depth within the aquifer;
3. To quantify and assess the structure of cultivable and total SRB communities across the contaminant gradient within the aquifer at borehole 59.

## 3.2 Materials and Methods

### 3.2.1 Visualisation of Attached Microbes

Quantitative analysis of cells attached to mineral surfaces is problematic as the cells have to be removed and placed into solution. Methods by which this is possible are gentle vortexing, sonication and/or stirring (Ferris *et al.*, 1989). These methods are not preferred as vigorous disruption may lead to cell lysis and therefore unreliable cell counts.

Within this study, cells attached to grains were assessed qualitatively. Grains were stained using a solution containing Syto 9 (Invitrogen Ltd, Paisley, UK). Where appropriate, a dual-staining technique, using Syto 9 and DAPI (Sigma-Aldrich Company Ltd, Dorset, Engand, UK), was used. This ensured that only microbial cells were imaged as both stains only fluoresce when bound to the DNA helix (some fluorescent stains, such as acridine orange, are less specific and stain particulates). The grains were stained by gently covering them in stain solution (DAPI - 45μM, Syto 9 – 7.5μM), before incubation in the dark for 15 minutes. After incubation, the staining solution was removed and the grains were gently rinsed 3 times with sterile UHQ, before being placed onto a glass slide and covering with an adhesive Grace Biolabs CoverWell™ imaging chamber (Stratech Scientific Ltd, Newmarket, Suffolk, UK). The grains were then imaged at 20x and 40x magnification using an Olympus BX50WI Upright Fluorescence Microscope (Olympus Optical Co. Ltd, London, UK) fitted with CoolSnap colour camera (Princeton Instruments, Buckinghamshire, UK). Extended depth of focus images of the grains were taken using Image-Pro Plus (v. 4.5.1.29, from Media Cybernetics Inc., Bethesda, MD, USA). Emission was detected in the green at 530nm using a U-MWB (Olympus Optical Co. Ltd, London, UK) filter cube. Bright field images of the grains were also taken and merged with fluorescence images using Image-Pro to show microbial attachment in a colour composite image.

### 3.2.2 DNA Extraction

#### 3.2.2.1 Groundwater and Grains

Total DNA was isolated for use in studies of microbial diversity using PCR amplification and DGGE (Section 3.2.4 and 3.2.5). 1L of groundwater was aliquoted to 375ml in 500ml polypropylene centrifuge bottles (Beckman Coulter UK Ltd., Buckinghamshire, UK) and centrifuged at 22,000*g* for 20 minutes using a Beckman Avanti™ J-25I centrifuge fitted with a JA-14 rotor (Beckman Coulter UK Ltd., Buckinghamshire, UK). The supernatant was discarded and the bacterial pellet was re-suspended in 1ml of sterile UHQ water (Goldenberger and Altwegg, 1995) and transferred to a sterile 2ml centrifuge tube, before further centrifugation at 15,000*g*. The supernatant was discarded and the pellet was stored at –20°C.

DNA was extracted using the Ultraclean™ Microbial DNA Isolation Kit (Mobio, San Diego, CA, USA). For extraction from bacterial pellets, the manufacturer's protocol was followed, including bead-beating for 10 minutes using the 13000-V1 MoBio Vortex Adapter and a heating step at 60°C for 15 minutes to increase DNA yields. To extract DNA from grains, one modification to the manufacturer's protocol was made. 0.2g of grains was weighed directly into the 2ml microbead tube before the addition of the bead solution and solution MD1. The subsequent steps in the manufacturer's protocol were then followed, resulting in the elution of DNA in 50µl of Tris buffer.

#### 3.2.2.2 Liquid Cultures

One ml of liquid culture was centrifuged at 15, 000*g* and the supernatant was discarded. The resulting pellet was then frozen at –20°C. DNA was extracted from the pellet using a modified bead-beating and CTAB/phenol chloroform method (Griffiths *et al.*, 2000). The bacterial pellet was re-suspended with 0.5ml of 120 mM phosphate buffer (pH=8) containing 5% CTAB and added to a 2ml grinding tube containing 0.5g of sterile 106µm glass beads (Sigma-Aldridge Company, Ltd, Poole, UK). 0.5ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the grinding tube. The tubes were shaken in an 8000M grinding mixer/mill (Glen Creston Ltd., Stanmore, Middlesex, UK) for 60 seconds before centrifugation at 10,000*g* for 10 minutes at 4°C in a micro-centrifuge. The

supernatant was transferred to a sterile 1.5ml tube and 0.5ml of chloroform:isoamyl alcohol (24:1) was added, and the tube was gently vortexed for 5 seconds. The tube was centrifuged at 10,000*g* for 10 minutes at 4°C. The resulting supernatant was transferred to another sterile 1.5ml tube and 0.6 volumes of isopropanol (100%) (approximately 240µl) and 1µl glycogen was added. The samples were left overnight at 4°C to allow the DNA to precipitate. The samples were centrifuged at 14,000*g* for 10 minutes at 4°C. The supernatant was discarded and 150µl of ethanol (100%) was used to re-suspend the pellets. The samples were further centrifuged at 14,000*g* for 10 minutes at 4°C. The supernatant was discarded and the pellet was dried using a vacuum centrifuge for 15 minutes to remove any remaining ethanol. Finally, the pellet was re-suspended in 20µl sterile UHQ water (Goldenberger and Altwegg, 1995) and stored at –20°C.

### 3.2.3 Quantification of Extracted DNA

Two methods were used to quantify the DNA extracted from the groundwater, sand samples and liquid cultures; spectrophotometry using a Nanodrop™ 8000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and fluorimetry using Quant-it™ PicoGreen® dsDNA reagent (Molecular Probes, Inc., Eugene, OR, USA).

The former is a high-throughput method to quantify dsDNA concentrations in up to eight 1µl samples simultaneously in just a few minutes, as no instrument calibration is required. The instrument was set-up by running a blank of the DNA carrying solution, Solution MD5 of the Ultraclean™ Microbial DNA Isolation Kit (Mobio, San Diego, CA, USA). 1µl of sample was loaded onto the instrument for measurement. Information on the concentration of total DNA and the absorbance at 260 and 280nm is provided. This allowed conclusions to be made with respect to the contribution of proteins and salts to the concentration reading. As this is a spectrophotometric method, it is not specific to DNA, and is therefore sensitive to other organic molecules.

For fluorimetric determination, 98µl of TE buffer (10mM Tris, 1mM EDTA at pH 7.5) was added to a 96-well plate. 2µl of sample/standard was added before 100µl of the PicoGreen (0.005% v/v in TE buffer) staining solution. The samples

were analysed using a FluoStar Optima plate reader (BMG Labtech Ltd., Aylesbury, UK), which was calibrated using known concentrations of herring DNA, using excitation and emission wavelengths of 500nm and 525nm, respectively (Rizoulis, 2008). The fluorescence intensity value for each unknown sample was converted into DNA concentration using the polynomial regression generated from the calibration standards (Figure 3-1). The analysis of each sample and calibration standard was replicated.

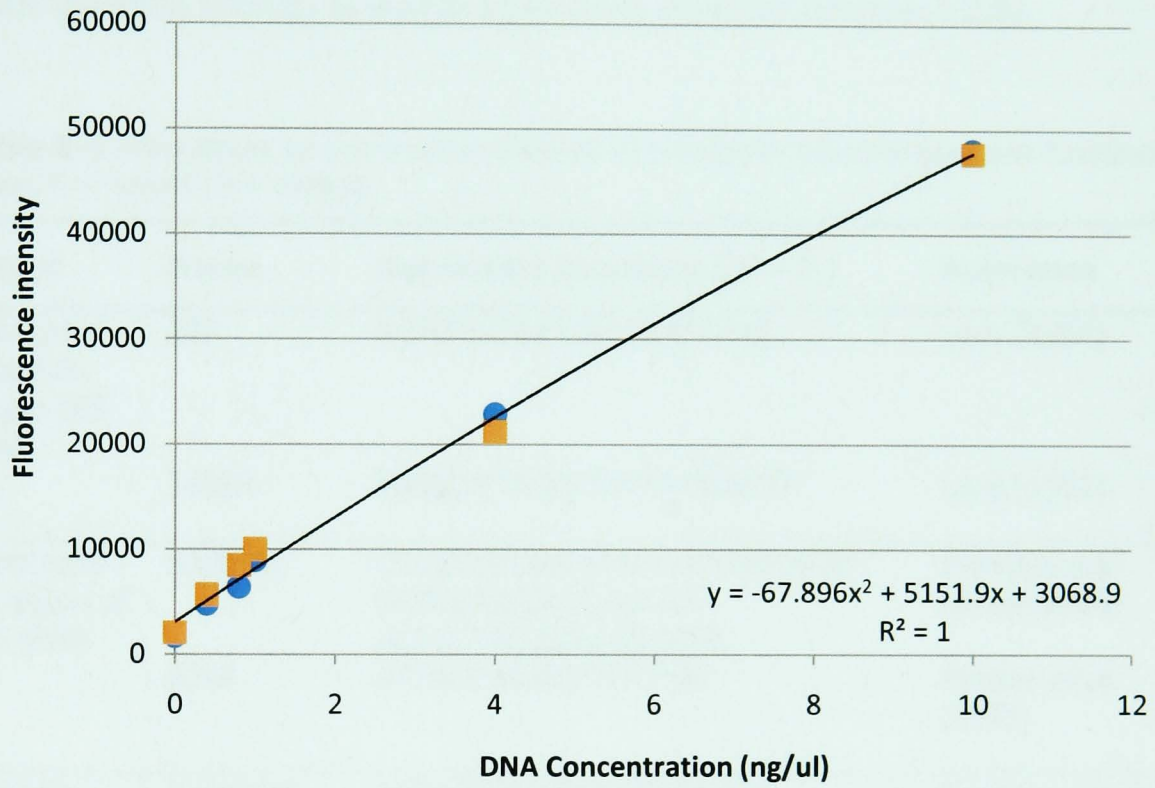



**Figure 3-1** Fluorimetric calibration curve for PicoGreen, showing reproducible replicate analyses (● = Replicate one, ■ = Replicate two).

The two methods were compared to assess which was the most reliable and applicable for the quantification of DNA extracted from the environmental matrices encountered in the characterisation of the Four Ashes site.

### 3.2.4 Polymerase Chain Reaction

The Polymerase chain reaction (PCR) was used to amplify DNA extracted both from environmental samples and from liquid culture. Bacterial colonies isolated on solid media were amplified directly, without DNA extraction (Elliott, 2004). For this, colonies were picked using a sterile cocktail stick and mixed into 50µl sterile UHQ water (Goldenberger and Altwegg, 1995), vortexed and frozen at -

20°C. After defrosting and vortexing for 5 seconds, 10µl of the colony suspension was added to the PCR reaction (Section 3.2.4.2). The freezing and initial PCR denaturation released enough DNA for amplification.

### 3.2.4.1 PCR Primer Sets

Primers used in this study (Table 3-1) targeted a variety of different microorganisms and functional genes. A 40bp GC-clamp (Muyzer *et al.*, 1993) was added to primers used to amplify targets for use in denaturant gradient gel electrophoresis (DGGE) to assess community diversity (Section 3.2.5).

**Table 3-1** PCR primers for universal amplification of eubacterial 16S rRNA gene and functional genes. F = forward, R = reverse.

| Target | Name | Nucleotide sequence (5′ – 3′) | Reference |
|---|---|---|---|
| Eubacteria - Near full-length 16S rRNA | 27F | AGAGTTTGATCMTGGCTCAG | Lane (1991) |
| | 1492R | TACGGYTACCTTGTTACGACTT | Lane (1991) |
| Eubacteria - V3 region of 16S rRNA | 338F-GC | CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGG-ACTCCTACGGGAGGCAGC | Whiteley and Bailey (2000) |
| | 519R | ATTACCGCGGCTGCTGG | Muyzer *et al.* (1993) |
| Archaea | Arch344F | ACGGGGCGCAGCAGGCGCGA | Casamayour *et al.* (200) |
| | Arch915R | GTGCTCCCCCGCCAATTCCT | |
| Nitrate-Reduction (*napA*) | *napA*-F1 | C TGG ACI ATG ATG GGY TTI AAC CA | |
| | *napA*-R1 | CC TTCYTT YTC IAC CCA CAT | Alcantara-Hernandez *et al.* (2009) |
| Sulphate-Reduction (*drsB*) | DSRp2060f | CAA-CAT-CGT-YCA-YAC-CCA-GGG | |
| | DSR4R | GTG TAG CAG TTA CCG CA | Geets *et al.* (2006) |

The primers used for the detection of *dsrB* target the β-subunit of dissimilatory sulphate-reduction. *dsrA* (the α-subunit) and *dsrB* are near-exact replicas, with only minor variations in DNA sequences. The primer pair targeting nitrate-

reduction amplifies the *napA* gene that codes for the periplasmic nitrate-reductase enzymes. The primers probing for periplasmic nitrate-reduction were chosen as *napA* was found to be widely distributed within a variety of proteobacteria (Alcantara-Hernandez *et al.,* 2009), a selection of which were successfully isolated from Four Ashes groundwater by Rizoulis (2008). The archaeal primers were designed by Raskin *et al.* (1994) using eight complimentary oligonucleotide groups of phylogenetically-defined methanogens, for the use in environmental microbiology.

### 3.2.4.2 PCR Conditions

PCR amplification was performed using a Thermo Px2 thermal cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). A positive and negative control was used to verify results. Below is an example of the PCR recipe for the amplification of the eubacterial 16S rRNA gene (Table 3-2). Sterile UHQ was prepared by pre-filtering through a sterile 25mm diameter 0.22µm syringe filter (Acrodisc®, Pall Life Sciences, USA) into a 1.5ml tube, before autoclaving at 121°C for 20 minutes. The water was then stored at -20°C along with other PCR reagents (Goldenberger and Altwegg, 1995).

**Table 3-2.** PCR recipe for one sample for 16S rRNA amplification (Rizoulis, 2009)

| Ingredient | Supplier | Working Concentration | Volume (µl) |
|---|---|---|---|
| Sterile UHQ water | | | 39.35 |
| Buffer (10x) | Bioline | 1x | 5 |
| $MgCl_2$ (50mM) | Bioline | 1.5mM | 1.5 |
| dNTPs (25mM) | Bioline | 0.2mM | 0.4 |
| 338F-GC (10µM) | Invitrogen | 1µM | 1.5 |
| 519R (10µM) | Invitrogen | 1µM | 1.5 |
| Taq (5U $\mu l^{-1}$) | Bioline | 0.75U | 0.15 |
| Template | | | ~3ng |
| | | Total | 50 |

The PCR cycling for the amplification of the eubacterial 16S rRNA gene is shown in Table 3-3. The recipes and PCR cycles used to amplify anaerobic functional genes are given in Appendix 1.

**Table 3-3.** Standard PCR cycles for amplification of universal full-length 16S rRNA gene and 200bp V3 region of 16S rRNA gene for subsequent DGGE analysis (Rizoulis, 2008).

| PCR Stage | 16S rRNA | V3 region of 16S rRNA (DGGE) |
|---|---|---|
| Initial denaturation | 94°C for 5 minutes | 95°C for 4 minutes |
| | 35 cycles of: | 35 cycles of |
| Denaturation | - 94°C for 1 min | - 95°C for 30 sec |
| Annealing | - 54°C for 1 min | - 60°C for 30 sec |
| Elongation | - 72°C for 30 sec | - 72°C for 1 min |
| Final Elongation | 72°C for 5 min | 72°C for 6 min |
| | Hold at 15°C | Hold at 15°C |

### 3.2.4.3 Analysis of PCR amplification products

Amplification products were analysed by electrophoresis through 1.5% w/v agarose-TAE (40mM Tris- base, 20mM acetic acid, 1mM EDTA, pH 8) gels containing 0.8µg $ml^{-1}$ ethidium bromide. A 2% w/v agarose-TAE gel was used to assess the amplification products intended for DGGE (products less than 600bp). 2µl of 5x gel loading buffer (Sigma-Aldridge Company, Ltd, Poole, UK) was added to 5µl of PCR product and subjected to electrophoresis (90v for 40 minutes) alongside a DNA molecular weight standard (Hyperladder I, Bioline, London, UK). Gels were imaged using a GelDoc UV system with digital camera (Whatman plc, Maidstone, Kent, UK).

## 3.2.5 Denaturant Gradient Gel Electrophoresis (DGGE) for the Assessment of Microbial Community Structure

To assess eubacterial community structure, samples were analysed by DGGE. A 28 x 18cm 10% polyacrylamide gel was poured using a 30-60% urea parallel denaturing gradient (Table 3-4, Whiteley and Bailey, 2000).

**Table 3-4** Composition of the denaturing solutions used for one DGGE analysis for eubacterial 16S V3 region PCR amplicons

| Component | 30% | 60% |
|---|---|---|
| 30% Acrylamide/Bisacrylamide (1:37.5) (ml) | 9.16 | 9.16 |
| Formamide (ml) | 3.3 | 6.6 |
| Urea (g) | 3.47 | 6.94 |
| 50 x TAE buffer (µl) | 275 | 275 |
| UHQ water (ml) | Up to 27.5ml | Up to 27.5ml |

The gel was polymerised by adding 120µl of 20% w/v ammonium per sulphate in 0.5x TAE and 12µl of TEMED (N,N,N',N'-Tetramethylethylenediamine) to 27.5ml of each denaturing solution before pouring. PCR products were added to 5x loading buffer (Sigma-Aldridge Company, Ltd, Poole, UK) at 2:1 concentration and electrophoresed (16h at 100 V and 60°C) in 0.5% TAE using an Ingeny PhorU system (Ingeny, Goes, The Netherlands). The DGGE conditions used for the community analysis of PCR amplifications targeting anaerobic functional genes are listed in Table 3-5.

**Table 3-5** DGGE conditions used for the community analysis of PCR amplifications targeting anaerobic functional genes. All were performed at 60°C in 0.5x TAE

| Function | Gradient | Voltage | Run-time (hrs) | Reference |
|---|---|---|---|---|
| Nitrate-reductase (*napA*) | 40-80% | 90 | 16 | Alacantara-Hernandez *et al.* (2009) |
| Sulphate-reductase (*dsrB*) | 40-70% | 120 | 15 | Geets *et al* (2006) |
| Total Archaeal community | 10-80% | 200 | 3.5 | Casamayour *et al.* (2000) |

All gels were stained using 1x concentrated SYBR® Gold (Invitrogen, Ltd, Paisley, UK) in 0.5x TAE buffer for 30 minutes in the dark, prior to imaging using a GelDoc UV system with digital camera (Whatman Plc, Maidstone, Kent, UK).

### 3.2.5.1 DGGE Markers

Seven species markers (Table 3-6) were used for comparison and to aid cluster analysis (Section 3.2.5.2). Markers were created by PCR amplification of DNA extracted from 7 different colonies isolated by Dr. David Elliott from Four Ashes groundwater, using primers targeting the V3 region of the 16S rRNA gene. All isolates migrated to different positions on the DGGE gel and produced one band. Equal amounts of amplification products for each isolate were mixed together to create one single marker, hereafter referred to as M7. Markers were loaded into outer wells of DGGE gels, and in the middle where necessary (Rizoulis, 2008).

**Table 3-6** List of isolates used to create a species standard marker for DGGE (Rizoulis, 2008)

| Isolate Number | Phylogenetic Affiliation |
|---|---|
| **282** | *Pseudomonas sp.* |
| **264** | *Pseudomonas sp.* |
| **245** | *Stentrophomonas sp.* |
| **273** | *Pseudomonas sp.* |
| **297** | *Bacillus sp.* |
| **272** | *Methylibium sp.* |
| **235** | *Rhizobium sp.* |

### 3.2.5.2 Cluster Analysis of DGGE Profiles

DGGE profiles were analysed using Phoretix 1D gel analysis software (Total Lab Ltd., Newcastle, UK). Images were analysed to assess band migrations. Slant lines were chosen based on the orientation of the species markers ran at either end of the gels. Cluster dendrograms were created and exported from the software. Matrix data was also exported into Community Analysis Package 2.04 (Pisces Conservation Ltd., Lymington, Hants, UK) to generate principle component analysis plots to further assess cluster similarity of profiles.

## 3.2.6 Sequencing of Isolates

Isolates were amplified using full-length eubacterial 16S rRNA primers, as described in Section 3.2.4. Products were sent to the Core Genetics Facility (Medical School, University of Sheffield, UK) for purification, amplification in sequencing reactions and capillary sequencing. Sequence files were checked for misreads using Chromas Lite Version 2.01 (www.technelysium.com.au). Checked sequences were assembled using the CAP3 Contig Assembly Program incorporated into BioEdit Sequence Alignment Editor Version 7.0.5.3 (www.mbio.ncsu.edu/BioEdit/bioedit.html). The closest relatives of the near full-length 16S rRNA sequences were identified in June 2010 by nucleotide BLAST search (blastn, http://blast.ncbi.nlm.nih.gov/Blast) using the nucleotide collection database and the default parameters.

### 3.2.7 Culturing of Anaerobic Organisms

#### 3.2.7.1 Planktonic Microorganisms

The most probable number (MPN) method (American Health Assoc., 1992) of cultivation was used to enumerate cultivable bacteria within groundwater samples. Modified Postgate medium B (Pickup *et al.*, 2001) and a mineral salts medium (Evans *et al.*, 1965), supplemented with 0.5mM or 5mM phenol as the carbon-source, was used (Table 3-7). The Postgate medium B was prepared by adding all ingredients (except sodium thioglycollate and sodium ascorbate) to 990ml of de-ionised water, and autoclaving at 121°C for 20 minutes. Sodium thioglycolate (175mM) and sodium ascorbate (100mM) solutions were pre-filtered through 0.22µm syringe filters (Acrodisc®, Pall Life Sciences, USA), before addition to the autoclaved solution under aseptic conditions, to a final concentration of 0.875mM and 0.505mM (both 0.1g in 1L), respectively. The final medium was adjusted to pH 7.1 ± 0.1.

The mineral salts medium was prepared by adding all salts to de-ionised water and autoclaving at 121°C for 20 minutes. Phenol was sterilised by filtration through a sterile 0.22µm nylon syringe filter (VWR International Ltd., West Sussex, UK) before addition to the autoclaved salt solution.

**Table 3-7** Recipes for 1L of Postgate medium B (top) and mineral salts medium

| Postgate Medium B | |
|---|---|
| $KH_2PO_4$ | 0.5g |
| $NH_4CL$ | 1.0g |
| $Na_2SO_4$ | 1.0g |
| $CaCl_2.6H_2O$ | 0.1g |
| $MgSO_4.7H_2O$ | 2.0g |
| Yeast Extract | 1.0g |
| $FeSO_4.7H_2O$ | 0.5g |
| Sodium lactate (60-70%) | 5.0ml |
| De-ionised water | Up to 990ml |
| Sodium thioglycolate solution (175mM) | 5.0ml (0.1g) |
| Sodium ascorbate solution (101mM) | 5.0ml (0.1g) |

| Mineral Salts Medium | |
|---|---|
| $K_2H(PO)_4$ | 1g |
| $(NH_4)_2SO_4$ | 1g |
| $MgSO_4.7H_2$ | 0.3g |
| $FeSO_4.7H_2O$ | 0.02g |
| $CaCl_2.2H_2O$ | 0.136g |
| De-ionised water | Up to 998 or 980ml |
| Phenol | 0.5mM or 5mM |

All media was dispensed into sterile 20ml vials, degassed with $Ar_2/N_2$ using a gas manifold system (Figure 3-2) and capped with sterile butyl rubber stoppers. This ensured that the thioglycollate and ascorbate reducing agents (Postgate medium B only) were not oxidised prior to use. Samples were then injected using sterile needles and syringes through the stoppers within an anaerobic environment before incubation at 10°C.

Samples were assessed over a period of weeks to document the change in FeS production, an indicator of SRB activity in the modified Postgate B media, and turbidity of the phenol mineral salts media. After adequate incubation time, results were compared with the most-probable number table (American Water Standards, 1997) to derive the MPN of cells per ml of sample.

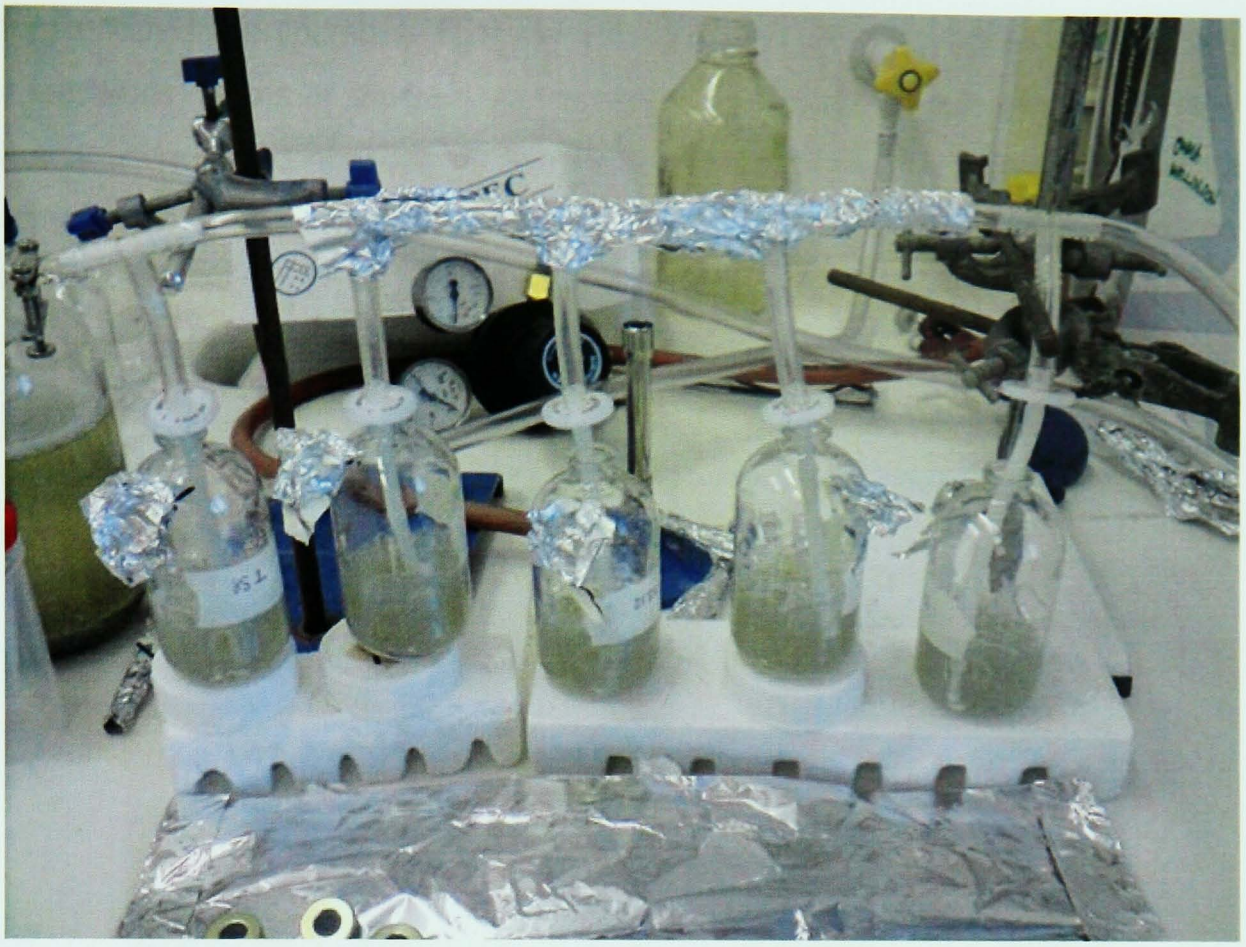


**Figure 3-2** Gas manifold system used to degas media prior to cultivation. This method was an adaptation of the method used by Miller and Molin (1974).

### 3.2.7.2 Attached Microorganisms

For the cultivation of cells attached to mineral and rock grains, a qualitative high- and low-inoculation density method was used (Postgate, 1984). This method was used to assess the presence of cultivable sulphate-reducing bacteria in the attached phase without quantification. 0.5g of grains was added to 4.5ml of degassed ($Ar_2/N_2$), pre-reduced (for minimum 24 hours) and sterile media, equalling a $10^{-1}$ dilution factor (high-inoculation density). Approximately 10 grains were added to 1ml of media for low-inoculation density cultivations. This method allowed both fast-growing and slow-growing microorganisms to be isolated as competition was reduced in the low-inoculation density cultivations.

Cultivation was performed within an anaerobic chamber (Figure 3-3, PlasLabs 815-Glove Box, PlasLabs, Inc, Lansing, MI, USA), containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide (Suihko *et al.*, 2005; Elliott, 2004; Postgate, 1984; Leach *et al.*, 1977). Gas entering the chamber was pre-filtered using a sterile 47mm diameter nylon 0.2µm filter (Whatman UK Ltd, Maidstone, Kent, UK). Fans with attached palladium catalysts were used to remove any oxygen

that entered the chamber. Fans were turned off during any manipulation with open tubes, plates or media to prevent the disturbance of the atmosphere and the subsequent contamination of samples. Silica gel crystals were used to remove any water from the chamber generated from the reaction between oxygen and hydrogen catalysed by the palladium catalysts. Both the palladium catalysts and silica gel were recharged at 35°C overnight when spent. All apparatus was sterile, either autoclaved prior to use or pre-sterilised and packaged by the manufacturer (e.g. sterile loops). Appropriate negative controls were used at all times.

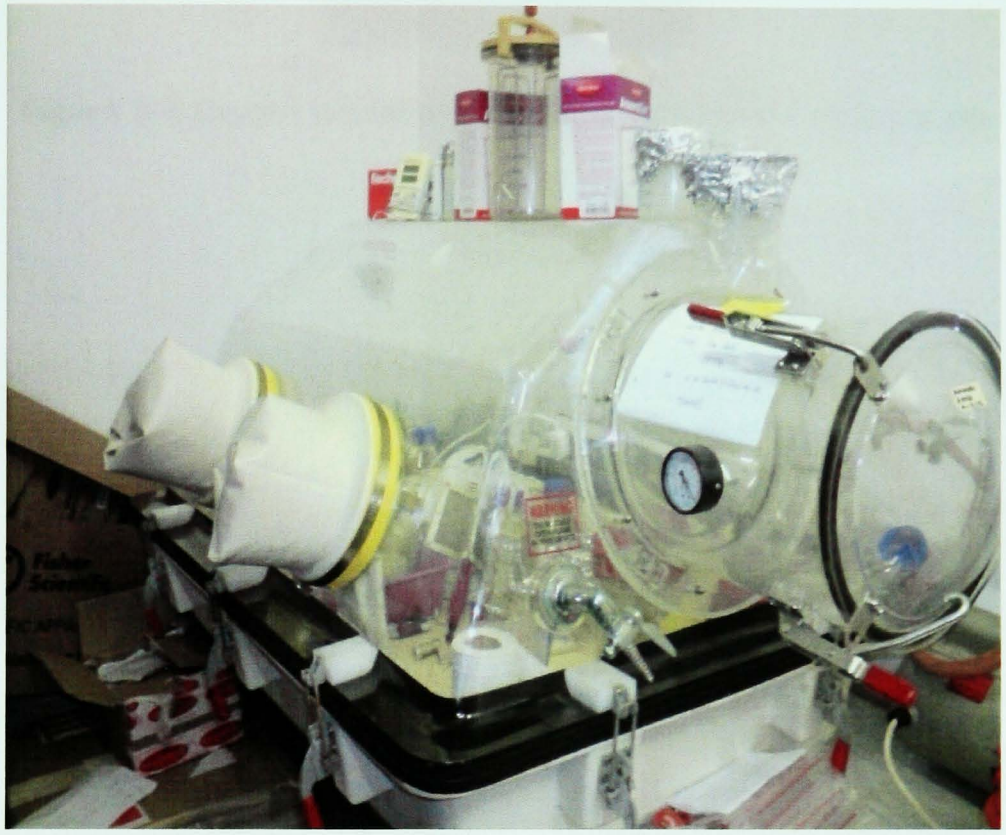


**Figure 3-3** Anaerobic chamber used for the culture of anaerobic organisms

The oxygen concentration within the chamber was monitored frequently using a Hach HQ10 handheld dissolved oxygen meter (Hach Company, Loveland, Colorado, USA), which monitors oxygen levels using luminescence technology. For more accurate oxygen measurements prior to sample manipulation, a PreSens Fibrox 3 Minisensor Oxygen Meter (PreSens Precision Sensing GmbH, Regensburg, Germany) was used. A sample of the atmosphere in the chamber was collected in a gas-tight vial containing an oxygen saturation patch (Figure 3-4). The patch was analysed using luminescence by the Fibrox minisensor to calculate oxygen concentration, which was logged for 5 minutes using the

Oxyview software (Figure 3-5). Oxygen concentrations were always kept below 0.2mg $L^{-1}$.

**Figure 3-4** Oxygen saturation patch inside atmosphere sampling vial

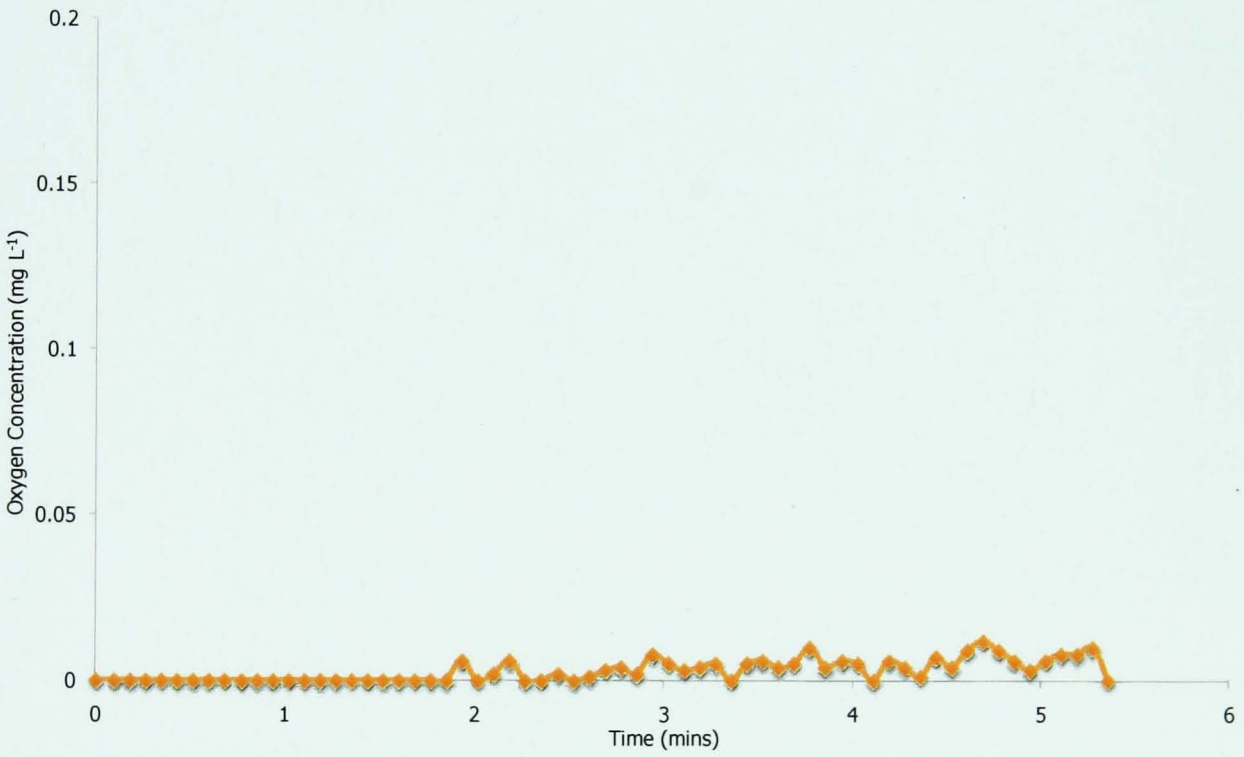



**Figure 3-5** Graph showing oxygen concentration within a gas sample taken from the anaerobic chamber. The increase in oxygen concentration after 2 minutes was due to oxygen infiltration into the sampling vial.

### 3.2.7.3 Sub-culture of Liquid Cultures onto Solid Media

Mixed-community liquid cultures were sub-cultured onto solid media to isolate bacterial colonies. Modified Postgate B media, and phenol mineral salts media (Section 3.2.7.1) were supplemented with 1.5% Noble agar (Kamimura and Araki, 1989). The plates were then pre-reduced in an anaerobic atmosphere (Section 3.2.7.2) for a minimum of 24 hours before use.

## 3.3 Results

### 3.3.1 Visualisation of Attached Microbes

Attached micro-organisms were stained and imaged (Section 3.2.1) to assess whether sufficient biomass was present for molecular (DNA) studies and cultivation.

Borehole 59

The quartz sand suspended in 2006 by Rizoulis (2008) was removed in January 2009 from 30mbgl and the grains were stained. Microbial cell attachment was visible on the quartz grains (Figure 3-6). Although the cells were not quantified, there were approximately 10 cells attached to each grain.

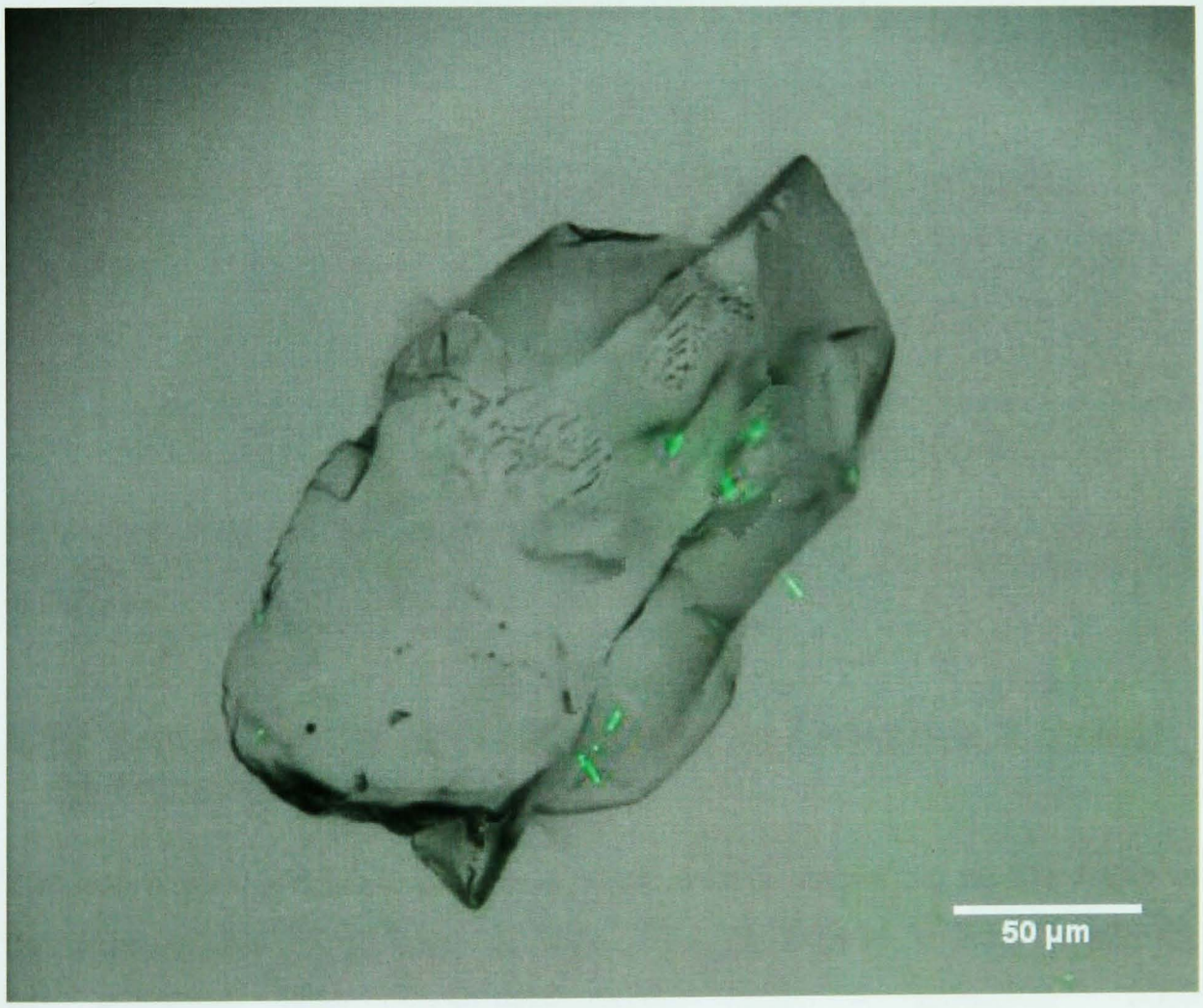



**Figure 3-6** Microorganisms (green) attached to quartz sand grain after incubation within borehole 59 (30mbgl) from August 2006 to Jan 2009.

Borehole 60

The sand bag containing local quarry sandstone suspended in 2003 by Wu (2002) was removed from 45mbgl at borehole 60 in August 2007. After fluorescent staining and imaging by epifluorescence microscopy, it was evident that there were no cells attached to the grains (Figure 3-7), which therefore prevented any downstream molecular analysis.

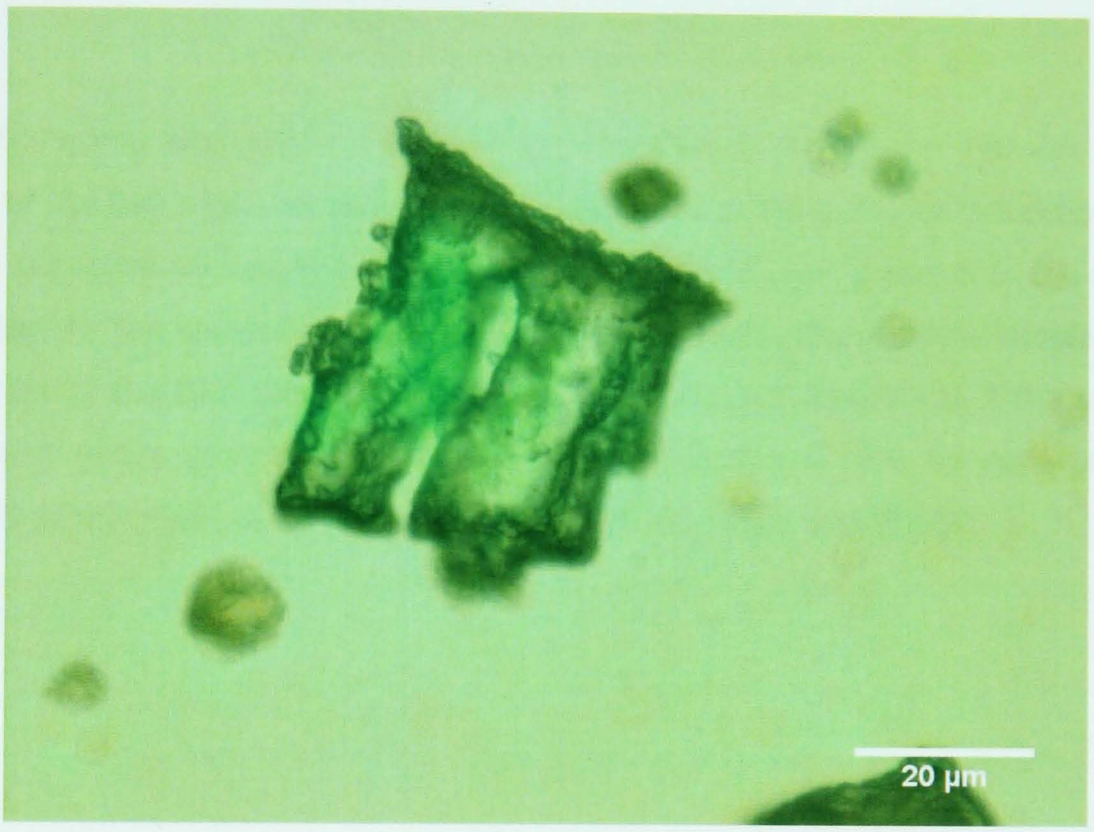



**Figure 3-7** Image of sand grain removed from borehole 60 in August 2007 and stained with Syto 9. No microbes are visible on the grain; the colour is due to auto-fluroescence of the grain.

### 3.3.2 DGGE Profiles of the Planktonic and Attached Microbial Communities at Borehole 59

DNA extractions yielded a varying amount of DNA depending on the depth in the borehole that the sample was taken. Two methods were used to quantify the extracted DNA; spectrophotometry using a Nanodrop™ 8000 Spectrophotometer and fluorimetry using Quant-it™ PicoGreen® dsDNA reagent (Section 3.2.3). Using fluorimetry, extracted DNA (Table 3-8) ranged from 0.6ng $ml^{-1}$ (8mbgl) to 7.6ng $ml^{-1}$ (12mbgl). Using spectrophotometry (Figure 3-8), however, the DNA concentration ranged between 3.3ng $ml^{-1}$ (8mbgl) to 43.6ng $ml^{-1}$ (12mbgl).

**Table 3-8** Quantity of DNA extracted from groundwater and quartz sand samples taken from borehole 59.

| Depth (mbgl) | Quantity (ng $\mu l^{-1}$) |
|---|---|
| 8 | 0.6 |
| 12 | 7.6 |
| 21 | 1.5 |
| 26 | 1.4 |
| 30 | 1.75 |
| Sand | 0.8 |

The Nanodrop also provides data on the absorbance at 260 and 280 nm. The ratio of the two measurements gives an indication of the purity of the extracted DNA, providing an insight into the contribution of salts and proteins to the DNA reading. As the spectrophotometer is not DNA specific, these ratios assess the accuracy of the DNA measurement. Results (Figure 3-8) indicate that the ratios for most of the samples are not near 1.8 (the optimum ratio for pure DNA). Only 3 samples had ratios within this region (21B1, 30B1 and BDHB).

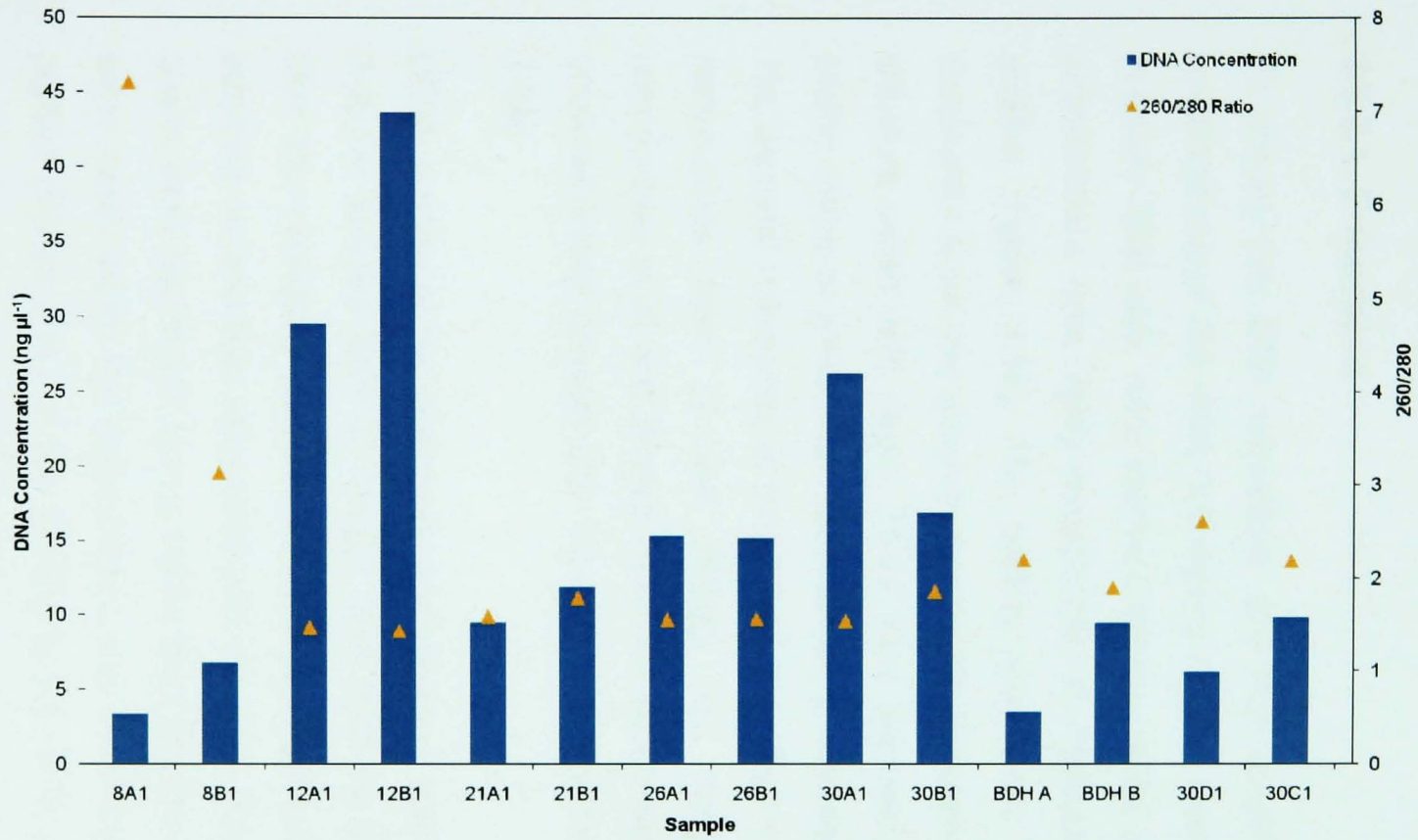



**Figure 3-8** Quantity of extracted DNA and 260/280nm ratios measured by spectrophotometry for samples removed from borehole 59

Extracted DNA was used as template for the PCR amplification of 16S rRNA (V3 region), *dsrB*, *napA* and Archaeal genes in order to profile the planktonic microbial communities in groundwater (2008 and 2009) and communities attached to quartz sand (January 2009). PCR was performed using approximately 3ng of DNA to ensure that DGGE band intensities were related to presence and not PCR drift.

### 3.3.2.1 Eubacteria

To ensure that DNA extractions and PCR amplifications were reproducible, amplifications of 16S rRNA (V3 region) isolated from groundwater samples from February 2008 only, were assessed initially. DGGE profiles showed that the PCR amplifications were highly reproducible, as replicates produced almost identical profiles (Figure 3-9a). The profiles also show that there was a diverse community down the groundwater profile at borehole 59, and that community structure varies with depth. There were approximately 10-20 bands on each profile, some of which were common at all sampled depths within the aquifer. The bacterial community at 8mbgl had the lowest number of strong bands, and communities from 12 and 30mbgl had more strong bands. Bacterial communities at 21 and 26mbgl had more strong bands than the communities in uncontaminated groundwater (8mbgl), but less than at 12 or 30mbgl (Figure 3-9a).

DGGE profiles of the planktonic and attached communities at the site (Figure 3-9b) in January 2009 at 30mbgl show that the attached community differed from the planktonic community. Common bands between the planktonic and attached communities were observed. However, the community attached to the quartz sand had fewer strong bands than the planktonic community from the same depth within the groundwater, but more less intense bands. The DGGE profiles (Figure 3-9c) of the planktonic microbial communities in August 2009 showed that the communities had more strong bands at 12 and 30mbgl than at 21 and 26mbgl. Included in Figure 3-9c is a comparison of the eubacterial community structure at 30mbgl between February 2008 and August 2009. It shows that even though band intensities differ, the community structure remained stable between samplings.

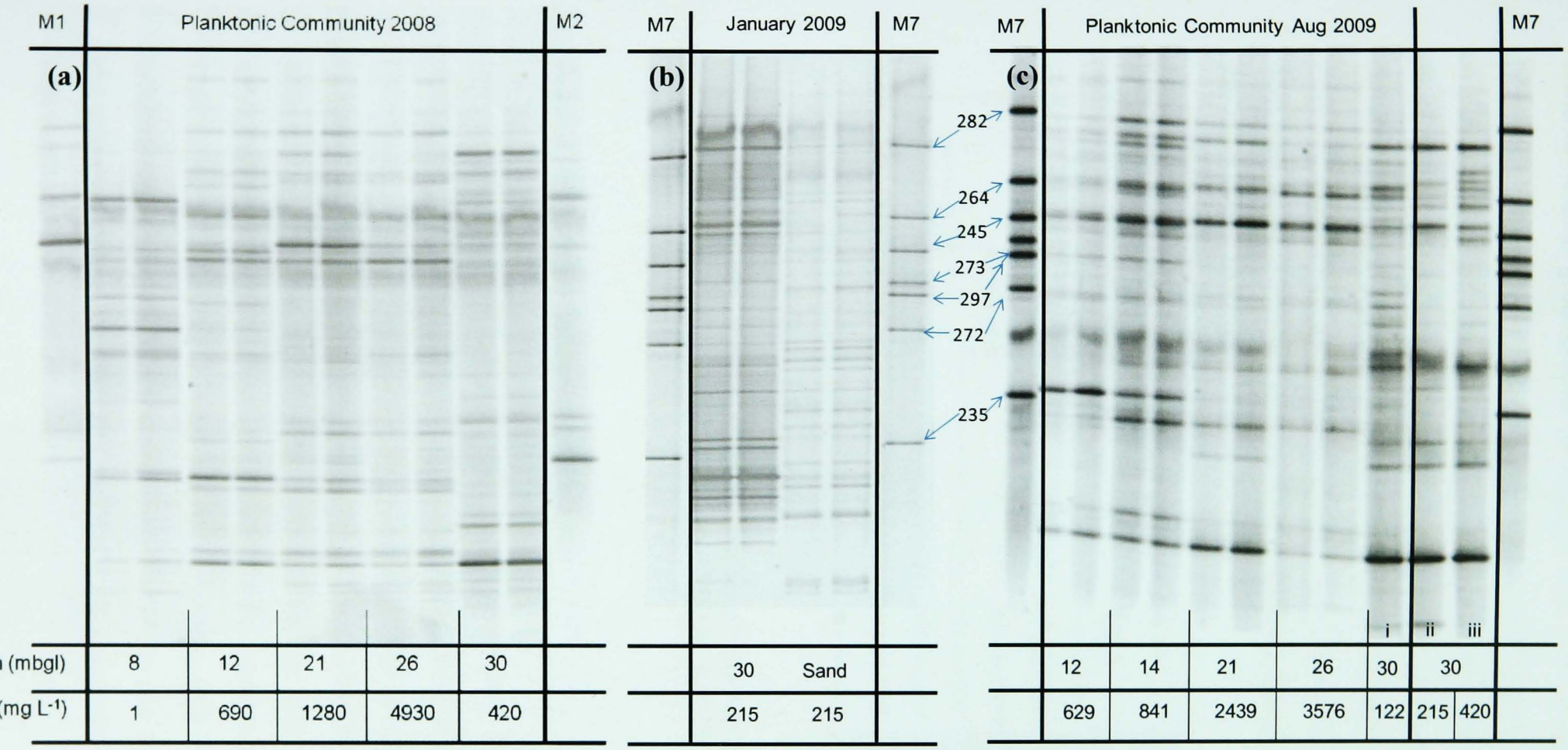



**Figure 3-9** DGGE profiles of planktonic and attached eubacterial communities within borehole 59. (a) Planktonic communities in February 2008 (b) planktonic and attached communities at 30mbgl in January 2009, (c) planktonic communities in August 2009; 30i = Aug 2009; 30ii = Jan 2009; 30iii = Feb 2008. M1 is a marker from the SRB cultivations from 12mbgl, and M2 is a marker from SRB cultivation inoculated with 30mbgl groundwater. M7 is a species marker containing PCR amplified DNA from Four Ashes isolates, which are numerically labelled (Section 3.2.5.1). Units of TPC and depth equal for all figures.

Cluster analysis (Figure 3-10) reveals that the planktonic community in uncontaminated groundwater (8mbgl) is only 44% similar to the communities found on the plume fringes and within the contaminant plume. The community on the plume fringe at 12mbgl is only 50% similar to the other depths sampled. At 21 and 26mbgl, the communities have an 81% similarity. 30mbgl is only 62% similar to 21 and 26mbgl. The communities at 30mbgl in February 2008 and January 2009 have a 74% similarity. The attached community is highly dissimilar to all the other communities, including the planktonic community at the same depth (30mbgl).

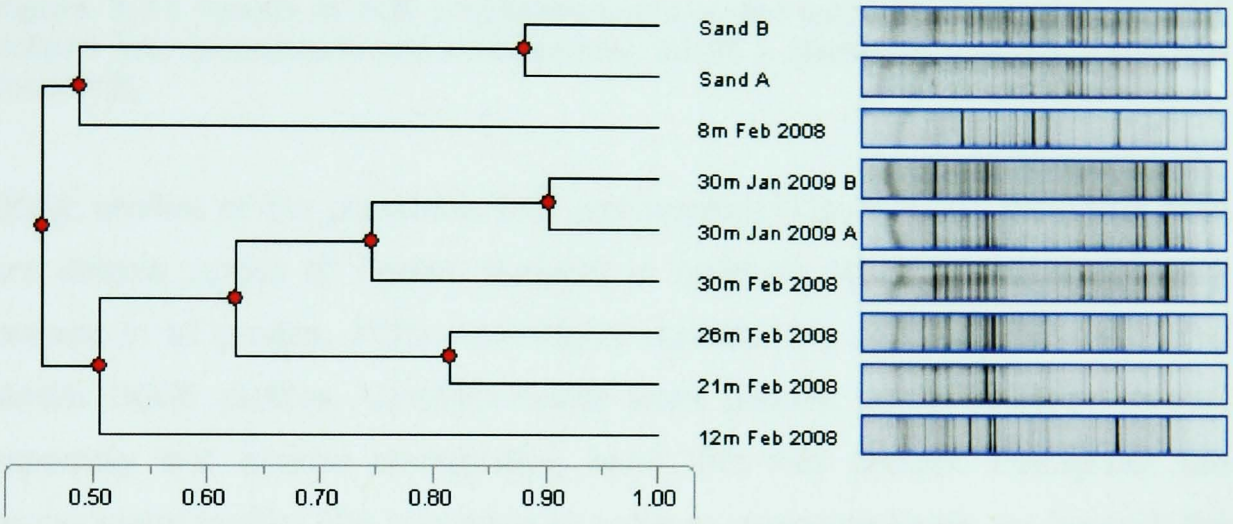



**Figure 3-10** Cluster analysis of DGGE profiles of planktonic communities from the borehole 59 groundwater profile and the attached community (Sand A and B) at 30mbgl.

### 3.3.2.2 Sulphate-Reducing Bacteria

PCR amplification and DGGE was performed using SRB-specific primers to assess the SRB community structure. Amplification of *dsrB* was possible for the planktonic communities (except in uncontaminated groundwater at 8mbgl), but PCR was unable to detect any SRB in the DNA isolated from the quartz sand. To ensure that this was not due to inhibition of the PCR by contaminants, DNA extracted from the attached communities was mixed with an equal volume of DNA extracted from the 30mbgl planktonic phase, and amplified. This yielded a positive result (Figure 3-11), suggesting that there are either no SRB present in the attached phase, or that SRB numbers are below the detection limits of the PCR method.

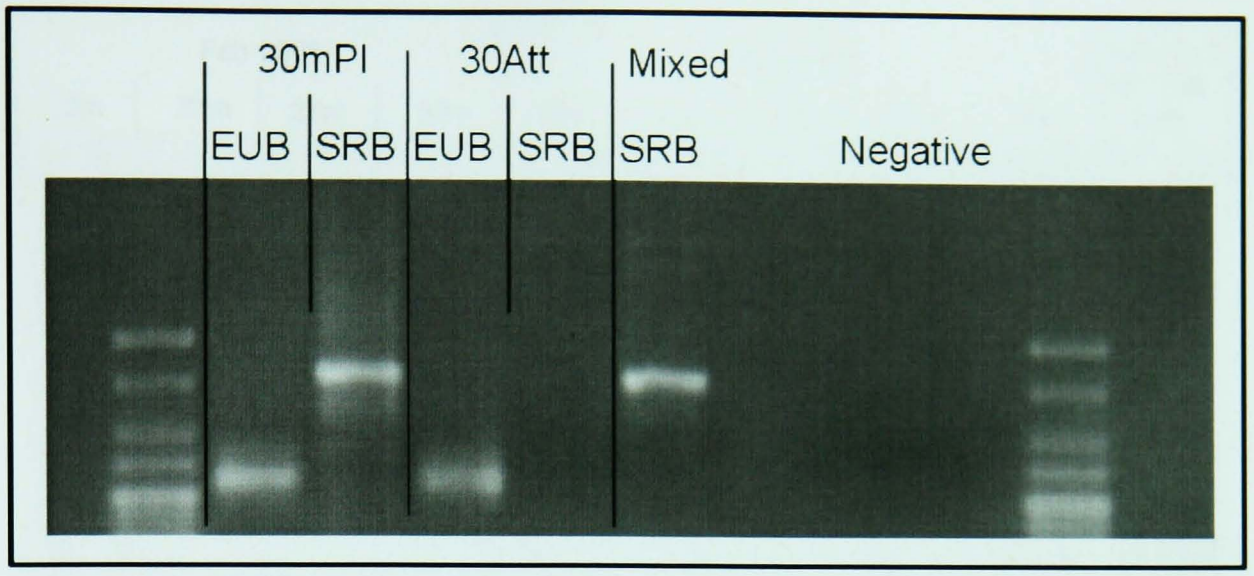



**Figure 3-11** Results of PCR amplification of the *dsrB* and 16S rRNA genes in DNA isolated from groundwater and sand samples. 30mPl = planktonic, 30m Att = attached community

DGGE profiles of the planktonic SRB communities (Figure 3-12) show that they are diverse across all depths sampled at borehole 59 as 10-15 bands were present in all profiles. PCRs were highly reproducible as they produced highly similar DGGE profiles. Common bands were present across multiple depths, especially one intense co-migrating band that was present throughout the groundwater profile. The intensities of some co-migrating bands are lower in the profiles from 21 and 26mbgl than in the profiles for 12, 14 and 30mbgl. The profiles also show that the SRB community contained a higher number of intense bands at 12, 14 and 30mbgl than at 21 or 26mbgl. DGGE Profiles of the SRB community at 30mbgl in February 2008, January 2009 and August 2009, show that the community was stable throughout sampling.

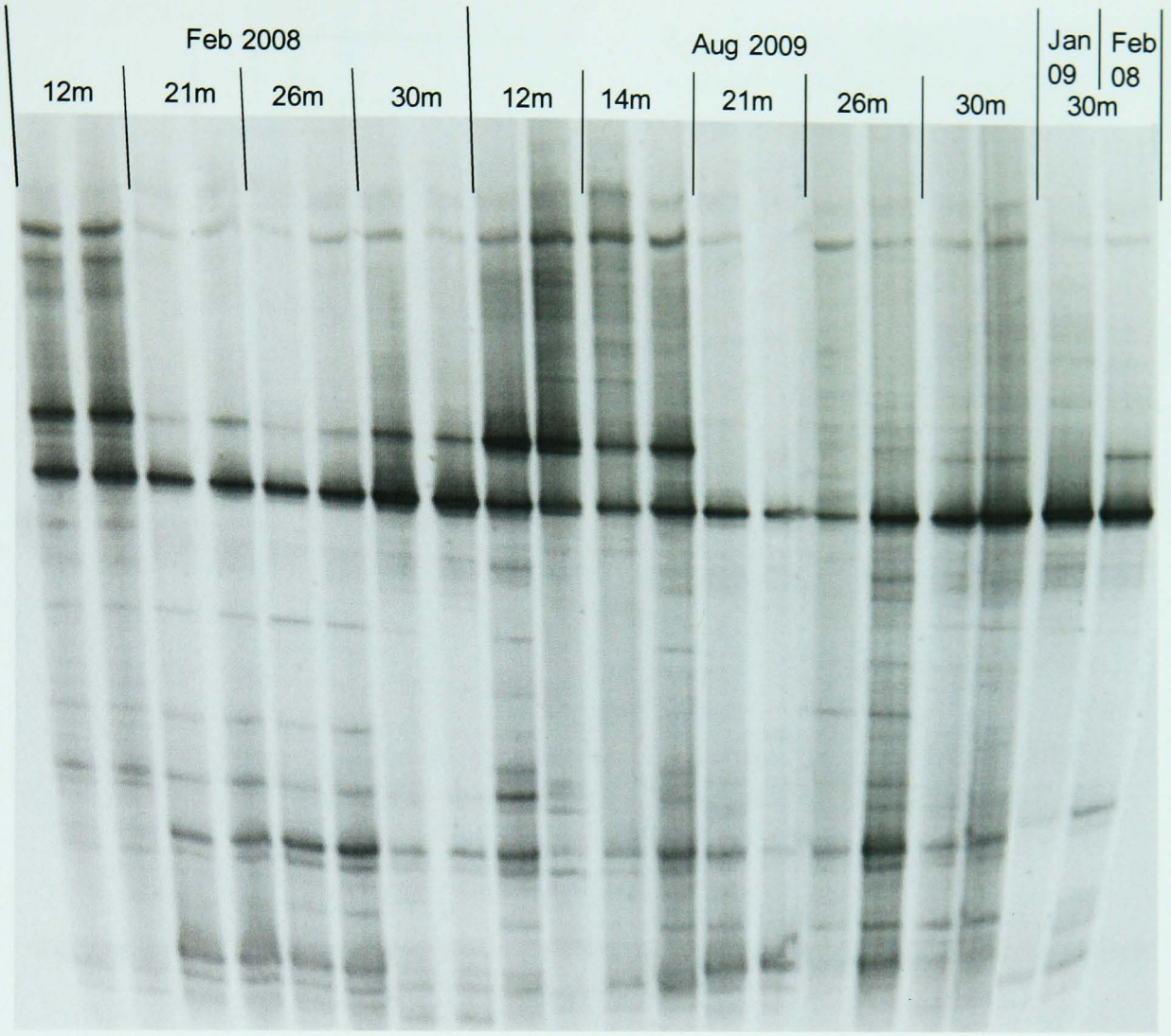



**Figure 3-12** DGGE profiles of planktonic SRB communities within borehole 59.

Cluster analysis (Figure 3-13) of the SRB DGGE profiles highlighted that the replicates from February 2008 clustered with a high degree of similarity. The SRB community from 12mbgl clustered with a 70% similarity to the SRB communities from 21 and 26mbgl. The SRB community at 30mbgl was 57% similar to the SRB communities found at 12, 21 and 26mbgl. In January 2009, the SRB community at 30mbgl was only 40% similar to the community at 30mbgl in February 2008, and only 47% similar to the community at 30mbgl in August 2009. In August 2009, the SRB communities at 12 and 14mbgl were only 56% similar to the SRB community found at 12mbgl in February 2008. The SRB community profile at 21mbgl in August 2009 was very dissimilar to previous samplings, clustering with only 35% similarity. Finally, the SRB communities found at 26 and 30mbgl were only 45% similar to the communities found at previous samplings.

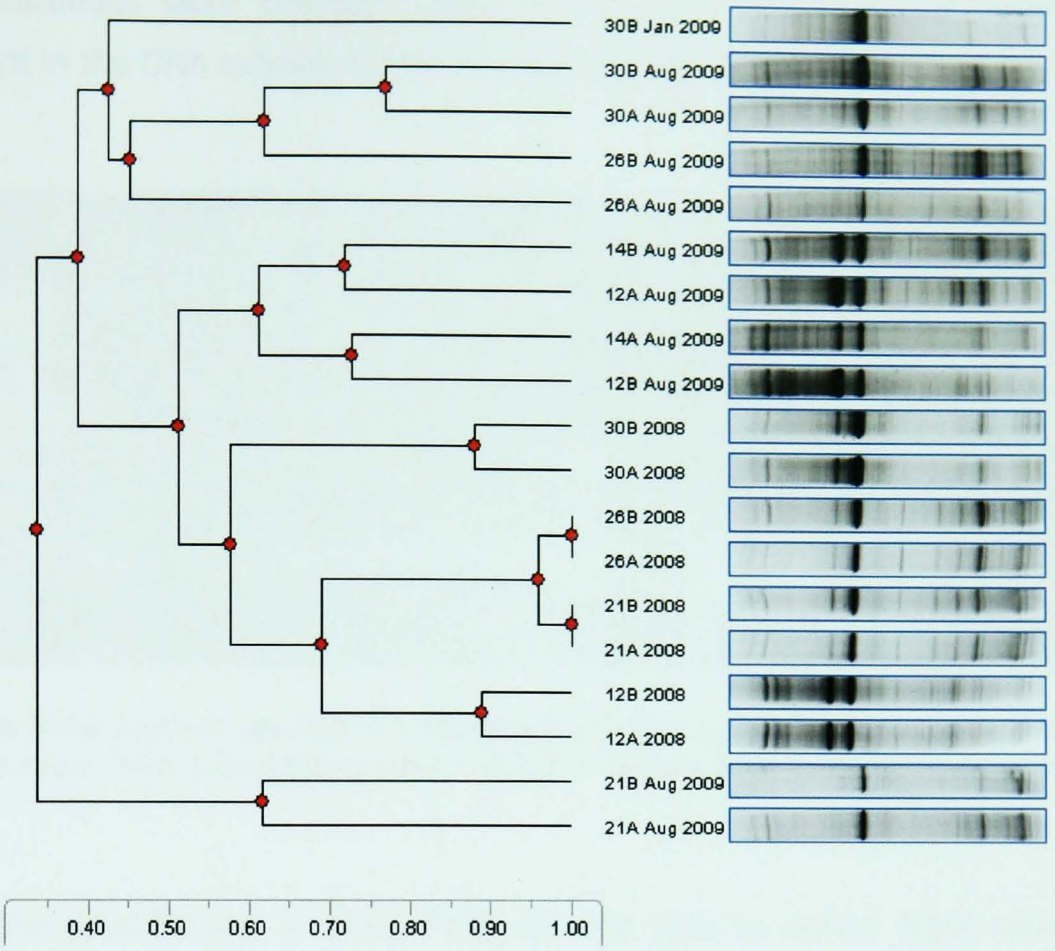



**Figure 3-13** Cluster analysis of the planktonic SRB community DGGE profiles within borehole 59

Bands on the dsrB DGGE were cut from the gel and the PCR amplification products were eluted (as described by Cunliffe and Kertesz, 2006). Attempts were made to reamplify the sequences using the unclamped reverse primer (DSR4R) only and the same PCR cycling method in preparation for sequencing. However, re-amplification was unsuccessful. This prevented the sequencing of the fragments to assess whether the primer pair from Geets *et al.* (2006) amplified sulphate-reducing bacteria only.

### 3.3.2.3 Nitrate-Reducing Bacteria

Initially, PCR amplification of the *napA* gene in extracted DNA was not possible, as the published conditions (Appendix 1, Alcantara-Hernandez *et al.*, 2009) were not suitable. Method development was therefore performed to find the best PCR recipe and thermocycling conditions for amplification of the *napA* gene. Nine combinations of primer (1, 5, 10mM) and $MgCl_2$ (1.5, 3, 5mM) concentration were tested (Figure 3-14). Results revealed that 3mM $MgCl_2$ and 5mM of each primer (recipe B) was the most suitable condition for use in PCR. These

concentrations were therefore used in the amplification of the *napA* gene present in the DNA extracted from the groundwater and quartz sand samples.

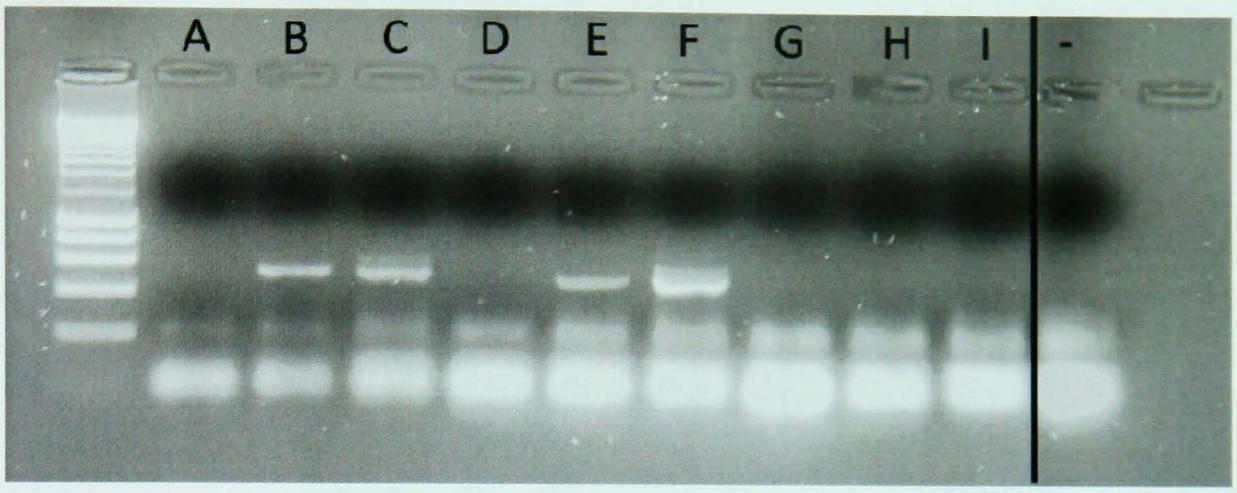



**Figure 3-14** Agarose gel showing results of amplification of the *napA* gene present in DNA isolated from groundwater from 14mbgl in borehole 59 using different recipes (A-I).

Like the amplification of *dsrB*, PCR was not able to detect *napA* within the community attached to the quartz sand. DGGE profiles (Figure 3-15) of the amplified *napA* gene in the planktonic communities show that they were diverse at all depths in the groundwater profile. PCR was found to be reproducible, as shown by the almost identical DGGE profiles for 30mbgl (August 2009). The amplification products of other samples were pooled to minimise the effects of PCR drift. A higher number of intense bands were found at 12 and 30mbgl and fewer intense bands were present in the profiles of the communities from 21 and 26mbgl. Co-migrating bands were present at all depths within the groundwater, suggesting that some dominant species are present throughout the aquifer.

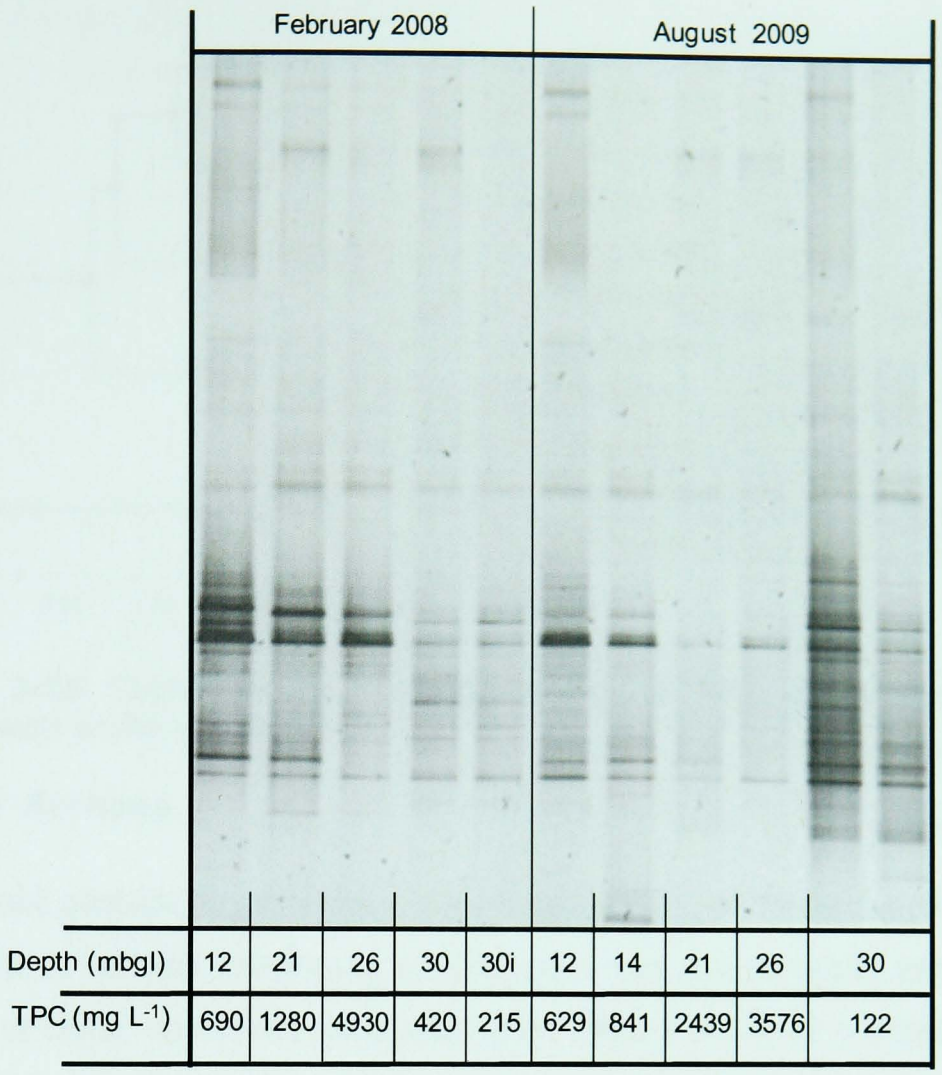



**Figure 3-15** DGGE profiles of planktonic NRB down the groundwater profile at borehole 59 in February 2008, January 2009 (30i) and August 2009.

Cluster analysis of the DGGE profiles (Figure 3-16) revealed that the NRB communities from 12mbgl in February 2008 an August 2009 were 80% similar. In February 2008, the NRB communities from 21 and 26mbgl were 82% similar, but this decreased to 66% in August 2009. The NRB communities at 21 and 26mbgl in February 2008 were only 35% similar to those NRB in August 2009. Also, the NRB communities from 30mbgl in February 2008 and January 2009 were 77% similar. However, in August 2009, the NRB community at 30mbgl was only 53% similar to previous samplings.

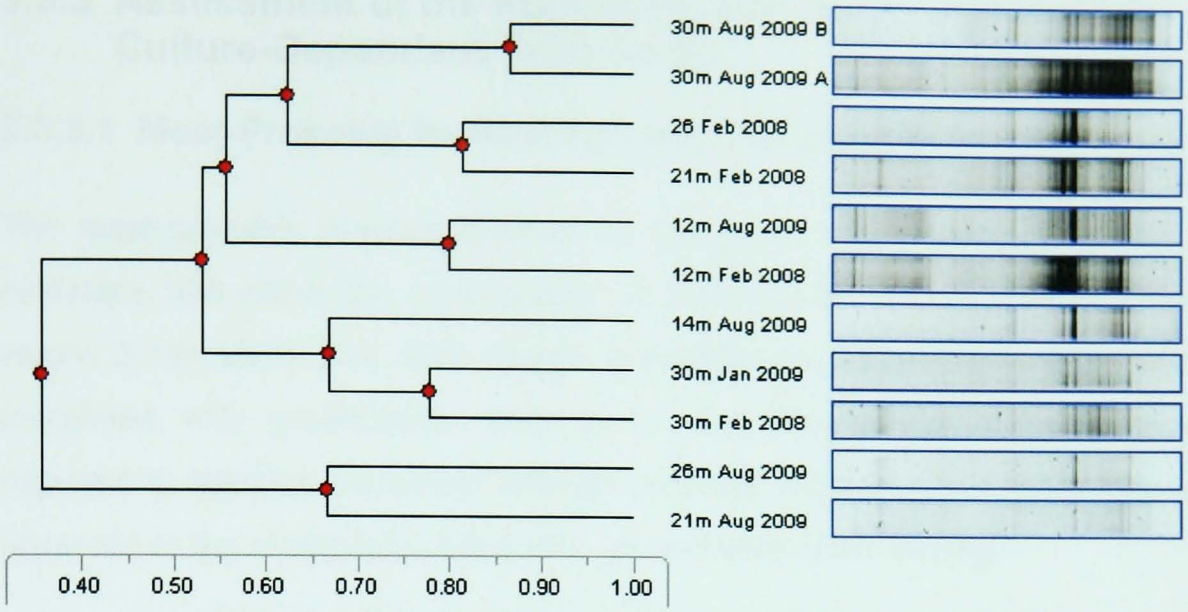



**Figure 3-16** Cluster analysis of DGGE profiles of NRB communities down the groundwater profile at borehole 59.

### 3.3.2.4 Archaea

It was not possible to generate a meaningful DGGE of the Archaeal community structure as suitable separation of amplicons was not possible, even though a variety of DGGE conditions were attempted (Figure 3-17). It is possible that the community contained few species, resulting in the separation and migration of only two strong bands. However, as no optimum conditions were found, the DGGE of archaeal fragments was dismissed.

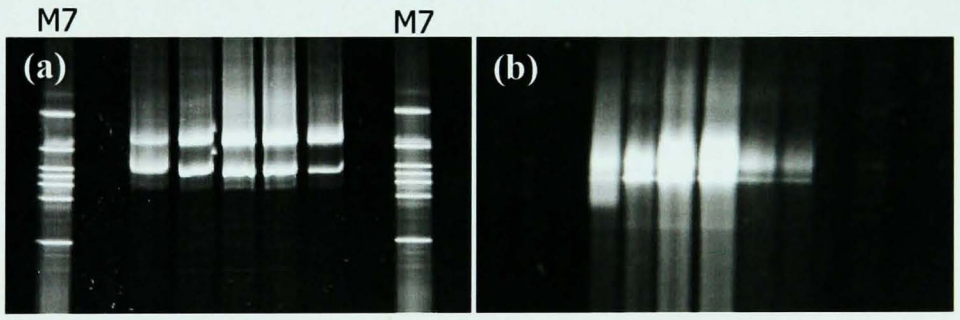



**Figure 3-17** DGGE profiles of archaeal fragments showing poor separation. (a) Samples were electrophoresed using the conditions published by Casamayour *et al.* (2000). (b) Samples were electrophoresed using the conditions used for eubacterial primers (30-60% denaturing gradient, 100v for 16 hours).

### 3.3.3 Assessment of the Planktonic SRB Community using Culture-Dependant Techniques

#### 3.3.3.1 Most-Probable Number Method of Cultivation

The most-probable number method of cultivation was used to quantify the cultivable SRB within the groundwater at borehole 59. Results (Table 3-9 and Figure 3-18) show that SRB growth predominantly occurred in the samples inoculated with groundwater from 8, 12 and 30mbgl. Less, slower growth occurred in samples inoculated with groundwater from 21mbgl. No growth was observed in the media inoculated with groundwater from 26mbgl.

**Table 3-9** MPN results of sulphate-reduction activity detected in Postgate B medium inoculated with groundwater.

| Depth (mbgl) | MPN (Cells $ml^{-1}$) |
|---|---|
| 8 | >180 |
| 12 | >180 |
| 21 | 6 |
| 26 | 0 |
| 30 | >180 |

**Figure 3-18** MPN cultivation of SRB showing black FeS precipitate characteristic of bacterial sulphate-reduction

Growth in the phenol mineral salts media was difficult to see due to the amount of suspended sediment within the groundwater inoculum. This prevented the

use of optical density as a measurement of growth. Therefore, direct counts using epifluorescence microscopy and DAPI (Section 2.2.4) were used to assess the extent of any growth. Results showed that the highest amount of growth, as indicated by a cell count greater than that of the groundwater inoculum, occurred in the cultivations inoculated with groundwater from 8mbgl and 30mbgl. The most growth also occurred in the low phenol media (0.5mM). No growth was observed in samples inoculated with groundwater from 21 or 26mbgl. Also, in the cultivations containing the most growth, black precipitates formed, signalling reducing conditions and SRB activity.

The samples that contained growth were sub-cultured onto solid Postgate B media (Section 3.2.7.3). Also, 1ml of colony suspension was removed for community analysis using PCR amplification of 16S rRNA and DGGE (Section 3.2.4/5), to assess the cultivable community structure down the groundwater profile at borehole 59. A final sample was taken and stored at -80C with 20% v/v glycerol (Franco *et al.*, 2005).

### 3.3.3.2 DGGE Profiling of Cultivable Planktonic SRB

DNA was extracted from the mixed cultures using the methods outlined in Section 3.2.2.2. The V3 region of the 16S rRNA gene was amplified and DGGE profiling was performed to assess community structure in each high- and low-inoculation density cultivation. It was evident that although co-migrating bands were present and each sample contained at least 3 intense bands (Figure 3-19), there was a difference in community structure with depth. Cultivations inoculated with groundwater from 8mbgl had fewer bands, whereas cultivations inoculated with groundwater from 12mbgl had a higher number of bands on the DGGE. High- and low-inoculation density samples showed high variation in community structure at every depth, excluding 8mbgl.

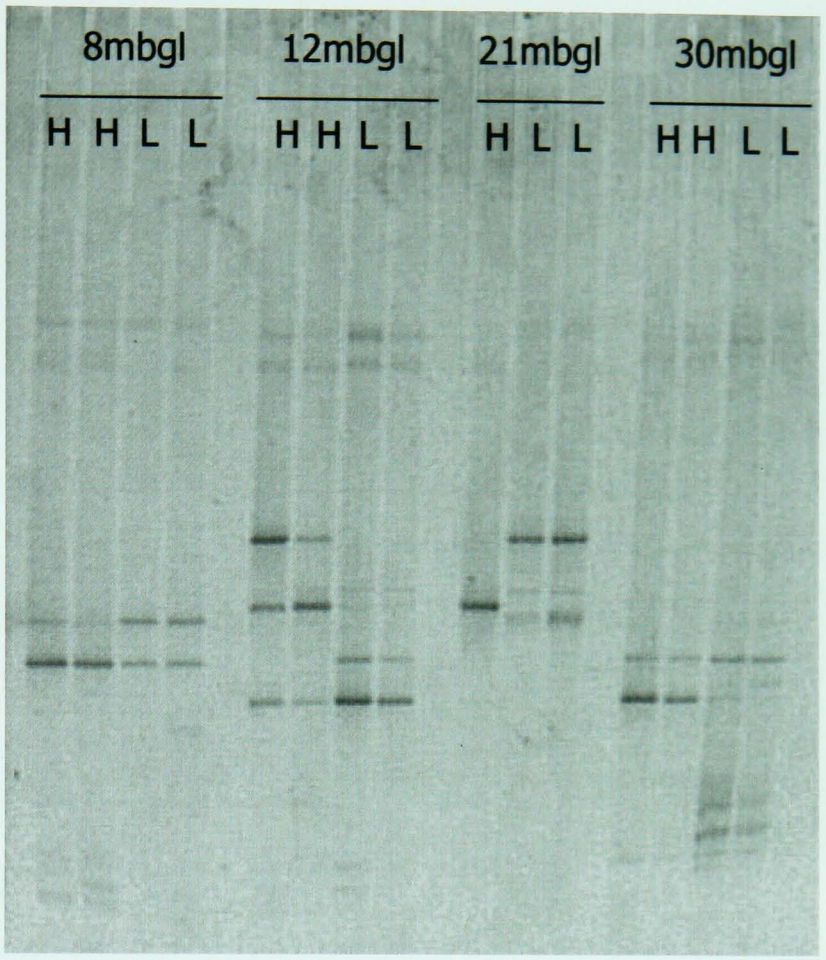



**Figure 3-19** DGGE profiles of cultivable SRB in groundwater from borehole 59. H = high inoculation density samples; L = low inoculation density samples

Cluster analysis of the DGGE profiles (Figure 3-20a) found that all samples were reproducible, as profiles were nearly identical. Samples cultivated from similar depths clustered together. The cultivable SRB community from 8mbgl was markedly dissimilar to other depths within the groundwater profile (22% similarity). Cultivable communities from the plume fringes (12 and 30mbgl) clustered with only 40% similarity. The high-inoculation density cultivation of 21mbgl was more similar (67%) to the low-inoculation density samples of 12mbgl than its own low-inoculation density samples. Intra-depth high and low inoculation density variation had a similarity of between 55 and 67%. Principle component analysis confirmed these observations (Figure 3-20b), showing the clustering of 12mbgl and 21mbgl (HID) samples, 30mbgl and 8mbgl.

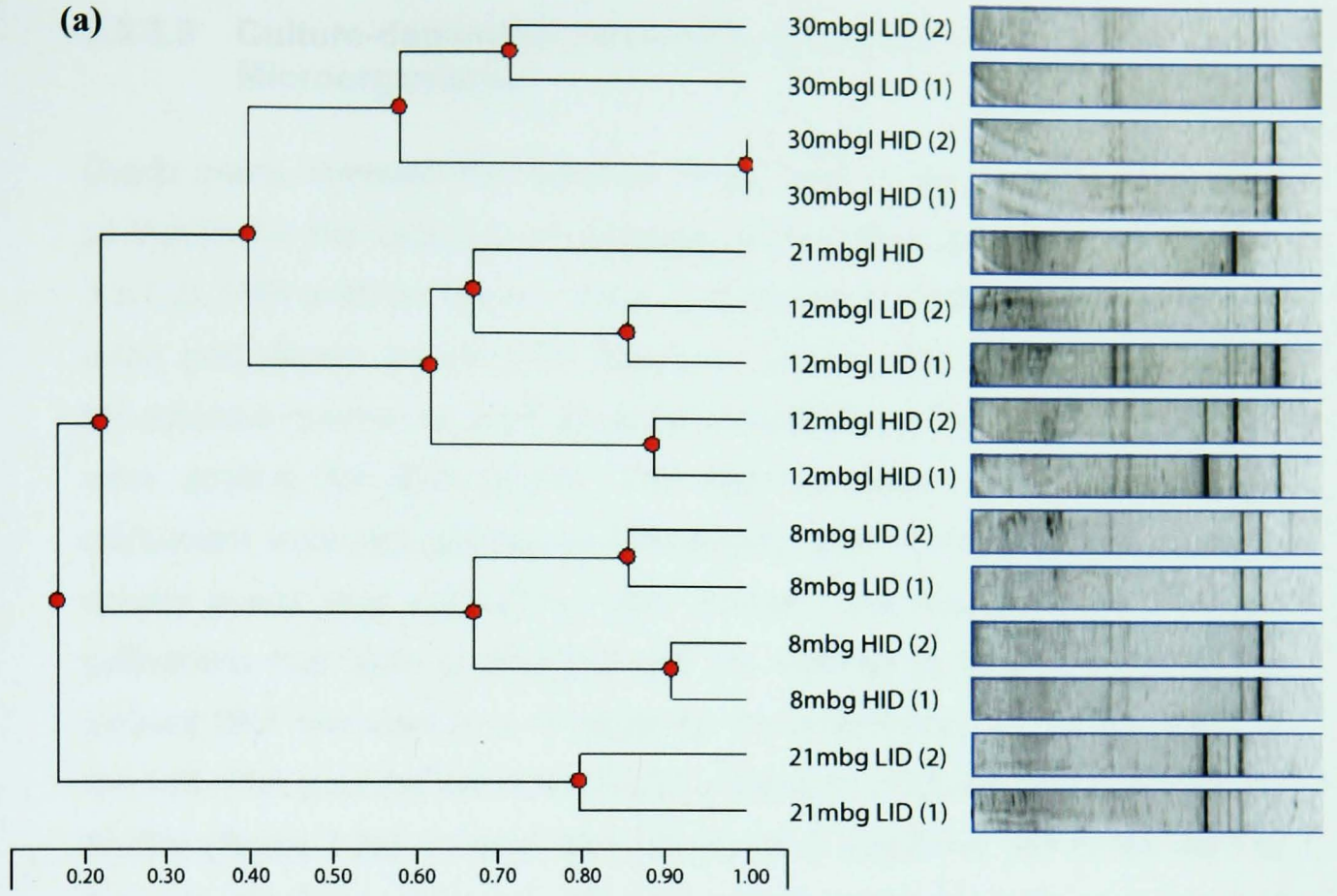



**Figure 3-20** Cluster analysis (a) and principle component analysis (b) of cultivable SRB DGGE profiles from down the groundwater profile at borehole 59.

### 3.3.3.3 Culture-dependant Assessment of Attached Microorganisms

Quartz grains, removed from borehole 59 (30mbgl) in January 2009, were used as inocula for the detection of cultivable SRB in Postgate medium B (Section 3.2.7.2). MPN analyses (Section 3.2.7.1) of planktonic SRB were also performed using groundwater purged from 30mbgl in January 2009, as a comparison to the attached community. After 10 weeks of incubation, all planktonic cultivations were positive for SRB growth. The high-inoculation density quartz sand cultivations were also positive for SRB activity. Only 4 of the 6 low-inoculation density quartz sand cultivations were positive. DNA was extracted from the cultivations that were positive for SRB (as outlined in Section 3.2.2.2). The isolated DNA was used as a template for the amplification of the V3 region of the 16S rRNA gene for use in the DGGE profiling of cultivable SRB communities. Profiles (Figure 3-21) revealed that cultivations of planktonic SRB contained 3 to 5 bands, showing low diversity. High- and low-inoculation density cultivations of planktonic SRB were almost identical. Also, the profiles were similar to the cultivable SRB community at 30mbgl in February 2008 (Figure 3-19). Cultivations inoculated with the quartz grains contained more bands in the DGGE profiles than the cultivations inoculated with groundwater, some of which were common with the cultivable planktonic SRB community. Low-inoculation density cultivations of attached SRB had fewer bands than the high-inoculation density cultivations. Most bands in the profiles of the cultivable attached SRB (aside from 1 or two) had very low intensity.

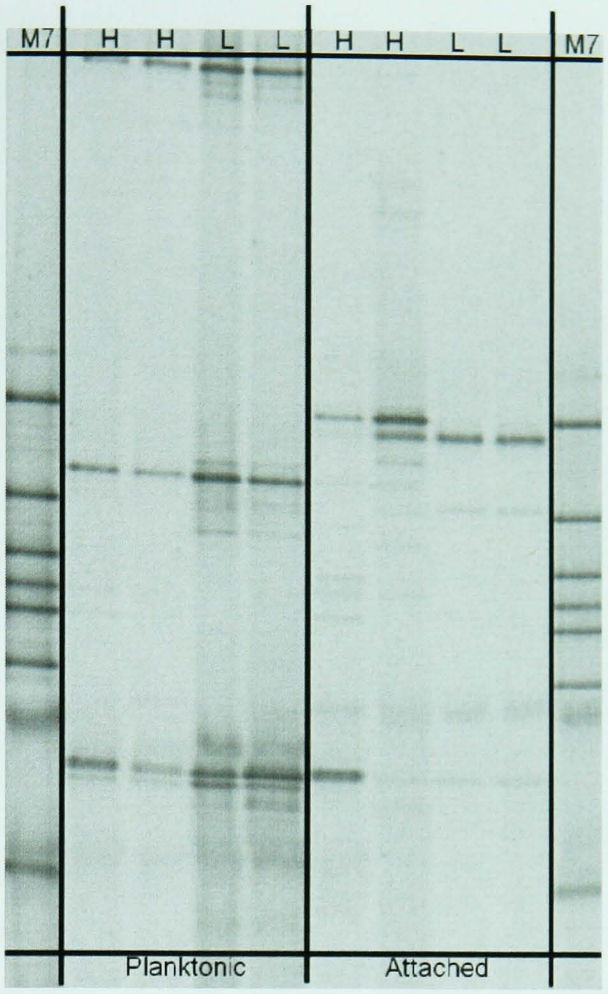



**Figure 3-21** DGGE profiles of cultivable SRB detected in Postgate medium 'B' after inoculation with groundwater from, and quartz grains incubated at, 30mbgl in borehole 59. H = high inoculation-density, L= low inoculation-density, M7 = eubacterial species marker.

### 3.3.3.4 Analysis of Isolated SRB Colonies

Primary liquid cultures showing evidence of SRB activity were sub-cultured onto solid SRB media. It was possible to isolate different colonies from liquid cultivations inoculated with groundwater from 8, 12 and 30mbgl, but not from the cultivations inoculated with groundwater from 21mbgl or the quartz sand. A sample of each colony was picked and used for community profiling (using DGGE) and direct sequencing (as outlined in Section 3.2.6). DGGE profiling (Figure 3-22) reveals that all colonies were multiply banded. The profiles of the isolates are comparablewith the profiles of the mixed-community cultivations, showing that the multiple bands on the profiles of the isolates account for many of the bands in the DGGE profiles of the mixed-communities from liquid culture (Figure 3-19).

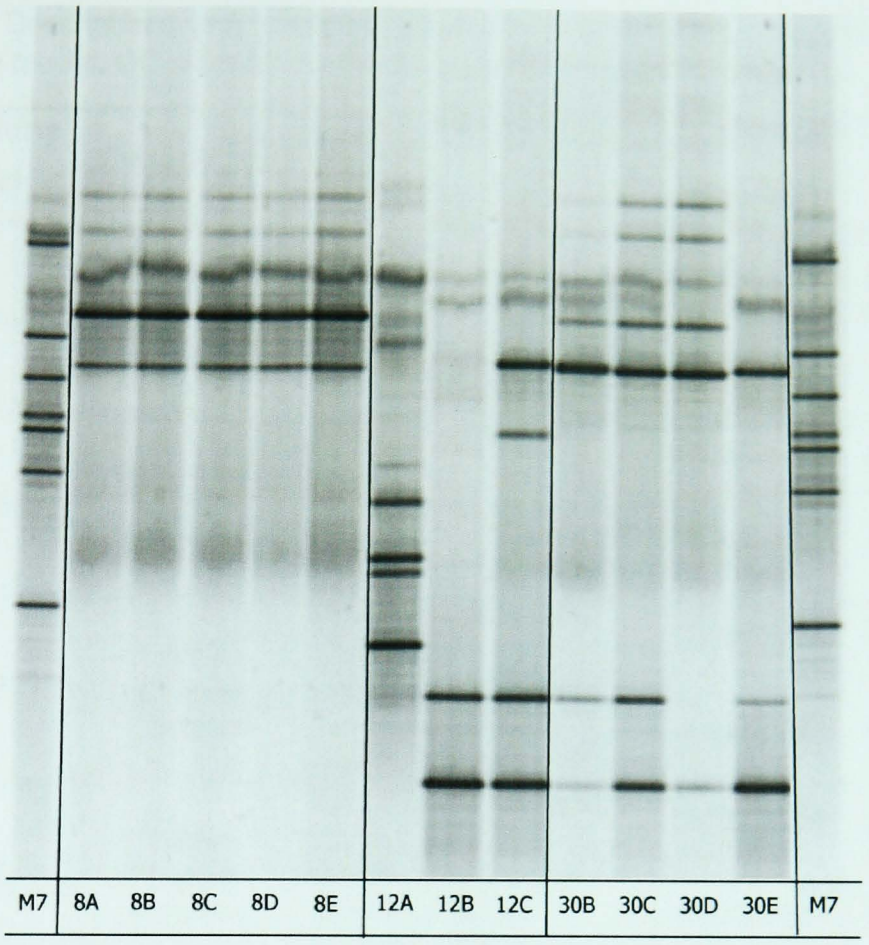



**Figure 3-22** DGGE profiles of SRB colonies isolated on Postgate media from SRB cultivations. Number prefix on each lane corresponds to the depth from which the groundwater was collected for the inoculation of primary cultivations. M7 = species marker (see Section 3.2.5.1).

Identification of the closest phylogenetic relative using nucleotide blast search revealed that the colonies isolated from SRB cultivations inoculated with groundwater from 8mbgl were closely related to *Firmicutes* sp. and *Desulfosporomusa polytropa*, a lake-sediment sulphate-reducer (Table 3-10). Colonies isolated from the cultivations of SRB from 12mbgl revealed that the closest relatives were not SRB, but psychrophillic organisms or phenol-degraders. One colony was closely related to the iron-reducing organism *Georgenia ferrireducans.* Colonies isolated from the cultivations of SRB from 30mbgl were anaerobic phenol degraders, fermenters and arctic bacteria (Table 3-10). The table also includes basic descriptions of the colonies.

**Table 3-10** Descriptions and closest relatives of the sequenced bacteria isolated on SRB-selective media. QC = query coverage, Max SS = sequence similarity.

| Depth | Colony ID | Description | Closest Phylogenetic Relative (GenBank) | Accession Number | QC | Max SS |
|---|---|---|---|---|---|---|
| 8m | 8A-1 | Yellow, irregular colonies | *Firmicutes* (iron-rich creek soil) | GQ342340 | 98% | 98% |
| | | | *Desulfosporomusa polytropa* (lake-sediment SRB) | AJ006606 | 95% | 88% |
| | 8A-3 | Clear, round convex colonies | *Firmicutes* (iron-rich creek soil) | GQ342340 | 98% | 98% |
| | | | *Desulfosporomusa polytropa* (lake-sediment SRB) | AJ006606 | 96% | 89% |
| | 8B-2 | Yellow, irregular colonies | *Firmicutes* (iron-rich creek soil) | GQ342375 | 98% | 98% |
| | | | *Desulfosporomusa polytropa* (lake-sediment SRB) | AJ006606 | 96% | 89% |
| | 8C-1 | Yellow, irregular colonies | *Firmicutes* (iron-rich creek soil) | GQ342375 | 98% | 98% |
| | | | *Desulfosporomusa polytropa* (lake-sediment SRB) | AJ006606 | 96% | 89% |
| | 8C-4 | Clear, round convex colonies | *Firmicutes* (iron-rich creek soil) | GQ342375 | 98% | 99% |
| | | | *Desulfosporomusa polytropa* (lake-sediment SRB) | AJ006606 | 96% | 89% |
| 12m | 12A-1 | Large, clear amorphous colonies | *Rahnella sp.* (proteobacteria) | AM167519 | 98% | 99% |
| | | | *Tiedjeia arctica* (Arctic waters) | DQ107523 | 98% | 99% |
| | 12B-2 | Small, clear, round and convex | Uncultured bacterium clone | EF575711 | 93% | 93% |
| | | | *Pseudomonas veronii* (phenol-degrader) | AB494445 | 93% | 91% |
| | 12C-1 | Small, clear, round and convex | *Georgenia sp.* (nickel mine) | EU863843 | 98% | 94% |
| | | | *Georgenia ferrireducans* (Fe(III)-reducer) | EU095256 | 98% | 94% |

| | | | | | | |
|---|---|---|---|---|---|---|
| **30m** | 30A-1 | Clear, round, convex colonies | *Pseudomonas sp.* (lake-water) | GU932965 | 91% | 67% |
| | | | *Pseuodomonas veronii* (arctic bacterium) | AM933507 | 83% | 67% |
| | 30B-1 | | Uncultured bacterium clone | FJ674543 | 94% | 95% |
| | | | *Vellonellacae* bacterium (fermentative bacteria) | AB298734 | 98% | 89% |
| | 30C-1 | | *Pseudomonas sp.* | GQ417855 | 98% | 97% |
| | | | *Pseudomonas veronii* (phenol degrader) | AB494445 | 98% | 87% |
| | 30D-1 | | Uncultured bacterium clone (anaerobic wastewater treatment) | HM346803 | 98% | 93% |

## 3.4 Discussion

### 3.4.1 Methods of Detection and Profiling of Microbial Communities

The structure of planktonic microbial communities down the groundwater profile, and the communities attached to quartz sand suspended at 30mbgl, was investigated using PCR amplification and DGGE profiling. Groundwater samples were taken in February 2008 (8, 12, 21, 26, 30mbgl), January 2009 (30mbgl) and August 2009 (12, 14, 21, 26, 30mbgl), and the quartz sand suspended in 2006 by Rizoulis (2008) was removed in January 2009.

DNA was extracted (Section 3.2.2), before quantification using two methods (Section 3.2.3). This was to find the most suitable technique for quantifying DNA isolated from these environmental matrices. Results (Figure 3-8 and Table 3-8) show marked differences in measured DNA concentration. DNA concentrations measured using fluorimetry were 5-10x less than concentrations measured by spectrophotometry. However, the ratio of 260 and 280nm measurements from the spectrophotometer revealed that the DNA was not pure, in all but 3 samples. This suggests that proteins and salts in the sample contributed to the high DNA concentrations found by the spectrophotometer. Fluorimetry using a DNA-specific stain is not susceptible to interference from impurities. Therefore, the results suggest that the quantification of DNA extracted from environmental matrices is best performed using a DNA-specific technique such as fluorimetry. Also, the impurities present in the sample suggest that the use of a proprietary kit (Section 3.2.2) to isolate DNA does not completely remove all impurities. Other methods may be more suitable for the isolation of microbial DNA from contaminated samples. However, Rizoulis (2008) found the MoBio Microbial DNA Isolation kit to be more sensitive than phenol-chloroform extraction methods.

The isolated total DNA was used as a template for PCR amplification of the V3 region of eubacterial 16S rRNA, *dsrB* (dissimilatory sulphate-reductase), *napA* (periplasmic nitrate-reductase) and Archaeal genes for use in DGGE community profiling. For ease of analysis, all chosen primer pairs were published sets. To probe for the presence of SRB, primers targeting the whole *dsrAB* gene were

available from the literature (Wagner *et al.*, 1998; Schmalenberger *et al.*, 2007). However, these were designed for use in terminal-restriction length polymorphism (TRFLP) studies of community structure, due to the length of the amplified product (1.9 kb). The work presented in this thesis is concerned only with DGGE, and therefore published primers that amplify *dsrB,* the beta subunit of the dissimilatory sulphate-reduction gene, were chosen. *dsrA* and *dsrB* are near exact replicates, however (Geets *et al.*, 2006).

Also, nitrate-reduction mechanisms other than *napA* exist, including *narGH*, which is membrane-bound nitrate-reduction. However, the PCR amplification targeting *narGH* has, like the *dsrAB* primers, been used for RFLP studies due to the length of the target sequence (1.9kb, Martinez-Espinosa *et al.*, 2006). Attempts have been made to amplify the *narG* subunit for use in DGGE studies, but these were unsuccessful (Alacantara-Hernandez *et al.*, 2009). Therefore, the successful *napA* primer pair was chosen. Given more time, it would have been possible to attempt the amplification the *narG* gene using published primers (Philipot *et al.,* 2002), before performing T-RFLP or RFLP analysis to assess basic community structure. Also, RFLP could have also been performed to assess the archaeal community structure, as DGGE profiling was not possible due unsuitable separation of amplicons under a variety of conditions.

DGGE was chosen as the technique to profile the microbial communities at Four Ashes as previous work at the site (Rizoulis, 2008) used DGGE. This enabled past results to be compared with the results in this study. Also, DGGE has been successfully applied in many studies to assess microbial community structure, both in environmental and clinical microbiology (Ercolini, 2007; Webster *et al.*, 2007). It is a cheap and rapid technique that can be used in conjunction with other techniques, such as Southern blotting (Ovreas *et al.*, 1997) and sequencing to generate a huge data set to compare the diversity and structure of mixed communities, cultures and isolates. However, DGGE is a technique that is limited in terms of resolution, as it can only detect organisms that form greater than 1% of the community. Also, it is difficult to reproduce DGGE gels, as conditions are difficult to recreate from gel to gel, making comparisons difficult. Finally, bands within a DGGE do not always indicate the present of one phenotype, and multiple bands can be due to the presence of a single organism

(as shown in the results of the DGGE profiling of anaerobic isolates, Section 3.3.3.4), it is not possible to infer any trends in microbial community diversity using DGGE profiling. More accurate methods have been used to detect rare organisms and to assess community diversity.

An alternative method used to assess the structure and diversity of the microbial communities is clone libraries. This involves the PCR amplification of 16S rRNA prior to cloning in a plasmid vector, preparation and sequencing. The sequences generated can be used to identify the phylogenetic groups and functional diversity present within the microbial communities. Previusly, this method has been successfully applied to investigate the microbial communities in phenol contaminated environments (Rizoulis, 2008; Manefield *et al.,* 2005; Padmanabhan *et al.*, 2003). Therefore, the use of clone libraries would provide more information and facilitate a deeper investigation of the aneorbic microorganisms present at Four Ashes in comparison to the sole use of community profiling.

Pyrosequencing could also be used to sequence and taxonomically identify a large number of microorganisms using PCR amplified fragments, providing a near complete analysis of the microbial community (Ronaghi, 2001). The use of this technique would allow the direct sequencing of rare organisms present within the attached community at Four Ashes, allowing inference to be made with respect to the variations between the attached and planktonic community structure at the site.

### 3.4.2 Planktonic Microbial Community Structure down the Groundwater Profile at Borehole 59

The structure of the eubacterial and anaerobic communities was investigated at 6 depths (8, 12, 14, 21, 26, 30) at borehole 59, over 3 separate samplings. This is the first study to target a variety of genes using DGGE at one single contaminated site. Comparison of the communities was performed using cluster analysis of DGGE profiles. Samples were taken based on the groundwater hydrochemistry profile, so that communities in uncontaminated groundwater and at depths containing varying amounts of TPC and TEA were investigated.

DGGE profiles from February 2008 (Figure 3-9) of the total eubacterial communities indicate that the planktonic community varies with depth and contaminant concentration in the groundwater profile. Cluster analysis (Figure 3-10) confirms the observations from the DGGE profiles, as the community at 8mbgl clusters separately to the communities at depths where contamination is present. Also, the communities found at depths where contamination is highest (21 and 26mbgl) cluster separately to communities found at the contaminant plume fringes (12 and 30mbgl). Finally, the fact that communities at 12 and 30mbgl cluster together, even though they are 18m apart, suggests that the TPC has an influence on the planktonic eubacterial community structure. Other research has found that contaminant load influences microbial community structure. Evans *et al.* (2004) found that petroleum and nutrient levels influenced *Pseudomonas* populations in contaminated soils. Also, Fahy *et al.* (2005) found that microbial communities within a BTEX-contaminated aquifer were influenced by the benzene contamination, as the communities found in contaminated water differed from those of uncontaminated groundwater. It was also found that communities within areas of contamination varied due to the differences in other groundwater geochemical parameters (e.g. oxygen, methane, sulphate).

DGGE profiles of the total eubacterial planktonic communities from January 2009 and August 2009 (30mbgl only) indicate that the community structure within the groundwater profile has remained stable throughout samplings (Figure 3-9c), despite changes in band intensities. This observation was confirmed by cluster analysis, as the communities from 30mbgl in 2008 and January 2009 cluster with 77% similarity (Figure 3-10). Communities from 21 and 26mbgl in August 2009 differ from the communities in February 2008, suggesting that the changes in TPC due to the onset of the pump and treat system (see Chapter 2) affected the community structure (Figure 3-9).

DGGE profiling of the planktonic SRB community (Figure 3-12) reveals similar results to that of the eubacterial community, as the community structure is different on the plume fringes (12 and 30mbgl) than in highly contaminated groundwater (21 and 26mbgl). The amplification of *dsrB* in DNA extracted from uncontaminated groundwater (8mbgl) was not possible, suggesting that SRB

numbers were below the detection limits of the PCR method. At 21 and 26mbgl, where TPC ranged from 1280 to 4930mg $L^{-1}$, communities cluster together and are different those on the fringes (Figure 3-13). This could be explained by the high levels of TPC, resulting in a toxic effect on the SRB and inhibiting their activity (Spence *et al.,* 2001).

Differences between the NRB community structure on the plume fringes (12 and 30mbgl) and in the plume core (21 and 26mbgl) were observed. Once again, this could be due an influence of the contaminant load. However, nitrate is undetectable within the plume core. Variation in nitrate levels could account for the variation in NRB community structure throughout the groundwater profile. Nitrate was present on the fringes due to the transverse mixing of uncontaminated and contaminated water, and the infiltration of fertilisers into the aquifer from the agricultural field above (Thornton *et al.,* 2001; Williams *et al.*, 2001). Inside the plume, nitrate levels were undetectable, resulting in poor conditions for the growth of NRB.

The NRB and SRB communities found in August 2009 clustered separately to the communities found in previous samplings, and on the whole, were highly dissimilar. This was especially the case for the community at 21 and 26mbgl, where the greatest change in TPC was observed due to the start of operation of the pump and treat system at the site. This clearly had a marked affect on the NRB and SRB communities, causing a shift in the microbial population due to changes in groundwater hydrochemistry.

### 3.4.3 Comparison of the Planktonic and Attached Communities at 30mbgl in Borehole 59

#### 3.4.3.1 Eubacterial Communities

To compare the planktonic and attached microbial communities, DGGE profiling of the microbial community attached to quartz sand grains removed from borehole 59 in January 2009 was performed. The profiles of the eubacterial community reveal that the attached community differs markedly from the planktonic community at the same depth (30mbgl). Cluster analysis confirms this observation, as the attached community clusters separately to the planktonic community at 30mbgl. The attached community also clusters separately to all other depths (with a maximum of 48% similarity). This suggests that the attached community differs to any of the eubacterial planktonic communities within the contaminant plume. However, due to technical constraints, it is not possible to investigate this further, by suspending surrogate substrata at multiple depths within the groundwater profile, as it is only possible to suspend substrata at 30mbgl where the only well-screen is located (Figure 2-1). Variation in the attached and planktonic community could be due to the partitioning of organisms into the attached phase in order to seek protection from the contaminant load (up to 420mg $L^{-1}$). To do this, the microbes must form a biofilm structure within an exopolymeric substance (EPS) matrix in order to gain protection, as EPS inhibits the diffusion of toxic substances into the biofilm (O'Toole *et al.,* 2000; as discussed in Section 1.4). This is not the case for the bacteria attached to the quartz sand, as microscopic observations of fluorescently-labelled cells (Section 3.3.1) show that an extensive biofilm was not formed on the quartz grains, resulting in no particular protection for the attached cells. Differences between attached and planktonic communities have been found by other researchers. Reardon *et al.* (2004) found distinctly different microbial communities in the planktonic phase and attached to surrogate hematite, shale and saprolite suspended within pristine groundwater.

There are no direct nutritive benefits of attaching to the quartz grains, as it is purified mineral made of $SiO_2$ only. At the pH of the groundwater (pH 6.8), quartz is also net negatively charged (Papirer, 2000), and is therefore repulsive

to bacteria (Baker *et al.,* 2009). This would reduce the likelihood of attachment. It is possible that conditioning films, formed on the surface of the quartz during incubation, could provide a suitable habitat for microbes that are in a starved state. Thornton *et al.* (2001) reported that the sandstone grains of the Four Ashes aquifer have Fe and Mn coatings, potentially formed from passing contaminated groundwater. This may have happened on the quart grains, thus providing a suitable surface for microbial attachment. Jass *et al.* (1995) found that *Legionella pneumophila* formed biofilms when it was in starved conditions, i.e. without a suitable carbon source or macronutrients. This concept could be applied to the bacteria attaching to the surface of the quartz grains.

One point worth noting is that the microbial community attached to the quartz grains was not formed by chance. If planktonic bacteria had got 'stuck' between the grains within the bags during the flow of groundwater through the bags, it could be assumed that the community would have remained the same as the planktonic community. As this is not the case, some ecological advantage must account for the difference in community structure between the attached and planktonic communities. One way to test this is to incubate more than one surrogate substratum within the aquifer. Each surface could provide a different nutrient, and then the communities could be compared (see Chapter 4).

### 3.4.3.2 Anaerobic Communities

PCR was unable to detect either the *dsrB* or *napA* genes in the DNA isolated from the quartz sand. This could have been the result of a methodology issue, for example due to inhibitors present in the DNA extracted from the quartz grains. Investigations proved this not to be case (Figure 3-11). Therefore, either very low numbers of anaerobic organisms were present in the attached community, or no anaerobic organisms were attached to the quart sand. However, low levels of SRB were detected in the SRB cultivations inoculated with the quartz sand. 4 of the 6 low inoculation-density cultivations showed SRB activity, suggesting that there were low numbers in comparison to the high inoculation-density cultivations where all vials were positive for SRB activity. Also, no SRB were detected in the DNA extracted from 8mbgl, the planktonic community most like that of the attached community at 30mbgl. These results

suggest that bacterial sulphate-reduction activity was not high in the attached communities at 30mbgl. This is understandable considering the aerobic-microaerophillic nature of the hydrochemistry at 30mbgl in borehole 59. Oxygen and nitrate levels were seen to be high on the plume fringes (12 and 30mbgl) and outside the contaminant plume (Section 2.3.1 and Thornton *et al.*, 2001), therefore aerobic and nitrate-reduction processes will dominate. However, even though oxygen is toxic to strict anaerobes such as SRB, their presence in the attached phase would be expected. This would be due to the development of micro-niches within an attached community, which could result in a proliferative environment for a variety of anaerobic microbial processes, due to oxygen depletion within the biofilm. This would only occur if biofilm growth was substantial enough to prevent oxygen diffusion into the biofilm, resulting in the formation of anaerobic micro-colonies within the attached community. If biofilm growth was not extensive, and grew only as a monolayer, then the growth of strictly anaerobic communities would not be possible. Sparse growth was seen from epifluorescence imaging of the microbes attached to the quartz-grains (Section 3.3.1).

Another explanation would be due to the quartz sand itself. The use of quartz may not have permitted an accurate assessment of the attached community at the site, as it did not provide a like-for-like surrogate for the Permo-Triassic sandstone forming the aquifer at Four Ashes. Even though sandstones usually contain over 95% quartz, the varying matrices within (including calcite, clay minerals and hematite) can provide nutrients and alternative attachment conditions, such as surface hydrophobicity, charge and topography (Geoghegan *et al.*, 2008; Cook and Kirk, 1995). The use of an unrepresentative surface may therefore have resulted in an unrepresentative attached microbial community. Further investigation using other geological surfaces is therefore required. This is explored in Chapter 4.

If SRB and NRB are in extremely low numbers in the attached phase, PCR amplification and DGGE may not be suitable techniques for the detection and profiling of attached communities. Also, the use of primers targeting the β-subunit of the *dsrAB* gene may not amplify the SRB gene in the attached phase. Attached cells may contain the α-subunit of the *dsrAB* gene. Although the two

subunits are near exact replicates, minor variations in the attached community could prevent amplification, leading to the misrepresentation that there are no SRB in the attached community. Alterative methods would therefore have to be used, such as the amplification of the whole *dsrAB* gene using the full-length primers, which can be used to profile the community by terminal-restriction length polymorphism (T-RFLP).

Also, if SRB (and NRB) were present in lower proportions than this, they will not be detected PCR and DGGE. Although PCR and DGGE are rapid methods, Muyzer *et al.*, (1993) stated that PCR-DGGE is only able to detect organisms that form greater than 1% of the total community.

### 3.4.4 Cultivable SRB Enumeration and Community Structure

Cultivable sulphate-reducing bacteria were quantified using the most-probable number method (Section 3.2.7.1). Groundwater, pumped from borehole 59 was used as inocula for the growth of bacteria in SRB-selective media. Results revealed that MPN counts were >180 cells $ml^{-1}$ at 8, 12 and 30mbgl at all samplings. At 21mbgl, the MPN of cultivable SRB was 6 cells $ml^{-1}$, whereas at 26mbgl, no SRB were detected. These results suggest, like the DGGE profiles of total communities, that the groundwater hydrochemistry has an influence on the SRB community, as high MPN were detected in areas where TPC was up to 690mg $L^{-1}$. Low numbers were detected at 21mbgl where TPC was 1280-2439mg $L^{-1}$. The lack of detection of SRB at 26mbgl suggests a toxic effect of the TPC on growth. This reflects the work of Garland *et al.* (2001), who found that the culturability of organisms relates to the successional state of microorganisms, relating to their performance in the environment. Also, Spence *et al.* (2001) noted that TPC concentrations of greater than 2000mg $L^{-1}$ inhibit SRB activity. This was evident in the results of this study, as the TPC at 26mbgl ranged from 3576 to 4930mg $L^{-1}$, nearly 2x the threshold of SRB inhibition noted by Spence *et al.* (2001). DGGE profiles (Figure 3-19) of the cultivable planktonic SRB community also highlight that TPC affects SRB growth. Communities cultivated from areas of lower TPC on the plume fringes (12mbgl and 30mbgl) differed from the cultivable SRB community from 26mbgl, where contaminant concentration is high. Also, the lack of carbon-source in uncontaminated

groundwater has influenced the community structure of the cultivable community at 8mbgl.

Comparison of the attached and planktonic cultivable SRB community was also performed. DGGE profiles (Figure 3-21) reveal that the planktonic community at 30mbgl remained stable over time. Profiles of the cultivable SRB community attached to the quartz sand revealed that the community differed from the cultivable planktonic SRB community. Co-migrating bands between the cultivable planktonic and attached SRB communities suggest that either specific phylotypes are living both in the free-living and attached phase, or that pore-waters from within the quartz sand inoculum contained planktonic SRB. Also, many of the bands in the DGGE profiles of the SRB cultivations inoculated with quartz sand were extremely weak, suggesting that even after cultivation many of the phylotypes were in low concentrations. This, and the fact that PCR was unable to detect *dsrB* (and *napA*) in total DNA isolated from the quartz sand, agrees with the idea that PCR may not be suitable for directly detecting rare functional genes in isolated total DNA. The results here concrete the requirement for total SRB/NRB community assessment using a technique with better detection limits, allowing the investigation of rare functional genes.

Sequencing of isolated colonies highlighted that the methods only isolated a few sulphate-reducing bacteria from the liquid cultures. Mainly anaerobic, psychrophilic bacteria and iron-reducers were isolated. Such bacteria would proliferate in a complex community containing SRB, resulting in Fe cycling and growth by respiration of the metabolic products generated by the SRB. As few SRB were isolated, this suggestes that the cultivation methodology may not be suitable for the organisms at Four Ashes.

## 3.5 Conclusion

The microbial community structure of eubacterial, sulphate-reducing and nitrate-reducing communities was investigated (by DGGE fingerprinting) down the groundwater profile at borehole 59 in the Four Ashes aquifer. The structure of planktonic communities was seen to vary with depth due to changes in hydrochemical conditions. Also, the planktonic and attached communities from 30mbgl were compared using groundwater and quartz sand suspended in the aquifer, respectively. DGGE profiling revealed that the attached community differed markedly from the planktonic community. PCR was not able to detect any SRB or NRB in DNA isolated from the quartz sand, suggesting numbers below the detection limits of the PCR methods. Quantification of cultivable SRB in the groundwater was also performed using the most-probable number method of cultivation. Results reveal that cultivable SRB numbers were greater on the plume fringes and in uncontaminated groundwater than inside the plume. No growth was observed in cultivations inoculated with groundwater from 26mbgl, due to the high TPC concentrations. DGGE profiles of the cultivations revealed that cultivable communities from 12 and 30mbgl differed from the cultivable SRB communities in uncontaminated groundwater or at 21mbgl, suggesting that growth is influenced by the levels of contamination. Cultivable SRB were also detected in media inoculated with the quartz sand that had been suspended in the aquifer. DGGE profiles of planktonic and attached cultivable SRB communities revealed that they too differed markedly. Overall, results suggest that TPC has a strong influence on microbial growth and anaerobic community structure. Also, results suggest that there is a major distinction between planktonic and attached communities within the aquifer. Further assessment is required to investigate this, as quartz sand is not a representative surrogate for the aquifer mineral assemblage at Four Ashes, and therefore the attached community on quartz may be unrepresentative of the indigenous microbial community. This is explored further in Chapter 4.

# 4

# *In situ* Microbial Attachment Studies Using Surrogate Geological Substrata

## 4.1 Introduction

It is widely accepted that over 99% of all microbial biomass is attached to, or associated with, a surface (Costerton and Wilson, 2004; Fletcher, 1996; Powelson and Mills, 1996). This has led to the realization that indigenous attached microbial communities are extremely important in controlling geochemical processes at the cell-mineral interface, and that microbial ecology, mineralogy and biogeochemistry are intimately linked processes (Davis and Luttge, 2005; Bennett *et al.*, 2001). As a consequence, studying microbial communities in the planktonic (or free-living) phase provides only a glimpse into the true microbial population. Research has already confirmed that microbial populations vary between the attached and planktonic phase (as discussed in Chapter 3; Rizoulis, 2008). However, studies of microbial attachment to surfaces can be limited, as access to the subsurface is often difficult and costly (Hendrickx *et al.*, 2005; Reardon *et al.*, 2004). Also, the variety of different surfaces found within the environment results in a huge variation in cell-surface interaction. As such, much research into the controls on microbial attachment takes place within laboratory studies where samples and techniques are accessible (Andrews *et al.*, 2010; Hendrickx *et al.*, 2005), or uses artificial surfaces that are not wholly representative of the environment.

Microbial attachment to surfaces may be spatially heterogeneous within a complex geological ecosystem such as an aquifer (Goldscheider *et al.,* 2006). To fully understand the biogeochemical processes that control contaminant natural attenuation in groundwater, investigation into the conditions influencing microbial attachment and biofilm formation is important. Fundamentally, the variation in microbial attachment depends on 3 principal factors; the species/genus of the attaching microbes, the physiochemical factors of the biological and stationary phase (e.g. hydrophobicity/surface roughness), and the chemical interactions on the bio-mineral interface (Davey and O'Toole, 2000). Moreover, in an oligotrophic environment, and where toxic levels of organic contamination are present, cell growth and biofilm formation can be limited. Therefore, assessing how the physical and chemical properties of the substratum influences *in situ* microbial attachment can provide information on the ecology of the attached communities. Research into *in situ* microbial

attachment has historically involved the use of permeable vessels containing surfaces representative of the geological ecosystem to assess microbial attachment over time, prior to removal and analysis in the laboratory. This method has proven useful for many researchers (Rizoulis, 2008; Mauck and Roberts, 2007; Reardon *et al.*, 2004; Bennett *et al.*, 2001).

Previous research at the Four Ashes has assessed the microbial ecology of both aerobic and anaerobic planktonic communities (Chapter 3; Rizoulis, 2008; Pickup *et al.*, 2001). Also, some research was undertaken to compare the attached and planktonic communities at borehole 59 (130m from the point source of contamination) by suspending permeable vessels containing clean commercial quartz sand in the well-screen at 30mbgl (Chapter 3; Rizoulis, 2008). However, although the Permo-Triassic sandstone aquifer underlying the site will be predominately composed of quartz (up to 95%), the varying matrices within (e.g. minerals such as hematite, clays, calcite, feldspars) could result in a variation in microbial attachment and biofilm formation at the site (Thornton *et al.*, 2001; Cook and Kirk, 1995). Investigating the mineralogical controls on microbial attachment using a variety of substrata will deepen the knowledge of the indigenous microbial community at the site, ultimately leading to a better understanding of the phenol natural attenuation and bioremediation potential. This work aims to investigate the following hypothesis:

"Substratum physiochemical properties influence microbial attachment and community structure within oligotrophic, phenol-rich groundwater".

The aims of the experiments were to:

1. Investigate any potential mechanisms of physical, chemical or bioweathering of the geological substrata after incubation in the organic-rich groundwater;
2. Investigate the influence of surface hydrophobicity, chemistry and charge on microbial attachment;
3. Investigate the geochemical controls on attached microbial community structure.

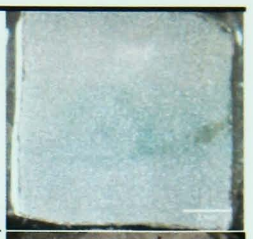
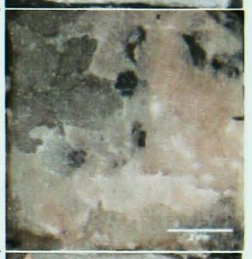
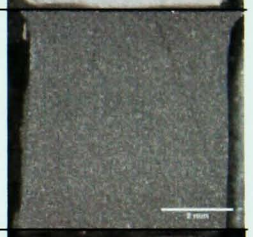
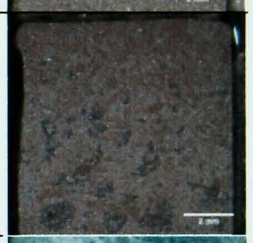
## 4.2 Materials and Methods

### 4.2.1 Surrogate Geological Substrata

The rocks and minerals used within the experiments (Table 4-1) were chosen based on their potential to add particular nutrients and/or physical conditions to the aquifer system, which may influence microbial attachment and growth. Quarry sandstone, local to Four Ashes, was also used to mimic the aquifer substrata. This allowed the microbial attachment and community structure on the different surrogate surfaces to be compared to the actual aquifer. It was not possible to use sandstone from the aquifer formation at Four Ashes as the core samples removed during the construction of the multi-level sampling boreholes in 1998 had already been contaminanted with phenolic compounds, and had been vaccum stored for several years. Chemical and microbiological modification of the surfaces may have occurred in that time, causing bias during the incubation experiments. It would have also been costly to drill new boreholes to retrieve fresh material from an uncontaminated area of the aquifer. Therefore, fresh material was sourced from a quarry near to Four Ashes (discussed further in Section 4.2.3).

Pure quartz-sand (BDH: VWR International Ltd, Lutterworth, Leicestershire, UK) was also used in the *in situ* incubation experiments as a comparison to previous work performed at the site (as discussed in Chapter 3 and Rizoulis, 2008).

**Table 4-1** Table of chosen substrata, basic descriptions and properties and/or conditions that they provided for microbial growth.

| Substratum | Description | Supplier | Condition/Nutrient Added | Image |
|---|---|---|---|---|
| **Quartz** | $SiO_2$, hard silicate mineral (Moh's Scale = 7), inert mineral. | Geosupplies, Sheffield, UK | Inert chemistry; hydrophillic; point of zero-charge (PZC) at very low pH |  |
| **Shap granite** | Igneous rock from NW England (GR 30U 517757 60361 65) containing quartz, plagioclase feldspar, orthoclase K-feldspar, biotite. | Weathering Consortium, U. Of Bristol, UK | Trace metals including phosphorous; heterogenous chemistry and surface properties |  |
| **Orthoclase** | Alkali feldspar mineral, with general formula $KAlSi_3O_8$. | Rockshop, Huddersfield, UK | macronutrients, less hydrophilic; low PZC |  |
| **Oil Shale** | Very fine-grained, extremely porous sedimentary rock. | Geosupplies, Sheffield, UK | Less hydrophilic due to organic content, trace chemistry |  |
| **Hematite** | Raw ore of iron. | Rockshop, Huddersfield, UK | Positively charged at pH <7.8<br>Provides Fe for redox processes |  |
| **Apatite** | Calcium and phosphate-rich mineral. | Geosupplies, Sheffield, UK | Phosphorous |  |

## 4.2.2 Rock and Mineral Coupon Design

Both microscopic and molecular biological techniques were to be used to investigate microbial attachment and community structure on the different surrogate surfaces. Therefore, two methods of substrata preparation were used. The first was designed for use in micro/spectroscopic assessment of the influence of surface properties on microbial attachment and the effect of the groundwater hydrochemistry on mineral weathering. The second was used for molecular (DNA) assessment where high cell biomass was required.

Method 1

Minerals and rocks (Table 4-1) were cut (to approximately 64mm$^2$) using a water-flushed Diamant Boart saw with diamond cutting blade (Diamant Boart, Ath, Belgium). 12 sliced sections (6 minerals in duplicate) were glued to a glass microscope slide in a random pattern using araldite DBF resin (Hunstman Advanced Materials, Basel, Switzerland) with Crayamid 140 hardener (Cray Valley, Stallingborough, North East Lincolnshire, UK). The slide-mounted surfaces were ground to 1-2mm using a flat steel lap using 220µm grit silicon carbide (Figure 4-1). The substrata mounted on the glass slides are hereafter referred to as coupons.

The surfaces were attached to the glass slide using the least amount of resin possible, being careful to prevent the resin rising and becoming level with the ground coupon surface. The small surface area allowed the glass slide to be scored using a diamond pen and broken quickly for downstream manipulation and assessment.

All the mineral slides were prepared using the same grinding equipment, resulting in a consistent mineral surface roughness (as much as possible). This prevented bias towards a particular surface that may result in greater microbial adhesion. Confocal laser scanning microtopography (CLSM) and vertical scanning interferometry (VSI) were used to quantitatively assess the surface roughness ($R_a$) of the minerals (Section 4.2.3.7).

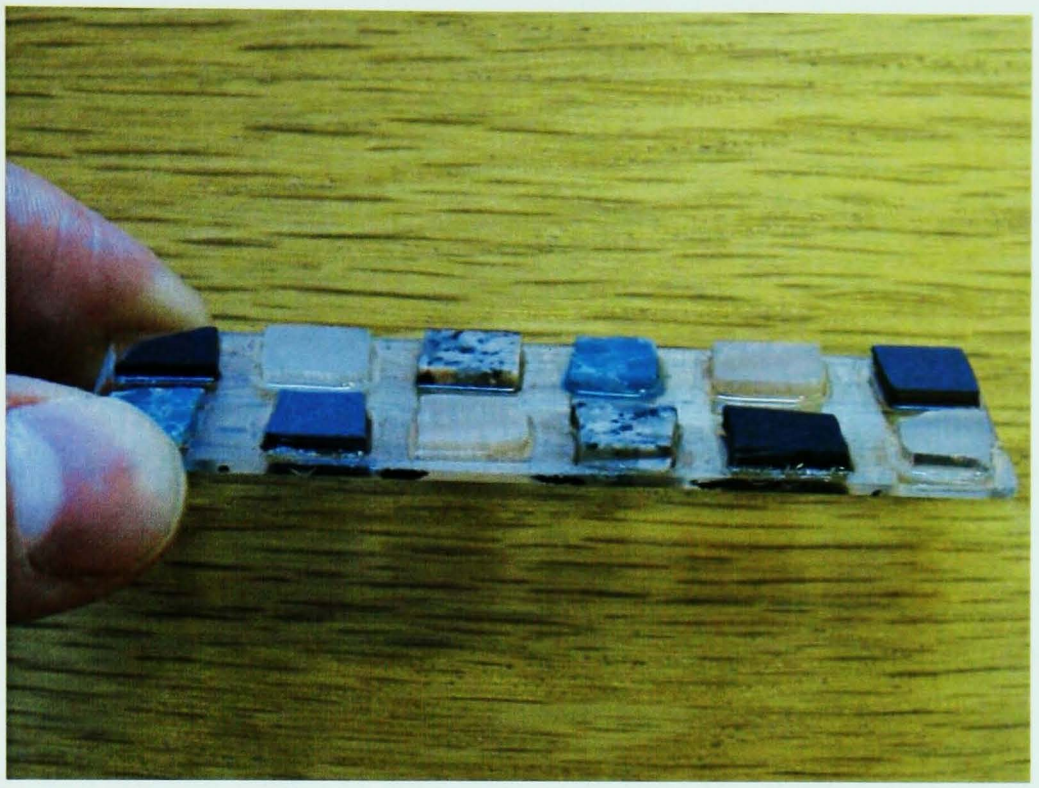


**Figure 4-1** Design of mineral slides to be used the micro/spectroscopic investigation of microbial attachment and mineral weathering.

Prior to characterisation and incubation, coupons were cleaned by sonication in 2% w/v sodium dodecyl sulphate using a Decon FS100 sonication bath (Decon Laboratories Ltd., Hove, UK) for 45 minutes, followed by 45 minutes sonication in de-ionised water and air-drying. This removed major dust and dirt from the cutting procedure and carbon deposited on the surface during the silicon carbide grinding. This method has been proven to remove organic contamination and sterilize the surfaces without weathering the minerals or altering surface chemistry (Buss *et al.*, 2003).

Method 2

5g of the local quarry sandstone (Section 4.3.1.2), Shap granite and the pure quartz-sand (BDH: VWR International Ltd, Lutterworth, Leicestershire, UK) were added to bags created from two layers of 100µm polyester multi-woven mesh sheet (Plastok, Ltd., Birkenhead, UK). The quarry sandstone and the Shap granite were ground using a percussion mortar and sieved to a grain-size representative of the aquifer (150-300µm, see Section 4.3.1.3). Each bag was labelled using coloured cable ties to ensure that each surface could be identified after removal from the aquifer. Four replicate bags of each substrata were used in the investigations.

### 4.2.3 Coupon Characterisation

All rocks and minerals were characterised before experiments began and after incubation in the aquifer, to assess the effects of the groundwater hydrochemistry on the physicochemical properties of the coupons.

Three local quarry sandstones were compared to assess which was the most representative of the sandstone at Four Ashes. The two most local quarries to Four Ashes that work the Permo-Triassic red sandstone, which forms the aquifer at the site, were Penkridge Pottal Pool (Hanson Aggregates, Penkridge, UK) and Hints (Tarmac Ltd., Tamworth, UK). Samples from both of these quarries were collected in February 2008. It was clear that the sandstone taken from Pottal Pool quarry contains distinct red and grey-coloured banding (Figure 4-2), suggesting a variation in chemical composition. These bands were separated and characterised independently.

Four Ashes core material from 8, 12, 21, 26 and 30mbgl, which was collected in 1999 by Thornton *et al.* (2001) during the construction of the borehole 59 multi-level sampler system, was analysed and compared to the quarry sandstone. This was performed to find the most suitable surrogate for the aquifer substrata in the *in situ* incubation studies. Samples were collected in plastic windowless sampling tubes that were cut with a clean scalpel blade to access the samples. A 2cm outer-shell of sandstone was removed and discarded as it may have been contaminated by drilling fluids during the rotary core drilling process. The central part of the core was then homogenised in preparation for grinding and analysis.

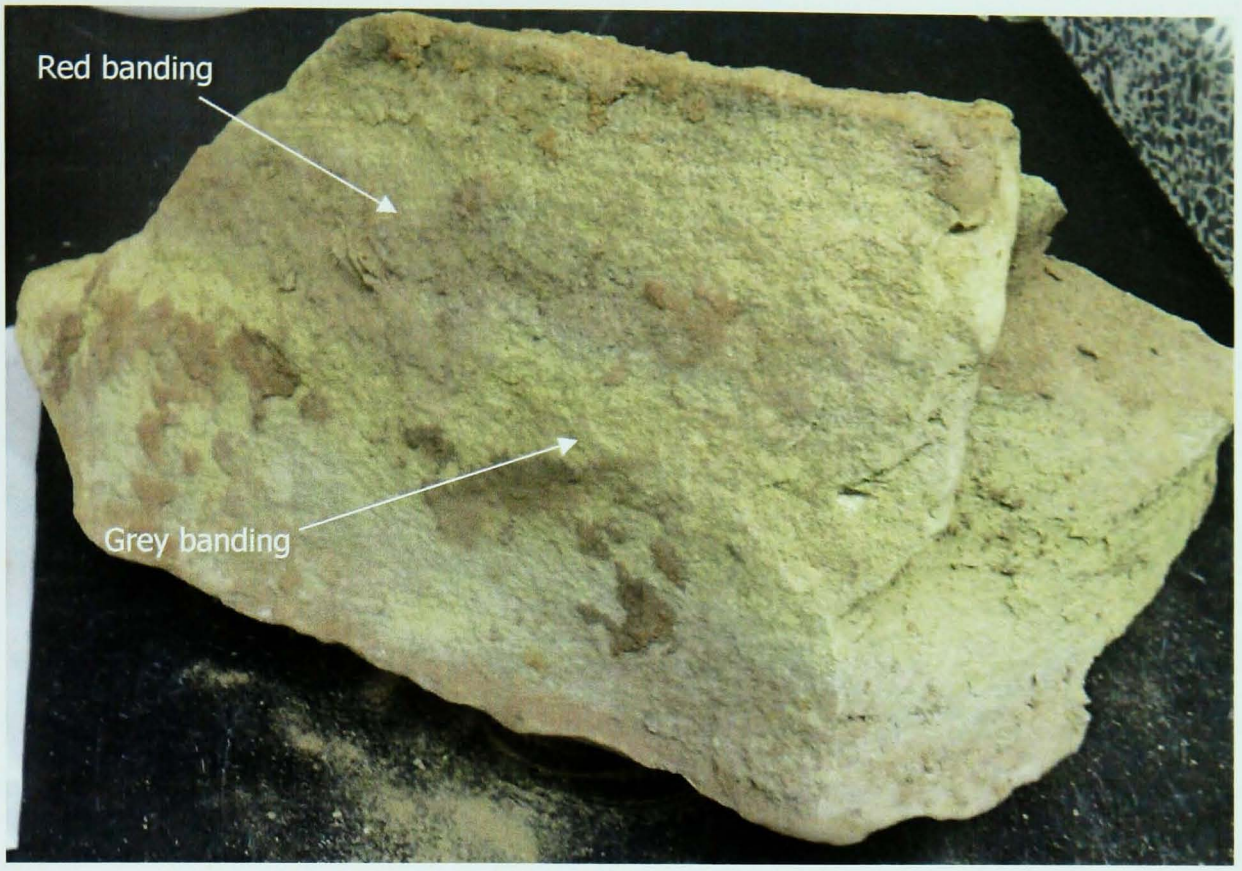



**Figure 4-2** Banding present in the sandstone unit derived from Penkridge quarry

### 4.2.3.1 Mineralogical Phase Identification

X-ray powder diffraction using a STOE NT-2 diffractometer (STOE & Cie GmbH, Darmstadt, Germany) with a Cu-K alpha radiation source was used to assess the mineral assemblage of the Four Ashes and local quarry sandstones. Diffraction patterns were collected over the complete 2θ range. Prior to analysis, samples were ground to a fine powder (<63μm) using a TEMA T100 chrome-steel mill (TEMA Machinary, Ltd., Northants, UK). The mill was washed between each sample with water and ethanol, and a small amount of the following sample was ground and discarded to remove any remnants of the previous sample, preventing cross-contamination. The powdered samples were then mounted using polyvinyl acetate (PVA) glue onto an acetate film within a sample holder and allowed to dry before analysis (Figure 4-3). Phase identification was performed using the WinX$^{POW}$ search-match package (STOE & Cie GmbH, Darmstadt, Germany) combined with the International Centre for Diffraction Data database (ICDD). Where phase identification was not possible using the database, literature sources were used (Jain *et al.,* 2009; Hajpal and Torok, 2008).

**Figure 4-3** Sampling disc used in XRD analysis of rock samples.

#### 4.2.3.2 Bulk Chemical Analysis of Quarry and Four Ashes Sandstones

X-ray fluorescence (XRF) using a PANalytical Axios Sequential X-ray Fluorescence Spectrometer (PANalytical B.V., Nelyweg, The Netherlands) was used to assess the bulk chemistry of the sandstones and granite. The analysis provides the concentrations of the major oxides and trace elements. The data was used to assess the variations in bulk rock chemistry both down the aquifer succession at Four Ashes (by analysing the core material), and between the Four Ashes aquifer and the local quarry sandstones. This allowed the correct quarry sandstone to be chosen as a representative surrogate of the aquifer for use in the *in situ* microbial attachment experiments.

Prior to analysis, samples were ground as previously described (Section 4.2.3.1). All analyses were performed by Mr. Paul Lythgoe of the School of Earth, Atmospheric and Environmental Sciences, University of Manchester, UK.

#### 4.2.3.3 Grain-Size Analysis

The quarry and Four Ashes core sample (30mbgl) were dry sieved according to BS 1377:1990 to assess grain-size distribution. Samples were oven-dried (110°C) before being placed into a nested sieve set with particle retention sizes of 0.063, 0.15, 0.212, 0.3, 0.425, 0.6, 1.18 and 2mm. Samples were shaken using a M14 Vibratory sieve shaker (Nash and Thompson Ltd., Surrey, UK) for 20 minutes and the fraction retained on each sieve was weighed and calculated as percentage of the total mass. Any agglomerates were broken softly by rubbing against the sieve mesh to separate the grains.

#### 4.2.3.4 Chemical Analysis of Coupons

The coupons mounted on the glass slides (Section 4.2.2 and Figure 4-1) were analysed using a FEI Quanta scanning electron microscope fitted with an EDAX energy-dispersive spectrometer (FEI, Hillsboro, Oregon, USA) to assess bulk chemistry. The coupons were bonded to aluminium stubs using adhesive silver paint and carbon coated using a Speedivac Carbon Coating unit (Edwards High Vacuum Ltd., West Sussex, UK) for 45 minutes. Wide scan spectra (20kV accelerating voltage, 1100 counts per second) were taken at varying magnifications to collect major elemental abundance. Elemental mapping of the surfaces was also performed to assess the variation in bulk chemistry across the surfaces. Maps were taken at 1024 x 800 resolution and at 400x magnification for 11 minutes per frame.

#### 4.2.3.5 Surface Chemical Characterisation

To assess the surface chemistry of the coupons X-ray Photoelectron Spectroscopy (XPS) was performed using an AXIS Ultra DLD XPS (Kratos Analytical, Manchester, UK). XPS analyzes the top 2nm of surface elements, and can therefore characterise the active sites available to attaching microorganisms. No special sample preparation was necessary prior to XPS analysis. Samples were analysed using wide scan mode in an operating vacuum of $2 \times 10^{-7}$ Torr. XPS spectra were processed using CasaXPS, available from Casa Software Ltd. (available from www.casaxps.com).

#### 4.2.3.6 Determination of Surface Hydrophobicity

To determine the wetting characteristics (hydrophobicity, hydrophillicty) of the different coupons, goniometry using a Kruss DSA100 Drop Shape Analyser (KRÜSS GmbH, Hamburg, Germany) was performed. A static 2µl sessile drop of UHQ water (Figure 4-4) was used to measure the contact angle (CA) for each surface. The CA is inversely related to the ability of the rock/mineral to permit a liquid to spread across its surface. The wetting ability is related to the cohesive and adhesive forces involved in the liquid-surface-embedding phase (the atmosphere in this case) interaction (the 3-phase interface), as described by Shafrin and Zisman (1960).

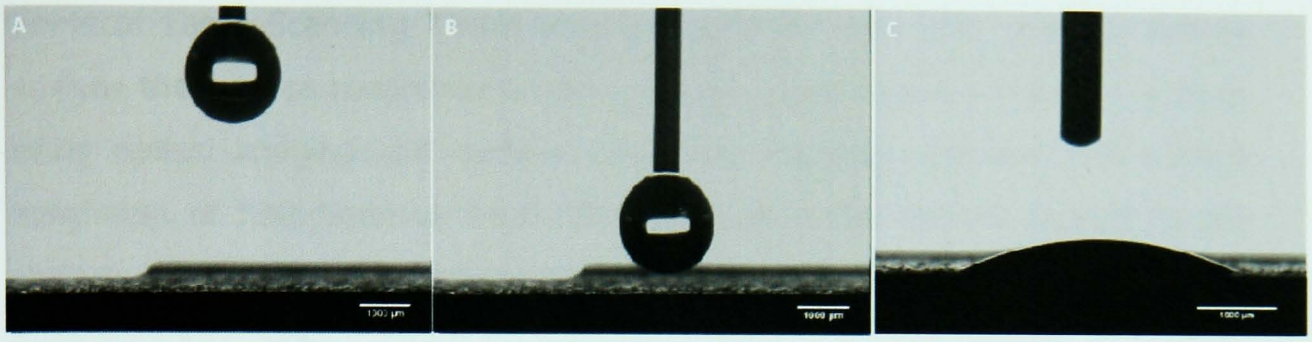



**Figure 4-4** Stages of drop shape analysis, including needle dosing with liquid (A), drop release onto surface (B) and needle retraction.

Angles were measured using two separate methods; Tangent-2 method which measures the slope of the droplet at the 3-phase interface, on the left and right hand side of the drop. The contact angle is then calculated according to a polynomial regression, with an average generated from the angles at either side of the drop. This was used when the drop on the surface of the coupon was asymmetric. The Young-Lapace (sessile-drop) fitting was used to analyse the contact angle of symmetrical drops where the whole contour of the drop could be analysed, returning the most exact method of calculation. Once again the contact angle was calculated using the slope of the drop at the 3-phase interface.

#### 4.2.3.7 Analysis of Surface Topography

The surface topography and roughness of the mineral coupons was analysed by vertical scanning interferometry using a Veeco Wyko NT9100 Surface Optical Profiler (Veeco Instruments Inc., Plainview, NY, USA). This technique uses a Mirau interferometer to split a beam of light into two separate beams. One beam excites the surface and the other reflects from a reference mirror, and when the two beams recombine, the interference from the surface is measured to create an interferogram, which is related to the surface topography (Waters *et al.*, 2008; Davis and Luttge, 2005). This information is then fed into a computer via a charged couple device where the interferograms are used to produce topographic maps of the mineral surface (Davis and Luttge, 2005). Average surface roughness ($R_a$) is then calculated as the mean height of the scanned surface in the z-direction, and is quoted in micrometres/nanometres.

Confocal Laser Scanning Microtopography (CLSM) was also used to further analyse the surface roughness of the coupons. CLSM allows combined analysis using optical imaging and surface roughness mapping, allowing the surface roughness of heterogenous rocks and minerals to be directly related to the surface properties, e.g. mineral assemblage. This was performed for the heterogeneous Shap Granite and apatite coupons. For all surface roughness analyses, samples were completely dry and 10x or 50x magnifications were used.

### 4.2.4 The Leaching Behaviour of the Coupon Mounting Resin

As a resin was used to mount the mineral coupons onto the glass slides, the leaching behaviour of the resin was investigated using a standard leaching test (BS EN 12457 -2). Microbial macronutrients could leach out of the resin into the groundwater system during incubation, which could therefore influence microbial attachment. 3 glass microscope slides coated with a known amount of araldite resin and hardener, and 1 blank (glass slide only), were place into a 50ml plastic tube. Two volumes (by weight of resin and glass slide) of UHQ water were added and the tubes were end-on-end inverted using an Elmi RM-2L Intelli-mixer (Elmi Ltd., Riga, Latvia) for 24 hours. Metal concentrations and dissolved ions in the leachate were measured using ion chromatography as previously described (Section 2.2.3).

It was unnecessary to analyse the organics in the leachate as the contribution to the system would be unsubstantial given the high concentration of organics already in the groundwater due to the contamination (see Chapter 2).

### 4.2.5 Suspension of the Substrata within Borehole 59

The samples were well supported within permeable vessels to allow groundwater flow over/through the substrata. The mineral slides were housed within a plastic microscope slide box, which was modified to fit in the inner well casing of borehole 59 (73mm ID). Holes of varying sizes were drilled through the slide box to maximise groundwater flow over the coupons (Figure 4-5), which were evenly spaced within the box to prevent bias. A clean glass microscope slide was placed in the top slot of the slide box to prevent

particulates settling onto the top mineral coupon slide and preventing downstream analysis. All slides were cleaned and sterilised using the method outlined in Section 4.2.2 prior to incubation in the aquifer.

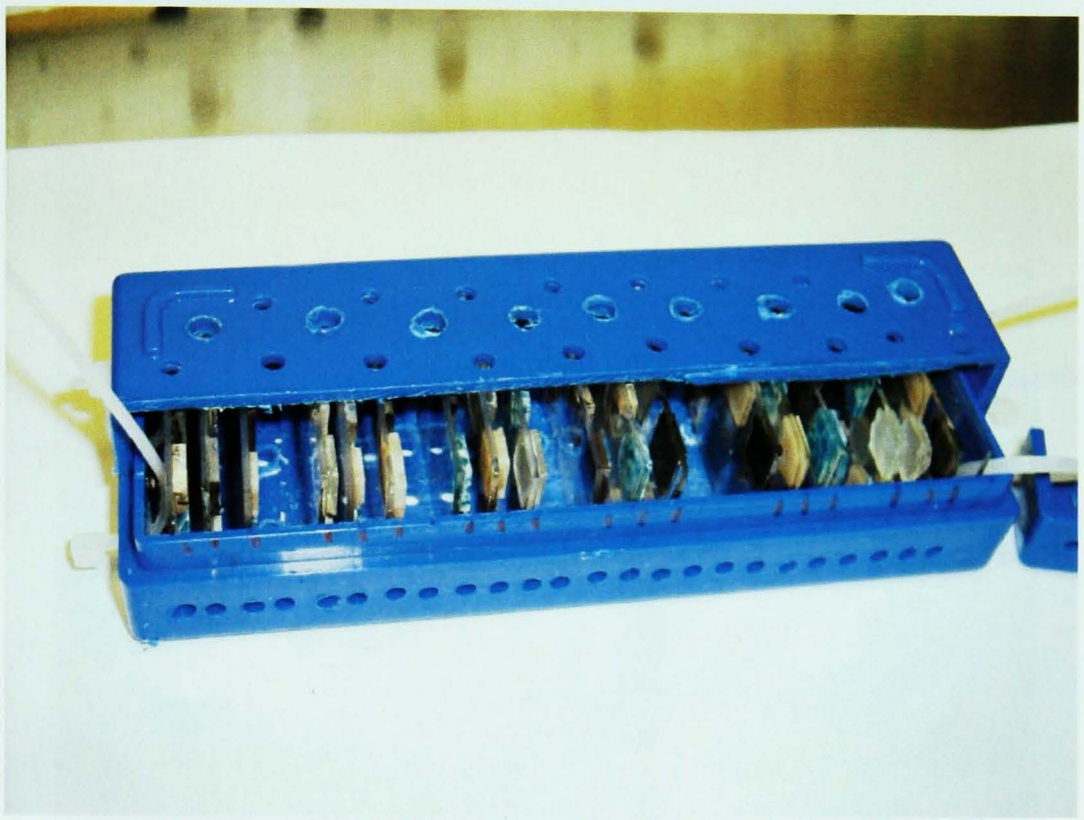


**Figure 4-5** Image of slide box showing even distribution of slides and drilled holes to allow groundwater flow over the mineral coupons.

The 12 bags containing 5g of rock grains (sandstone, quartz-sand and Shap granite) were housed within a 34cm length of polypropylene pipe (32mm ID, Marley Plumbing and Drainage Ltd, Maidstone, Kent, UK) with drilled holes to ensure good groundwater infiltration through the bags. The bags were separated randomly at 2cm intervals using crossed cable-ties. The top of the pipe was capped using a plastic stop-end fitting to prevent particulates from settling onto the bags. The piping (Figure 4-6) was autoclaved at 121°C for 15 minutes to sterilize.

The slide box was attached in a laminar flow hood to the underneath of the piping using cable ties. These were placed through holes drilled in the top of the slide box and hooked around the bottom platform of the piping. The total length of the piping and attached slide-box was 50cm, equal to the length of the well-screen at 30mbgl in borehole 59.

**Figure 4-6** Final construction of vessels used to suspend surrogate substrata in borehole 59 at 30mbgl

A 30m length of polypropylene rope was used to suspend the piping and slide-box within the inner well-casing of the borehole. Polypropylene was chosen for both the piping and the suspension line as it has excellent compatibility with phenolic compounds. This ensured that they would not degrade during incubation.

The substrata were suspended within the borehole in January 2009.

### 4.2.6 Removal of the Substrata from Borehole 59

The surrogate substrata were removed after 8 months of incubation in August 2009. They were slowly withdrawn from the borehole to ensure that removal did not adversely affect cell attachment. The coupons were immediately separated into two halves. One half was placed into a sterile tub and fixed with 4% formaldehyde (in phosphate buffer saline - PBS) in preparation for imaging by epifluorescence microscopy and ESEM (Section 4.2.8). The remainder were placed individually into sterile agar plates and left to air dry at 4°C in preparation for physiochemical analyses.

The bags of sandstone, quartz-sand and Shap granite were removed from the polypropylene tubing and placed into sterile plastic pots. 3 bags of each substratum were frozen on site using dry ice, and subsequently stored at -80°C. The remainder were placed on ice for transport to the laboratory, where 5g was aliquoted into sterile 1.5ml tubes for molecular (DNA) assessment.

### 4.2.7 Groundwater Sampling

To compare the community structure of the planktonic microbial community to the attached communities, 6 aliquots of 120ml of groundwater from 30mbgl were filtered on-site through a 45mm nylon 0.22µm filters (VWR International Ltd., Lutterworth, Leicestershire, UK). The filter was immediately frozen on dry-ice and stored at -80°C on return to the lab in preparation for DNA extraction and molecular (DNA) studies (Section 4.2.9).

### 4.2.8 Imaging of Attached Microbes

#### 4.2.8.1 Epifluorescence Microscopy

Mineral coupons were stained using 0.0001% Syto 9 (Invitrogen Ltd., Paisley, UK) in DI-water and covered with a coverslip before incubation in the dark for 15 minutes. The coupons were imaged and counted according to Section 3.2.1 using an Olympus BX50WI Upright Fluorescence Microscope (Olympus Optical Co. Ltd, London, UK) fitted with CoolSnap colour camera (Princeton Instruments, Buckinghamshire, UK). Extended depth of focus images were taken using Image Pro (Media Cybernetics Inc., Bethesda, MD, USA). Emission

was detected in the green at 530nm using a U-MWB (Olympus Optical Co. Ltd, London, UK) filter cube.

#### 4.2.8.2 Environmental Scanning Election Microscopy

Coupons were imaged using a FEI Quanta 200 ESEM (FEI, Hillsboro, Oregon, USA) to further investigate the extent of microbial attachment to the coupons. Samples were removed from 4°C storage and mounted immediately onto the ESEM cooling stub using sterile tweezers and adhesive carbon tape. The imaging chamber was cooled to 4°C and a light vacuum was applied. Images were taken using scanning mode and at an accelerating voltage of 20kV, at varying magnifications.

### 4.2.9 DNA Extraction, PCR and DGGE

DNA was extracted from the grains using the Ultraclean™ Microbial DNA Isolation Kit (Mobio, San Diego, CA, USA), as outlined in Section 3.2.2. DNA was extracted from the filters using the same kit by adding one quarter of one nylon filter to the microbead tube, prior to the addition of the bead solution and solution MD1. To increase DNA yields, a heating step at 60°C for 15 minutes was performed using a water bath, followed by a 10 second pulse in a MP Fast-Prep homogenizer (MP Biomedicals Inc., Solon, Ohio, USA). This ensured that all the microbial cells had been removed from the filter and lysed. The subsequent steps in the manufacturer's protocol were followed. DNA was quantified using Quant-it™ PicoGreen® dsDNA reagent (Molecular Probes, Inc., Eugene, OR, USA), as outlined in Section 3.2.3.

To ensure that the extra bead beating stage did not adversely affect the length of the extracted DNA fragments, 8µl of eluted DNA was added to 2µl of 5x loading buffer (Sigma-Aldridge Company, Ltd, Poole, UK) and electrophoresed for 50 minutes at 90v in a 1% agarose-TAE (40mM Tris- base, 20mM acetic acid, 1mM EDTA, pH 8) gel stained with ethidium bromide. A 10kb molecular weight standard (Hyperladder I, Bioline, London, UK) was used to find to the length of the extracted DNA fragments. The gel was imaged according to Section 3.2.4.3. It was found that the extracted DNA was not smaller than 10kb

(Figure 4-7), and could therefore be used in downstream applications, including PCR amplification of long target sequences.

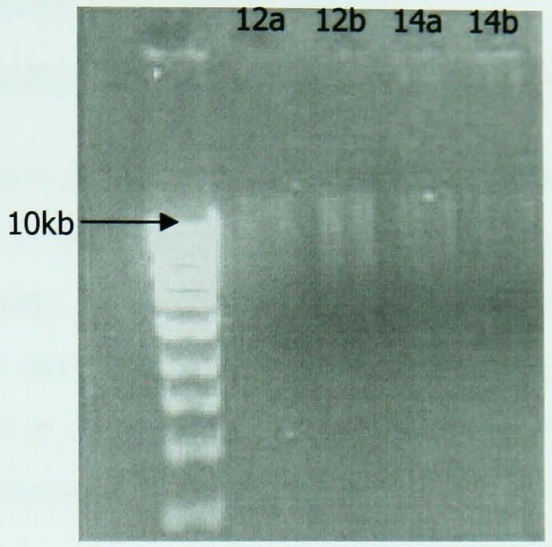



**Figure 4-7** Image of agarose gel showing base pair length of DNA extracted from the groundwater filters of borehole 59 at 12mbgl and 14mbgl.

DGGE profiling and cluster analysis was performed using the methods outlined in Sections 3.2.5.

## 4.3 Results

### 4.3.1 Mineralogical Characterisation

#### 4.3.1.1 X-Ray Powder Diffraction of Local Quarry Sandstones

The diffraction patterns (Figure 4-8) show that quartz dominates the mineralogy both at the site and in the quarry sandstones. It is also evident that there are a small number of peaks representative of calcite, haematite and clay minerals (Figure 4-9). These data show there is little variation in terms of mineralogy down the succession at Four Ashes. Results also suggest that the dark banding of the Penkridge sandstone is most similar to the Four Ashes core sample at 30mbgl.

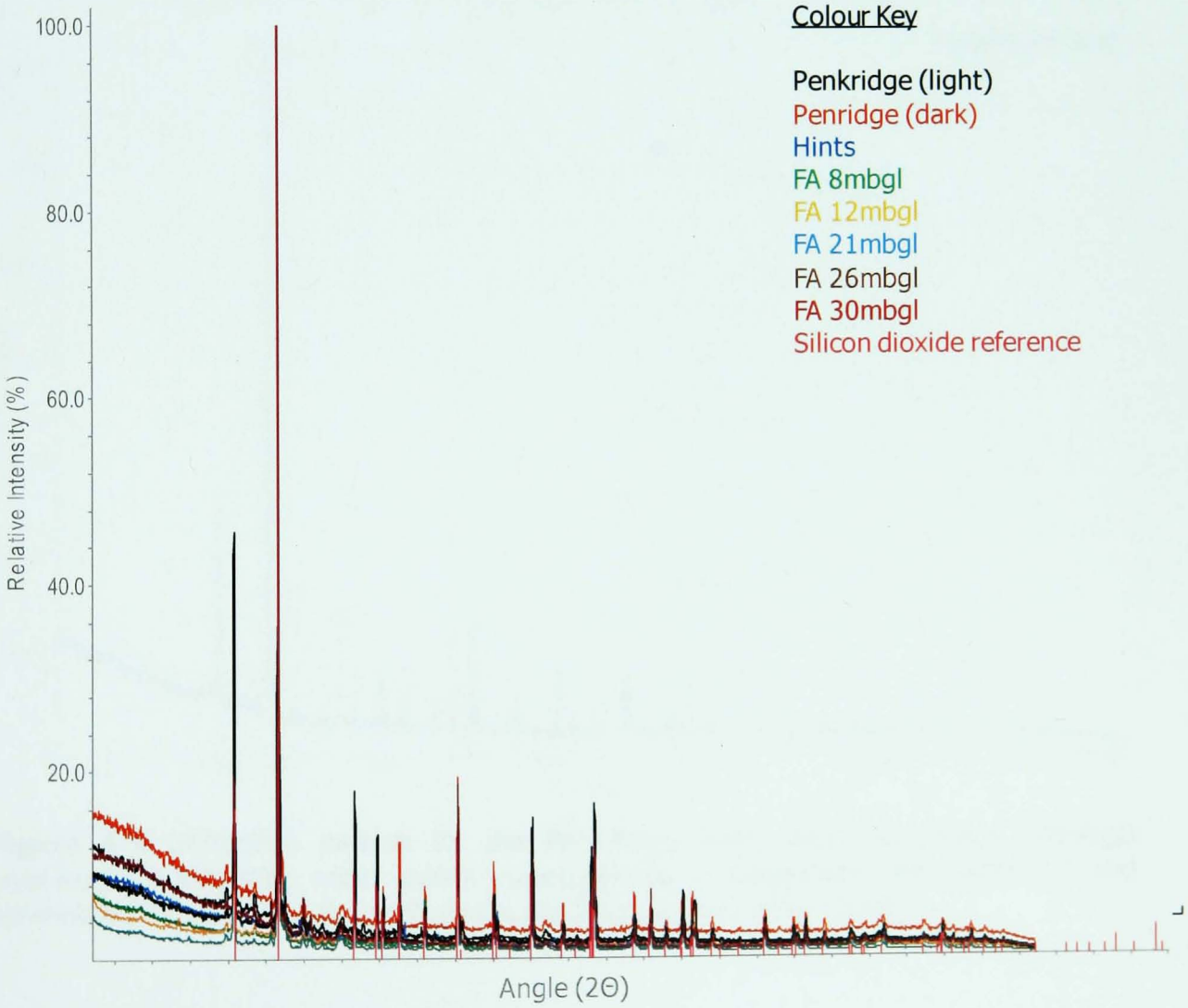



**Figure 4-8** Diffraction patterns for the sandstones from Four Ashes and the local quarries. Also shown are the locations of the quartz (silicon dioxide) reference peaks (vertical lines at 2Θ) from the ICDD database. FA in figure legend refers to Four Ashes, followed by the depth from which the core sample was taken.

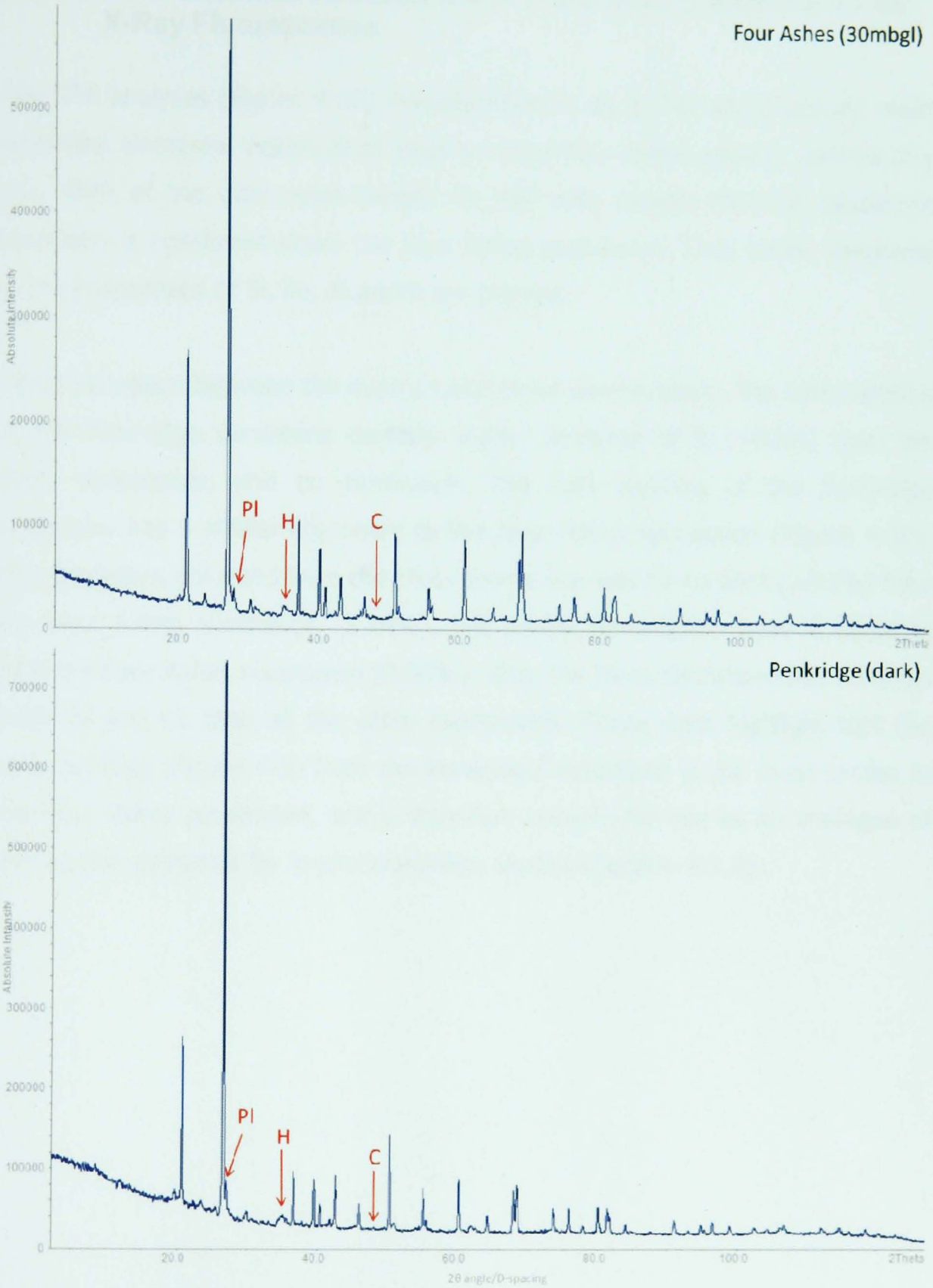
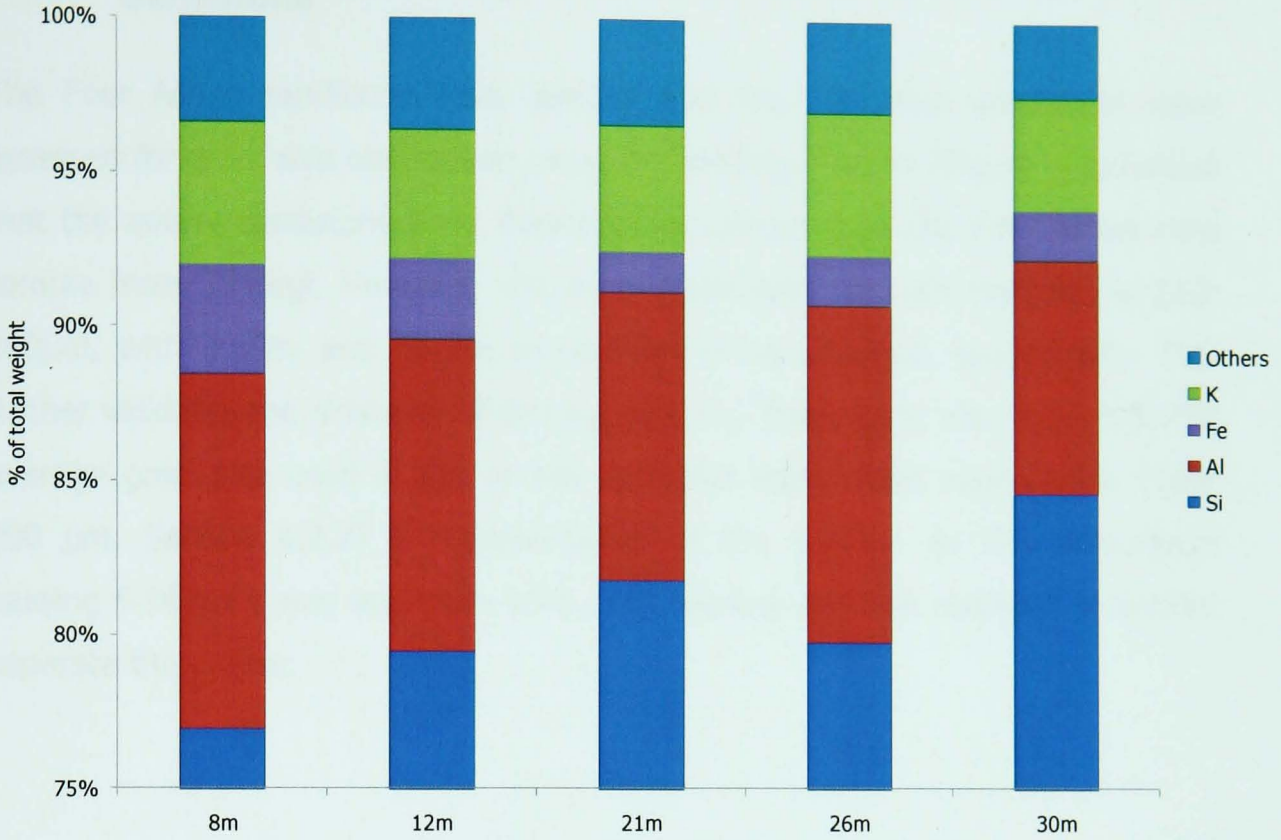



**Figure 4-9** Diffraction pattern for the Penkridge (dark) and Four Ashes (30mbgl) sandstones highlighting minor peaks corresponding to plagioclase (Pl) calcite (C) and haematite (H). 2θ angles for each phase are found in Jain *et al.* (2009).

#### 4.3.1.2 Bulk Chemical Assessment of Local Quarry Sandstones by X-Ray Fluorescence

The XRF analyses (Figure 4-10) revealed that Si, Al, K, Fe, and C are the most dominant elemental oxides at all depths in the Four Ashes aquifer, contributing over 96% of the total oxide weight %. XRF data reveals that the sandstone chemistry is consistent down the Four Ashes succession. Only minor variations in the proportions of Si, Fe, Al and K are present.

Major variations between the quarry sandstones were evident. The light banding of the Penkridge sandstone contains higher amounts of Si (~93%) than the other sandstones, and no aluminium. The dark banding of the Penkridge sandstone has a similar chemistry to the Four Ashes succession (Figure 4-10). The sandstone collected from the Hints quarry has less Fe content (0.03%) than the Four Ashes succession (2.14%), but nearly three-times more C (2.68%) than the Four Ashes succession (0.98%). Also, the Hints sandstone has 10 times more Ni and Ca than all the other sandstones. These data highlight that the dark banding (Figure 4-2) from the Penkridge sandstone is the most similar to the Four Ashes succession, and is therefore suitable for use as an analogue of the aquifer substrata for *in situ* incubation studies (Section 4.2.2).



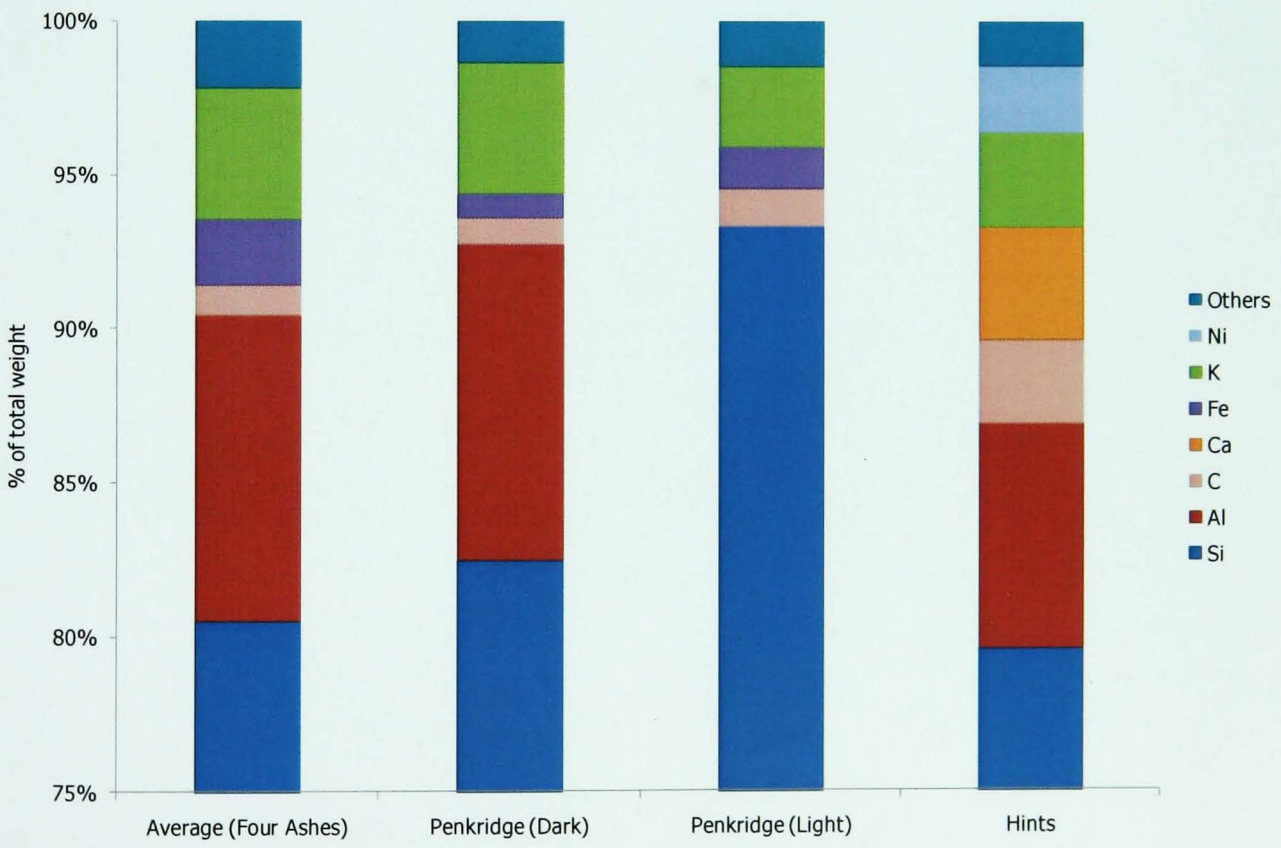
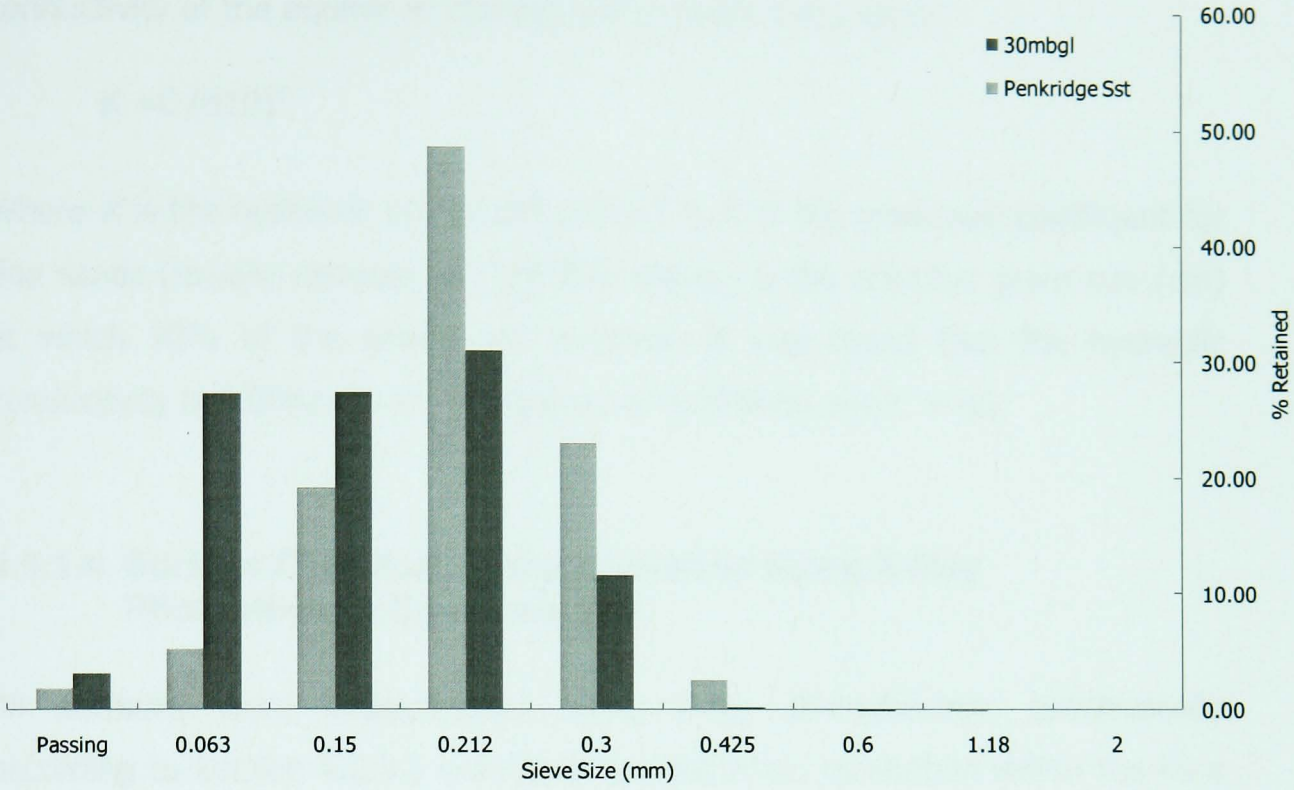




**Figure 4-10** Stacked chart showing the proportions of dominant elemental oxides within the Four Ashes sandstones (top), and a comparison of the average chemistry of the Four Ashes succession and the quarry sandstones.

#### 4.3.1.3 Grain-Size Analysis of Four Ashes Core Material and Quarry Sandstone

The Four Ashes sandstone from 30mbgl and the Penkridge sandstone were assessed for grain size distribution using dry sieving. Results (Figure 4-11) show that the quarry sandstone from Penkridge is coarser than the Four Ashes core sample from 30mbgl. However, the mode grain-size for both samples is 212-300µm, with 30.8% and 48.3% of particles being retained, respectively. This further validates the similarity of the sandstones. These data also show that the average grain-size used in the *in situ* microbial attachment experiments (150-300 µm, Section 4.2.2) is representative of the aquifer. As the percentage passing 0.063mm was less than 10%, wet sieving was not required to further separate the grains.




| Coarse | Fine | Medium | Coarse |
|---|---|---|---|
| **SILT** | **SAND** | | |

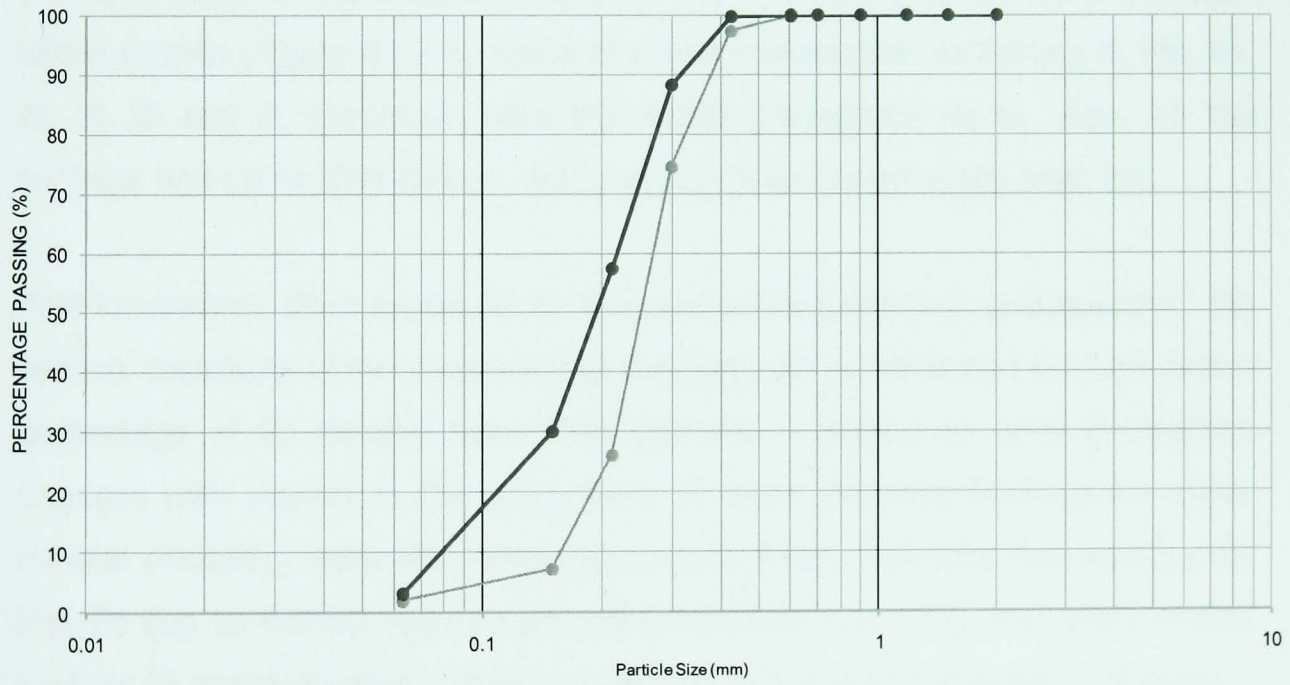



**Figure 4-11** Top: Grain-size distribution of the Four Ashes core material (30mbgl) and Penkridge sandstone. Bottom: Cumulative grain-size distribution of both samples. Key applicable to both graphs.

The grain-size analysis data were also used to estimate the hydraulic conductivity of the aquifer at 30mbgl, using Hazen's equation:

$$K = C\,(d10)^2$$

Where *K* is the hydraulic conductivity (cm $s^{-1}$), C is the grain-size coefficient for fine sands (usually between 40 and 80) and $d_{10}$ is the effective grain size (cm) at which 90% of the grains are retained. It was found that the hydraulic conductivity is 0.0062 cm $s^{-1}$, using a $d_{10}$ of 0.0786cm and C = 60.

#### 4.3.1.4 Surface Chemical Characterisation using X-Ray Photoelectron Spectroscopy

All surfaces were characterised using x-ray photoelectron spectroscopy according to Section 4.2.3.5 both prior to, and after, incubation within the Four Ashes aquifer.

Pre-Incubation, the surfaces had the expectant chemical composition based on the mineralogy of the coupons (Figure 4-12). For example, the biotite crystals within granite (Figure 4-12 A) had a chemical composition containing K, Mg, Fe, Al, Si, O and F, consistent with the biotite-phlogopyte series. Also, all the surfaces had up to 30% carbon, and varying minor contributions from Na.

Post-incubation, after exposure to the phenol-contaminated groundwater, the surface chemistry of the coupons had changed. All surfaces had a much higher percentage of C, ranging from 53% (granite – biotite) to 64% (hematite). Changes with respect to the proportions of other elements forming the major mineral chemistry were also observed (Figure 4-12). Hematite had significantly less Fe (up to 0.3%), quartz had proportionately less $SiO_2$, and trace metals such as Ca and Al formed a lower percentage of the total composition of shale.

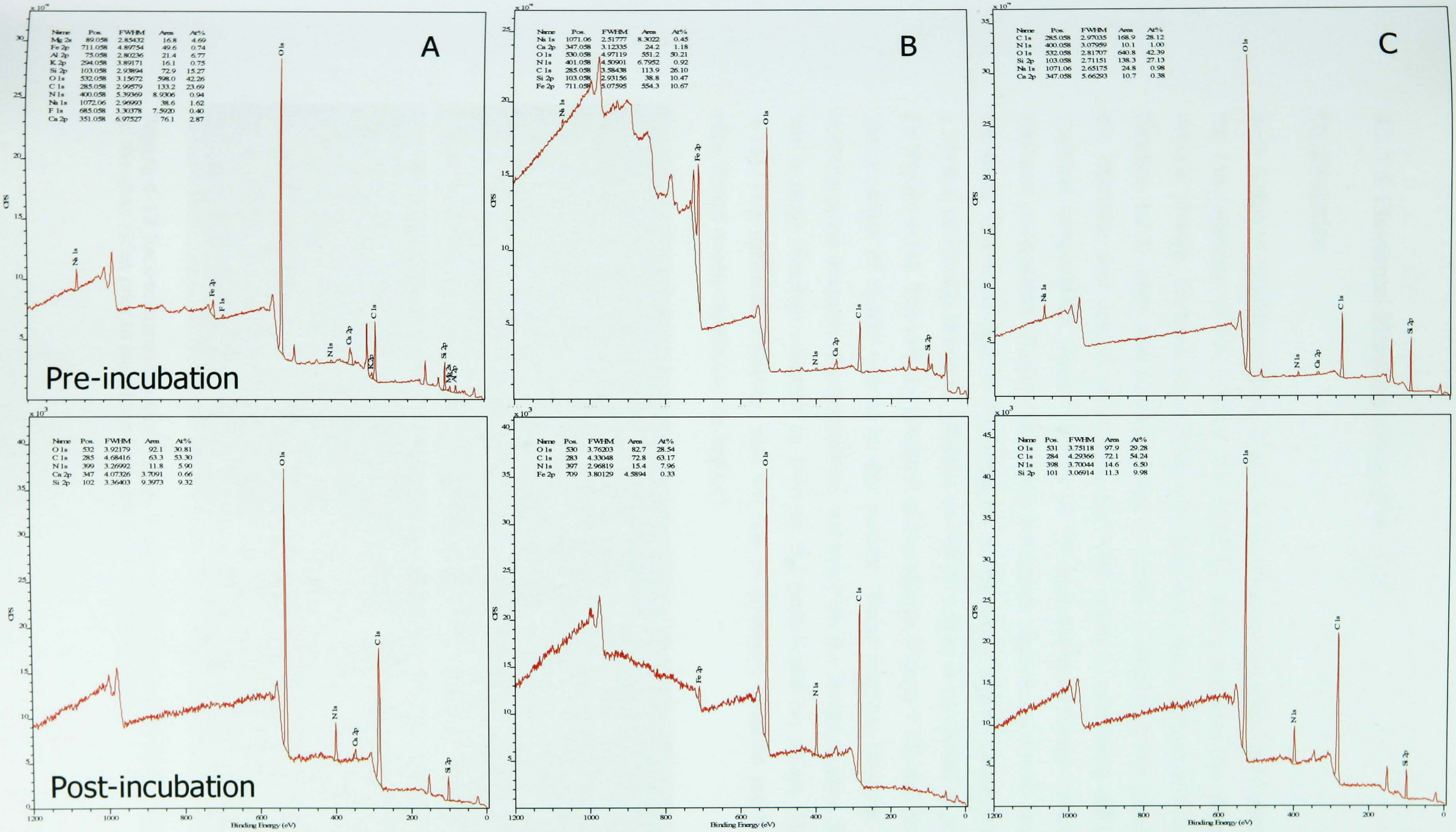



**Figure 4-12** XPS spectra for granite (A), hematite (B) and quartz (C). Top row: pre-incubation; Bottom row: post-incubation

### 4.3.1.5 Elemental Mapping using SEM-EDS

Pre-Incubation

Back-scattered electron imaging (Figure 4-13) provided valuable information on the heterogeneity of the mineral and rocks by clearly showing the different mineral phases. Surfaces seen to be heterogeneous using optical techniques (Section 4.2.1) were also heterogeneous in terms of chemistry. For example, the hematite and apatite show light and dark regions, confirming different chemical compositions. Darker regions on the back-scattered image indicate a chemical composition of lighter elements, and lighter regions are due to heavier elements. This is the case for the hematite, as the darker regions correspond to a mineral containing Si and O, whereas the lighter region is composed of purely Fe (Figure 4-14). The raft-like structure of the apatite (Figure 4-13) was due to the presence of quartz veining in the mineral. These regions, however, still contains small amounts of phosphorous, but less than the lighter regions. Shale had a large chemical variation as seen by the back-scattering image (Figure 4-13). This variation, however, was not due to dominant minerals within the rock, it was evenly distributed throughout.

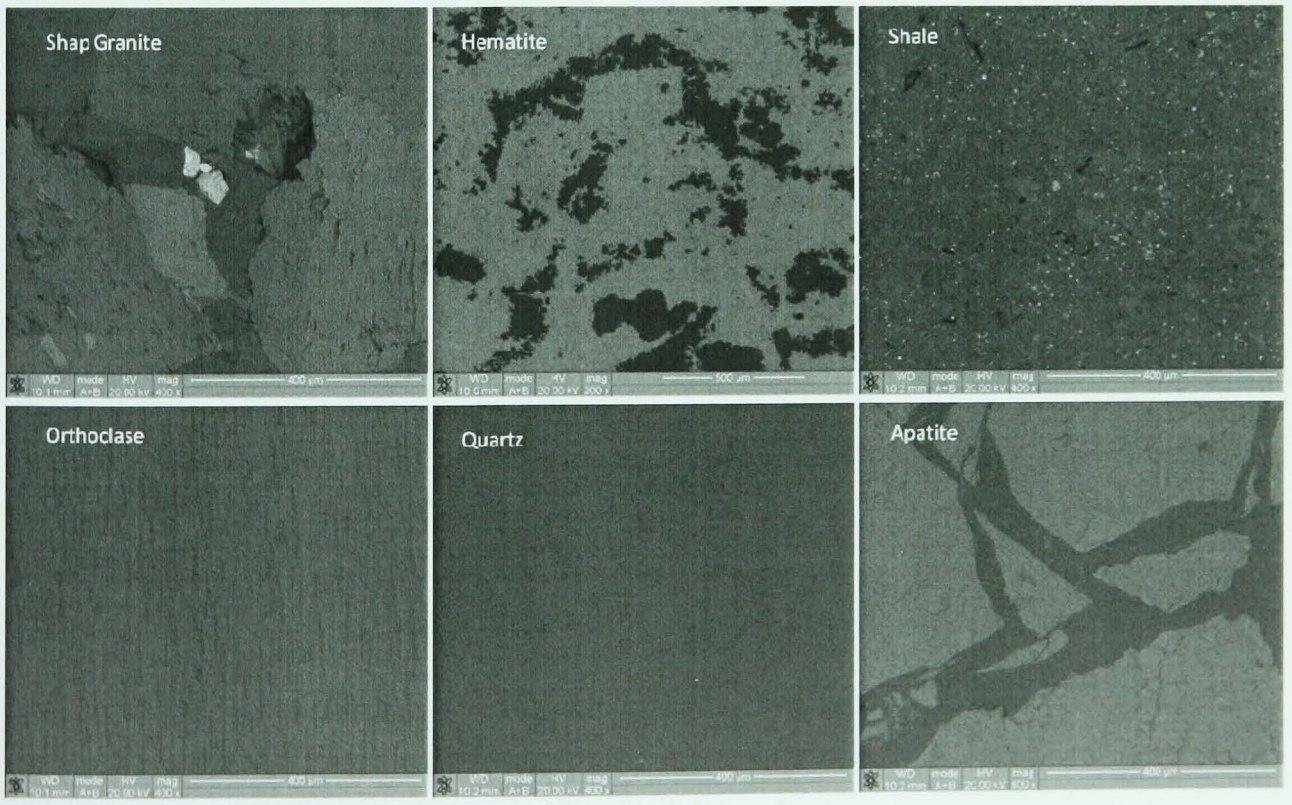



**Figure 4-13** Back-scattered electron images of the mineral and rock surfaces mounted on the glass slides prior to incubation in borehole 59 in January 2009.

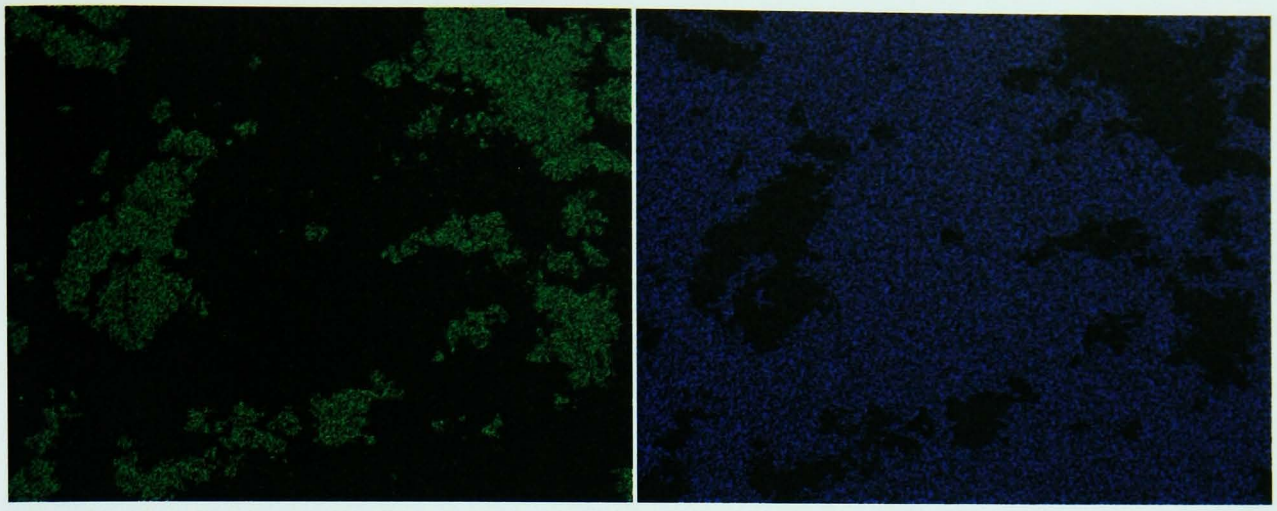


**Figure 4-14** Elemental maps of the dark and light regions of hematite. The left image shows the Si present within voids interspersed within the Fe (right)

Elemental mapping shows that the Shap Granite (Figure 4-15) has the most heterogeneous chemistry, highlighting the spatial variability of the elements with respect to the different mineral phases. Results also confirmed the presence of phosphorous in the rock, as previously found by XRF (Section 4.3.1.2). Large amorphous inclusions of phosphorus were present either individually between mineral phases (alongside pure iron inclusions), or within plagioclase crystals (Figure 4-15).

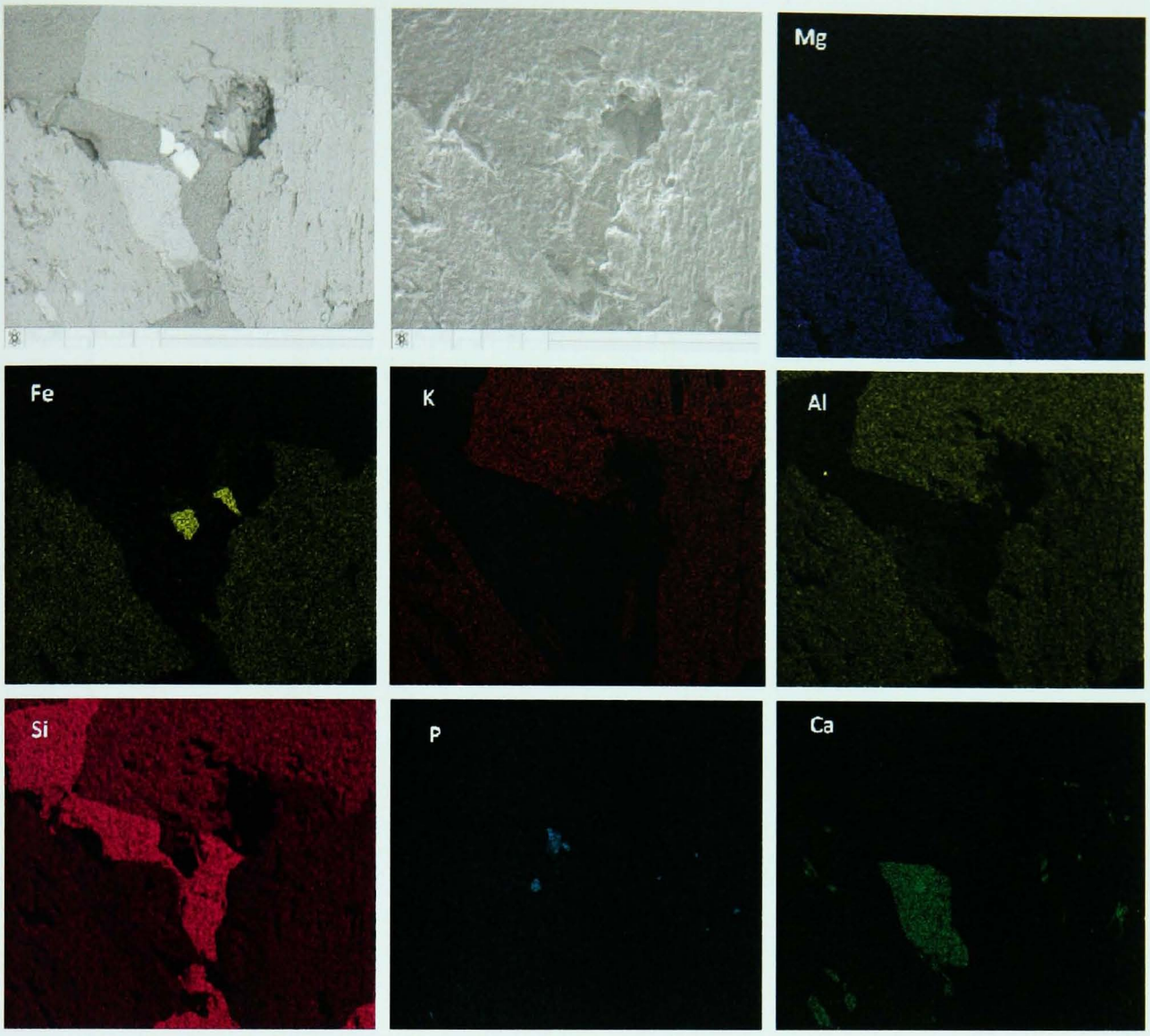



**Figure 4-15** SEM-EDS analysis of Shap granite surfaces showing back-scattered image (top left), SEM image of surface (top, middle), and elemental maps (scale equal for all images).

Post-Incubation

After incubation within borehole 59 at 30mbgl, the substrata were removed and re-analysed according to Section 4.2.3.4. Elemental mapping was not necessary at this stage, as spectral analysis was suitable to assess any changes in geochemistry. Results show that the bulk chemistry of the substrata had not changed during incubation and interaction with the contaminated groundwater. The results for orthoclase and apatite are presented (Figure 4-16), showing the chemistry for each mineral surface.

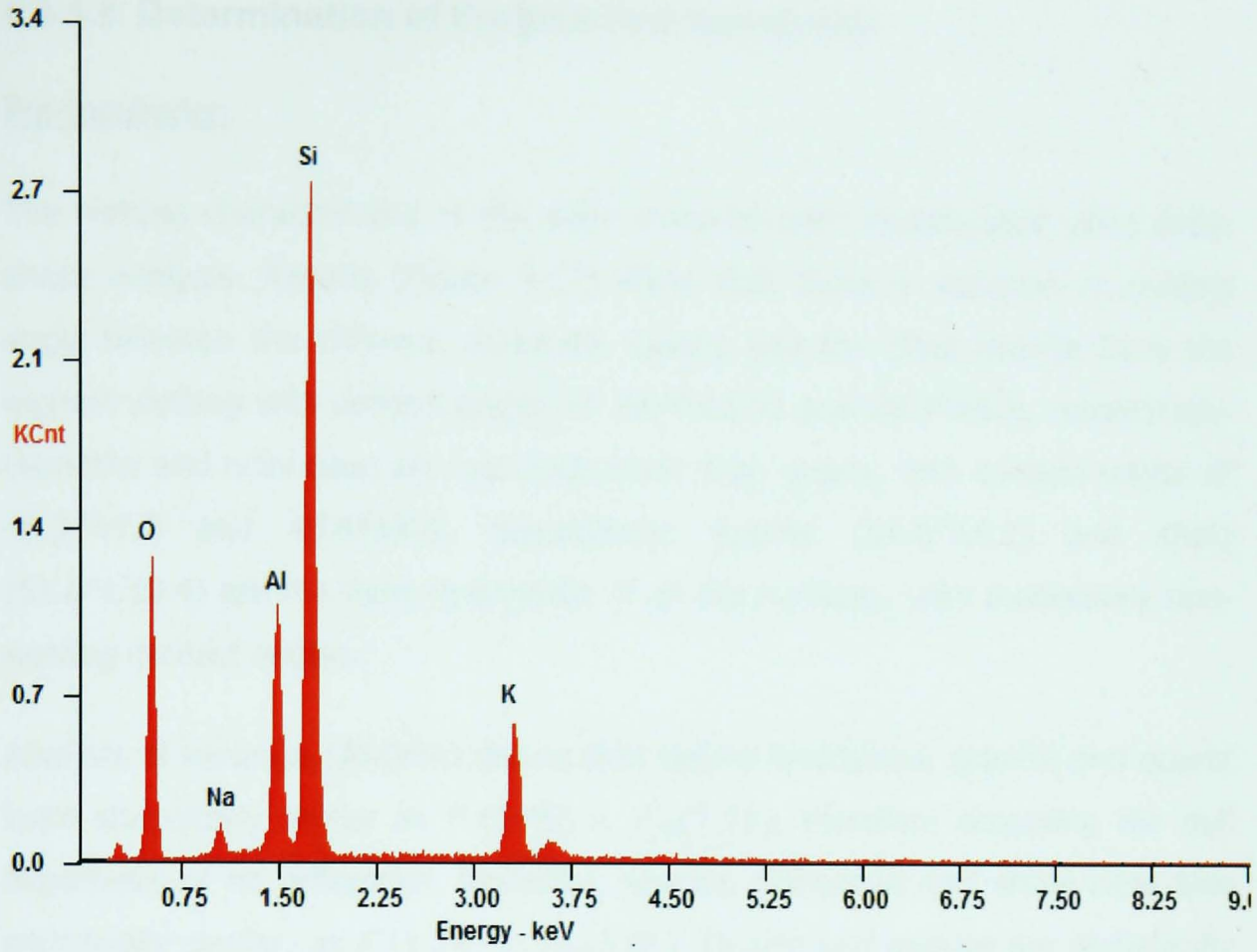



**Figure 4-16** SEM-EDS spectra of orthoclase (top) and apatite after 9 months of incubation within the aquifer at borehole 59.

### 4.3.1.6 Determination of Surface Hydrophobicity

Pre-Incubation

The wetting characteristics of the clean surfaces were investigated using drop-shape analysis. Results (Figure 4-17) show that there is variation in contact angle between the different substrata. Quartz and the Shap granite have the highest wetting with contact angles of 28.4°±0.91 and 28.9°±6.9, respectively. Hematite and orthoclase are less hydrophilic than quartz, with contact angles of 46.2°±7.2 and 46.6°±8.5, respectively. Apatite (54.8°±5.7) and shale (51.6°±10.4) are the least hydrophilic of all the surfaces, with moderately non-wetting contact angles.

Analysis of variance (ANOVA) shows that before incubation, granite and quartz were statistically similar as $F$ (0.45) < $F_{crit}$(7.71), therefore accepting the null hypothesis of no difference. Hematite, apatite, orthoclase and shale were also statistically similar, as $F$ (1.74) < $F_{crit}$(3.86). Quartz and granite are statistically different from apatite, hematite, orthoclase and shale, as $F$(47.21) > $F_{crit}$(7.71).

Post-Incubation

Drop-shape analysis of the substrata after 9 months incubation in the phenol-contaminated aquifer (Figure 4-17) shows that all surfaces were less wetting than prior to incubation. The contact angle of a 2µl drop of UHQ water on the Shap granite had increased from 28.9°±6.9 to 65.5°±5.21. ANOVA analysis indicates that the incubated granite surface has a contact angle statistically different to the clean surface ($F$ (107.3) > $F_{crit}$ (7.7)). This trend was the same for all the other surfaces (Table 4-2).

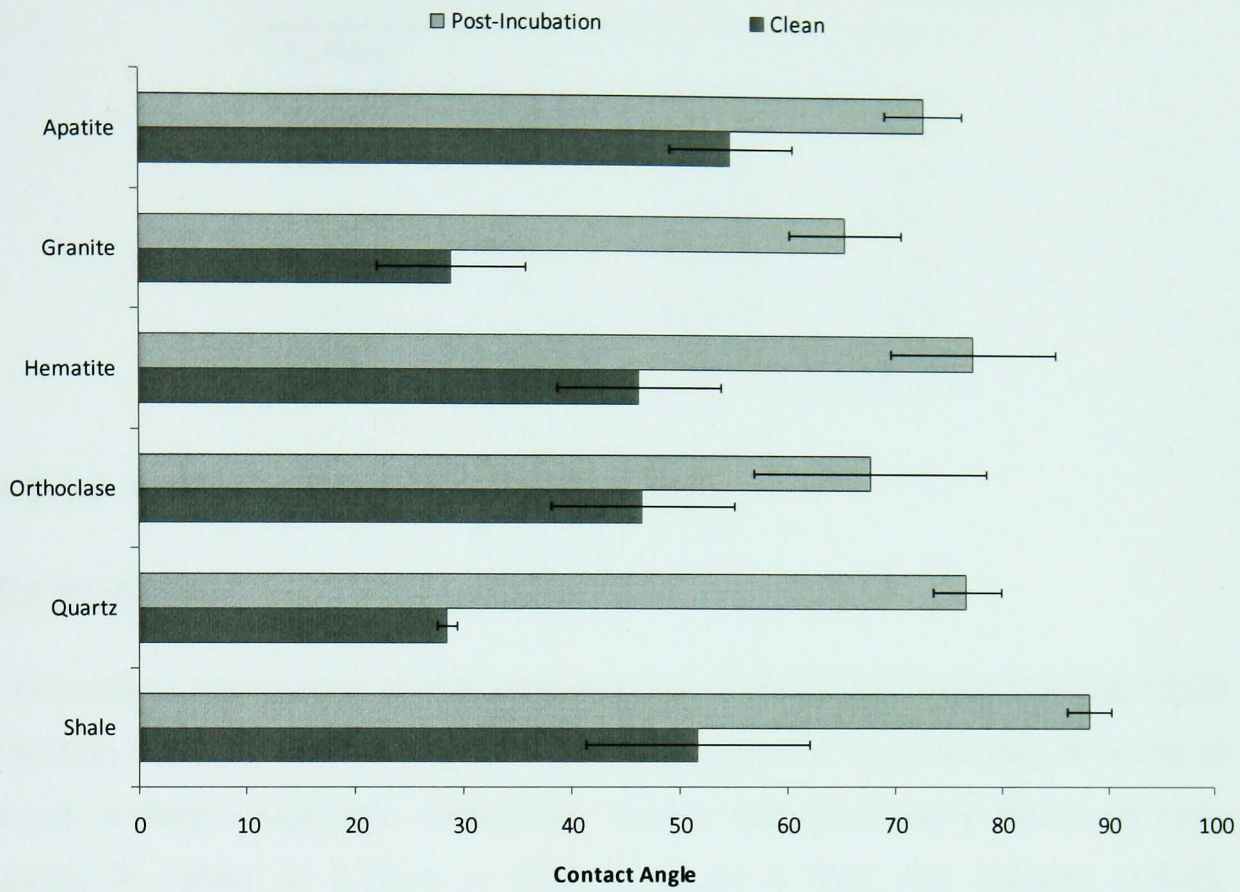



**Figure 4-17** Graph showing the average contact angle of both clean and incubated substrata (top) and optical images of the sessile UHQ droplet on quartz and shale before and after incubation.

Further ANOVA between the incubated surfaces shows that apatite, granite, hematite, orthoclase and quartz are statistically similar, as $F(3.39) < F_{crit}(3.84)$, but statistically different to the shale, as $F(14.04) > F_{crit}(5.32)$.

**Table 4-2** Results of the analysis of variance of the contact angle data

| Surface | $F$ | $F_{crit}$ |
|---|---|---|
| Apatite | 12.33 | 10.13 |
| Granite | 107.33 | 7.71 |
| Hematite | 28.94 | 10.13 |
| Orthoclase | 19.25 | 6.61 |
| Quartz | 250.97 | 7.71 |
| Shale | 27.66 | 5.99 |

### 4.3.1.7 Analysis of Surface Topography

Pre-Incubation

The surface topography of the substrata was investigated using VSI and CLSM (Section 4.2.3.7). Results (Figure 4-20) show that the surfaces vary in terms of mean surface roughness. Orthoclase, quartz and shale were found to have similar $R_a$ values of 2.32μm ± 0.17, 2.08 μm ± 0.21 and 2.22μm ± 0.25, respectively. Granite, apatite and hematite were found to have the highest mean surface roughness of 5.27μm ± 1.24, 3.86μm ± 1.1 and 4.14μm ± 0.19 respectively.

The results from the CLSM show that, for the granite (Figure 4-18), areas of higher roughness were due to pits on the biotite surface. Smoother areas were localised on the quartz and orthoclase crystals. This was also visible from images taken using the scanning electron microscope (Figure 4-19). A similar heterogeneity in surface roughness was seen for the apatite due to pits in the coupon surface.

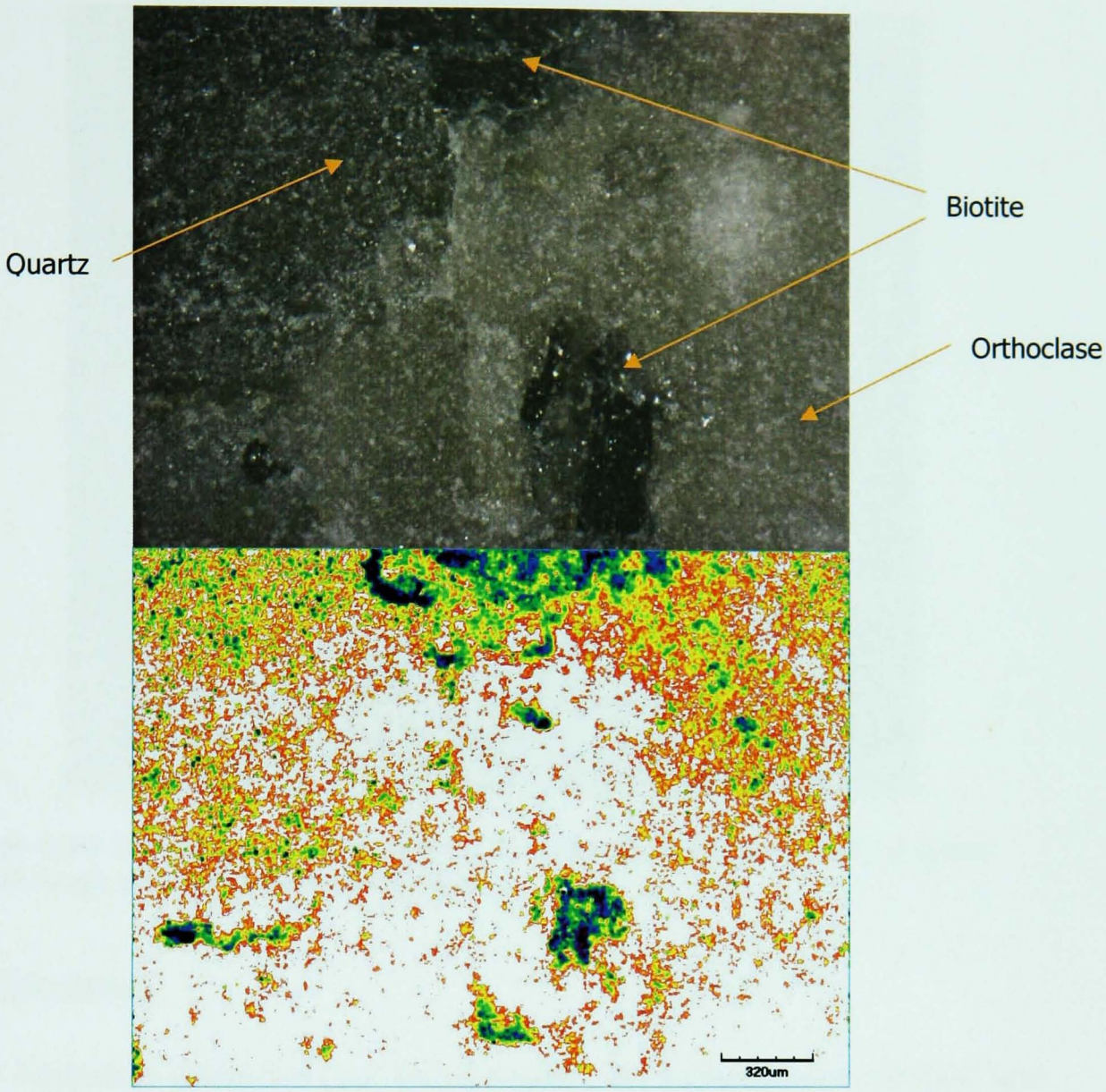



**Figure 4-18** CLSM optical image of granite coupon surface (top) and topographic image corresponding to the same field of view, showing deeper pits on the biotite (dark regions). Scale equal for both images.

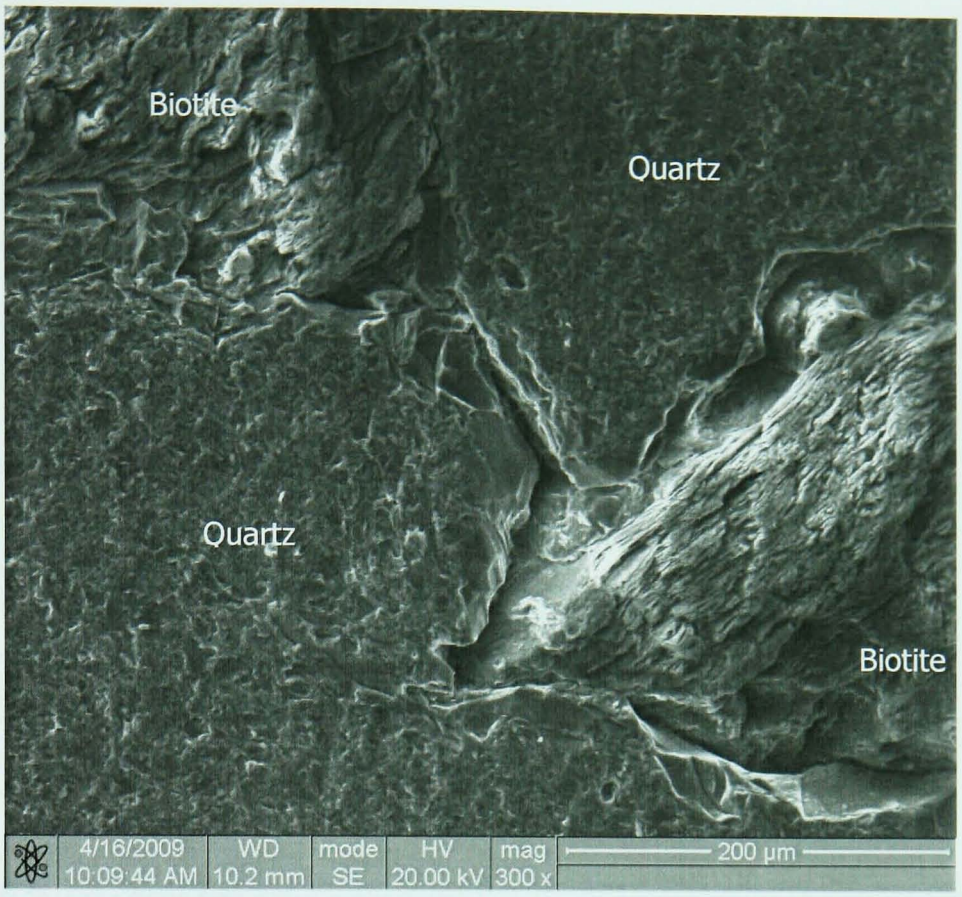



**Figure 4-19** SEM image of granite surface, showing smoother topography of quartz around rough, cleaved and chipped biotite.

Post-Incubation

After incubation within the Four Ashes aquifer, the surfaces were removed and analysed by VSI to assess the surface roughness ($R_a$). Results (Figure 4-20) show that only hematite and shale had changed significantly during incubation within the phenol-contaminated groundwater. The most prominent change in surface roughness was for hematite, which was 4.14µm ± 0.19 prior to incubation, but was 1.99 µm ± 0.09 after incubation within the aquifer. The 3D images generated from the VSI (Figure 4-21) confirm the observation from the $R_a$ data, as the many of the surfaces had stable surface topography throughout incubation. The grain boundaries observed on the heterogeneous granite surface prior to incubation were still present after 9 months incubation (Figure 4-21).

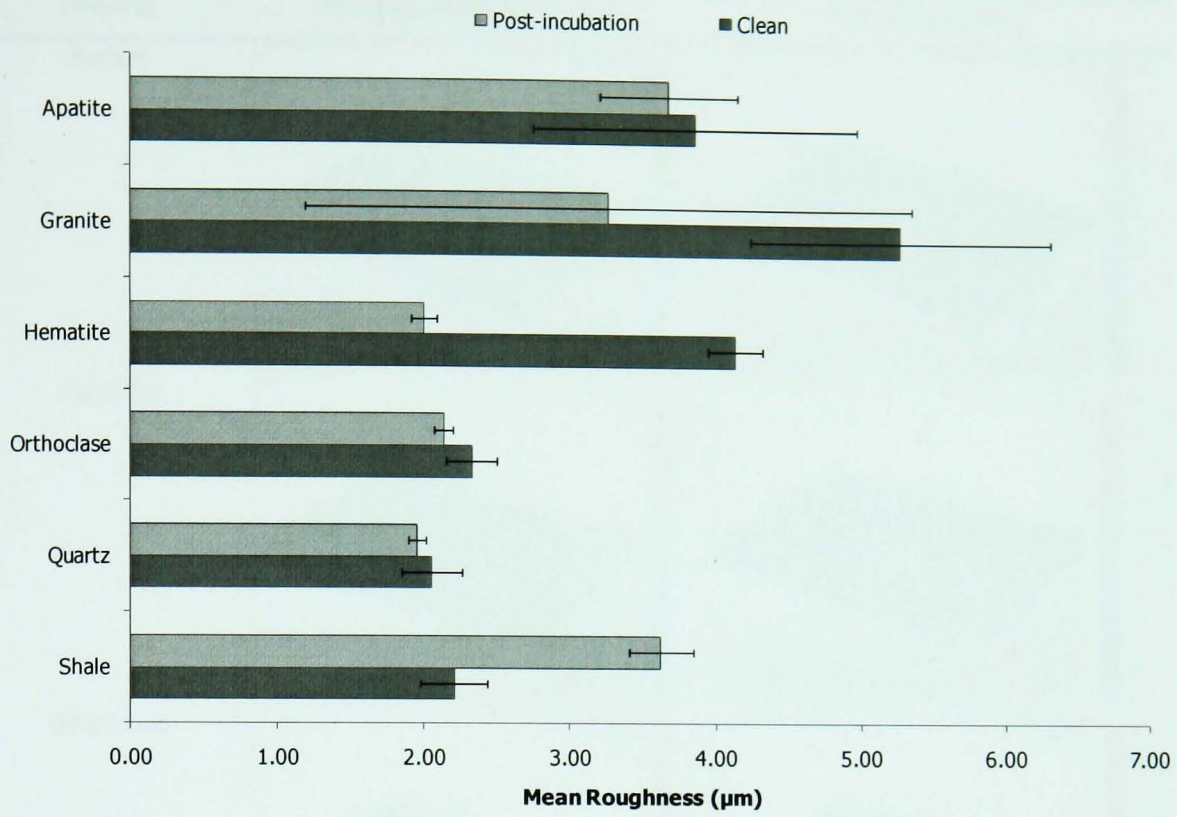



**Figure 4-20** Mean surface roughness ($R_a$) of both clean and incubated coupons

ANOVA analysis of the surface roughness measurements show that there is a statistical difference in surface roughness between the clean and incubated surfaces of hematite and shale, as $F$ was greater than $F_{crit}$ in both cases (Table 4-3). Moreover, ANOVA confirms that the apatite, granite, orthoclase and quartz maintained stable surface topography, as $F_{crit}$ was higher than $F$ in all cases. Finally, ANOVA indicates that prior to incubation there were two distinct groups of surface roughness. Apatite, granite and hematite had statistically similar surface topography, as did orthoclase, quartz and shale. After incubation, apatite, granite and shale were statistically similar, quartz and hematite were statistically similar, and orthoclase was dissimilar to all the other surfaces.

**Table 4-3** ANOVA analysis of the different surfaces before and after incubation within the Four Ashes aquifer.

| Surface | $F_{crit}$ | $F$ |
|---|---|---|
| Apatite | 4.84 | 1.14 |
| Granite | 5.59 | 2.28 |
| Hematite | 5.32 | 586.22 |
| Orthoclase | 6.61 | 3.22 |
| Quartz | 5.59 | 2.02 |
| Shale | 5.59 | 87.73 |

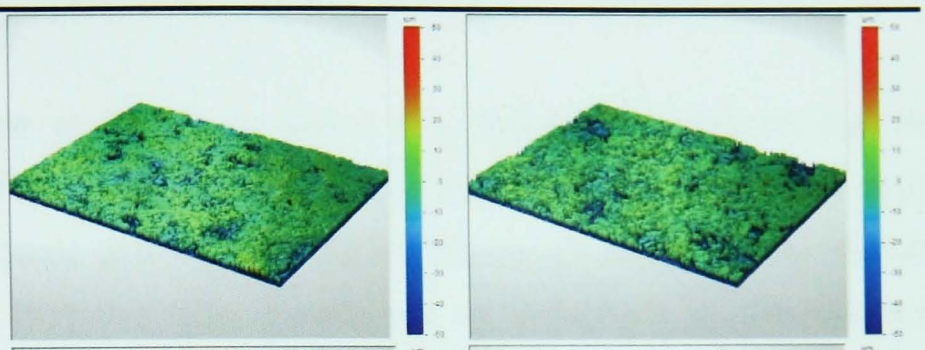
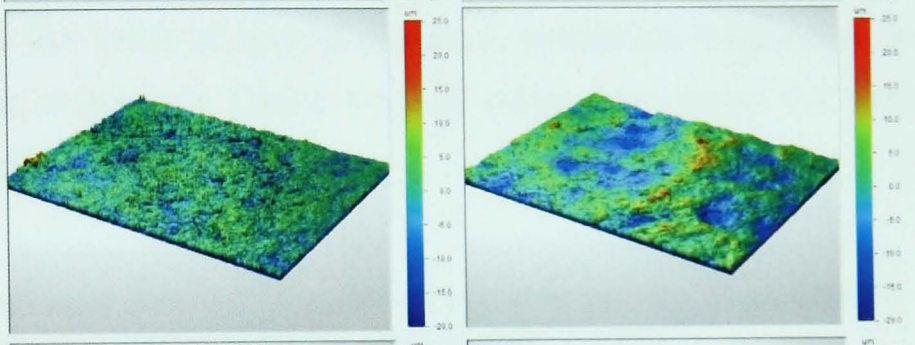
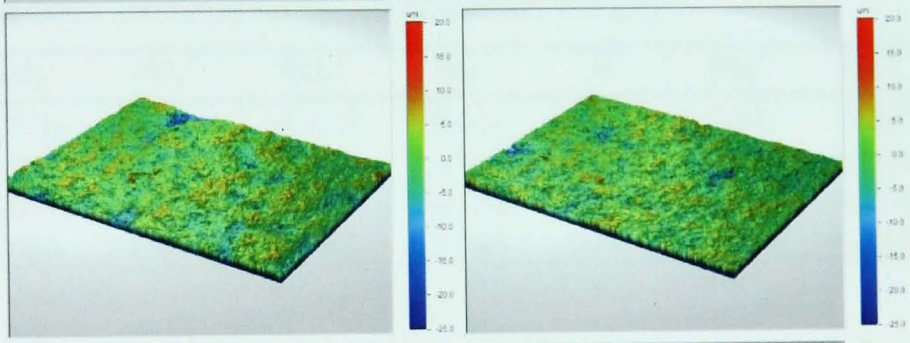
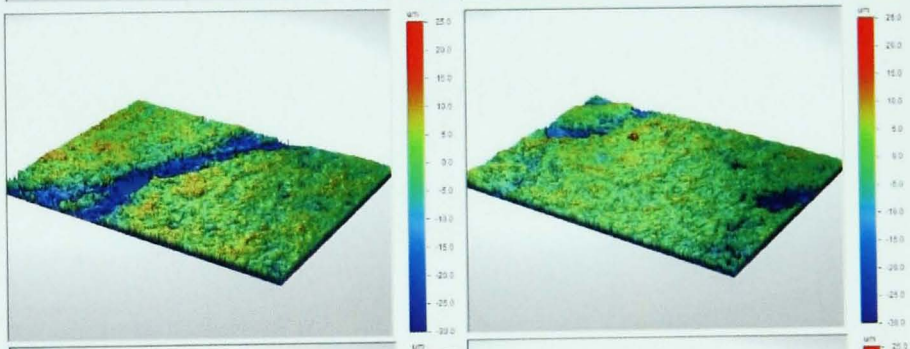
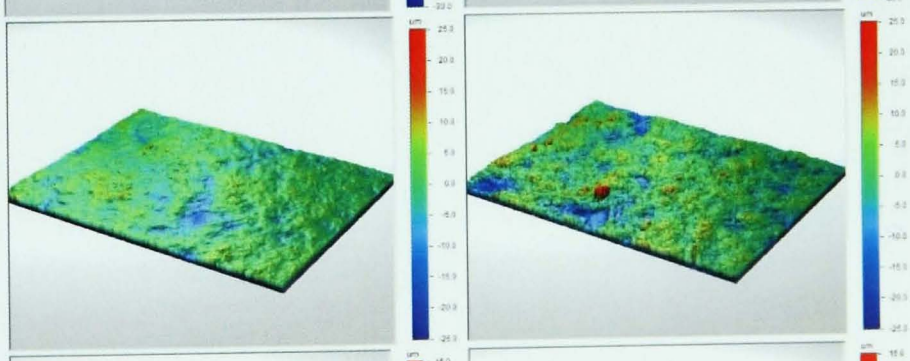
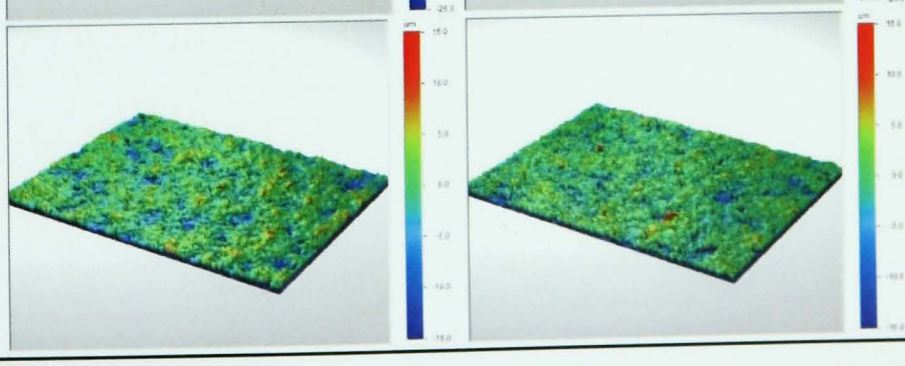
| Mineral | Pre-Incubation (clean) | Post-Incubation |
|---|---|---|
| Apatite |  | |
| Hematite |  | |
| Orthoclase |  | |
| Granite |  | |
| Shale |  | |
| Quartz |  | |

**Figure 4-21** 3D surface images of each coupon before and after incubation within the aquifer.

### 4.3.2 Effect of Coupon Bonding Resin on Groundwater Chemistry

Leaching tests were performed to assess the potential contribution that the resin used to bond the surrogate geological substrata to the glass slides might make to local inorganic geochemistry during incubation in the aquifer. Results (Table 4-4) show that the resin contributed no phosphate to the leachate. However, extremely low levels of sulphate, sodium, potassium, magnesium and calcium leached out of the resin (Table 4-4). All values were below the lowest standard used to calibrate the instrument.

**Table 4-4** Concentrations of dissolved inorganic species after leaching of the bonding resin. All samples have been corrected for the blank and are in mg $g^{-1}$.

| | $NO_3^-$ | $PO_4^{2-}$ | $SO_4^{2-}$ | $Na^+$ | $K^+$ | $Mg^{2+}$ | $Ca^{2+}$ |
|---|---|---|---|---|---|---|---|
| **Leachate Rep 1** | 0.000 | 0.000 | 0.018 | 0.056 | 0.003 | 0.007 | 0.057 |
| **Leachate Rep 2** | 0.100 | 0.000 | 0.102 | 0.220 | 0.011 | 0.025 | 0.091 |
| **Leachate Rep 3** | 0.051 | 0.000 | 0.050 | 0.133 | 0.005 | 0.013 | 0.035 |
| **Average** | 0.050 | 0.000 | 0.057 | 0.136 | 0.006 | 0.015 | 0.061 |

### 4.3.3 Microbial Attachment to Surrogate Substrata

#### 4.3.3.1 Rock and Mineral Coupons

Fluorescence Microscopy

Microbes attached to the surrogate geological substrata suspended within the Four Ashes aquifer at 30mbgl in borehole 59 between January and August 2009 were stained and imaged according to Section 4.2.8.1. Cells counts (Figure 4-22) highlight that most cells were attached to the Shap granite (2.2 x $10^6$ cells $cm^{-2}$). A similar number of cells were attached to the orthoclase (3.02 x $10^6$ cells $cm^{-2}$) and hematite (2.61 x $10^6$ cells $cm^{-2}$). The lowest number cells were attached to apatite (2.19 x $10^6$ cells $cm^{-2}$), shale (1.92 x $10^6$ cells $cm^{-2}$) and quartz (1.1 x $10^6$ cells $cm^{-2}$).

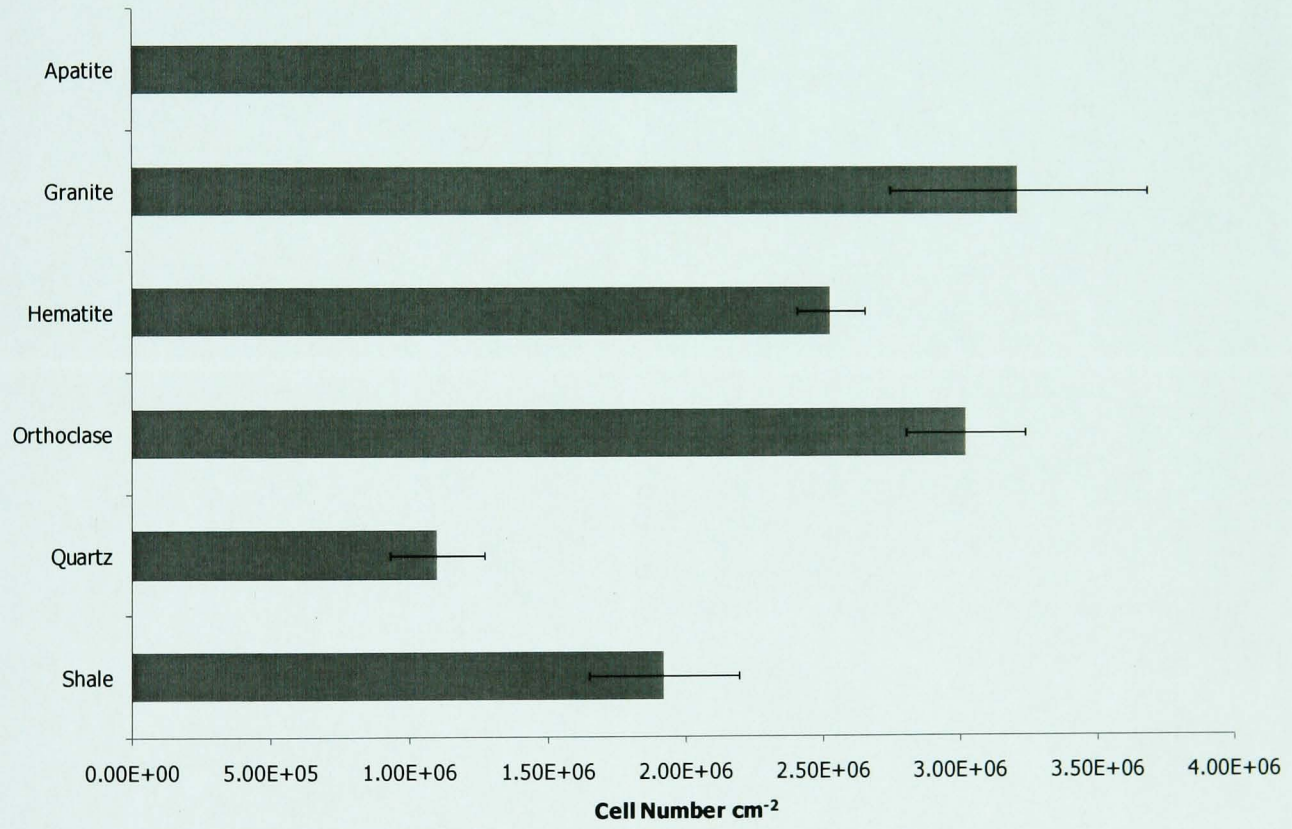



**Figure 4-22** Number of cells attached to the surrogate geological surfaces after 9 months of incubation within the Four Ashes aquifer.

Figure 4-23 shows that there was a large amount of attachment to each surface. Cells generally attached in clusters rather than individually, and they were seen to be wrapped in an EPS matrix on surfaces where extensive attachment was observed (all except quartz). Although imaging was performed using a reflected fluorescence source, the optical light source is transmission

only, therefore it was not possible to assess whether the clustering of cells was related to heterogeneities in the coupons.

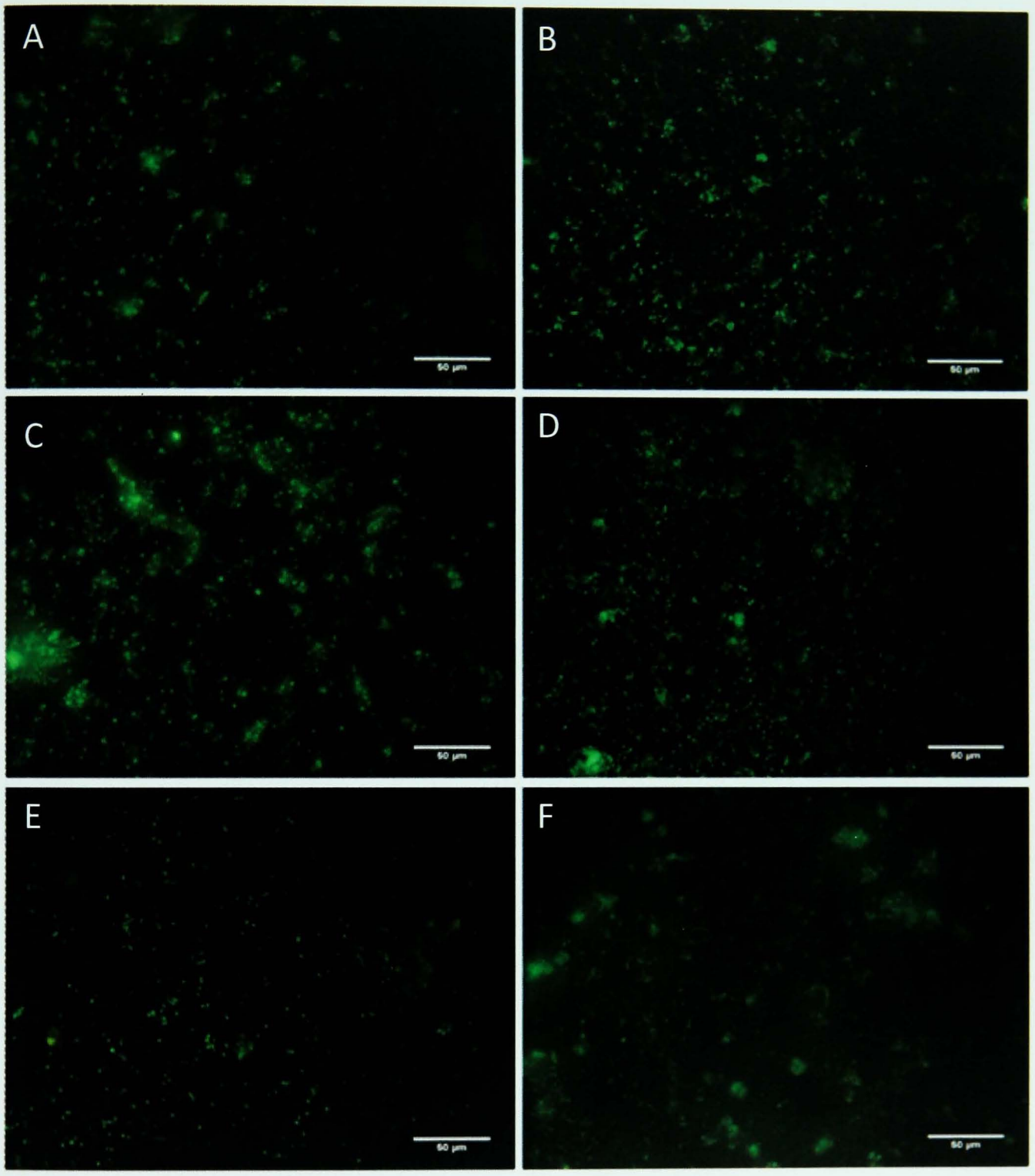



**Figure 4-23** Epifluorescence images of microbial attachment to each surface using syto 9 staining after incubation in the Four Ashes aquifer for 9 months. A, apatite; B, Shap granite; C, hematite; D, orthoclase; E, quartz; F, shale.

ANOVA analysis indicates that granite, hematite and orthoclase had a statistically similar number of attached microbes, as $F$ (1.17) was less than $F_{crit}$ (9.55). Apatite and shale also had statistically similar numbers of attached microbes, as $F$ (1.97) was less than $F_{crit}$ (18.51). The number of microorganisms attached to quartz was statistically dissimilar to all the other surfaces.

Environmental Scanning Electron Microscopy (ESEM)

ESEM imaging was undertaken to validate the microbial attachment seen using epifluorescence microscopy. Results confirm that the cells seen under epifluorescence microscopy were attached to the coupons, most of which were surrounded by white precipitates (Figure 4-24). Also, microbial cells were seen to be dividing on the Shap granite surface prior to fixation (Figure 4-24). However, prior to imaging by ESEM, technical problems with the fridge where the samples were stored resulted in the freezing of the samples. This incident could have skewed the results of the ESEM due to a loss of cells due to bursting during the freezing process.

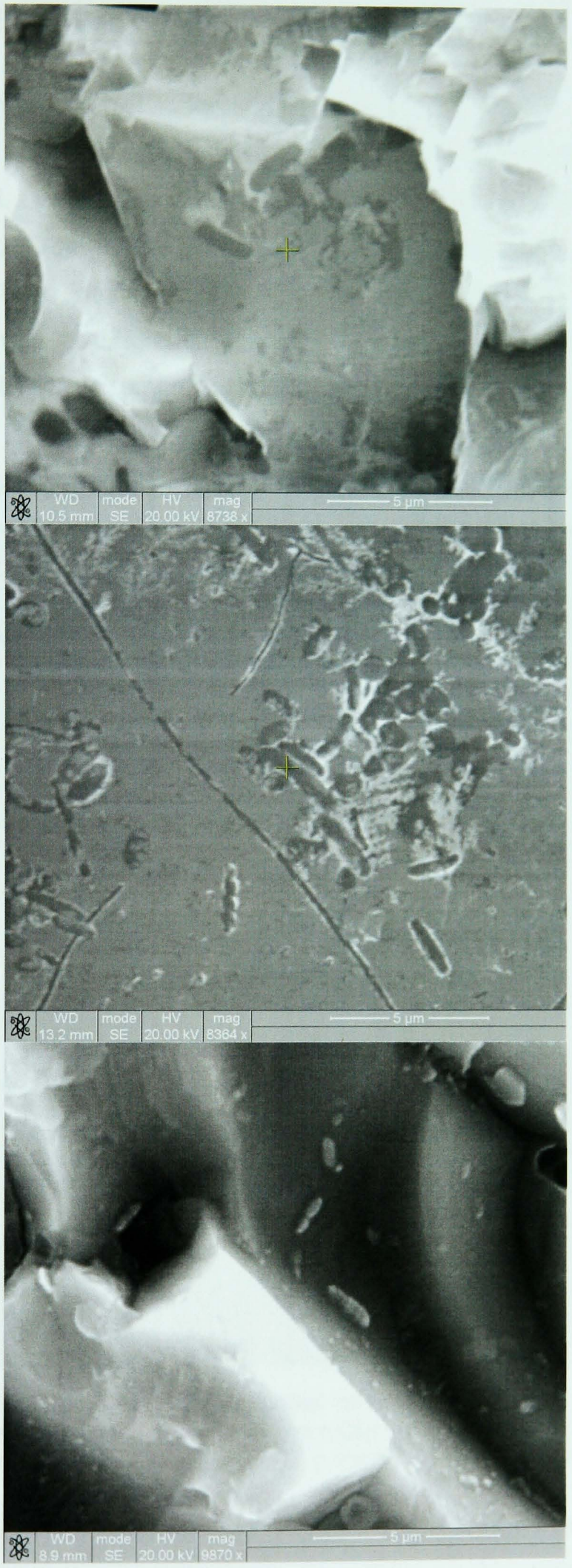



**Figure 4-24** ESEM micrographs of microbes attached to orthoclase (top), apatite (centre) and Shap granite.

### 4.3.4 DGGE Profiles of Attached Communities

Analysis of the microbial communities attached to quartz, Shap granite, sandstone and within the groundwater at 30mbgl in borehole 59 using DGGE was performed. Profiles (Figure 4-25) indicate that the community was diverse on each surface. There were no major variations in community structure, only differences in band intensities. Although common bands were evident on the DGGE, there was a difference between the attached community and the groundwater planktonic community at 30mbgl.

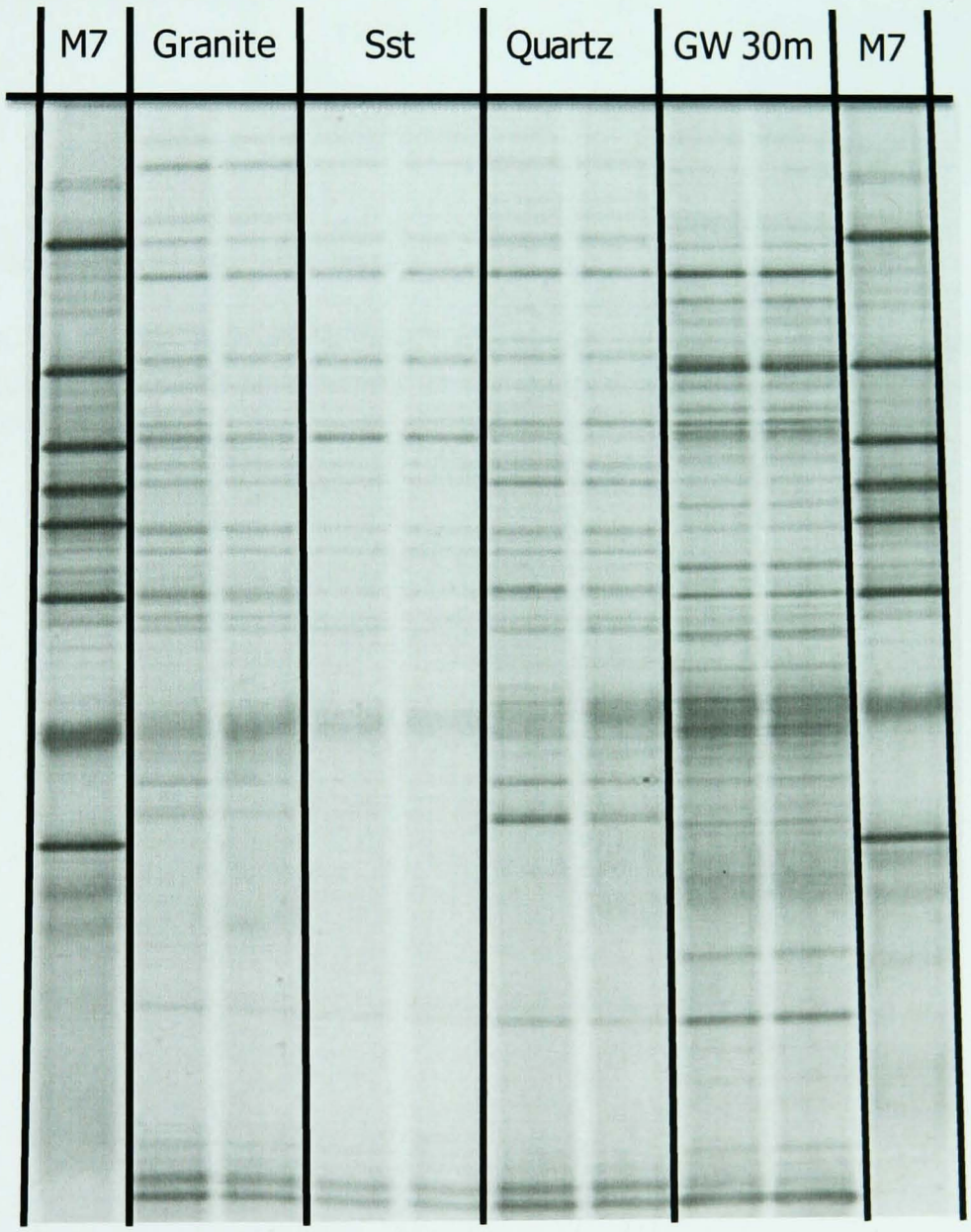



**Figure 4-25** DGGE profiles of the microbial communities attached to quartz, Shap granite and sandstone, and the planktonic microbial community within the groundwater at 30mbgl in borehole 59 in August 2009. M7 = species marker (Section 3.2.5.1).

Cluster analysis of the DGGE profiles (Figure 4-26) indicates that the replication of each sample was extremely good, as profiles cluster with at least 96% similarity. The planktonic microbial community within the groundwater at

30mbgl in borehole 59 clusters separately to the attached community on all surfaces, and is very dissimilar, having only 55% similarity. The microbial communities attached to sandstone and the Shap granite cluster with 83% similarity. These communities cluster with 77% similarity to the community attached to quartz.

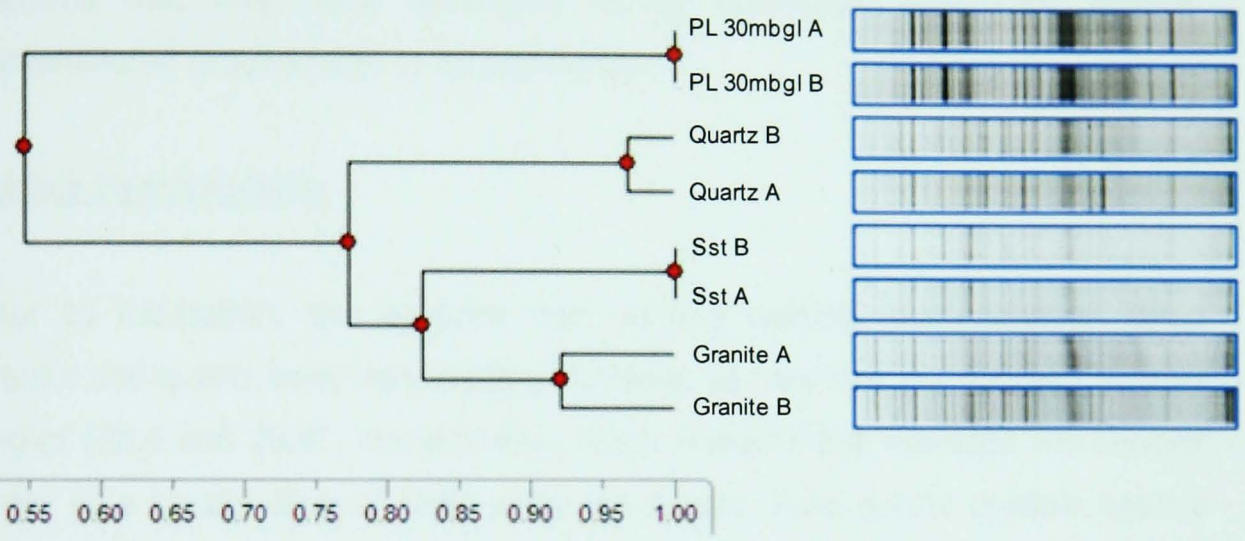



**Figure 4-26** Cluster analysis of DGGE profiles of microbial communities attached to granite, quartz and sandstone, as well as the planktonic microbial community within the groundwater at 30mbgl.

## 4.4 Discussion

### 4.4.1 Coupon Weathering

Prior to, and after, incubation in the aquifer, the surrogate geological coupons were characterised using a variety of techniques. This allowed any weathering patterns that may have developed during incubation within the phenol-contaminated groundwater to be highlighted.

Surface Hydrophobicity

Prior to incubation, the coupons had varying wetting characteristics. Shap granite and quartz were very wetting surfaces, as they had low average contact angles (28.4 and 28.9°, respectively). Much research has assessed the contact angle of a sessile drop of UHQ water on quartz. Pure quartz crystals have a contact angle of 0° due to the surface OH groups resulting in a perfectly wetting surface (Tschapek *et al.,* 1983). However, natural quartz has been found to have a contact angle of 6-40°, agreeing with the results of this study (Kowalczyk *et al.*, 1996; Janczuk *et al.*, 1990). Analysis of variance confirmed that the hydrophobicity of the Shap granite was statistically similar to quartz. The greater error associated with Shap granite is due to the heterogeneity of the mineral assemblage in the rock. Shap granite contains both wetting (e.g. quartz) and moderately non-wetting surfaces (orthoclase, apatite, hematite), resulting in a variation in contact angle measurements.

The contact angles of orthoclase and hematite were comparable to published values analysed on both natural and synthetic surfaces (Baker *et al.*, 2009; Iveson *et al.*, 2003). Iveson *et al.* (2003) found that the contact angle of natural hematite varies depending on the composition of the ore, as an increased amount of quartz and clay minerals can result in a more wetting surface. The contact angles of apatite were unusually high, showing moderately non-wetting behaviour. Sis and Chander (2003) found that the average contact angle of apatite was 10° using distilled water in the absence of any surfactants.

Shale was less-wetting prior to incubation in comparison to the other coupons as it is of oil original. As shale is a fine-grained argillaceous rock, it is very

porous (up to 80%), resulting in absorption of oil (mainly kerogen) into the void spaces (Duff, 2002). This causes an elevated average contact angle due to the hydrophobic nature of the oil. Had the shale not have been of oil origin, the average contact angle would be expected to be similar to those of quartz and granite, as shale typically contains around 30% quartz, 18% feldspars and 18% mica, a mineral assemblage similar to granite (Duff, 2002). As the contact angle for shale was significantly higher than quartz, it was used as a comparison to quartz in terms of the effects of hydrophobicity on microbial attachment (Section 4.4.2).

After incubation within the aquifer, all the coupons had statistically less-wetting surfaces. Apatite, granite, hematite, orthoclase and quartz had statistically similar contact angles, whereas shale was statistically dissimilar to the other surfaces. The increase in contact angles for all the surfaces could be attributed to the major change in coupon chemistry seen in the top 2nm of the surfaces using XPS (Figure 4-12). It was found that there was a significant increase in carbon on the surface of each coupon. This indicates that the organic compounds in the groundwater may have sorbed to the surfaces. Organic molecules are generally hydrophobic, unless a polar group is included in the structure, forming an amphiphilic molecule (both hydrophilic and hydrophobic) (Schwarzenbach *et al.*, 1993). Sorption would therefore result in an increase in hydophobicity of the surfaces. The porous nature of shale will have resulted in more absorption of the contaminated groundwater (Duff, 2002). This may have resulted in the coupon being statistically more hydrophobic than the other surfaces.

It is possible to quantify the sorption of organic compounds onto surfaces by calculating the compound retardation factor, or $R_f$ (Schwarzenbach *et al.*, 1993). Lighter organic compounds are more soluble and mobile within groundwater systems, resulting in lower distribution coefficients ($K_{oc}$). Therefore, it would be more likely that the heavier phenolic compounds (xylenols), which have high $K_{oc}$ values, would sorb onto the surfaces of the coupons, as the lighter phenol and cresols would be in the dissolved phase (Fetter, 1988). It is not possible to calculate $R_f$ for the coupons, or the bags of grains, as the fraction of organic

carbon on the surfaces was not measured after incubation, and the bulk density of the samples are not known.

Microbially-mediated mechanisms could also result in a change in hydrophobicity of the coupons. Microbes have been found to precipitate extracellular compounds (e.g. polysaccharides) that can increase the wettability of the surface, as well as mineralise elements (e.g. S) that can result in a decreased wetting behaviour (Somasundaran *et al.*, 1998). Further analysis, such as characterising the sorbed organic compounds directly using Fourier transform infra-red sprectroscopy could provide information on whether the organics are of microbial origin (e.g. exoplymeric substances) or due to the sorption of xenbiotics onto the surfaces. Extraction of the sorbed organics followed by GC-MS could also be used to characterise the surface organics. Finally, simple techniques, such as Nile red staining in conjunction with epifluorscence microscopy could be used to investigate the association of the microbial cells with any lipids present on the coupon surfaces (Andrews *et al.,* 2010). If lipids were found after staining with Nile red, this would suggest that microbial processes are contributing to the changes in hydrophobicity of the coupons due to the build up of hydrophobic lipids.

Ultimately, such data could lead to a conclusion on whether microbial or chemical processes are dominatant in accounting for the changes in surface hydrophobicity.

Surface Topography

Measurements of surface topography using VSI (Section 4.3.1.7) show that the coupons had varying surface roughness ($R_a$). Prior to incubation, apatite, Shap granite and hematite had statistically similar $R_a$, as did orthoclase, quartz and shale. The variation between the two groups of surfaces is due to the heterogeneities of the coupons. Orthoclase, quartz and shale are homogenous surfaces that did not have any surface defects. This resulted in a consistent and lower surface roughness than apatite, Shap granite and hematite. Post-incubation, orthoclase and quartz maintained statistically similar surface roughness. Moh's scale describes the hardness of minerals based on scratch

tests. Data (Table 4-5) shows that orthoclase and quartz are hard surfaces (6 and 7 respectively in Moh's scale), which are more likely to have stable surface topography as they are more resistant to physical weathering (Gorbushina, 2007).

**Table 4-5** Moh's scale of hardness for rocks and minerals (Cook and Kirk, 1995). *Minerals used in this study.

| Hardness | Mineral |
|---|---|
| 1 | Talc |
| 2 | Gypsum |
| 3 | Calcite |
| 4 | Fluorite |
| 5 | Apatite* |
| 6 | Orthoclase* |
| 7 | Quartz* |
| 8 | Topaz |
| 9 | Corundum |
| 10 | Diamond |

Shale was the only surface to become rougher after incubation (Figure 4-20). Changes in surface roughness are related the stability of the coupons. Due to its porosity, shale will have become saturated with the groundwater, resulting in the swelling and subsequent cracking/pitting of the surface, which was visible by SEM (Figure 4-27). This commonly occurs with other porous rocks, such as sandstones (Gorbushina, 2007). de la Torre *et al.* (1993) also found from *in vitro* studies that microbial attachment can influence the deterioration of substratum surfaces by influencing the dissolution of surfaces by the release of organic acids, resulting in biopitting.

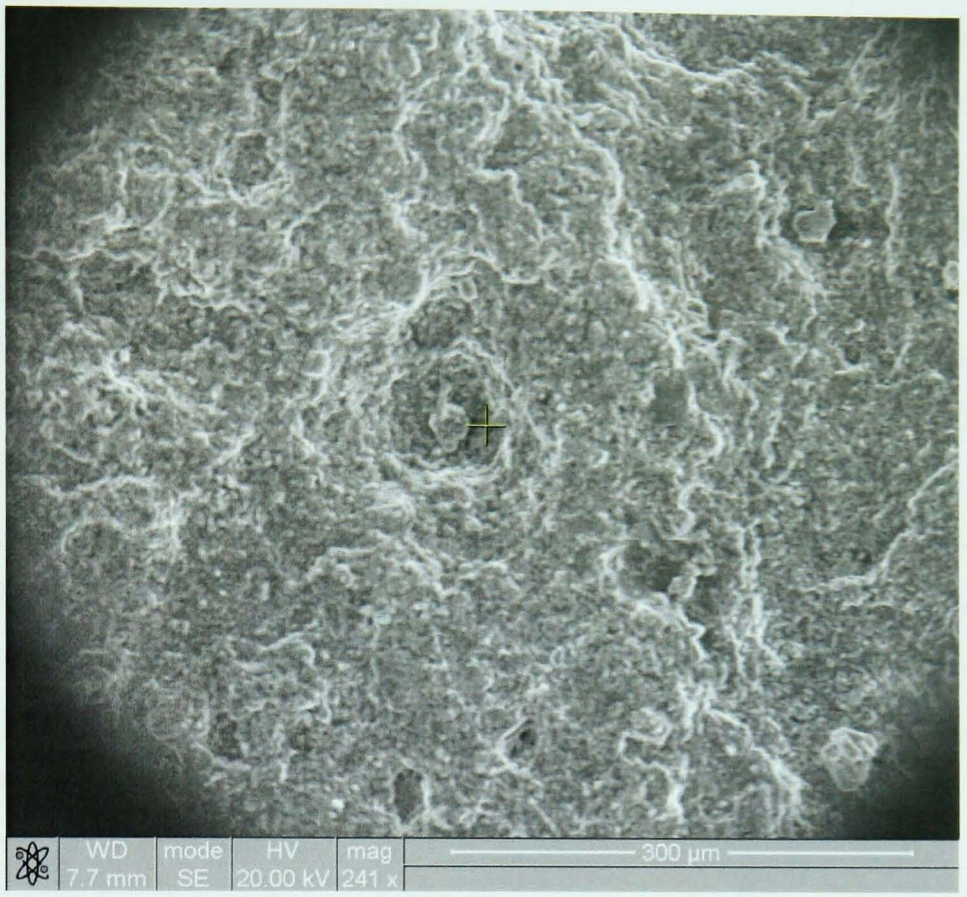



**Figure 4-27** Image showing pitted surface of shale after incubation within the aquifer.

The Shap granite is very heterogeneous, and is composed of both hard and soft minerals, resulting in variations is surface roughness. Shap granite contains quartz, orthoclase, apatite and plagioclase (Section 4.3.1.5), as well the soft mineral biotite (2 - 2.5 on Moh's scale). This varied assemblage can result in preferential grinding and surface perturbation during coupon preparation, ultimately leading to a high $R_a$ prior to incubation. Also, the rough cleavage planes of biotite (Figure 4-19) may have contributed to the higher $R_a$ of Shap granite, as well as pits found at mineral grain boundaries, which were imaged by the VSI (Figure 4-21). After incubation in the aquifer, the $R_a$ of Shap granite was lower (3.26 ± 2.08µm), suggesting that the soft minerals (e.g. biotite) had been preferentially weathered. This is not due to physical weathering, as the groundwater is only flowing at an extremely low rate (~2.5cm $day^{-1}$, Williams *et al.,* 2001). However, chemical changes in the surface may result in preferential leaching of elements from minerals. Mineral dissolution and leaching occurs at the highest rates when the pH is greater than 8 or less than 5 (Bennett *et al.,* 2001; McMahon *et al.*, 1995). The groundwater pH is circum neutral in the Four Ashes groundwater (Thornton *et al.,* 2001). This would result in a rate minimum for remineralisation. However, microorganisms attached to the mineral surfaces can change local pH, and therefore influence mineral dissolution. A reduction in

local pH is caused by the release of organic ligands associated with expolymeric substance, which significantly enhance silicate dissolution (Mailloux *et al.,* 2005; Bennett *et al.,* 2001). The microbes attached to the Shap granite were found to be enveloped in an EPS-matrix (Figure 4-23), indicating that bioweathering may have caused a change in the surface topography, resulting in the release of important macronutrients. Evidence of this was present on the ESEM images (Figure 4-24), which showed white precipitates around the microbial cells, suggesting remineralisation of the surface to form secondary minerals. A similar phenomenon was found by Bennett *et al.* (2001) who attributed the precipitates to the biomineralisation of $CaCO_3$ after mineral dissolution by the release of organic ligands. Microorganisms can also physically perturb a surface due to mineral precipitation, hydration and disaggregation (Gorbushina, 2007; Banfield *et al.,* 1999).

Optical imagining of apatite confirmed that the surface was pitted prior to incubation (Table 4-1). This is a property related to the formation of the mineral. It is clear that a source of uncrystallized quartz infiltrated the mineral after its precipitation, causing quartz 'veining' and the raft-like structure seen under backscattered SEM (Figure 4-13; Vapnik *et al.*, 2007). This disturbed the structure of apatite, resulting in voids and pits that contributed to a higher $R_a$ prior to incubation in the groundwater. Apatite maintained a statistically similar surface roughness after incubation. Again, this is most probably due to the hardness of the mineral (5 on Moh's scale, Table 4-5).

Hematite had a rough surface (4.14µm ± 0.19) prior to incubation due to the formation of small pits on the surfaces after grinding (Figure 4-14). This could be due to inclusions of quartz, which is common in hematite mineral deposits (or ores). Quartz forms from the cooling magmatic fluid after the precipitation of other minerals at higher temperatures (Atkinson and Atkinson, 1979). Hematite is softer than quartz (approximately 6.5 on Moh's scale), which may have resulted in physical disturbance of the surface during the grinding procedure. Hematite would have been preferentially ground before the quartz inclusions, resulting in an uneven surface, and therefore a higher $R_a$ and the noticeable peaks seen on the 3D images generated by the VSI (Figure 4-21). However, after incubation in the aquifer, the $R_a$ of hematite changed significantly to 1.99

μm ± 0.09. VSI imaging (Figure 4-21) of the surface shows that it has become homogenous. Microbially-mediated dissolution could account for the decrease in $R_a$ of the hematite. This could be a result of silicate dissolution due to the build up of microbial organic acids coupled to microbial iron-chelating mechanisms that increase electron transfer to mineral-associated iron for use as a TEA (McMahon *et al.,* 1995). They can also mobilize the iron, resulting in dissolution of Fe-bearing minerals (Hersman *et al.,* 1995). Even if attachment conditions are not perfectly suited to the attachment of Fe (III)-reducing bacteria, dissolution mechanisms can still be significant. Caccavo *et al.* (2002) found that the rate of Fe (III)-reduction by the Fe (III)-reducing bacterium *Shewanella alga* is independant of the attachment ability, as an attachment deficient strain of the species reduced Fe (III) at the same rate as the attached strain. Although mineral-bound Fe (III)-reduction usually occurs in anaerobic environments, and as the groundwater at 30mbgl is aerobic, local microniches on the hematite surface may have developed due to rapid oxygen consumption by aerobes. This would promote the respiration of anaerobic TEA resulting in enhanced electron transfer and mineral dissolution (Mailloux *et al.,* 2005; Newman, 2001). However, as a microbial mat was not formed across the hematite surface during incubation, it is not possible that microbial mechanisms could account for the consistent weathering across the surface. Chemical leaching and dissolution due to interaction with the organic groundwater is the most likely cause.

The change in surface chemistry of the hematite supports the idea of leaching of Fe from the hematite. XPS spectra (Figure 4-12) reveal that the atomic % of Fe on the surface decreased significantly (by a factor of 20 on average) after incubation in the aquifer. Also, the C:Fe ratio increased from an average of 3.8 before incubation to 176.25 after incubation, suggesting that the sorption of C on the surface is not responsible for the decrease in the surface fraction of Fe. Mechanisms, such as leaching and dissolution, are likely causes for the removal of iron from the surface and the change in surface roughness of the coupon.

Overall, environmental weathering mechanisms are complex. It is therefore difficult to highlight the exact mechanisms of coupon weathering in the Four Ashes aquifer. Without further analysis, the reasons behind the changes in surface roughness, chemistry and hydrophobicity are speculative. Mesocosms

designed to investigate both biotic and abiotic weathering mechanisms will need to be used. For this, coupons could be suspended within large volumes of both filter-sterilized and unfiltered groundwater. This would allow microbial bioweathering patterns to be distinguished from chemical weathering mechanisms. Moreover, further measurements, such as nanomechanics using atomic force microscopy or non-invasive surface measurements using VSI, are required to assess the controls on microbial mineral respiration and degradation (Davis and Luttge, 2005; Lower *et al.,* 2001).

### 4.4.2 Geochemical Controls on Microbial Attachment

The geochemical controls on microbial attachment were assessed using surrogate geological substrata that were suspended within borehole 59 at Four Ashes. Microbial attachment to the coupons was quantified using epifluorescence microscopy and imaged by ESEM.

Results show that the highest numbers of microbes were attached to the Shap granite and orthoclase, followed by the hematite, apatite, shale, with the least attachment observed on quartz (Figure 4-22). These results differ from the work of Mills and Maubey (1981), who found that quartz was more colonized than hematite and limestone in a pond and mountain lake after 24 days incubation.

Where the highest attachment was witnessed at Four Ashes, cells generally attached in clusters, enveloped in an exopolymeric substance (EPS) matrix, but did not form a microbial mat across the surface. This cell aggregation has also been reported by other researchers (Baker *et al.* 2009; Davey and O'Toole, 2000; Decho, 2000; Ransom *et al.,* 1999). Forming close communities within EPS is an integral part of biofilm formation. EPS acts as a protection system from the prevailing environmental conditions, a diffusion channel for inorganic nutrients, and regulates waste metabolite movement around the microbial community, increasing the chances for complete contaminant degradation (Davey and O'Toole, 2000).

Before highlighting the potential reasons behind the variation in microbial attachment, it is important to assess whether the properties of the coupons before, or after, incubation were important factors controlling microbial

attachment. The data within this study does not provide any information on the time taken for microbial attachment to occur. It is therefore difficult to ascribe any variations in attachment to the conditions of the coupons after incubation. Microbial bioweathering after attachment on the clean surfaces may have also influenced the changes in surface properties. It is therefore more appropriate to consider the influence of the initial coupon properties on microbial attachment.

Greater microbial attachment to Shap granite may have been a result of the mineral assemblage in the rock. The rock contains positive (hematite) and negative surfaces (quartz, orthoclase), providing a variety of attachment conditions for the microbial cells. Research has shown that positive surfaces decrease the interaction energies that the microbes experience on approach to the surface (Hermansson, 1999). This results in more extensive attachment. Baker *et al.* (2009) used Extended Derjaguin-Landau-Verwey-Overbeek (xDLVO theory) to determine the interaction energies ($G_{tot}$) experienced by sulphate-reducing bacterial (SRB) cells on approach to positive and negative mineral surfaces. Results (Figure 4-28) show that the total interaction energies experienced by the SRB are attractive for the positively charged hematite (pH 4), showing that cell attachment is likely. On approach to a negatively charged surface (e.g. quartz at pH 4), the interaction energies are repulsive, suggesting that the cells must overcome the energies before attachment can occur. This model highlights that attaching to a positive surface is much easier for microbial cells.

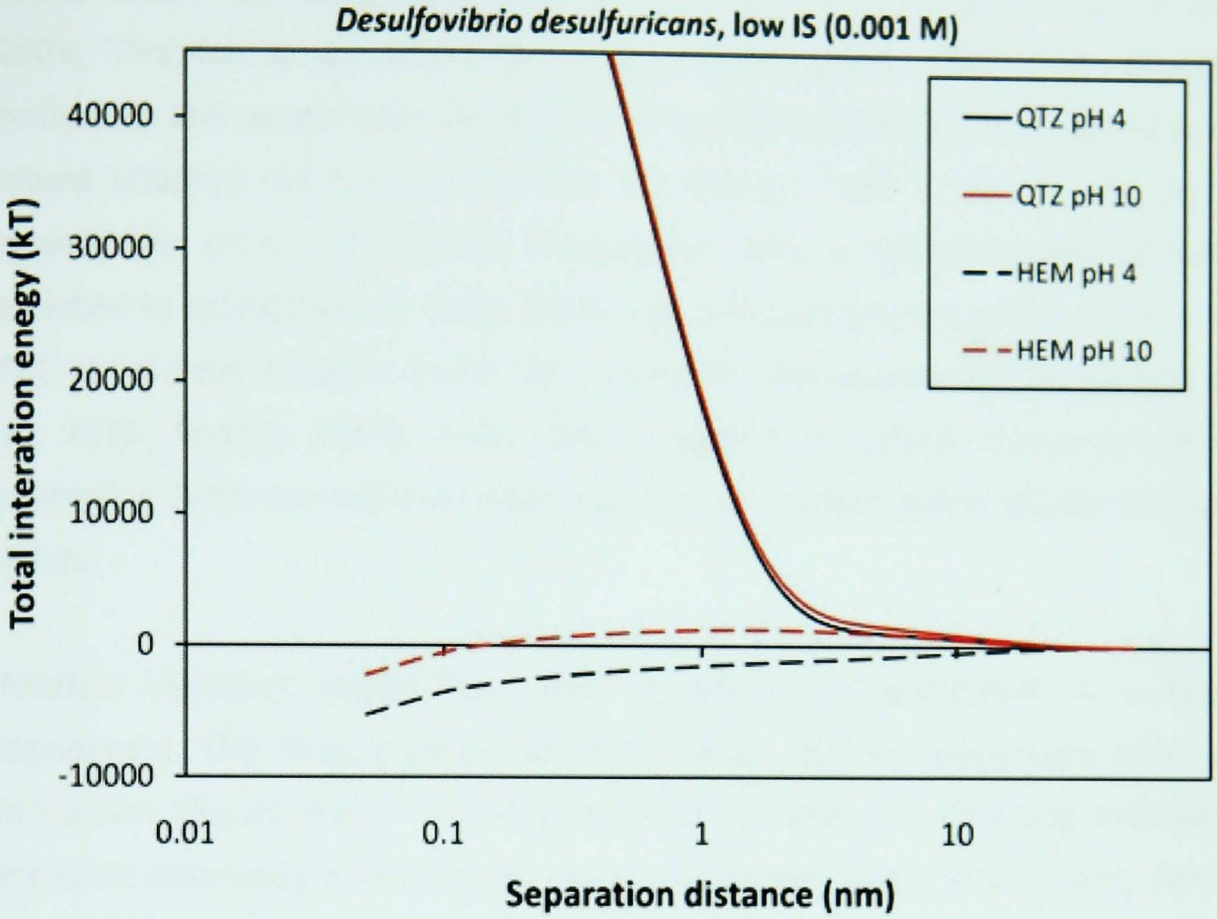



**Figure 4-28** Plots of interaction energies experienced by D.desulfricans approaching quartz and hematite surfaces (Baker *et al.*, 2009).

It is not known, however, how the surface organic coating will have affected the surface charge of the coupons. Phenolic compounds deprotonate in water, resulting in a negative charge. Therefore, after sorption to the coupon, the surface may have become negatively charged. This would remove surface charge as a controlling factor that influences microbial attachment, as all the surfaces have a similar amount of sorbed carbon, as shown by the XPS analyses (Section 4.3.1.4). Further analysis would be required to assess the impact of the sorption of phenol on the point of zero charge of the surfaces, using potentiometric titration and zeta-potential measurements (Appel *et al.*, 2002; Yukselen and Kaya, 2002). These data could then be used to model the interaction energies experienced by organisms known to be present in the Four Ashes aquifer.

There are other results that suggest that surface charge is not a factor controlling microbial attachment. Both orthoclase and quartz have a point of zero charge (PZC, the point at which charge density on a surface is zero) at pH 2-3. As the groundwater pH is 6.8 (at 30mbgl in borehole 59), orthoclase and

quartz would have been very negatively charged during incubation (Lin *et al.*, 2009; Thornton *et al.*, 2001; Domenico and Schwartz, 1998). Even though orthoclase and quartz have similar PZC, they have statistically the highest and lowest attached cell numbers ($3.02 \times 10^6$ cells $cm^{-2}$ and $1.1 \times 10^6$ cells $cm^{-2}$) respectively, of all the surfaces investigated. Also, a similar number of cells attached to orthoclase and Shap granite, even though granite is likely to have a PZC of between 3 and 7, due to the varying mineral assemblage (Barrientos *et al.*, 2010; Papelis, 2001). These data suggest that surface charge is not a controlling factor on microbial attachment in the contaminated aquifer at Four Ashes.

Another important factor that could influence cell attachment is surface topography. The Shap granite had the roughest surface topography prior to incubation (Figure 4-20), providing more active sites for attaching microbes therefore potentially enhancing microbial attachment (Wang *et al.,* 2009; Davis and Luttge, 2005). Taylor *et al.* (1998) found that small increases in surface roughness increase the attachment of *P. aeroginosa.* However, the surfaces investigated by Taylor *et al.* (1998) had a 2 orders of magnitude difference in roughness. The surfaces used in this study all have similar micron-scale roughness. Also, a statistically similar number of cells were attached to Shap granite and orthoclase. Orthoclase was statistically smoother than the Shap granite, both before and after incubation in the groundwater. Also, if surface topography was a controlling factor, cell numbers attached to shale would have been statistically similar to the Shap granite, as the two coupons had statistically similar surface roughness after incubation. This was not the case, as statistically lower a number of cells attached to shale ($1.92 \times 10^6$ cells $cm^{-2}$) in comparison to Shap granite.

Another property of the coupons that was measured before and incubation is hydrophobicity. Research has found that hydrophobicity of surfaces influences microbial attachment (Liu *et al.*, 2004; Keffard and Marshall, 1986; Pringle and Fletcher, 1983; Fletcher and Loeb, 1979). Microbial cells attached to the coupons used within this study show no preference for any particular surface wetting characteristic. The highest number of cells were attached to the hydrophilic granite. However, the least number of cells were attached to quartz,

which was also hydrophilic prior to incubation. Furthermore, fewer cells attached to the moderately non-wetting (more hydrophobic) shale. As quartz and shale were opposites with respect to surface wetting characteristics, and a similar number of cells attached to the two surfaces, this further suggests that the microbial attachment within the aquifer is independent of substratum hydrophobicity. After incubation, most of the surfaces had statistically the same contact angle measurements. Depsite this, attached cell numbers varied markedly, suggesting that the less hydrophilic surfaces post-incubation also did not influence attachment, as cell numbers would have been expected to be similar.

Finally, microbial attachment could be controlled by substratum chemistry. The extensive attachment to the Shap granite could be a result of the complex mineral assemblage and heterogeneity within the rock. SEM-EDS (Section 4.3.1.5) analysis shows that a variety of macronutrients (P, K, Ca, Al) integral to growth are present in the rock. It is also evident from XRF analysis that the Shap granite contains Mn (0.031%) and Fe (1.87%), which are important thermodynamically-favourable TEA. This results in a perfect environment for microbial attachment and growth (Bennett *et al.*, 2000). Many studies have found that surfaces containing abundant macronutrients, as well as P, are more extensively colonised than nutrient-poor surfaces (Boyd *et al.*, 2006; Bennett *et al.*, 2001; Roger *et al.*, 1998).

Also, XRF analysis of the orthoclase (Table 4-6) reveals that it not only contained the expectant mineral chemistry ($KAlSi_3O_8$), but other elemental oxides and trace metals important for microbial growth (Perry and Staley, 1997). This explains why extensive microbial colonisation, similar to Shap granite, could occur on the orthoclase, even though the coupons differ in terms of hydrophobicity, charge and roughness.

**Table 4-6** Results of XRF analysis of orthoclase showing bulk chemistry. Values in weight % unless otherwise stated.

| Element | Weight % |
|---|---|
| Si | 64.67 |
| Al | 19.07 |
| K | 11.34 |
| Fe | 0.13 |
| C | 0.04 |
| Na | 2.92 |
| Ca | 0.36 |
| Ni | 0.19 |
| Ba | 0.90 |
| Sr | 0.21 |
| Pb | 0.01 |
| Mn | 2.6ppm |
| Co | 1.6ppm |
| Cu | 4.2ppm |

Interestingly, shale also has a diverse chemistry. SEM (EDS) analysis found that shale contains S, Mg, Si, Fe, K, Ca and Al, most of which are important for microbial growth. It would have therefore been expected that the attachment to shale would have been as extensive as Shap granite and orthoclase. However, surface topography measurements and SEM reveal that shale was unstable in the groundwater due to its porosity, which may have affected microbial attachment. Gorbushina (2007) found that surface microbial growth only occurs extensively on surfaces that are stable for long periods.

The chemical analyses of the minerals and the number of attached cells on each coupon provides some inference into the importance of phosphorous in controlling microbial attachment and proliferation. Phosphorous is important for microbial growth as it is required for the generation of adenosine tri-phosphate (ATP) during microbial respiration. ATP is the energy source of cell, and is integral to all cellular processes. Phosphorous is also an important constituent of the cell membrane, forming phospholipids (Perry and Staley, 1997). Some authors found that phosphorous is an attachment-limiting nutrient, as colonization was only extensive on p-bearing surfaces (Bennett *et al.,* 2001). Other authors have proven this not to be the case, as p-bearing substrata were only found to be important in extremely reducing (methanogenic) environments (Mauck and Roberts, 2007). This could explain why attachment to orthoclase

and hematite is extensive, even though they contain no phosphorous. The conditions at 30mbgl are not reducing, and therefore according to Mauck and Roberts (2007), the presence of phosphorous in the coupons may not affect attachment.

Comparing the attachment on the nutrient-rich surfaces to quartz shows that coupon chemistry is the most important mineralogical control on microbial attachment. Quartz had statistically less attachment than all the other surfaces, even though it has a similar roughness and point of zero charge as orthoclase, and a similar hydrophobicity to the Shap granite. The only difference between quartz and the other surfaces is the surface and bulk chemistry. Quartz is nutrient-poor, due to a chemistry consisting of only Si and O. This resulted in poor attachment to the quartz coupon, indicating that chemistry is the most important control governing microbial attachment in the aquifer.

### 4.4.3 Geochemical Controls on Microbial Community Structure

The microbial community structure attached to Shap granite, sandstone and quartz incubated within the aquifer between January and August 2009 were compared to the planktonic community at the same depth (30mbgl) in the groundwater using DGGE profiling. Results (Figure 4-26) show that the Shap granite, quartz and sandstone cluster separately to the planktonic microbial community. The planktonic community is highly dissimilar to the attached community, having only 55% similarity. This agrees with the work in Chapter 3 and Rizoulis (2008). Results from this study further indicate that the attached community was extremely different to the groundwater planktonic community, suggesting that the organisms are potentially partitioning into the attached phase due to the advantages of living as part of mixed attached community (Davey and O'Toole, 2000). However, Reardon *et al.* (2004) found that groundwater communities in a uranium-contaminated aquifer were more similar (~80%) to the microbial community attached to the quartz than to the other suspended geological substrata, including natural sediments.

From the data, it is also clear that that the microbial communities attached to the sandstone and Shap granite cluster separately to the community attached to the quartz, with 77% similarity. This further highlights that geochemistry may be an important factor that controls microbial attachment (as seen above in Section 4.4.2). As mentioned above, XRF analysis reveals that the dark banding of the Penkridge sandstone and Shap granite contained nutrients important for microbial growth (Na, Ca, P, S, K) and respiration (Fe, Mn). Quartz, however, contains no nutrients that are integral to microbial growth. Also, the variety of different physiochemical conditions that the microbial cells would experience on approach to the surfaces (e.g. hydrophobicity and surface charge) are more variable on the Shap granite and sandstone due to the mineral assemblage and heterogeneity. Quartz has no variation in physiochemical properties, as it possesses negative surface charge and is very wetting (average contact angle of 28°), which will influence attachment of some microorganisms. This may have resulted in the different microbial community structures that were witnessed on quartz in comparison to Shap granite and sandstone. This supports the work of Boyd *et al.* (2006), who found that surface heterogeneity influences microbial

community structure on hematite, quartz and saprolite surfaces suspended in groundwater for 8 weeks.

The Penkridge sandstone used within this study was chosen as a surrogate for the Permo-Triassic sandstone forming the aquifer beneath the Four Ashes site. XRF and grain-size analyses show that the Penkridge sandstone is very similar to the sandstone at 30mbgl. This suggests that the community attached to the Penkridge sandstone suspended in the aquifer could be representative of the attached community in the aquifer. Moreover, XRF data shows that there is little variation with respect to chemistry down the Four Ashes succession. Therefore, the attached community on the Penkridge sandstone could be representative of the attached community throughout the Four Ashes succession. The attachment conditions would be similar as both the rocks have similar mineral assemblages, chemistry and grain-size. However, due to technical constraints (only one well-screen located at 30mbl), it is not known how the contaminant load influences attached community structure. Hydrochemical profiles from borehole 59 (Chapter 2) show that the total phenolics concentration (TPC) varies with depth, with the majority of the contaminant load being located in the plume core (25-28mbgl). Concentrations in this region were found to be up to 5800mg $L^{-1}$ (27mbgl, Thornton *et al.*, 2001). Planktonic communities in the groundwater were found to be influenced by contaminant load down borehole 59 (Chapter 2 and 3; Rizoulis, 2008), and other research has found that phenol affects microbial activity and growth (Yong *et al.*, 1997; Spence *et al.*, 2001). Therefore, it is possible that attached community structure could be influenced by contaminant load. Vrionis *et al.* (2005) found that attached communities varied with depth inside a uranium-contaminated aquifer, after analysing samples collected over the hydrochemical gradient in the contaminant plume. An influence of hydrochemistry on the attached communities was also seen at borehole 60 at Four Ashes, as no microbial cells were attached to the sandstone grains removed in August 2007 (Chapter 2). This was due to a marked increase in TPC, from 100mg $L^{-1}$ in 1998 to 10000mg $L^{-1}$ in August 2007. This increase seemingly sterilised this part of the aquifer, as a statistically innumerable number of cells were also found in the groundwater.

It is also not possible to assess the differences in the attached and planktonic communities at other depths within the aquifer, including uncontaminated groundwater (Chapter 3). A multi-level borehole containing multiple well-screens that sample the complete hydrochemical profile of the groundwater would be required to assess this, as only one well-screen is present at borehole 59 (30mbgl). However, Iribar *et al.* (2008) investigated the variation in community structure at various depths in the Garrone River in France. Results show that planktonic and attached communities were significantly different irrespective of their location of sampling. Microcosm studies could be used to investigate the variation in attached community structure using Four Ashes groundwater (from multiple levels) as inocula. Surrogate geological substrata could be suspended in groundwater pumped from multiple levels in the aquifer (including uncontaminated groundwater) to perform a time-series analysis of community development using both community profiling and phylogenetic methods. However, microcosms could result in the preferential growth of certain organisms, potentially resulting in the communities being unrepresentative of those produced in the true environment.

## 4.5 Conclusion

The geochemical controls on microbial attachment and community structure were investigated using surrogate geological substrata that were suspended within permeable vessels in the Four Ashes aquifer. Epifluorescence microscopy and environmental scanning electron microscopy (ESEM) were used to quantify and image the attached microbial communities. Community structure was investigated using DGGE fingerprinting. Results show that the most extensive microbial attachment was witnessed on the Shap granite coupon ($3.21 \times 10^6$ cells $cm^{-2}$), which has the most nutrient-rich chemistry due to the varying mineral assemblage of quartz, plagioclase, K-feldpar, hematite, biotite and apatite. Extensive attachment was also witnessed on orthoclase, hematite and apatite, all of which provide at least one macronutrient essential for microbial growth to the system. The least attachment was seen on quartz ($1.1 \times 10^6$ cells $cm^{-2}$), which was nearly 3 times less extensive than on Shap granite. This can be explained by the lack of nutrients for microbial growth within the mineral ($SiO_2$). This variation in attachment was also seen in the DGGE profiles of the microbial communities attached to Shap granite, quartz and sandstone grains suspended in the aquifer. Shap granite and sandstone clustered with 82% similarity. The community attached to these surfaces clustered with only 73% similarity to quartz, and the planktonic community in the groundwater was extremely dissimilar to all the attached communities. This shows that the chemical properties of the surface, in particular nutrient availablility, influences microbial attachment and community development within organic-rich groundwater, and that the attached community on a variety of surfaces differs significantly to the planktonic community.

# 5

# General Discussion

## 5.1 Summary of Results Presented in this Thesis

Geological ecosystems such as aquifers contain a range of surfaces that microorganisms can attach to and interact with for growth and proliferation. Such interactions can be enhanced by biostimulation to increase microbial attachment and biofilm formation, which can influence the bioremediation potential of contaminants in groundwater. It has been demonstrated that microbial communities attached to or associated with surfaces have a more efficient processing capability for contaminants (Davey and O'Toole, 2000). Also, research has found that over 99% of microbial communities live associated to a surface, and therefore studying how the physiochemical properties of surfaces influence microbial attachment is fundamental to the implementation of a suitable bioremediation strategy. However, much research only investigates either the planktonic (or free-living) microbial community, or the attached microbial community on model or artificial surfaces, due to limitations in investigating the subsurface (Hendrickx *et al.*, 2005). Also, many studies only consider the influence of aerobic communities on contaminant degradation, but many contaminated aquifers have a majority of the contaminant load located in anaerobic groundwater, and research has highlighted the importance of anaerobic communities on contaminant degradation in natural environments (Weidemeier, 1999). The aim of this study was to investigate the effect of groundwater hydrochemistry and substratum physiochemistry on planktonic and attached anaerobic microbial ecology. The work was performed at the Four Ashes site, Wolverhampton, UK, as the underlying Permo-Triassic sandstone aquifer was contaminated with phenolic compounds during plant operation between the 1950s and the 1980s.

The first objective of the study was to investigate how the groundwater hydrochemistry and the contaminant plume has developed with time at two boreholes; borehole 59 (130m from point source) and 60 (350m from the point-source of contamination). The objective was performed by analysing groundwater samples pumped from the boreholes at depth-discrete intervals. The hydrochemical profiles of organic contaminants indicated that the groundwater hydrochemistry at borehole 59 did not change significantly between 1998 (when investigations began) and July 2009. However, data from

August 2009 shows that there was a marked change in contaminant distribution within one month. These data suggest that the pump and treat remediation system, implemented at borehole 60 by the site owners, is having a significant effect on groundwater hydrochemistry at borehole 59. Further groundwater sampling at the site, in conjunction with pumping information from the site owners, would provide further information on the effect of pumping on contaminant distribution. This would also aid conceptual modelling to assess mass discharge and biodegradation rates before and after the implementation of the pump and treat system. At borehole 60, the contaminant plume remained stable down to 41mbgl. Below this, a marked increase in phenolic compounds was seen at all samplings. This indicates that a more concentrated spillage early in site operation resulted in a deeper source of contamination, and that the plume may extend deeper than 45mbgl, validating the estimate made by Williams *et al.* (2001). The ratios of each phenolic solute to 2,6-xylenol (an internal tracer) in July 2009 confirmed that there were multiple sources of contamination released from the site, as the ratios of the compounds differed with depth in the aquifer. These data agree with the work of Thornton *et al.* (2001). Overall, the data from borehole 60 raises questions about the potential size of the contaminant plume and its development with time.

Total microbial cell counts were performed to assess the influence of contaminant load on microbial abundance using the same groundwater samples from borehole 59 and 60. Results indicate that the contaminant load had a significant influence on microbial numbers, as a 100-fold increase in microbial abundance was seen between uncontaminated groundwater and the plume fringe, where a steep hydrochemical gradient existed. Inside the contaminant plume, microbial numbers decreased 10-fold from the plume fringe to the core, coinciding with further increases in contaminant load. Extremely low (uncountable) numbers of cells were present in samples taken from the bottom of borehole 60 (43-45mbgl), and the clustering of particulates on the filter membrane prevented the filtration of high volumes of groundwater to assess microbial abundance. This suggests that the marked increase in contamination at these depths has significantly affected microbial abundance. This was reflected by the lack of cells attached to the sand grains that were removed from borehole 60 in August 2007.

The second objective of the study was to assess the influence of contaminant load on anaerobic microbial community structure in the planktonic phase (i.e. within groundwater samples taken from the whole groundwater profile) using DGGE profiling. This study of anaerobic communities generated novel data for the site, as past community profiling considered total eubacteria only. Results indicate that contaminant load has an influence on anaerobic microbial community structure (nitrate-reducing and sulphate-reducing). Communities located on the plume fringes where contamination was low, and where there was a high concentration of terminal electron acceptors, were different to the communities found in the most grossly contaminant areas of the plume and in uncontaminated groundwater. The profiling of anaerobic communities has provided new insights in to the influence of contaminant load on microbial ecology at the site, and will prove useful for the future assessments of natural attenuation. It is not possible to infer any variations in community diversity using the DGGE profiles, as DGGE can only detect organisms that form greater than 1% of a community (Muyzer *et al.,* 1993). Also, species within a DGGE can co-migrate, resulting in one band representing more than one organism, and a single organism can produce more than one band on a DGGE. The eubacterial microbial community attached to a model geological surface (quartz sand) suspended at 30mbgl in borehole 59 was also compared to the planktonic community at the same depth. Results show that the planktonic community was highly dissimilar to the attached community, agreeing with the results of other researchers (Iribar *et al.*, 2008; Rizoulis, 2008).

The importance of assessing the attached community arises from research suggesting that attached communities have a better potential to degrade toxic levels of contamination, due to the benefits of growing within a complex consortia (Davey and O'Toole, 2000). However, investigating the attached community using a single model surface is not suitable, as the physiochemical properties of a surface have been found to have a strong influence on attachment (Bennett *et al.,* 2001; Davey and O'Toole, 2000). Therefore, the third objective of the study was to investigate the geochemical controls on microbial attachment and community structure. This was performed by suspending different geological substrata (apatite, Shap granite, hematite, orthoclase, quartz, sandstone and shale) in borehole 59 at 30mbgl. The

substrata provided different physical (roughness) and chemical (charge, hydrophobicity, nutrient availability) conditions to the system, with the intention of enhancing microbial attachment and biofilm formation. Many researchers have used different geological surfaces to investigate the controls on microbial attachment. However, studies usually only consider one or two substratum properties, such as nutrient availability (Bennett *et al.*, 2001) or charge (Scholl *et al.*, 1990). Very few consider multiple substratum factors that may influence microbial attachment, and this study was the first of its kind to be used at Four Ashes, as only model or representative surfaces (e.g. quartz) have been used previously.

Microscopic imaging (epifluorescence and ESEM) and DGGE fingerprinting of the attached microbial communities was performed to assess the attached microbial numbers and the structure of the attached microbial communities (Shap granite, quartz and sandstone only). Microscopy revealed that there was significantly more attachment on the Shap granite, orthoclase, hematite and apatite, in comparison to the shale and quartz. Although substratum surface roughness and hydrophobicity were measured before and after incubation in the aquifer, results indicate that attachment was independent of these factors. The similarity in attachment of the former group of surfaces was found to be due to them providing at least one nutrient essential to microbial growth. Shap granite attracted the most extensive microbial attachment, which is due to its varied mineralogy and greater nutrient content. Quartz has no nutrients, resulting in poor attachment. Therefore, results suggest that the most important control on microbial attachment at Four Ashes is substratum chemistry, particularly microbial nutrient availability.

DGGE profiling revealed that the planktonic community was highly dissimilar to all the attached communities, as it clustered separately. Also, the community attached to quartz clustered separately to the communities attached to Shap granite and sandstone. This again indicates the selective cell attachment to surfaces that contain nutrients important for microbial growth, supporting the results of the epifluorescence microscopy that geochemistry is an important control on microbial attachment. PCR was not able to detect any anaerobic

communities (nitrate/sulphate-reducers) in the attached phase. This further highlights the limitations of using PCR and DGGE.

## 5.2 Implications for Contaminant Remediation at Four Ashes

The hydrochemical data presented in this study have important implications for the treatment of the contamination at the site, as remediation efforts will have to take into account this increased plume size (potentially deeper than 45mbgl). Morever, the results of the microbial studies have shown that both total eubacterial and anaerobic communities are diverse at all levels of contamination in the Four Ashes aquifer. Therefore, *in situ* remediation strategies that utilise the microbial community may be successful.

The simplest and cheapest method to remediate the contaminants at Four Ashes is likely to be biostimulation. The data in this study have shown that both aerobic and nitrate-reducing organisms are present at all areas within the contaminant plume. Therefore, the groundwater could be sparged with oxygen and nitrate using the exisiting multi-level and single-screen boreholes to enhance the growth of thermodynamically favoured organisms, subsequently increasing the rate of biodegradation of the phenolics. However, sparging is limited to the solubility of the gases, as only a maximum of 30mg $L^{-1}$ oxygen and 50mg $L^{-1}$ nitrate could be supplied to the groundwater (in equilibrium with pure reservoirs of each gas). This supply could limit the speed at which degradation would occur. Also, lack of phosphorous, which was to be below detectable limits in this study, could be limiting microbial degradation in the aquifer. It is a key nutrient required to make adenosine tri-phosphate, the energy of microbial cell processes. If such a nutrient is limited (as at Four Ashes), then microbial cell growth and contaminant degradation will be minimal. Sparging the groundwater with soluble P may therefore be integral to enhancing microbial degradation of the contamination.

However, the intense contaminant loads found at the site may result in extremely low degradation rates due to the toxic effects of the phenolics on the microbes, even in the presence of these high energy TEA and growth nutrients.

Therefore, the re-circulation of groundwater from different depths in conjunction with biostimulation could be beneficial. This would involve mixing highly contaminated water from within the plume core with less contaminated water on the fringes, whilst still sparging with TEA. This would reduce the toxic effects by diluting the contamination, further promoting microbial degradation. This technology is usually used in aquiers that have conifining layers resulting in the formation of multiple aquifer systems.

*In situ* engineering solutions could also be used at Four Ashes to utilise the diverse microbial community found within the aquifer in this study. Permeable reactive barriers could be installed and used to re-circulate water and to inject soluble growth nutrients into the aquifer perpendicular to the groundwater flow direction. These 'reaction barriers' could be installed at the leading edge of the plume to ensure that all the contamination is degraded by microorganisms prior to reaching the groundwater extraction well. Multiple rows of permeable reactive barriers could be used to ensure maximum degradation and contaminant dispersion/dilution. It is unlikely that an *in situ* remediation method would fail at Four Ashes, as the geology at Four Ashes is very homogenous. It has no channel flow zones, as similar hydraulic conductivity was found throughout with no confining layers or evidence of fracture networks. Therefore, no groundwater would be able to circumvent the permeable reactive barriers and be left untreated. Much research would still be required to ensure that sparging and re-circulation rates matched the microbial degradation rates.

The above remediation options are only examples of many methods of treating organic contaminated groundwater. Furthermore, much more information and understanding is required to truly assess the contaminant distribution, microbial ecology and bioremediation potential at Four Ashes. The data found in this study has shown, however, that the hydrochemical conditions and microbial communities are present that have the capability to degrade the toxic contaminants in the groundwater given the application of the correct methodology.

## 5.3 Future Work

Although the geology at Four Ashes is relatively homogenous, discrepancies still exist in terms of the source term input of the contamination and the lateral migration of the contamination from the site. More information on contaminant distribution at the site could be gained from analysing groundwater from more boreholes. Borehole 59 and 60 are along the groundwater flow path, but horizontal dispersion or heterogeneities in the aquifer may have spread the contaminants laterally (Brewster *et al.*, 2005). There are a number of screened boreholes at the site that could be used to assess this (Williams *et al.*, 2001). Although these sample groundwater from one depth (averaged over the order of metres), they will provide up to date information on the contaminant distribution in the aquifer. A better, more depth-discrete analysis of the groundwater would require the construction of more multi-level sampling boreholes. The data from which could be used to assess both the 2D and 3D plume thickness using isoconcentration mapping (Fetter, 1999). It would also be appropriate to collect hydrochemical data from a borehole upstream of the contamination source, so that the hydrochemistry of an uncontaminated groundwater profile can be compared to the groundwater downstream.

Other methods could be used to assess the 3D distribution of contaminants in the groundwater. Geophysical techniques such as electromagnetic induction, ground penetrating radar and resistivity have been proven to be effective in the delineation of contaminant plumes (Jin *et al.*, 2008; Kaya *et al.*, 2007; Benson, 1995). These techniques would be more efficient and cheaper than the construction of more multi-level boreholes. However, they will not provide information on mass concentrations; the data therefore could not be used to assess mass discharge from the aquifer or to estimate biodegradation rates.

Although DGGE is a rapid technique that proved useful in investigating the influence of contaminant load on community structure at Four Ashes, no assessment of diversity in the aquifer can be made. Alternative techniques should now be used to follow up the work using DGGE to assess the community in greater detail. The use of clone libraries would provide an extensive data set on the microbial population in the Four Ashes aquifer. Phylogenetic analysis of

the communities using clone libraries would allow the diversity of aerobic and anaerobic communities (iron-, manganese-reducers, methanogens) to be characterised and compared at different loci within the groundwater profile. This would prove useful in the investigation of the effect of groundwater contamination on the variation and distribution of dominant microbial species at Four Ashes.

Another technique that would be valuable in assessing community diversity is pyrosequencing, a high-throughput sequencing-by-synthesis method that can assess a full community in a few hours after amplification using broad eubacterial/archaeal primers. Pyrosequencing would provide an extensive data set on the anaerobic communities at the site, providing information on the presence and diversity of all the microbial groups in the attached and planktonic communities (Fierer *et al.,* 2008). Pyrosequencing data would provide information on the both the rare and dominant microbial species in the aquifer, allowing the investigation of whether rare organisms found at one area of the contaminant plume (and under certain hydrochemical conditions) are dominant at other areas under different hydrochemical conditions.

It was not possible within this study to assess the differences between the attached and planktonic communities at other depths within the borehole as only one well-screen is located in borehole 59. This limitation could be overcome by gaining access to other boreholes at the site, which are single-screen monitoring wells drilled to a variety of depths (Williams *et al.,* 2001). These monitoring wells have long screens, and would not be depth-discrete, as an average groundwater sample over a few metres would be sampled, giving unreliable results. Additional multi-level boreholes could be installed with multiple well-screens, but would be extremely costly. This would, however, give an insight into the influence of contaminant load on the attached community structure at the site, as well as assessing the attachment characteristics of microorganisms within the anaerobic parts the plume. Also, during the construction of new multi-level boreholes, aquifer core samples could be collected to directly assess the community attached to the aquifer sandstone. Profiling techniques (DGGE) and clonelibraries/pyrosequencing could be used to assess the community structure and diversity. This would provide a more

complete assessment of the attached community in comparison to the surrogate substrata used as a representative of the aquifer sandstone. The attached communities on core samples taken both downstream and upstream of the contaminant source could be compared to assess the impact of the contamination and oligotrophic, uncontaminated groundwater on attached community dynamics.

The attachment processes of indigenous microorganisms to the aquifer and surrogate mineral coupons could be further assessed by performing a time-series analysis of microbial attachment, both *in situ* and in the laboratory. Coupons suspended in mesocosms containing both filter-sterilized and unfiltered groundwater from Four Ashes could provide data on cell attachment rates, and assess the development of both biotic and abiotic substratum weathering patterns, as it was not possible to infer any specific individual chemical/biological weathering from the data. Also, a novel flow cell that incorporates mineral coupons has been developed by Baker *et al.* (2009) to quantify the attachment rates of microbes to different surfaces in real-time using epifluorescence microscopy (Appendix 2). This work, in conjunction with xDLVO modelling theory, could be continued to assess the influence of different environmental conditions (pH, ionic strength, nutrient status and phenol concentration) on microbial attachment.

Confocal Raman microspectroscopy could be used to directly assess the diversity of organisms attached to the different substrata by comparing the spectra of individual microbial cells (Huang *et al.,* 2009). This would be possible using the coupons, and would not require total DNA extraction methodologies, which could bias results. Single-cell PCR could be performed using Raman optical tweezers to amplify rare organisms found attached to the surfaces, or to isolate unculturable anaerobic organisms (Huang *et al.,* 2009; Wagner, 2009). Furthermore, Raman microscopy in conjunction with fluorescent *in situ* hybridisation (FISH) could be used to assess the community structure and abundance of attached microorganisms directly using function-specific fluorescent rRNA-targeted oligonucleotide probes. FISH could be used not only to detect function-specific species in the complex communities attached to the coupon surfaces, but also to assess the activity of the organisms by correlating

the fluorescence intensity to ribosomal content. However, much method development would be required to ensure that the natural fluorescence of any of the coupons does not affect the fluorescence-based detection by FISH.

## 5.4 Conclusion

The work presented in this study has provided a better understanding of the sources and distribution of contaminants in the phenol-contaminated aquifer at Four Ashes, UK. Contaminant distribution was seen to have changed both downstream at borehole 60 and upstream at borehole 59, the latter being due to the onset of the pump and treat strategy at the site. For the first time at Four Ashes, anaerobic microbial communities were profiled and geochemical controls on microbial attachment were investigated. Results show that groundwater hydrochemistry has a strong influence on anaerobic community structure, and that coupon geochemistry is an important factor controlling microbial attachment. Under the same hydrochemical conditions, planktonic and attached communities were seen to differ, on more than one surrogate geological surface. This highlights the requirement for studying both aerobic and anaerobic planktonic and attached communities using different surrogate substrata to fully assess the indigenous microbial population. Such a holistic approach provides a better understanding of how geochemistry and geological heterogeneity affect microbial ecology, which could prove beneficial in the assessment of natural attenuation and for the design of bioremediation methods.

# 6

# Appendix 1

Methods for the PCR Amplification of Anaerobic Genes

PCR amplification of *napA*

a) Original published method by Alcantara-Hernandez *et al.* (2009)

| Ingredient | Working Concentration | Rxn Volume (µl) |
|---|---|---|
| Buffer (10x Bioline) | 1x | 2.5 |
| $MgSO_4$ (50mM) | 2.5mM | 1.25 |
| dNTP (25mM) | 200µM | 0.2 |
| F-Primer (100uM) | 1mM | 2.5 |
| R-Primer (100uM) | 1mM | 2.5 |
| Taq (5 U $\mu l^{-1}$) | 1.3 | 0.13 |
| Bovine Serum Albumin | 6µg | 60 |
| DNA Vol | 3ng | |
| Mol.grade water | | To 25 |

b) Modified recipe developed after method development:

| Ingredient | Rep E Conc. | Rxn Vol (µl) |
|---|---|---|
| Buffer (10x Bioline) | 1x | 2.5 |
| $MgCl_2$ (50mM) | 3mM | 1.5 |
| dNTP (25mM) | 200µM | 0.2 |
| F-Primer (100uM) | 5uM | 0.25 |
| R-Primer (100uM) | 5uM | 0.25 |
| Taq (5 U $\mu l^{-1}$) | 0.65 | 0.13 |
| Bovine Serum Albumin | 6µg | 0.25 |
| Template | 3ng | |
| Mol. Grade water | | To 25 |

c) Thermocycling conditions:

| Stage | Temperature (˚C) | Time (mins) |
|---|---|---|
| Initial Denaturation | 93 | 4 |
| 35 cycles of | 93 | 1 |
| | 52 | 1 |
| | 72 | 1 |
| Final Elongation | 72 | 7 |
| Hold | 10 | - |

PCR amplification of *dsrbB* (Geets *et al.*, 2006)

a) PCR recipe:

| Ingredient | Working Concentration | Volume (µl) |
|---|---|---|
| Buffer (10x Bioline) | 1x | 10 |
| $MgCl_2$ (50mM) | 2mM* | 4 |
| dNTP (25mM) | 100µM | 0.4 |
| F-Primer (10µM) | 1µM | 10 |
| R-Primer (10µM) | 1µM | 10 |
| Taq (5 U $µl^{-1}$) | 2.5 U | 0.5 |
| DNA Vol | | 4 |
| Mol. grade water | | To 100 |

* = Ex Taq reaction buffer used in reference, which contains 20mM $MgCl_2$ at 10x concentration. The recipe here was modified accordingly.

b) Thermocycling conditions:

| Stage | Temperature (˚C) | Time (mins) |
|---|---|---|
| Initial Denaturation | 94 | 4 |
| 35 cycles of | 94 | 1 |
| | 55 | 1 |
| | 72 | 1 |
| Final Elongation | 72 | 10 |
| Hold | 10 | - |

PCR amplification of achaea (Casamayour *et al.*, 2000)

a) PCR recipe:

| Ingredient | Working Concentration | Volume (µl) |
|---|---|---|
| Buffer (10x Bioline) | 5x | 10 |
| $MgCl_2$ (50mM) | 1.5mM | 1.5 |
| dNTP (25mM) | 100µM | 0.2 |
| F-Primer (10µmol) | 300nM | 1.5 |
| R-Primer (10µmol) | 300nM | 1.5 |
| Taq | 0.75 | 0.15 |
| Template | 3ng | |
| Mol. grade water | | To 50 |

a) Thermocycling conditions:

| Stage | Temperature (˚C) | Time (mins) | |
|---|---|---|---|
| Initial Denaturation | 94 | 5 | |
| 20 cycles of | 94 | 1 | |
| | 71 | 1 | drop 0.5°C every subsequent cycle (touchdown PCR) |
| | 72 | 3 | |
| 15 cycles of | 94 | 1 | |
| | 61 | 1 | |
| | 72 | 3 | |
| Final elongation | 72 | 10 | |
| Hold | 10 | - | |

# 7

# Appendix 2

Development of a Novel Flow Cell for the Real-Time Imaging of Microbial Attachment to Geological Substrata

On the following page is a copy of abstract A76 of the 19th Annual Goldschmidt Conference in Davos, Switzerland, published in Geochimica et Cosmochimia Acta in 2009, outlining the work on the novel flow cell developed to quantify cell attachment rates to mineral surfaces by Baker *et al.* (2009). A copy of the conference poster presentation is available on the compact disk below.

Please ensure that your abstract fits into one column on one page and complies with the *Instructions to Authors* available from the Abstract Submission web page.

# Mineral-specific attachment of sulphate-reducing bacterial consortia: combined experimental and xDLVO modelling approach

K. BAKER[1,2*], J. BRIDGE[2], D. BROWN[2], S. ROLFE[2,3], J. SCHOLES[2,3], R. EDYVEAN[1,2], S. BANWART[2]

[1]Chemical and Process Engineering, University of Sheffield, UK (*correspondence: k.m.baker@shef.ac.uk)
[2] Cell-Mineral Research Centre, Kroto Research Institute, University of Sheffield, UK
[3]Animal and Plant Sciences, University of Sheffield, UK

99% of all microbial life is attached to, or assocatied with, a surface [1]. The association of microogranisms with substrata (Fig.1) is critical to understanding the role of biogeochemical processes in natural attenuation, but cell-mineral interactions, and the formation of biofilms, may be highly variable and spatially heterogeneous over many scales within a geological ecosystem such as an aquifer [2].

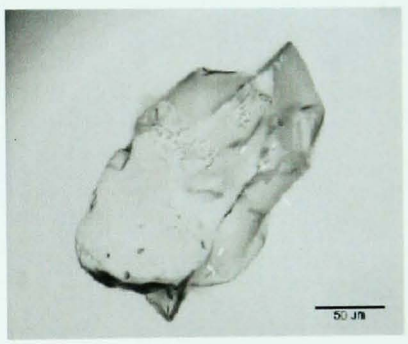



**Figure 1** Microbes (white) attached to quartz grain after *in situ* incubation.

In this work, we adopt a multi-faceted approach to investigate the mineral attachment characteristics of sulphate-reducing bacteria (SRB) isolated from a deep sandstone aquifer contaminated with phenolic compounds. We assess the formation of biological conditioning films on the solid phase, substratal bio-weathering patterns, and the forces involved in anchoring a sessile community within microcosms containing various minerals. Extended (x)DLVO theory [3] is used to estimate the forces experienced by planktonic SRB approaching the different mineral surfaces and assess the role of physicochemical conditions in the initiation of mineral colonisation by SRB.

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