# Diversity of planktonic and attached microbial communities in a phenol polluted aquifer

**A thesis submitted by**

**Athanasios Rizoulis**

**In partial fulfilment of the requirement for admittance to the degree of**

**Doctor of Philosophy**

**at the University of Sheffield**

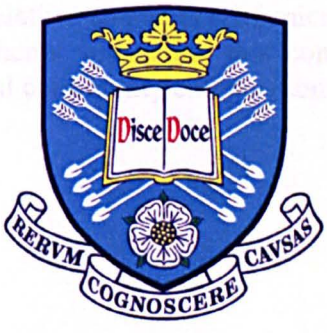
**Department of Animal and Plant Sciences**

**University of Sheffield**

**Sheffield**

**S10 2TN**

**United Kingdom**




**October 2008**

## Abstract

The sandstone aquifer underlying the Four Ashes industrial site near Wolverhampton, UK, is contaminated with high concentrations of organic pollutants, in particular phenol, cresols and xylenols. Although in the past the geochemistry of the site has been studied extensively, relatively little is known about the *in situ* microbial communities despite their potential for bioremediation. The aim of this thesis was to investigate the effect of groundwater pollution on the diversity of planktonic and attached microbial communities and to make comparisons between the two.

This aim was investigated by sampling planktonic microbial communities at different positions within the fringes of the plume and the planktonic and attached communities at one plume depth (30 metres below ground level in borehole 59). Denaturing Gradient Gel Electrophoresis (DGGE) analysis of PCR amplified 16S ribosomal RNA (rRNA) gene fragments indicated that diversity of planktonic microbial communities varied with depth across the steep geochemical gradient of the plume whilst under the same geochemical conditions the planktonic and attached microbial communities differed markedly. The latter result was investigated further by 16S rRNA gene cloning and sequencing. Phylogenetic analysis of the two clone libraries demonstrated that there was limited overlap between the two communities and that the planktonic community was less diverse than the attached community. The 'groundwater' clone library was dominated by four bacterial phylogenetic groups ($\alpha$-, $\beta$-*Proteobacteria*, *Firmicutes* and *Bacteroidetes*) whilst the 'sand' clone library was characterised by the presence of $\alpha$-, $\beta$-, $\gamma$-*Proteobacteria*, *Bacteroidetes* as well as a large number of clones (29%) that could not be classified or belonged to minor bacterial phyla. Thirteen percent of the groundwater and 5% of the sand clones had 100% 16S rRNA gene sequence identity to a phenol degrading *Azoarcus* strain, while 14.7% of the sand clones were closely related (98% sequence identity or more) to members of the *Acidovorax* genus that have been isolated or detected in phenol contaminated environments.

In addition to the *in situ* studies, laboratory microcosms were inoculated with mixtures of bacteria isolated from the Four Ashes site (with known functional characteristics regarding their abilities to degrade or tolerate phenol and to attach to sand) in order to investigate the influence of different phenol concentrations or changes in phenol concentration on microbial community composition of both planktonic and attached communities. These studies revealed that the relative abundance of microbial isolates within the microcosms altered in response to phenol suggesting that complex metabolic and cell-cell interactions may influence microbial community composition.

## Acknowledgments

There are many people who helped to make the completion of this thesis possible. Firstly, I would like to thank my two main supervisors, Prof. Julie Scholes and Dr. Stephen Rolfe (Department of Animal and Plant Sciences) for their continued support, advice, accessibility, patience and encouragement throughout my PhD and until the very intensive last few months. I also thank my other two supervisors, Prof. Steven Banwart (Dept. of Civil and Structural Engineering) and Prof. Roger Pickup (Centre for Ecology & Hydrology, Lancaster), as well as everyone involved in the Cell Mineral Interface (CMI) research programme for their useful input and for giving me the opportunity to be part of a stimulating research environment. Many thanks to Dr. David Elliott for all his valuable help and advice during my first steps in the field of microbial ecology and to Ammar Abdul Razak for his contribution to the development of the laboratory microcosms and the HPLC method. I also thank Dr. Steven Thornton for help with groundwater sampling, Kieran Baker for assistance with the HPLC, and Maggi Killion for technical assistance in the lab.

I would also like thank my brother Giorgos and my parents, Konstantinos and Fevronia, for their love and support all these years.

In addition, I thank all the people that I had the pleasure of knowing and helped me during my stay in Sheffield and as a PhD student at the Department of Animal and Plant Sciences. I particularly thank Bleddyn Hughes, Kan Huang, Cindy Smith and Ashley Houlden for useful discussions and advice and I am grateful to Dionysia, Lisette, Isabelle, Felipe, Alain and Juan for all their patience and encouragement.

This study was part of the CMI research programme of The University of Sheffield, which was primarily funded by the Engineering and Physical Sciences Research Council (EPSRC), with additional support from the Biotechnology and Biological Sciences Research Council (BBSRC) and The University of Sheffield. They are gratefully acknowledged, as well as the Greek State Scholarships Foundation, which provided personal funding.

## Abbreviations

| | |
|---|---|
| A C G T | Adenine / Cytosine / Guanine / Thymine |
| bp | Base pairs |
| BTEX | Benzene / Toluene / Ethylbenzne / Xylene |
| CTAB | Hexadecyltrimethylammonium bromide |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonuceoside triphosphate |
| IPTG | Isopropyl $\beta$-D-1-thiogalactopyranoside |
| $OD_{600}$ | Optical density at 600 nanometres |
| PAH | Polycyclic aromatic hydrocarbon |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| SDS | Sodium dodecyl sulphate |
| TCE | Trichloroethene |
| TPC | Total phenolics concentration (= sum of phenol and cresols) |
| T-RFLP | Terminal-Restriction Fragment Length Polymorphism |
| v/v | Volume/volume |
| w/v | Weight/volume |
| X-Gal | 5-Bromo-4-chloro-3-indonyl-$\beta$-D-galactoside |

# Table of contents

# Chapter 1

# General Introduction

## Introduction

The pollution of groundwater by industrial activity has resulted in a legacy that will persist for generations to come. However, native microorganisms in contaminated natural environments can show wide metabolic versatility and exhibit appetite even for toxic compounds. Therefore, in recent years a number of studies have focused on understanding these microbial communities, which constitute our best allies in our efforts to clean up groundwater pollution.

The aim of this thesis is to investigate microbial community structure in a polluted aquifer and in laboratory microcosms. In the following introduction a number of topics are discussed. The introduction starts by defining groundwater, groundwater pollution and *in situ* bioremediation approaches. Then, biodegradation of phenolic compounds (and particularly phenol) via aerobic and anaerobic pathways is discussed in more detail. Some of the available methods for assessing microbial diversity are then presented and the role of biofilm communities in polluted environments is stressed. Finally, the experimental site and the scope of this thesis are described.

# 1.1 Polluted aquifers

### 1.1.1 Defining groundwater, water table and aquifers

A common misconception is that groundwater occurs in underground lakes with water flowing along subsurface rivers. In reality, groundwater is mainly stored in the pores and fissures of the various rocks that make up the earth's crust. When all pores and fissures are filled with water, the rock or soil is described as saturated and the water contained within it is called groundwater. The upper limit of the saturated zone is called the water table. The zone above the water table, where pore spaces contain both air and water (films of water are held around grains by surface tension and by molecular forces), is known as the unsaturated or vadose zone (Price, 1996).

According to Price (1996), an aquifer is "*a geological formation, group of formations, or part of a formation that contains sufficient saturated permeable material to yield significant quantities of water to wells and springs*". When its upper surface is at atmospheric pressure, it is called unconfined. However, in some cases areas of low

permeability (such as layers of clay or shale) may overlay a part of the aquifer, forming a confined aquifer, where the groundwater is under pressure. Alternatively, when water infiltration is prohibited by impermeable layers that are situated above the water table, perched aquifers are formed.

The rock types of aquifers are usually classified into three groups according to their origin. Igneous rocks are formed by the solidification of magma and metamorphic rocks by the alteration of other rocks under the action of heat or pressure. Water in these rocks can be found mainly in sizeable cracks, most commonly found in basalt lavas. The other major aquifers are of sedimentary origin. They were formed after the deposition of particles which derived from the weathering and erosion of other rocks. Thus, sedimentary rocks have large porosity, with layers of coarse sands and gravels being the most permeable. However, fine sediments, such as clays and silts or fine sandstones, may have high porosities but the pores are so small that surface tension or molecular forces prevent water movement so that in these materials permeability is low.

The most important aquifers in the United Kingdom are situated in the south and east where large quantities of water are yielded mainly from Chalk aquifers and from sandstones of the Permian and Triassic Systems (Price, 1996). Chalk is a soft white limestone composed of calcium carbonate and usually consists of the skeletal remains of aquatic organisms. Permo-Triassic sandstones originated from sand which became partially cemented. Aquifers of minor importance are the unconsolidated ones. They are of relatively recent origin and lack of compaction and cementation means that they include some of the most permeable natural materials (sand and gravels).

Groundwater is a valuable source since significant amounts of the public water supply comes from groundwater. It is estimated that one third of the drinking water in England, but as high as 70% in Southern England, comes from groundwater. Groundwater is extracted at a rate of 7 million $m^3$ $day^{-1}$ but pollution and increasing demand for water is putting the resource under pressure. (Environment Agency, 2006. The state of groundwater in England and Wales. Available at *www.environmentagency.gov.uk).*

### 1.1.2 Pollutants in aquifers and pollutant movement

It is estimated that there are 300,000 potentially contaminated sites in England and Wales. Of these ~33,000 require action and ~21,000 sites have received some action to remove contamination or prevent harm from contamination (Environment Agency, 2005. Indicators for land contamination. Environment Agency, Science Report SC030039/SR. Available at *www.environmentagency.gov.uk*). A wide variety of environmental pollutants can leach through soil and enter groundwater systems particularly from waste pits, landfills, mine wastes, buried containers and leaking storage tanks and pipelines. Moreover, intensive agriculture practices can result in elevated concentrations of nitrates and pesticides in groundwater, much higher than the limits set by the European Community Drinking Water Directive (50 mg $L^{-1}$ for nitrates and 1 $\mu g$ $L^{-1}$ for pesticides).

Common pollutants in groundwater include inorganic cations and anions, hydrocarbons, synthetic organic chemicals and radionuclides, as shown in Table 1.1. A full list of groundwater contaminants and examples of uses can be found in Fetter (1993). Apart from nitrates and pesticides, there is special concern about contamination derived from fuel leakages and industrial wastes. Fuels consist of a mixture of hydrocarbons, such as benzene, toluene, ethylbenzene, xylene (BTEX) and methyl tert-butyl ether (MTBE), which is used as a replacement of tetraethyl lead and can comprise as much as 15% of the fuel volume (Chapelle, 2001). The most commonly found industrial pollutants are the chlorinated hydrocarbons, such as carbon tetrachloride, trichloroethene (TCE), perchloroethene (PCE) and polychlorinated biphenyls (PCBs). These compounds are used in metalworking industries as degreasants, in the electronics industry, in the tanning of animal skins and as dry-cleaning solvents.

The majority of the organic contaminants are almost insoluble in water and when they enter the groundwater they form a plume which moves along with the groundwater flow. Concentrations of pollutants are normally highest at the core of the plume with lower concentrations at the plume fringes. There are two types of plumes, depending on their composition. Plumes with organic contaminants that are denser than water (dense non-aqueous phase liquids or DNAPLs) penetrate the water table and form pools along geologic layers while organic contaminants that are lighter than water (light NAPLs or

LNAPLs) accumulate near the water table and follow the water flow. In general, the movement of a contaminant in groundwater is controlled by many transport mechanisms, such as advection (travels in the same direction and at the same speed as the groundwater), diffusion (moves towards regions of lower concentration), dispersion (spreads to all directions, becoming diluted in the process), adsorption (on mineral surfaces) and gravity (Alexander, 1999).

**Table 1.1.** Categories of subsurface contaminants, frequency of occurrence and sources (adapted from the National Research Council, 2000).

| Chemical class | Example compounds | Occurrence frequency[a] | Examples of industrial sources or applications |
|---|---|---|---|
| **Inorganic** | | | |
| Metals | Cr, Cu, Ni, Pb, Hg, Cd, Zn | F | Mining, gasoline additives, batteries, paints, fungicides |
| Non-metals | As, Se | F | Mining, pesticides, irrigation drainage |
| Oxyanions | Nitrate, (per)chlorate, phosphate | F | Fertilisers, paper manufacturing, disinfectants, aerospace |
| Radionuclides | Tritium ($^{3}H$), $^{238,239,240}Pu$, $^{235,238}U$, $^{99}Tc$, $^{60}Co$, $^{137}Cs$, $^{90}Sr$ | I | Nuclear reactors, weaponry, medicine, food irradiation facilities |
| **Organic** | | | |
| Hydrocarbons | | | |
| Low molecular weight | BTEX, alkanes, phenolic compounds | F | Crude oil, refined fuels, dyestuffs, solvents |
| High molecular weight | Polycyclic aromatic hydrocarbons (PAHs), non-volatile aliphatic hydrocarbons | C | Creosote, coal tar, crude oil, dyestuffs, lubricating oils |
| Oxygenated hydrocarbons | Alcohols, ketones, esters, ethers, phenols, MTBE | F | Fuel oxygenates, solvents, paints, pesticides, adhesives, pharmaceuticals, fermentation products, detergents |
| Halogenated aliphatics | | | |
| Highly chlorinated | Tetrachloroethene, trichloroethene, 1,1,1-trichloroethene | F | Dry cleaning fluids, degreasing solvents |
| Less chlorinated | 1,1-Dichloroethene, 1,2-dichloroethene, vinyl chloride, methylene chloride | F | Solvents, pesticides, landfills, biodegradation by-preducts, plastics |
| Halogenated aromatics | | | |
| Highly chlorinated | Pentachlorophenol, PCBs, polychlorinated dioxins, polychlorinated dibenzofurans, chlorinated benzenes | C | Wood treatment, insulators, heat exchangers, by-products of chemical synthesis, combustion by-products |
| Less chlorinated | Chlorinated benzenes, PCBs | C | Solvents, pesticides |
| Nitroaromatics | TNT, RDX | C | Explosives |

BTEX = benzene, toluene, ethylbenzene and xylene; MTBE = methyl tert-butyl ether; PCBs = polychlorinated biphenyls; TNT = trinitrotoluene; RDX = royal Dutch explosive (1,3,5-trinitrohexahydro-s-triazine)

[a]F = very frequent; C = common; I = infrequent

### 1.1.3 Bioremediation of polluted aquifers

*Organic pollutants as a source of carbon and energy*

Remediation of polluted groundwater is a field of increased scientific interest because degradation of organic contaminants in particular can occur by the indigenous microbial population (bioremediation) in the presence of available electron donors, electron acceptors and nutrients (Scow and Hicks, 2005; Weiss and Cozzarelli, 2008). The organic contaminants are utilised by the microorganisms as a source of carbon (and in most cases as electron donors) while the required energy for these reactions is yielded by catalysing redox reactions. Under aerobic conditions, oxygen serves as electron acceptor (aerobic degradation). Once oxygen is consumed, the required energy is yielded by the reduction of other electron acceptors such as nitrate, Mn (IV), Fe(III), sulphate and carbon dioxide (anaerobic degradation). The energy yielded from the oxidation of organic compounds by these different Terminal Electron-Accepting processes (TEAPs) varies as shown in Table 1.2, with the most negative Gibb's energy of reaction [$\Delta G^0$ ($W$)] being the most favourable (Christensen *et al.*, 2000).

**Table 1.2.** Gibb's energy of reaction calculated for oxidation of organic carbon ($CH_2O$) by various electron acceptors and by methane fermentation of organic carbon. Adjusted from Christensen *et al.* (2000).

| Process | Electron acceptor | Product | $\Delta G^0$ ($W$) (kJ/eq$^{-1}$) |
|---|---|---|---|
| Aerobic respiration | $O_2$ | $H_2O$ | – 125 |
| Denitrification | $NO_3^-$ | $N_2$ | – 119 |
| Mn reduction | Mn(IV) | Mn(II) | – 98 |
| Fe reduction | Fe(III) | Fe(II) | – 42 |
| Sulphate reduction | $SO_4^{2-}$ | $S^{2-}$ | – 25 |
| $CO_2$ reduction/methane fermentation | $CO_2$ | $CH_4$ | – 23 |

As a consequence, the microbial activity often leads to the formation of distinct geochemical redox zones within hydrocarbon-polluted aquifers (e.g. Lendvay *et al.*, 1998; Bekins *et al.*, 1999), where the aerobic head of the plume is succeeded by $NO_3^-$, Mn(IV), Fe(III), $SO_4^{2-}$ reducing zones, while in the core of the plume methanogenic processes predominate (Fig. 1.1). This distribution of the TEAP zones is usually documented by the successive depletion of the pre-mentioned electron acceptors and the increased

concentrations of the reduced forms ($N_2$ or $NO_2^-$, Mn(II), Fe(II), sulphide and $CH_4$ respectively). However, as Chapelle (2001) emphasises, in occasions that the electron donors are abundant (e.g. constant supply of hydrocarbons in the polluted aquifer), this redox zonation is less distinct and concomitant methanogenesis, Fe(III) and sulphate reduction may occur. The latter is in agreement with what was observed in an aquifer polluted with high concentrations of phenolic compounds (Thornton *et al.*, 2001b).

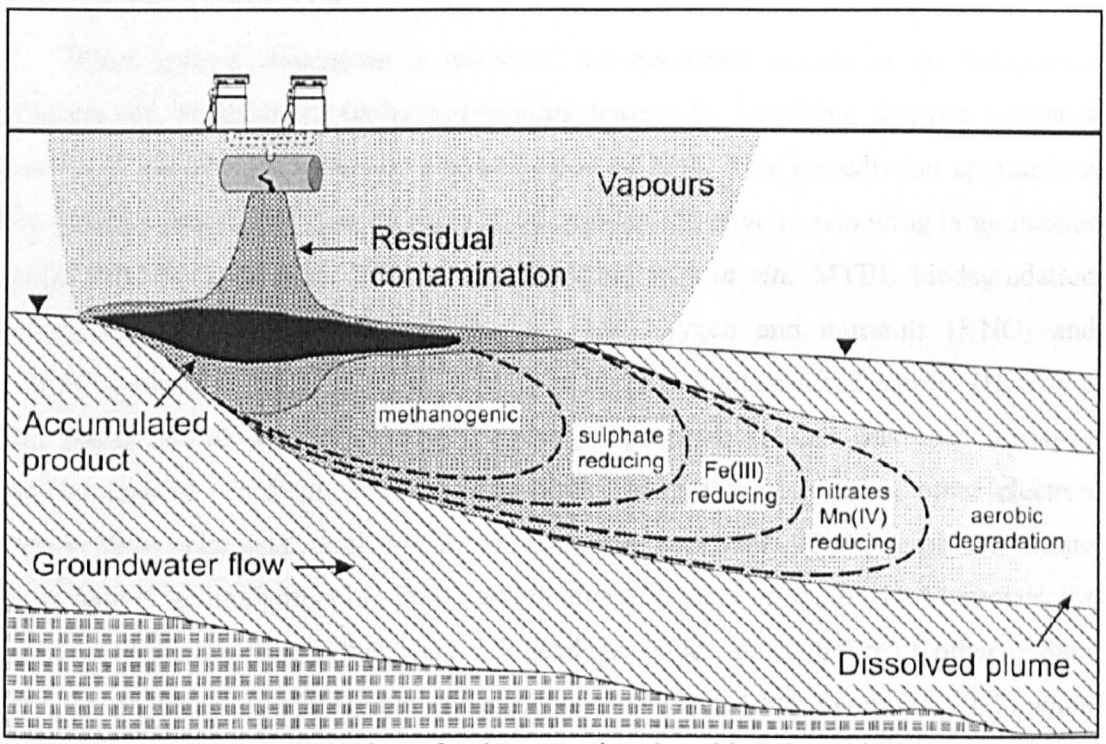



**Figure 1.1.** Typical distribution of redox zonation (aerobic, nitrate / Mn(IV) / Fe(III) / sulphate reducing, and methanogenic) that is formed within hydrocarbon polluted aquifers. Taken from Franzmann *et al.* (2002).

*Natural attenuation and enhanced bioremediation*

In general, biodegradation of polluted groundwater is a complex process which depends on the nature and the amount of the pollutants, the environmental conditions and the composition of the microbial community (Wikström *et al.*, 2000). In some occasions, the biodegradation rates are high enough and groundwater pollution does not pose a foreseeable threat (for example the plume is away from a public water supply or a

freshwater ecosystem), so that no human intervention is needed other than regular monitoring of the pollution. This remediation approach is usually called Monitored Natural Attenuation (MNA) but it is also referred to as intrinsic or passive bioremediation (Farhadian *et al.*, 2008). Natural attenuation processes were detected in a number of different sites, including aquifers contaminated with BTEX (Bekins *et al.*, 2001; Cozzareli *et al.*, 2001), with volatile organic compounds (Eganhouse *et al.*, 2001) and with chlorinated solvents (Davis *et al.*, 2002).

When natural attenuation is too slow, the microbial activity of the indigenous population can be enhanced (enhanced bioremediation) by supplying electron acceptors ($O_2$ or $NO_3^-$ usually), nutrients or a combination of both. This remediation approach is called biostimulation and in many cases it was proved effective in removing large masses of pollutants. For example, diffused oxygen stimulated *in situ* MTBE biodegradation (Wilson *et al.*, 2002), and the addition of both oxygen and nutrients ($KNO_3$ and $NH_4H_2PO_4$) stimulated the microbial activity in a diesel contaminated aquifer (Hunkeler *et al.*, 2002). As an alternative to $O_2$, hydrogen peroxide ($H_2O_2$) was used on some occasions instead (Pardieck *et al.*, 1992). Under anaerobic conditions, other electron acceptors have been used, such as nitrate (Schreiber and Bahr, 2002), combined nitrate and sulphate (Cunningham *et al.*, 2001) and methane (Eguchi *et al.*, 2001). Moreover, for some recalcitrant pollutants that were not biodegradable, the addition of other carbon sources enabled their co-metabolism (Hopkins and McCarty, 1995; Semprini, 1997) while the addition of surfactants facilitated the bioavailability of some hydrophobic pollutants (Ron and Rosenberg, 2002; Garcia-Junco *et al.*, 2003).

Bioremedation can also be enhanced with the introduction of allochthonous microorganisms in the site/aquifer of interest (bioaugmentation). These microorganisms (single strains or microbial consortia) are chosen on the basis that they carry the necessary catabolic genes/properties for the degradation of the pollutants. Bioaugmentation has been successfully applied in activated sludge of industrial wastewater (reviews by Stephenson and Stephenson, 1992; Van Limbergen *et al.*, 1998) and in some polluted aquifers. For instance, it was shown that the addition of a dechlorinating microbial consortium that included *Dehalococcoides ethenogenes* strains led to the successful degradation of chlorinated ethenes *in situ* and the progressively increased abundance of these strains

within the polluted aquifer (Major *et al.*, 2002). Sometimes the bioaugmentation of a polluted environment was followed by the acquisition of the catabolic properties by indigenous microorganisms due to transfer of catabolic genes (review by Top *et al.*, 2002). However, in other cases bioaugmentation did not have the desired results (e.g. Bouchez *et al.*, 2000) and the choice of the appropriate organisms (and conditions) for bioaugmentation that they will persist in a polluted environment is always a challenge (Thompson *et al.*, 2005).

Specifically for xenobiotic compounds, i.e. manmade chemicals that are not otherwise found in the environment (Lovley, 2003), naturally occurring bacteria may not have the ability to biodegrade them. Therefore, bioaugmentation using genetically engineered microorganisms has been considered instead (Chen *et al.*, 1999; Pieper and Reineke, 2000). So far, the application of such genetically engineered microorganisms in the environment has been limited due to the risks associated with uncontrolled growth and proliferation of the introduced biocatalyst and horizontal gene transfer (Paul *et al.*, 2005). Programming for rapid death of these microbial biocatalysts soon after the depletion of the pollutant could minimise the risks in applying these technologies for successful bioremediation (Paul *et al.*, 2005). In any case, the use of bioaugmentation (with or without genetically engineered microbes) as remediation approach remains a source of controversy (Thompson *et al.*, 2005).

Although there are many studies describing bioremediation processes in polluted aquifers, the majority of them describe natural attenuation processes using geochemical data that confirm the mass loss of contaminants, the production of metabolites, changes in redox-sensitive species (production of reduced forms), and isotopic fractionation of reactants or products (for a review on stable isotope fractionation see Meckenstock *et al.*, 2004). However, few investigators combine these observations with microbiological studies, something which Weiss and Cozzarelli (2008) stated is "*essential in evaluating and implementing remediation strategies which are inherently driven by the composition and function of the microbial community*".

## 1.2 Microbial degradation of phenolic compounds

### 1.2.1 Utilisation of phenolic compounds by microorganisms

Phenolic compounds (phenol, cresols and xylenols) are common environmental pollutants because of their presence in the effluents of many industrial processes, such as oil refineries, petrochemical plants, coal conversion plants and phenolic resin industries (Hinteregger *et al.*, 1992). The chemical structures of phenol, cresols and xylenols are shown in Fig. 1.2. Phenol consists of one benzene ring (6 carbon aromatic hydrocarbon) with an attached hydroxyl group. Cresols (methylphenols) have one methyl group substituted on the phenolic ring. Depending on the position of the carboxyl group, there are three forms of cresols: *ortho*-cresol (*o*-cresol), *meta*-cresol (*m*-cresol) and *para*-cresol (*p*-cresol). Xylenols (dimethylphenols) have 2 methyl groups attached to the phenolic ring; depending on the position they attach, there are 6 xylenol isomers (position 1 is the carbon where the hydroxyl group is attached). The toxicity of phenolic compounds is well reported (phenol toxicity in particular is discussed in 4.4.2) and they have long been used as antimicrobial agents because of their antiseptic, disinfectant or preservative properties, depending on the compound (McDonnell and Russell, 1999; Lucchini *et al.*, 1990).

Nevertheless, many microorganisms such as Archaea (Tor and Lovley, 2001) and fungi (Santos and Linardi, 2004; Atagana, 2004; Leitão *et al.*, 2007; Ryan *et al.*, 2007) including yeasts (Bergauer *et al.*, 2005; Jiang *et al.*, 2005) were shown to have the ability to utilise phenolic compounds. The majority of the microorganisms that can metabolise phenol are Bacteria and are equipped with the necessary enzymes for phenol catabolism. The genes coding for the catabolism of aromatic compounds (such as phenol) are usually clustered together and found in mobile genetic elements such as transposons and plasmids, so that horizontal gene transfer and consequently rapid adaptation of microorganisms to new pollutants is facilitated (Díaz, 2004).

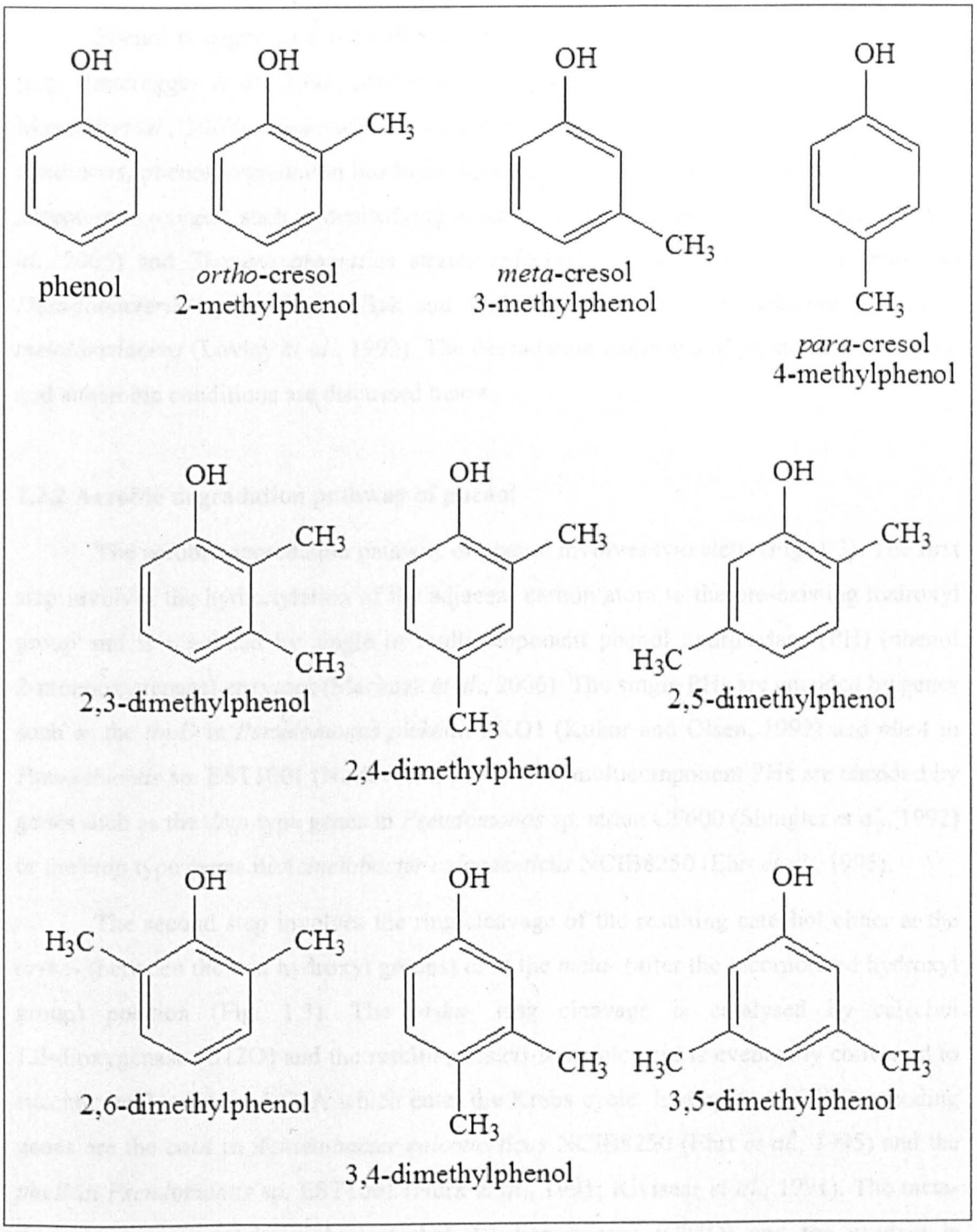



**Figure 1.2.** The chemical structure of phenol, cresols (methylphenols) and xylenols (dimethylphenols).

Phenol is degraded aerobically by a wide range of bacteria such as *Pseudomonas* (e.g. Hinteregger *et al.*, 1992; Merimaa *et al.*, 2006), *Acinetobacter* (Ehrt *et al.*, 1995; Mazzoli *et al.*, 2007), *Comamonas testosteroni* (Yap *et al.*, 1999) etc. Under anaerobic conditions, phenol degradation has been reported by isolates that use alternative electron acceptors to oxygen, such as denitrifying *Azoarcus* (van Schie and Young, 1998; Rabus *et al.*, 2005) and *Thauera aromatica* strains (Mechichi *et al.*, 2002), sulphate reducing *Desulfobacterium phenolicum* (Bak and Widdel, 1986), and iron reducing *Geobacter metallireducens* (Lovley *et al.*, 1993). The degradation pathways of phenol under aerobic and anaerobic conditions are discussed below.

### 1.2.2 Aerobic degradation pathway of phenol

The aerobic degradation pathway of phenol involves two steps (Fig. 1.3). The first step involves the hydroxylation of the adjacent carbon atom to the pre-existing hydroxyl group and is regulated by single or multicomponent phenol hydroxylase (PH) (phenol 2-monooxygenase) enzymes (Merimaa *et al.*, 2006). The single PHs are encoded by genes such as the *tbuD* in *Pseudomonas pickettii* PKO1 (Kukor and Olsen, 1992) and *pheA* in *Pseudomonas* sp. EST1001 (Nurk *et al.*, 1991). The multicomponent PHs are encoded by genes such as the *dmp* type genes in *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1992) or the *mop* type genes in *Acinetobacter calcoaceticus* NCIB8250 (Ehrt *et al.*, 1995).

The second step involves the ring cleavage of the resulting catechol either at the *ortho-* (between the two hydroxyl groups) or at the *meta-* (after the incorporated hydroxyl group) position. (Fig. 1.3). The *ortho-* ring cleavage is catalysed by catechol 1,2-dioxygenase (C12O) and the resulting *cis,cis*-muconic acid is eventually converted to succinic acid and acetyl-CoA which enter the Krebs cycle. Examples of C12O encoding genes are the *catA* in *Acinetobacter calcoaceticus* NCIB8250 (Ehrt *et al.*, 1995) and the *pheB* in *Pseudomonas* sp. EST1001 (Nurk *et al.*, 1991; Kivisaar *et al.*, 1991). The meta-ring cleavage is catalysed by catechol 2,3-dioxygenase (C23O) and the product is 2-hydroxymuconic semialdehyde. Examples of C23O encoding genes include the *xylE* in *Pseudomonas putida* H (Herrmann *et al.*, 1995) and the *dmpB* in *Pseudomonas* sp. CF600 (Nurk *et al.*, 1991). Shingler *et al.* (1992) showed that in *Pseudomonas* sp. CF600 the

2-hydroxymuconic semialdehyde can be further catalysed to 2-hydroxy-oxovalerate either directly (by 2-hydroxymuconic semialdehyde hydrolase) or via the intermediate 4-oxalocrotonate (by 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase). The 2-hydroxy-oxovalerate is finally converted to pyruvate and acetaldehyde which are utilised for cellular metabolism.

In general, as Díaz (2004) points out, under aerobic conditions structurally diverse aromatic compounds are degraded via many different peripheral pathways (carried out by hydroxylating monooxygenases and/or dioxygenases) to a few intermediate dihydroxy aromatic compounds (such as catechol, substituted catechols, protocatechuate), which are then cleaved and enter central catabolic pathways. Therefore, it is not uncommon that some microorganisms are capable of utilising a wide range of (mono-) aromatic compounds. For example, *Pseudomonas* sp. strain CF600 could grow efficiently on phenol, cresols and 3,4-dimythylphenol (3,4-xylenol) and the fifteen genes encoding for their degradation pathways (*dmpKLMNOPQBCDEFGHI*) were detected in a single operon structure (Shingler *et al.*, 1992). Another example is *Pseudomonas pickettii* PKO1, which could metabolise *o*-cresol and *p*-cresol when cells were previously exposed to phenol or *m*-cresol (Kukor and Olsen, 1990; Kukor and Olsen, 1991). Versatile bacteria also exist under anaerobic conditions, such as a denitrifying *Azoarcus* strain EbN1 that could utilise a wide range of aromatic compounds, including phenol, *p*-cresol, ethylbenzene and benzoate (Rabus *et al.*, 2005). However, it is worth pointing out that when mixtures of aromatic carbon sources are present, microorganisms tend to degrade the pollutants in sequential order. For example, *Acinetobacter radioresistens* S13 could utilise benzoate > acetate > phenol (Mazzoli *et al.*, 2007).

### 1.2.3 Anaerobic degradation pathway of phenol

Under oxygen limited conditions it has been shown that some *Pseudomonas* isolates can degrade phenol via the meta- ring cleavage pathway, using nitrate as electron acceptor (Kukor and Olsen, 1996; Su and Kafkewitz, 1994). However, under anaerobic conditions phenol is mainly degraded by *para* carboxylation to 4-hydroxybenzoate (Fig. 1.3; Zhang and Wiegel 1994; Lupa *et al.*, 2005). An alternative pathway is followed by a

denitrifying *Thauera aromatica* strain, as phenol is firstly phosphorylated to phenylphosphate (Fig. 1.3), before being converted by *para* carboxylation (by phenylphosphate carboxylase genes) to 4-hydroxybenzoate (Breining *et al.*, 2000; Schühle and Fuchs, 2004; Schmeling *et al.*, 2004). In both occasions, the 4-hydroxybenzoate is then converted to benzoyl-CoA and finally acetyl-CoA, which enters the citric acid (Krebs) cycle (Boll *et al.*, 2002).

Cresols can also be degraded anaerobically by a range of pathways, depending on the position of the hydroxyl group (Rudolphi *et al.*, 1991; Schink *et al.*, 2000). Anaerobic degradation occurs via anaerobic oxidation of the methyl groups (*m, p, o*-cresols) or para-carboxylation (*m*- and *o*-cresols). Many of the intermediates produced are also intermediates (or simple derivatives) of phenol degradation pathways. For example, 4-hydroxybenzoate is also an intermediate of *p*-cresol metabolism. To our knowledge, there are no studies on the anaerobic degradation of xylenols by single isolates. However, biodegradation of 3,4-dimethylphenol (3,4-xylenol) in anaerobic wastewater has been reported (Puig-Grajales *et al.*, 2003).

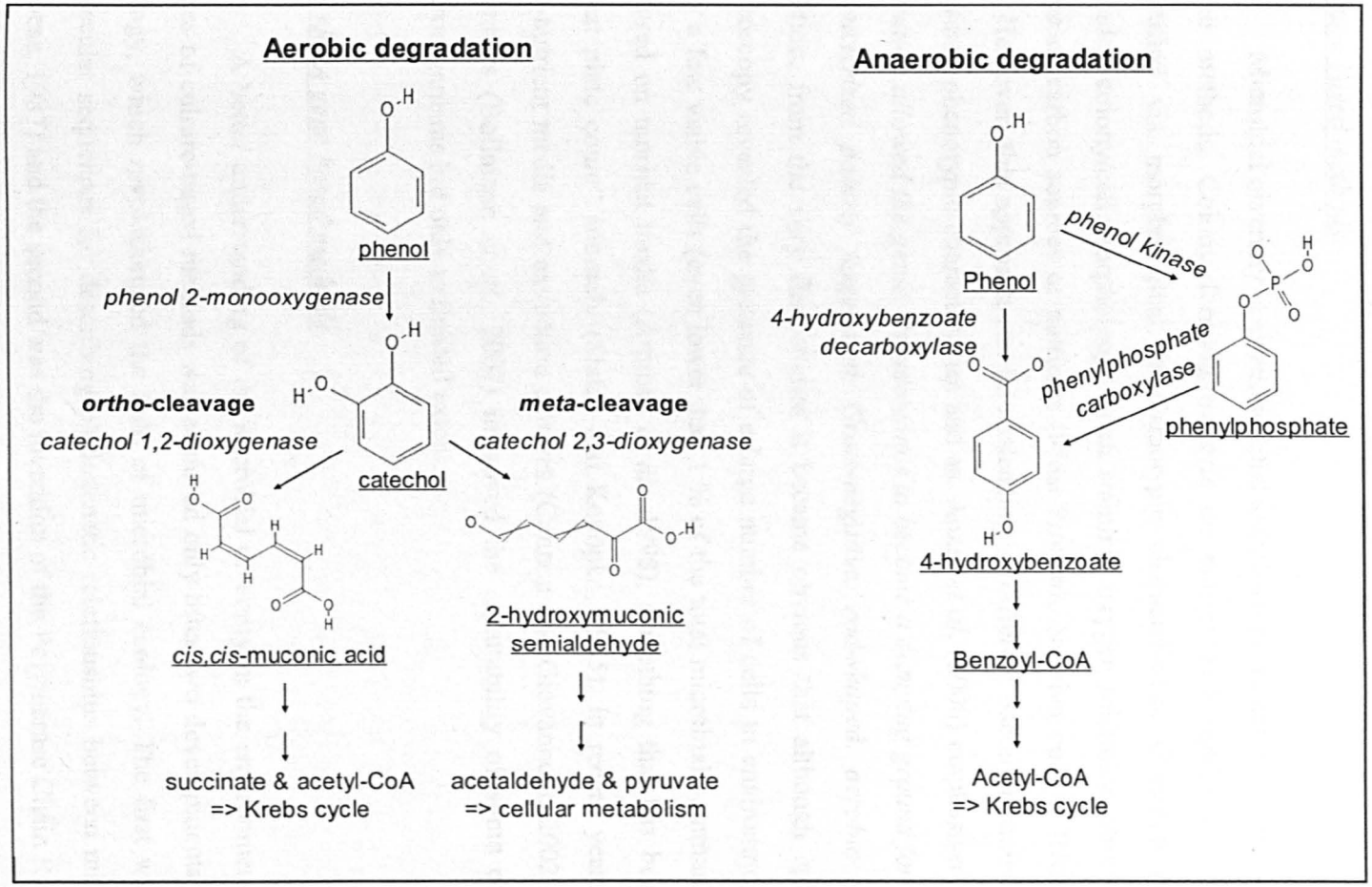



**Figure 1.3.** The aerobic and anaerobic degradation pathways of phenol. Under aerobic conditions phenol is hydroxylised to catechol which is then cleaved by the *ortho-* or the *meta-* ring cleavage pathways. Under anaerobic conditions, phenol is carboxylated to 4-hydroxybenzoate which is then converted to benzoyl-CoA.

## 1.3 Methods for describing microbial diversity

*Culture based methods*

Microbial diversity in environmental samples was traditionally assessed by culture based methods. Colony forming bacteria on nutrient-rich media were isolated and identified via morphological or phenotypic characteristics. Common characteristics included colony/cell morphology, Gram staining, oxygen tolerance, ability to utilise a range of carbon sources or nutrients (Most Probable Number counts, BIOLOG plates), etc. However, this approach can be misleading as different bacterial strains may exhibit the same phenotypic characteristics and as, Anzai *et al.* (2000) emphasised, "*phenotypic definition allowed the genus Pseudomonas to become a dumping ground for incompletely characterized polarly flagellated, Gram-negative, rod-shaped, aerobic bacteria*". In addition, from the very first studies it became obvious that although epifluorescence microscopy revealed the presence of a large number of cells in environmental samples, only a few viable cells (even lower than 1 % of the total microbial community) could be cultured on nutrient media (Amman *et al.*, 1995), something that has been called the "great plate count" anomaly (Staley and Konopka, 1985). In recent years, the use of low-nutrient media and extinction cultures (Connon and Giovannoni, 2002) or diffusion chambers (Bollmann *et al.*, 2007) improved the culturability of some environmental microorganisms but only to limited extent.

*16S rRNA gene based methods*

A better understanding of the microbial diversity in the environment without the biases of culture-based methods was achieved only after two developments in molecular biology, which revolutionised the field of microbial ecology. The first was the use of molecular sequences for describing phylogenetic relationships between microorganisms (Woese, 1987) and the second was the invention of the Polymerase Chain Reaction (Saiki *et al.*, 1988).

In the late 1970s, using 16S rRNA gene sequences to describe phylogenetic affiliations between organisms, Carl Woese and colleagues identified that the Archaea

(called Archaebacteria at that time) was a distinct and separate Kingdom to the Eubacteria and Eukaryotes (Woese and Fox, 1977). Ten years later, again based on the information of 16S rRNA gene sequences to date, the Bacteria were grouped into 11 different phylogenetic groups (Woese, 1987). In the next 2 decades bacterial taxonomy based on 16S rRNA gene phylogenetic affiliations became the common standard (also adopted by Bergey's Manual of Systematic Bacteriology). The number of 16S rRNA gene sequences in public repositories (such as GenBank and EMBL) expanded exponentially and up to 35 different phylogenetic groups within the Bacteria were recognised (Hugenholtz, 2002). The main reasons that the 16S rRNA gene is used as phylogenetic marker are because a) it is present in all prokaryotic organisms, b) it is a component of ribosomes that are required by all organisms to synthesise proteins, c) it is long enough (~1.5 kb) to document evolutionary history, d) horizontal transfer of rRNA genes is limited and e) it consists of universally conserved domains (Osborn and Smith, 2005).

The development of PCR using thermostable DNA polymerases (Saiki *et al.*, 1988) enabled the culture-independent analysis of environmental microbial communities. Even low quantities of isolated nucleic acids (DNA or RNA) from diverse environmental microbial communities could now be amplified using gene-specific primers. For the purposes of prokaryotic phylogeny/diversity studies, "universal" primers targeting either the (almost) full length of the 16S rRNA gene or its hypervariable V3 region (positions 341 – 534 in the 16S rRNA gene of *E. coli*; Muyzer *et al.*, 1993) are normally used. The amplified fragments can then be inserted into cloning vectors that are used to transform *E. coli* cells. The PCR fragments can then be re-amplified from the individual clones and 16S rRNA gene sequencing can reveal the microbial community diversity in the original sample. This procedure is described in more detail elsewhere (3.2.5).

Cloning of 16S rRNA genes was employed in a number of studies describing microbial diversity in polluted aquifers, including aquifers contaminated with coal-tar (Bakermans and Madsen, 2002), crude oil (Watanabe *et al.*, 2000), chlorinated solvents (Hohnstock-Ashe *et al.*, 2001; Lowe *et al.*, 2002; Davis *et al.*, 2002), and uranium (Holmes *et al.*, 2002; North *et al.*, 2004). Most of these studies showed that different microbial communities inhabited polluted and pristine areas of the aquifer, indicating that pollutants can affect microbial diversity of natural occurring microorganisms.

*Profiling of microbial diversity using DNA fingerprinting methods*

When identification of bacteria is not necessary but a rapid comparison of the microbial diversity within different samples is needed, DNA fingerprinting techniques (based on the 16S rRNA gene phylogeny) are used instead, such as Denaturing Gradient Gel Electrophoresis (DGGE; Muyzer *et al.*, 1993), Temperature Gradient Gel Electrophoresis (TGGE; Rosenbaum and Riesner, 1987; Felske *et al.*, 1997), Terminal-Restriction Fragment Length Polymorphism (T-RFLP; Liu *et al.*, 1997; Osborn *et al.*, 2000), and single-strand-conformation polymorphism (SSCP; Lee *et al.*, 1996; Schwieger and Tebbe, 1998). These methods separate 16S rRNA gene fragments based on differences in nucleotide sequence without the need to read the sequence.

The DGGE method was introduced in molecular ecology by Muyzer *et al.* (1993) and it rapidly became one of the most widely used methods for profiling microbial diversity. The first step for DGGE analysis, involves the PCR amplification of isolated environmental DNA with a primer pair targeting a variable region of the 16S rRNA gene. DGGE analysis requires that one of the primers should have a 40 bp GC clamp incorporated at its 5′ tail. The PCR products are then electrophorised in a polyacrylamide gel with a denaturing chemical gradient which is usually formed by different urea and formamide solutions (as described elsewhere - 2.2.7). DGGE exploits the fact that when these PCR products run into the gel, the DNA strands will migrate until the concentration of denaturant causes the double stranded DNA to denature (although the GC clamp remains folded). The distance that the PCR products will migrate in the gel depends on their GC content. 16S rRNA gene fragments rich in double A-T bonds will denature faster than 16S rRNA gene fragments rich in triple G-C bonds. So, although the PCR products of the mixed microbial community are of (approximately) the same length, the DGGE gel facilitates their separation in the gel, according to their GC content. TGGE is based on the same principle with DGGE, with the difference that a temperature gradient is used for denaturation (Rosenbaum and Riesner, 1987). Both DGGE and TGGE are sensitive enough to detect even single point mutations.

DGGE has been used to monitor the effect of biostimulation in a TCE contaminated aquifer on microbial community structure over time (Iwamoto *et al.*, 2000),

differences in microbial diversity in different compartments of an industrial phenol remediation system (Whiteley and Bailey, 2000), in oil contaminated benthic sediments (Powell *et al.*, 2005), in lake sediment samples (Cummings *et al.*, 2003; Koizumi *et al.*, 2003) and in different pristine groundwater samples (Cho *et al.*, 2003). Moreover, by attaching a GC clamp to one of the primers targeting the BTEX mono-oxygenase genes, Hendrickx *et al.* (2006a) used PCR-DGGE to profile diversity of functional (BTEX mono-oxygenase) genes.

The other DNA fingerprinting methods have been used less often in microbial ecology studies and some examples are the use of TGGE for profiling microbial structure in different soils (Felske *et al.*, 1997) and in wastewater microcosms (Cortés-Lorenzo *et al.* 2005), the use of T-RFLP analysis to compare microbial diversity in groundwater samples from pristine and BTEX contaminated sandstone aquifer (Fahy *et al.*, 2005) and between different PAH contaminated soils (Muckian *et al.*, 2007) and the use of SSCP to describe microbial diversity in soil samples (Bharathkumar *et al.*, 2008) and in a phenol polluted aquifer (Lin *et al.*, 2007).

*Biases introduced during PCR amplification of 16S rRNA gene*

Although 16S rRNA gene based methods can describe microbial diversity more efficiently than culture-based methods, PCR-based methods are not bias free. It is well established that PCR amplification can result in a number of different artefacts, such as mutations, deletions and chimeric sequences. Point mutations during PCR amplification are introduced because DNA polymerase enzymes such as *Taq* polymerase are not 100 % accurate; thermostable DNA polymerases that contain a 3′→5′ exonuclease (proofreading) activity (such as *Pfu*) can decrease 10-fold the miscorporation rate of nucleotides (Lundberg *et al.*, 1991). Deletions are caused when PCR amplification proceeds without including stable hairpin secondary structures, resulting in smaller PCR fragments (Cariello *et al.*, 1991). Chimeric sequences are formed as a result of PCR co-amplification of 16S rRNA genes from different bacterial species (partially amplified fragments anneal to other homologous fragments) and are thought to increase in the presence of highly fragmented nucleic acids caused by rigorous cell lysis practices

(Liesack *et al.*, 1991). The occurrence and detection of chimeric sequences are discussed more extensively elsewhere (3.4.1).

In addition to these biases, PCR can lead to preferential amplification. When DNA extracted from an environmental sample is subjected to PCR amplification, it is expected that the relative abundance of the microbial species in the original sample is reflected in the PCR products. However, this is not strictly the case as nucleic acid molecules are not equally accessible to primer hybridisation, primer-template hybrids do not form with equal efficiencies, and extension efficiency of DNA polymerase is not the same for all templates (Wintzingerode *et al.*, 1997). Moreover, limitation by substrate exhaustion (primers, nucleotides) does not affect the extension of all templates equivalently; it has been demonstrated that in this case, preferential amplification of less abundant sequences occurs (Suzuki and Giovannoni, 1996), something which has been called the $C_0t$ effect (Mathieu-Daudé *et al.*, 1996). In addition, the heterogeneity in rRNA (*rrn*) gene copy numbers between different bacterial species (from 1 and up to 15 *rrn* copy numbers; Klappenbach *et al.*, 2001) constitutes another source of systematic error in community analysis (Farrelly *et al.*, 1995; Crosby and Criddle, 2003), while within some species different rRNA operons may exist (Nübel *et al.*, 1996). Finally, as 16S rRNA genes with lower G+C content dissociate more easily than G+C rich genes during the PCR denaturation step, this can eventually lead to the preferential amplification of low G+C containing genes (Reysenbach *et al.*, 1992).

From all the above, it becomes obvious that during PCR amplification, isolated nucleic acids from a mixed microbial community are not amplified at the same rate. Therefore, the quantitative interpretation of all downstream analyses following PCR amplification such as 16S rRNA gene cloning libraries and DNA fingerprinting methods (DGGE, TRFLP, etc) should be done with caution.

*Metagenomic approaches*

The limitations of the PCR based methods can be overcome by emerging high-throughput metagenomic approaches (also called as community genomics, environmental genomics, and population genomics), i.e. the techniques that characterise

the genomes of whole communities of organisms rather than individual species (Handelsman, 2004; Tringe and Rubin, 2005). These approaches avoid the PCR step and the isolated genomic DNA from mixed microbial communities (present in environmental samples) is firstly fragmented into smaller pieces and then multiple sequence reads are reassembled using bioinformatics tools.

In one of the first metagenomic studies, using "whole-genome shotgun sequencing", Venter *et al.* (2004) recovered 1.045 billion base pairs of non-redundant sequences from surface water samples from the Sargasso Sea, which were estimated to derive from more than 1,800 different bacterial species. Another metagenomic study from the same research group (Rusch *et al.*, 2007) resulted in 6.5 billion base pairs and demonstrated once again the extent of microbial diversity in natural habitat, as most sequence reads were unique.

In the last few years, the development of second-generation ultrafast and cost effective sequencing technologies such as 454-pyrosequencing and the parallel advances in computational tools (e.g. Huson *et al.*, 2007; Raes *et al.*, 2007; Markowitz *et al.*, 2008a; Markowitz *et al.*, 2008b) that are capable of assembling and processing the generated sequence fragments have led to a number of metagenomic projects in different environments. For example in the last two years (2007 and 2008) 454-pyrosequencing technology was used to assess the microbial diversity in: termite hindgut (Warnecke *et al.*, 2007), human gut (Andersson *et al.*, 2008), chronic wounds (Dowd *et al.*, 2008), a wastewater treatment plant (note: plasmid metagenome; Szczepanowski *et al.*, 2008), a biogas plant (Schlüter *et al.*, 2008), soil (Roesch *et al.*, 2007; Urich *et al.*, 2008) and coral atolls (Dinsdale *et al.*, 2008).

At the same time, "whole-genome shotgun sequencing" of single bacterial strains has resulted in a large number of fully sequenced bacterial genomes (more than 1,000 genomes to date – DOE Joint Genome Institute, September 2008). As sequences from (cultured) bacterial genomes and metagenomic studies continue to be generated, our understanding of the structure and function of environmental microbial communities will be enhanced greatly in the future.

## 1.4 Environmental biofilms

Bacteria can exist in nature either as free-floating within the aqueous environment (planktonic) or attached to surfaces, forming matrix enclosed communities called biofilms (Fig. 1.4). Biofilms are composed of structured microbial assemblages and secreted extracellular polymeric substances or exopolysaccharides (EPS). The EPS are a slime-like matrix which provides structural support for the biofilm and consists of polysaccharides, proteins, nucleic acids and phospholipids. The composition of biofilm matrices is shown in Table 1.3. Biofilms exhibit the properties of a viscoelastic fluid (Costerton and Wilson, 2004), with cell clusters becoming elongated in the downstream direction, forming filamentous streamers (Stoodley *et al.*, 2002).

**Table 1.3.** Range of composition of biofilm matrices (Taken from Sutherland, 2001).

| Component | % of biofilm matrix |
|---|---|
| Water | up to 97% |
| Microbial cells | 2–5% (Many species) |
| Polysaccharides | 1–2% (Neutral and polyanionic) |
| Proteins (extracellular and resulting from lysis) | <1–2% (Many, including enzymes) |
| DNA and RNA | <1–2% (From lysed cells) |
| Ions | ? (Bound and free) |

The role of physico-chemical interactions between cells or cell envelope appendages and substrata in initial attachment is described elsewhere (4.4.3). Cell-surface attachment is also mediated by flagellar movement and twitching motility by type IV pili (Stoodley *et al.*, 2002), although under certain conditions biofilms can be formed without the apparent presence of flagellar- and pili- mediated movement (Pratt and Kolter, 1999). When the cells are irreversibly attached to mineral surfaces, biofilm formation proceeds by binary division of attached cells, redistribution of attached cells by surface twitching motility (i.e. movement along the surface) and aggregation of cells (secondary attachers) from the bulk fluid to the developing biofilm (Hall-Stoodley and Stoodley, 2002). These mechanisms lead to the formation of clonal mosaics (clusters of genetically identical bacteria) within the multispecies biofilms (Rickard *et al.*, 2003). Maturation of biofilms is reached within a few days and mature biofilms are characterised by a complex architecture (as revealed by confocal laser microscopy), with channels, pores and discrete

mushroom-shape multicellular microcolonies (Webb *et al.*, 2003). Mature biofilms are not static microbial communities; there is constant dynamic motion of single cells over surfaces by twitching motility, within biofilm microcolonies by flagellar motility, the flow of entire microcolonies along surfaces, and the continual detachment of single cells and entire microcolonies from mature biofilms (Stoodley *et al.*, 2002). Detachment occurs not only by shear forces within the aquatic environment, but also it may be regulated by the release of matrix-degrading enzymes when cell density reaches a high level in biofilm microcolonies (Stoodley *et al.*, 2002).

It is well established that biofilm functions are regulated by quorum-sensing systems (cell-density dependent gene regulation) and intercellular signalling (Webb *et al.*, 2003; Stoodley *et al.*, 2002). The role of the *lasI* gene product acyl-HSL (or acylated homoserine lactone-AHL) is of particular importance, as it is thought to facilitate cell communication between Gram-negative bacteria (Stickler, 1999; Kjelleberg and Molin, 2002) and it was shown to induce the formation of differentiated biofilms by *P. aeruginosa* PAO1 (Davies *et al.*, 1998). Moreover, it was shown that the degree of structure complexity of *Pseudomonas aeruginosa* is regulated by alginate, which is a bacterial product of the EPS. Hentzer *et al.* (2001) showed that when alginate was added to undifferentiated, flat, *P. aeruginosa* biofilms, it induced the structural complexity causing the development of mound- and mushroom- shaped cell clusters. Disruption of EPS results in less structural complexity and greater susceptibility to antimicrobial agents (Stoodley *et al.*, 2002). The activation of the critical alginate promoter *algD* occurs in response to a variety of environmental factors, including nitrogen limitation, membrane perturbation induced by ethanol, and cell exposure to media of high osmolarity.

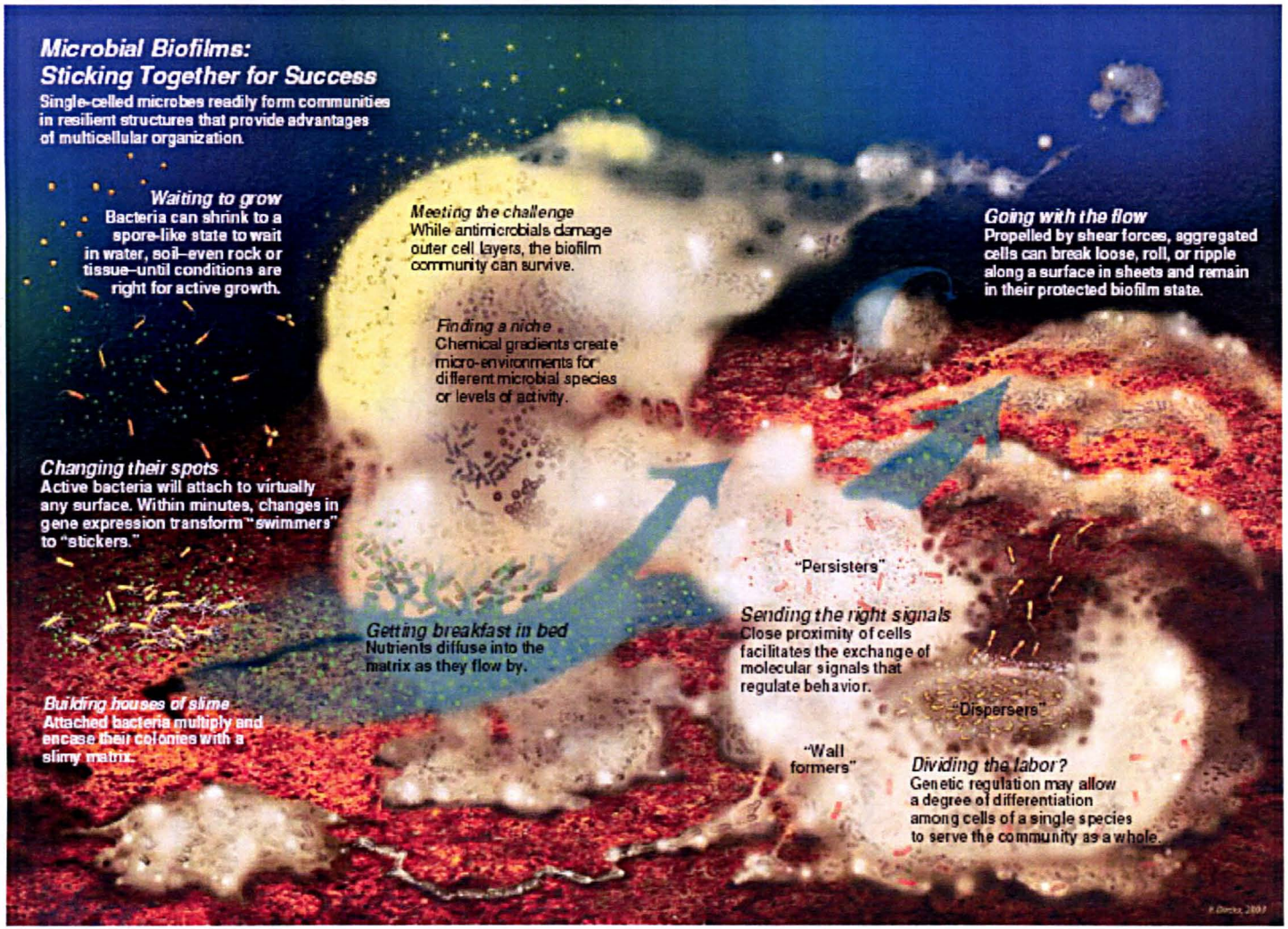



**Figure 1.4.** Bacterial biofilms and the benefits of sticking together (taken from Costerton and Wilson, 2004).

Biofilm formation appears to be triggered by environmental stresses, such as nutrient deprivation and predation stress during protozoan grazing (Webb *et al.*, 2003), while in the presence of antimicrobial agents, microbial growth in biofilms appears to be the preferred state (Costerton *et al.*, 1999; Mah and O'Toole, 2001; Hogan and Kolter, 2002). It is generally thought that the development of microscale chemical gradients and the presence of genetic and physiological heterogeneity inside biofilm structures can enhance microbial adaptation to local environmental conditions (Stewart and Franklin, 2008).

Therefore, in nutrient-limited environments that contain concentrations of toxic compounds such as aquifers contaminated with aromatic hydrocarbons, attached microbial growth should be regarded as of particular importance. The advantage that the attached phase offers to microorganisms in polluted environments could be due to the physical structure of the biofilm itself. For example, anaerobic bacteria will establish in anoxic zones of the attached communities (Stewart and Franklin, 2008), while the three-dimensional arrangement of bacterial cells within a biofilm may facilitate the absorption and utilisation of recalcitrant xenobiotics (Wuertz *et al.*, 2004), and the formation of channels may counteract diffusion limitations by increasing the biological surface area (Massol-Deya *et al.*, 1995). However, the role of genetic heterogeneity could be an even more important adaptation mechanism of environmental bacteria to the presence of toxic compounds, not least because of the exchange of genetic material via horizontal gene transfer that is facilitated by the proximity of cells (Marri *et al.*, 2007; Nojiri *et al.*, 2004).

From all of the above it becomes clear why biofilms are regarded as more advantageous for bioremediation purposes than free cells (Singh *et al.*, 2006). Hence, it is not surprising that biofilm-based reactors are used for hydrocarbon bioremediation purposes, such as fluidised bed reactors (Puhakka *et al.*, 1995) and granular activated carbon reactors (Caldeira *et al.*, 1999; Carvahlo *et al.*, 2001). Therefore, it is imperative that microbiological studies on polluted aquifers should include the characterisation of attached microbial communities.

## 1.5 The experimental site (Four Ashes)

*History and description of the experimental site*

The experimental site is the 33 hectare Four Ashes industrial complex which is located 10 km north of Wolverhampton, UK (Fig. 1.5). The site was initially developed in the 1950s primarily to distil and fractionate coal tars. Following the closure of the coal-tar distillation plant in the mid 1980s and the caustic soda lime kilns in the early 1980s, the industries on site have been used for the manufacturing of speciality organic chemicals using feedstocks brought in from other chemical plants (Williams *et al.*, 2001; Thornton *et al.*, 2001a).

The Four Ashes site overlies part of the Permo-Triassic Sherwood sandstone aquifer, which is the second most important in the UK. Locally, the aquifer is a quite uniform fluviatile red-bed quartzo-feldspathic sandstone, containing abundant Fe and Mn oxides as grain coatings and minor particulate organic carbon (Thornton *et al.*, 2001a). In the vicinity of the site, the aquifer is 250 m thick, with a porosity of about 26% and bulk hydraulic conductivity of about 0.7 m $day^{-1}$. The water table is less than 5 metres below ground level (mbgl), with a seasonal variation of 1 – 2 m. The westerly groundwater flow has a velocity between 4 and 10 m $year^{-1}$ and it is mainly controlled by pumping from a public supply borehole which is installed 2 km SW of the Four Ashes site.

*The pollutant plume, the installed boreholes and initial site investigations*

Since the development of the Four Ashes industrial complex and until the mid-1980s, spillages of mixtures of organic compounds from the acid-tar distillation plant, Cl from de-icing activities, NaOH from alkali storage facilities and $SO_4$ from mineral acid spills (Thornton *et al.*, 2001a) have resulted in severe groundwater pollution that was first discovered in 1987 by consultants Aspinwall and Co. The extent of the groundwater pollution was investigated by installing 22 conventional boreholes (with screens up to 10 m), nested in groups of three and terminating at different depths in the aquifer (Fig. 1.6). In addition, two multilevel sampling boreholes (borehole 59 and borehole 60) were installed 130 and 350 m away from the plume source (Figure 1.7). The design of these boreholes (Thornton *et al.*, 2001b) allows groundwater sampling at 1 m depth intervals

and to a depth of 30 and 45 m respectively for borehole 59 and borehole 60. The initial site investigations by the consultants showed that the plume had moved along with the groundwater flow 500 m away from the source and to a depth of 60 mbgl as defined by the 1 mg $L^{-1}$ phenol contour (Figure 1.7). The total concentration of organic pollutants was 24,800 mg $L^{-1}$ near the source area of the plume, consisting mainly of phenolic compounds: 12,500 mg $L^{-1}$ of phenol, 8,900 mg $L^{-1}$ of cresols (methylphenols) and 2,400 mg $L^{-1}$ of xylenols (dimethylphenols). The complete list of the organic compounds found by the consultants in the plume is given in Table 1.4.

*Natural attenuation processes in the Four Ashes plume*

Since the late 1990s, studies on the Four Ashes site led by the Groundwater Protection and Restoration Group (GPRG) of the University of Sheffield (www.sheffield.ac.uk/gprg) have resulted in a series of papers published primarily in a special issue of the Journal of Contaminant Hydrology (2001, Volume 53, Issue 3-4), as well as in other peer-reviewed journals. These studies provided a better understanding of the geochemistry and the natural attenuation processes occurring at the Four Ashes site.

The composition of the pollutant plume at the Four Ashes site and its historical causes was described by Williams *et al.* (2001) after groundwater samples were taken from all 22 conventional boreholes. Williams *et al.* (2001) identified that the composition of the pollutant plume is not homogenous but consists of 3 distinct sub-plumes or "Suites" (Figure 1.8). The major plume (Suite B) consists of organic contaminants and sulphate and reflects early inputs (since the 1950s) from the acid-tar distillation plant, as it extends 500 m away from the source area. However, closer to the distillation plant (up to 100 m away from the source) sodium rather than calcium becomes the dominant cation in the plume (Suite C). This shift reflects changes in the pollutant input which probably coincided with the demolition of the distillation plant in the early 1980s. In the shallow aquifer above these two plumes and underneath the site of a caustic soda/lime kiln plant that was demolished in 1987 (originally situated 100 m away from the distillation plant), a third sub-plume (Suite D) was identified. This plume consists of alkaline sodium bicarbonate/sulphate water with a pH up to 9.9, which is significantly higher than the pH 5.5 – 6 at the core of the main plume and the neutral pH in the uncontaminated water.

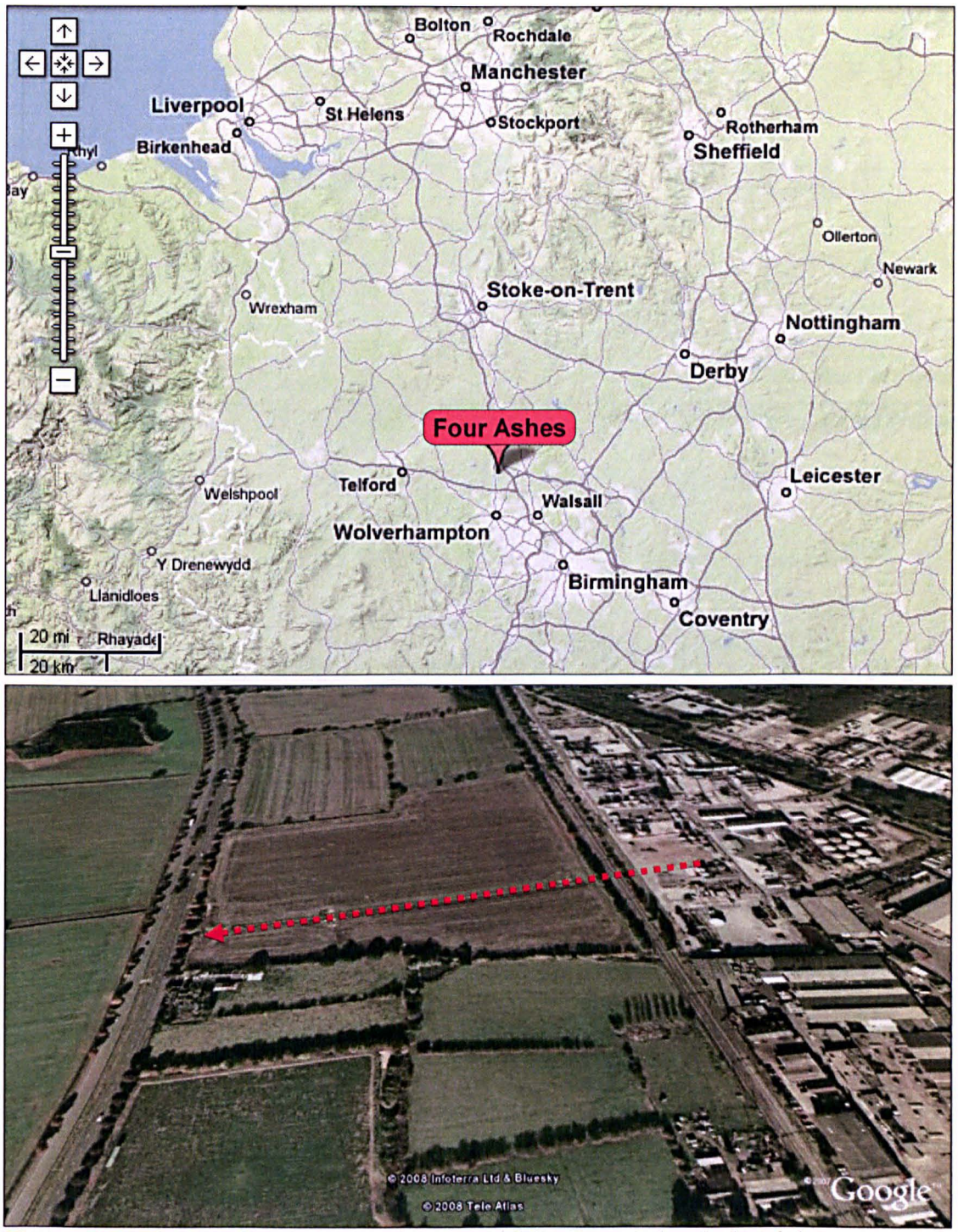



**Figure 1.5.** The location and an aerial view of the Four Ashes industrial estate and the surrounding area. The arrow indicates the direction of the groundwater flow, along which the boreholes are installed. The map was adjusted from Google maps and the image taken from Google Earth.

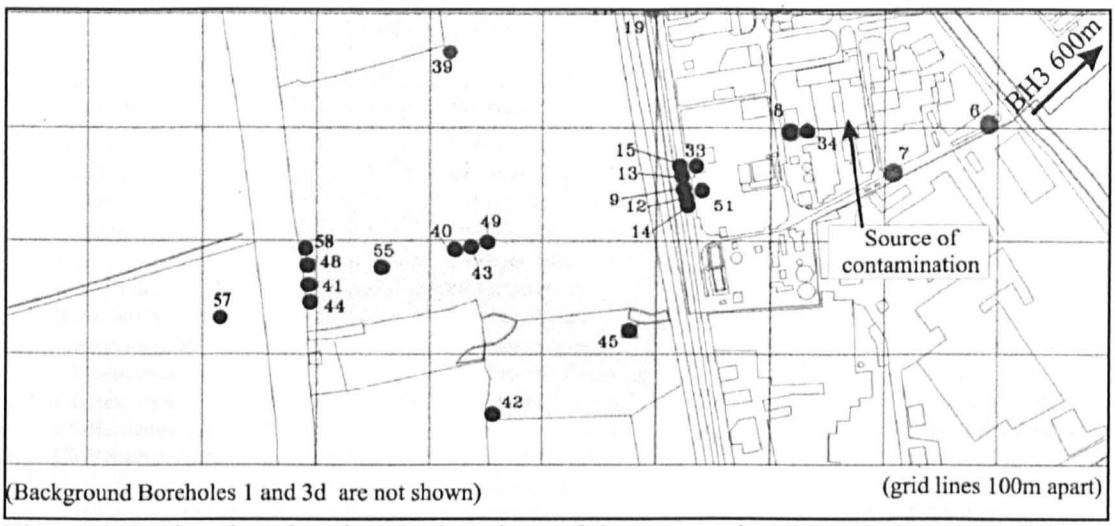



**Figure 1.6.** Site plan showing the locations of the conventional boreholes. From Williams *et al.* (2001).

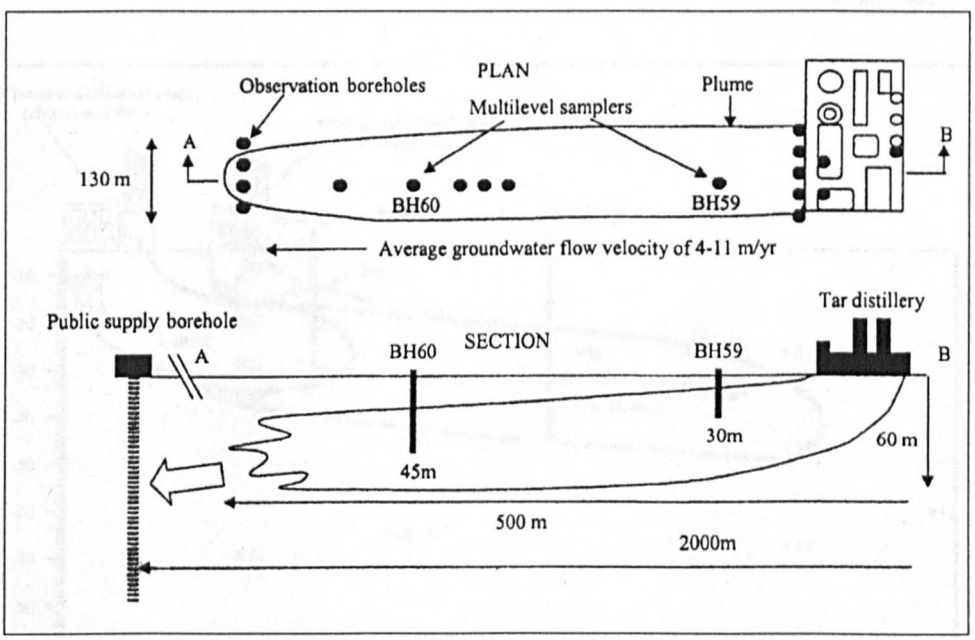



**Figure 1.7.** Schematic plan and cross section of the Four Ashes site, showing conventional observation boreholes, multilevel boreholes 59 and 60 (BH59 and BH60), and approximate location of plume (1 mg $L^{-1}$ phenol contour). Taken from Lerner *et al.* (2000).

**Table 1.4.** Organic compounds previously identified within the Four Ashes plume. Taken from Williams *et al.* (2001).

| Tar neutrals | Tar acids | Tar bases |
|---|---|---|
| Toluene | Phenol | Pyridine |
| Butyl acetate | *o*-Cresol | Picoline |
| Ethylbenzene | *m/p*-Cresols | 3/4-Methylpyridine |
| *o*-Xylene | 2,6-Xylenol | 4-Heptanone |
| *m/p*-Xylenes | 2-Ethylphenol | 2,6-Lutidine |
| Benzofuran | Ethylphenol | 2-Ethylpyridine |
| Benzonitrile | 2-Propylphenol | Dimethylpyridine |
| Methyl anisole | Ethylmethylphenol | 3-Ethylpyridine |
| C3-Benzenes | Ethylphenol Ethanone | Aniline |
| C4-Benzenes | C1-phenol | Trimethylpyridine |
| C5-Benzenes | 2,3,6-Trimethylphenol | Ethylmethypyridine |
| C6-Benzenes | sec-Butylphenol | Methylaniline |
| | Ethylbenzaldehyde | Dihydroindole |
| | C4-phenols | 2-Ethylaniline |
| | | Dimethyaniline |
| | | Isoquinoline |
| | | Propylpyridine |
| | | C4-pyridine |
| | | 2-vinylpyridine |
| | | 4-vinylpyridine |

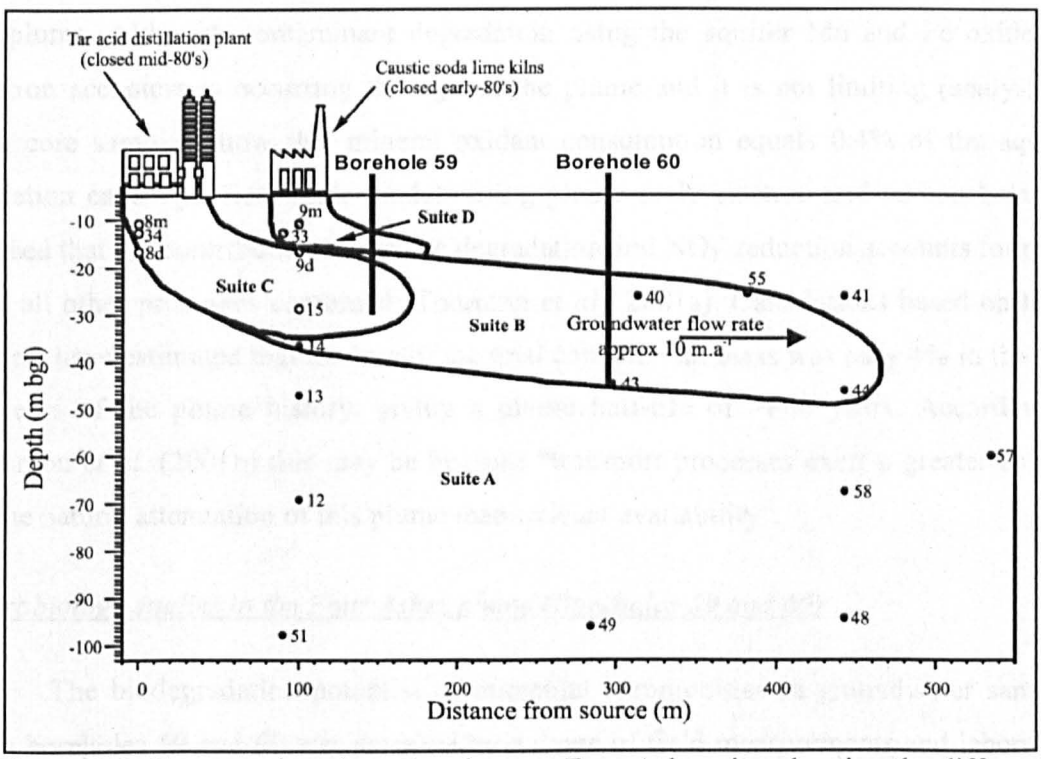



**Figure 1.8.** Cross section along the plume at Four Ashes site, showing the different pollutant compositions (Suites B, C, D) and the position of multilevel boreholes 59 and 60. The dots correspond to the positions of 20 conventional boreholes. Image adjusted from Williams *et al.* (2001).

The redox zonation and the natural attenuation processes within the plume were assessed by using data from the 22 conventional boreholes and the two multilevel boreholes (boreholes 59 and 60) as well as field scale modelling. The aquifer is characterised by low vertical dispersion, resulting in the formation of a very sharp upper plume boundary, where within a 2 m vertical interval, the concentration of the organic pollutants increases from 0 to 6000 mg $L^{-1}$. The lower boundary of the plume is generally broader, with a pollutant gradient of 4000 mg $L^{-1}$ within a vertical interval of 11 m (Williams *et al.*, 2001). Within these plume fringes dissolved oxygen and nitrate become rapidly depleted, suggesting that aerobic degradation and nitrate reduction are confined only to the outer thin layer of the plume fringes. Deeper in the plume, the elevated concentrations of reduced inorganic species ($Mn^{2+}$, $Fe^{2+}$, $S^{2-}$) and $\delta^{34}S\text{-}SO_4$ isotope fractions in comparison to the background groundwater indicate concomitant iron-, manganese- and sulphate-reducing processes, whereas accumulation of methane, acetate and $H_2$ indicate methanogenic and fermentative conditions, in particular near the source of the plume. Although contaminant degradation using the aquifer Mn and Fe oxides as electron acceptors is occurring throughout the plume and it is not limiting (analysis of rock core samples show that mineral oxidant consumption equals 0.4% of the aquifer oxidation capacity), field scale models using plume scale electron and carbon balances showed that the contribution of aerobic degradation and $NO_3^-$ reduction accounts for more than all other processes combined (Thornton *et al.*, 2001a). Calculations based on these models have estimated that the loss of the total contaminant mass was only 4% in the first 50-years of the plume history, giving a plume half-life of >800 years. According to Thornton *et al.* (2001b) this may be because "transport processes exert a greater control on the natural attenuation of this plume than oxidant availability".

*Microbiology studies in the Four Ashes plume (Boreholes 59 and 60)*

The biodegradation potential of microbial communities in groundwater samples from boreholes 59 and 60 was assessed by a range of field measurements and laboratory experiments. Increased $\delta^{13}C$ isotope fraction of the TDIC (total dissolved inorganic carbon) indicated that the excess inorganic carbon derived from *in situ* degradation processes (Thornton *et al*, 2001b). Moreover, enrichment in $\delta^{34}S\text{-}SO_4^{2-}$ and $\delta^{18}O\text{-}SO_4^{2-}$

indicated degradation by sulphate reduction (Spence *et al.*, 2001a) but the contribution of sulphate reduction to biodegradation was very limited because of sulphate exhaustion (in borehole 59) or because of phenol toxicity (at > 2000 mg $L^{-1}$ TPC, in borehole 60). Further laboratory experiments using the MPN (Most Probable Number count) method, API 20NE test strips, PCR targeting methanogenic genes (Pickup *et al.*, 2001) showed that microbial communities in most groundwater samples possessed sulphate reducing, denitrifying and methanogenic potential respectively. However, the metabolic activity (as assessed by API 20NE strips containing a range of substrates) and the degradation potential of $^{14}$C-phenol (assessed under both aerobic and anaerobic conditions) indicated that the microbial communities from the core of the plume exhibited low or no activity (Lerner *et al.*, 2000; Pickup *et al.*, 2001). The lowest microbial numbers were also observed in samples from the core of the plume (Pickup *et al.*, 2001). In addition, the microbial diversity within these samples was assessed by ERIC-PCR and TGGE (Pickup *et al.*, 2001) and the results indicated the presence of diverse microbial communities that varied with depth and no particular species dominated both boreholes 59 and 60.

## 1.6 Aim and objectives

The aim of this thesis is to investigate the effect of groundwater pollution on the diversity of planktonic and attached microbial communities. This aim is investigated firstly *in situ*, in a phenol polluted aquifer (Four Ashes) and then more specific hypotheses are investigated by studies of microbial consortia in laboratory microcosms.

Specific objectives of this study are:

- To investigate how the abundance and diversity of microbial communities differs across a geochemical pollutant gradient.
- To compare the microbial diversity of planktonic and attached communities under the same geochemical conditions.
- To examine how different phenol concentrations and shifts in phenol concentration affect microbial community composition and function in defined microcosms.

In Chapter 2, microbial abundance and diversity across a geochemical gradient in a phenol polluted aquifer (Four Ashes) are investigated using fluorescence microscopy and DGGE fingerprinting. The latter is also used to compare the structure of planktonic and attached microbial communities at one depth within the contaminant plume.

In Chapter 3, both culture-based and culture-independent approaches are used to compare the composition of planktonic and attached microbial communities from the same depth in the phenol polluted aquifer.

In Chapter 4, a number of bacterial strains isolated from the polluted aquifer are characterised functionally with regard to their ability to degrade or tolerate 2 different concentrations of phenol and to attach to sand grains.

In Chapter 5, laboratory microcosms (using sand columns) are established and inoculated with mixtures of characterised bacterial isolates to test (i) the effect of different phenol concentrations on planktonic and attached microbial community structure and (ii) the ability of bacteria to establish in pre-existing microbial communities.

This study should provide a greater understanding of the diversity of planktonic and attached microbial communities in polluted environments and the impact of organic pollution on their composition.

# Chapter 2

# Microbial abundance and diversity within the Four Ashes aquifer

## 2.1 Introduction

There are a number of studies that have investigated microbial diversity in polluted aquifers, usually in comparison to background uncontaminated groundwater. By using molecular based techniques such as 16S rRNA gene cloning and DNA fingerprinting methods, they often demonstrated that microbial community composition or structure differed at different parts of the aquifer (e.g. Rooney-Varga *et al.*, 1999; Röling *et al.*, 2001; Feris *et al.*, 2004; Haack *et al.*, 2004; Fahy *et al.*, 2005; Takahata *et al.*, 2006; Alfreider and Vogt, 2007; Winderl *et al.*, 2008).

Due to technical constraints (e.g. long-screened monitoring wells), most of these studies have compared the microbial diversity in groundwater samples taken from different parts of the aquifer/plume using a number of different wells/boreholes rather than sampling at vertical transect through a particular part of a pollutant plume. This often results in failure to describe efficiently the structure of microbial communities across plume boundaries (= plume fringes). Within plume fringes it has been shown that natural attenuation is higher (Thornton *et al.*, 2001b; Prommer *et al.*, 2006; Bauer *et al.*, 2008), mainly because this is where pollutants and electron acceptors with high energy yields (dissolved oxygen, nitrate) mix. In recent years it has become clear that within polluted aquifers geochemical conditions (Lendvay *et al.*, 1998; Cozzarelli *et al.*, 1999; Anneser *et al.*, 2008) and microbial physiological types (Smith *et al.*, 1991; Bekins *et al.*, 1999; Winderl *et al.*, 2008) can vary on sub-meter scales. Therefore, many studies have stressed the importance of closely spaced vertical sampling in delineating chemical and microbiological gradients in groundwater studies (Smith *et al.*, 1991; Wilson *et al.*, 2004; Kappler *et al.*, 2005), something which is achieved by multilevel sampling wells/boreholes with vertical distance between ports of 1 m (Thornton *et al.*, 2001b) or lower (0.125 m, Prommer *et al.*, 2006; 0.03 m, Anneser *et al.*, 2008).

Furthermore, most of the studies on subsurface environments (including polluted aquifers) focus on the characterisation of planktonic microbial communities only, because groundwater samples can be collected more easily than sediment/core samples and changes at the same sampling point can be studied over time. However, it is well reported that in aquifers the majority of microbial biomass is within the attached phase and only a

small proportion is in the planktonic (free-living) phase (Hazen *et al.*, 1991; Holm *et al.*, 1992; Alfreider *et al.*, 1997). Therefore, in order to overcome the technical difficulties of core drilling or sediment sampling, a number of studies have taken the approach of suspending different substrata within the aquifer over a period of time and analysing them (e.g. Hirsch and Rades-Rohkohl, 1990; Holm *et al.*, 1992; Alfreider *et al.*, 1997; Lehman *et al.*, 2004; Hendrickx *et al.*, 2005).

The microbiological studies at the Four Ashes site in particular (Pickup *et al.*, 2001) showed that microbial diversity differed within different areas of the plume. However, these studies were performed with groundwater samples from various plume depths (e.g. from 5, 8, 10, 17, 23, and 30 mbgl in Borehole 59), omitting the plume boundaries (fringe of the plume), where microbial activity is expected to be higher and account for the larger proportion of biodegradation in the Four Ashes plume (Thornton *et al.*, 2001b). Moreover, although with their study Pickup *et al.* (2001) indicated the presence of functionally diverse microbial communities with the potential to degrade phenol, they recognised that groundwater does not necessarily reflect microbial densities and activities in the solid aquifer matrix and therefore "*some caution should be exercised when extrapolating results from pumped water samples to core samples unless comparative experiments have been carried out*". So, it becomes obvious that very little is known about the microbial communities inhabiting the Four Ashes plume.

Therefore the aims of this study are (i) to investigate the microbial community structure and abundance across a geochemical gradient in the Four Ashes aquifer and (ii) to compare microbial community structure between planktonic and attached communities. Due to technical constraints, the second aim will be explored by using suspended material (sand) in one plume depth only. Specific hypotheses are:

- Microbial abundance and activity will be higher in the presence of organic contaminants due to carbon availability.
- Microbial diversity of planktonic communities will differ across the geochemical gradient.
- Microbial structure will differ between planktonic and attached communities.

## 2.2 Methods

### 2.2.1 Sampling from the Four Ashes site

*Groundwater samples*

Groundwater samples from various depths in the aquifer were pumped from multilevel boreholes 59 and 60 in June 2005 and in August 2006. The depths sampled for this study are shown in Table 2.1.

**Table 2.1.** Depths that groundwater samples were pumped from.

| Borehole | Year | Sampled depth (mbgl*) |
|---|---|---|
| 59 | 2005 | 6, 8, 9, 10, 11, 30 |
| | 2006 | 6, 8, 9, 10, 11, 12, 30 |
| 60 | 2005 | 12, 15, 18, 19, 20, 44 |
| | 2006 | 12, 15, 16, 17, 18, 44 |

*mbgl = meters below ground level

Prior to sample collection, 2 L of groundwater was purged from each depth using a vacuum pump in order to remove stagnant water. Then, 2 L of groundwater was collected from each sampling depth using a multiple-head peristaltic pump into 2 x 1 L sterilised, $N_2$-flushed amber Winchester glass bottles (Fisher Scientific UK Ltd, Loughborough, UK) and filled up to the brim. At the same time, an additional 60 ml sample from each depth was filtered through a 0.2 $\mu$m pore size syringe filter (Nalge Nunc International, Rochester, NY, USA) and aliquoted into three 20ml scintillation vials (Sarstedt AG & Co, Nümbrecht, Germany) for chemical analyses. All bottles and vials were transferred on ice and eventually stored at 4°C and processed within 6 days as follows.

From each sampling depth, one of the 2 Winchester bottles was inverted repeatedly and shaken vigorously, to ensure that the sample was mixed. Then, the lid was opened and groundwater sub-samples were taken for further analyses:

- For direct total cell counts (2.2.3), 20 ml of groundwater from each sampling depth was fixed with 1.150 ml of 36.5% formaldehyde solution

(Sigma-Aldrich, Inc, St Louis, MO, USA) so that the final solution contained 2% (v/v) formaldehyde.

- For frozen glycerol stocks, 800 μl of groundwater was mixed with 200 μl of 80% (v/v) sterilised glycerol solution. Samples were vortexed and then frozen at -80 °C.
- For DNA extractions from the planktonic microbial communities, 150 ml of groundwater was centrifuged in 250 ml polypropylene Beckman centrifuge bottles (Beckman Instruments, Inc., Fullerton, CA, USA) at 22,095 x g (12,000 rpm) for 1h at 4°C using a Beckman Avanti™ J-25I centrifuge with a JA-14 rotor (Beckman Coulter, Inc., Fullerton, CA, USA). The pellet was resuspended in 1.5 ml of nuclease-free $H_2O$ (Ambion, Inc., Austin, TX, USA) and transferred into a sterile 1.5 ml microtube (Sarstedt AG & Co, Nümbrecht, Germany). The cells were spun down once more for 10 min at 13,000 x g (12,000 rpm) at 4°C using a refrigerated Hawk 15/05 centrifuge (Sanyo Electric Co., Ltd., Osaka, Japan). The supernatant was removed and the pellet was kept at -20°C for the extraction of DNA. Three replicate samples were prepared from each depth.

*Sand samples*

After the groundwater sampling in 2005, 3 sand bags were left at the bottom of Borehole 59, at 30 mbgl. Each sand bag contained 50 g of sand (purified by acid, 0.1 – 0.3 mm, 50 – 150 mesh, from BDH: VWR International Ltd, Lutterworth, UK), wrapped with 2 layers of 100 micron polyester multiwoven mesh (Plastok, Ltd., Birkenhead, UK). The three sand bags were placed in a punched 500 ml plastic bottle that would allow the groundwater to flow freely through them. The bottle containing the 3 sand bags was autoclaved and transferred in a sterile box before being suspended with a 30.2 m rope into the aquifer, in the centre of the 30 m screen of Borehole 59.

The sand bags were retrieved anaerobically from Borehole 59, during the 2006 sampling. Anaerobic sampling was achieved by purging the groundwater at depth 30 with

$N_2$ gas while having the head of the borehole sealed with a plastic glove-bag (Model X-27-27; $I^2R$, Instruments for Research & Industry Inc., Cheltenham, PA, USA) as shown in Fig. 2.1. Inside the glove-box bag, the bottle containing the sand bags was transferred into a sterile zip bag and stored at 4°C. Within 3 days the sand bags were opened in a laminar flow cabinet and from each sand bag, wooden swabs were used to inoculate R2A agar plates (OXOID Ltd., Basingstoke, UK) and frozen glycerol stocks were prepared in tubes containing 0.2 g of sand, 800 µl of AB minimal medium (Table 2.2; Heydorn *et al.*, 2000) and 200 µl of 80% glycerol. The remaining sand was stored in 50 ml plastic tubes (Sarstedt, Germany) at -20°C.

**Table 2.2** The composition of AB minimal medium.

| **AB medium** | **g $L^{-1}$** | **Molarity in the AB medium (mM)** |
|---|---|---|
| $Na_2HPO_4.2H_2O$ | 6 | 33.7 |
| $KH_2PO_4$ | 3 | 22.1 |
| $(NH_4)_2SO_4$ | 2 | 14.7 |
| NaCl | 3 | 51.7 |
| $CaCl_2$ | 0.011 | 0.1 |
| $MgCl_2.6H_2O$ | 0.203 | 1 |
| pH was adjusted to 6.5 & 1 ml of the trace elements solution (below) was added | | |
| **Trace elements solution** | **mg $L^{-1}$** | **Final molatity in the AB medium (µM)** |
| $CaSO_4.2H_2O$ | 200 | 1163 |
| $FeSO_4.7H_2O$ | 200 | 719 |
| $MnSO_4.H_2O$ | 20 | 118 |
| $CuSO_4$ | 20 | 126 |
| $ZnSO_4.7H_2O$ | 20 | 70 |
| $CoSO_4.7H_2O$ | 10 | 36 |
| $NaMoO_4.H_2O$ | 10 | 50 |
| $H_3BO_3$ | 5 | 80 |

**Figure 2.1.** Sand bags were retrieved anaerobically from the bottom of Borehole 59, during the 2006 sampling.

### 2.2.2 Chemical analyses of the groundwater samples

All chemical analyses of the groundwater samples were performed by Dr Steven F. Thornton and his colleagues at the Department of Civil and Structural Engineering of the University of Sheffield, as described previously (Thornton *et al.*, 2001b).

Triplicate samples from each depth were analysed for the inorganic and organic compounds that are shown in Table 2.3. Analysis of inorganic compounds was performed by ion chromatography using a Dionex DX-120 system (Dionex Ltd, Camberley, UK). The Dionex system is connected to a Dionex AS40 Automated Sampler, a LC30 Chromatography Oven, AD20 Absorbance Detector, GP50 Gradient Pump, PC10 Pneumatic Controller. The concentration of all the major organic pollutants and some of the most important organic metabolites was determined by High-Pressure Liquid Chromatography, using a Perkin Elmer Series 200 HPLC System (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA, USA) which comprised of an autosampler, two

micro pumps, a vacuum degasser and a UV/VIS detector. All the sub-systems were connected through a 600 Series LINK chromatography interface to a PC workstation. One ml of the filtered groundwater sample was transferred into small glass vials which were loaded onto the autosampler rack alongside appropriate standards. Then, 10 $\mu$l of the samples was pumped at 1 ml $h^{-1}$ in a 50% (v/v) acetonitrile/water solution through an Alltech® Allsphere™ ODS-2 5$\mu$m (internal diameter x length = 4.6 mm x 250 mm) HPLC column (W. R. Grace & Co.-Conn., Deerfield, IL, USA) and the organic components were detected at 280 nm.

**Table 2.3.** Inorganic and organic compounds that were analysed in the groundwater samples from the Four Ashes site.

| **Inorganic compounds** | | **Major organic pollutants** | **Organic metabolites** |
|---|---|---|---|
| Sodium | Nitrate | Phenol | Catechol* |
| Calcium | Nitrite | *o*-cresol | 4-hydroxybenzoic acid |
| Potassium | Ammonium | *m/p*-cresol | 4-hydroxybenzoic alcohol |
| Magnesium | Phosphate | 3,4 xylenol* | 4-hydroxybenzaldehyde |
| Fluoride | Sulphate | 3,5 xylenol* | Acetate** |
| Chloride | | 2,3 xylenol* | |
| Bromide | | 2,4/2,5 xylenol* | |
| | | 2,6 xylenol* | |

*= 2005 samples only, **=2006 samples only

### 2.2.3 Determination of total cell numbers by acridine orange staining

Abundance of microbial cells in the groundwater samples was measured by direct total cell counts following staining with acridine orange (Sigma-Aldrich, Inc, St Louis, MO, USA), based on the procedure described by Fry (1990) and Pickup *et al.* (2001).

For the staining and the filtration of the groundwater samples, a Whatman filtration system (Whatman International, Maidstone, UK) was used. The filtration system (Fig. 2.2) comprised of a 250 ml collection conical flask, a 25 mm diameter glass filter holder fitted on a conical flask stopper, and a 25 ml cylindrical reservoir which was clamped onto the filter holder. Before staining, a black (0.2 μm pore size) Nuclepore® polycarbonate membrane (Whatman, UK) was placed on the top of the glass filter holder and the filtration system was connected to a Laboport mini diaphragm N86 KN.18 vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) as shown in Fig. 2.2.

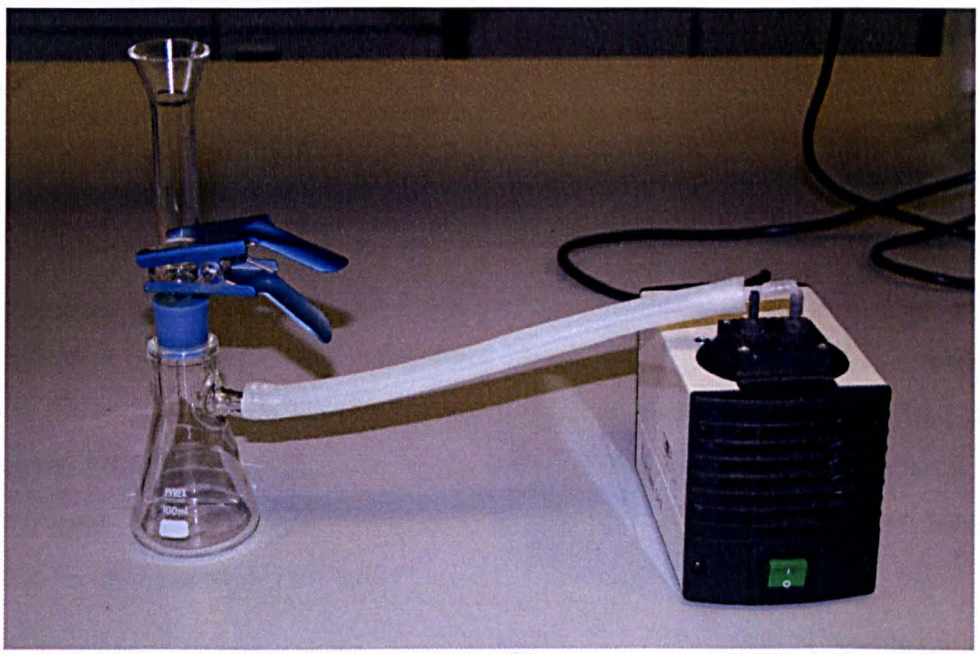


**Figure 2.2** The Whatman filtration system connected to the Laboport mini vacuum pump that was used for acridine orange staining and filtration of the groundwater samples taken from the Four Ashes site.

Five hundred $\mu$l of formaldehyde fixed groundwater (described in 2.2.1; 10 ml for samples with low bacterial numbers) was mixed with 9.5 ml of 2% (v/v) formaldehyde and 50 $\mu$l of acridine orange stock solution (125 mg $L^{-1}$, prepared in UHQ $H_2O$) in the 25 ml cylinder of the filtration system and incubated for 3 minutes in the dark (the cylinder was covered with foil). Following staining, the vacuum pump was switched on so that the solution was drawn through the membrane, which was washed in the same way with an additional 10 ml of 2% (v/v) formaldehyde. The membrane was then transferred to a microscope slide, and 10 $\mu$l of UHQ $H_2O$ and a 22 x 64 mm coverslip were placed on the top. All formaldehyde solutions and UHQ $H_2O$ used during this procedure had been previously filtered through a 0.2 $\mu$m pore size cellulose acetate syringe filter (Nalge Nunc International, Rochester, NY, USA) to remove particulates.

The stained cells were viewed under an epifluorescence Olympus BX50WI microscope (Olympus Optical Co., Ltd, London, UK) using either 40x (40x/0.55 SLCPlanFl, Olympus, Japan) or 100x (100x/1.30 Oil Ph3, UPlanFl, Olympus, Japan) objectives. One drop of UV-transparent, fluorescence-free immersion oil (Sigma-Aldrich Company, Ltd, Poole, UK) was used between the coverslip and the 100x objective. Excitation in the blue region (470 nm) was provided by a xenon ebx75 lamp (Leistungselektronik Jena, GmbH, Jena, Germany) and emission was detected in the green at 530 nm using a super wide band (U-MSWB) fluorescence filter cube (Olympus, UK). For each membrane, multiple images of view were taken using a CoolSNAP K4 camera (Roper Scientific, GmbH, Ottobrunn, Germany). The mean number of cells in 40 different fields of view or after 2000 cells counted (whichever was reached first), as well as the standard deviation from the mean were calculated for each membrane. These measurements were ultimately expressed in cells per ml of groundwater sample as described by the following equation:

$$\text{Total cell counts (cell ml}^{-1}) = C \times \frac{1\text{ml}}{\text{Volume of sample filtered}} \times \frac{\text{Area of membrane}}{\text{Area of field of view}},$$

where $C$ is the mean number of cells per field of view.

### 2.2.4 DNA extraction from the groundwater and sand samples taken from Borehole 59 in 2006.

*DNA extraction using the MoBio UltraClean™ Microbial DNA Kit*

Total genomic DNA was extracted from groundwater and sand samples taken from Borehole 59 in 2006 using the MoBio UltraClean™ Microbial DNA Kit (Mo Bio Laboratories, Inc, Carlsbad, CA, USA), following the manufacturer's protocol including the additional optional heating step (for 10 min at 65°C). The kit lyses bacterial cells and cellular components by combining mechanical action (bead-beating) and chemical lysis with guadinine thiocyanate and Sodium Dodecyl Sulfate (SDS).

The frozen bacterial pellets from the centrifuged groundwater samples were thawed, resuspended in 300 µl of MicroBead solution (Guanidine Thiocyanate) and transferred into the MicroBead tubes which contained quartz beads. After the addition of 50 µl of solution MD1 (Sodium Dodecyl Sulfate), the MicroBead tubes were inverted gently a few times and left in a 65°C water bath for 10 minutes. Then the Microbeads were vortexed at full speed for 10 min using the 13000-V1 MoBio Vortex Adapter tube holder and centrifuged at 10,000 x g for 30 sec using an Eppendorf 5417C microcentrifuge (Eppendorf UK Ltd, Cambridge, UK). The supernatant was transferred into new 1.9 ml microcentrifuge tubes (provided with the kit), in which 100 µl of solution MD2 (acetate) was added. The new tubes were vortexed for 5 seconds, incubated at 4°C for 5 min and centrifuged at 10,000 x g for 1 min. About 450 µl of the supernatant was transferred into new microtubes, in which 900 µl of solution MD3 (aqueous solution of Guanidine HCl and Isopropanol) was added. The tubes were vortexed for 5 sec and then 700 µl of their content was loaded into spin filters, which were centrifuged at 10,000 x g for 30 sec. The flow through was decanted and two more loadings were performed, before the bound DNA was washed once with 300 µl of solution MD4 (ethanol). An additional centrifugation step for 1 min was performed, before the spin filter was placed in a new 1.9 tube. The bound DNA was eluted in 50 µl of solution MD5 (Tris (hydroxymethyl) aminomethane / Hydrochloride), which was added to the centre of the filter membrane. The extracted DNA was stored at –20°C.

For the sand samples the same procedure was followed, with the exception that DNA was extracted from 0.2 g of sand which was weighed into the bead beating tube, before the addition of the MicroBead (Guanidine Thiocyanate) and the MD1 (Sodium Dodecyl Sulfate) solutions.

*DNA extraction using hexadecyltrimethylammonium bromide (CTAB)*

A second extraction method was used for the extraction of DNA from one replicate of the 2006 groundwater samples. This method was based on Griffiths *et al.* (2000) and it combined mechanical action (bead-beating) and chemical lysis. The centrifuged microbial pellets were resuspended in 0.5 ml of CTAB extraction buffer, containing equal volumes of a 10% (w/v) CTAB (hexadecyltrimethylammonium bromide, Sigma-Aldrich Company, Ltd, Poole, UK) in 0.7M NaCl solution and 240mM potassium phosphate buffer at pH 8 (made with 22.56 ml of 1M $K_2HPO_4$ and 1.44 ml of 1M $KH_2PO_4$). The resuspended cells were transferred into 2 ml microtubes (Sarstedt, Germany) which contained 1 g of sterilised (overnight at 160°C) 106 $\mu$m glass beads (Sigma-Aldrich Company, Ltd, Poole, UK). Then, 0.5 ml of a phenol-chloroform-isoamyl alcohol (25:24:1) solution was added and the microtubes were placed in an 8000M Mixer/Mill grinding machine (Glen Creston Ltd, Stanmore, Middlesex, UK) for 1 minute. The upper aqueous phase was removed and placed in a 1.5ml microtube, to which 0.5 ml of chloroform-isoamyl alcohol (24:1) was added. These tubes were then shaken vigorously and centrifuged at 13,000 g (12,000 rpm) for 10 min at 4°C. The new upper aqueous solution was then transferred into a new 1.5 ml microtube, 0.6 volumes of propan-2-ol was added and mixed, and samples were incubated for 2 hours at room temperature, followed by centrifugation for 10 minutes at 13,000 g (12,000 rpm) at 4°C. The supernatant was decanted and the pellets were washed by being resuspended in 150 $\mu$l of ice-cold 70% (v/v) ethanol and centrifuged again for 10 minutes. The ethanol was removed and the pellets were air-dried for 10 minutes before eventually being resuspended in 50 $\mu$l nuclease-free water (Ambion, Inc., Austin, TX, USA).

For the sand samples the same procedure was followed, with the exemptions that:

- DNA was extracted from 5g of sand and no glass beads were added.

- Extractions were done in 50ml tubes (Sarstedt, Germany) instead of 2 ml/1.5 ml microtubes.
- The volumes of the solutions used were 10-fold higher and centrifugations were performed for 20 min at 6,000 rpm (3,904 g) at 4°C using a refrigerated Hermle Z 300 K centrifuge (HERMLE Labortechnik, GmbH, Wehingen, Germany) with a 220.97 V02 rotor (6 x 50 ml).

### 2.2.5 DNA quantification using PicoGreen

Initially, the DNA from the environmental samples was quantified using a Shimadzu UV-2101PC, UV-VIS Scanning Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 260 nm. However, because the $OD_{260}$ readings were close to the detection limits of the device ($OD_{260}$ readings below 0.002, which corresponded to DNA concentration lower than 20 ng $\mu l^{-1}$), a fluorimetric method using Quant-iT™ PicoGreen® dsDNA reagent (Molecular Probes, Inc., Eugene, OR, USA) as the nucleic acid stain was developed for the quantification of the extracted DNA from the environmental samples.

A 200-fold dilution of PicoGreen in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was prepared as the PicoGreen working solution. The TE buffer was previously filtered through a 0.2 $\mu$m cellulose acetate syringe filter (Nalge Nunc International, Rochester, NY, USA) to remove particles that could interfere with the fluorimeter readings. Samples and standards were aliquoted into a black 96 well microplate (BMG LABTECH, Ltd, Aylesbury, UK). In each well, 98 $\mu$l of TE buffer, 2 $\mu$l of DNA standard or DNA extract, and 100 $\mu$l of PicoGreen working solution were loaded. The DNA standards were prepared from Herring sperm DNA and ranged from 0 to 100 ng $\mu l^{-1}$ of DNA. The plate was incubated for 5 min at room temperature in the dark, before the fluorescence from each well was measured with a FluoStar Optima (BMG LABTECH, Ltd, Aylesbury, UK) spectrofluorimeter (excitation at 485 nm, fluorescence emission measured at 545 nm). The concentration of the extracted DNA was calculated using a standard curve. Fig. 2.3 shows an example of a standard curve made with DNA standards

ranging from 0 to 1 ng $\mu l^{-1}$. The fluorimetric method could quantify accurately DNA concentrations above 0.05 ng $\mu l^{-1}$, which was adequate for the purposes of this study.

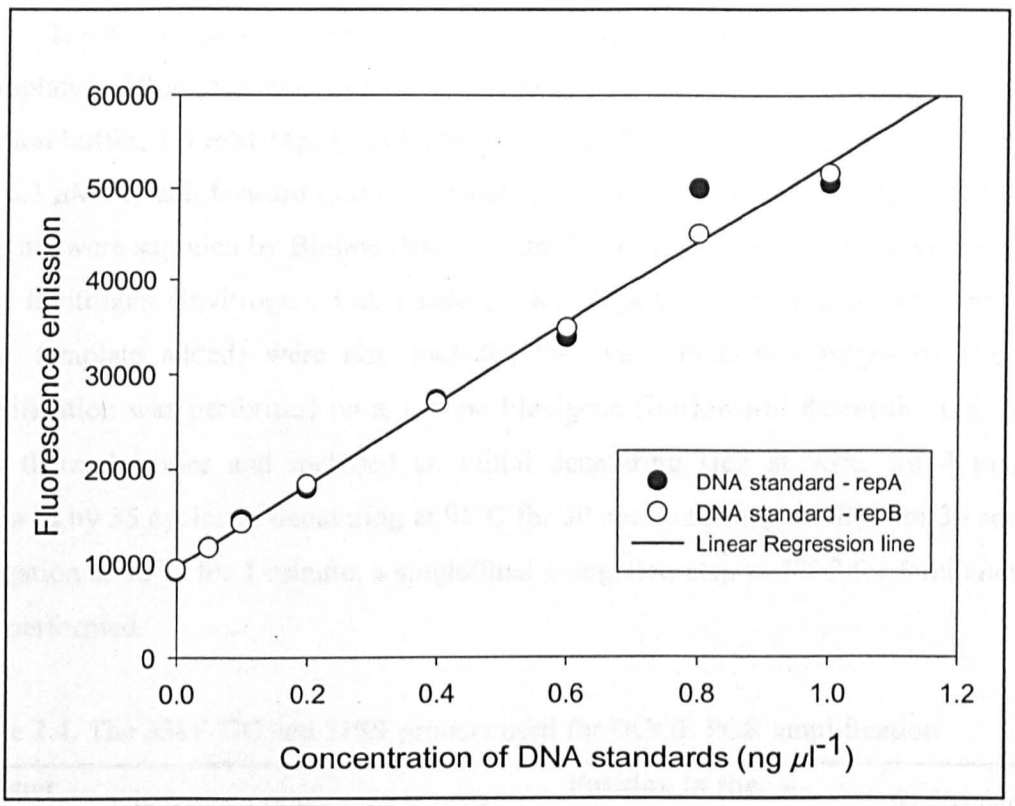



**Figure 2.3.** An example of a standard curve produced by plotting the fluorescence emissions of duplicate 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ng $\mu l^{-1}$ DNA standards, illustrating the detection limits of the developed PicoGreen fluorimetric method for DNA quantification.

### 2.2.6 Amplification of 16S rRNA gene for Denaturing Gradient Gel Electrophoresis (DGGE) analysis

Three nanograms of extracted DNA from the environmental samples was used as a template in 50 $\mu$l PCR reactions containing 0.75 units of Taq DNA polymerase, 1 x $NH_4$ reaction buffer, 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.3 $\mu$M of each forward (338F-GC) and reverse (519R) primers (Table 2.4). All PCR reagents were supplied by Bioline (Bioline, Ltd, London, UK) and primers were obtained from Invitrogen (Invitrogen, Ltd, Paisley, UK). A positive and a negative control (no DNA template added) were also included for every PCR mix prepared. The PCR amplification was performed on a Techne Flexigene (Barloworld Scientific, Ltd, Stone, UK) thermal cycler and included an initial denaturing step at 95°C for 4 minutes, followed by 35 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 1 minute; a single/final elongation step at 72°C for 6 minutes was also performed.

**Table 2.4.** The 338F-GC and 519R primers used for DGGE PCR amplification

| Primer name | Nucleotide sequence (5' – 3') | Position in the 16S rRNA of E. coli | Reference |
|---|---|---|---|
| 338F-GC | CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGG ACTCCTACGGGAGGCAGC | 338 – 355 (+ 40 bp GC clamp) | Whiteley and Bailey, 2000 |
| 519R | ATTACCGCGGCTGCTGG | 519 - 534 | Muyzer *et al.*, 1993 |

Following PCR, 5 $\mu$l from each PCR product was mixed with 2 $\mu$l of 5x gel loading buffer (Sigma-Aldrich Company, Ltd, Poole, UK) and subjected to electrophoresis (80V for 50 min) in a 2% (w/v) agarose-TAE gel (40mM Tris base, 20mM acetic acid, 1 mM EDTA, pH 8). DNA molecular weight standards were run on each gel (Hyperladder IV, Bioline, London, UK; Appendix 1). Following electrophoresis, gels were stained for 20 min with 0.005% (v/v) ethidium bromide-TAE buffer and visualised on an Epi Chem II Darkroom (UVP Lab products, Cambridge, UK), using the fitted ethidium bromide filter.

*Avoidance of contamination in PCR reactions*

Although it is rarely mentioned in the literature, internet forums and personal communication with other molecular biologists reveal that it is not uncommon to have problems with PCR contamination when using universal bacterial primers. Rigorous procedures were developed to eliminate contamination of PCR reactions, which is defined here as amplified PCR products of the correct size present in the negative control.

Numerous combinations of procedures were tried to ensure that contamination was avoided in PCR reactions. These procedures included:

- purchasing new laboratory equipment (pipettors, laboratory coat) that were used exclusively for pre-PCR reagent handling
- re-locating to a laboratory in which post-PCR reactions were not handled
- working in a class II laminar flow cabinet that was exposed to UV light when not in use and using barrier tips (source) for all pipetting
- exposing all PCR reagents to UV light in a BLX-E254 crosslinker (Vilber Lourmat, France)

Although these procedures reduced the frequency of contamination, amplification of PCR products in negative controls was still common. The source of this contamination was traced to be via exposure of reagents to the air as there was a strong correlation between the number of times a reagent tube was opened and the occurrence of contamination. Eventually, contamination was eliminated by incorporating the following procedures:

- All reagents and primers were kept in small aliquots (for example enough for the preparation of forty 50 $\mu$l PCR reactions)
- All pipetting was performed near a Bunsen burner flame.
- Reagent mixes were prepared for a maximum of 10 reactions at a time.

### 2.2.7 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE analysis was performed by loading 10 μl of PCR amplified 16S rRNA gene fragments mixed with 5 μl of 5x DNA loading buffer (Bioline, London, UK) into a 16cm x 16cm 10% (v/v) acrylamide gel. The 30% to 60% parallel denaturing gradient was formed by mixing 15 ml from each of the denaturing solutions shown in Table 2.5 and polymerised by adding 100 μl of 10% (w/v) ammonium persulfate in UHQ $H_2O$ and 16.5 μl of TEMED (N,N,N',N'-tetra-methyl-ethylenediamine) beforehand in each denaturing solution.

**Table 2.5.** The composition of the denaturing solutions used for DGGE analysis.

| | Denaturing solutions | |
|---|---|---|
| **Components** | **High – 60%** | **Low – 30%** |
| Urea (g) | 25.2 | 12.6 |
| Formamide (ml) | 24 | 12 |
| 30% Acrylamide* (ml) | 33.3 | 33.3 |
| 50x TAE Buffer (ml) | 2 | 2 |
| + UHQ $H_2O$ (ml) | up to 100 ml | up to 100 ml |

*30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio)

The gel was electrophorised at 62°C at a constant voltage of 80V for 16h in a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK) which contained 7L of TAE-buffer (40mM Tris base, 20mM acetic acid, 1 mM EDTA, pH 8). Following electrophoresis, gels were stained with a 0.0001% (v/v) SYBR® Gold in TAE-buffer solution for 20 min in the dark, before being visualised on an Epi Chem II Darkroom (UVP Lab products, Cambridge, UK), using the fitted SYBR® Gold CY™ 3 filter.

SYBR® Gold was obtained from Invitrogen (Invitrogen, Ltd, Paisley, UK); TEMED from BioRad (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK); 30% Acrylamide (Proto Gel) from Geneflow (Geneflow, Ltd, Fradley, UK); ammonium persulfate and formamide from Sigma-Aldrich; Tris-base ultrapure from Melford (Melford Laboratories, Ltd, Ipswich, UK).

The optimum gradient of denaturant was determined in a preliminary experiment where PCR amplicons of an environmental sample (59/11 from the 2005 groundwater samples) were electrophorised in a perpendicular DGGE gel of 10% to 80% denaturing gradient (Fig. 2.4). The 30% to 60% denaturing gradient was chosen because within this gradient all bands were separated clearly from each other.

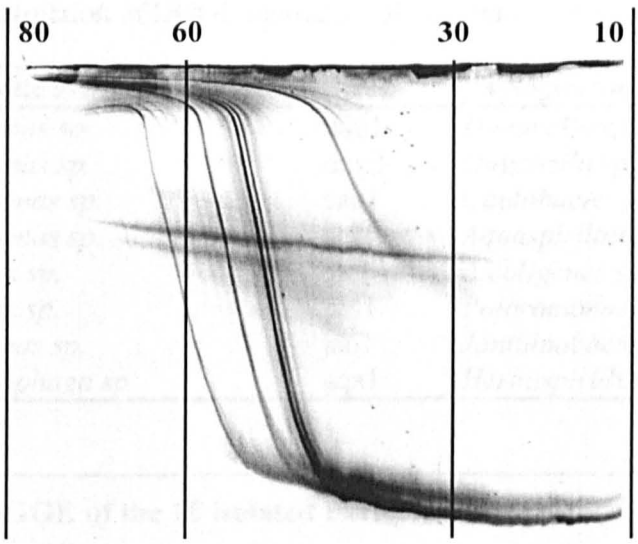



**Figure 2.4.** A perpendicular 10% to 80% DGGE gel that was used in order to determine the optimum denaturing gradient for DGGE analysis (30% to 60%). The lines indicate the corresponding denaturing gradient.

### 2.2.8 DGGE markers

From the groundwater samples taken from different depths in Borehole 59 in 2005, 16 different bacterial strains (Table 2.6) were isolated aerobically on R2A agar by Dr David Elliott who was a post-doctoral researcher of the Cell-Mineral Interface programme of the University of Sheffield. Genomic DNA was extracted with the CTAB method described in 2.2.4 and PCR amplification for DGGE fingerprinting was performed as described in 2.2.6. The amplified 16S rRNA gene fragments were run independently (4 $\mu$l of PCR products mixed with 2 $\mu$l of 5x DNA loading buffer) in a

DGGE gel and eleven out of the 16 isolated strains produced single bands that migrated to different distances in the DGGE gel (Fig. 2.5.a). These eleven strains were then divided into two groups and mixed in order to create two DGGE marker sets (Fig. 2.5.b); DGGE marker set 1 consisted of pse2, acv1, gla1, sph1, cau1, alc1 whereas DGGE marker set 2 consisted of pse1, var1, sph2, hyd1, pol1.

**Table 2.6.** The 16 bacterial strains isolated from different groundwater depths sampled from Borehole 59 in 2005 and their phylogenetic affiliations. A selection of these strains was used for the construction of DGGE markers. All 16 strains were provided by Dr David Elliott.

| Strain | Phylogenetic affiliation | Strain | Phylogenetic affiliation |
|---|---|---|---|
| pse1 | *Pseudomonas sp.* | dug1 | *Duganella sp.* |
| pse2 | *Pseudomonas sp.* | dug2 | *Duganella sp.* |
| sph1 | *Sphingomonas sp.* | cau1 | *Caulobacter sp.* |
| sph2 | *Sphingomonas sp.* | col1 | *Aquaspirillum sp.* |
| acv1 | *Acidovorax sp.* | alc1 | *Alcaligenes sp.* |
| var1 | *Variovorax sp.* | pol1 | *Polaromonas sp.* |
| gla1 | *Polaromonas sp.* | jan1 | *Janthinobacterium sp.* |
| hyd1 | *Hydrogenophaga sp.* | aqa1 | *Herbaspirillum sp.* |

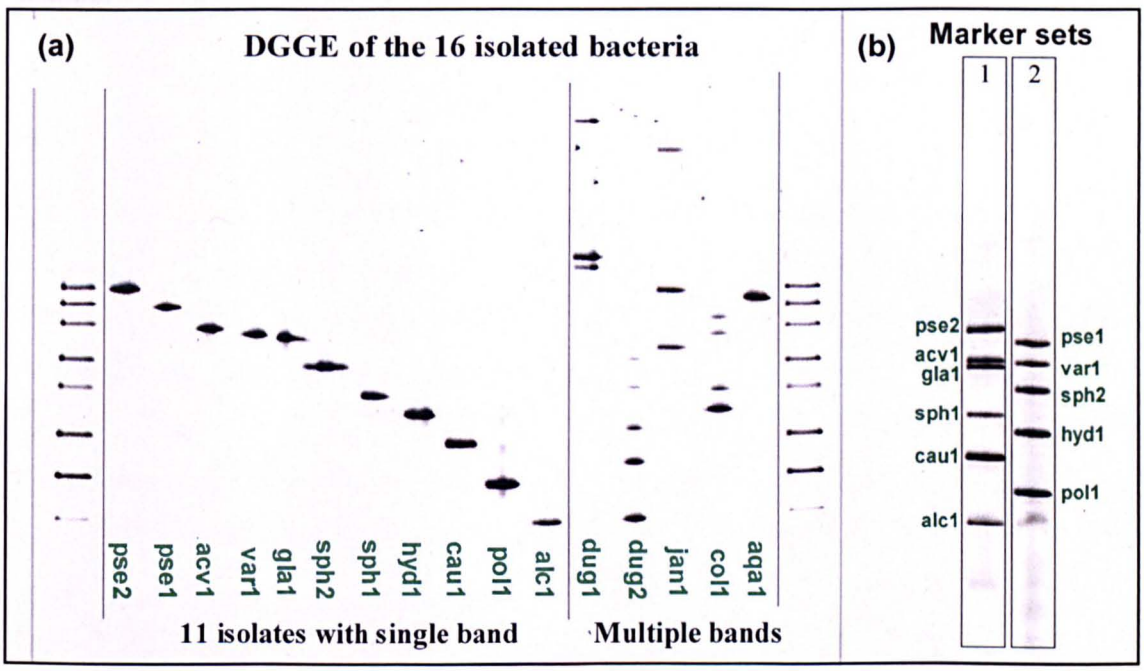



**Figure 2.5. a.** DGGE gel of 16 isolated bacterial strains from the 2005 groundwater samples from borehole 59 and **b.** the two DGGE marker sets that were created by using eleven of these strains.

### 2.2.9 Cluster analysis of DGGE profiles

The DGGE image (Fig. 2.14) was analysed with the LabWorks™ Image Acquisition and Analysis software (UVP, Inc., Upland, CA, USA). Bands that have migrated to the same distance in the gel were identified using the following settings: minimum band weight 1%, band numbers represent rows of equal Rf ± 0.50 % and minimum band separation at ± 0.10% of Rf. Alignment of the bands was also assisted by adding slant lines that joined the DGGE markers. In cases that the software falsely recognised smear or spots as bands, they were removed manually. The results were presented as a table, where the relative distance of each band in the gel (in a scale of 0 to 1) and its maximum OD (or absolute integrated OD) were shown.

This table was used as an input file in the Primer v6 software package (PRIMER-E Ltd, Ivybridge, UK). The data was transformed to presence/absence and the resemblance matrixes were calculated using the S17 Bray Curtis similarity resemblance measure. The hierarchical cluster analysis was performed using the average cluster method.

## 2.3 Results

### 2.3.1 The geochemistry across two pollutant gradients at the Four Ashes site

The geochemistry across two pollutant gradients at the Four Ashes site was examined by pumping groundwater from different depths from boreholes 59 and 60 (see 2.2.1), which are located 150 and 350 m away from the source of the plume respectively. Groundwater samples were taken in two consecutive years (2005 and 2006) and chemical analyses were performed for a range of organic and inorganic compounds, as described in 2.2.2. The most important results are summarised in Fig. 2.6 (Bh 59) and in Fig. 2.7 (Bh 60), which show how the concentration of the principal organic pollutants of the plume (phenol, *o-/m-/p*-cresols and xylenols) and six of the analysed inorganic compounds (nitrate, sulphate, magnesium, calcium, sodium and chloride) varied with depth in 2005 (grey symbols) and in 2006 (black symbols). Further details about all the analysed compounds can be found in Appendix 2. Since the 2006 samples were not analysed for xylenols, the Total Phenolic Concentration (TPC) is defined as the sum of phenol, *o*-cresol and *m-/p*-cresols. The TPC was plotted together with phenol because the latter is the major constituent of the plume.

*Borehole 59*

The groundwater samples pumped from borehole 59 revealed that there was little phenol or TPC in the aquifer above a depth of 10 mbg in both 2005 and 2006. Thereafter there was a steep organic pollutant gradient at the upper plume fringe. In 2005 the TPC at 10 mbgl was 5mg $L^{-1}$ but within one metre this had risen to 233mg $L^{-1}$ (Fig 2.6a). At the bottom of the borehole (30 mbgl) TPC was 350 mg $L^{-1}$. In 2006, total phenolic concentration was again negligible at depths 6, 8 and 9 and very low at 10 mbgl (0.5 mg $L^{-1}$). Within the next 2 metres, TPC rose steeply to 280 mg $L^{-1}$ at 11 mbgl and 662 mg $L^{-1}$ at 12 mbgl. At 30 mbgl TPC was much higher than one year ago, at 1220 mg $L^{-1}$. In both 2005 and 2006 samples, phenol accounted for about 30 – 40% of the TPC, the sum of *m-/p*-cresols 40 – 50% and *o*-cresol 10 – 20%. The concentration of xylenols (Fig. 2.6.c) was measured only in the 2005 samples; the total concentration of the different xylenol isomers ranged between 25 and 43% of the TPC (sum of phenol and cresols only)

at depths 10, 11 and 30, constituting a significant component of the pollutant load in borehole 59.

Nitrate concentration (Fig. 2.6.d) followed a reverse trend to the organic pollutant concentration. In uncontaminated groundwater nitrate was present at 80 mg $L^{-1}$ (in 2005) or 120 mg $L^{-1}$ (in 2006). Nitrate decreased at 10 mbgl (the first depth contaminated with low concentration of phenolics) and it became depleted at 11 mbgl. At the bottom of borehole 59 low concentrations of nitrate were measured in both years (28 mg $L^{-1}$ in 2005 and 15 mg $L^{-1}$ in 2006).

The concentration of inorganic compounds (Fig. 2.6.e and Fig. 2.6.f) increased significantly within the plume compared to background concentrations. However, in contrast to the phenolic compounds where the highest TPC was measured at 30 mbgl, the highest concentrations of all inorganic compounds were measured at the upper fringe of the plume, at 11 and 12 mbgl. For example in the 2006 samples, at 12 mbgl sulphate, magnesium, sodium and chloride concentrations were approximately 12x, 7x, 42x and 6x higher respectively compared to background concentrations (Fig. 2.6.e and Fig. 2.6.f). Calcium concentration (Fig. 2.6.f) remained at about the same levels in all sampled depths (only at 12 mbgl it was 25 % lower).

*Borehole 60*

In borehole 60 (Fig. 2.7), both 2005 and 2006 samples indicated the presence of a very steep pollutant gradient between 18 and 20 mbgl and very high concentrations of organic pollutants (TPC > 5500 mg $L^{-1}$) at 45 mbgl (Fig. 2.7.a). Phenol, *m-/p*-cresols, and *o*-cresols accounted for about 45%, 42% and 13% of the TPC respectively in all polluted samples. The combined concentration of xylenols was about 15% of the TPC, indicating that xylenols were not as significant component of the organic pollutant load as in borehole 59. Following the appearance of organic pollution (at 18 mbgl), nitrate became immediately depleted (Fig. 2.7.d). The other inorganic compounds were found in much higher concentrations than in uncontaminated groundwater but contrary to borehole 59, where concentration of calcium was quite stable (Fig. 2.6.f), in borehole 60 increased concentrations of calcium were measured (Fig. 2.7.f).

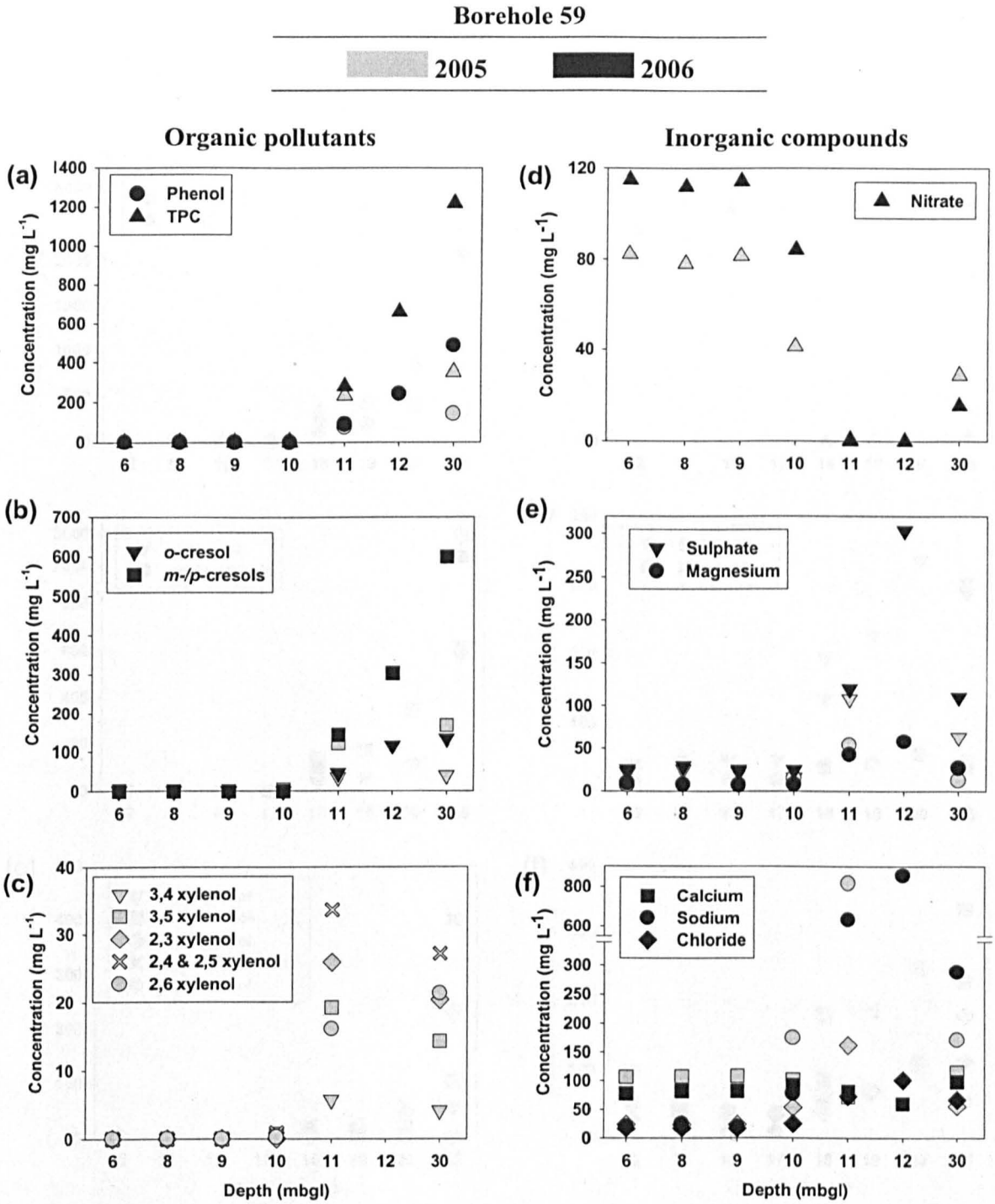



**Figure 2.6.** The concentration of the most important organic and inorganic compounds measured in groundwater samples pumped from different depths in Borehole 59 in 2005 (grey symbols) and in 2006 (black symbols).

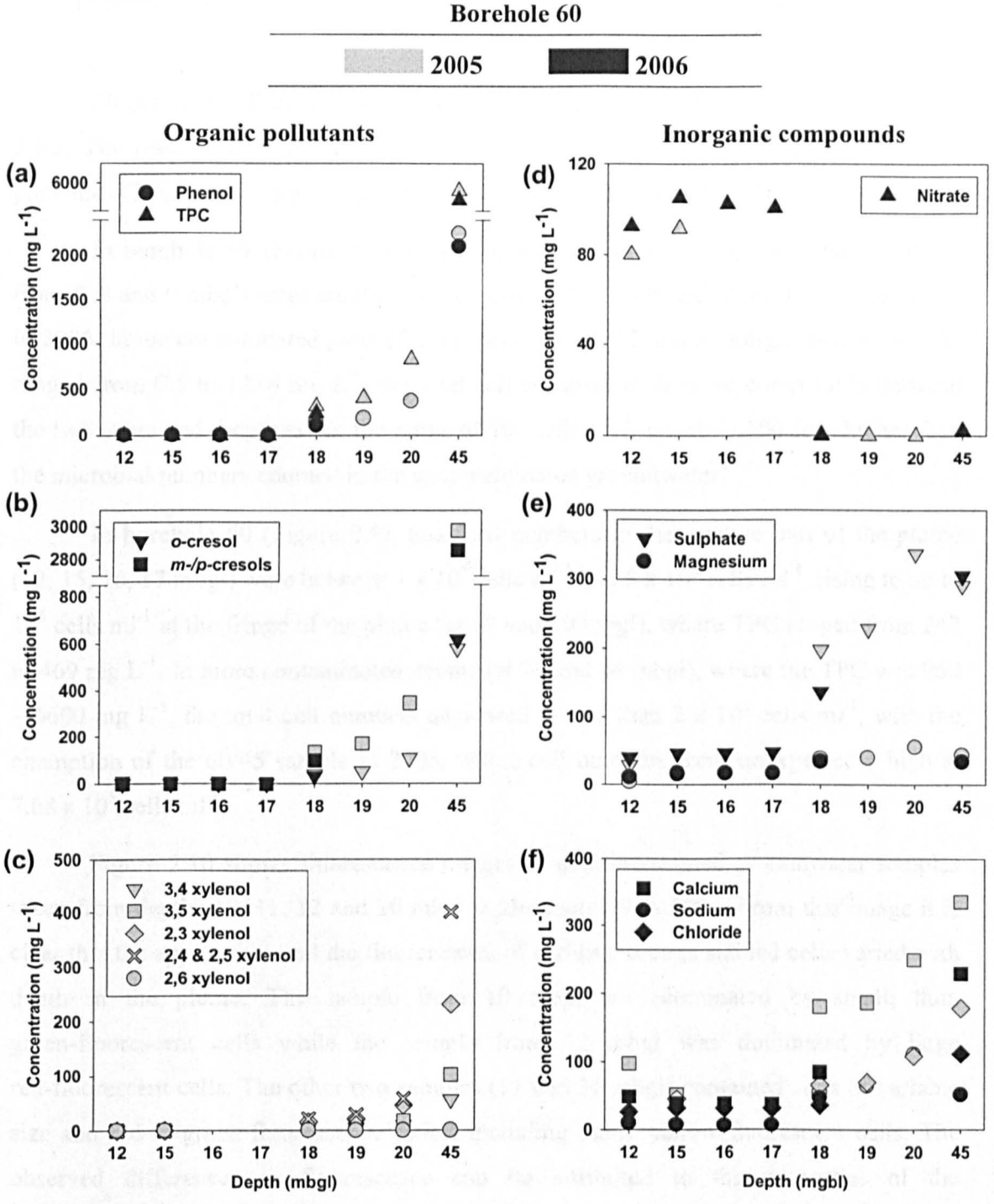



**Figure 2.7.** The concentration of the most important organic and inorganic compounds measured in groundwater samples pumped from different depths in Borehole 60 in 2005 (grey symbols) and in 2006 (black symbols).

### 2.3.2 Determination of total cell numbers by acridine orange staining

Direct total cell counts were taken with acridine orange staining as described in 2.2.3. The results of the direct total cell counts in relation to the concentration of total phenolics (TPC) are shown in Figures 2.8 and 2.9 for boreholes 59 and 60 respectively.

In borehole 59 (Figure 2.8), total cell numbers in pristine groundwater (pumped from 6, 8 and 9 mbgl) were around $5 \times 10^5$ cells $ml^{-1}$ in 2005 and about $1 \times 10^5$ cells $ml^{-1}$ in 2006. In the contaminated parts of the plume (10, 11, 12 and 30 mbgl), where the TPC ranged from 0.5 to 1220 mg $L^{-1}$, the total cell numbers were more comparable between the two years and they were in the range of $10^7$ cells $ml^{-1}$, which is 100-fold higher than the microbial numbers counted in the uncontaminated groundwater.

In borehole 60 (Figure 2.9), total cell numbers in the pristine part of the plume (12, 15, 16, 17 mbgl) were between $1 \times 10^5$ cells $ml^{-1}$ and $5 \times 10^5$ cells $ml^{-1}$, rising to up to $10^6$ cells $ml^{-1}$ at the fringe of the plume (at 18 and 19 mbgl), where TPC ranged from 242 to 469 mg $L^{-1}$. In more contaminated depths (at 20 and 44 mbgl), where the TPC was 960 – 6600 mg $L^{-1}$, the total cell numbers decreased to less than $2 \times 10^5$ cells $ml^{-1}$, with the exemption of the 60/45 sample in 2005, where cell numbers were unexpectedly high at $7.68 \times 10^5$ cells $ml^{-1}$.

Figure 2.10 shows fluorescence images of acridine-stained groundwater samples taken from depths 10, 11, 12 and 30 mbgl in Borehole 59 in 2006. From this image it is clear that the appearance and the fluorescence of acridine-orange stained cells varied with depth in the plume. The sample from 10 mbgl was dominated by small, thin, green-fluorescent cells while the sample from 12 mbgl was dominated by large red-fluorescent cells. The other two samples (11 and 30 mbgl) contained cells of variable size and red-to-green fluorescence ratios, including many yellow-fluorescent cells. The observed differences in fluorescence can be attributed to the properties of the acridine-orange stain, because according to its manufacturer (Molecular Probes), acridine orange is a cationic dye which has a maximum emission wavelength at 525 nm when bound to DNA (green fluorescence) and 650 nm when it is associated with RNA (red fluorescence).

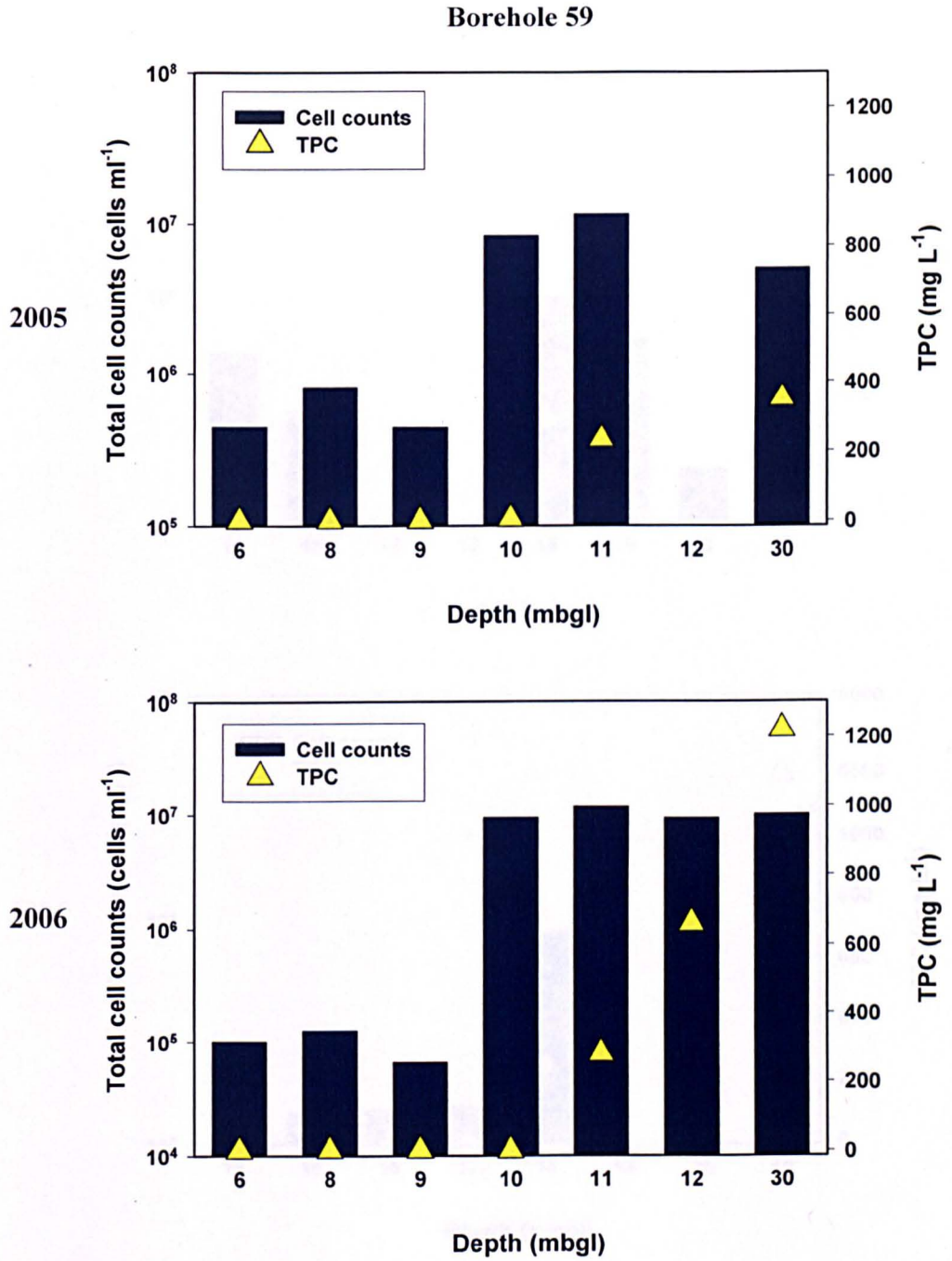



**Figure 2.8.** Direct total counts of cells in the groundwater samples pumped from borehole 59 in 2005 and in 2006 in relation to the Total Phenolics Concentration (TPC, the sum of phenol and *o*-/*m*-/*p*-cresols).

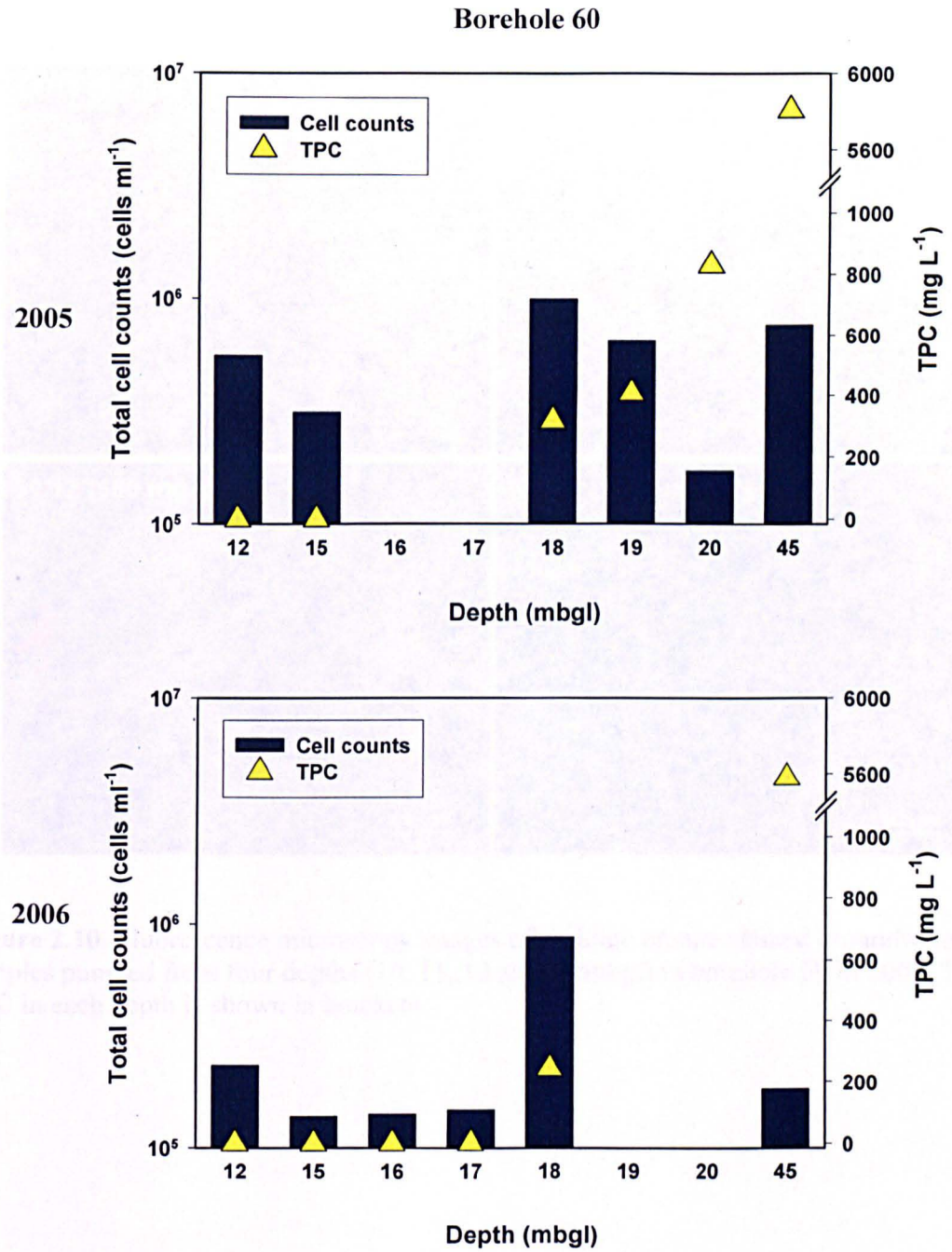



**Figure 2.9.** Direct total counts of cells in the groundwater samples pumped from borehole 60 in 2005 and in 2006 in relation to the Total Phenolics Concentration (TPC, the sum of phenol and *o*-/*m*-/*p*-cresols).

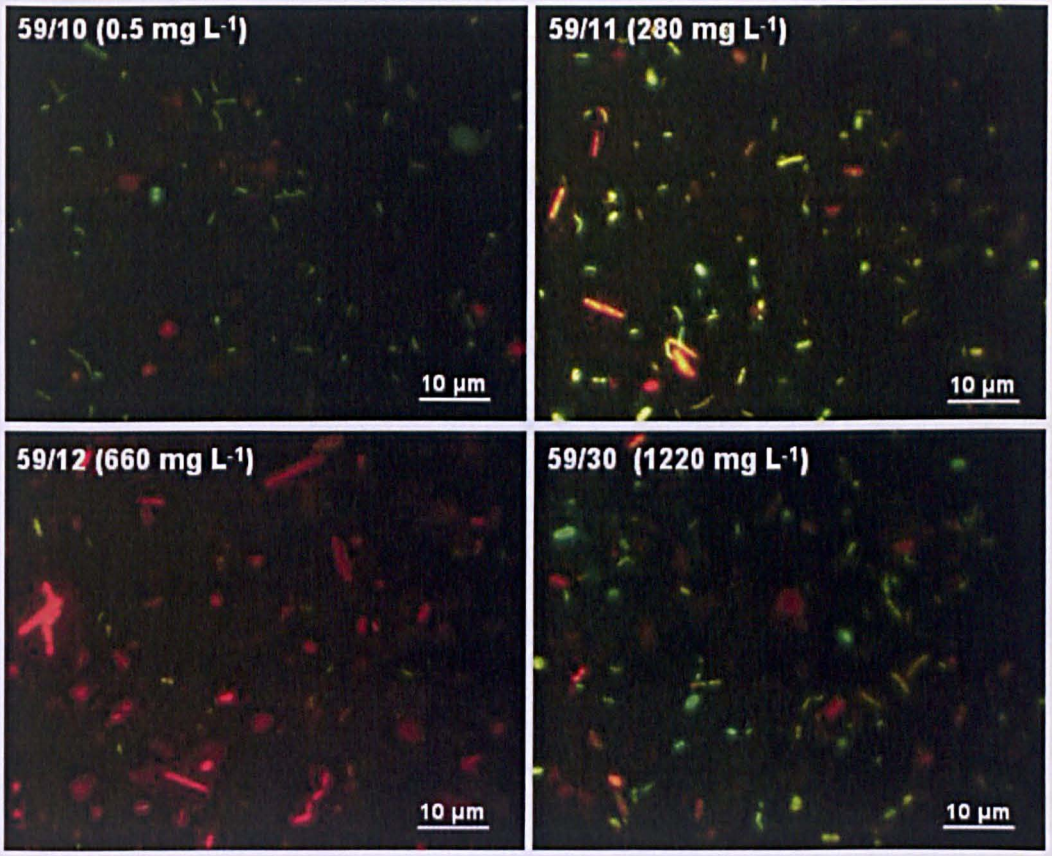



**Figure 2.10.** Fluorescence microscopy images of acridine orange stained groundwater samples pumped from four depths (10, 11, 12 and 30 mbgl) in borehole 59 in 2006. The TPC in each depth is shown in brackets.

### 2.3.3 DNA extraction and 16S rRNA gene PCR amplification of the groundwater and sand samples taken from Borehole 59 in 2006.

*Groundwater samples*

DNA was extracted from all the groundwater samples pumped from borehole 59 in 2006 using the CTAB method (2.2.4) and PCR amplification was performed as described in 2.2.6. Amplification was successful only for samples from depths 10, 11, 12, and 30 mbgl; faint bands were obtained from depths 6 and 9 and no PCR amplicons from depth 8 (Fig. 2.11).

In addition, DNA was extracted from depths 10, 11, 12 and 30 using the MOBIO kit and the PCR amplification generated amplicons of the correct size (Fig. 2.12).

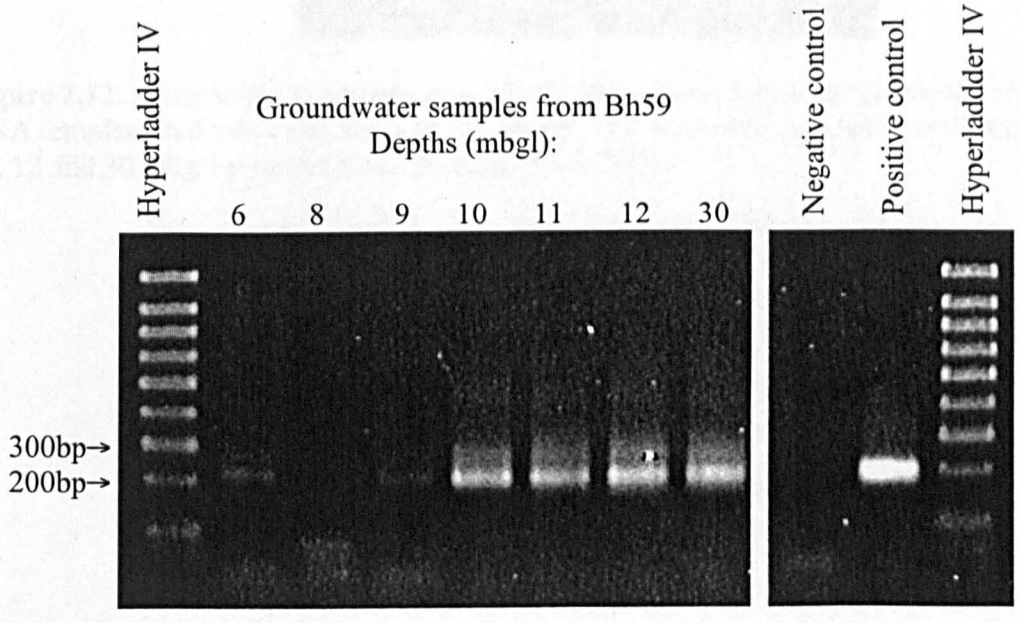



**Figure 2.11.** Agarose gel electrophoresis of 16S rRNA gene fragments generated using DNA template that was extracted with the CTAB-phenol-chloroform method from 7 different depths sampled from Borehole 59 in 2006.

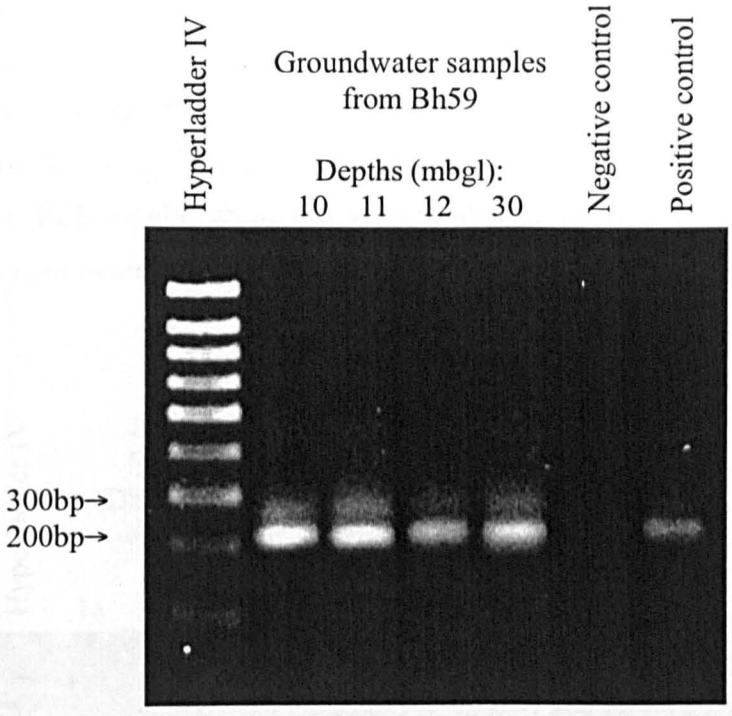



**Figure 2.12.** Agarose gel electrophoresis of 16S rRNA gene fragments generated using DNA template that was extracted with the MOBIO kit from four groundwater depths (10, 11, 12 and 30 mbgl) sampled from Borehole 59 in 2006.

*Sand samples*

DNA was isolated from two different sand bags that were left at the bottom of Borehole 59 for 14 months, using the CTAB method or the MoBio kit, as described in 2.2.4. However, PCR amplification was successful only when DNA extracted with the MoBio kit was used as template in the PCR reactions (Fig. 2.13).

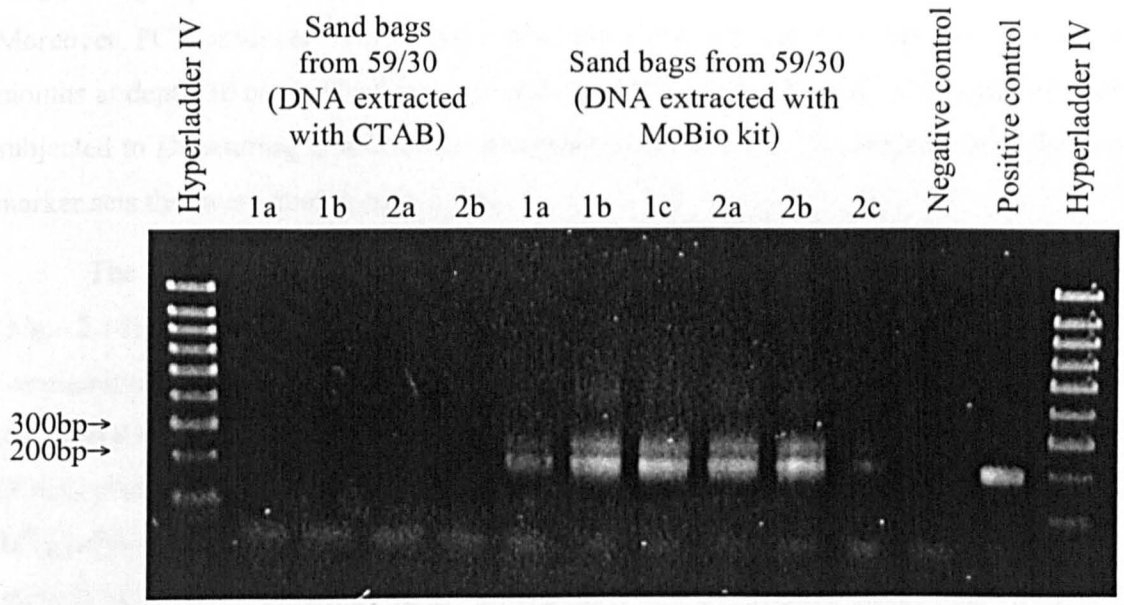



**Figure 2.13.** Agarose gel electrophoresis of 16S rRNA gene fragments generated using DNA template that was extracted from 2 different sand bags that were left for 14 months at depth 30 in Borehole 59, using the CTAB method or the MOBIO kit.

### 2.3.4 DGGE profiles of groundwater and sand samples taken from Borehole 59 in 2006

DGGE of PCR amplified 16S rRNA gene fragments was used to profile the diversity of planktonic and attached microbial communities present respectively in groundwater and sand samples taken from Borehole 59 in 2006. As described in 2.3.3, PCR products were obtained from four groundwater samples (depths 10, 11, 12 and 30 mbgl) using DNA extracted with either the CTAB method (C) or the MoBio kit (M). Moreover, PCR products were obtained from two different sand bags that were left for 14 months at depth 30 using DNA isolated with the MoBio kit. All these PCR products were subjected to Denaturing Gradient Gel Electrophoresis (see 2.2.7), alongside with the two marker sets that were described in 2.2.8.

The multiple bands in the DGGE profiles of all groundwater and sand samples (Fig. 2.14) indicated the presence of diverse planktonic and attached microbial communities. Although two different methods were used for the isolation of DNA from the groundwater samples, the two replicates from each depth produced almost identical DGGE profiles. However, groundwater samples taken from different depths produced different banding patterns, indicating that the planktonic community structure varied with depth in the plume. The observed banding patterns across the four different groundwater depths can be summarised into 3 categories. There were some intense co-migrating bands present in all groundwater samples, indicating that there are a few dominant bacterial species across the aquifer, from relatively pristine (TPC was 0.5 mg $L^{-1}$ at 10 mbgl) down to heavily contaminated groundwater (TPC = 1220 mg $L^{-1}$ at 30 mbgl). Secondly, there were some co-migrating bands with either increasing or decreasing intensities across the pollutant gradient, suggesting that some bacterial species became more or less dominant respectively with depth in the plume. Finally, the presence of some bands only at certain depths is a possible indication that the growth of specific microorganisms was restricted to environmental niches. At 30 mbgl, the DGGE profiles of 2 different sand bags were identical to each other but significantly different to the groundwater sample taken from the same depth. This difference suggests that under the same pollutant and geochemical

conditions, the attached microbial community was markedly different to the planktonic community.

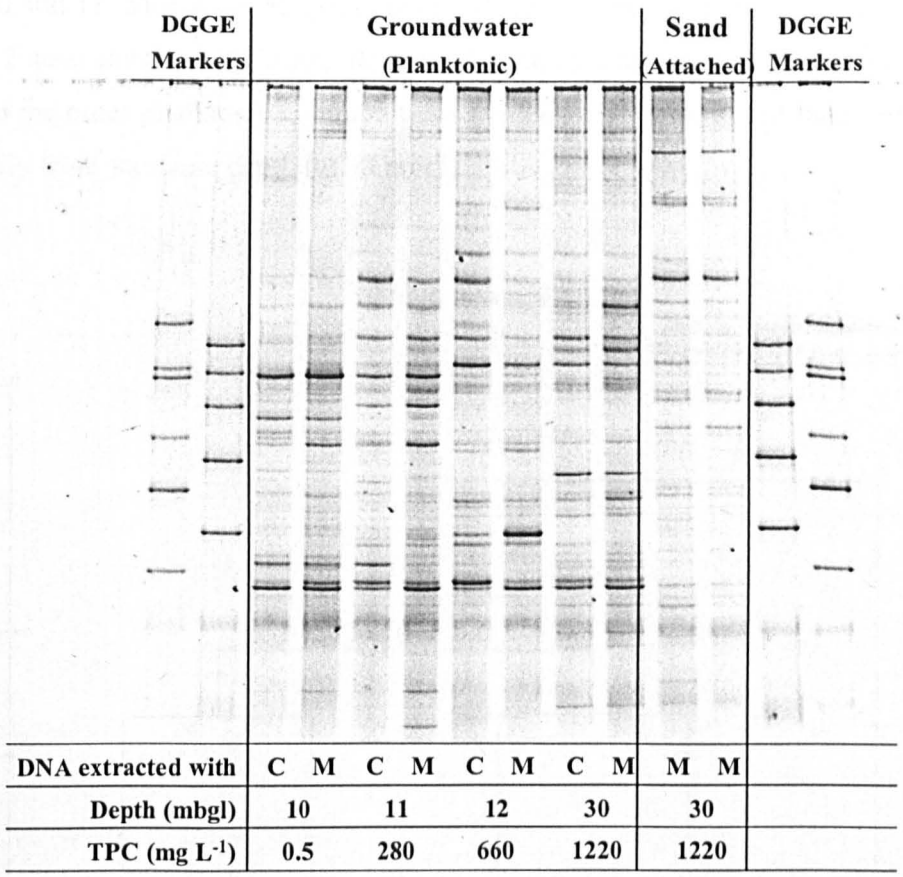



**Figure 2.14.** DGGE profiles of groundwater samples from 10, 11, 12 and 30 mbgl, and of two different sand bags from depth 30, taken from Borehole 59 in 2006. DNA extractions were performed with either the CTAB method (C) or the MoBio kit (M). The 2 DGGE marker sets were prepared from bacteria isolated from the Four Ashes site.

The DGGE image was further analysed with the LabWorks software (see 2.2.9) and in total 68 bands migrating to different distances in the gel have been identified. The number of major DGGE bands in each sample varied from 14 to 22 (Fig. 2.15). Then, cluster analysis based on the presence or absence of co-migrating bands was performed with the Primer software package, as described in 2.2.8. The similarity between replicates from the same groundwater depth was consistently high (between 84% and 95%),

highlighting the efficiency of both DNA extraction methods used. Groundwater samples 12 and 30 which contained very high concentrations of pollutants (660 and 1220 mg $L^{-1}$ respectively) clustered separately to the less contaminated groundwater samples from depths 10 and 11. Moreover, all groundwater samples were clustered closer together than with the 2 sand samples, indicating that the planktonic community at depth 30 was more similar to the other planktonic communities sampled rather than to the attached microbial community from the same depth (at 30 mbgl).

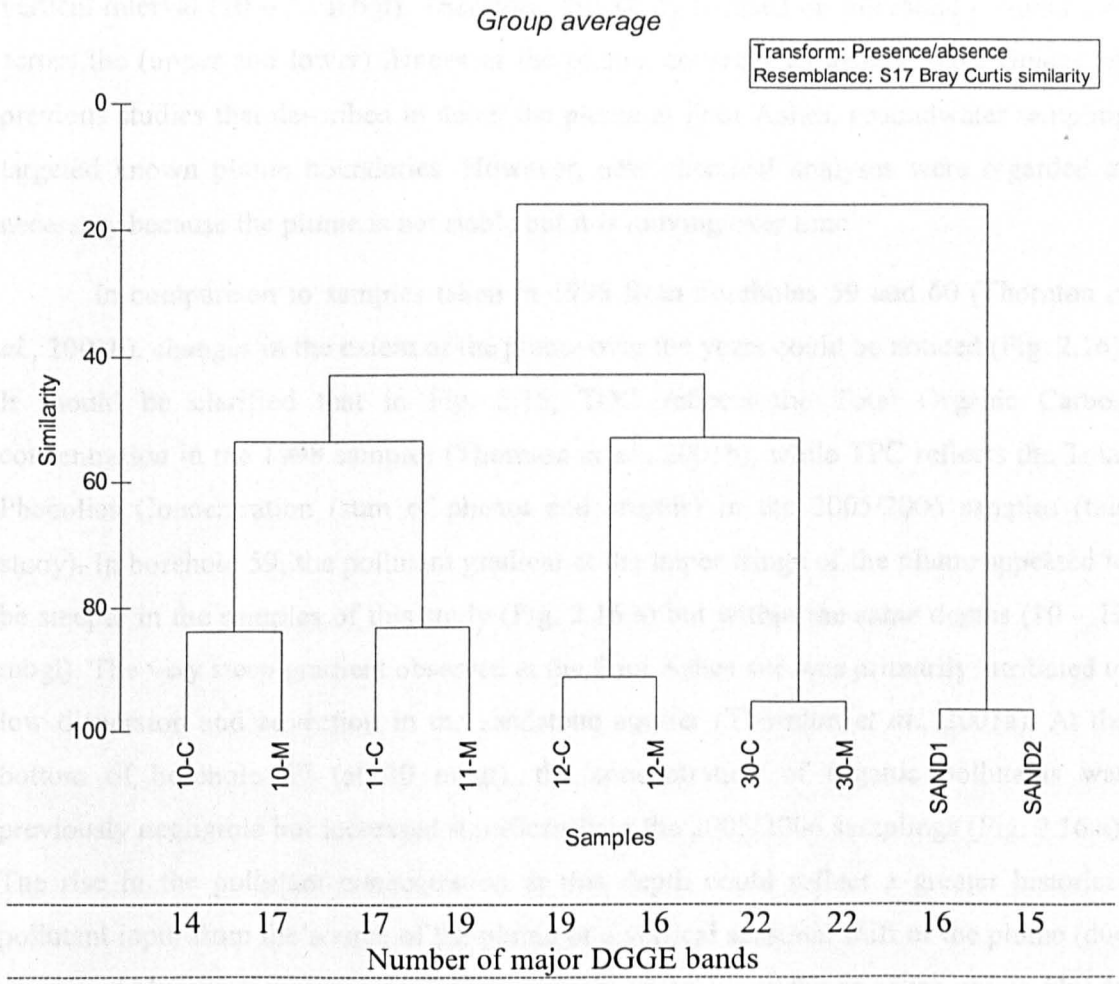



**Figure 2.15.** Cluster analysis of DGGE profiles of groundwater samples from 10, 11, 12 and 30 mbgl, and of two different sand bags from depth 30, taken from borehole 59 in 2006. DNA extractions were performed with either the CTAB method (C) or the MoBio kit (M).

## 2.4 Discussion

### 2.4.1 The geochemistry at the Four Ashes site over time

The main aim of this study was to investigate how pollutant concentration affects abundance and diversity of *in situ* microbial communities. It has been shown previously (Thornton *et al.*, 2001b) that aerobic degradation and $NO_3^-$ reduction account for more than all other natural attenuation processes combined and they are restricted to a 2 – 3 m vertical interval (10 – 12 mbgl). Therefore, this study focused on microbial communities across the (upper and lower) fringes of the plume, covering its aerobic part. Guided by previous studies that described in detail the plume at Four Ashes, groundwater sampling targeted known plume boundaries. However, new chemical analyses were regarded as necessary because the plume is not stable but it is moving over time.

In comparison to samples taken in 1998 from boreholes 59 and 60 (Thornton *et al.*, 2001b), changes in the extent of the plume over the years could be noticed (Fig. 2.16). It should be clarified that in Fig. 2.16, TOC reflects the Total Organic Carbon concentration in the 1998 samples (Thornton *et al.*, 2001b), while TPC reflects the Total Phenolics Concentration (sum of phenol and cresols) in the 2005/2006 samples (this study). In borehole 59, the pollutant gradient at the upper fringe of the plume appeared to be steeper in the samples of this study (Fig. 2.16.a) but within the same depths (10 – 12 mbgl). The very steep gradient observed at the Four Ashes site was primarily attributed to low dispersion and advection in the sandstone aquifer (Thornton *et al.*, 2001a). At the bottom of borehole 59 (at 30 mbgl), the concentration of organic pollutants was previously negligible but increased significantly in the 2005/2006 samplings (Fig. 2.16.a). The rise in the pollutant concentration at this depth could reflect a greater historical pollutant input from the source of the plume or a vertical seasonal shift of the plume (due to water table variations) or a gradual depression of the lower fringe of the plume (due to pollutant movement by gravity and dispersion). In borehole 60, the extent of the plume changed even more dramatically (Fig. 2.16.c). The upper fringe of the plume was detected at shallower depths (at 18 mbgl instead of 21 mbgl) and the previously uncontaminated

bottom of borehole 60 (at 45 mbgl) contained very high concentrations of organic pollutants, similar to the ones normally measured in the core of the plume (Fig. 2.16.c).

As the pollutant concentration increased with depth across the upper fringe of the plume in borehole 59 (10 – 12 mbgl), a reverse gradient of decreasing nitrate concentrations was detected (Fig. 2.16.b). In pristine groundwater, nitrate concentration was about 80 mg $L^{-1}$ in 2005 and 120 mg $L^{-1}$ in 2006. This difference probably reflects different farming practices over time, considering the fact that the main source of nitrate in the aquifer is likely to be the application of fertilisers and/or manure on the overlying field. Immediately after the appearance of organic pollutants at 10 mbgl, nitrate decreased significantly in both 2005/2006 samplings and it was depleted at depths 11 and 12. Thornton *et al.* (2001b) have also shown that within this 2 – 3 m vertical interval $NO_3^-$ and $O_2$ became depleted (Fig. 2.16.b) as degradation of organic pollutants was occurring by aerobic bacteria and nitrate reducers. Although dissolved $O_2$ was not measured during this study, the fact that $NO_3^-$ becomes depleted within the same depths, allows us to conclude with confidence that within these depths (10 – 12 mbgl) groundwater becomes anaerobic and that the aerobic upper fringe of the plume was sampled successfully.

Even though the 2005/2006 samplings were not very extensive (only 12/13 depths were sampled respectively), the presence of 3 sub-plumes (see Fig. 1.8) could be identified, in accordance with Williams *et al.* (2001). The plume in borehole 60 was characterised by high concentrations of calcium (Fig. 2.7.f) while closer to the source of the plume (borehole 59) sodium was the dominant cation (Fig. 2.6.f). The differences observed in the main plume have been attributed to different historical pollutant inputs from the coal-tar distillation plant that followed its demolition in the early 1980s (Fig. 1.8; Williams *et al.*, 2001). Taking into account that groundwater flow at the Four Ashes site is about 10 m $year^{-1}$ and that Bh60 is situated 200 m down gradient from Bh59, the plume in borehole 60 reflects pollutant inputs of about 20 years prior to that in Bh59. Moreover, in the shallow aquifer of borehole 59, the presence of particularly elevated concentrations of inorganic pollutants (Fig. 2.6.e and 2.6.f) are in agreement with Thornton *et al.* (2001b) who also detected the highest concentrations of sodium (700 mg $L^{-1}$ at 12 mbgl) and chloride (around 250 mg $L^{-1}$) at the upper fringe of the plume and indicate a secondary plume origin, deriving from the soda/lime kiln plants (Fig. 1.8; Williams *et al.*, 2001).

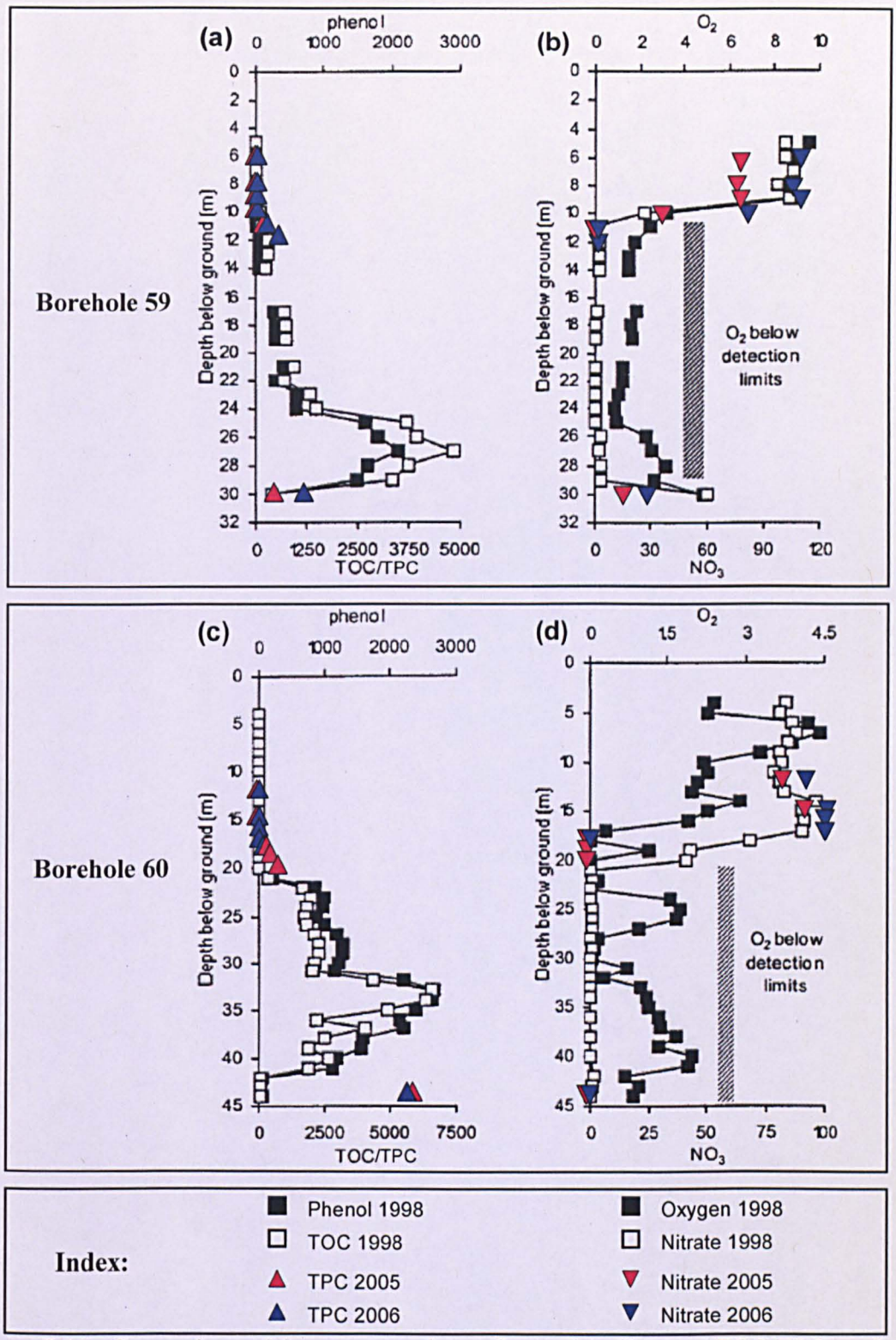



**Figure 2.16.** Comparison of the concentrations of organic pollutants (TOC/TPC) and nitrate in the 1998, 2005 and 2006 samples taken from Borehole 59 and 60 in the Four Ashes aquifer. Graphs adjusted from Thornton *et al.* (2001b).

### 2.4.2 The influence of pollutant concentration on microbial abundance

The influence of pollutant concentration on microbial abundance was investigated by direct total cell counts. It is widely recognised that viable counts generally underestimate microbial numbers in environmental samples because only a small proportion of bacteria can be cultured, which is estimated to be 0.1% or at most 10% of the total population (Amann *et al.*, 1995). Therefore the total number of cells in environmental samples, including non-viable and non-culturable microorganisms, is usually determined by direct epifluorescent microscopic counts. In the past, acridine orange (3,6-bis[dimethylamino]acridinium chloride) and DAPI (4′,6-diamidino-2-phenylindole) have been the most commonly used fluorochromes applied for the direct enumeration of bacteria in a wide range of samples (Kepner and Pratt, 1994) while in recent years other fluorescent nucleic acid dyes have also been used, such as SYBR Green I & II (Weinbauer *et al.*, 1998) and SYBR Gold (Helton *et al.*, 2006). For this study, total cell counts were taken using acridine orange.

Cell counts were taken from 25 different groundwater samples pumped from multilevel boreholes 59 and 60 in two consecutive years (2005 and 2006) and microbial numbers ranged from $6 \times 10^4$ to $10^7$ cells $ml^{-1}$ (Fig. 2.8 and 2.9). The highest microbial numbers (100-fold higher than in uncontaminated groundwater) were measured at the periphery of the plume in borehole 59 (at 10, 11, 12 and 30 mbgl), where TPC was between 0.5 and 1220 mg $L^{-1}$ (Fig. 2.8). However, samples with similar pollutant load in borehole 60 did not contain increased microbial numbers. Despite the fact that at some depths the microbial numbers were slightly lower in 2006 than in 2005, the measurements were remarkably comparable not only between the two samplings of this study but also with previous measurements from the Four Ashes site. In samples pumped from 22 conventional boreholes from various parts of the plume at the Four Ashes site Williams *et al.* (2001) found that microbial numbers ranged from $10^4$ to $3.6 \times 10^6$ cells $ml^{-1}$, while Pickup *et al.* (2001) measured $2 \times 10^5$ to $7 \times 10^6$ cells $ml^{-1}$ in samples from BH59 and $7 \times 10^4$ cells $ml^{-1}$ to $6 \times 10^6$ cells $ml^{-1}$ in samples from BH60. From all these measurements no obvious trend with respect to the effect of phenol concentration could be observed but the lowest bacterial numbers were usually counted in uncontaminated groundwater possibly

due to lack of carbon availability and in the core of the plume due to toxicity. Occasionally cell counts could not even be performed in heavily contaminated samples due to the formation of an opaque precipitate (Pickup *et al.*, 2001; Williams *et al.*, 2001).

Microbial numbers in the Four Ashes aquifer were also comparable to other polluted environments. In a wastewater-processing system (with concentrations of phenolics between 5 and 500 mg $L^{-1}$) total cell counts were between 9.1 x $10^6$ and 2.9 x $10^7$ cells $ml^{-1}$ (Whiteley and Bailey, 2000) while in an aquifer contaminated with fuel oil, Aamand *et al.* (1989) measured 7.2 x $10^4$, 3.3-5 x $10^5$ and 2.2 x $10^6$ cells $ml^{-1}$ in unpolluted, polluted and heavily polluted groundwater respectively.

Although during this study similar numbers of cells (around $10^7$ cells $ml^{-1}$) were counted at the boundaries of the plume in borehole 59 (10, 11, 12 and 30 mbgl), fluorescence microscopy images illustrated big differences in the ratio of red-to-green fluorescence of these acridine orange stained cells (Fig. 2.10). The property of acridine orange to distinguish between double and single stranded nucleic acids (the ratio of green to red fluorescence intensity) has been exploited in the past to measure cellular RNA content (Darzynkiewicz *et al.*, 1979) and to describe the cytoplasmic ribosome state (Gordon *et al.*, 1997). Therefore, the appearance of acridine orange-stained cells could be a useful indicator of their functional state (transcriptional activity), considering the fact that bacteria at high growth rates fluoresce red due to the predominance of RNA while inactive bacteria have mostly DNA and fluoresce green (Yu *et al.*, 1995). Thus the planktonic communities from different depths in Borehole 59 appear to have different degrees of (transcriptional) activity, as illustrated clearly in Fig. 2.10. Microbial cells were relatively inactive at 10 mbgl, probably due to limited carbon availability (TPC = 0.5 mg $L^{-1}$). Within the next two metres, as the TPC rose to 280 mg $L^{-1}$ (at 11 mbgl) and 660 mg $L^{-1}$ (at 12 mbgl), a parallel increase in microbial (transcriptional) activity occurred as detected by the increasing cell size and red-to-green fluorescence. The fact that nitrate became depleted at depth 11, implies that the active bacteria at 12 mbgl could utilise other electron acceptors than oxygen or nitrate. At depth 30, the TPC was even higher (TPC = 1220 mg $L^{-1}$) and nitrate was not depleted but microbial activity appeared to decrease, probably due to toxicity effects.

### 2.4.3 Microbial community structure and diversity across a pollutant gradient

The structure of planktonic microbial communities from four different depths across the pollutant gradient in borehole 59 (TPC ranging from 0.5 to 1220 mg $L^{-1}$) was investigated by DGGE. The DGGE profiles (Fig. 2.14) indicated that the planktonic community structure varied with depth in the plume. Interestingly enough, most of the changes in the banding pattern and the intensity of the bands appeared to occur between depths 11 and 12 (Fig. 2.14). This visual observation was also confirmed by cluster analysis based on the presence or absence of bands (see 2.3.5). As it is shown by the dendrogram in Fig. 2.15, groundwater samples from depths 10 and 11 were similar and clustered separately to samples from more contaminated depths (12 and 30 mbgl). The fact that groundwater sample 12 clustered together with a sample pumped from 18 m away and not with the two other samples from the upper fringe of the plume implies that the observed changes in microbial community structure should be attributed to the geochemical environment rather than the location in the plume. The concentration of inorganic compounds did not appear to have a significant effect on microbial community structure because in that case sample 12 which contained considerably high concentration of inorganic pollutants should have clustered separately from samples 11 and 30 which had similar composition of inorganic compounds. On the other hand, the only parameter that differentiated both samples 12 and 30 from sample 11 was the elevated concentration of phenolics. Therefore, from all the environmental factors that could have been responsible for the shift in microbial community structure, the increased concentration of phenolics at depths 12 and 30 should be regarded as the most important. Previous studies have also investigated the influence of pollutants on microbial community structure, sometimes with contradictory results. Ferguson *et al.* (2007) found that in PAH contaminated soils the pollutant concentration rather than spatial location was the main driver for the observed differences in the microbial community composition, whereas Juck *et al.* (2000) showed that clustering of DGGE profiles of hydrocarbon-contaminated arctic soils occurred on the basis of geographical location rather than pollutant concentration.

In addition to the pollutant concentration, the type of the pollution and the general geochemical environment have been regarded as equally or more important factors determining microbial community composition. For example, Fahy *et al.* (2005) using multivariate analysis of geochemical data and bacterial community structure (T-RFLP profiles) in groundwater samples from a BTEX contaminated aquifer showed that other "anoxia-related parameters" (oxygen, carbon dioxide, methane, sulphate, nitrate, dissolved iron and manganese) accounted for 60 % of the variance in bacterial community structures while benzene concentration accounted for only 7.7 %. The influence of the specific type of the pollution was demonstrated by Muckian *et al.* (2007) who found that irrespectively to the pollutant load, microbial communities in soil samples containing 3 or 4 ring PAHs clearly differentiated from uncontaminated samples and those containing 2-ring PAHs. Furthermore, strong microbial community structuring was observed across the different compartments of a phenol-fed wastewaster process system (Whiteley and Bailey, 2000) and in a PAH-bioreactor system (Ferguson *et al.*, 2007) while in a BTEX contaminated aquifer, Rooney-Varga *et al.* (1999) showed that microbial community composition differed markedly among the aerobic, Fe(III)-reducing, and methanogenic zones of the aquifer. As discussed in 2.4.1, the outer thin layer of the plume (10 and 11 mbgl) is likely to be dominated by aerobic and nitrate-reducing microbial communities while deeper in the plume concomitant iron-, manganese-, and sulphate-reducing natural attenuation processes have been shown to occur (Thornton *et al.*, 2001b). Therefore, the shift in microbial community structure observed across the pollutant gradient in borehole 59 could correspond to different functional microbial communities with depth in the plume, performing different degradation processes.

Although there was strong evidence that pollutant concentration and the geochemical environment influenced the structure of planktonic microbial communities at the Four Ashes site, due to inherent limitations of DGGE no clear conclusion could be drawn whether pollutant concentration increased or decreased microbial diversity. DGGE is generally regarded as a very good tool for rapid assessments of shifts in microbial community structure and has been applied to describe microbial diversity in a wide range of environments, from agricultural soils to deep-sea vents and Arctic permafrost soils. In principal (Muyzer *et al.*, 1993), bacterial species that constitute more than 1% of the total

population can be detected by DGGE and each DGGE band corresponds to a single species. However, in practice one species may produce several bands in the gel (Fig. 2.5) and more than one bacterial species can co-migrate to the same distance in the gel because denaturing gradients are too steep to resolve sufficiently the banding patterns (Gafan and Spratt, 2005). Moreover, the intensity of the bands does not directly correspond to the relative abundance of one species in the total population, due to PCR amplification biases (discussed in 1.3) and differences in rRNA gene copy numbers between bacterial species (Wintzingerode *et al.*, 1997; Crosby and Criddle, 2003). Therefore, quantitative interpretation of DGGE gels should be carried out with caution (Watanabe *et al.*, 2000). From all the above, it becomes apparent that the slightly higher number of DGGE bands identified (see 2.3.5) in the more contaminated groundwater samples does not necessarily reflect higher microbial diversity. Using ERIC-PCR to profile the microbial diversity in samples from borehole 59, Pickup *et al.* (2001) also concluded that phenol concentration did not markedly affect the number of bands within each fingerprint. However, other studies have reported clear effects of pollutant concentration on microbial community diversity. In comparison to background uncontaminated samples, increased microbial diversity was noticed in a petroleum-contaminated aquifer (Rooney-Varga *et al.*, 1999) whereas reduced species diversity was observed in TCE-contaminated groundwater (Lowe *et al.*, 2002), highly PAH-contaminated soils (Ferguson *et al.*, 2007), and hydrocarbon-contaminated arctic soils (Juck *et al.*, 2000).

### 2.4.4 Structure of planktonic and attached microbial communities within the same depth

In order to compare the structure of planktonic and attached communities inhabiting the same plume depth, bags containing quartz sand were left at the bottom of borehole 59 in the Four Ashes aquifer. The microbial communities that developed on the incubated sand do not necessarily represent the actual attached microbial community in the aquifer, because the indigenous aquifer sandstones contain Fe and Mn oxides as grain coating (Thornton *et al.*, 2001a) that can influence microbial community composition. However, the fact that sterile sand was incubated for a period of time (14 months) and the developed microbial communities differed markedly to the planktonic community

indicates strongly that natural planktonic and attached communities differ significantly. The difference in the composition of attached and the planktonic microbial communities observed in the Four Ashes aquifer could be due to the fact (as discussed in 1.4) that microorganisms that could not tolerate the elevated concentration of phenolics in borehole 59 (TPC = 1220 mg $L^{-1}$), may be able to exist within the heterogeneous (genetically and physiologically) biofilm structures (Stewart and Franklin, 2008).

Due to technical constraints (we could suspend the sand bags only at the bottom of borehole 59), it is also not clear whether differences would be observed between attached and planktonic communities in other aquifer depths. However, it is interesting that Iribar *et al.* (2008) demonstrated by DGGE analysis that attached and free-living microbial communities were significantly different to each other, irrespectively of the location that were sampled from (different depths in the Riparian zone of Garonne River in France). This was shown even more clearly by the study of Vrionis *et al.* (2005) who examined the microbiological and geochemical heterogeneity in an *in situ* uranium contaminated aquifer which was supplied with acetate to enhance microbial U(VI) reduction. By using closely spatial sampling (sediments taken from 4.0, 4.6 and 5.2 mbgl), they showed that microbial diversity of attached communities (as determined by 16S rRNA gene cloning) varied with depth in the plume, across the steep geochemical gradient. The planktonic microbial communities from approximately the same depths (5 mbgl) were different to the attached ones.

Contrary to the pre-mentioned studies, using a similar experimental approach to this study, Lehman *et al.* (2004) incubated in a TCE contaminated aquifer, chambers containing distilled water or crushed (sterile) basalt and after DGGE analysis and 16S rRNA gene sequencing of excised DGGE bands they concluded that the planktonic communities were compositionally similar to the attached ones. However, they noticed that the two communities exhibited different potential functions (e.g. aerobic mineralization, iron-reducing, $H_2$-oxidising, methanotrophs) and that increased organic contamination increased biomass associated with core/basalt samples.

### 2.4.5 Conclusions

Microbial abundance and diversity of planktonic communities was investigated across the plume fringes of borehole 59 in the Four Ashes aquifer (10, 11, 12 and 30 mbgl). Total cell counts by acridine orange staining showed that the presence of organic pollutants (TPC = 0.5 – 1220 mg $L^{-1}$) increased approximately 100-fold the microbial numbers in the fringes of borehole 59 (10, 11, 12 and 30 mbgl) compared to microbial numbers in uncontaminated groundwater. Within the same depths microbial activity (assessed by the fluorescence of acridine orange stained cells) and planktonic community structure (assessed by DGGE fingerprinting) varied with depth, as a result of the different geochemical conditions. In addition, the DGGE profiles revealed that under the same geochemical conditions (30 mbgl), the attached microbial community differed significantly to the planktonic one. This finding is explored more extensively in Chapter 3, where a culture based and a culture independent (16S rRNA gene cloning) approach are used to compare the composition of attached and planktonic microbial communities at the bottom of borehole 59 (30 mbgl).

# Chapter 3

# Diversity of planktonic and attached microbial communities from the Four Ashes aquifer

## 3.1 Introduction

In marine/aquatic environments there are a large number of studies that have compared the diversity of free-living (planktonic) and particle-associated (attached) microbial communities (that colonise macroscopic aggregates of particulate organic matter). The findings regarding which of the two communities is more diverse are some times contradictory, probably because different environments were studied, different methods to separate free-living from particle methods were followed (e.g. size of the particles was > 0.5 mm in DeLong *et al.*, 1993 and > 8 mm in Acinas *et al.*, 1999), and different techniques for assessing microbial diversity were used (16S rRNA gene sequencing or DNA fingerprinting). However, all these studies agreed that distinct phylogenetic/compositional differences exist between free-living and attached communities in various environments, such as in the Santa Barbara Channel, California (DeLong *et al.*, 1993), in the Aegean Sea, Greece (Moesender *et al.*, 2001), in Western Mediterranean, Spain (Acinas *et al.*, 1999), NW Mediterranean, France (Ghiglione *et al.*, 2007), in the Wadden Sea, Germany (Stevens *et al.*, 2005), at Victoria Harbour area, Hong Kong (Zhang *et al.*, 2007), in a coastal lagoon in Santa Barbara, California (LaMontagne and Holden, 2003), in the floodplain of river Danube, Austria (Besemer *et al.*, 2005), and in samples from Columbia river (USA), its estuary, and the adjacent coastal ocean (Crump *et al.*, 1999). It is also characteristic that hierarchical cluster analysis using UPGMA (unweighted pair-group method using arithmetic averages) analysis of T-RFLP patterns of samples taken from different locations in the Aegean Sea, Greece (Moesender *et al.*, 2001) or SSCP (single strand conformation polymorphism) patterns of samples taken from different depths in NW Mediterranean, France (Ghiglione *et al.*, 2007) showed that all the attached microbial communities clustered separately to the free-living communities, irrespective of the location/depths they sampled from.

In polluted aquifers, some studies suggest that only a small proportion of the microbial biomass exists in the planktonic phase (Holm *et al.*, 1992; Alfreider *et al.*, 1997; Griebler *et al.*, 2002) and that different functional communities may exist in the attached and planktonic phase (Lehman and O'Connell, 2002). However, phylogenetic characterisation of microbial communities in polluted aquifers has mainly focused on

samples of either groundwater or core/sediment samples and rarely both. For example, the diversity of planktonic microbial communities (groundwater samples) has been investigated by 16S rRNA gene sequencing in coal-tar (Bakermans and Madsen, 2002), crude oil (Watanabe *et al.*, 2000), TCE (Hohnstock-Ashe *et al.*, 2001; Lowe *et al.*, 2002) and BTEX (Fahy *et al.*, 2005; Alfreider and Vogt, 2007) polluted aquifers while the diversity of attached microbial communities (sediments/core samples) has been investigated in BTEX (Winderl *et al.*, 2008), petroleum (Rooney-Varga *et al.*, 1999), chlorinated ethenes (Davis *et al.*, 2002), and uranium (Holmes *et al.*, 2002; North *et al.*, 2004) polluted aquifers. Comparison of the phylogenetic diversity of both the attached and planktonic communities has been attempted by only a few studies, including BTEX (Hendrickx *et al.*, 2005), TCE (Lehman *et al.*, 2004), petroleum (Pombo *et al.*, 2005) and uranium (Vrionis *et al.*, 2005; Reardon *et al.*, 2004) polluted aquifers.

In this study, it has already been demonstrated by DGGE fingerprinting that the attached and planktonic communities from 30 mbgl in the Four Ashes aquifer differed markedly (Fig. 2.14). The microbial diversity of these two communities will be investigated further using a culture independent (16S rRNA gene cloning) and a culture based approach. To our knowledge this is the first study that employs a molecular based method (16S rRNA gene cloning) to describe the microbial diversity in an aquifer polluted with phenolic compounds. To date microbial diversity in phenol polluted environments using culture-independent techniques has been studied only in a wastewater treatment plant (Manefield *et al.*, 2005), industrial wastewater (Cortes-Lorenzo *et al.*, 2006), and soil (Padmanabhan *et al.*, 2003; DeRito *et al.*, 2005).

The objectives of this study are:

1. To generate a clone library of planktonic and attached microbial communities sampled from the same depth in the Four Ashes aquifer
    a. to compare the diversity of these two communities
    b. to infer functions where possible and to relate diversity to the geochemical environment within the Four Ashes aquifer
2. To isolate and identify microorganisms from both the groundwater and the sand sample in order to compare them to the clone libraries and to use in microcosm studies.

## 3.2 Methods

### 3.2.1 Overall framework

Two different strategies (Fig. 3.1) were followed to describe the diversity of planktonic and attached microbial communities at the same depth (30 mbgl) in borehole 59 of the Four Ashes aquifer. Firstly, bacteria were cultured on R2A agar, isolated and identified by 16S rRNA gene sequencing. Secondly, a culture-independent approach was taken that involved the construction of 16S rRNA gene clone libraries. Clones were sequenced from isolated plasmid DNA or following direct amplification of the vector insert by PCR.

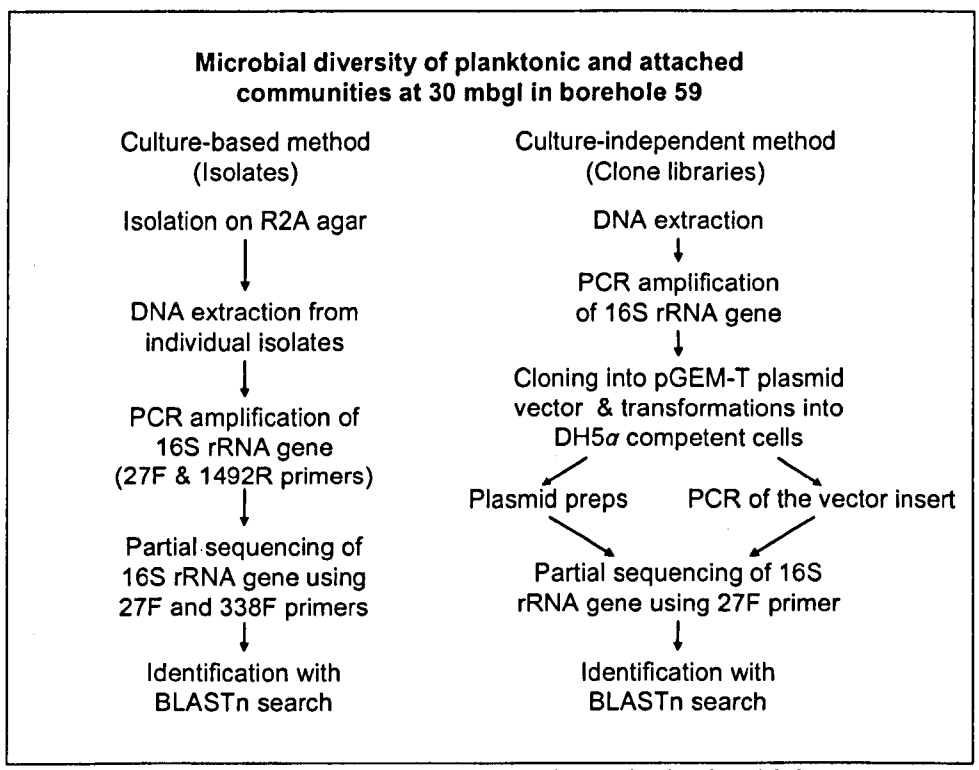



**Figure 3.1.** The diversity of planktonic and attached microbial communities at 30 mbgl in borehole 59 was described by following a culture-based and a culture-independent approach.

### 3.2.2 Isolation of bacteria on R2A agar

Fifty $\mu$l of frozen glycerol stocks that were prepared from groundwater and sand samples taken from 30 mbgl from Borehole 59 in 2006 (2.2.1) were spread on triplicate plates containing R2A agar (OXOID Ltd., Basingstoke, UK) and incubated for 2 weeks at 11°C. Distinct single bacterial colonies with various morphologies were picked and used for streaking onto new R2A agar plates. After incubation at 20°C for a period of 3 – 7 days (depending on bacterial growth), the morphological characteristics of the colonies (including colony size, margins, elevation, consistency, opacity, pigmentation) from each isolate were recorded. Then, single bacterial colonies were picked with disposable plastic loops and used to inoculate 3 ml of liquid Luria Bertani (LB) broth (10g $L^{-1}$ tryptone, 5g $L^{-1}$ yeast extract, 10g $L^{-1}$ NaCl, pH 7.5). The liquid cultures were placed on an IKA-VIBRAX-VXR S17 orbital shaker (IKA® Werke GmbH & Co. KG, Staufen, Germany) at 600 rpm at 20°C. Two days later, 800 $\mu$l of the liquid cultures was mixed with 200 $\mu$l of sterile 80 % glycerol in a 2 ml sterile cryogenic vial (Nalge Nunc Int. Corp., Rochester, NY, USA) and kept at -80°C as frozen glycerol stocks. 1.5 ml of the remaining liquid culture was centrifuged at 10,000 x g for 10 min using an Eppendorf 5417C microcentrifuge and the bacterial pellets were stored at -20°C prior to DNA extraction.

### 3.2.3 DNA extraction from the isolated bacteria

DNA extractions from the pelleted bacterial cultures (3.2.2) of all isolated bacteria were performed using the CTAB method (2.2.4) and quantified with PicoGreen (2.2.5).

### 3.2.4 Full length 16S rRNA gene PCR amplification and purification

DNA extracted from the bacterial isolates (3 – 40 ng) was used as a template in 50 $\mu$l PCR reactions containing 0.75 units of *Taq* DNA polymerase, 1 x $NH_4$ reaction buffer, 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.3 $\mu$M of each 27F (5'–AGAGTTTGATCMTGGCTCAG–3') and 1492R (5'–

TACGGYTACCTTGTTACGACTT-3') primers (Lane, 1991). This primer pair amplifies almost full length of the 16S rRNA gene (~1.5 kb; positions 8–27 and positions 1492–1513 in 16S rRNA gene of *E. coli*). All PCR reagents were supplied by Bioline (Bioline, UK) and primers were obtained from Invitrogen (Invitrogen, UK). A positive and a negative control (no DNA template added) were also included for every PCR mix prepared. The PCR amplification was performed on a Techne Flexigene (Barloworld Scientific, UK) thermal cycler and included an initial denaturing step at 94°C for 5 minutes, followed by 30 cycles of denaturing at 94°C for 60 sec, annealing at 54°C for 60 sec, and elongation at 72°C for 90 sec; a final elongation step at 72°C for 6 minutes was also performed. Following PCR, the size of the amplicons in relation to DNA Hyperladder I (Bioline, UK; Appendix 1) was determined by electrophoresis in 1.5% (w/v) agarose gels as described previously (2.2.5).

The amplified PCR products were purified (from primers, nucleotides, *Taq* DNA polymerase and salts) using a QIAquick® PCR purification kit (QIAGEN Ltd., Crawley, UK), following manufacturer's instructions. The purified PCR products were eluted in 50 $\mu$l of nuclease-free $H_2O$ (Ambion, USA), quantified with PicoGreen (2.2.5) and stored at -20°C until sequencing.

### 3.2.5 Preparation of the clone libraries (from the groundwater and sand sample from 30 mbgl in Borehole 59)

*DNA extractions and PCR amplifications*

Two 16S rRNA gene clone libraries were created during this study; one from the groundwater sample (planktonic community) and one from a sand sample (attached community) taken from 30 mbgl in borehole 59. Total community genomic DNA from each sample was extracted using the MoBio UltraClean™ Microbial DNA Kit (see 2.2.4) and used as template in PCR reactions which amplified full-length 16S rRNA gene sequences (as described in 3.2.4). PCR products were then purified with the QIAquick® PCR purification kit (3.2.4) and quantified with PicoGreen (2.2.5).

*Cloning into pGEM-T vector and transformation in* E. coli *DH5α competent cells*

Purified PCR products were ligated into pGEM®-T Easy Vector System I (Fig. 3.2; Promega Corporation, Madison, WI, USA), according to manufacturer's instructions. For the ligations, 30 ng of PCR products was used, to give a molar ratio of the insert DNA to the vector close to 1:

$$\frac{\text{Insert DNA}}{\text{Vector}} = \frac{\dfrac{\text{ng of insert}}{\text{kb size of insert}}}{\dfrac{\text{ng of vector}}{\text{kb size of vector}}} = \frac{\dfrac{30\text{ ng}}{1.5\text{ kb}}}{\dfrac{50\text{ ng}}{3\text{ kb}}} = 1.2$$

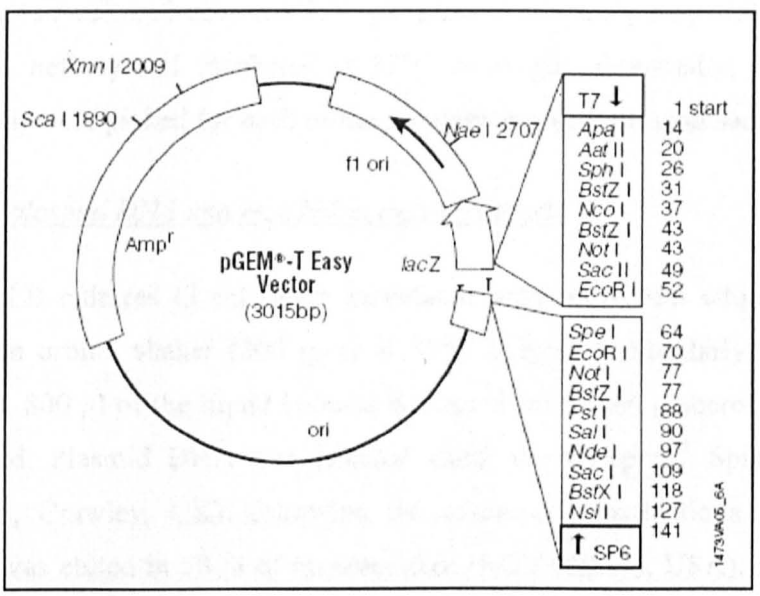



**Figure 3.2.** pGEM®-T Easy Vector circle map and sequence reference points. Image taken from the pGEM®-T Easy Vector Systems Technical manual (Promega, USA).

Following incubation at room temperature for one hour, 1 μl of the ligation reaction was added to 100 μl of *E. coli* Library Efficiency® DH5α™ Competent Cells (Invitrogen, UK) and incubated on ice for 30 min. The cells were then heat-shocked at 42°C for 45 sec and placed on ice for 2 min, before being added to 900 μl of room temperature S.O.C. medium (supplied with the competent cells; Invitrogen, UK) and shaken on an orbital shaker at 220 rpm at 37°C for 1 hour. Various volumes of the transformed cells (50-200 μl) were plated on LB agar plates which contained 100 μg $ml^{-1}$ ampicillin and had been spread with 10 μl of 1M IPTG (Isopropyl β-D-1-

thiogalactopyranoside) and 40 μl of X-Gal (20 mg $ml^{-1}$, prepared in formamide; 5-Bromo-4-chloro-3-indonyl-β-D-galactoside). The plates were incubated at 37°C overnight and successful ligation of an insert (PCR product) into the pGEM-T plasmid vector was identified by the presence of white colonies of ampicillin-resistant *E. coli* DH5α cells. The presence of IPTG induces the expression of β-galactosidase (*lacZ*), which hydrolyses X-Gal to galactose and an intense blue precipitate (5,5'-dibromo-4,4'-dichloro-indigo), resulting in blue-coloured colonies. However, successful ligation of an insert (PCR product) into the vector disrupts the coding sequence of β-galactosidase (*lacZ* in Fig. 3.2) and cell colonies appear white. Thus, only white colonies that contained the ligated PCR fragments were sub-cultured onto new LB agar plates (containing ampicillin and spreaded with X-Gal, as before) and incubated at 37°C overnight. Eventually, more than 250 colonies (clones) were picked for each of the groundwater and the sand samples.

*Preparation of plasmid DNA and restriction enzyme digests*

Liquid LB cultures (3 ml) were inoculated with individual white colonies and incubated on an orbital shaker (200 rpm) at 37°C overnight. Similarly to the isolated bacteria (3.2.2), 800 μl of the liquid cultures was used for frozen glycerol stocks and 1.5 ml was pelleted. Plasmid DNA was isolated using the QIAprep® Spin Miniprep kit (QIAGEN Ltd., Crawley, UK), following manufacturer's instructions. The extracted plasmid DNA was eluted in 50 μl of nuclease-free $H_2O$ (Ambion, USA), quantified with PicoGreen (2.2.5) and stored at -20°C until sequencing.

In order to examine whether the vector inserts were of the correct size, restriction enzyme digests were performed with *Eco*R I (Promega, USA), which cleaves the pGEM-T vector at two points, from each side of the insert (Fig. 3.2). In 20 μl reactions containing 2 μl of 10 x Buffer H and 0.2 μl of 10 μg $\mu l^{-1}$ acetylated BSA, 5 μl of plasmid DNA was digested with 0.5 μl of *Eco*RI for 1 hour at 37°C and the enzyme was de-activated by transferring at 65°C for 15 min. The size of the vector inserts in relation to Hyperladder I (Bioline, UK; Appendix 1) was determined by electrophoresis in 1.5% (w/v) agarose gels, as described previously (2.2.5). Only clones with the correct insert size were processed for sequencing.

*PCR amplification of the cloned 16S rRNA gene fragment*

Sterile toothpicks were used to pick individual white colonies and inoculate the wells of flat-bottom Costar-3595 96-well plates (Corning Incorporated, Corning, NY, USA) that contained 200 μl of liquid LB supplemented with 100 μg $ml^{-1}$ ampicillin. The plates were sealed with gas permeable adhesive membrane (AB gene, Limited, Epsom, UK) and left on an orbital shaker (100 rpm) at 37°C overnight. The 200 μl liquid cultures were then split into two 96 well plates. In the first plate, 40 μl of 80% sterile glycerol was added in each well and the plate was stored at -80°C as frozen glycerol stocks. The second plate was centrifuged at 3,000 x g for 40 min and then the supernatant was removed and 50 μl of nuclease-free $H_2O$ (Ambion, USA) was added. After gentle vortexing, the plate was heated for 8 min at 99°C and then centrifuged at 3,000 x g for 50 min. Two μl of the supernatant was used as a template in 25 μl PCR reactions, containing 0.5 units of *Taq* DNA polymerase (Bioline, UK), 1 x $NH_4$ reaction buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.3 μM of each M13F (5'–GTAAAACGACGGCCAGTG–3') and M13R (5'–GGAAACAG CTATGACCATG–3') primers. M13F and M13R primers bind to the pGEM-T vector and amplify the whole length of the insert. PCR conditions included an initial denaturing step at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 min, 55°C for 60 sec, 72°C for 2 min, and a final elongation step at 72°C for 10 minutes. The size of the amplicons in relation to DNA Hyperladder I (Bioline, UK; Appendix 1) was checked in 1% (w/v) agarose gels, as described previously (2.2.5).

The PCR products were purified by adding an equal volume (20 μl) of SureClean reagent (Bioline, UK) in each well. After incubation at room temperature for 10 min, the plate was centrifuged at 3,000 x g at 4°C for 50 min and the supernatant was removed by vortexing at 190 x g for 3 sec. Following 2 washes with 40 μl of 70% ethanol, the plate was air-dried and the PCR products were resuspended in 10 μl of nuclease-free $H_2O$. The plates were kept at -20°C until sequencing.

### 3.2.6 Sequencing of isolated bacteria and environmental clones

Cycle sequencing reactions were set up in low-profile 96-well microplates (Greiner Bio-One Ltd, Stonehouse, UK). As DNA template, about 40 ng of PCR amplified 16S rRNA gene from the isolated bacteria, and 200 ng of plasmid DNA or 2 μl of M13 PCR products of the clones was used. Each 10 μl reaction contained DNA template, 0.5 μl of ABI PRISM® BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA, USA), 1.75 μl of 5 x Buffer, and 0.64 μl of one 1 μM primer. The environmental clones were sequenced with the 27F primer only (Lane, 1991), whereas the isolates were sequenced with 338F (Whiteley and Bailey, 2000) as well as 27F primers to obtain a longer 16S rRNA gene sequence. PCR amplification was performed on a Techne Flexigene (Barloworld Scientific, UK) thermal cycler, using an initial denaturing step at 96°C for 10 min, which was followed by 30 cycles of 94°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Following PCR amplification, 10 μl of $H_2O$, 2 μl of 3M sodium acetate (pH 5.5), 2 μl of 0.125M EDTA, and 70 μl of 95% ethanol were added to each well. The plate was incubated for 30 min in the dark and then centrifuged at 3,500 rpm (1,424 x g) using a Hermle Z 300 K centrifuge (HERMLE Labortechnik, Germany) with a 220.50 V06 Swing out 2 x Microtiter rotor. Following centrifugation, the supernatant was removed and two washes with 150 μl of 70% (v/v) ethanol were performed. The precipitated DNA was air-dried in the dark for 20 min, resuspended in 20 μl of Hi-Di™ formamide (Applied Biosystems, USA) and then 10 μl was transferred in a new 96-well microplate. The plate was covered with clean rubber septa and after a denaturing step at 95°C for 3 min, it was placed on ice for about 10 min. Finally, the plate was loaded to an AB/Hitachi 3730 DNA analyser (Hitachi High Technologies Corporation, Tokyo, Japan).

The raw chromatograms from the DNA analyser were viewed using Sequencing Analysis Software Version 5.1 (Applied Biosystems, USA) and good quality nucleotide sequences were copied into FastA files. Using BioEdit sequence alignment editor version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html), multiple reads of the same sequence with different primers were aligned and joined into a single, long 16S rRNA gene sequence.

All 16S rRNA gene sequences obtained were checked for chimera formation with the Mallard version1.02 software programme (Ashelford *et al.*, 2006), using the default procedures and settings. Sequences were firstly aligned together with the reference sequence of *Escherichia coli* K12 MG1655 [Accession number: U00096] using the ClustalW v1.4 multiple sequence alignment programme (Thomson *et al.*, 1994), which was incorporated in BioEdit. The aligned file was then imported to Mallard and a window size of 300, a step size of 25 and a 99.9% cut-off value were used to identify anomalous sequences. These potentially chimeric sequences were further analysed by pairwise comparisons to their closest GenBank matches using Pintail (Ashelford *et al.*, 2005), to evaluate that the sequences were truly chimeric.

### 3.2.7 Phylogenetic analysis of the sequences

The closest relatives for non-chimeric 16S rRNA gene sequences of this study were identified by nucleotide Blast search (http://blast.ncbi.nlm.nih.gov/Blast) on the 20$^{th}$ of June 2008, using the default settings (blastn algorithm, nr database, expect threshold = 10, word size = 11, match/mismatch scores = 2/-3, gap costs = existence 5/extension 2). In addition, the closest type strain relative was found using the "sequence analysis" tool of the Ribosomal Database Project II (Release 9.61; http://rdp.cme.msu.edu) (Cole *et al.*, 2007). The RDP search was performed using both uncultured & isolated microorganisms but only for good quality sequences over 1,200 bp length.

Phylogenetic trees were constructed with MEGA version 4 (Tamura *et al.*, 2007), following alignment with the incorporated ClustalW v1.4. Evolutionary relationships were inferred by the Neighbour-Joining method (Saitou and Nei, 1987) and the evolutionary distances were computed using the Jukes-Cantor model (Jukes and Cantor, 1969) in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Bootstrapping (Felsenstein, 1985) was performed for 1000 replicates.

## 3.3 Results

### 3.3.1 Colony morphology of isolated bacteria from the Four Ashes site

Bacteria were isolated on R2A agar from groundwater and sand samples taken from 30 mbgl in borehole 59. In total, 80 bacterial colonies from groundwater and 45 from sand were isolated and named with the prefix "ig" (isolates groundwater) and "is" (isolates sand) respectively. The isolates were characterised by a range of colony morphologies, including different colour (red, pink, orange, yellow, cream, and white), size (large, medium, small, pinpoint), margins and consistency (smear, viscous, butyrous, dry/rough, wrinkled). Based primarily on the colony colour and secondly on other distinct characteristics (size, margins, consistency), the observed colony morphologies could be divided roughly into 18 groups/morphotypes (Table 3.1). Representative isolates from each morphotype are shown in Figures 3.3 – 3.5. Four isolates that could not be subcultured from the frozen glycerol stocks (ig221, ig225, ig228, ig238) are not included in Table 3.1, and isolates that were not identified by 16S rRNA gene sequencing are indicated with an asterisk. Sequencing of the remaining isolates, showed that some of the morphotypes consisted of more than one organism (e.g. the vaguely grouped morphotypes 11 and 18), whereas other distinctly unique morphotypes consisted of a single strain. For example all isolates with large white colonies of butyrous consistency (morphotype 8) were identified as the same *Bacillus* sp., and all white colonies with dry/rough surface but orange bottom (morphotype 13) were identified as *Nocardia* sp.

Apart from differences in colony morphology, isolate ig264 (later identified as *Pseudomonas* sp.) was fluorescent when grown in R2A liquid broth compared to non-fluorescent isolates (Fig. 3.6; image taken using a UV benchtop transilluminator; UVP, LLC, Upland, CA, USA).

**Table 3.1.** The observed colony morphotypes of the isolated bacteria from the groundwater and sand samples from the Four Ashes site. All isolates have been identified by 16S rRNA gene sequencing, apart from the ones indicated with an asterisk.

| Morphotype | Colony colour | Other characteristic | Isolates |
|---|---|---|---|
| 1 | Red | Pinpoint | ig208* |
| 2 | Pink | Fungus-like | ig263 |
| 3 | | Smear | ig232 |
| 4 | | Pinpoint | ig252, ig241* |
| 5 | Orange | Bright orange | ig217, ig223 |
| 6 | | Dark orange | ig220, ig254 |
| 7 | Yellow | Medium colony, wrinkled surface | ig253 |
| 8 | | Small, very bright yellow | ig207, ig214, ig215, ig226, ig227, ig247, ig249, ig265, ig269, ig271, ig272, is283, is288, is292, is293, is296, is03, is04b, is06, is07, is12 |
| 9 | | Small/pinpoint | ig212, ig219, ig233, ig236, ig237, ig239, ig243, ig240, ig244, ig248, ig250, ig255, ig257, ig258, ig259, ig262, ig266*, ig267, ig268 |
| 10 | Cream | Medium and smear | ig204, ig205*, ig206, ig231*, ig234, ig245, ig59/30-1, ig59/30-3, ig59/30-5, |
| 11 | | Medium/small and viscous | ig59/30-4, ig59/30-6, ig59/30-7, ig59/30-8, ig211, ig213, ig222, ig224, ig235, ig251, ig260, is01*, is05, is13, is15, is19 |
| 12 | | Distinct small | ig246, ig261, ig264 |
| 13 | White | White dry/rough surface but orange bottom | is280, is291, is04a |
| 14 | | Large, butyrous (buttery) | ig203, is274, is276, is277, is278, is297 |
| 15 | | Smear | ig242, ig256 |
| 16 | | Large viscous | is273, is279*, is281, is285, is287, is289, is09, is10, is11, is17 |
| 17 | | Medium | ig201, ig202, ig230, ig282, ig290, ig59/30-2 |
| 18 | | Small | ig209, ig210, ig216, ig218, ig229, ig270, is275, is284, is286, is290, is294, is02, is08, is14, is16, is18 |

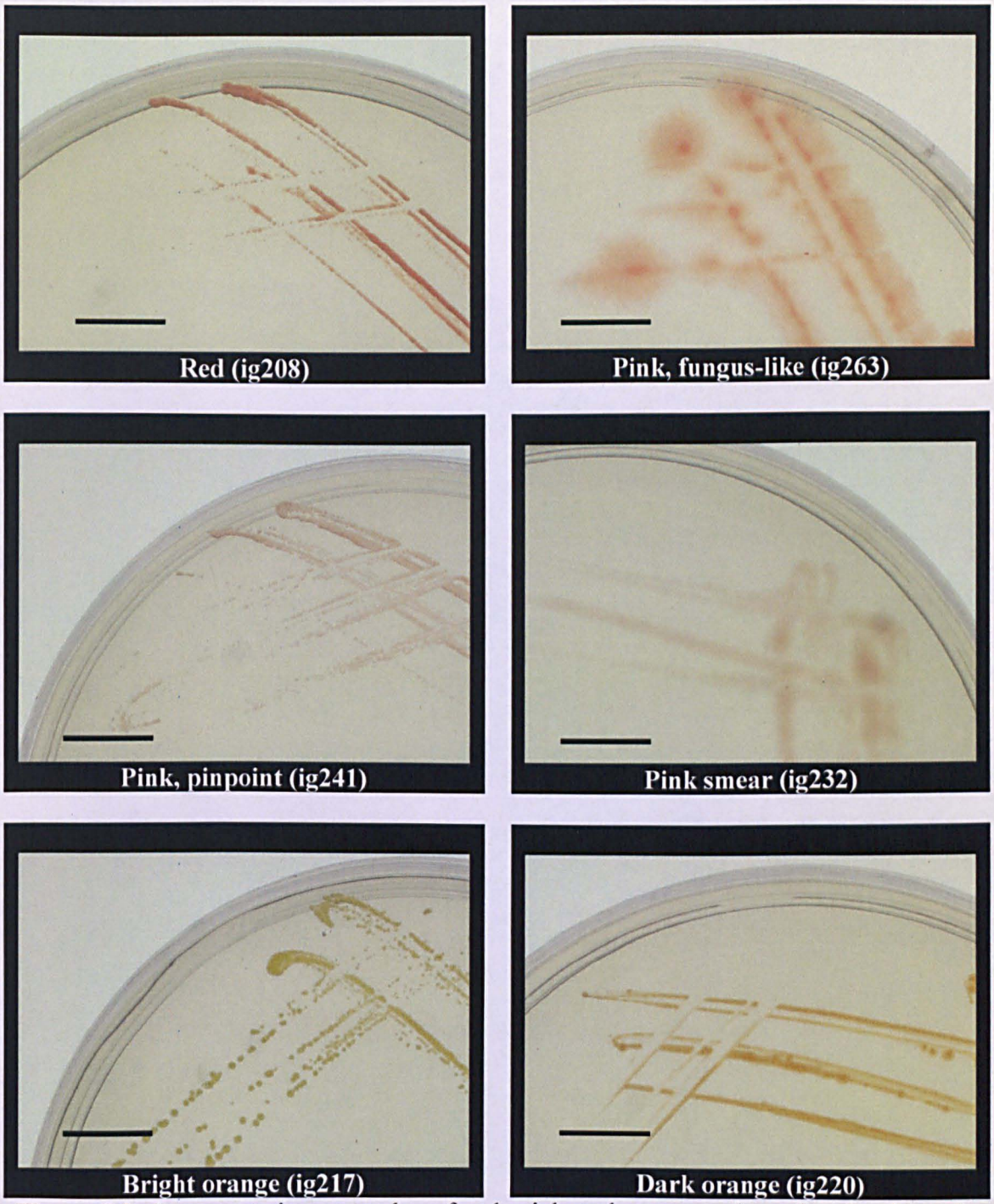



**Figure 3.3.** Representative examples of red, pink and orange colony forming bacteria from the Four Ashes site. Each image represents an area of approx. 5 x 3.5 cm and the scale bar equals 1 cm.

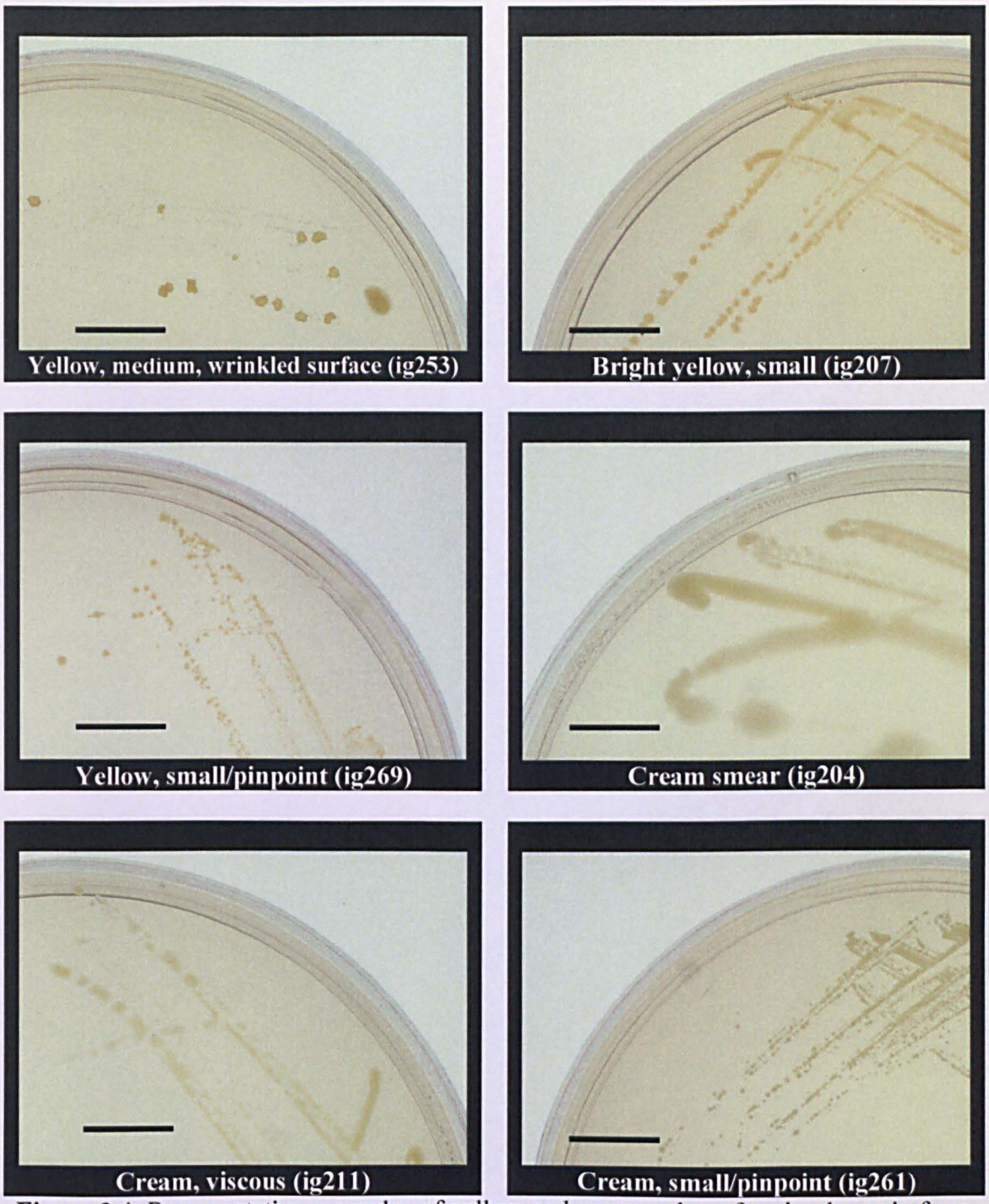



**Figure 3.4.** Representative examples of yellow and cream colony forming bacteria from the Four Ashes site. Each image represents an area of approx. 5 x 3.5 cm and the scale bar equals 1 cm.

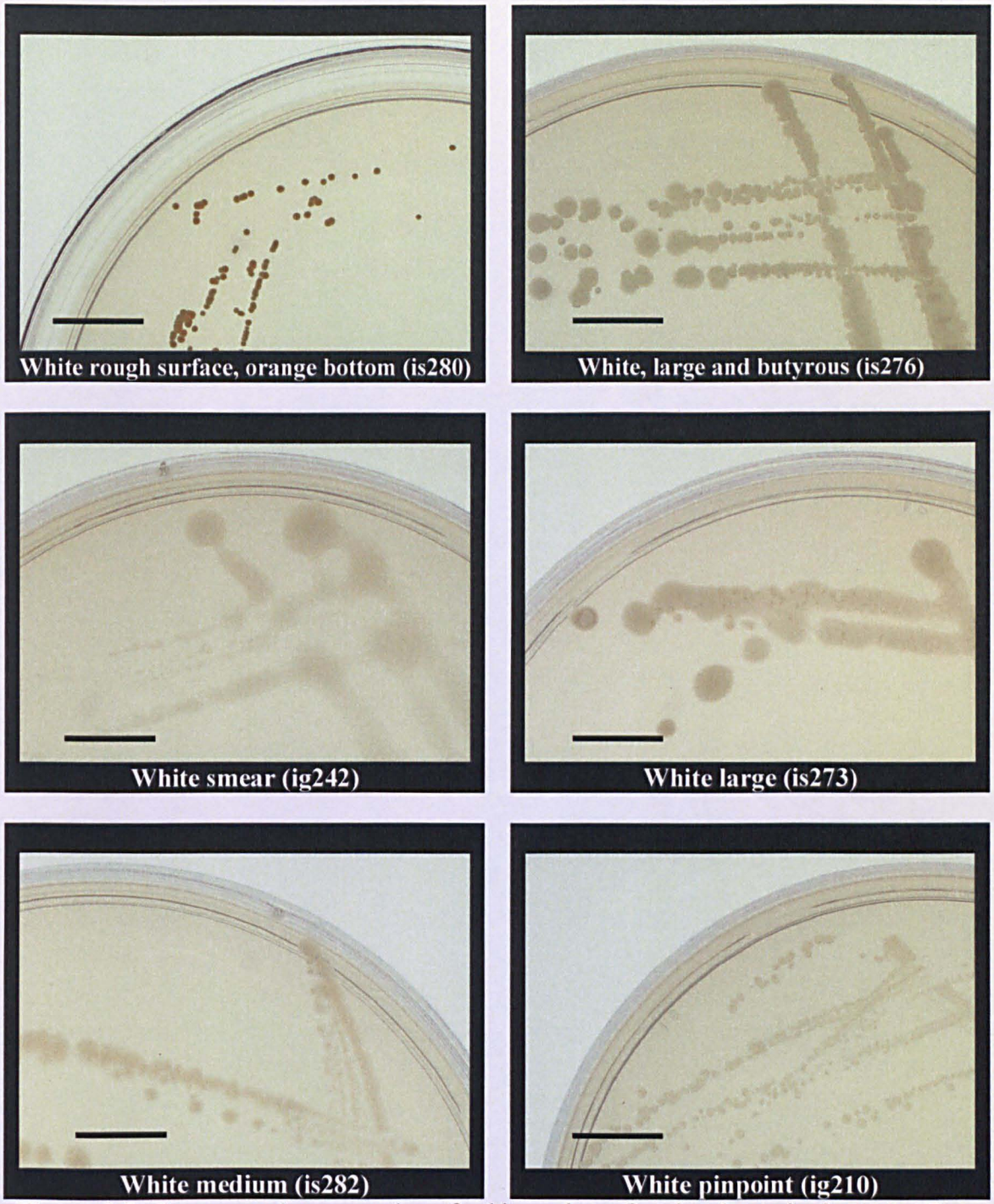



**Figure 3.5.** Representative examples of white colony forming bacteria from the Four Ashes site. Each image represents an area of approx. 5 x 3.5 cm and the scale bar equals 1 cm.

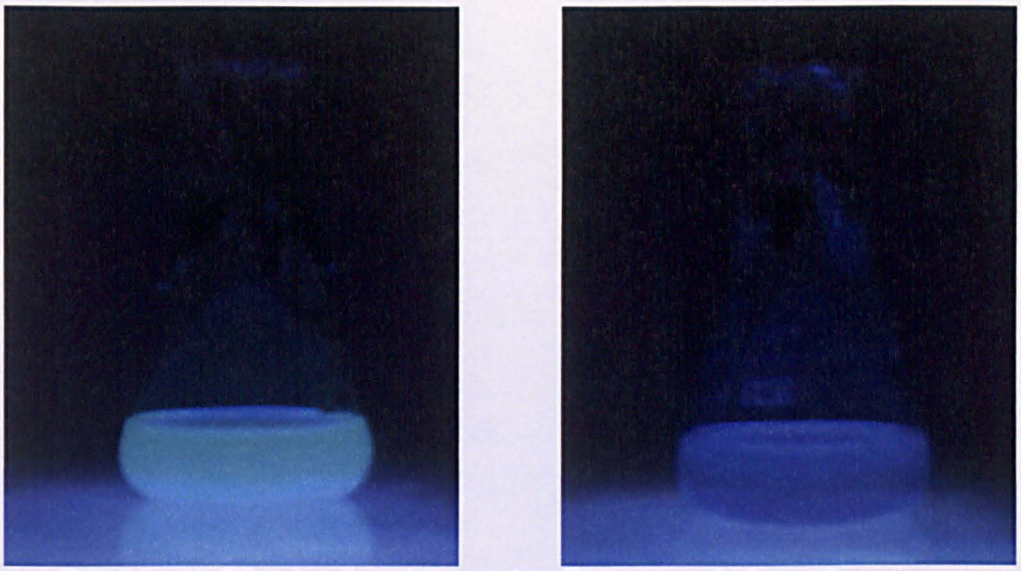


**Figure 3.6.** Liquid R2A batch cultures of a fluorescent isolate (*Pseudomonas* sp. ig264, on the left) and a non-fluorescent isolate (*Acidovorax* sp. is19, on the right).

### 3.3.2 Sequencing of isolated microorganisms and environmental clones

*Isolates*

Genomic DNA was extracted from each isolate and used as template in PCR reactions that generated almost full-length 16S rRNA gene amplicons (Fig. 3.7). Purified amplicons were then sequenced as described in 3.2.6 and long 16S rRNA gene sequences were obtained from 71 groundwater isolates and 43 sand isolates.

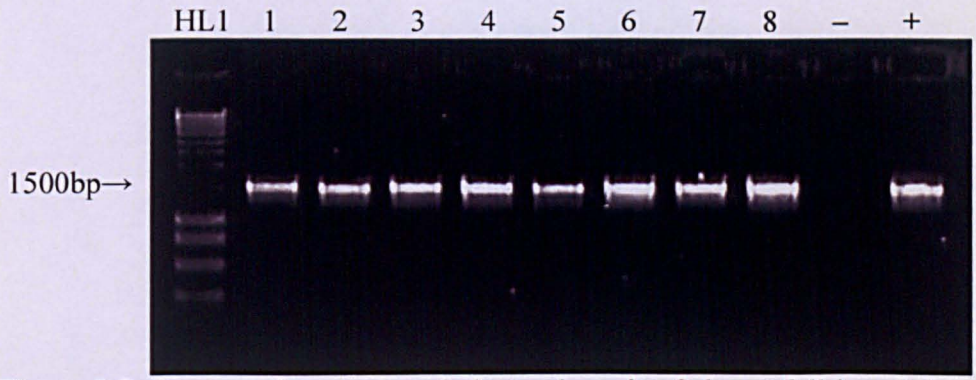



**Figure 3.7.** An example agarose gel electrophoresis of almost full-length (approx. 1500 base pairs long) 16S rRNA gene PCR amplicons from isolated bacteria ig251, ig254, ig257, ig258, ig259, ig262, ig264, ig267 (lanes 1 – 8 respectively). HL1=hyperladder 1 (Bioline, UK), negative (–) and positive (+) controls.

*Clones*

In addition to the culture dependent method, two clone libraries were created from the groundwater and the sand samples taken from 30 mbgl in borehole 59. As described in 3.2.5, two different routes were followed for the sequencing of the clone libraries.

For most of the sequenced clones (65%), the plasmid DNA was extracted and restriction enzyme digests were performed with *Eco*R I, in order to examine the size of the vector inserts. In many cases *Eco*R I cleaved not only the pGEM-T vector but also the inserts themselves and at different sites, indicating a first sign of diversity amongst the clones (Fig. 3.8). Only clones with plasmid inserts of the correct size (adding up to approx. 1500 base pairs) were processed to sequencing.

For the remaining 35% of the sequenced clones, PCR using M13F and M13R primers was performed to amplify the full length of the vector inserts. Once again, only PCR amplicons of the correct size (approx. 1500 base pairs long; Fig.3.9) were processed to sequencing. In total 132 groundwater clones and 143 sand clones were sequenced. Groundwater clones were labelled with the prefix "G" (in capital) whereas sand clones were labelled with prefix "S".

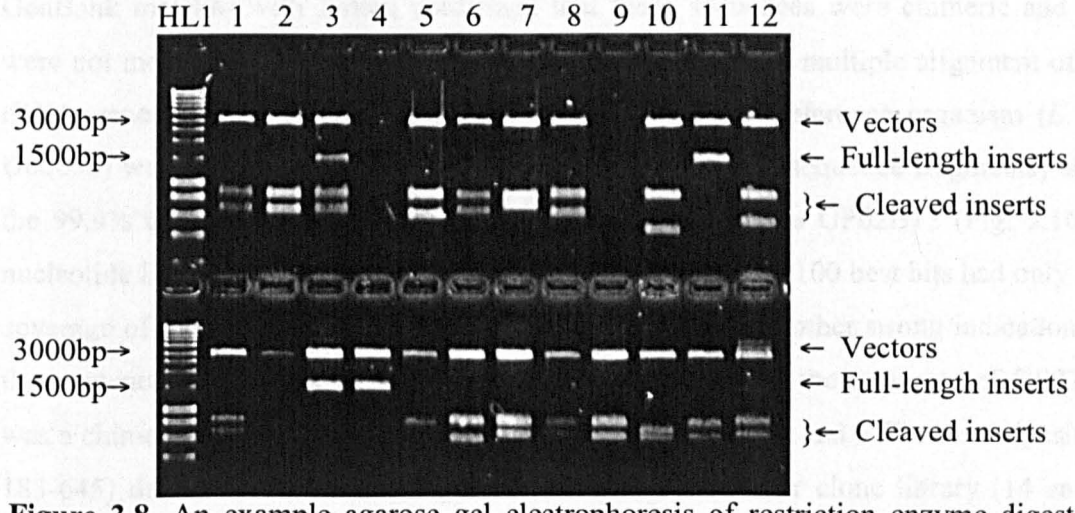



**Figure 3.8.** An example agarose gel electrophoresis of restriction enzyme digests of isolated plasmid DNA with *Eco*R I. Upper row: sand clones S01 to S12 (lanes 1 to 12 respectively). Lower row: sand clones S13 to S24 (lanes 1 to 12 respectively). HL1 = hyperladder 1 (Bioline, UK).

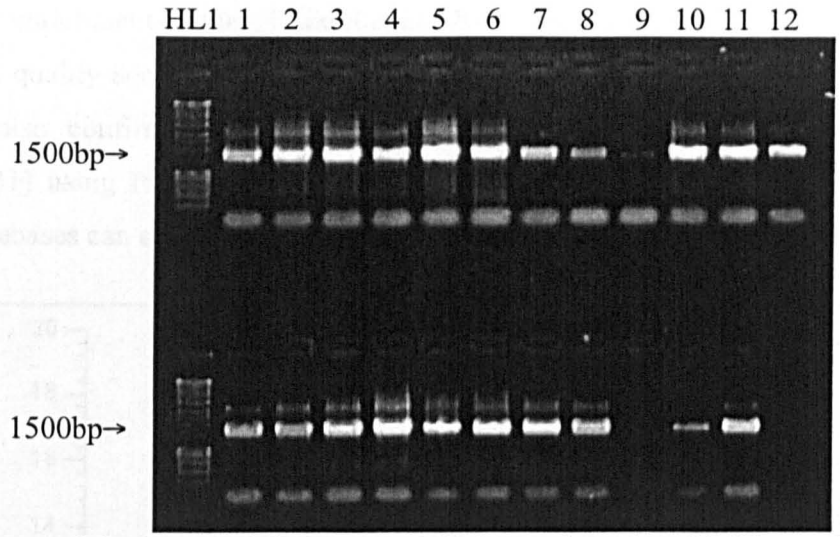



**Figure 3.9.** An example agarose gel electrophoresis of PCR amplified clone vector inserts. Upper row: sand clones SP01C01 to SP01C12 (lanes 1 to 12 respectively). Lower row: sand clones SP01D01 to SP01D12 (lanes 1 to 12 respectively). HL1 = hyperladder 1 (Bioline, UK).

*Chimera check*

All 16S rRNA gene sequences obtained were checked for anomalies using Mallard (see 3.2.6) and 10 sequences from groundwater and 7 sequences from sand clones were identified as anomalous. Further analysis of these sequences against their closest GenBank matches with Pintail confirmed that these sequences were chimeric and they were not included in further analyses. As an example, when a multiple alignment of 16S rRNA gene sequences from 60 groundwater clones and a reference organism (*E. coli* U00096) was analysed with Mallard, 19 outliers (anomalous sequence fragments) above the 99.9% cut-off line were identified, all belonging to clone GP02B12 (Fig. 3.10). A nucleotide Blast search of GP02B12 showed that 99 out of the 100 best hits had only 71% coverage of the length of its sequence (positions 183-645), another strong indication that the sequence was chimeric. Subsequent analysis showed that the sequence of GP02B12 was a chimera between a *β-Proteobacterium* (positions 1-182) and a *Firmicute* (positions 183-645) that were both found repeatedly in the groundwater clone library (14 and 12 times respectively).

Interestingly, the first GenBank match of GP02B12 with 88% identity (and 99% coverage) was the uncultured beta proteobacterium NE62 [AJ575696] that was found in

lake water enrichment culture (Burkert *et al.*, 2003). However, this environmental clone is of suspect quality according to RDP II database (which incorporates Pintail), something that we also confirmed by pairwise comparison with its closest GenBank match [DQ017931] using Pintail (not shown here). The deposition of chimeric sequences in public databases can compromise their reliability (discussed in 3.4.1).

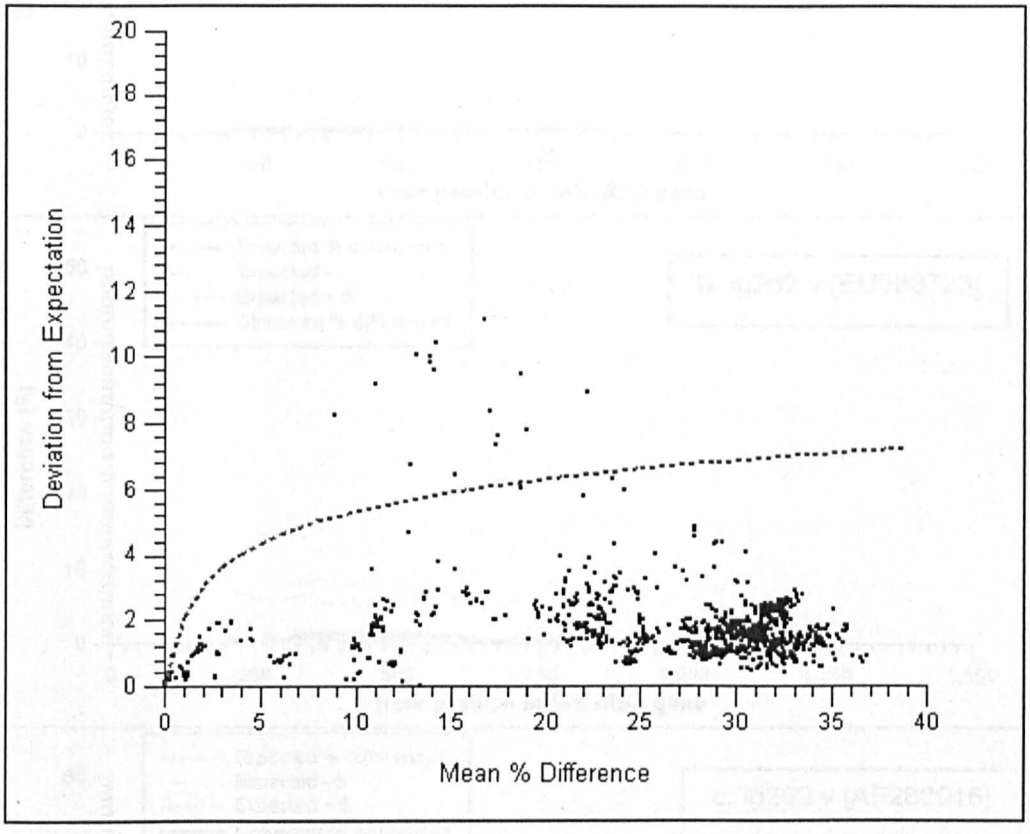



**Figure 3.10.** An example of a Mallard plot, where a multiple alignment of 16S rRNA gene sequences of 60 groundwater clones was analysed and 19 outliers above the cut-off line were identified, all belonging to clone GP02B12.

In addition to the sequences from 17 clones, Mallard found strong evidence that the sequence of isolate ig262 was also chimeric. However when ig262 was subjected to pairwise comparisons in Pintail with its closest matches, no sequence anomaly could be detected (Fig. 3.11), suggesting that the sequence ig262 was falsely recognised as chimeric by Mallard. Isolate ig262 was checked against its closest uncultured and cultured GenBank matches (with accession numbers of EF540435 and EU583723 respectively), as well as its closest type strain relative, *Pigmentiphaga kullae* (T) K24 [AF282916].

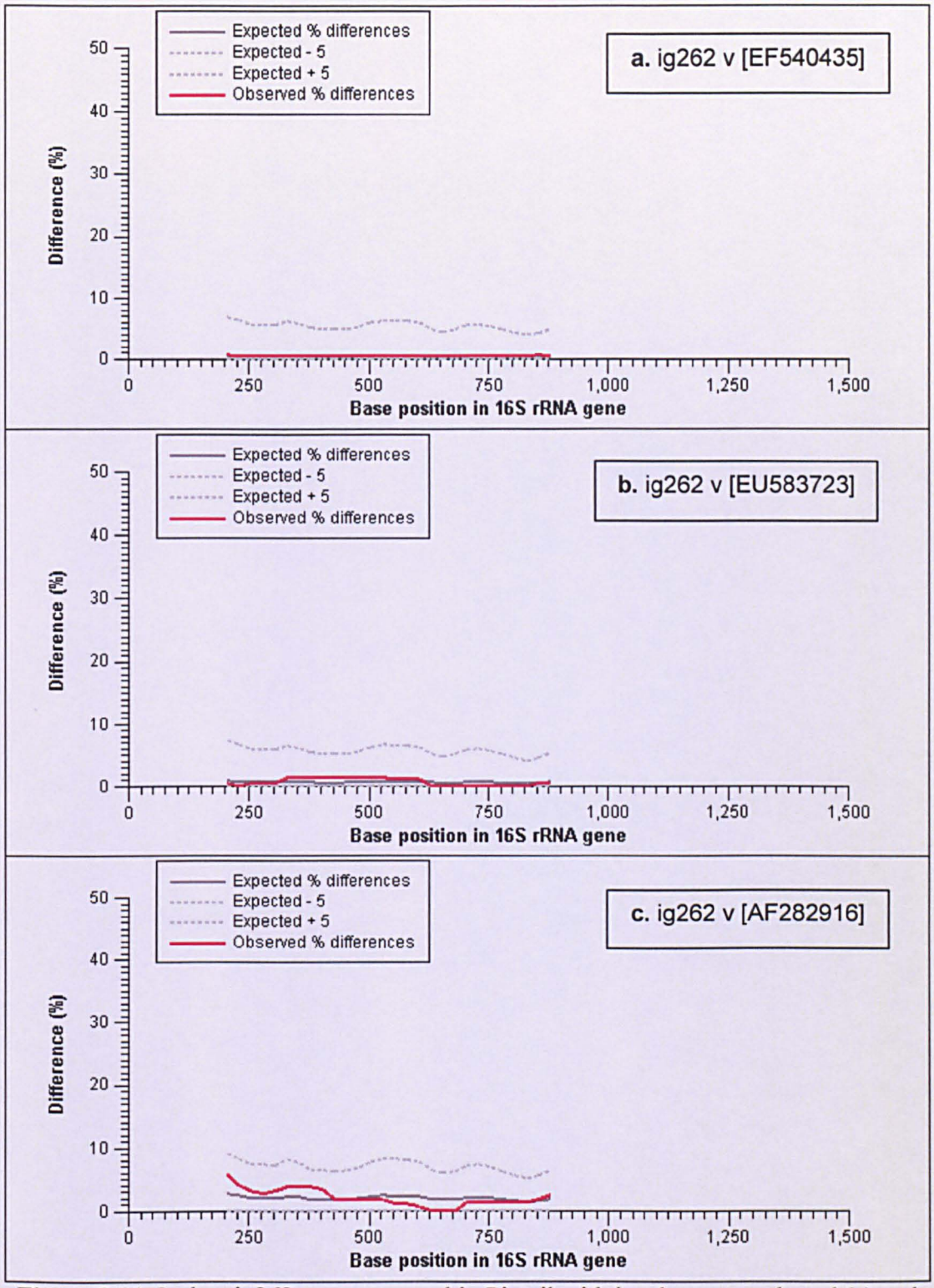



**Figure 3.11.** Isolate ig262 was compared in Pintail with its closest uncultured (a) and cultured (b) GenBank matches, as well its closest type strain relative (c) and no sequency anomalies could be detected. The accession numbers of these matches are shown in square brackets.

After removing all the chimeric sequences, 114 sequences from isolates (71 from groundwater and 43 from sand isolates), 122 from groundwater clones and 136 from sand clones remained (Table 3.2).

**Table 3.2.** The initial and the final number of sequences in each one of the four sequence groups (groundwater and sand isolates, groundwater and sand clones), after removal of the chimeric sequences. The number of the chimeric sequences detected as well as their percentage in the total sequence library is also shown.

| | Isolates | | Clones | |
|---|---|---|---|---|
| **No. of sequences** | **Groundwater** | **Sand** | **Groundwater** | **Sand** |
| Initial | 71 | 43 | 132 | 143 |
| Chimeric (%) | - | - | 10 (7.6%) | 7 (4.9%) |
| Final | 71 | 43 | 122 | 136 |

### 3.3.3 Identification of the sequenced 16S rRNA gene fragments and ribotype groups

Preliminary analyses (not shown) of all non-chimeric 16S rRNA gene sequences showed that in many cases more than 2 sequences were identical over their overlapping lengths (> 99 % sequence identity) when aligned with ClustalW and clustered together in a Neighbour-Joining phylogenetic tree that was constructed with MEGA4. These sequences were grouped together into what we define as a "ribotype" and normally the longest sequence was kept as the representative of this ribotype group. For each ribotype, the closest type strain relative was identified using RDP II. These matches are shown in Appendix 3, together with the closest GenBank matches (cultured organisms or/and uncultured environmental clones) only when the latter were different and had equal or higher % identity similarity than the type strains. Throughout this study, type strains are indicated by an uppercase T enclosed in parentheses next to the species name and sequence accession numbers are shown in square brackets.

In total, the groundwater isolates grouped into 38 ribotypes, the sand isolates in 10, the groundwater clones in 28 and the sand clones in 75 (Table 3.3). Table 3.3 also shows the sequence frequency distribution within these identified ribotypes. It was noticeable that within the 6 largest ribotypes of the groundwater clone library belonged 85 clones (about 70% of the total number of groundwater clones), whereas the 6 largest

ribotypes of the sand clone library totalled 44 clones (32% of the total). Based on the number of the identified ribotypes and the number of the sequences belonging to each of them, community diversity indices for the isolated bacteria and the clone libraries (both groundwater and sand) were calculated (Table 3.3). For the isolates, both Shannon and Simpson diversity indices showed that the groundwater isolates were more diverse. On the other hand, the diversity indices showed that the sand clone library was more diverse than the groundwater clone library. Furthermore, species (ribotypes in this study) accumulation curves were plotted for the groundwater and sand clone libraries (Fig. 3.12) using UGE analyses (Ugland *et al.*, 2003) for 999 permutations in the PRIMER 6 software programme (PRIMER-E Ltd, UK). Both curves indicated that the maximum number of expected species was not covered within the sequenced number of clones, and particularly for the sand clone library (its species accumulation plot is less curved).

**Table 3.3.** The total number of sequences, the number of identified ribotypes, the sequence frequency distribution within the ribotypes, and the calculated community diversity indices, for each sequence group (groundwater and sand isolates, groundwater and sand clones).

| Parameter | | Isolates | | Clones | |
|---|---|---|---|---|---|
| | | Groundwater | Sand | Groundwater | Sand |
| Total no. of sequences ($N$) | | 71 | 43 | 122 | 136 |
| No. of ribotypes ($S$) | | 38 | 10 | 28 | 75 |
| No. of ribotypes with X sequences | X = 25 seq. | - | - | 1 | - |
| | 17 | - | - | - | 1 |
| | 16 | - | - | 1 | - |
| | 14 | - | - | 1 | - |
| | 12 | - | - | 1 | - |
| | 9 | 1 | 2 | 2 | - |
| | 8 | - | - | - | 1 |
| | 7 | - | - | - | 1 |
| | 6 | 1 | - | - | - |
| | 5 | - | 3 | - | 1 |
| | 4 | 4 | 1 | 2 | 1 |
| | 3 | - | 1 | 2 | 7 |
| | 2 | 8 | - | 5 | 11 |
| | 1 | 24 | 3 | 13 | 52 |
| Shannon index ($H'$) | | 3.364 | 2.074 | 2.707 | 3.954 |
| Evenness index ($H'/ H'_{max}$) | | 0.925 | 0.901 | 0.813 | 0.916 |
| Simpson index (1-$D$) | | 0.953 | 0.857 | 0.902 | 0.967 |

Shannon index $H' = -\Sigma_i p_i \ln(p_i)$
Shannon evenness index $= H'/ H'_{max} = H'/ \ln S$
Simpson index 1-D = $1-\Sigma p_i^2$

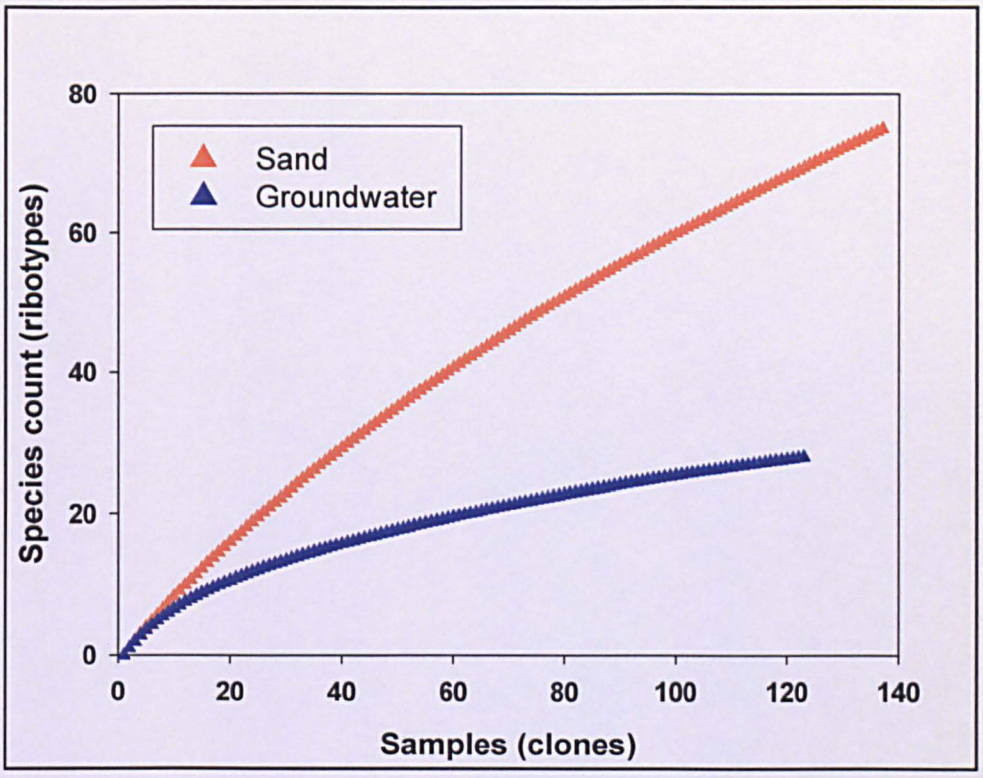



**Figure 3.12.** Species accumulation curves were plotted for the groundwater and the sand clone libraries.

The overlap between the identified ribotypes of all 4 sequence groups was very limited and it is shown graphically in Fig. 3.13. In detail, there were only 2 common ribotypes between the groundwater and the sand isolates, 5 common ribotypes between the sand isolates and the sand clone library and 3 ribotypes between the groundwater and the sand clones (Fig. 3.13 and Table 3.4). No overlap was found between the isolates and the groundwater clone library. More detailed information about these ribotypes can be found in Appendix 3 as well as in the corresponding tree and paragraph describing each phylogenetic group (in 3.3.4).

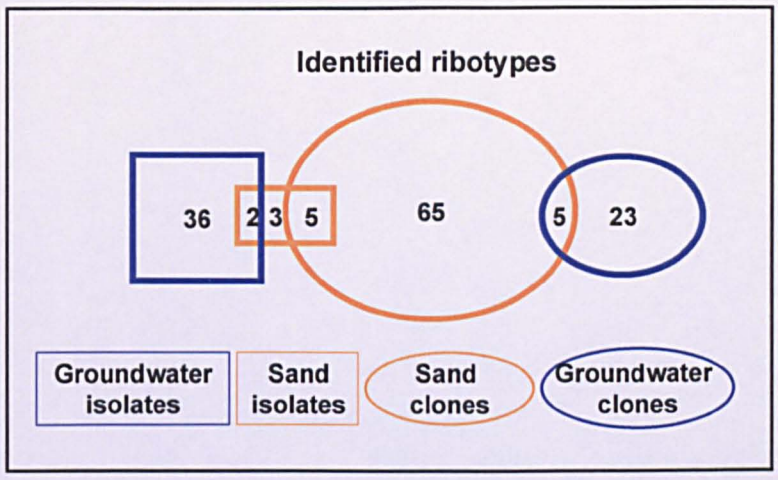



**Figure 3.13.** The number of identified ribotypes for each sequence group (groundwater isolates, sand isolates, groundwater clones and sand clones) and their overlaps.

**Table 3.4.** Common ribotypes shared between groundwater and sand isolates and clones.

| | Isolates | | Clones | | Phylogenetic | Closest phylogenetic |
|---|---|---|---|---|---|---|
| **Ribotype ID** | **GW** | **Sand** | **Sand** | **GW** | **group** | **affiliation** |
| is292 | 2 | 9 | - | - | *Actinobacteria* | *Rhodococcus fascians* |
| is297 | 1 | 5 | - | - | *Firmicutes* | *Bacillus* sp. |
| is19 | - | 4 | 3 | - | *β-Proteobacteria* | *Acidovorax* sp. |
| is14 | - | 5 | 17 | - | *β-Proteobacteria* | *Acidovorax* sp. |
| is17 | - | 9 | 1 | - | *γ-Proteobacteria* | *Pseudomonas* sp. |
| is282 | - | 1 | 1 | - | *γ-Proteobacteria* | *Pseudomonas* sp. |
| is290 | - | 1 | 1 | - | *γ-Proteobacteria* | *Pseudomonas* sp. |
| SP11F11 | - | - | 7 | 16 | *β-Proteobacteria* | *Azoarcus* sp. |
| SP12B04 | - | - | 8 | 14 | *β-Proteobacteria* | uncult. *Rhodocyclaceae* |
| SP12A07 | - | - | 3 | 1 | *α-Proteobacteria* | *Caulobacter* sp. |
| SP01C12 | - | - | 3 | 1 | *γ-Proteobacteria* | *Methylobacter* sp. |
| GP12E06 | - | - | 2 | 1 | *ε-Proteobacteria* | *Sulfurimonas* sp. |

GW = Groundwater

### 3.3.4 Phylogenetic analysis of the isolates and the clone libraries

The overlap between the isolated bacteria from the Four Ashes site and the clone libraries was limited (Fig. 3.13) to the 5 ribotypes that were shown in Table 3.4. Therefore for the purposes of the phylogenetic analysis, the sequences of all isolates (114) were grouped together and compared to the groundwater and the sand clone libraries, which were of comparable size (122 and 136 clones respectively). The phylogenetic analysis of the isolates, the groundwater clones and the sand clone libraries revealed differences in the composition of these 3 sequence groups (Fig. 3.14). With the exception of two *Deinococci* and one *Bacteroidete*, all the isolated microorganisms from the Four Ashes site belonged to 5 phylogenetic groups: *α-Proteobacteria* (7.9%), *β-Proteobacteria* (23.7%), *γ-Proteobacteria* (20.2%), *Actinobacteria* (38.6%) and *Firmicutes* (7%) and were members of a diverse range of genera and species (the identified number of ribotypes within each phylogenetic group is shown next to the barchart). Nevertheless, most of the isolates grouped within the *Comamodanaceae* family (*Variovorax, Acidovorax, Simplicicpira, Xylophilus, Rhodoferax* genera) of the β-Proteobacteria (22%), and the *Pseudomonas* (15%), *Rhodococcus* (12.3%), *Frigoribacteria* (8.8%), *Bacillus* (5.3%), and *Stenotrophomonas* (5.3%) genera. The groundwater clones were dominated by *β-Proteobacteria* (35%), *Bacteroidetes* (30%) and *Firmicutes* (22%), and their main characteristic was that they all clustered in different Bacterial orders than the isolated microorganisms (Figures 3.15 – 3.21). Orders with high number of groundwater clones included the *Rhodocyclales* (33.6%), the *Bacteroidales* (29.5%) and the *Clostridiales* (20.5%). Most of the groundwater clones were affiliated to uncultured *Bacteroidales* (20.5%), 2 uncultured *β-Proteobacteria* (11.5% and 7.4%), *Azoarcus* (13%), *Acetobacterium* (9.8%), and *Desulfosporisinus* (7.4%) species. The sand clones were characterised by the presence of *β-Proteobacteria* (41%), *α-Proteobacteria* (15.4%), *γ-Proteobacteria* (5.1%), *Bacteroidetes* (7.35%), as well as a large number (29%) of sequences that could not be classified or belonged to other bacterial phyla. Contrary to the groundwater clones, the sand clones were more diverse, spanning over many bacterial orders. Sand clones that were sequenced repeatedly were all within the *β-Proteobacteria*,

including the *Acidovorax-Simplicispira* (12.5%), uncultured *β-Proteobacterium* (5.9%), *Azoarcus* sp. (5.1%), and *Rhodoferax* (4.4%) genera.

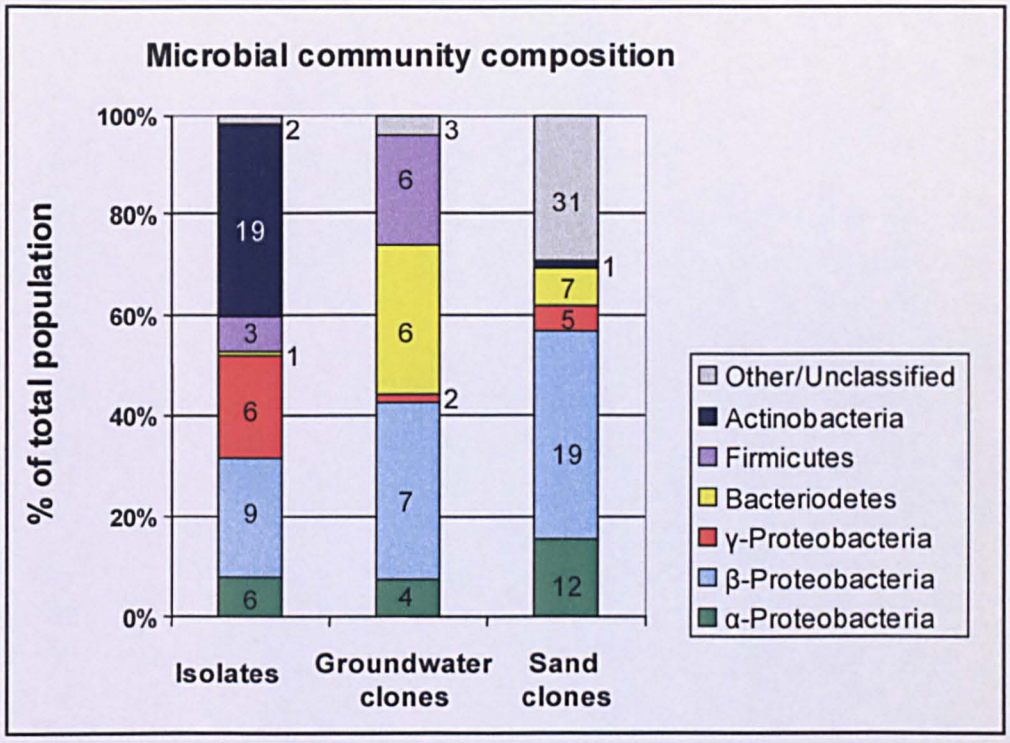



**Figure 3.14.** Phylogenetic comparison of the identified isolated microorganisms and the groundwater and sand clone libraries. The numbers indicate the no. of ribotypes belonging in each phylogenetic group.

Using the identified ribotypes (from all 3 sequence groups) and a selection of their closest matches (type strains or cultured microorganisms, where possible), a Neighbour-Joining phylogenetic tree was constructed with MEGA4 as described in 3.2.6. For illustrative purposes, the phylogenetic tree was split into six subtrees, one for each of the *α-, β-, γ-Proteobacteria, Firmicutes, Bacteroidetes,* and *Actinobacteria* (Figures 3.15 to 3.20 respectively), which were the largest phylogenetic groups in this study. The overall topology of the tree can be seen in Fig. 3.21 which includes the ribotypes that belonged to other bacterial phyla or could not be classified. A detailed description of the members of for each phylogenetic group follows (accession numbers are shown in square brackets).

**α-Proteobacteria**

Nine of the isolated bacteria from the Four Ashes as well as 9 groundwater and 22 sand clones (15.4% of the total) grouped within the α-Proteobacteria class and distributed along 5 different orders (Fig. 3.15). Isolates were members of the *Rhizobiales* (3 isolates), the *Sphingomonadales* (5) and the *Rhodobacterales* (1) while most of the groundwater clones belonged to the *Rhodospirillales* (8 out of 9). The sand clones were more diverse, with members in *Rhizobiales* (7), *Caulobacteriales* (5), *Sphingomonadales* (5), and *Rhodospirillales* (4). No overlap was observed between the isolates and the clones, and only one ribotype in the *Caulobacterales* (SP12A07) was shared by groundwater and sand clones.

In the *Rhizobiales* order, 3 groundwater isolates and 3 sand clones belonged to the *Rhizobium* genus. Ribotype ig209 (2 isolates) had 99% sequence identity to *Rhizobium radiobacter* (T) (Yang *et al.*, 1985) and isolate ig235 was closely related (97% identity) to *Rhizobium huautlense* (T) SO2 (Wang *et al.*, 1998); both these *Rhizobium* species are usually associated with plants and agricultural soils. On the other hand, 2 of the 3 sand clones belonging to the *Rhizobium* genus (ribotype SP01C11) had 100% identity to the phenol-degrading nitrate-reducing *Rhizobium radiobacter* C4 (Baek *et al.*, 2003). The remaining members of the *Rhizobiales* order (4 sand clones) were affiliated to *Devosia neptuniae* (T) J1 (Rivas *et al.*, 2002), *Bradyrhizobium* sp. BTA-1 (T) (Jarabo-Lorenzo *et al.*, 2003), *Blastochloris viridis* (T) ATCC19567 (Hiraishi, 1997) and to the methylotrophic *Methylocystis* sp. SV97 (T) (99% identity) that was isolated from an Arctic wetland soil (Wartiainen *et al.*, 2006b).

In the Rhodocyclales, ig260 was affiliated (97% identity) to *Rhodobacter ovatus* (T) JA234T, an isolate from an industrially polluted fresh water pond (Srinivas *et al.*, 2008) and to the *Rhodobacter sphaeroides* (T) 2.4.1 (96% identity), a facultative photoheterotrophic bacterium (Dryden and Kaplan, 1990).

In the *Caulobacterales*, ribotype SP12A07 (1 groundwater and 3 sand clones) was closely related (98%) to *Caulobacter fusiformis* (T) ATCC 15257 (Abraham *et al.*, 1999) while clone S48 was affiliated (99% identity) to *Brevundimonas* sp. FWC04 (Abraham *et al.*, 1999). Clone S30 was related (96% identity) to *Phenylobacterium lituiforme* (T)

FaiI3, a facultative anaerobic strain isolated from a thermal aquifer (Kanso and Patel, 2004).

In the *Sphingomonadales*, one isolate from sand (is12) was closely related (99% identity) to the chlorophenol-degrading *Sphingopyxis chilensis* (T) S37 (Godoy *et al.*, 2003) and the remaining 4 isolates (all from groundwater) belonged to the *Sphingomonas* genus. Two of them (ribotype ig217) were closely related (98% identity) to *Sphingomonas aquatilis* (T) JSS-7 which was isolated from natural mineral water (Lee *et al.*, 2001) while ribotype ig220 had 99% sequence identity to *Sphingomonas faeni* (T) MA-olki, an air-borne bacterium from Antarctica (Busse *et al.*, 2003). All the 5 sand clones belonging to the *Sphingomonadales* (ribotype SP01A08) were affiliated (97% identity) to the nonylphenol-degrading bacterium *Sphingomonas cloacae* (T) S-3 [AB040739] (*Sphingobium cloacae* according to the RDP II taxonomy) that was isolated from the wastewater of a sewage-treatment plant (Fujii *et al.*, 2001).

Finally, in the *Rhodospirillales*, 4 groundwater clones (ribotype GP01H11) were closely related (97% identity) to *Magnetospirillum magnetotacticum* (T) DSM 3856 (Schleifer *et al.*, 1991), a microaerophilic magnetotactic denitrifier (Bazylinski and Frankel, 2004), while 2 others (ribotype GP01F09) were related (97% identity) to *Magnetospirillum gryphiswaldense* (T) MSR-1/DSM 6361 (Schleifer *et al.*, 1991). Ribotype SP12C09 (3 sand clones) was only distantly related (95% identity) to bacterium Ellin328 [AF498710] (Sait *et al.*, 2005) and ribotype GP01B07 (2 groundwater clones) and clone SP11G10 had 98% and 97% identities respectively to *Azospirillum brasilense* (T) ATCC 49958 [AY150046].

**β-Proteobacteria**

β-Proteobacteria constituted a sizeable proportion of the isolates (24%) and was the largest phylogenetic group of the groundwater (35%) and the sand clone libraries (41%) (Fig. 3.14). The importance of this phylogenetic group is also highlighted by the fact that 3 out of the 4 ribotypes with the highest number of clones belonged to β-Proteobacteria. All isolated β-Proteobacteria belonged to the *Burkholderiales*, whereas almost all the groundwater clones belonged to the *Rhodocyclales*. Sand clones were

present in both these orders and they overlapped with 2 ribotypes of the isolated bacteria and with 2 ribotypes of the groundwater clones (Fig. 3.16).

In the *Burkholderiales*, the highest number of isolates and sand clones grouped within the *Comamonadaceae* family, where 5 ribotypes closely affiliated to the Acidovorax-Simpicispira-Variovorax genera were identified. Ribotype is14 consisted of 5 isolates and 17 sand clones and it was closely related (99% identity) to Bacterium rJ1 [AB021320], an isolate from phenol-digesting activated-sludge (Watanabe *et al.* 1999). Ribotype is19 (4 isolates and 3 sand clones) was closely related (98% identity) to *Acidovorax defluvii* (T) BSB411 that was isolated from activated sludge of a municipal wastewater treatment plant (Schulze *et al.*, 1999) and ribotype is08 (5 isolates) had 100% ID similarity to another isolate [AB076846] from activated sludge (Khan *et al.*, 2002). Eight more isolates of this study (split between ribotypes 227 and 247) were closely related to *Variovorax sp.* 44/31 [AY571831] that derived from a hydrocarbon-contaminated soil in Antarctica (Saul *et al.*, 2005) and 7 other members of the *Comamonadaceae* family (1 isolate and 6 sand clones) were affiliated closely (98% identity) to *Rhodoferax ferrireducens* (T) T118, an iron-reducing, facultative anaerobic bacterium (Finneran *et al.*, 2003). In addition to the *Comamonadaceae*, *Burkholderiales* included isolate ig272 which was affiliated (97% identity) to the MTBE (methyl tert-butyl ether) degrading *Methylibium petroleiphilum* (T) PM1 (Nakatsu *et al.*, 2006; Kane *et al.*, 2007), isolate ig262 which was related to *Pigmentifaga* sp., and 4 sand clones that were not related closely to any cultured microorganism.

In the *Rhodocyclales*, the majority of the clones clustered within 3 large ribotype groups (SP11F11, SP12B04 and GP12E08). The first ribotype (SP11F11) comprised of 16 groundwater and 7 sand clones and had 100% identity to *Azoarcus* sp. PbN1, an anaerobic denitrifying isolate that can degrade phenol and other aromatic hydrocarbons (Rabus and Widdel, 1995). On the other hand, the other two large ribotype groups were only very distantly related (93% identity) to a cultured microorganism (*Denitratisoma oestradiolicum* AcBE2-1; Fahrbach *et al.*, 2007). Ribotype SP12B04 (14 groundwater and 8 sand clones) was closely related (98% identity) to an environmental clone [AY532566] that was detected in a uranium-contaminated aquifer while the closest match (95% identity) of ribotype GP12E08 (9 groundwater clones) was a clone [DQ110036] detected

in freshwater sediments (Weber *et al.*, 2006). The remaining 4 clones within the *Rhodocyclales* order (2 from groundwater and 2 from sand) had very low sequence identities (90-92%) to *Sterolibacterium denitrificans* (T) Chol-1S (Tarlera and Denner, 2003).

From the clones that were not classified within the two orders that were described above (*Burkholderiales* and *Rhodocyclales*), 3 clones were distantly related (93% identity) to the iron-oxidising *Gallionella ferruginea* (Hallbeck *et al.*, 1993) and 2 sand clones were affiliated (96% identity) to the facultative anaerobe *Thiobacillus denitrificans* (T) NCIMB 9548, a denitrifying, sulphur-oxidising strain (Kelly and Wood, 2000).

### γ-Proteobacteria

About 20% of the isolated bacteria (n = 23) but only 2 groundwater and 7 sand clones were affiliated with the γ-Proteobacteria class. With the exception of two clones which could not be classified, all the other sequences fell within the *Pseudomonas* (5 ribotypes, with 17 isolates and 3 sand clones), *Stenotrophomonas* (1 ribotype with 6 isolates) and *Methylobacter* (1 ribotype, consisting of 1 groundwater and 3 sand clones) genera (Fig. 3.17). It is worth highlighting that all 3 sand clones that were members of the *Pseudomonads* overlapped with cultured isolates from the four Ashes site, and they were presented in Table 3.4.

Almost all the sequences belonging to the *Pseudomonas* genus were closely related to species that are associated with phenol degradation or hydrocarbon contamination. Two isolates (ribotype ig264) had 99% sequence identity to *Pseudomonas putida* PC36 [DQ178233] which has been reported as phenol-degrader (Merimaa *et al.*, 2006) and 4 other isolates (ribotype ig59/30-4) had also 99% identity to the phenol-degrading *Pseudomonas sp.* BFXJ-8 [EU013945]. Ribotype is17 (9 isolates and 1 sand clone) shared 100% identity with *Pseudomonas* sp. 'ARDRA PS2' [AY364086], an isolate from a BTEX contaminated soil (Junca and Pieper, 2004) and was closely related (99% identity) to the phenanthrene-degrading *Pseudomonas frederiksbergensis* (T) JAJ28 that was isolated from a coal-gasification site (Andersen *et al.*, 2000). Ribotype is282 (1 isolate and 1 sand clone) had 99% similarity to an isolate [AY512612] from a BTEX

contaminated industrial site (Hendrickx *et al.*, 2006b) and ribotype is290 (1 isolate and 1 sand clone) was closely related (99% identity) to *Pseudomonas* sp. JN18_A60_A4 [DQ168654], a PCB-dechlorinating isolate (Bedard *et al.*, 2006).

In the *Xanthomonadales*, ribotype ig245 (6 isolates, all from groundwater) had 100% ID similarity with a large number of GenBank entries, including an isolate from hydrocarbon contaminated soil in Antarctica [DQ109991], *Stenotrophomonas sp.* A09 from arsenic-contaminated soil (Achour *et al.*, 2007), and many isolates from agricultural fields.

Finally, in the *Methylococcales*, ribotype SP01C12 (1 groundwater and 3 sand clones) was closely related (99% identity) to the methane-oxidizing type strain *Methylobacter tundripaludum* (T) SV96, that was isolated from Arctic wetland soil (Wartiainen *et al.*, 2006a).

**Actinobacteria**

Out of the 258 clones sequenced, only 2 sand clones belonged to the Actinobacteria phylum and they were closely related (97% identity) to an environmental clone [EU266892] which derived from a coal-tar polluted aquifer (Winderl *et al.*, 2008). On the other hand, the largest number of the isolated microorganisms (44 isolates or 38% of the total) as well the largest number of ribotypes (19) belonged to Actinobacteria. Most of the isolated Actinobacteria grouped within the *Nocardiaceae* and *Nocardiodiaceae* families, and the *Micrococcineae* suborder (Fig. 2.18).

In the *Nocardiaceae*, 11 isolates (ribotype is292) were closely related (99% identity) to *Rhodococcus fascians* (T) ATCC 12974 (Ruimy *et al.*, 1995) and 2 others (ig242, ig256) to *Rhodococcus erythropolis* (T) ATCC 4277T (Ruimy *et al.*, 1995). In the past, *Rhodococci* have been often isolated or detected by molecular methods in a diverse range of environments and it is characteristic that the first 100 GenBank matches had similarity IDs of 98% or higher to the isolated *Rhodococci* of this study. Amongst the first few matches, *Rhodococci* that have been detected in hydrocarbon-contaminated environments were included; is292 was closely related (99% identity) to *Rhodococcus sp.*

AP17 [EU374915] that was isolated from hydrocarbon polluted sand while isolate ig242 had 100% sequence identity *to Rhodococcus erythropolis* Ri81 [AM905948], a benzene-degrading isolate that derived from a BTEX contaminated aquifer (Fahy *et al.*, 2008). The remaining 3 isolates in the *Nocardiaceae* (ribotype is291) were closely related (99% identity) to *Nocardia ignorata* DSM 44496 (Yassin *et al.*, 2001).

Amongst the *Nocardioidaceae*, ribotypes ig213 and ig218 had 98% and 99% sequence identity respectively to *Nocardioides plantarum* (T) DSM 11054T that was cultured from Antarctic sandstone (Schumann *et al.*, 1997) and ribotype ig269 (4 isolates) had 97% identity to *Nocardioides ganghwensis* (T) JC2055 that was isolated from tidal flat sediments (Yi and Chun, 2004). The remaining 2 isolates in the *Nocardioidaceae* family were related to the *Marmoricola* (ig250) and *Aeromicrobium* (ig202) genera.

In the *Micrococcineae* suborder, 10 of the isolates (all from groundwater) were members of the *Frigoribacterium* genus, closely related (99% identity) to *Frigoribacterium* sp. GIC6 [AY439262] that was isolated from a glacial ice core (Miteva *et al.*, 2004) and to the psychrophilic *Frigoribacterium faeni* (T) 801 [Y18807], whose optimal growth is at temperatures between 4 – 10 °C (Kämpfer *et al.*, 2000). Other members of the *Micrococcineae* suborder belonged to the *Cellulomonas* (ig216), *Micrococcus* (ig261), *Arthrobacter* (ig201, ig230), *Clavibacter* (ig257), and *Rathaybacter* (ig252) genera, the majority of them usually detected either in agricultural fields or in cold environments.

The remaining 2 isolates belonging to Actinobacteria were affiliated with the *Blastococcus* (ig270) and *Nakamurella* (ig253) genera.

**Bacteroidetes**

Only one isolate belonged to the Bacteroidetes phylum, which enumerated 36 groundwater clones (30% of the total) and 10 sand clones (Fig. 2.19). The isolate (ig263) had 98% sequence identity to *Taxeobacter gelupurpurascens* (T) Txg1 [Y18836], which was isolated from Antarctic soil. On the other hand, the majority of the clones had particularly low identities with cultured microorganisms and there was no overlap

between the groundwater and the sand clone libraries. With the exception of one unclassified clone, all groundwater clones belonged to the *Bacteroidales* (36), whereas sand clones were either unclassified Bacteroidetes (7) or members of the *Sphingobacteriales* (4) and the *Flavobacteriales* (2).

Amongst the *Bacteroidales*, the largest ribotype group (GP01H05) in the whole groundwater library was identified (25 clones, 20.5% of the total). This ribotype had 100% sequence identity to an environmental clone [EU266902] that was detected in sediments from a tar-oil contaminated aquifer (Winderl *et al.*, 2008). However, its second closest GenBank match had 95% ID similarity and the closest cultured organism only 85%. From the remaining ribotypes in the *Bacteroidales*, 2 ribotypes (7 clones in total) had 96% identity to the strictly anaerobe *Proteiniphilum acetatigenes* (T) TB107 that was isolated from a sludge reactor treating brewery wastewater (Chen and Dong, 2005) and the remaining 2 clones were distantly related (92% identity) to *Paludibacter propionicigenes* (T) WB4 (Ueki *et al.*, 2006).

The 3 sand clones belonging to *Sphingobacteriales* (SP11H01) were distantly related (91% identity) to *Terrimonas ferruginea* (T) [M62798]. Similarly, the unclassified Bacteroidetes sand clones were distantly affiliated to any cultured microorganisms, although they all had high sequence identity similarities (97% or higher) to other uncultured environmental clones.

**Firmicutes**

In the Firmicutes phylum belonged 8 of the isolates, 27 groundwater clones (22% of the total) but no sand clones. All 8 isolates grouped within the *Bacillales* order whereas all groundwater clones belonged to the *Clostridiales*, with the exception of 2 unclassified clones (Fig. 2.20).

Six of the isolated bacteria (ribotype is297) belonged to the *Bacillus* genus and their closest type strain relative was *B. muralis* (T) LMG 20238 (Zou *et al.*, 2007). Amongst the first 100 GenBank matches of ribotype is297 (all with identities of 100% or 99%), there were strains isolated mainly from soil but also from other environments

(Mediterranean coast, marine sponge, deep-sea sediments, asphalt seeps). Similarly, ig211 was closely related to bacteria that are detected predominantly in soil and had 99% identity to *Paenibacillus amylolyticus* (T) NRRL NRS-290T (Shida *et al.*, 1997). On the other hand, isolate ig59/30-2 had 99% similarity to *Sporosarcina macmurdoensis* (T) CMS 21w, which was isolated from a cyanobacterial mat from a pond in Antarctica (Reddy *et al.*, 2003).

The majority of the clones belonging to the Firmicutes clustered within the *Clostridiales* order and were split between the *Acetobacterium* and *Desulfosporisinus* genera. Ribotype GP01D11 (12 groundwater clones, 9.8% of the total) had 99% sequence identity to an environmental clone [AF422686] found in trichloroethene-contaminated groundwater (Lowe *et al.*, 2002) and 96% identity to the acetogen *Acetobacterium carbinolicum* (T) DSM 2925 (Willems and Collins, 1996; Paarup *et al.*, 2006). Ribotype GP01C04 (9 groundwater clones, 7.4% of the total) had 99% ID similarity to an environmental clone [AF407196] detected in monochlorobenzene contaminated groundwater (Alfreider *et al.*, 2002) and 96% to the sulfate-reducing *Desulfosporosinus meridiei* (T) S10, which was isolated from a shallow aquifer contaminated with gasoline (Robertson *et al.*, 2000). Three more clones (ribotype GP12E10) were distantly related to the *Desulfosporisinus* genus and had 99% ID similarity to a clone [AF414571] detected in uranium mine sediment. Finally, one clone (ribotype GP02B05) was affiliated (96% identity) to the strictly anaerobe *Anaerovorax odorimutans* (T) NorPut (Matthies *et al.*, 2000).

**Other phylogenetic groups and unclassified clones**

Apart from the major phylogenetic groups described above, 2 isolates, 5 groundwater clones and a noteworthy 29% of the sand clones (40 clones) belonged to other phylogenetic groups or could not be not be affiliated to any of the present bacterial phylogenetic groups when a 80 % confidence threshold was used in the RDP classifier (Fig. 3.21).

Both isolates were members of the Deinococcus-Thermus phylum. Isolate ig232 (sequence of only 530 base pairs long) had 100% sequence identity to *Deinococcus*

*radiopugnans* (T) ATCC 19172T (Rainey *et al.*, 1997) while ig252 was closely related (97% identity) to *Deinococcus radiomollis* PO-04-20-144, which was isolated from an alpine environment and its optimum growth is at 10°C (Callegan *et al.*, 2008).

From the 5 groundwater clones, 2 of them (ribotype GP01E12) belonged to the Spirochaetes and they were distantly related (88% identity) to *Spirochaeta stenostrepta* (Paster *et al.*, 1991) while clone G41 had 98 % identity to environmental clone ADK-WSe02-91 [EF520621] that was detected in an acid-impacted lake (Percent *et al.*, 2008). The remaining 2 clones as well as 1 sand clone (ribotype GP12E06) were closely related (99% identity) to uncultured epsilon proteobacterium CC1_CL33 [DQ295573] that was detected in microbial mat from sulfidic cave spring and had 92% identity to *Sulfurimonas denitrificans* (T) DSM 1251 [L40808], an isolate from a deep-sea hydrothermal vent (Muyzer *et al.*, 1995).

The classified sand clones belonged to various phylogenetic groups (Fig. 3.21), including δ-Proteobacteria (3 sand clones), Chloroflexi (3), Nitrospira (1), Verrucomicrobia (2), ϵ-Proteobacteria (1), phylum TM7 (ribotype S07; 3 sand clones), Acidobacteria (SP11E11), and Planctomycetes (4). In many cases these clones were closely related either to uncultured environmental clones or to cultured bacteria from polluted environments. For example, clone S20 had 100% sequence identity to uncultured delta proteobacterium D12_01 [EU266811] that was detected in tar-oil contaminated aquifer sediments (Winderl *et al.*, 2008), clone S12 was closely related (99% sequence identity) to an uncultured bacterium GOUTB20 [AY050605] found in monochlorobenzene contaminated groundwater (Alfreider *et al.*, 2002). Moreover, clone SP11E11 had 98% sequence identity to *Geothrix fermentans* H5, an Fe(III)-reducing isolate (Lonergan *et al.*, 1996) and clone SP01C04 had 98% sequence to nitrate-oxidising Candidatus *Nitrospira defluvii* Contig5882 [EU559167], which was isolated from a wastewater treatment plant (Maixner *et al.*, 2008).

The unclassified clones were normally not affiliated to any cultured microorganisms (less than 80% sequence identity) but in some cases, they were very closely related to other (uncultured) environmental clones. For example, clone SP12D04 had 99% identity to uncultured bacterium LWS-T4601 [EU546359] from lake sediments,

clone SP11E03 had 98% identity to uncultured bacterium Pia-s-69 [EF632929] from freshwater sediments, and clone SP11H08 had 97% sequence identity to uncultured bacterium 656054 [DQ404610] that was detected in heavy metal and radionuclide contaminated subsurface sediments (Abulencia *et al.*, 2006).

**Figures 3.15 – 3.21. (Following pages).** Phylogenetic analysis of all 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from the Four Ashes site, as well as a selection of their closest affiliated bacterial type strains (T) or cultured/uncultured GenBank matches. Sequences that clustered together on the phylogenetic tree were grouped under the same "ribotype" ID. Sequences/ribotypes from this study are shown in bold. The brackets indicate the number of "isolates / groundwater clones / sand clones" that belong within each ribotype, bacterial order or phylogenetic group.

Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The percentages of replicate trees (1000 replicates) in which the associated taxa clustered together in the bootstrap test (Felsenstein, 1985) are shown next to the branches. Bootstrap values lower than 50% were omitted. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1727 positions in the final dataset.

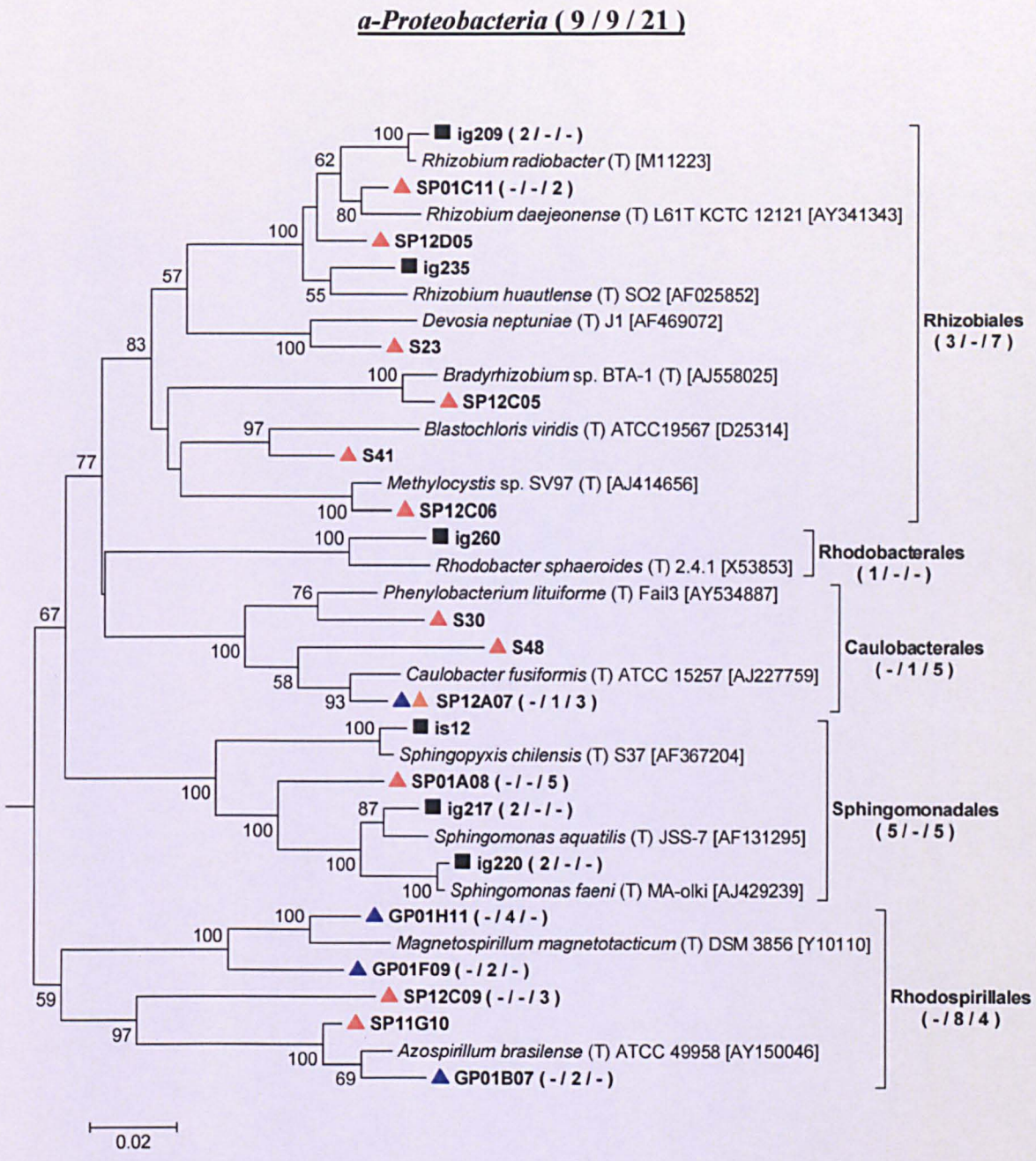



**Figure 3.15.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from Four Ashes belonging to *α-Proteobacteria*. The scale bar represents 0.02 base changes per nucleotide position.

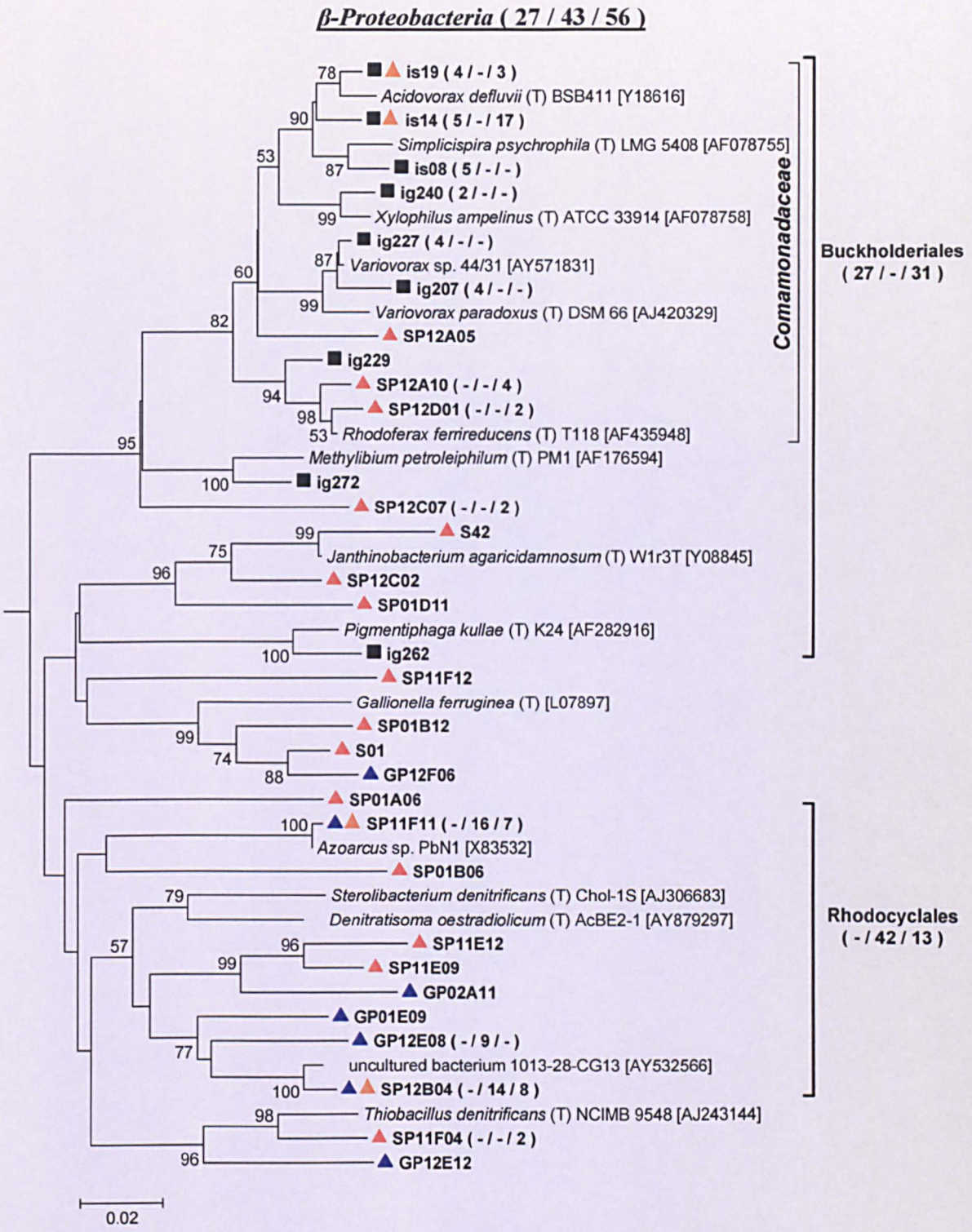



**Figure 3.16.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from Four Ashes belonging to *β-Proteobacteria*. The scale bar represents 0.02 base changes per nucleotide position.

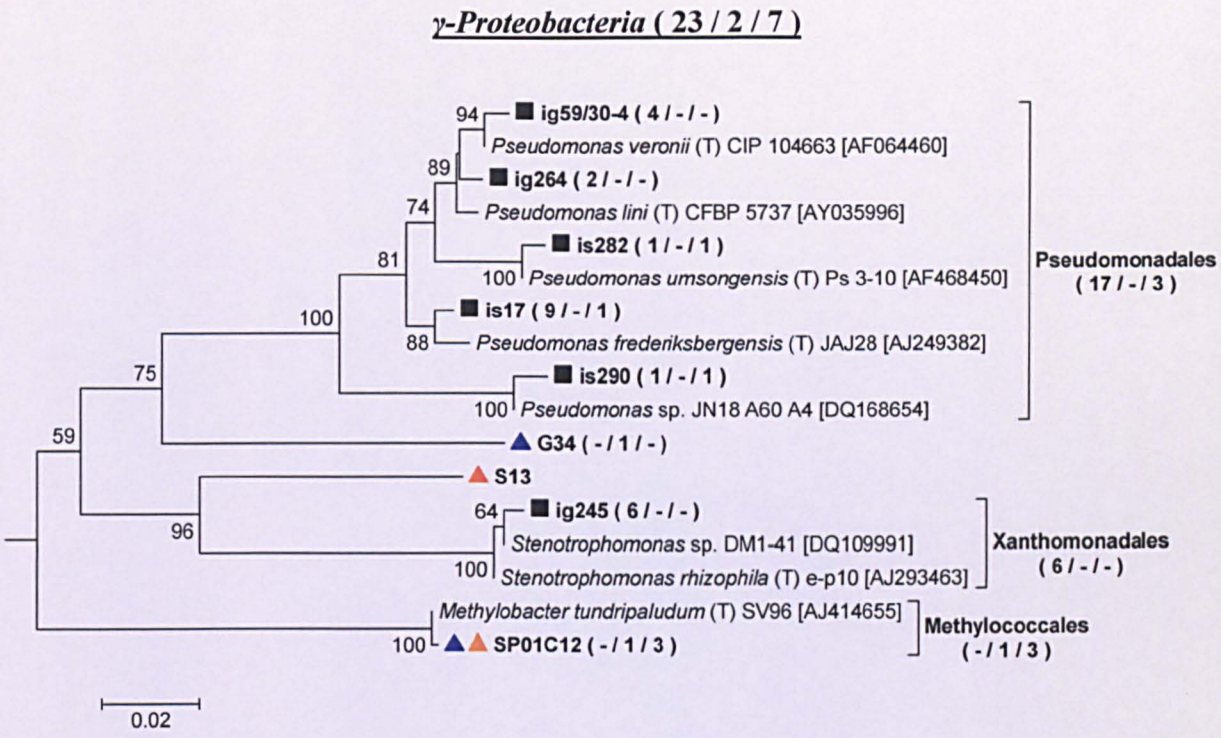



**Figure 3.17.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from Four Ashes belonging to *γ-Proteobacteria*. The scale bar represents 0.02 base changes per nucleotide position.

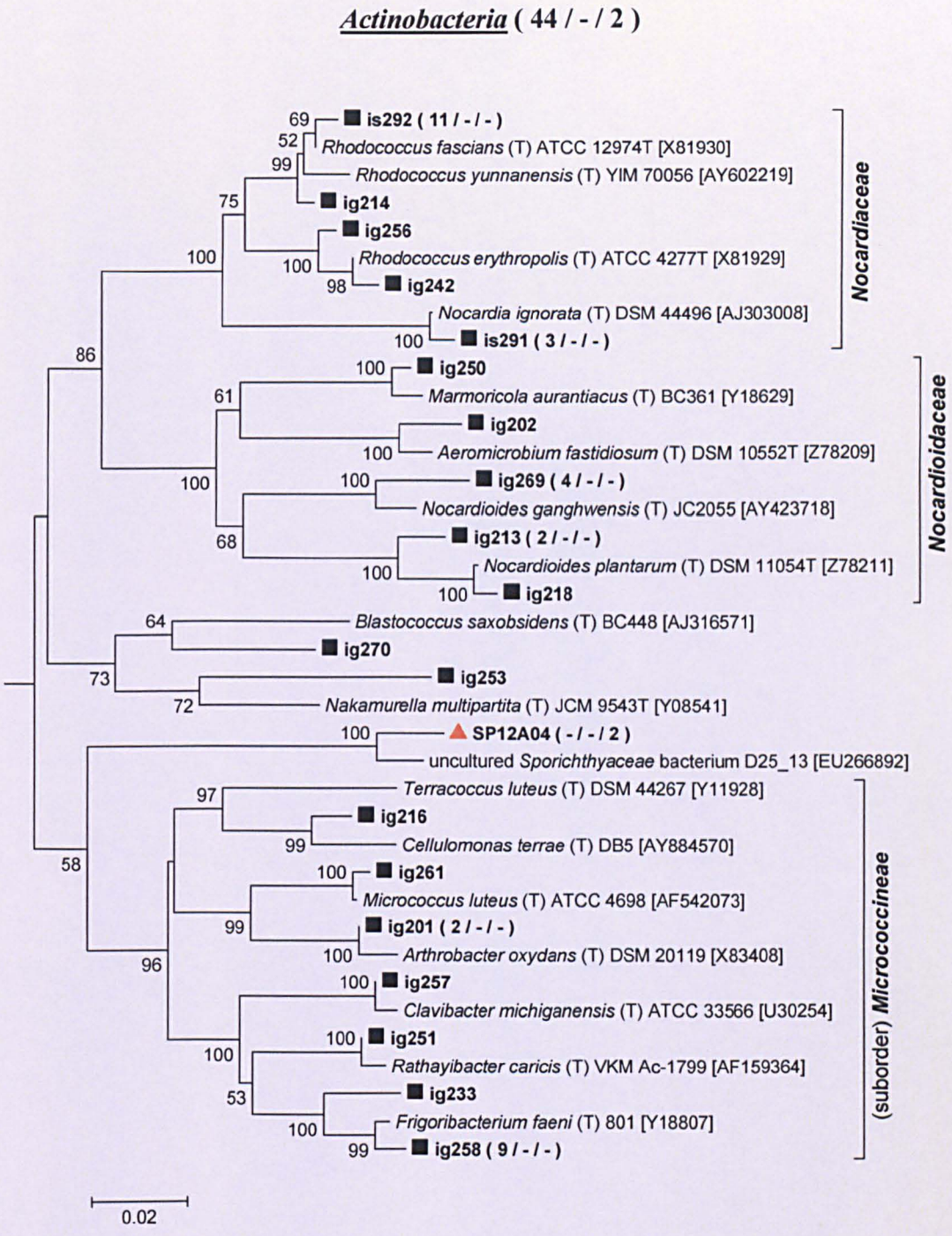



**Figure 3.18.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■) and sand clones (▲) from Four Ashes belonging to *Actinobacteria*. The scale bar represents 0.02 base changes per nucleotide position.

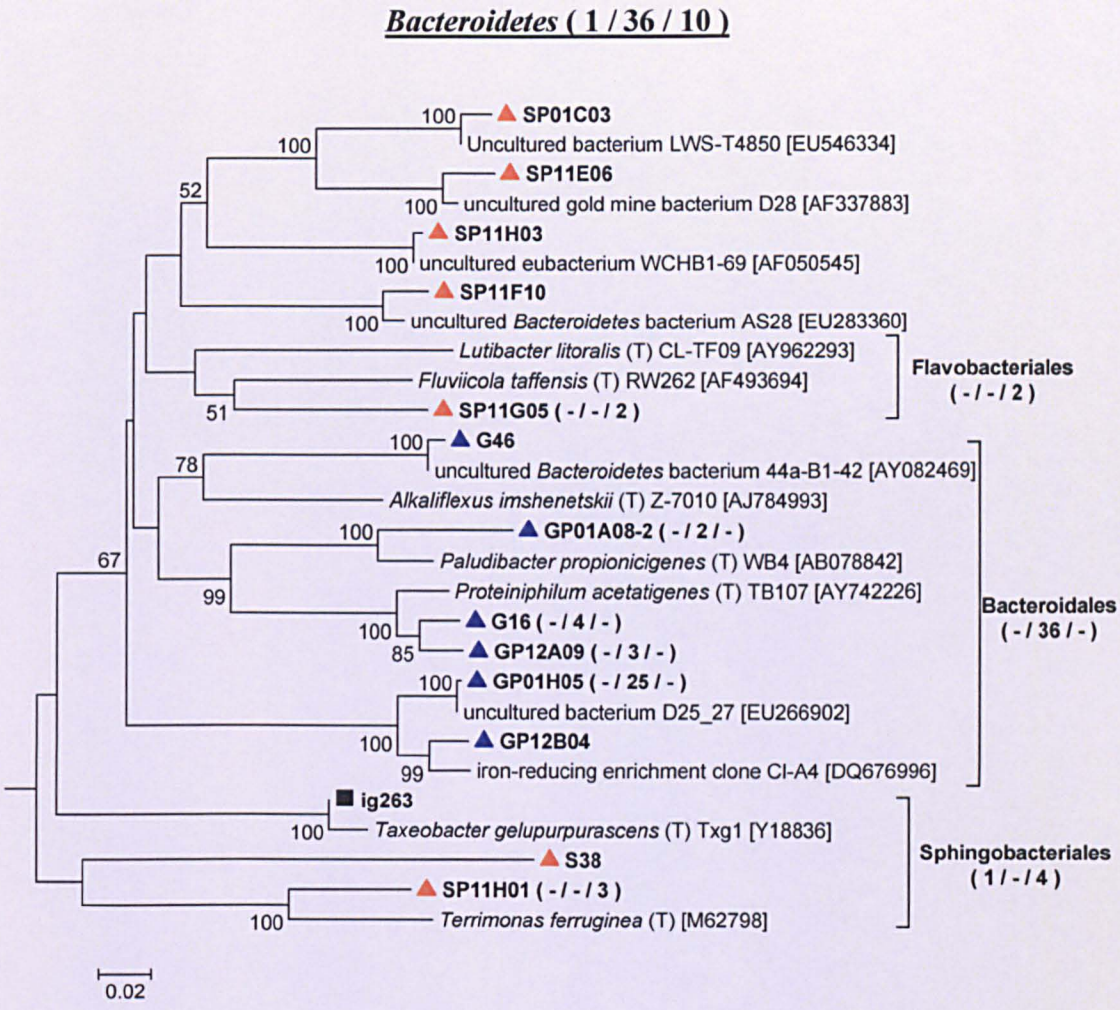



**Figure 3.19.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from Four Ashes belonging to *Bacteroidetes*. The scale bar represents 0.02 base changes per nucleotide position.

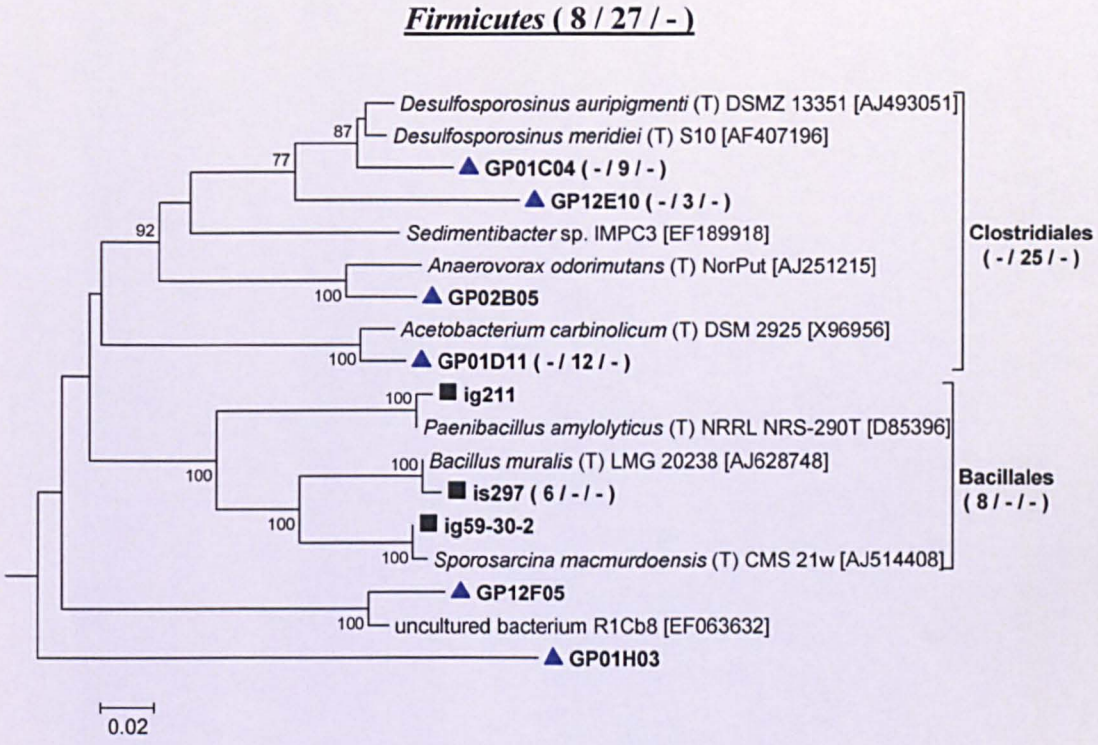
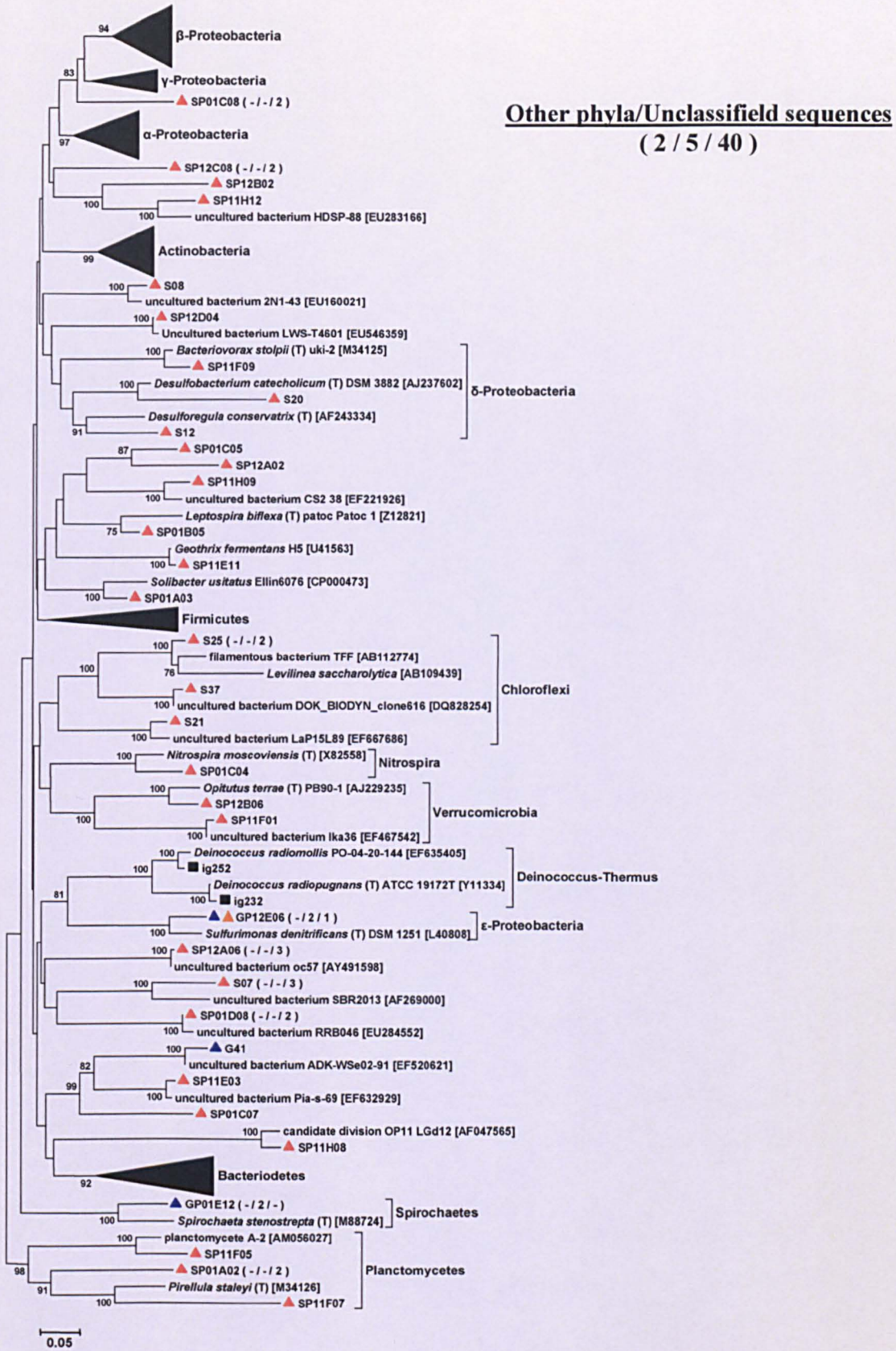



**Figure 3.20.** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■) and groundwater clones (▲) from Four Ashes belonging to *Firmicutes*. The scale bar represents 0.02 base changes per nucleotide position.

**Figure 3.21. (Next page).** Neighbour-joining phylogenetic analysis of 16S rRNA gene sequences from isolated bacteria (■), groundwater clones (▲), and sand clones (▲) from Four Ashes belonging to minor bacterial phylogenetic groups or could not be classified. The overall topology of the tree (including the collapsed phylogenetic groups that have been already presented) can be seen. The scale bar represents 0.05 base changes per nucleotide position.


β-Proteobacteria
γ-Proteobacteria
SP01C08 ( - / - / 2 )
α-Proteobacteria
SP12C08 ( - / - / 2 )
SP12B02
SP11H12
uncultured bacterium HDSP-88 [EU283166]
Actinobacteria
S08
uncultured bacterium 2N1-43 [EU160021]
SP12D04
Uncultured bacterium LWS-T4601 [EU546359]
Bacteriovorax stolpii (T) uki-2 [M34125]
SP11F09
Desulfobacterium catecholicum (T) DSM 3882 [AJ237602]
S20
Desulforegula conservatrix (T) [AF243334]
S12
δ-Proteobacteria
SP01C05
SP12A02
SP11H09
uncultured bacterium CS2 38 [EF221926]
Leptospira biflexa (T) patoc Patoc 1 [Z12821]
SP01B05
Geothrix fermentans H5 [U41563]
SP11E11
Solibacter usitatus Ellin6076 [CP000473]
SP01A03
Firmicutes
S25 ( - / - / 2 )
filamentous bacterium TFF [AB112774]
Levilinea saccharolytica [AB109439]
S37
uncultured bacterium DOK_BIODYN_clone616 [DQ828254]
S21
uncultured bacterium LaP15L89 [EF667686]
Chloroflexi
Nitrospira moscoviensis (T) [X82558]
SP01C04
Nitrospira
Opitutus terrae (T) PB90-1 [AJ229235]
SP12B06
SP11F01
uncultured bacterium Ika36 [EF467542]
Verrucomicrobia
Deinococcus radiomollis PO-04-20-144 [EF635405]
ig252
Deinococcus radiopugnans (T) ATCC 19172T [Y11334]
ig232
Deinococcus-Thermus
GP12E06 ( - / 2 / 1 )
Sulfurimonas denitrificans (T) DSM 1251 [L40808]
ε-Proteobacteria
SP12A06 ( - / - / 3 )
uncultured bacterium oc57 [AY491598]
S07 ( - / - / 3 )
uncultured bacterium SBR2013 [AF269000]
SP01D08 ( - / - / 2 )
uncultured bacterium RRB046 [EU284552]
G41
uncultured bacterium ADK-WSe02-91 [EF520621]
SP11E03
uncultured bacterium Pia-s-69 [EF632929]
SP01C07
candidate division OP11 LGd12 [AF047565]
SP11H08
Bacteriodetes
GP01E12 ( - / 2 / - )
Spirochaeta stenostrepta (T) [M88724]
Spirochaetes
planctomycete A-2 [AM056027]
SP11F05
SP01A02 ( - / - / 2 )
Pirellula staleyi (T) [M34126]
SP11F07
Planctomycetes
0.05
Other phyla/Unclassifield sequences
( 2 / 5 / 40 )

## 3.4 Discussion

During this study, a culture-based and a culture independent method were followed to investigate the microbial diversity of planktonic and attached microbial communities at 30 mbgl in Borehole 59. In total, 16S rRNA gene fragments from 114 isolates (71 from groundwater and 43 from sand) and 275 clones (132 and 143 respectively) were sequenced and non-chimeric ribotypes were identified by nucleotide Blast search and by using the RDP II database. The sequencing results indicated that there was limited overlap between groundwater and sand clones as well as between the clone libraries and the isolated microorganisms. Here I will discuss the issues of detection of chimeric 16S rRNA gene sequences and the reliability of 16S rRNA databases (3.4.1), the observed differences between the planktonic and attached microbial communities (3.4.2), the inferred functions of these communities and their relationship to the geochemical environment at the Four Ashes site (3.4.3). I will also consider the differences between the clone libraries and the isolated bacteria, the biases of the culture-based method that was followed (3.4.4), and the relevance of the isolated bacteria to other environments (3.4.5).

### 3.4.1 Detection of chimeric sequences and reliability of 16S rRNA databases

Before further analysis, all sequenced 16S rRNA gene fragments were subjected to chimera formation check. Chimeras are formed as a result of PCR co-amplification of 16S rRNA genes from different bacterial species and can occur with frequencies of 30% or more (Wang and Wang, 1997). In the past, Chimera_Check (http://35.8.164.52/cgis/chimera.cgi) of RDP II (Cole *et al.*, 2003) was the most frequently used programme for chimera detection. However, Chimera_Check is currently based on a largely outdated database and recent versions of the RDP II (Release 9.42 or later; Cole *et al.*, 2007) have adopted Pintail for testing for sequence anomalies. Moreover, Mallard (which is based on the Pintail algorithm) was shown (Ashelford *et al.*, 2006) to correctly detect chimeric sequences consistently better than Bellerophon (Huber *et al.*, 2004), which is the alternative software programme for screening whole clone

libraries. Thus, in this study Mallard was employed for chimera screening of all the obtained sequences in this study and the results were verified with Pintail. As shown in Table 3.2, 7.6% and 4.9% of the groundwater and the sand clones respectively were detected as chimeric, percentages that are comparable to the findings of other studies that used Mallard to identify sequence anomalies. Lehours *et al.* (2007) found that out of 357 sequences of bacterial and archaeal clones from anoxic lake samples 23 were chimeric (6.4%), while Gomez-Alvarez *et al.* (2007) found that chimeric sequences constituted 0%, 7.3%, 8% and 10.1% of four clone libraries from different volcanic deposits. Despite the recognised problem of chimera formations, in many studies with 16S rRNA gene sequencing results there is no mention whether chimera checks have been performed. More importantly, already submitted clone libraries appear to contain high percentages of chimeric sequences. In general, the makers of Mallard and Pintail estimated that at least 5% of the sequence records in public repositories (such as GenBank, EMBL, and RDP) contain substantial anomalies (Ashelford *et al.*, 2005). Later, when Ashelford *et al.* (2006) used Mallard to screen 25 of the largest clone libraries that were submitted to GenBank in 2005 (>100 sequences each, sequences of 1200 bases at least), they identified 543 (9% of the total) anomalous sequences with the chimeric content within each clone library ranging from 0 to 45.8%, and concluded that "serious anomalies are polluting the public repositories to such an extent that their usefulness is being surreptitiously and progressively compromised".

Reliability of public 16S rRNA libraries such as GenBank is also compromised by the submission of sequences that are named arbitrarily, without taking into account widely accepted reference standards (Bergey's Manual of Systematic Bacteriology, RDP II). Moreover, although prokaryotic nomenclature is regulated by certain rules (Tindall *et al.*, 2006) and a species definition has been proposed by an ad hoc committee (Stackebrandt *et al.*, 2002), prokaryotic taxonomy is constantly evolving as the continuous expansion of 16S rRNA databases improves our understanding of the phylogenetic affiliations between known species. As a result, in the past different genera were grouped into one (e.g. *Rhizobium*, *Agrobacterium* and *Allorhizobium* were merged under the *Rhizobium* genus by Young *et al.*, 2001), established genera were divided into new ones (e.g. genus *Sphingomonas* was split into 4 genera by Takeuchi *et al.*, 2001) or more often mistakenly

named species were reclassified to different genera or even phyla (e.g. *Pseudomonas spp.* in Anzai *et al.*, 2000; Humphry *et al.*, 2003; Xie and Yokota, 2005). From all the above it becomes apparent that identification of a 16S rRNA gene sequence is a challenging procedure, especially when no close relative can be identified or at bushy areas of the RDP trees (e.g. *Comamonadaceae* family, *Pseudomonas* genus), where clear taxonomic groups with defining boundaries for genera is still problematic (Bergey's Taxonomic Outline; http://141.150.157.80/bergeysoutline/main.htm). Therefore, during this study a dual approach was followed for the identification of the closest relatives of the sequenced isolates and clones. The curated non-chimeric database of RDP II (Cole *et al.*, 2007), which uses the Naïve Bayesian classification method (Wang *et al.*, 2007a) to classify a large number of 16S rRNA gene sequences (513,272 in Release 9.61) into the new higher-order taxonomy proposed in Bergey's Taxonomic Outline of the Prokaryotes (Garrity *et al.*, 2004), was used to identify the closest type strain relatives. In addition, the closest GenBank matches were taken into account but normally only when they had higher similarity identities to the type strains.

### 3.4.2 Comparison between the diversity of planktonic and attached microbial communities

One of the main aims of this study was to compare the diversity of *in situ* planktonic and attached microbial communities. Being unable to retrieve sediments from the Four Ashes plume, sterile sand was placed at 30 mbgl in borehole 59 and left over a period of time (14 months) in order for attached growth to develop. Then, the established attached microbial community was compared to the planktonic community that was sampled from the same depth, using a variety of techniques (DGGE, culturing on R2A, 16S rRNA gene cloning).

There are a limited number of published studies which have examined the diversity of both planktonic and attached communities in polluted groundwater using 16S rRNA gene sequencing and their findings are quite contradictory. In a uranium contaminated aquifer treated with acetate, Vrionis *et al.* (2005) demonstrated that microbial diversity of attached communities (assessed by 16S rRNA gene cloning) varied

with depth and the planktonic communities from approximately the same depth were different to the attached ones; however they noticed that direct comparison between the two communities should not be carried out because of the existence of the very steep geochemical gradient. In background groundwater (not treated with acetate) and sediment samples from the same depth, they found that the planktonic community was dominated by β-Proteobacteria (41%) while in the attached community Acidobacteria, α-Proteobacteria, and Actinobacteria comprised about 10% to 15% each. However, other studies in polluted aquifers have not found significant differences between attached and planktonic communities. Hendrickx *et al.* (2005) followed a similar approach to this study and they placed sterile and non-sterile material (sediment from a pristine aquifer) into a BTEX contaminated aquifer. By limited 16S rRNA gene cloning (up to 25 clones in each library) they observed that the introduced sediments were rapidly colonised by the bacterial community present in the contaminated aquifer and hydrocarbon-degrading *Pseudomonas* spp. dominated both attached and planktonic communities. Lehman *et al.* (2004) incubated in a TCE contaminated aquifer, chambers containing distilled water or crushed (sterile) basalt and after DGGE analysis and 16S rRNA gene sequencing of excised DGGE bands they also concluded that the planktonic communities were compositionally similar to the attached ones; however they did observe different functional potential between the 2 communities. Pombo *et al.* (2005) applied $^{13}C$ labelled acetate in a petroleum-hydrocarbon contaminated aquifer and they also demonstrated that functional activity differed between planktonic and attached microbial communities, as the $\delta^{13}C$ of PLFA (phospholipid fatty acids) increased on average by 27‰ and 4‰ respectively. Moreover, it appeared that planktonic populations of *Desulfotomaculum acetoxidans*, *Desulfobacter* sp. and acetoclastic methanogens were utilising the $^{13}C$ labelled acetate, while in the attached community only PLFA belonging to *Desulfotomaculum acetoxidans* were labelled.

In this study, every approach that was followed showed clear differences in the diversity of the planktonic and the attached microbial communities sampled from 30 mbgl in Borehole 59. The DGGE profile of the groundwater sample from 30 mbgl was evidently more similar to planktonic communities from other depths than to the attached community from the same depth (Fig. 2.14 and cluster analysis in Fig. 2.15). Moreover,

different bacteria cultured on R2A were isolated from the groundwater sample and the sand sample, and two largely different clone libraries were obtained from these samples (Fig. 3.13). The limitations of the DGGE in profiling microbial diversity have been already discussed (in 2.4.3). The limitations of culture based methods are also well established (see 1.3) and the biases of the culture based method that was followed in this study is discussed elsewhere (in 3.4.4). Therefore, the differences between the diversity of attached and planktonic microbial communities will be discussed primarily based on the results of the 16S rRNA gene phylogenetic analysis of the cloning and sequencing results, which should reflect (without being bias-free due to biases introduced during PCR amplification, see 1.3) more accurately the actual diversity and composition of these natural microbial communities.

The cloning and sequencing results indicated not only that the 2 communities differed in composition (at every phylogenetic level) but also that the planktonic community was less diverse than the attached community. At the phylum level, the groundwater clones were dominated by only 4 phylogenetic groups (35% β-Proteobacteria, 30% Bacteroidetes, 22% Firmicutes, 7.4% α-Proteobacteria) while the sand clones were characterised by the presence of β-Proteobacteria (41%), α-Proteobacteria (15.4%), γ-Proteobacteria (5.1%), Bacteroidetes (7.35%) and a large number of sequences (29%) that belonged either to other phylogenetic groups (see Fig. 3.21) or could not be assigned (at the 80 % confidence threshold) to any of the known bacterial phyla by the RDP II classifier (Cole *et al.*, 2007). The fact that some of the species could not be classified should not surprise us because in a recent phylogenetic study of volcanic deposits (Gomez-Alvarez *et al.*, 2007) 3 out of the 4 16S rRNA gene clone libraries contained 59.6%, 74.2% and 81% unclassified clones. At the level of bacterial orders, once again large differences could be detected between the two communities, as the sand clones belonged to many more bacterial orders than the groundwater clones (for example in the *Buckholderiales* of the β-Proteobacteria and in the *Rhizobiales* of the α-Proteobacteria).

The differences between the two communities became very clear at the species level, where although as many as 28 and 75 ribotypes/species were identified within the groundwater and the sand clone library respectively (Table 3.4), only 5 species/ribotypes

(defined by > 99% sequence similarity in this study) were present in both communities (Fig. 3.13 and Table 3.4). The common ribotypes included 2 species that were sequenced repeatedly in the clone libraries (one in the *Azoarcus* genus and an uncultured clone, both belonging in the *Rhodocyclales* order of β-Proteobacteria) and 3 species (in the *Caulobacter*, *Methylobacter*, *Sulfiromonas* genera) that were sequenced up to 4 times (see Table 3.4). Given the limitations of the sampling method (the attached community was studied on suspended quartz sand and not on aquifer sediment material), the biases of the PCR based methods (discussed in 1.3) and the fact that species accumulation curves were not flattened out (Fig. 3.12) we cannot conclude that the 16S rRNA gene cloning approach of this study reflects the total microbial composition present in the Four Ashes plume. However, these sequence results suggest strongly that in the Four Ashes aquifer the planktonic microbial community was less diverse than the attached one. It is characteristic that 70% of the groundwater clones grouped within 6 ribotypes/species only (28 in total were identified; Table 3.3), while amongst the sand clones more species were identified (75 ribotypes), diversity indices were higher (Table 3.3), and the species accumulation curve was less flattened out (Fig. 3.12). This finding may be explained by the fact that attached growth (biofilms) can offer some ecological advantages to microorganisms that exist in highly polluted environments (1220 mg $L^{-1}$ at that depth in the Four Ashes plume), such as (physical) protection from the environment, nutrient availability and metabolic cooperativity, and acquisition of new genetic traits (Davey and O'Toole, 2000).

Nevertheless a paradox remains, considering the fact that sterile material (quartz sand) was placed into the aquifer, the attached microbial community that developed should be a subset of the planktonic community and not different and (in addition) more diverse. However, this is not necessarily the case because the sand had been left over a period of time (14 months in our case) in the aquifer, before it was recovered at the same time with the groundwater sample. As it was already discussed, the plume at the Four Ashes site is not stable. It moves horizontally along with the groundwater flow (4-10 m $year^{-1}$) and it moves vertically (1-2 metres) following the seasonal shifts of the water table (Thornton *et al.*, 2001b). As a result, the concentration of pollutants constantly changes over time. Specifically for 30 mbgl in Borehole 59, when the sand was suspended in the

Four Ashes aquifer in June of 2005, the TPC was about 500 mg $L^{-1}$, at a concentration that the plume is expected to be micro-aerobic, and when it was retrieved the TPC was 1220 mg $L^{-1}$ (anaerobic). As it was demonstrated with the DGGE (Fig. 2.14) different microbial communities inhabit the Four Ashes aquifer at different concentrations of phenol. Thus, although the groundwater sample that was taken in August of 2006 reflects the composition of the planktonic microbial community of that time point, the sand sample should be regarded as a time capsule which recorded these microorganisms that were able to establish on the sand (or in the biofilm communities developed on the sand) at any point during its 14-month suspension in the aquifer. These microorganisms could be bacteria (aerobic or not) that could attach to the sand physically even if they cannot degrade phenol, and bacteria that although they cannot degrade the phenolic pollutants they can grow on the metabolites that are produced from other microorganisms existing in the biofilm communities. On the other hand, the reason that the planktonic community was less diverse, could be because the toxic phenol levels in combination with the lack of the protective microenvironments found in biofilm communities, has led to the dominance of a few species that could either degrade the phenolic compounds anaerobically or at least tolerate them.

A number of these hypotheses are tested in the following chapters. Using a selection of the isolated microorganisms from the Four Ashes site, the ability of non-phenol degrading species to grow in the simultaneous presence of phenol and other organic compounds (i.e. to tolerate phenol) or to exist in attached phase are examined in Chapter 4. Moreover, the influence of pollutant concentration shifts on attached and planktonic microbial communities was investigated using a series of laboratory microcosm experiments in Chapter 5.

### 3.4.3 Linking microbial diversity (16S rRNA gene cloning results) to the geochemistry at the Four Ashes plume

Some of the sequenced clones from the Four Ashes site were distantly related to cultured microorganisms, although in most of the cases they would be closely related to uncultured environmental clones (for example 18.8% of groundwater and 5.9% of sand

clones within the *Rhodocyclales* order of β-Proteobacteria). However, a considerable number of the sequenced clones were related (≥96% sequence identity) to cultured organisms with known functional properties. Although inferring the functional properties of an organism and particularly of an environmental clone based on its phylogenetic affiliations bears a degree of uncertainty, a very diverse functional community appears to operate at this depth in the Four Ashes plume, which correlates with the geochemistry of the plume.

To begin with, many members of the clone libraries were closely related to strictly anaerobic bacteria. In the *Bacteroidetes*, 7 groundwater clones (ribotypes G16 and GP12A09) had 96% sequence identity to the strict anaerobe *Proteiniphilum acetatigenes* (T) TB107 (Chen and Dong, 2005), and the *Firmicutes* clone GP02B05 was affiliated (96% identity) to the strict anaerobe *Anaerovorax odorimutans* (T) NorPut (Matthies *et al.*, 2000). In addition, 9% of all the clones (16 from groundwater and 7 from sand) had 100% identity to *Azoarcus sp.* PbN1 (*β-Proteobacteria*), a well characterised (and with complete sequenced genome) isolate that amongst other aromatic hydrocarbons can degrade phenol and *p*-cresol (major pollutants in the Four Ashes plume) via anaerobic pathways only (Fig. 3.22; Rabus *et al.*, 2005). Therefore, the presence of these clones indicates strongly that the plume at 30 mbgl in borehole 59 was anaerobic, in accordance with the geochemical studies, where the TPC was measured at particularly high levels at this depth (1220 mg $L^{-1}$).

Secondly, clones that were closely related to phenol-degrading genera have been identified, indicating that a metabolically active microbial community (capable of utilising the organic pollutants) exists in this depth in the Four Ashes site, something that was expected by the fluorescence of acridine-orange stained cells from this depth (Fig. 2.10). In addition to the *Azoarcus* genus, clones closely related to the phenol degrading strains within the *Acidovorax* (14.7% of the sand clones), *Sphingomonas* and *Rhizobium* genera have been identified. In contrast, only 3 of the (sand) clones belonged to the *Pseudomonas* genus, although many phenol degrading *Pseudomonas* strains are usually isolated from phenol polluted environments (Heinaru *et al.*, 2000; Whiteley *et al.*, 2001). Other known phenol degrading species that are often detected or isolated from phenol polluted environments include the denitrifying β-Proteobacterium *Thauera* sp. (Manefield

*et al.*, 2002) and the γ-Proteobacterium *Acinetobacter* sp. (Watanabe *et al.*, 1998; Abd-El-Haleem *et al.*, 2002; Geng *et al.*, 2006) but they were not detected during this study.

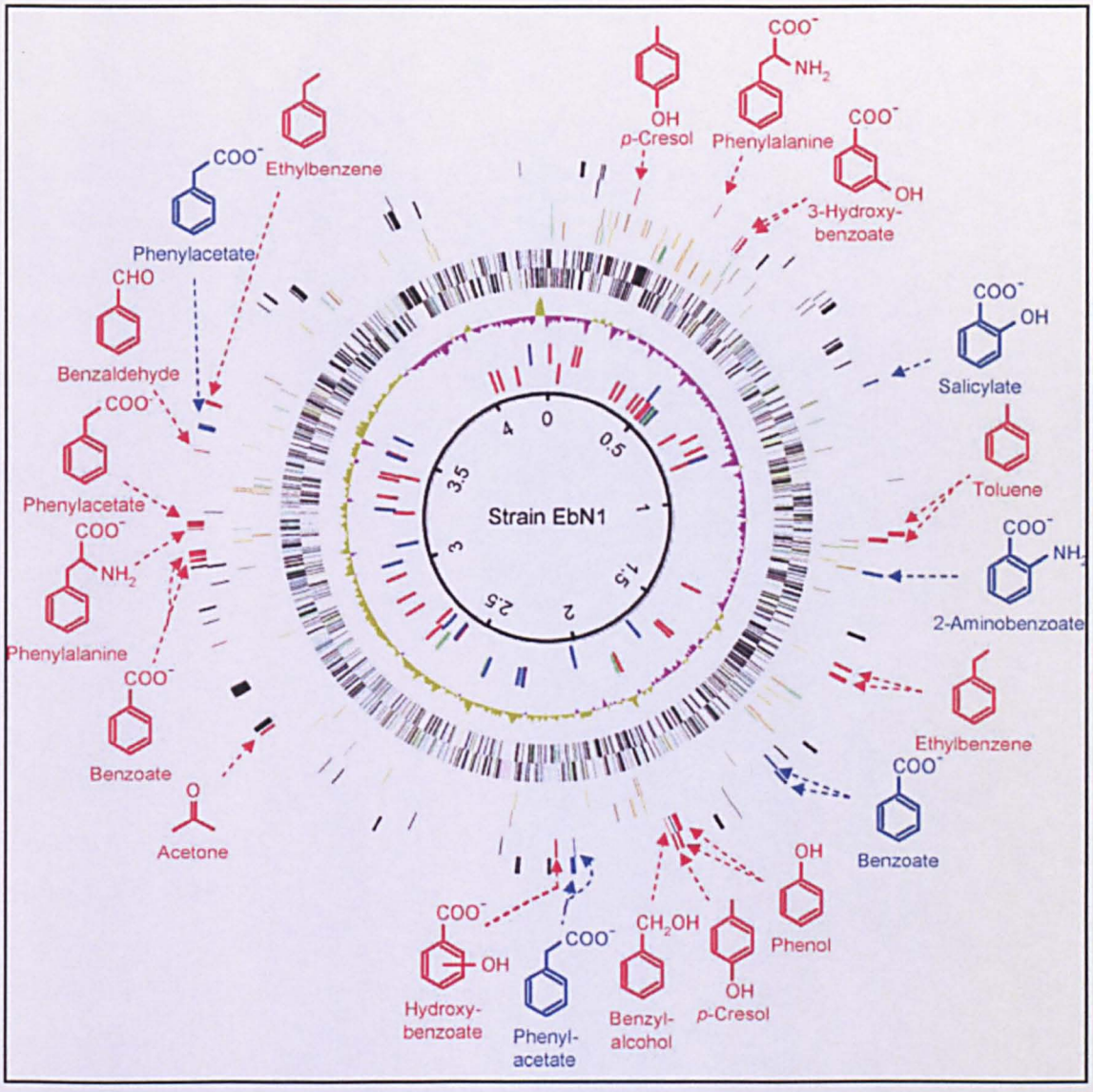



**Figure 3.22.** Structural representation of the chromosome of *Azoarcus sp.* EbN1, where the outer circle illustrates the distribution of catabolic genes of various aromatic hydrocarbons; red colour indicates anaerobic aromatic degradation (including phenol and *p*-cresol) and blue colour aerobic aromatic degradation. Image adjusted from Rabus *et al.*, 2005.

The 16S rRNA gene phylogeny also suggests that a very diverse functional community operates at this depth in the Four Ashes plume. Clones that were closely

related (sequence identities 96 – 100%) to denitrifying (*Azoarcus*, *Acidovorax*, *Magnetospirillum*), nitrate-oxidising (*Nitrospira*), iron-reducing (*Magnetospitillum*, *Rhodoferax*, *Geothrix*), sulphate reducing (*Desulfosporisinus*), denitrifying sulphur-oxidising (*Thiobacillus*, *Sulfurimonas*), acetogenic (*Acetobacterium*), and methane-oxidising (*Methylobacter, Methylocystis*) strains have been identified (details in 3.3.4). Quantitative interpretation of these results should not be attempted because of the known biases of the 16S rRNA gene sequencing approach and because the function of a large number of clones could not be inferred, since they were only distantly related to any cultured bacterial species (including the potentially dominant ribotypes GP01H05, SP12B04, GP12E08). Nevertheless, the fact that 16.6% of all the clones were closely related to denitrifying phenol-degrading *Azoarcus* and *Acidovorax* strains indicates that degradation of phenol by nitrate-reduction may be a dominant process at this depth in the Four Ashes aquifer. This is also supported by the fact that nitrate concentration is decreased (but not depleted) at 30 mbgl in borehole 59 (Fig. 2.6). It is noteworthy that when Manefield *et al.* (2005) followed an RNA-SIP based approach, they showed that 2 *Acidovorax* strains dominated phenol degradation in aerobic activated sludge reactors. What is more important is that qualitatively, these sequencing results are in agreement with the geochemical studies at the Four Ashes site, which showed that multiple biodegradation processes (nitrate-, iron-, manganese-, and sulphate-reduction, methanogenesis and fermentation) are occurring simultaneously within the plume at the Four Ashes (Thornton *et al.*, 2001b; Williams *et al.*, 2001), and that groundwater samples from different depths in borehole 59 exhibited denitrifying, sulphate reducing and methanogenic potential (Pickup *et al.*, 2001). Methanogens (i.e. methane-producing Archaea) were not identified during this study because Archaeal diversity was not investigated. In the past, methanogenic Archaea were shown to inhabit other hydrocarbon (Macbeth *et al.*, 2004) or phenol-polluted (Watanabe *et al.*, 2002; Chen *et al.*, 2008) environments. Specifically for the Four Ashes site, the presence of methanogens is supported not only by the elevated concentrations of $CH_4$ in the plume (Thornton *et al.*, 2001b) but also by the detection of clones closely affiliated to methane-oxidising strains.

### 3.4.4 Comparison between culture and culture-independent methods

So far the results of the phylogenetic analysis of the 16S rRNA gene cloning approach have been discussed. However, in addition to the environmental clones from the Four Ashes site, a large number of bacteria (71 from groundwater and 43 from sand) were isolated aerobically on R2A agar and identified by 16S rRNA gene sequencing. Isolation of bacteria from the Four Ashes site was employed during this study mainly because we wanted to get hold on a number of different bacterial strains that would enable the establishment of laboratory microcosm experiments (Chapter 5). It was not the intention of this study to undertake an exhaustive culture-based approach to describe the microbial diversity in the Four Ashes site, because it is well established that only a small percentage of environmental clones can be cultured anyway (Amman *et al.*, 1995). However, it is worth comparing the phylogenetic results of the two approaches.

*Limited overlap and major discrepancies between the isolated bacteria and the environmental clones from the Four Ashes site*

At the phylum level, with the exception of two *Deinococci*, all the cultured bacteria from the Four Ashes site belonged to 4 bacterial phyla (Fig. 3.14): *Proteobacteria* (51.8%), *Actinobacteria* (38.6%), *Firmicutes* (7%) and *Bacteroidetes* (only 1 isolate). This is not surprising, because it is well recognised that there is a cultivation bias towards the "big four" (Hugenholtz, 2002), which anyway consist the majority of the sequences of cultured bacteria deposited in public repositories (Fig. 3.23). In contrast, many of the groundwater clones grouped within the *Bacteroidetes* and *Firmicutes* (30% and 22% respectively) while many sand clones (29%) belonged either to minor phylogenetic groups or could not be classified. However, the most striking difference observed at the phylum level was the fact that although 38.6% of the isolates belonged to *Actinobacteria* including potentially phenol-degrading *Rhodococcus* strains, only 2 out of 258 clones belonged in the *Actinobacteria*. Other studies have also noticed low number of *Actinobacteria* in the clone libraries; *Actinobacteria* were not detected in 4 large clone libraries (1515 clones in total) from reservoir sediments (Wobus *et al.*, 2003)

and in a clone library from a mat sample of a lake in Antarctica, only 3 out of 325 clones belonged to *Actinobacteria*, although many *Actinobacteria* were isolated from the same sample (Brambilla *et al.*, 2001). There is some speculation that Gram-positive bacteria are under-represented in the clone libraries, mainly because they are harder to lyse than Gram-negative bacteria (Bürgmann *et al.*, 2001). However when Popp *et al.* (2006) constructed 2 clone libraries from the same hydrocarbon-contaminated soil (with BTEX and phenols) using 2 different DNA extraction methods (3 bead treatments compared to only 1 bead treatment) he did not observe significant differences (at least at the phylum level) and *Actinobacteria* consisted only 5% and 4% of the clone libraries respectively.

At the species level, the phylogenetic analysis of all sequences obtained indicated that although isolated microorganisms represented a wide range of genera, the overlap between the isolated microorganisms and the sequenced clones was limited to only 5 ribotypes (Table 3.4). As it was shown in Table 3.4, ribotype is19 (4 sand isolates) and ribotype is14 (5 sand isolates) were related to *Acidovorax* strains that were found repeatedly within the sand clone library (17 and 3 sand clones respectively). On the other hand, although many *Pseudomonas* strains were isolated from the Four Ashes site and other studies have reported the dominance of *Pseudomonas* strains in phenol/hydrocarbon contaminated environments (Popp *et al.*, 2006; Whiteley and Bailey, 2000), only 3 *Pseudomonas* strains were detected amongst the clones from the Four Ashes site.

In the past, numerous studies in a range of environments observed big discrepancies between cultured microorganisms and clone libraries (Felske *et al.*, 1999; Brambilla *et al.*, 2001; Kisand and Wikner, 2003; Pearce *et al.*, 2003; Blaire *et al.*, 2007) and they all recognised the limitations of the culture-based methods. Similarly, the differences observed during this study can be explained by the biases that the culture-based method of this study involved.

*Biases of the culture-based method that was followed*

To begin with, it is important to recognise the fact that only a small proportion of environmental microbial communities can be cultured anyway. The number of culturable bacteria can be even less than 1% of the total number of bacteria as determined by direct

microscopy counts (Amman *et al.*, 1995), something which has been called as the "great plate count anomaly" (Staley and Konopka, 1985). In the last 2 decades, the use of molecular techniques (16S rRNA gene) revealed that unknown (uncultured) microorganisms can dominate natural microbial communities (Hugenholtz *et al.*, 1998). This became evident during this study, since a large number of clones had low sequence identities ( < 90%) to cultured microorganisms, although in most of the cases they were closely related ( > 97% identities) to uncultured environmental clones that were detected by other molecular-based studies (details in 3.3.4). Even apparently dominant members of the microbial community at the Four Ashes site (ribotypes GP01H05, SP12B04, GP12E08) appeared not to be closely related to cultured microorganisms. All the above indicate that currently unidentified bacterial species that have not been cultured yet may constitute a big proportion of the microbial communities at the Four Ashes plume.

In order to increase culturability of representative oligotrophic bacteria from polluted environments, in some cases minimal media supplemented with organic pollutants have been used. For example, Watanabe *et al.* (1998a) used minimal media supplemented with 500 mg $L^{-1}$ (5.3 mM) phenol to isolate phenol-degrading bacteria from phenol-containing activated sludge and Heinaru *et al.* (2000) used minimal media supplemented with 2.5 mM phenol and other organic compounds to isolate bacteria from river-water polluted with phenol leachate. Remarkably, another study showed that the gelling reagent may also have a significant effect on the successful isolation of representative environmental clones. Compared to a clone library (112 clones) from lake sediments, Tamaki *et al.* (2005) demonstrated that 11 out of 30 isolates growing on gellan gum but only 1 out of 30 isolates growing on agar medium had more than 95% sequence similarity to the environmental clones. The agar medium appeared to favour the growth of *α-Proteobacteria*, *Actinobacteria* and *Firmicutes*, which were minor groups in the clone library. Moreover, another study (Sait *et al.*, 2002) demonstrated that previously uncultured bacteria could be isolated successfully on minimal agar medium using xylan (a heteropolysaccharide polymer) as the growth substrate. However, in this study culturing was performed on rich R2A medium. The incubation of plates at the temperature of the groundwater (11°C) appeared to have favoured the isolation of potentially psychrotolerant bacteria (e.g. 10 groundwater clones had 99% identity similarity to psychrophilic

Actinobacterium *Frigoribacterium faeni*). As a result, the microbes that were cultured were not necessarily representative of the microbial community at the Four Ashes site but probably instead what Hugenholtz (2002) called the "weeds" of the microbial world that are able to form colonies on rich artificial media. The potential of some of the isolated bacteria from the Four Ashes site to degrade and tolerate phenol was investigated further, as described in Chapter 4.

Finally, it appears that anaerobic isolation of bacteria would have been a more suitable method for the isolation of bacteria from that depth (30 mbgl) in borehole 59. For the purposes of this study, anaerobic isolation had not been planned due to technical constraints and because the plume at this depth was expected to be aerobic-microaerobic based on the chemical analyses of the 2005 samples (TPC 350 mg $L^{-1}$). However, in the 2006 sampling the TPC was measured at levels (1220 mg $L^{-1}$) that dissolved oxygen is expected to be depleted (see also Fig. 2.16). This is supported by the fact that amongst the clones, there were many clones closely related to known anaerobic species as discussed previously (in 3.4.3).

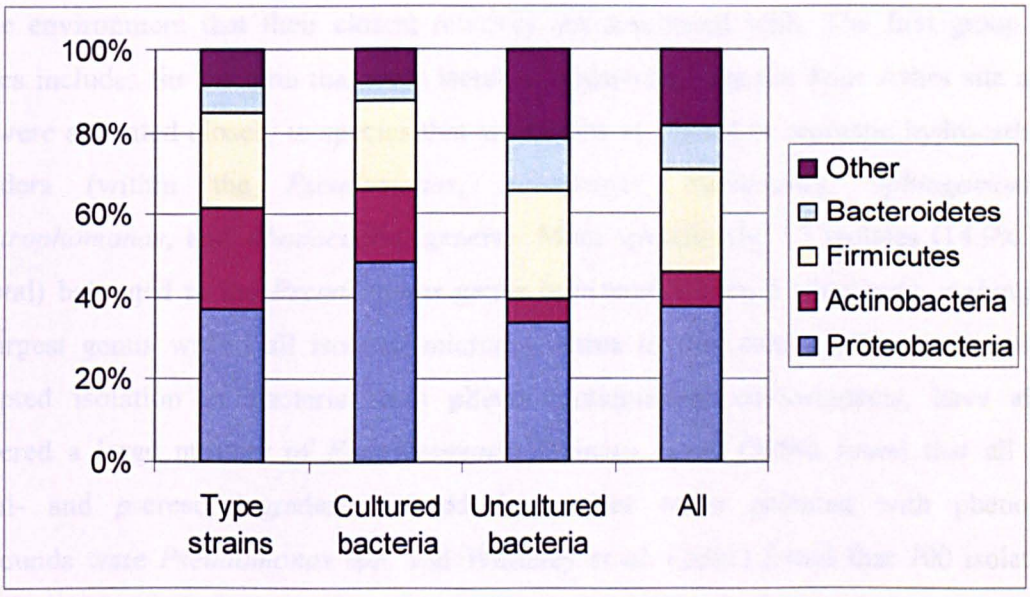



**Fig. 3.23.** Phylogenetic comparison of the 513,272 16S rRNA gene sequences contained in the RDP II database (Release 9.61; 22 May 2008).

### 3.4.5 Isolated microorganisms – the usual suspects

Since we made clear the limitations of the culturing method that was followed, it is interesting to investigate what microorganisms were actually cultured, whether they can be associated with a preferred state (planktonic or attached) and whether they are related to microorganisms isolated from other (phenol or aromatic hydrocarbon) polluted environments.

Although some of the cultured bacteria were isolated repeatedly from the groundwater sample only and some of them from the sand sample only, no definite conclusion should be drawn for their preferred habitat (aquatic or attached/terrestrial). For example 9 groundwater isolates were closely related to *Frigoribacterium faeni* that was isolated from Antarctic sandstone (Kämpfer *et al.*, 2000) and many other groundwater isolates were closely related to strains of the *Rhizobium, Paenibacillus, Bacillus* and *Rathaybacter* genera that are more commonly associated with agricultural soils than with aquatic environments. Hence, for the purposes of the phylogenetic analysis (in 3.3.4), no distinction was made between groundwater and sand isolates.

The isolated bacteria from the Four Ashes site can be divided into 2 groups, based on the environment that their closest relatives are associated with. The first group of isolates includes the bacteria that were isolated repeatedly from the Four Ashes site and they were affiliated closely to species that are known as phenol or aromatic hydrocarbon degraders (within the *Pseudomonas*, *Acidovorax*, *Variovorax*, *Sphingomonas*, *Stenotrophomonas*, and *Rhodococcus* genera). More specifically, 17 isolates (14.9% of the total) belonged to the *Pseudomonas* genus (clustered within 5 ribotypes), making it the largest genus within all isolated microorganisms in this study. Other studies that attempted isolation of bacteria from phenol-contaminated environments, have also recovered a large number of *Pseudomonads*. Heinaru *et al.* (2000) found that all 39 phenol- and *p*-cresol-degraders isolated from river water polluted with phenolic compounds were *Pseudomonas* spp. and Whiteley *et al.* (2001) found that 700 isolated bacteria from an operational phenolic remediating industrial treatment plant represented 59 species, of which 48 were *Pseudomonads* (although 15 of them were reclassified later as *Brevundimonas vesicularis*). Moreover, 22% of the isolates from the Four Ashes site

clustered within the *Comamonadaceae* family of the *β-Proteobacteria* and closely to known phenol or hydrocarbon degrading species of the *Acidovorax*, *Variovorax*, and *Rhodoferax* genera. Strains belonging to the *Comamonadaceae* are often isolated from phenol contaminated sites and it is characteristic that when Watanabe *et al.* (1998) isolated bacteria from a phenol-digesting activated sludge, strains belonging in the *Pseudomonas*, *Variovorax*, *Nocardioides*, *Rhodobacter*, *Rhodoferax*, *Acinetobacter*, *Xanthomonas*, *Cytophaga* and *Brevibacterium* genera were recovered (most of these genera were also isolated in this study) and functional analysis of phenol-hydroxylase genes showed that one *Variovorax paradoxus* strain was the principal phenol digester in the activated sludge. Six more isolates of this study were closely related to hydrocarbon degrading *Stenotrophomonas* strains (see 3.3.4). On the other hand, although many strains within the *Spingomonas* genus are known as degraders of polycyclic aromatic hydrocarbons (Leys *et al.*, 2004), the isolated bacteria from the Four Ashes aquifer were more closely related to *Sphingomonas* strains from uncontaminated environments (see 3.3.4). Apart from these Gram-negative isolates, a large number of (Gram-positive) *Actinobacteria,* including *Rhodococci* strains with known ability to degrade aromatic hydrocarbons were also isolated from the Four Ashes site; for example isolate ig242 had 100% sequence identity *to Rhodococcus erythropolis* Ri81, a benzene-degrading strain that was isolated from a BTEX contaminated aquifer (Fahy *et al.*, 2008).

The second group of isolates includes the bacteria that were affiliated to genera that are more often associated with agricultural soils (for example the *Rhizobium*, *Bacillus*, *Nocardia*, and *Rathaybacter* genera) and/or cold environments (*Frigoribacterium*, *Clavibacter, Taxeobacter, Sporosarcina* genera) rather than with hydrocarbon degradation (as described in 3.3.4). The detection of these isolates in the samples from the Four Ashes site can be principally justified by the presence of the overlying agricultural field and the culturing conditions that were followed (11°C). The ability of some of these isolated strains to degrade or tolerate phenol was investigated further, in Chapter 4.

### 3.4.6 Conclusions

The diversity of planktonic and attached microbial communities sampled from the same depth in the Four Ashes aquifer (30 mbgl in borehole 59) was assessed by 16S rRNA gene cloning and sequencing. The phylogenetic analysis showed that the two communities differed significantly (in agreement with their DGGE profiles in Fig. 2.14) and that the attached community was more diverse than the planktonic one. Based on the functions of the closest relatives of the identified clones, 16S rRNA gene phylogeny could be linked to the geochemical environment at the Four Ashes site, as the presence of anaerobic microorganisms, phenol degrading species (major pollutant at the Four Ashes site) or species with diverse functional properties has been detected.

In addition to the environmental clones, a large number of bacteria was isolated from the same samples and identified by 16S rRNA gene sequencing. The phylogenetic analysis showed that there was limited overlap between the clones and the isolated microorganisms and the biases of the culture method that was followed were recognised (in 3.4.4). However, some of the isolated microorganisms were closely related to known phenol degrading species.

# Chapter 4

# Ability of isolated bacteria to degrade or tolerate phenol and to attach to sand

## 4.1 Introduction

Organic solvents (including phenol) are toxic to microorganisms by disrupting the integrity of the cytoplasmic membrane (Heipieper *et al.*, 1994; Sikkema *et al.*, 1995). Microbial adaptation mechanisms to organic solvents in general (reviews by Heipieper *et al.*, 1994; Sikkema *et al.*, 1995; Weber and de Bont; 1996 Isken and de Bont; 1998; Ramos *et al.*, 2002) and phenol in particular (Keweloh *et al.*, 1991; Heipieper *et al.*, 1992) have been described while, as discussed in section 1.2, a range of microorganisms can degrade phenol aerobically. However, not all bacteria can degrade phenol; they will also differ in their affinity for this substrate and the maximum concentration that they can tolerate.

In Chapter 3, 114 bacterial strains were isolated from groundwater and sand samples from the phenol contaminated plume of the Four Ashes aquifer (borehole 59, 30 mbgl) and were identified by partial 16S rRNA gene sequencing. However, it is not known whether these bacteria were active within the Four Ashes aquifer at the point from which they were isolated as microorganisms can exist in dormant/inactive state in natural environments (Luna *et al.*, 2002) and be transported significant distances from other parts of the plume. For example, six *Bacillus* strains were isolated in this study (all belonging to the same ribotype) and *Bacillus* species are known to be able to survive for years as dormant spores (Setlow, 2007). Moreover, since these bacterial strains were isolated on carbon-rich R2A medium, it is not known whether they can degrade phenol at concentrations relevant to the ones found in the Four Ashes site (488 mg $L^{-1}$ or 5.2 mM phenol, in borehole 59, in 2006). Of course, there is the possibility that some of these isolates are able to tolerate the presence of phenol and grow on other carbon sources (other pollutants of the Four Ashes aquifer or metabolites produced by the action of other species).

In addition, it was not possible to determine based on their phylogenetic affiliations or the sample (groundwater or sand) whether the isolated microorganisms preferentially existed in the attached or the planktonic phase. Initial bacterial adhesion of single strains to mineral surfaces is thought to be governed by physicochemical

interactions (Bos *et al.*, 1999). Once irreversible attachment is achieved by bacterial envelope structures, such as fimbriae, flagella or adhesins (Lejeune, 2003) and by the production of EPS (Stoodley *et al.*, 2002; Palmer *et al.*, 2007), then biofilm formation is initiated by binary division of attached cells (Hall-Stoodley and Stoodley, 2002).

However, information on these functional properties (the ability to degrade or tolerate phenol, the ability to attach to sand grains) is needed as a selection of these isolates is to be used to inoculate the microcosm experiments described in Chapter 5 that will test specific hypotheses about microbial behaviour in complex microbial communities.

Therefore, the aim of this chapter is to investigate the diversity of functions exhibited by bacteria isolated from the Four Ashes aquifer (borehole 59, 30 mbgl). Specific objectives of this chapter are to examine whether isolated bacteria from the Four Ashes site can:

- degrade 0.5 or 5 mM phenol when supplied as the sole carbon source
- tolerate 0.5 or 5 mM phenol in the presence of other organic carbon sources
- attach to sand grains in microcosm columns

## 4.2 Methods

### 4.2.1 Selection of bacterial isolates from the Four Ashes site

As seen previously (Chapter 3, Table 3.3), 48 ribotypes were identified in the sequenced bacteria isolated from the Four Ashes site (borehole 59, 30 mbgl). To further characterise these isolates regarding their ability to degrade or tolerate phenol and to attach to sand, 18 isolates were selected on the basis of the criteria indicated in Table 4.1. The selected isolates included 4 strains (out of the 5) that were also found in the (sand) clone library (CL) and isolates that were closely related (≥98% sequence identity) to known phenol or hydrocarbon degrading species (●). Nine further isolates were selected including at least one representative from each identified phylogenetic group (R), isolates from known hydrocarbon-degrading genera (i.e. *Sphingomonas*, *Stenotrophomonas*), as

well as isolates with no apparent hydrocarbon catabolic properties. The selection included strains that were isolated (repeatedly or once) from the groundwater sample only (217, 220, 229, 232, 235, 245, 256, 257, 262, 263, 264, 272), from the sand sample only (273, 282, 284, is19) as well as the 2 strains that were isolated from both the groundwater and the sand sample (226 and 297).

**Table 4.1.** Isolated bacteria from the Four Ashes site selected for further characterisation, regarding their ability to degrade or tolerate phenol and to attach to sand.

| Isolate ID (ribotype ID) | Property | | Phylogenetic group | Closest phylogenetic affiliation |
|---|---|---|---|---|
| ig217 | | | *α-Proteobacteria* | *Sphingomonas aquatilis* |
| ig220 | | | *α-Proteobacteria* | *Sphingomonas faeni* |
| ig226 (is292) | | ● | *Actinobacteria* | *Rhodococcus fascians* |
| ig229 | | ● | *β-Proteobacteria* | *Rhodoferax sp.* |
| ig232 | | R | *Deinococcus-Thermus* | *Deinococcus radiopugnans* |
| ig235 | | | *α-Proteobacteria* | *Rhizobium huautlense* |
| ig245 | | | *α-Proteobacteria* | *Stenotrophomonas rhizophila* |
| ig256 | | ● | *Actinobacteria* | *Rhodococcus erythropolis* |
| ig257 | | | *Actinobacteria* | *Clavibacter michiganensis* |
| ig262 | | | *β-Proteobacteria* | *Pigmentifaga* sp. |
| ig263 | | R | *Bacteroidetes* | *Taxeobacter* sp. |
| ig264 | | ● | *γ-Proteobacteria* | *Pseudomonas* sp. |
| ig272 | | ● | *β-Proteobacteria* | *Methilibium* sp. |
| is273 (is17) | CL | ● | *γ-Proteobacteria* | *Pseudomonas* sp. |
| is282 | CL | ● | *γ-Proteobacteria* | *Pseudomonas* sp. |
| is284 (is14) | CL | ● | *β-Proteobacteria* | *Acidovorax* sp. |
| is297 | | R | *Firmicutes* | *Bacillus* sp. |
| is19 | CL | ● | *β-Proteobacteria* | *Acidovorax* sp. |

CL = sharing 100% sequence identity (common ribotype) with environmental clones from the Four Ashes site

● = related ( ≥98% sequence identity) to phenol or hydrocarbon degrading species

R = selected (or unique) representative from this phylogenetic group

### 4.2.2 Ability of the isolates to degrade or tolerate phenol

*Media*

The composition of the R2A broth (Reasoner and Geldreich, 1985) is shown in Table 4.2 and the composition of the AB minimal medium (Heydorn *et al*, 2000) can be seen in Table 2.2. In addition, a phenol stock solution (10 g $L^{-1}$) was prepared and was filtered sterilised through a 0.2 $\mu$m pore size syringe filter (Nalge Nunc, USA).

**Table 4.2.** The composition of R2A broth.

| R2A broth | Concentration (g $L^{-1}$) |
|---|---|
| Bacto™ Yeast extract | 0.5 |
| Proteose peptone | 0.5 |
| Casamino acids | 0.5 |
| Glucose | 0.5 |
| Starch | 0.5 |
| $K_2HPO_4$ | 0.3 |
| $MgSO_4.7H_2O$ | 0.024 |
| Sodium pyruvate | 0.3 |

*Preparation of bacterial isolates*

Plates containing R2A (OXOID, UK) were streaked with frozen stocks of the isolated bacteria (see 3.2.2) and grown for 3 days at 20°C. Single bacterial colonies were used to inoculate 10 ml of liquid R2A broth (Table 4.2) and the liquid cultures were placed on an orbital shaker at 200 rpm at 20°C. Two days later, 2 ml of these cultures was used to inoculate 100 ml R2A broth batch cultures in 250 ml conical flasks. Following incubation for 3 days on an orbital shaker at 200 rpm at 20°C, 10 ml of the batch cultures (Fig. 4.1) was spun down for 10 min at 2,200 x g at room temperature, the supernatant was decanted and the pellet was resuspended into 5 ml of 0.9% (w/v) NaCl. Two more washes with 0.9% NaCl were performed before the pellet was resuspended into 5 ml of AB medium (Table 2.2). The $OD_{600}$ of 1 ml of this solution was measured using a Shimadzu UV-1201 UV-VIS Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

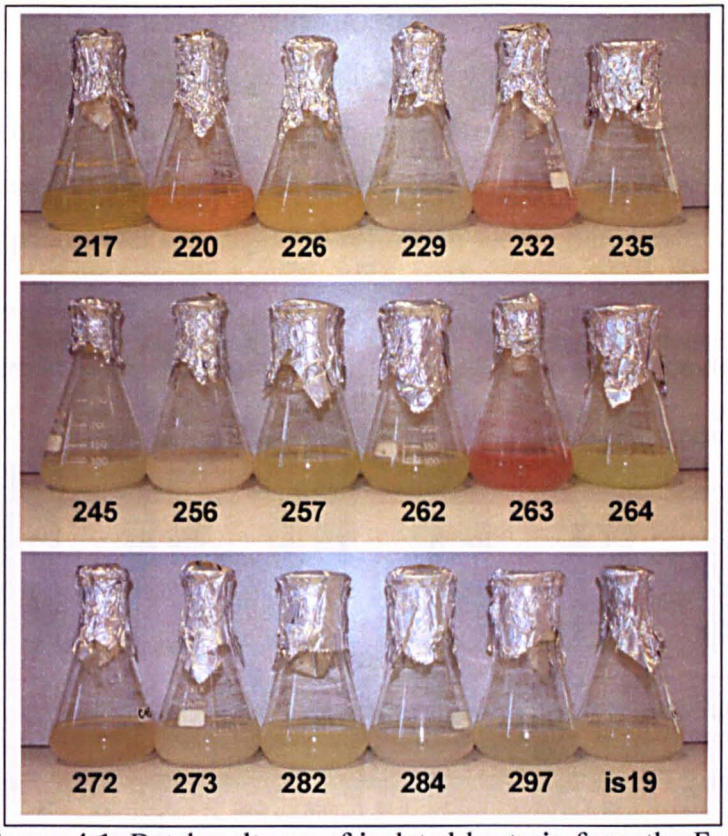



**Figure 4.1.** Batch cultures of isolated bacteria from the Four Ashes site in 100 ml of R2A broth.

*Experimental treatments*

Six different treatments were prepared in order to test the ability of single isolates to degrade or tolerate 0.5 or 5 mM phenol (Fig. 4.2). Their ability to degrade phenol was investigated in 10 ml shaken liquid cultures containing AB minimal medium supplemented with 0.5 or 5 mM phenol as the sole carbon source. At the same time, their ability to tolerate phenol was tested in shaken liquid cultures which contained a carbon-rich medium (R2A broth) supplemented with the same concentrations of phenol (0.5 or 5 mM). All liquid cultures were prepared in duplicate (Fig. 4.2) and they were inoculated simultaneously with an appropriate volume of washed bacterial cultures (see above) so that the starting $OD_{600}$ in all cultures was 0.01. Following incubation for 3 days on an orbital shaker at 200 rpm at 20°C, bacterial growth was assessed by measuring the OD at 600 nm (the $OD_{600}$ of sterile AB or R2A broth medium was used as zero) using a Shimadzu UV-1201 spectrophotometer.

In addition, 1.5 ml from all the bacterial cultures supplemented with 0.5 or 5 mM phenol was centrifuged for 10 min at 13,000 x g at 4°C using a refrigerated Hawk 15/05 centrifuge (Sanyo, Japan) and 1 ml of the supernatant was transferred into new sterile microtubes. These samples were kept at -20°C for HPLC analysis to determine the concentration of phenol that was degraded within the shaken liquid cultures (see below).

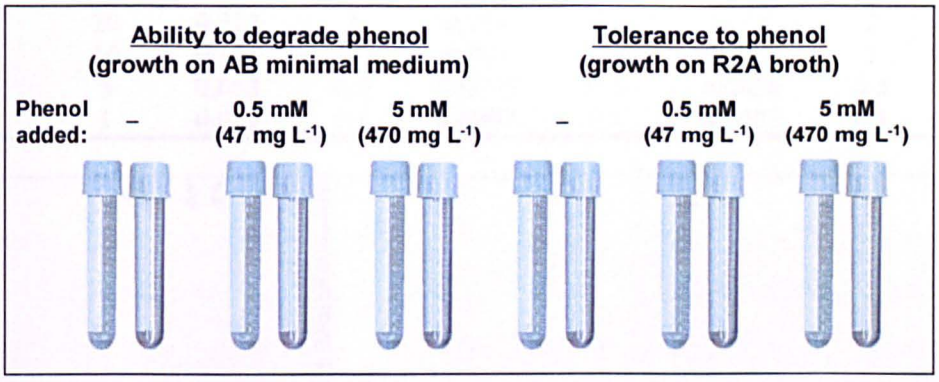



**Figure 4.2.** An overview of the experimental set-up (6 different liquid culture treatments) to explore the potential of isolated bacteria from the Four Ashes site to degrade or tolerate 2 different concentrations of phenol (0.5 and 5 mM).

*Determination of phenol concentration by HPLC*

The concentration of phenol in the samples from the liquid cultures was determined by High-Pressure Liquid Chromatography, using a Perkin Elmer Series 200 HPLC System and an Alltech® Allsphere™ ODS-2 5µm HPLC column (details of the system and the settings in 2.2.2). Appropriate HPLC standards (Table 4.3) were prepared in AB minimal medium for phenol and 3 possible metabolites: *cis,cis*-muconic acid, 4-hydroxybenzoic acid and catechol. Prior to HPLC analysis, each of these compounds was run independently so that their retention times could be determined. The retention times were approximately 2.4, 2.8, 3.1 and 3.7 minutes for *cis,cis*-muconic acid, 4-hydroxybenzoic acid, catechol and phenol respectively, and their peaks were readily resolved when present in AB medium. An example HPLC chromatograph (standard 4, in AB medium) is shown in Fig. 4.3, where the 4 peaks with the corresponding retention times can be seen. However, in R2A-containing cultures, only the detection of the phenol peak was possible because unidentified components of the R2A broth had similar retention times to those of the 3 phenol metabolites (Fig. 4.4).

**Table 4.3.** The concentration of phenol, *cis,cis*-muconic acid, 4-hydroxybenzoic and catechol within each of the 8 HPLC standards used.

| | Phenol | | *Cis,cis*-muconic acid | | 4-hydroxybenzoic acid | | Catechol | |
|---|---|---|---|---|---|---|---|---|
| **Standard** | **mg $L^{-1}$** | **mM** | **mg $L^{-1}$** | **mM** | **mg $L^{-1}$** | **mM** | **mg $L^{-1}$** | **mM** |
| 1 | 500 | 5.319 | 50 | 0.352 | 50 | 0.362 | 50 | 0.454 |
| 2 | 200 | 2.128 | 20 | 0.141 | 20 | 0.145 | 20 | 0.181 |
| 3 | 100 | 1.064 | 10 | 0.070 | 10 | 0.072 | 10 | 0.091 |
| 4 | 50 | 0.532 | 5 | 0.035 | 5 | 0.036 | 5 | 0.045 |
| 5 | 20 | 0.213 | 2 | 0.014 | 2 | 0.014 | 2 | 0.018 |
| 6 | 10 | 0.106 | 1 | 0.007 | 1 | 0.007 | 1 | 0.009 |
| 7 | 5 | 0.053 | 0.5 | 0.0035 | 0.5 | 0.0036 | 0.5 | 0.0045 |
| 8 | 1 | 0.011 | 0.1 | 0.0007 | 0.1 | 0.0007 | 0.1 | 0.0009 |

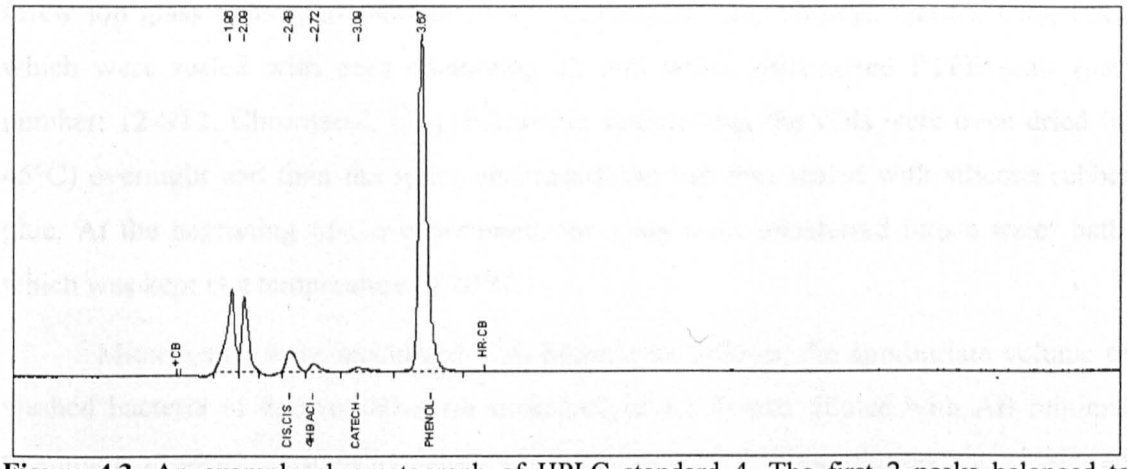



**Figure 4.3.** An example chromatograph of HPLC standard 4. The first 2 peaks belonged to unspecified components of the AB medium and the remaining 4 peaks were assigned (from left to right) to *cis,cis*-muconic acid, 4-hydroxybenzoic acid, catechol and phenol. The retention time (in minutes) for each of these peaks is shown on the top of the chromatograph.

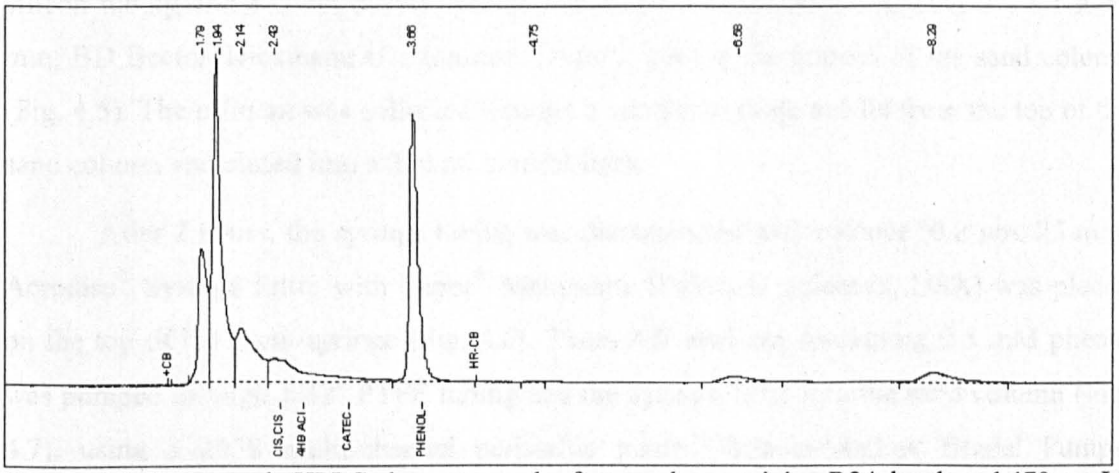



**Figure 4.4.** An example HPLC chromatograph of a sample containing R2A broth and 470 mg $L^{-1}$ phenol only (no phenol metabolites). Components of the R2A broth had similar retention times to the 3 phenol metabolites, making their quantification problematic.

### 4.2.3 Attachment of individual isolates on microcosm sand columns

*The design of the laboratory microcosms*

A model microcosm system was developed in collaboration with Dr David Elliott and Mr Ammar Abdul Razak (University of Sheffield) in order to investigate bacterial attachment to sand.

Eight grams (8 g) of acid washed (with 1M HCl) and oven (160°C) sterilised quartz sand (270 $\mu$m grain size; Quarzwerke, Germany) was weighed and placed into 4 ml screw top glass vials (part number: 4-SV; Chromacol Ltd, Welwyn Garden City, UK), which were sealed with caps containing 12 mm white silicone/red PTFE seals (part number: 12-ST2, Chromacol, UK). Following autoclaving, the vials were oven dried (at 45°C) overnight and then the space underneath the cap was sealed with silicone rubber glue. At the beginning of the experiment, the vials were transferred into a water bath, which was kept at a temperature of 20°C.

Microcosms were inoculated with bacteria as follows. An appropriate volume of washed bacteria of known $OD_{600}$ (as described in 4.2.2) was diluted with AB minimal medium to produce 10 ml of inoculum with an $OD_{600}$ of 0.1. This solution was transferred into 10 ml syringes and loaded on a 10-syringe pump (Cole-Palmer, Vernon Hills, IL, USA). The flow rate was set at 3 ml $h^{-1}$ and the inoculum was pumped through 1/32" silicon tubing and a 5 cm sterile syringe needle (BD Microlance™ 3, 19G 2", 1.1 x 50 mm; BD Becton Dickinson UK Limited, Oxford, UK) at the bottom of the sand column (Fig. 4.5). The effluent was collected through a smaller syringe needle from the top of the sand column and eluted into a 250 ml conical flask.

After 2 hours, the syringe tubing was disconnected and a sterile (0.2 $\mu$m, 25 mm) Acrodisc® Syringe Filter with Supor® Membrane (Pall Life Sciences, USA) was placed on the top of the 5 cm syringe (Fig. 4.6). Then, AB medium containing 0.5 mM phenol was pumped through 1/32" PTFE tubing and the syringe filter into the sand column (Fig. 4.7), using a 205S multi-channel peristaltic pump (Watson-Marlow Bredel Pumps, Falmouth, UK). All tubing, fittings and flasks were sterilised by autoclaving, apart from the Mediprene™ red-red 14 mm tubing (Elkay Laboratory Produts Ltd, Basingstoke, UK)

of the peristaltic pump which was sterilised with 10% (v/v) bleach and rinsed by passing through 60 ml of autoclaved $H_2O$.

To assess initial bacterial attachment the microcosm was run overnight (16 - 20 hours) before the attachment of microbial cells to sand grains was determined by fluorescent microscopy (see below).

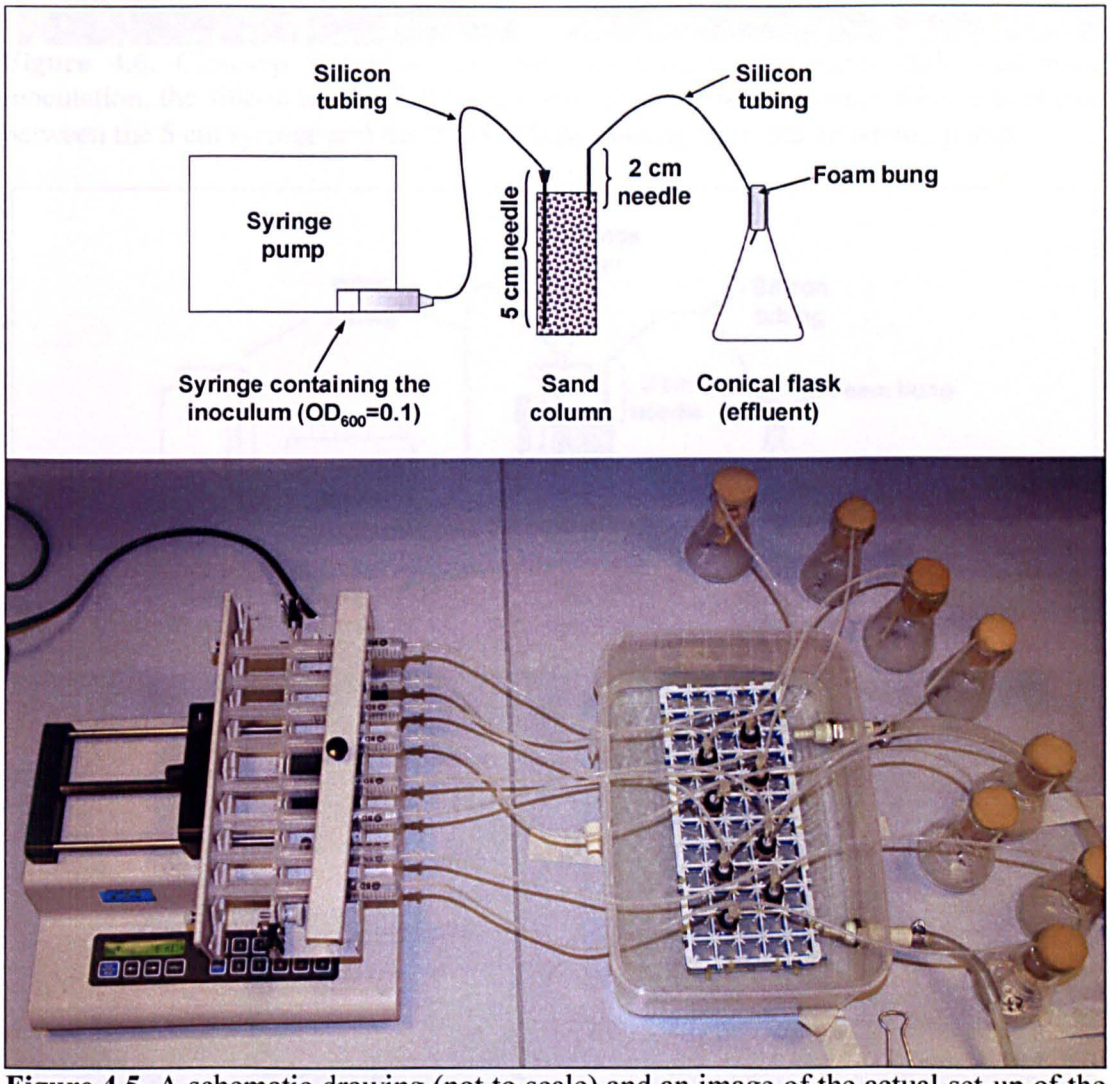



**Figure 4.5.** A schematic drawing (not to scale) and an image of the actual set-up of the system used for the inoculation of the sand columns with the bacterial inoculum.

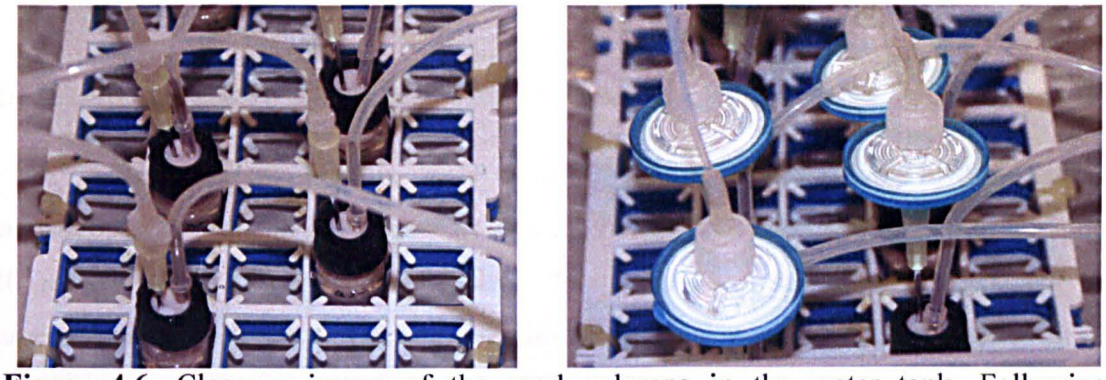


**Figure 4.6.** Close-up image of the sand columns in the water tank. Following inoculation, the silicon tubing (left) was removed and a sterile syringe filter was placed between the 5 cm syringe and the PTFE tubing coming from the peristaltic pump.

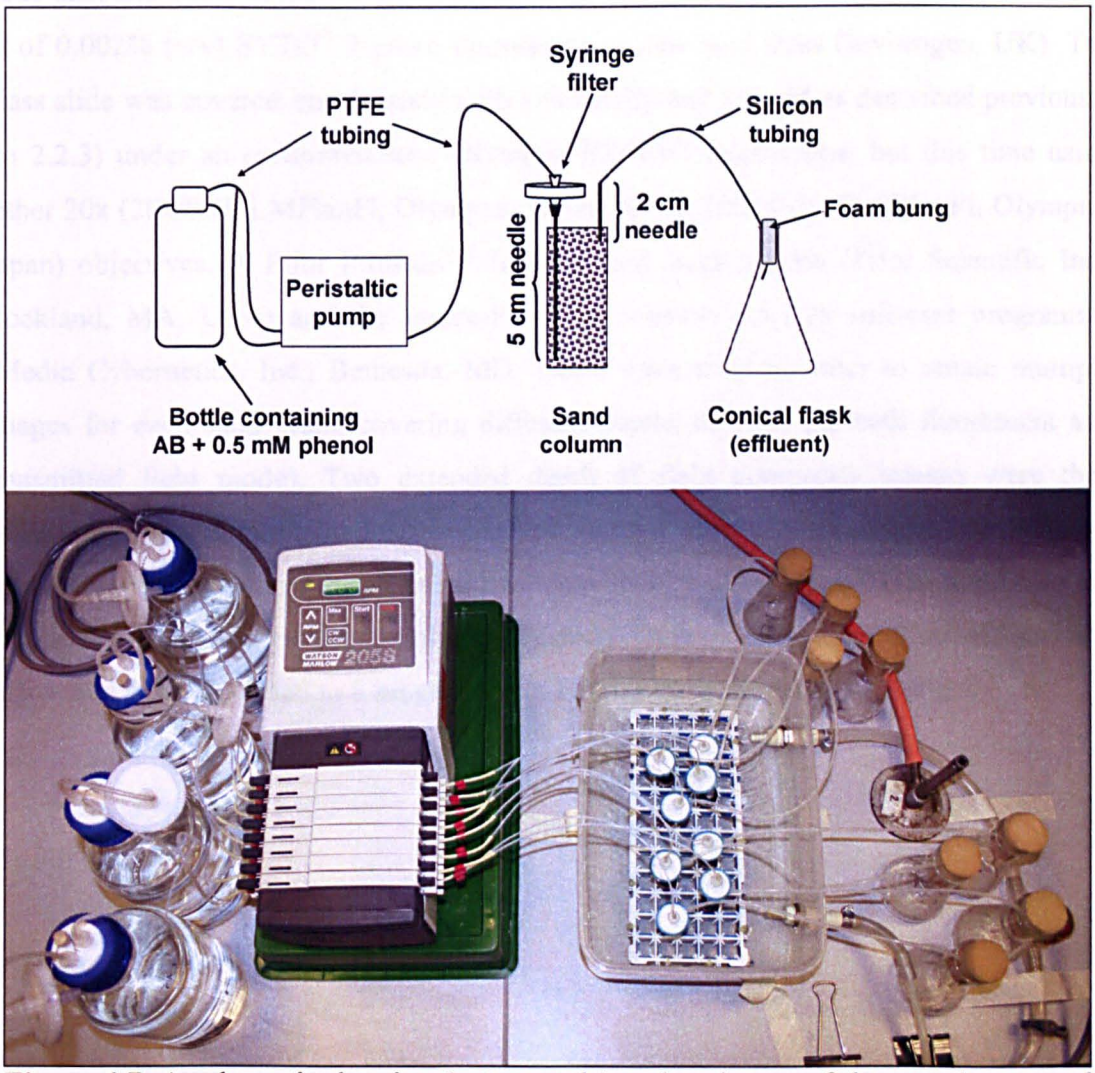



**Figure 4.7.** A schematic drawing (not to scale) and an image of the actual set-up of the system used for the circulation of the AB medium supplemented with 50 mg $L^{-1}$ phenol through the already inoculated sand columns.

*Visualisation of bacteria attached to sand grains*

Attachment of microbial cells to sand grains was determined by fluorescence microscopy. The sand in the glass vials was emptied into a sterile Petri-dish that contained 20 ml of sterile AB minimal medium. The Petri-dish was tilted slightly and the supernatant was discarded. The microorganisms attached to the sand were fixed with 2% (v/v) membrane-filtered formaldehyde in $H_2O$ for 3 min and then washed once more with 20 ml of AB medium. Using a flamed spatula, the sand grains were mixed in the Petri-dish and a few of them were transferred onto a microscope slide and stained with 50 $\mu$l of 0.002% (v/v) SYTO® 9 green fluorescent nucleic acid stain (Invitrogen, UK). The glass slide was covered immediately with a coverslip and viewed as described previously (in 2.2.3) under an epifluorescence Olympus BX50WI microscope, but this time using either 20x (20x/0.40 LMPlanFl, Olympus, Japan) or 10x (10x/0.30 SLCPlanFI, Olympus, Japan) objectives. A Prior ProScan™ II motorized stage system (Prior Scientific Inc., Rockland, MA, USA) and the Image-Pro Plus, version 4.5.1.29 software programme (Media Cybernetics, Inc., Bethesda, MD, USA) were used in order to obtain multiple images for each sand grain, covering different depths of view (in both fluorescent and transmitted light mode). Two extended depth of field composite images were then generated from the obtained sequences of images. For the fluorescent images the composite image was generated using the "Maximum local contrast" command. For the transmitted light images the "Minimum intensity" command was used instead and the image was then converted to 8 bit grey scale. Finally the 2 images were merged.

## 4.3 Results

### 4.3.1 Potential of isolated bacteria to degrade or tolerate phenol

The ability of 18 isolated bacteria from the Four Ashes site to grow on phenol as a sole carbon source was tested in shaken liquid cultures containing AB minimal medium supplemented with 2 different concentrations of phenol (0.5 mM or 5 mM). After incubation for 3 days, their growth was determined by OD measurements at 600 nm and the $OD_{600}$ of each isolate in AB medium alone (without phenol) was subtracted from these values (Fig. 4.8). The potential of the isolates to tolerate phenol in the presence of other carbon sources was examined by growing them in R2A broth, supplemented with 0.5 or 5 mM of phenol. These results were expressed as the ratio (%) of their growth ($OD_{600}$) in R2A liquid cultures supplemented with 0.5 or 5 mM phenol relative to their growth in R2A broth alone. Isolate 220 (*Sphingomonas* sp.) was excluded from the results presented here because its growth was very low ($OD_{600} < 0.050$) when grown on R2A broth alone. All other isolates had $OD_{600} > 0.200$ when grown in R2A broth alone.

For all liquid cultures supplemented with phenol, degradation of phenol was assessed by HPLC analysis. Results were expressed as the % of phenol degraded over the 3 days of the experiment. Due to limitations of the HPLC method (see 4.2.2 & Fig.4.4) the concentrations of 3 phenol metabolites (*cis,cis*-muconic acid, 4-hydroxybenzoic acid, catechol) were quantified in the AB medium liquid cultures only.

Based on their ability to degrade 0.5 mM phenol as the sole carbon source (in AB medium) or in the presence of other organic compounds (in R2A broth), the isolates could be categorised into five groups, as shown in Fig. 4.8.A. Group I consisted of 1 isolate (*Rhodococcus erythropolis* 256) that degraded phenol almost completely in both AB and R2A liquid cultures. This isolate was also capable of utilising 5 mM phenol (Fig. 4.8.B), although at this concentration its growth was lower than that observed at 0.5 mM phenol, indicating some toxicity. Group II included 4 isolates (*Pseudomonas* spp 282, 264, 273, and *Methylibium* sp. 272) that utilised completely 0.5 mM of phenol when supplied as the sole carbon source but in the R2A medium degraded phenol partially (28 – 55% of the

initial phenol concentration degraded). Group III consisted of one isolate (*Acidovorax* sp. 284) that also utilised 0.5 mM phenol completely when supplied as the sole carbon source; however, in the R2A cultures it grew exclusively on other carbon sources and did not utilise any of the phenol present. Group IV consisted of isolates *Pigmentifaga* sp. 262 and *Taxeobacter* sp. 263, which degraded phenol only in the R2A cultures. The remaining 9 isolates (Group V) were not capable of degrading phenol either in AB or R2A liquid cultures. In general, the addition of 0.5 mM of phenol decreased bacterial growth in R2A broth (with the exceptions of 256, 284, 262, and 232) but all isolates were able to tolerate it, since in all cases bacterial growth was at least 45% of the growth observed in R2A alone. In addition, HPLC analysis indicated that degradation of phenol in AB medium by isolates *R. erythropolis* 256 and *Acidovorax* sp. 284 was accompanied by the production of 3.5 and 7.2 mg $L^{-1}$ of *cis,cis*-muconic acid respectively. Nevertheless, this result should be treated with caution because we cannot exclude the possibility that another phenol metabolite that was not included in the standards of this study may have the same retention time with *cis,cis*-muconic acid.

When 5 mM of phenol was supplied either in AB minimal medium or in R2A broth (Fig. 4.8.B), only one isolate (*Rhodococcus erythropolis* 256) degraded some of it (2% and 35% of the initial amount in AB and R2A liquid cultures respectively). The remaining isolates did not degrade any of the phenol present in either media and exhibited different tolerances to 5 mM phenol. For 4 isolates (*Pseudomonas* sp. 282, *Pseudomonas* sp. 273, *Methylibium* sp. 272, and *Taxeobacter* sp. 263) the presence of 5 mM phenol in R2A broth completely inhibited their growth, while the remaining 12 isolates exhibited different degrees of tolerance and their growth in R2A supplemented with 5 mM phenol ranged between 8.5 and 85% of their growth in R2A alone.

**Figure 4.8. (Next page).** Growth of isolated bacteria from the Four Ashes site on phenol as sole carbon source (in AB minimal medium) and in the presence of other organic compounds (in R2A broth). Two different concentrations of phenol were tested, 0.5 mM (Fig. 4.8.A) and 5 mM (Fig.4.8.B) and phenol degradation was quantified by HPLC analysis and expressed as % of the initial concentration.

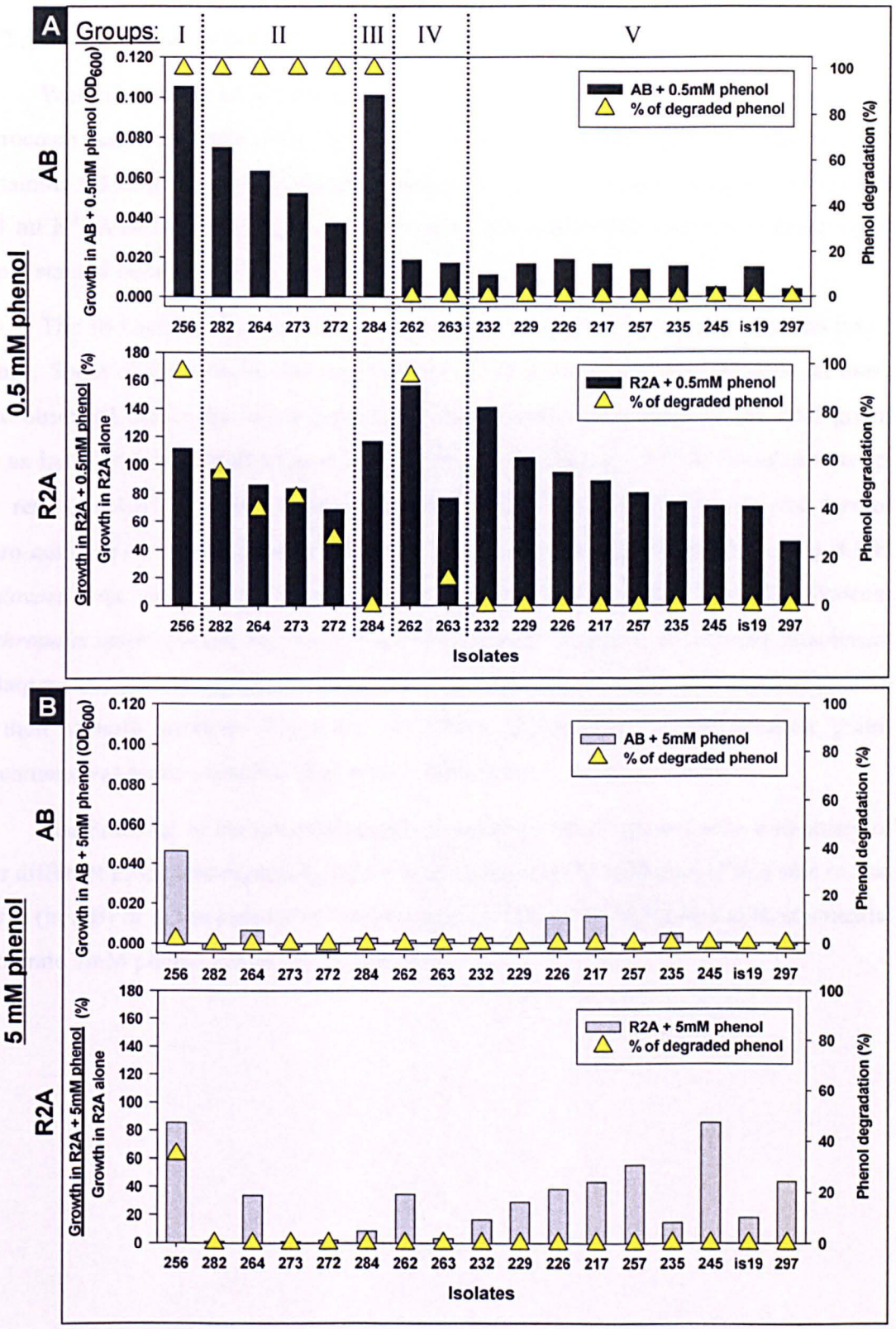



**Figure 4.8.** (Legend on the previous page)

### 4.3.2 Attachment of isolates to sand grains

Washed cultures of isolated bacteria ($OD_{600}$ adjusted to 0.1) were used to inoculate microcosm sand columns (see 4.2.3). Following inoculation AB minimal medium containing 0.5 mM phenol was pumped through the sand columns overnight at a flow rate of 3 ml $h^{-1}$. Attachment to the sand grains was assessed by fluorescence microscopy of Syto 9 stained cells (4.2.3).

The 18 bacteria showed different degrees of attachment to the sand that fell into 3 groups. Some of the isolates did not attach at all or a couple of attached cells, at most, were observed. Secondly, some isolates exhibited sparse attachment to the sand grains and as individual cells only (Fig. 4.9.A & 4.9.B – *Bacillus* sp. 297 & *Pseudomonas* sp. 264 respectively) and finally there were isolates that attached extensively and formed micro-colonies on the sand grains of various degrees of coverage (Fig. 4.9.C – Fig. 4.9.F: *Acidovorax* sp. is19, *Acidovorax* sp. 284, *Pigmentifaga* sp. 262 and *Rhodococcus erythropolis* respectively). For the isolates that showed extensive attachment, attachment on larger sand grains was usually observed across the edges/cracks of the grains and not on their smooth surfaces (Fig. 4.9.C & 4.9.D). In contrast, on the smallest grains attachment was more extensive (Fig. 4.9.F – *Rhodococcus erythropolis* 256).

The potential of the selected bacteria to attach to sand together with a summary of their different properties regarding their ability to degrade 0.5 mM phenol as a sole carbon source (in AB) or in the presence of other organic compounds (in R2A) and their potential to tolerate 5mM phenol are shown in Table 4.4.

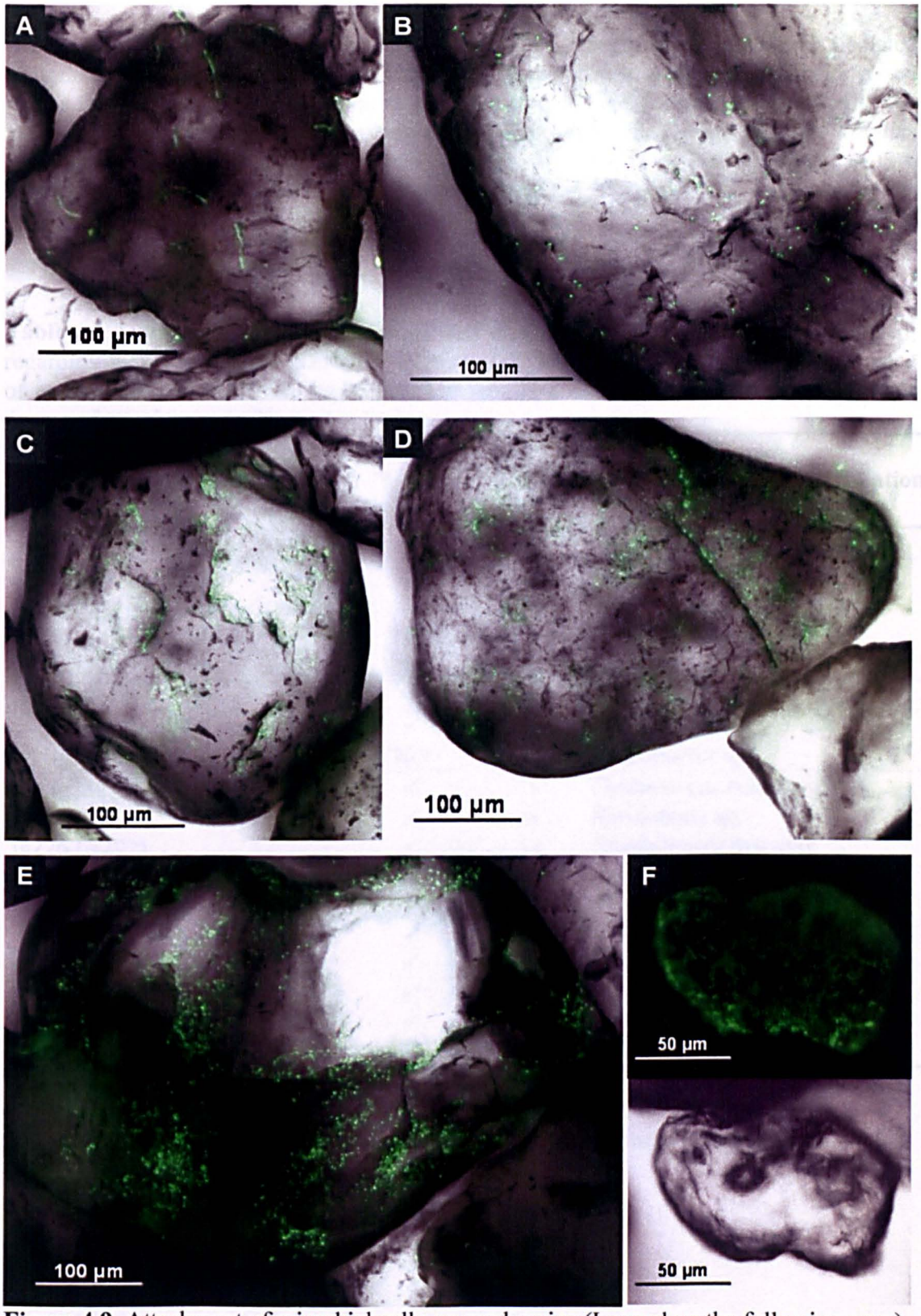



**Figure 4.9.** Attachment of microbial cells on sand grains (Legend on the following page).

**Figure 4.9. (Previous page).** Fluorescence microscopy images of microbial cells (isolated bacteria from the Four Ashes aquifer) attached to sand grains. Attachment of cells varied from a few individual cells (A, B) to more extended coverage, forming micro-colonies (C, D, E). Attachment was particularly extended on some small sand grains (F). A = *Bacillus* sp. 297; B = *Pseudomonas* sp. 264; C = *Acidovorax* sp. is19; D = *Acidovorax* sp. 284; E = *Pigmentifaga* sp. 262; F = *Rhodococcus erythropolis* 256.

**Table 4.4.** Summary of the different properties identified within the 18 isolates examined, regarding their ability to degrade phenol as sole carbon source (in AB) or in the presence of other organic compounds (in R2A), their potential to tolerate 5mM phenol and their degree of attachment to the sand columns.

| Isolate ID (ribotype ID) | Degraded 0.5 mM phenol | | Tolerated 5 mM phenol | Attached to sand | Closest phylogenetic affiliation |
|---|---|---|---|---|---|
| | in AB | in R2A | | | |
| ig256 | ● | ● | ✓ | ++ | *Rhodococcus erythropolis* |
| is282 | ● | ◍ | NO | – | *Pseudomonas* sp. |
| ig264 | ● | ◍ | ✓ | + | *Pseudomonas* sp. |
| is273 (is17) | ● | ◍ | NO | – | *Pseudomonas* sp. |
| ig272 | ● | ◍ | NO | – | *Methilibium* sp. |
| is284 (is14) | ● | | ✓ | ++ | *Acidovorax* sp. |
| ig262 | | ● | ✓ | ++ | *Pigmentifaga* sp. |
| ig263 | | ◍ | NO | – | *Taxeobacter* sp. |
| ig232 | | | ✓ | – | *Deinococcus radiopugnans* |
| ig229 | | | ✓ | + | *Rhodoferax sp.* |
| ig226 (is292) | | | ✓ | – | *Rhodococcus fascians* |
| ig217 | | | ✓ | – | *Sphingomonas aquatilis* |
| ig257 | | | ✓ | ++ | *Clavibacter michiganensis* |
| ig235 | | | ✓ | + | *Rhizobium huautlense* |
| ig245 | | | ✓ | ++ | *Stenotrophomonas rhizophila* |
| is19 | | | ✓ | ++ | *Acidovorax* sp. |
| is297 | | | ✓ | + | *Bacillus* sp. |
| ig220 | ? | ? | ? | – | *Sphingomonas faeni* |

● = degradation of phenol complete or > 95%
◍ = degradation of phenol incomplete (11 – 55%)
Attachment to sand = extended (++), sparse and as individual cells (+), no attachment (–).

## 4.4 Discussion

### 4.4.1 Degradation of phenol by single bacterial isolates

The ability of 17 isolated bacteria from the Four Ashes site to degrade 0.5 and 5 mM of phenol as the sole carbon source was examined in shaken liquid cultures containing AB minimal medium. The results indicated that 6 of the isolates (*Rhodococcus erythropolis* 256, *Acidovorax* sp. 284, *Methylibium* sp. 272, *Pseudomonas* sp. 264, *Pseudomonas* sp. 273 and *Pseudomonas* sp. 282) could grow and utilise completely 0.5 mM phenol (Fig. 4.8). However when grown at 5 mM phenol, toxicity effects were observed because only one of these isolates (*R. erythropolis* 256) exhibited limited growth and degradation of phenol (toxicity effects of phenol are discussed in 4.4.2). Bacterial strains capable of degrading even higher concentrations of phenol have been reported in the literature, though. For example an *Alcaligenes faecalis* strain could utilise an initial concentration of 12 mM phenol (Bastos *et al.*, 2000) while *Comamonas testosteroni* P15 and its mutant strain E23 could utilise up to 15 mM and 20 mM phenol respectively as the sole carbon source (Yap *et al.*, 1999).

As discussed earlier (1.2.2) the aerobic degradation of phenol by microorganisms involves two steps. The first step is the hydroxylation of the adjacent carbon atom to the pre-existing hydroxyl group and is catalysed by phenol hydroxylase genes. The resulting catechol is then cleaved by catechol 1,2-dioxygenase (C12O) or catechol 2,3-dioxygenase (C23O) genes (ortho- or meta-cleavage pathway respectively) producing *cis,cis*-muconic acid or 2-hydroxymuconic semialdehyde. During this study functional analysis on the genes involved in the degradation of phenol by the 6 phenol-degrading isolates was not performed. However, other studies have described the phenol degradation pathways of strains belonging to the same bacterial genera. Veselý *et al.* (2007) identified that within the gene cluster *catRABC* of *R. erythropolis* CCM2595 there were genes coding for small and large subunits of phenol hydroxylase (*pheA2*, *pheA1*) as well as the C12O gene *catA*. For *Acidovorax* strains no references regarding functional genes involved in degradation of phenol could be found but Meyer *et al.* (1999) identified very low C23O activity in a number of PAH-degrading *Acidovorax* strains. For a phenol-degrading *Methylibium petroleiphilum* PM1 strain, Kane *et al.* (2007) showed that it possessed two distinct

clusters of dimethylphenol (*dmp*)-like genes; the first cluster included the key structural gene *dmpP* coding for phenol hydroxylase as well as other genes similar to those encoded by pVI150 in *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1992), including a mPH (multicomponent phenol hydroxylase) and catechol 2,3-dioxygenase (*meta*-cleavage pathway). For *Pseudomonas* strains there is a extended literature on the diversity of their phenol hydroxylase and C12O or C32O genes (e.g. Merimaa *et al.*, 2006; Viggor *et al.*, 2008); most *Pseudomonas* strains can utilise catechol via the meta-cleavage pathway (Herrmann *et al.*, 1995) but degradation via the ortho-cleavage pathway has also been reported (Margesin *et al.*, 2004; Viggor *et al.*, 2008). In some cases, *Pseudomonas* strains were capable of expressing both ortho- and meta- pathways (Hamzah and Al-Baharna, 1994; Hinteregger *et al.*, 1992). In this study the accumulation of *cis,cis*-muconic acid in the liquid AB cultures of *Acidovorax* sp. 284 and *R. erythropolis* 256 implies the presence of the *ortho*-cleavage pathway for these two strains. Since 2-hydroxymuconic semialdehyde (the metabolite of catechol via the meta-cleavage pathway) was not included into the HPLC standards, there is no indication about the metabolic pathways followed by the other isolates. In any case, functional gene analysis is needed for a better understanding of their phenol catabolic properties.

In addition to the liquid AB cultures, all isolates were also grown in carbon-rich R2A broth cultures. The HPLC analysis of the AB and R2A cultures supplemented with 0.5 mM phenol revealed a range of different behaviours between the characterised bacteria that fell into six groups (Fig. 4.8.A); from bacteria that could utilise phenol both as a sole carbon source and in the presence of other organic carbon sources (isolate 256) to non-phenol degraders (10 isolates in group V). However, the isolates in between these two distinct types exhibited the most intriguing behaviour.

Group II consisted of 4 isolates (*Methylibium* sp. 272, *Pseudomonas* spp. 264, 273, 282) that utilised 0.5 mM phenol completely in AB medium but degraded it only partially in the R2A broth. This response is a typical display of carbon catabolic repression, a phenomenon that is well documented (reviews by: Stülke and Hillen, 1999; Görke and Stülke, 2008): in the presence of simple organic sources that are readily available for the bacteria and can yield them a high energy return, the degradation of other organic compounds (and particularly aromatic hydrocarbons) is usually suppressed by

inhibition of the transcription of the catabolic genes encoding the corresponding catabolic enzymes. Specifically for phenol degradation, it was shown that its utilisation by a *Pseudomonas putida* strain was dramatically reduced in the presence of glucose (minimal media cultures supplemented with 40 mM glucose and 2.5 mM phenol) as a result of reduced transcription of phenol hydroxylation (Müller *et al.*, 1996). Interestingly, glucose was a component of the R2A medium (Table 4.1) and phenol degradation was inhibited in all 3 *Pseudomonas* strains tested (Fig. 4.8).

An extreme case of catabolite-repression-like phenomenon was observed by *Acidovorax* sp. 284, which utilised completely 0.5 mM phenol as a sole carbon source but grew exclusively on other carbon sources in the R2A medium. However, this repression could also be attributed to the transcriptional inactivity of catabolic promoters during the exponential growth of bacteria on nutrient-rich media (such as LB and R2A), a phenomenon that has been called as "exponential silencing" (Cases *et al.*, 1996). For example, degradation of aromatic compounds such as 2-methylphenol (*o*-cresol) by *Pseudomonas* sp. strain CF600 (Sze *et al.*, 1996) or *m*-xylene by a *Pseudomonas putida* strain harbouring the catabolic TOL plasmid pWW0 (Cases *et al.*, 1996) was induced rapidly only after the bacteria reached a specific growth phase, usually after they entered stationary phase. Generally, it is believed that transcription of catabolic enzymes is modulated (amongst others) by both the growth medium as well as the growth phase of the culture (Cases and de Lorenzo, 2001).

In contrast to these isolates, two isolates (*Pigmentifaga* sp. 262 and *Taxeobacter* sp. 263) utilised phenol in the R2A liquid cultures only. The inability of these two isolates to utilise 0.5 mM phenol in the AB medium could be due to toxicity effects. However, it is not clear whether the induction of phenol catabolic genes in the R2A broth was due to a component in the R2A broth (carbon source or nutrient) or a consequence of their physiological state.

### 4.4.2 Toxicity and tolerance to phenol by single bacterial isolates

The cell membrane forms a permeable barrier that regulates the transport of solutes between the cell and the environment and performs an important role in the maintenance of the energy status of the cell (Sikkema *et al.*, 1995). Gram-negative bacteria have two membranes that are separated by a peptidoglycan (sugar polymers) layer (Fig. 4.11); the (inner) cytoplasmic membrane consists of a phospholipid bilayer with embedded enzymes and transport proteins while the outer membrane consists of phospholipids and lipopolysaccharides (LPS). Gram-positive bacteria have only the cytoplasmic membrane which is surrounded by a thick rigid cell wall consisting of peptidoglycan and teichoic acid (Fig. 4.11).

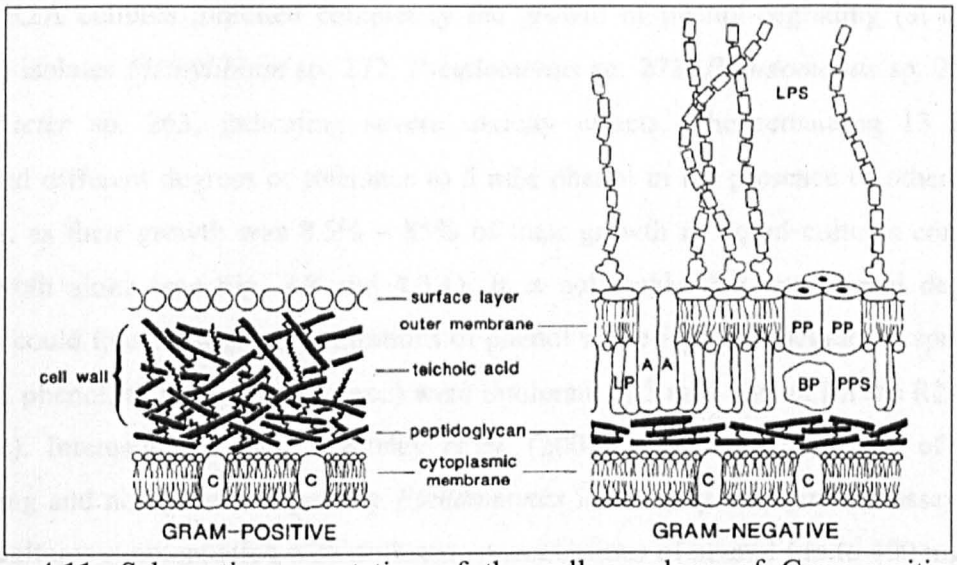



**Figure 4.11.** Schematic presentation of the cell envelope of Gram-positive and Gram-negative bacteria. PP = porin, C = cytoplasmic membrane protein, BP = binding protein, PPS = periplasmic space, A = outer membrane protein, LP = lipoprotein, LPS = lipopolysaccharides. Taken from Sikkema *et al.* (1995).

Organic solvents (including phenol) are toxic to microorganisms because due to their lipophilic nature they can partition into the cytoplasmic membrane and disrupt its integrity. The toxicity of these lipophilic compounds is related to their hydrophobicity as expressed by the logarithm of the partitioning coefficient of a solvent in a defined octanol-water mixture (log $P_{ow}$); organic solvents with log $P_{ow}$ values between 1 and 5 are the most toxic (Heipieper *et al.*, 1994). Phenol (with a log $P_{ow}$ of 1.45) as well as other

phenolic compounds exert their toxicity at the cell membrane level only (Sikkema *et al.*, 1995). Accumulation of phenol within the cytoplasmic membrane not only disrupts its integrity but it was also found to induce efflux of potassium ions (Heipieper *et al.*, 1991), although the mechanism for the latter was not specified.

The tolerance to phenol of 17 bacterial strains isolated from the Four Ashes site was investigated in shaken R2A broth cultures supplemented with 0.5 or 5 mM phenol. Following a similar experimental approach (tryptone soya broth supplemented with phenol), Brown *et al.* (2000) found that the halotolerant *Oceanomonas baumannii* could tolerate up to 35 mM phenol (although it could not utilise it) but its tolerance was reduced from 35 mM to 3 mM phenol as salinity increased from 1 % to 12 % NaCl (w/v). In this study, we found that all isolates were tolerant at 0.5 mM phenol but the presence of 5 mM in the R2A cultures inhibited completely the growth of phenol-degrading (at 0.5 mM phenol) isolates *Methylibium* sp. 272, *Pseudomonas* sp. 273, *Pseudomonas* sp. 282, and *Taxeobacter* sp. 263, indicating severe toxicity effects. The remaining 13 isolates exhibited different degrees of tolerance to 5 mM phenol in the presence of other carbon sources, as their growth was 8.5% – 85% of their growth in liquid cultures containing R2A broth alone (see Fig. 4.8 and 4.3.1). It is noticeable that non-phenol degrading species could tolerate high concentrations of phenol while 3 phenol-degrading species (at 0.5 mM phenol, as sole carbon source) were intolerant at 5 mM phenol (in the R2A broth cultures). Interestingly, when Whiteley *et al.* (2001) studied the tolerance of phenol degrading and non phenol degrading *Pseudomonas* isolates by a respiration assay in LB liquid cultures supplemented with different concentrations of phenol (up to 400 mg $L^{-1}$ or 4.3 mM), they also observed that the majority of phenol degrading *Pseudomonas* isolates exhibited lower tolerance than non phenol degrading *Pseudomonas* isolates. However, it is not clear whether non-phenol degraders in general are more tolerant than phenol degrading species.

Generally, it has been found that microorganisms can exert 5 different adaptation mechanisms (I – V in Fig. 4.12) to the toxicity effects of lipophilic compounds (Sikkema *et al.*, 1995). Although this study did not investigate mechanisms of phenol tolerance by these isolates, a number of studies have reported that microbial cells respond to phenol toxicity by increasing the saturated or the ratio of trans/cis-unsaturated fatty acids in the

cytoplasmic membrane (adaptation mechanism I in Fig. 4.12). For example, it was shown that exposure to phenol increased the degree of saturation in membrane lipids of *Escherichia coli* (Keweloh *et al.*, 1991) and the ratio of *trans/cis* unsaturated fatty acids of *Pseudomonas putida* P8 cells (Heipieper *et al.*, 1992). These changes result in a higher rigidity of the cytoplasmic membrane because the bent structure of the *cis*-unsaturated fatty acids (Fig. 4.13) confers relatively high fluidity on the membrane compared to the *trans*-unsaturated fatty acids which insert into the membrane in a similar way to saturated fatty acids (Heipieper *et al.*, 1994).

Further adaptation mechanisms of microbial cells to toxicity of other lipophilic compounds (not reported for phenol) include the modification of the LPS of the outer membrane of Gram-negative bacteria, modifications of cell wall constituents and cell wall hydrophobicity, increase in S-layer hydrophobicity, and active excretion of the toxic compound by energy-consuming transport systems (Sikkema *et al.*, 1995; Fig. 4.12).

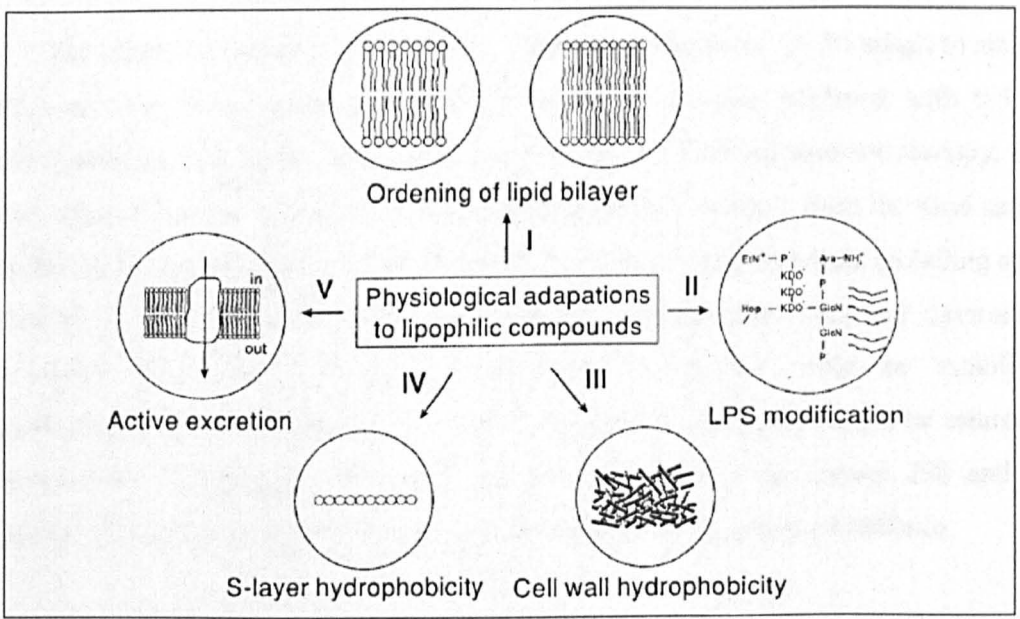



**Figure 4.12.** Schematic presentation of the adaptation mechanisms that protect microbial cells against the toxic effects of lipophilic compounds (such as phenol). Image taken from Sikkema *et al.* (1995).

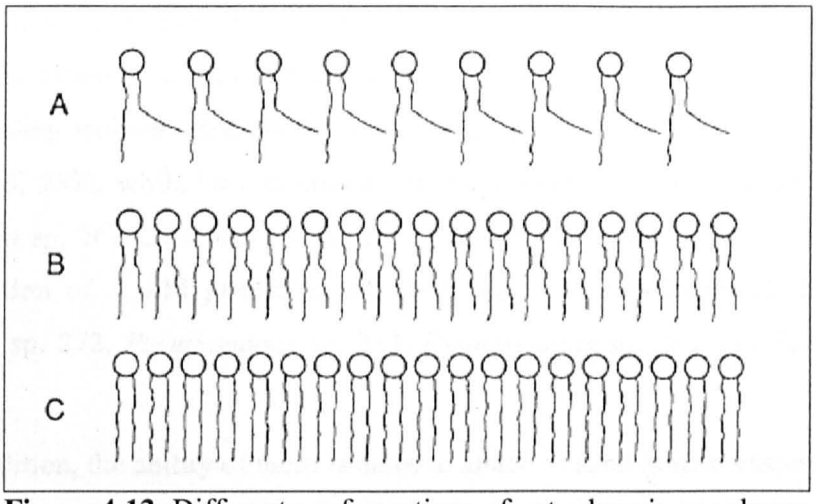



**Figure 4.13.** Different conformations of cytoplasmic membrane phospholipids. (A) *cis*-unsaturated, (B) *trans*-unsaturated and (C) saturated fatty acids. Taken from Sikkema *et al.* (1995).

### 4.4.3 Attachment of single bacterial strains of quartz sand grains

The ability of isolates from the Four Ashes site (Borehole 59, 30 mbgl) to attach to quartz sand was investigated using microcosm sand columns perfused with 0.5 mM phenol overnight. Microbial attachment was assessed by fluorescence microscopy. Eight isolates appeared not to attach (including strains originally isolated from the sand sample) while the remaining 10 isolates exhibited different degrees of attachment including strains isolated from the groundwater sample. Thus, no connection between the environment from which they were isolated and attachment behaviour could be established. Furthermore, no phylogenetic connection to the degree of attachment could be established because strains belonging in the same genus (e.g. *Rhodococcus* strains 226 and 256; *Pseudomonas* strains 264, 273, and 282) exhibited opposite attachment abilities.

### 4.4.4 Conclusions

In this study, the potential of 18 isolated bacterial strains to degrade or tolerate two concentrations of phenol (0.5 and 5 mM) was investigated in shaken liquid cultures. When phenol was provided as sole carbon source (in AB minimal medium), 6 of the isolates (*Rhodococcus erythropolis* 256, *Acidovorax* sp. 284, *Methylibium* sp. 272, and *Pseudomonas* spp. 264, 273, 282) could utilise it fully, while at 5 mM phenol only *R.*

*erythropolis* 256 could utilise phenol partially. When 0.5 mM phenol was added in R2A broth cultures, catabolic repression of phenol degradation was observed by most of the phenol-degrading isolates (*Acidovorax* sp. 284, *Methylibium* sp. 272, and *Pseudomonas* spp. 264, 273, 282), while two additional strains exhibited phenol degradation abilities (*Pigmentifaga* sp. 262, *Taxeobacter* sp. 263). All isolates could tolerate 0.5 mM phenol but the addition of 5 mM phenol in the R2A broth cultures inhibited the growth of *Methylibium* sp. 272, *Pseudomonas* sp. 273, *Pseudomonas* sp. 282 and *Taxeobacter* sp. 263.

In addition, the ability of these isolates to attach to sand grains was investigated by using microcosm sand columns perfused with 0.5 mM phenol overnight. Bacterial attachment was assessed by fluorescence microscopy and different degrees of attachment were observed; some isolates did not attach to the sand grains, while others attached either as independent cells or as clusters of cells.

The identification of bacteria with different functional abilities (Table 4.4) will allow appropriate strains to be selected for the establishment of microcosm experiments (Chapter 5) to test specific hypotheses about the behaviour individual species in assembled microbial communities.

# Chapter 5

# Microcosm studies of mixed microbial consortia

## 5.1 Introduction

Microcosm experiments have long been used as means to assess biodegradation potential of samples taken from polluted aquifers containing different pollutant loads. For example, using groundwater and sediments from another phenol polluted aquifer (east of Mansfield, UK) Broholm and Arvin (2000) studied degradation rates of phenolic compounds under aerobic and anaerobic conditions in microcosms containing different concentrations of pollutants, amended or not with nitrate. Similarly, Harrison *et al.* (2001) established laboratory microcosms with groundwater and sediments (acclimatised in the plume) from the Four Ashes aquifer. Under aerobic conditions they found that degradation occurred at total phenolic concentrations up to 660 mg $L^{-1}$ and that *o*-cresol was degraded once phenol and *p*-cresol were depleted. Under anaerobic conditions, degradation of *p*-cresol occurred at lower concentrations of total phenolics (up to 195 mg $L^{-1}$) and was followed by a non-stoichiometric decrease in nitrate and sulphate. Spence *et al.* (2001) also established laboratory microcosms with groundwater and sediments from the Four Ashes aquifer and demonstrated that addition of 1000 mg $L^{-1}$ nitrate could stimulate rapid degradation of 200 mg $L^{-1}$ *p*-cresol; phenol (present at lower concentrations) was also degraded but recalcitrant *o*-cresol was not degraded during the course of their study (35 days). However all these microcosm studies focused on the geochemistry and they did not study the microbial component.

There are a number of limitations to studies using batch culture microcosms seeded with natural inocula in examining the effects of different phenol concentrations on microbial community structure. Firstly, batch cultures cannot effectively reproduce the conditions in polluted aquifers, where attached and planktonic microbial communities experience continuous high concentrations of pollutants as they move with the groundwater flow. Secondly, because natural inocula are derived from an environment containing different organic carbon sources (organic pollutants) and diverse microbial communities (including many unidentified bacterial species), it is difficult to relate changes in microbial community structure to microbial function (i.e. ability to utilise the pollutants present). These limitations can be addressed using microcosm systems

inoculated with well-characterised bacterial isolates and supplied with a single carbon source. For example, Christensen *et al.* (2002) found that when the benzyl alcohol degrading *Pseudomonas putida* strain R1 and *Acinetobacter* strain C6 were grown together in limiting concentrations of benzyl alcohol, *Acinetobacter* strain C6 outcompeted *P. putida* R1 (500:1) in chemostat cultures (i.e. planktonic growth), whereas *P. putida* R1 was present in higher numbers than *Acinetobacter* strain C6 (5:1) in biofilms. It appeared that the dominance of *P. putida* R1 in the biofilm was due to metabolic interactions as it could utilise benzoate, a metabolite of benzyl alcohol that was produced by the *Acinetobacter* strain.

Therefore, this study has used the following approach:

- Laboratory microcosms were inoculated with mixtures of isolated bacteria from the Four Ashes site with known functional properties relating to their ability to degrade or tolerate 0.5 and 5 mM phenol and to attach to sand (as determined in Chapter 4). The relative abundance of these bacterial strains within the microcosms was assessed by DGGE analysis.
- In order to replicate the flow conditions of the Four Ashes sandstone aquifer, the laboratory microcosms were established with columns packed with quartz sand and then AB minimal medium containing different concentrations of one organic carbon source (phenol) pumped continuously through the sand columns at a low flow rate. This system allowed phenol degradation rates to be measured within the sand column and sampling of both planktonic and attached microbial communities.

The main aims of this study are to examine (i) the effects of phenol concentration (and shifts in phenol concentration) on planktonic and attached microbial community structure and phenol biodegradation rates and (ii) the importance of metabolic and cell-cell interactions in the persistence of microorganisms in polluted environments.

Specific hypotheses of this study are:

1. Different phenol concentrations, or shifts in phenol concentration, will affect both the composition of planktonic and attached microbial communities and phenol degradation rates.

a. Phenol degrading species will dominate both planktonic and attached phase
b. Isolates that are unable to degrade phenol will be able to persist in the attached phase due to metabolic interactions with phenol degrading microbes.

2. Phenol-degrading organisms that are unable to attach directly to the mineral substrate (quartz sand) in isolation may be able to attach in pre-existing attached microbial communities due to cell-cell and/or cell-EPS interactions

## 5.2 Methods

### 5.2.1 Establishment of the microcosms

*Overview of the experimental design of the microcosm studies*

For the microcosm studies, 10 different treatments each with duplicate sand columns were established and different concentrations of phenol were supplied for 1 or 2 weeks, as shown in Fig. 5.1. The microcosm sand columns were prepared as described previously (4.2.3) but instead of single bacterial strains, they were inoculated with mixtures of multiple isolates.

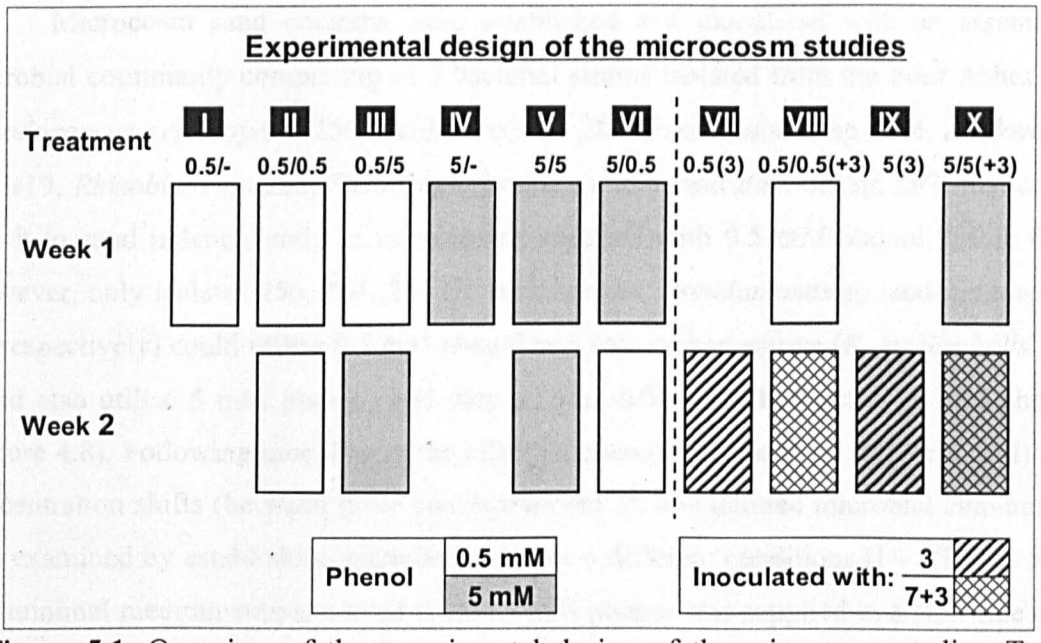



**Figure 5.1.** Overview of the experimental design of the microcosm studies. Ten different treatments were established (I – X) with duplicate sand columns each. The shorthand codes above the columns indicate the concentration of phenol (0.5 or 5 mM) that was pumped through the sand columns for 1 or 2 weeks (e.g. 5/- or 5/5 respectively). All sand columns were inoculated with a mixture of 7 isolated bacteria at the start of week 1 and sand columns of treatments VIII and X were re-inoculated with a mixture of 3 bacterial strains (+3). Two additional treatments (VII and IX) were established in the second week and were inoculated with the mixture of 3 isolates only (3).

The isolates to be used in the mixed inocula were grown independently in R2A broth, washed with 0.9 % NaCl and resuspended in AB medium to a final $OD_{600}$ of 0.1 (4.2.2). Then an appropriate volume from each bacterial culture was mixed in AB medium so that the final $OD_{600}$ of the mixed inoculum was 0.1. Inoculation was performed using a peristaltic pump for 2 hours (4.2.3). For the purposes of these microcosm studies, 2 different microbial mixtures were used. The first mixture (comprising 7 isolates) was used to inoculate all the sand columns in week 1 (16 sand columns – 8 treatments) while the second mixture (comprising 3 isolates) was used to inoculate 8 sand columns (4 treatments) at the start of week 2.

*Effect of phenol concentration and concentration shifts on microbial communities*

Microcosm sand columns were established and inoculated with an assembled microbial community comprising of 7 bacterial strains isolated from the Four Ashes site (*Rhodococcus erythropolis* 256, *Acidovorax* sp. 284, *Pseudomonas* sp. 264, *Acidovorax* sp. is19, *Rhizobium* sp. 235, *Stenotrophomonas* sp. 245, and *Bacillus* sp. 297) that could attach to sand independently in microcosms supplied with 0.5 mM phenol (Table 4.4). However, only isolates 256, 264, 284 (*R. erythropolis*, *Pseudomonas* sp. and *Acidovorax* sp. respectively) could utilise 0.5 mM phenol as a sole carbon source (*R. erythropolis* 256 could also utilise 5 mM phenol) and they all had different tolerances to 5 mM phenol (Figure 4.8). Following inoculation, the effect of phenol concentration (0.5 or 5 mM) and concentration shifts (between these concentrations) on this defined microbial community was examined by establishing microcosms under 6 different conditions (I – VI, Fig. 5.1). AB minimal medium supplemented with 0.5 mM phenol was supplied at a flow rate of 3 ml $h^{-1}$ for 1 week (treatment I), 2 weeks (II) or swapped to 5 mM after 1 week (III). Similarly, 5 mM of phenol was supplied for 1 week (IV), 2 weeks (V) or swapped to 0.5 mM after 1 week (VI). All sand columns were harvested at the end of the second week, apart from the sand columns of treatments I and IV that were harvested at the end of the first week.

*Establishment of bacteria in pre-existing attached microbial communities*

It was found previously (Table 4.4) that isolates 272, 273, 282 (1 *Methylibium* and 2 *Pseudomonas* spp.) could utilise 0.5 mM phenol as sole carbon source but they did not attach to sand when this concentration of phenol was pumped through the sand columns overnight (determined by fluorescence microscopy). Therefore, these 3 isolates formed a second microbial mixture (inoculum 3) that was used to test the ability of bacteria to establish in pre-existing attached microbial communities.

The experimental set-up involved 3 stages. Initially, sand columns were inoculated with the first microbial mixture (inoculum 7) composed of bacterial strains that could attach to sand and were supplied with 0.5 or 5 mM phenol (treatments VIII and X respectively in Fig. 5.1). After 1 week, the second microbial mixture (inoculum 3) was used to re-inoculate these sand columns (for 2 h with a syringe pump at 3 ml $h^{-1}$, as described previously); phenol was then re-supplied at the same concentration for an additional week and the sand columns were harvested at the end of the second week. As a control, the same microbial mixture (inoculum 3) was also used to inoculate sterile sand columns which were then supplied with the same concentrations of phenol (0.5 and 5 mM phenol - treatments VII and IX) for 1 week, after which they were harvested (see Fig. 5.1).

### 5.2.2 Sampling and harvesting procedure

For each sand column the same procedure was followed for the collection of samples for further analyses. The 2 cm outlet needle (Fig. 4.7) was replaced by a new needle with attached PTFE tubing (Fig. 5.2). In the next 1h 30 min, the effluent from the sand columns (approximately 4.5 ml) was collected into 7 ml bijou vials that were kept on ice (Fig. 5.2). Then the sand columns were removed from the system and, in a similar way, approximately 4 ml of the effluent from the needle at the inlet of the sand column was collected. Finally a third liquid sample was collected using a sterile 10 ml syringe directly from the bottles that contained the media.

From each effluent sample from the sand columns, 100 $\mu$l was used for viable counts and 2 x 500 $\mu$l was fixed with formaldehyde and kept for direct total cell counts with acridine orange, as described in 2.2.3. Moreover, 3 x 1.2 ml was transferred into microtubes and centrifuged at 13,000 x g at 4°C using a refrigerated Hawk 15/05 centrifuge (Sanyo, Japan). The pellets were kept for DNA extractions and the supernatant was pooled and filtered through sterile (0.2 $\mu$m, 25 mm) Acrodisc® Syringe Filter with Supor® Membrane (Pall Life Sciences, USA) into 3 new microtubes. In a similar way, the collected medium for the large needle and the bottles was filtered and aliquoted into 3 microtubes. These filtered media were used for determination of phenol concentration by HPLC analysis, as described in 4.2.2.

The sand within the column microcosms was emptied into a Petri-dish that contained 20 ml of sterile AB minimal medium and the plates were tilted slightly. The AB medium was decanted, the sand was mixed with a flamed spatula and some transferred into 3 microtubes and stored at -20°C for DNA extraction. The remaining sand in the Petri dish was fixed for 5 minutes with 2% (v/v) membrane-filtered formaldehyde and rinsed once more with AB medium. A few sand grains were stained with the nucleic acid stain SYTO 9 and the bacteria attached to the sand grains were viewed by fluorescence microscopy as described previously (4.2.3).

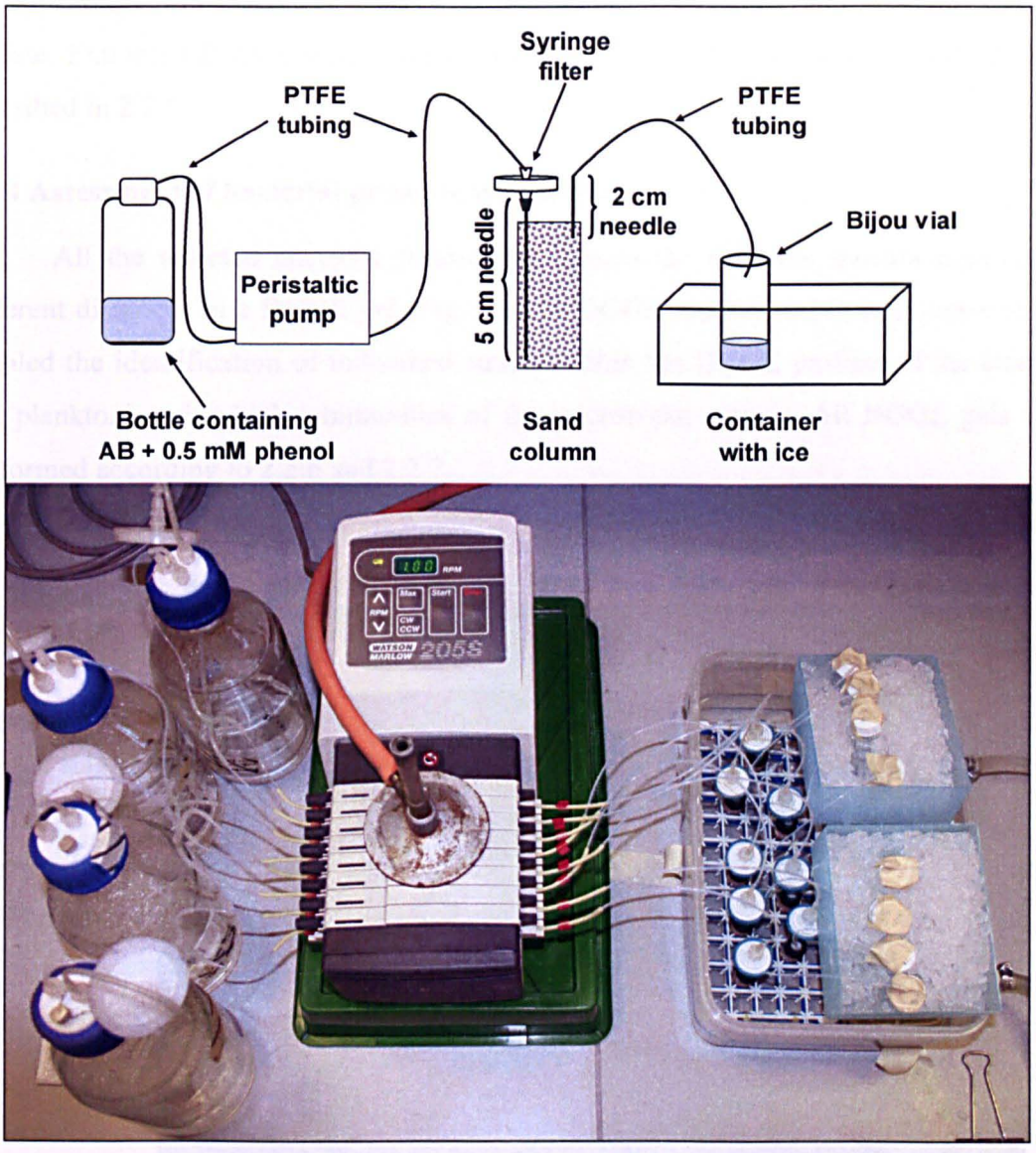



**Figure 5.2.** Effluent from the microcosm sand columns was collected into sterile bijou vials for further analyses (HPLC and planktonic community).

### 5.2.3 Isolation of genomic DNA from the microcosm samples (sand and effluent)

Total genomic DNA was extracted from 1.2 ml of the effluent (1 replicate) and 0.5 g of the sand columns (in triplicate) using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc, Carlsbad, CA, USA), following the manufacturer's protocol.

The kit lyses bacterial cells and cellular components by combining mechanical action (bead-beating) and chemical lysis with guadinine thiocyanate and Sodium Dodecyl Sulfate. Extracted DNA was quantified using the fluorimetric method (with PicoGreen) described in 2.2.5.

### 5.2.4 Assessment of bacterial presence with DGGE

All the selected bacterial strains that formed the different inocula migrated to different distances in a DGGE gel (Fig. 5.3). A DGGE marker (m10) was prepared that enabled the identification of individual strains within the DGGE profiles of the attached and planktonic microbial communities of the microcosm studies. All DGGE gels were performed according to 2.2.6 and 2.2.7.

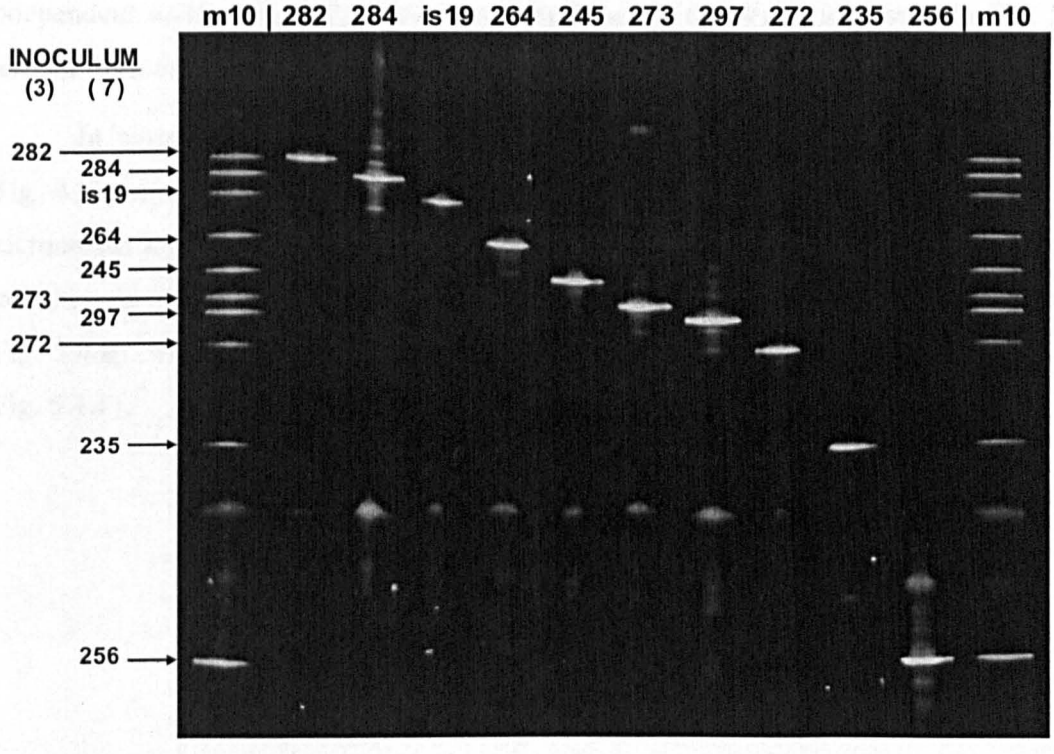



**Figure 5.3.** All the selected bacterial strains used in the microcosm experiments migrated to different distances in a DGGE gel. A DGGE marker (m10) was prepared from these 10 selected bacterial strains (*Pseudomonas* sp. 282, *Acidovorax* sp. 284, *Acidovorax* sp. is19, *Pseudomonas* sp. 264, *Stenotrophomonas* sp. 245, *Pseudomonas* sp. 273, *Bacillus* sp. 297, *Methylibium* sp. 272, *Rhizobium* sp. 235, *Rhodococcus erythropolis* 256)

## 5.3 Results

### 5.3.1 Attachment of assembled microbial mixtures to microcosm sand columns

Fluorescence microscopy with SYTO 9 nucleic acid stain was used to visualise cell attachment to the sand grains of the microcosm sand columns. In all treatments, cells attached to sand could be observed. In some occasions cells were attached independently (e.g. Fig. 5.4.A and 5.4.B) and in other occasions they produced more extended coverage, with biofilm–like formations (e.g. Fig. 5.4.C, 5.4.D, 5.4.E). However, no obvious trend between cell attachment and the different treatments could be established. Even within the same sand column different degrees of attachment were observed; for example the independent cells seen in Fig. 5.4.A and the biofilm-like formations seen in Fig. 5.4.E derived from the same sand column (0.5 mM phenol for 1 week, treatment I).

In comparison to the attachment assay of independent bacterial cells overnight (Fig. 4.9), more grains with attached cells and more clusters of cells were observed in microcosms incubated for 1 or 2 weeks. In some occasions attached cells formed chains, probably an indication of bacterial growth/division within these attached communities (Fig. 5.4.E). Moreover, some flocs of cells (loosely attached to sand grains) were seen (Fig. 5.4.F).

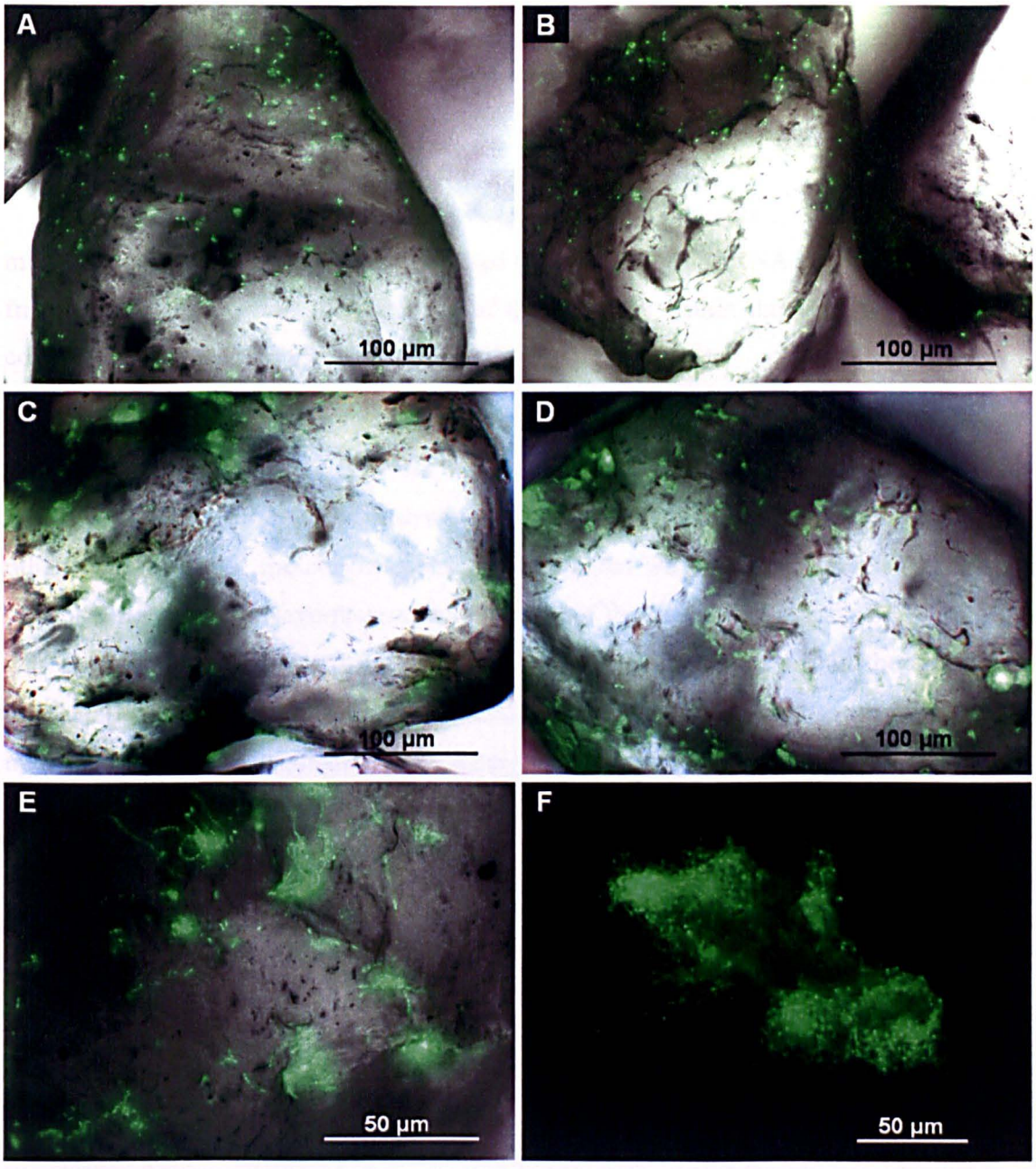



**Figure 5.4.** Fluorescence microscopy of microbial cells attached to sand grains derived from the microcosm sand columns. Attachment of cells varied from a few individual cells (A, B) to more extended coverage, forming micro-colonies (C, D, E). Flocs of bacterial cells loosely attached to the sand grains were also observed (F).
A = 0.5 mM phenol for 1 week (treatment I)
B = 5 mM phenol for 1 week (IV)
C = re-inoculated sand column, at 0.5 mM phenol (VIII)
D = 5 mM phenol for 1 week, 0.5 mM in the second week (VI)
E (= A) = 0.5 mM phenol for 1 week (I)
F = 0.5 mM phenol for 1 week, 5 mM in the second week (III)

### 5.3.2 Effect of phenol concentration and concentration shifts on attached microbial community structure

DNA was isolated from all sand columns of treatments I – VI (Fig. 5.1) and microbial community structure was profiled by DGGE of 16S rRNA gene PCR amplified fragments (Fig. 5.5). The identification of the members within the assembled microbial community was assisted by the DGGE marker m10 (see 5.2.4).

The DGGE profiles (Fig. 5.5) highlighted that there was high reproducibility between the 2 replicates (2 different sand columns) of each treatment. In addition, the DGGE profiles of all attached microbial communities were remarkably similar to each other, irrespective of the concentration of phenol that was pumped through or shifts in phenol concentration. Nevertheless, the intensity of the bands within each DGGE profile was significantly different to the (starting) intensity of the bands within the assembled microbial community (not shown here, but of similar intensity to the bands of the DGGE marker m10). In all treatments, the brightest band corresponded to the phenol-degrading isolate 264 (*Pseudomonas* sp.). Less bright bands of similar intensities corresponded to the phenol degrading isolate 284 (*Acidovorax* sp.), as well as isolates is19, 245 and 235 (*Acidovorax*, *Stenotrophomonas* and *Rhizobium* spp. respectively). The least bright bands in the DGGE profiles co-migrated with isolates *Bacillus* sp. 297 and *R. erythropolis* 256.

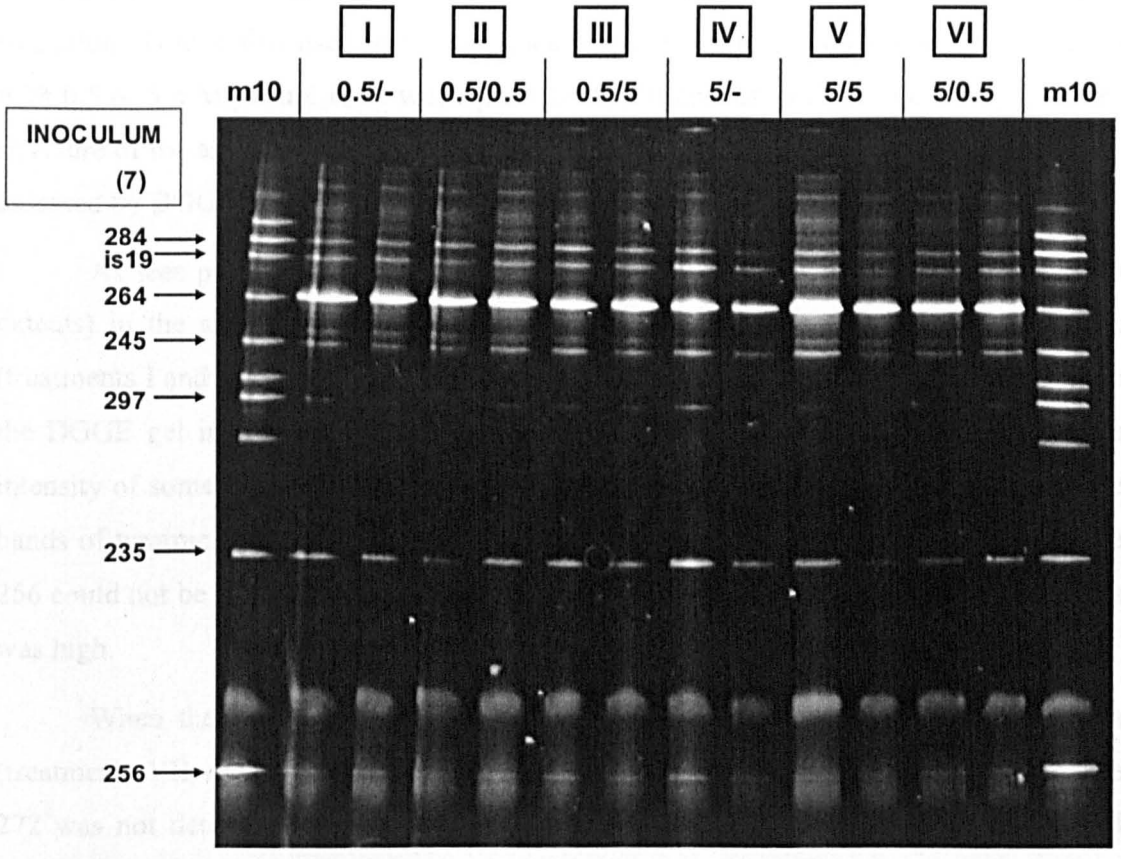



**Figure 5.5.** DGGE profiling of the attached microbial communities subjected to different concentrations of phenol as well as phenol concentration shifts (m10 = DGGE marker). For each treatment duplicate sand columns were initially inoculated with the same inoculum (7). (*Acidovorax* sp. 284, *Acidovorax* sp. is19, *Pseudomonas* sp. 264, *Stenotrophomonas* sp. 245, *Bacillus* sp. 297, *Rhizobium* sp. 235, *Rhodococcus erythropolis* 256)

### 5.3.3 Establishment of bacteria in existing attached microbial communities

Three isolates (282, 273 and 272 – 2 *Pseudomonas* and 1 *Methylibium* spp respectively) that could utilise 0.5 mM phenol as sole carbon source (Fig. 4.8) but could not attach to sand independently at the same concentration of phenol (as determined by fluorescence microscopy in Chapter 4) formed a second microbial mixture (Inoculum 3). This mixture of 3 isolates was used to re-inoculate microcosm sand columns that had been previously inoculated with inoculum 7 and supplied with 0.5 or 5 mM phenol for 1 week

(treatments VIII and X respectively in Fig. 5.1); phenol was then supplied at the same concentration for an additional week. As a control, this second microbial mixture (inoculum 3) was also used to inoculate sterile sand columns which were then supplied with 0.5 or 5 mM phenol for 1 week (treatments VII and IX in Fig. 5.1). Once again, the structure of the attached microbial communities in all these microcosm sand columns was assessed by DGGE profiling, in relation to DGGE marker m10.

As seen previously, all the isolates comprising inoculum 7 established (to different extents) in the sand columns when 0.5 or 5 mM of phenol was supplied for 1 week (treatments I and IV respectively in Fig. 5.5). The same PCR products were also loaded in the DGGE gel in Fig. 5.6 (treatments I and IV) but slight differences appeared in the intensity of some bands between these two DGGE gels. In the second gel (Fig. 5.6), the bands of treatment I samples appeared less bright and the band corresponding to isolate 256 could not be resolved. Once again the reproducibility between replicate sand columns was high.

When the second bacterial mixture was used to inoculate sterile sand columns (treatments VII and IX respectively in Fig. 5.6), isolate 273 attached weakly and isolate 272 was not detectable. On the other hand, isolate 282 (*Pseudomonas* sp.) established very strongly at in microcosms supplied with either 0.5 or 5 mM phenol.

When inoculum 3 was used to re-inoculate pre-existing attached microbial communities (treatments VIII and X), once again isolate 273 attached weakly and 272 was not detectable within both these microcosms. Isolate 282 established very strongly at 5 mM phenol (treatment X), where its DGGE band was almost as bright as the band corresponding to isolate 264 (another *Pseudomonas* strain). However at 0.5 mM phenol (treatment VIII) isolate 282 established to a markedly lesser extent.

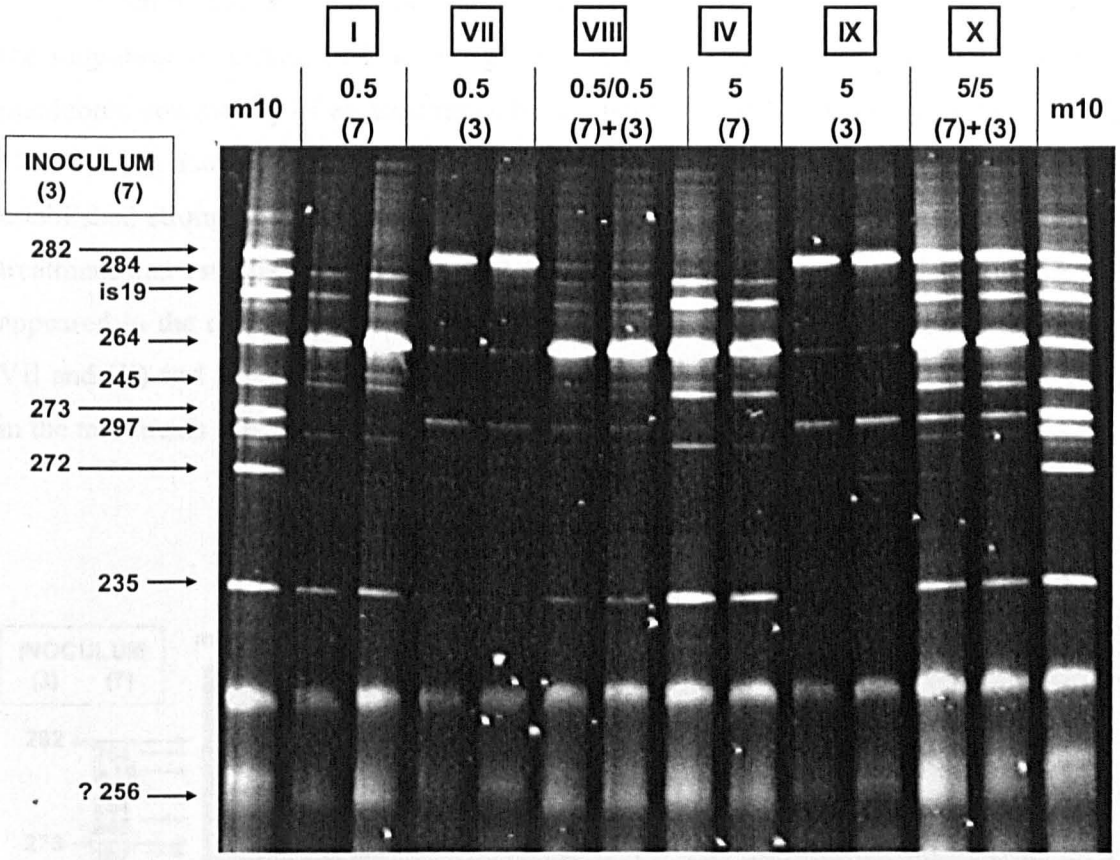



**Figure 5.6.** DGGE profiling of attached microbial communities which were inoculated with different assembled communities (7, 3, 7 and re-inoculated with 3 after 1 week) and supplied with 0.5 (treatments I, VII, VIII) or 5 mM phenol (treatments IV, IX, X). For each treatment duplicate sand columns were established (m10 = DGGE marker). (*Pseudomonas* sp. 282, *Acidovorax* sp. 284, *Acidovorax* sp. is19, *Pseudomonas* sp. 264, *Stenotrophomonas* sp. 245, *Pseudomonas* sp. 273, *Bacillus* sp. 297, *Methylibium* sp. 272, *Rhizobium* sp. 235, *Rhodococcus erythropolis* 256)

### 5.3.4 Planktonic microbial communities

In addition to the structure of the attached microbial communities, the structure of the planktonic microbial communities in the effluent from the 20 sand columns (10 treatments) was also profiled by DGGE (Fig. 5.7). Compared to the attached communities, where most of the isolates were detected (Fig. 5.5 and 5.6), the planktonic communities were characterised by the presence of fewer bacterial strains.

Even though in some lanes (treatments I and IV in Fig. 5.7) there was variation in the migration of isolate 264, it was evident that *Pseudomonas* sp. 264 dominated the planktonic community of all treatments inoculated with the first microbial mixture (I-VI, VIII and X). Isolate 282 was detectable in the effluent of the treatments where it had established strongly in the attached community (VII, IX, X) but was not detectable in the treatment that established to a lesser extent (VIII). Of the remaining isolates, isolate 273 appeared in the effluent of the microcosms inoculated with inoculum 3 only (treatments VII and IX) and isolate 245 (*Stenotrophomonas* sp.) could also be detected, more clearly in the treatments supplied with 5 mM phenol (III – VI and X).

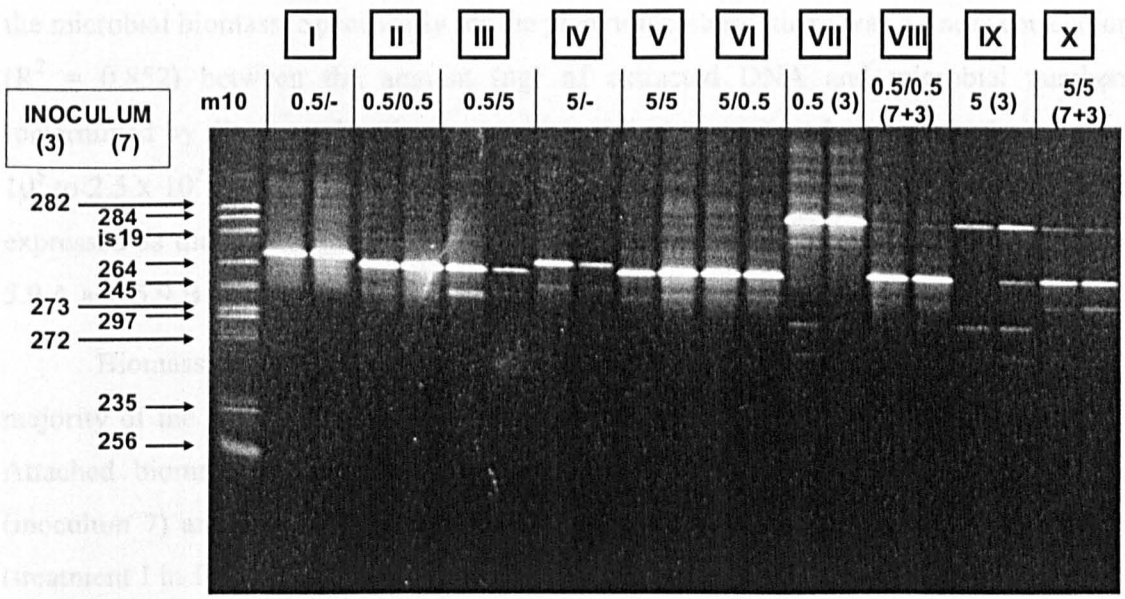



**Figure 5.7.** DGGE profiles of the planktonic microbial communities of the microcosm studies (m10 = DGGE marker). For each treatment, duplicate sand columns were established. (*Pseudomonas* sp. 282, *Acidovorax* sp. 284, *Acidovorax* sp. is19, *Pseudomonas* sp. 264, *Stenotrophomonas* sp. 245, *Pseudomonas* sp. 273, *Bacillus* sp. 297, *Methylibium* sp. 272, *Rhizobium* sp. 235, *Rhodococcus erythropolis* 256)

### 5.3.5 Microbial biomass and phenol degradation within the established microcosms

*Biomass of planktonic and attached communities*

In addition to the profiling of the structure of the attached and planktonic microbial communities, the biomass within each phase (attached and planktonic) was measured.

Quantification of the attached microbial biomass and parallel comparison to the planktonic biomass is challenging and prone to a series of biases. Thus, during this study the amount of the extracted DNA (ng) from each phase was used as a direct estimation of the microbial biomass. Specifically for the planktonic phase, there was a linear correlation ($R^2$ = 0.852) between the amount (ng) of extracted DNA and microbial numbers (determined by direct total cell counts with acridine orange), at least within the range of $10^5$ to 2.5 x $10^7$ cells $ml^{-1}$ (Fig. 5.8). So, attached and planktonic microbial biomass were expressed as the measured amount of DNA (ng) in 8 g of sand and 2 ml of liquid (Fig. 5.9.A and 5.9.B respectively) that were contained within each microcosm column.

Biomass estimations (based on the amount of DNA extracted) revealed that the majority of the biomass in all treatments existed in the attached phase (90 % or more). Attached biomass in the sand columns inoculated with the first microbial mixture (inoculum 7) and supplied with 0.5 mM phenol was about 1500 ng $vial^{-1}$ after 1 week (treatment I in Fig. 5.9.A). Attached biomass did not differ when during the second week phenol was supplied at the same concentration (II) or shifted to 5 mM phenol (III). In contrast, the accumulation of biomass was slower (about 600 ng $vial^{-1}$) when 5 mM phenol was supplied for 1 week (IV) but it reached similar levels when 5 or 0.5 mM phenol was supplied during the second week (V and VI respectively). Inoculum 3 on its own gave slightly more variable results but once again biomass was lower at 5 mM (IX) than at 0.5 mM phenol (VII). The attached biomass of the re-inoculated microcosms (VIII and X) was similar to that of microcosms inoculated with inoculum 7 alone.

The planktonic biomass in all microcosm sand columns was up to 100 ng $vial^{-1}$ (with the exception of 1 sand column) but was more variable between different treatments

(Fig. 5.9.B). When inoculum 7 was used, the planktonic biomass was about 100 ng $vial^{-1}$ after 1 week at 0.5 mM (I) but was lower when 0.5 or 5 mM phenol was supplied during the second week (II and III). In contrast, planktonic biomass was very low after 1 week with 5 mM phenol (IV) but increased noticeably when 5 or 0.5 mM phenol was supplied during the second week (V and VI). In the sand columns inoculated with inoculum 3 alone, after 1 week planktonic biomass was noticeably higher at 0.5 mM (VII) than at 5 mM phenol (IX), similar to the microcosms inoculated with inoculum 7 alone (I and IV). In the re-inoculated microcosms, planktonic biomass was also higher at 0.5 mM (VIII) than at 5 mM phenol (X).

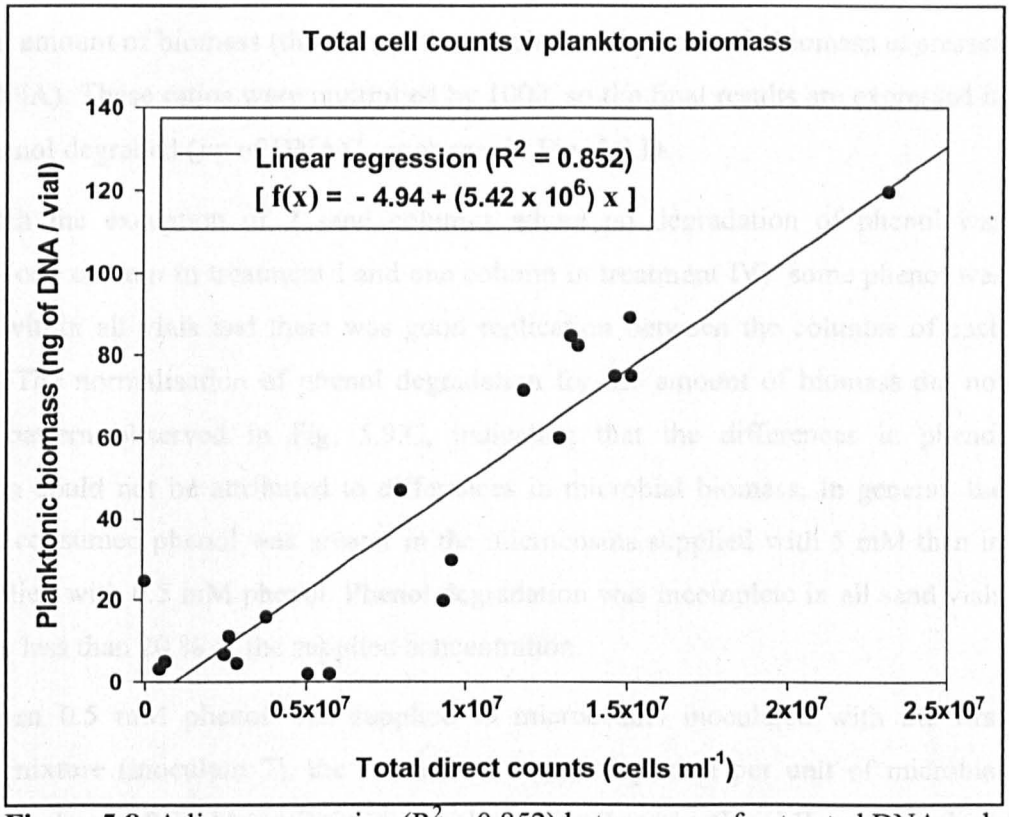



**Figure 5.8.** A linear regression ($R^2$ = 0.852) between ng of extracted DNA and total number of cells, within the range of $10^5$ to 2.5 x $10^7$ cells $ml^{-1}$.

*Phenol degradation within the microcosm sand columns*

Phenol degradation within each vial was calculated by taking the difference in phenol concentration (mM) between the inlet and the outlet needles of the microcosms at the time of the harvest. These results are shown in Fig. 5.9.C and error bars indicate the standard deviation from the mean between 3 HPLC measurements for each vial. A small amount of phenol (less than 5 % of the initial phenol concentration) was also lost between the bottle and the lower end of the large needle, probably consumed by microbial communities established within the large needle or at the lower surface of the syringe filter (data not shown). The concentration of phenol degraded within each vial was eventually normalised for the amount of biomass by taking the ratio of consumed phenol to the total amount of biomass (the sum of the attached and planktonic biomass expressed in ng of DNA). These ratios were multiplied by 1000, so the final results are expressed in mM of phenol degraded ($\mu$g of DNA)$^{-1}$, as shown in Fig. 5.9.D.

With the exception of 2 sand columns where no degradation of phenol was measured (one column in treatment I and one column in treatment IV), some phenol was degraded within all vials and there was good replication between the columns of each treatment. The normalisation of phenol degradation for the amount of biomass did not alter the pattern observed in Fig. 5.9.C, indicating that the differences in phenol degradation could not be attributed to differences in microbial biomass. In general, the amount of consumed phenol was greater in the microcosms supplied with 5 mM than in those supplied with 0.5 mM phenol. Phenol degradation was incomplete in all sand vials and always less than 20 % of the supplied concentration.

When 0.5 mM phenol was supplied to microcosms inoculated with the first microbial mixture (inoculum 7), the amount of degraded phenol per unit of microbial biomass was about 0.06 mM ($\mu$g DNA)$^{-1}$ after 1 week or 2 weeks (I and II respectively in Fig. 5.9.D) but it increased up to 0.40 mM ($\mu$g DNA)$^{-1}$ when 5 mM was supplied during the second week (III). When 5 mM phenol was supplied to the same microbial mixture (inoculum 7) for 1 week (IV), phenol degradation was very limited, around 0.04 mM ($\mu$g DNA)$^{-1}$. During the second week at 5 mM (V) phenol degradation increased to 0.12

mM ($\mu$g DNA)$^{-1}$ but when phenol was shifted to 0.5 mM during the second week (VI), phenol degradation remained low at 0.03 mM ($\mu$g DNA)$^{-1}$.

In the microcosms inoculated with the second microbial mixture only (inoculum 3) and supplied with 0.5 or 5 mM phenol (VII and IX), the amount of degraded phenol per unit of biomass was about 0.05 mM ($\mu$g DNA)$^{-1}$ and 0.14 mM ($\mu$g DNA)$^{-1}$ respectively. In the re-inoculated microcosms supplied with 0.5 or 5 mM phenol (VIII and X), degradation rates were about 0.02 and 0.12 mM ($\mu$g DNA)$^{-1}$ respectively. These values are lower but comparable to the degradation rates within treatments II and V, which were inoculated with the first assembled community (inoculum 7) and supplied with 0.5 or 5 mM phenol for 2 weeks, too.

Metabolites of phenol could be detected only in the effluent of 3 sand columns and at very low concentrations. *Cis,cis*-muconic acid (0.4 mg L$^{-1}$) and catechol (0.2 mg L$^{-1}$) were detected only in 1 replicate of treatment I, while *cis,cis*-muconic acid (about 0.4 mg L$^{-1}$) was detected in both replicates of treatment VII.

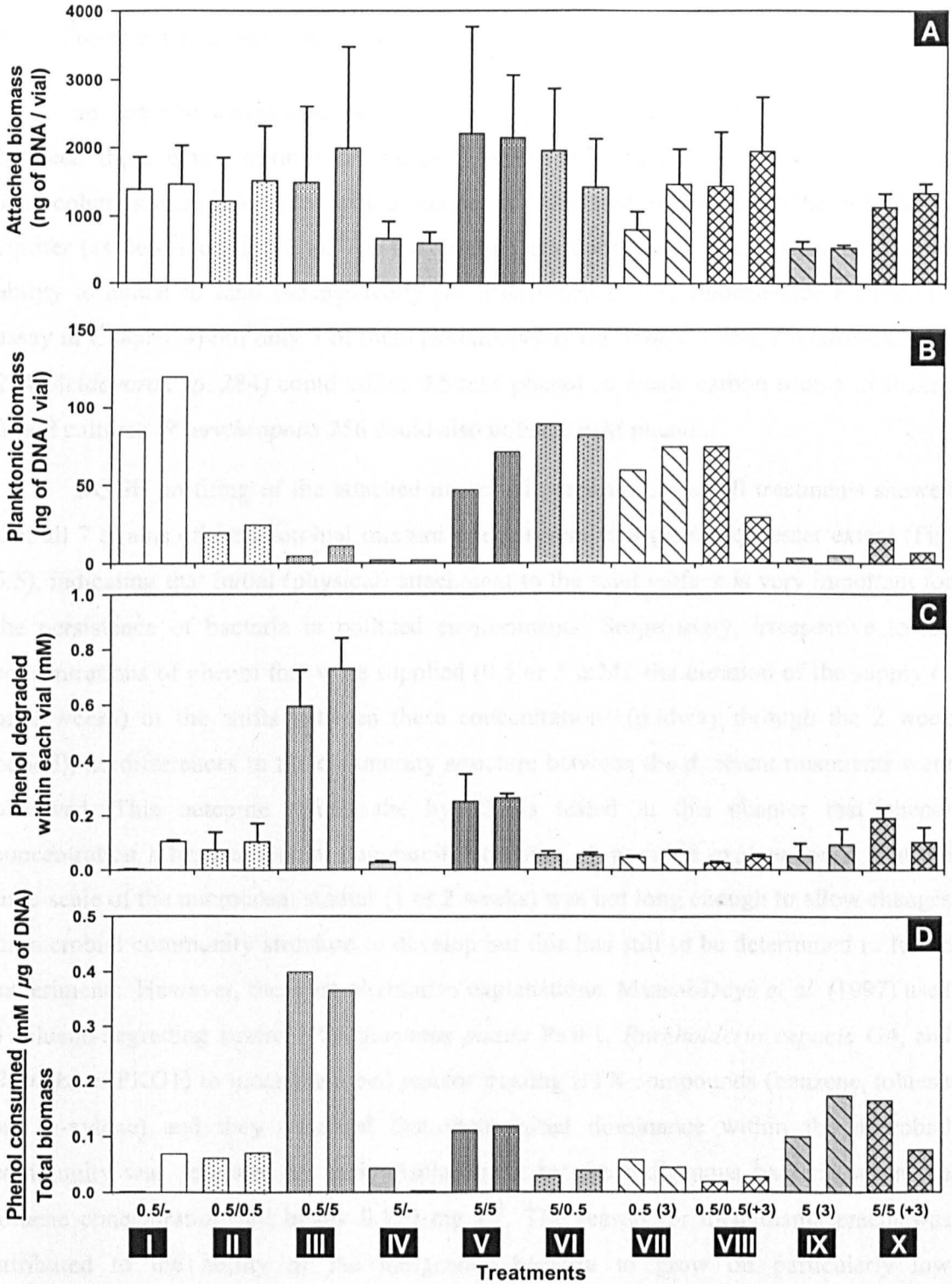



**Figure 5.9.** Biomass/growth of attached (A) and planktonic (B) microbial communities, phenol degradation (C), and the ratio of the consumed phenol to the total biomass (D) within each sand column of the 10 experimental treatments (I – X).

## 5.4 Discussion

### 5.4.1 The effect of phenol concentration on attached microbial community structure

In order to investigate the effect of supplying 0.5 or 5 mM phenol and shifts between these concentrations on attached microbial community structure, microcosm sand columns were inoculated with a mixture of 7 isolated bacteria from the Four Ashes aquifer (as described in 5.2.1). The bacterial strains comprising this mixture all had the ability to attach to sand independently (as determined by the fluorescence microscopy assay in Chapter 4) but only 3 of them (*Rhodococcus erythropolis* 256, *Pseudomonas* sp. 264, *Acidovorax* sp. 284) could utilise 0.5 mM phenol as a sole carbon source in shaken liquid cultures. *R. erythropolis* 256 could also utilise 5 mM phenol.

DGGE profiling of the attached microbial communities of all treatments showed that all 7 strains of the microbial mixture were present to a greater or lesser extent (Fig. 5.5), indicating that initial (physical) attachment to the sand surface is very important for the persistence of bacteria in polluted environments. Surprisingly, irrespective to the concentrations of phenol that were supplied (0.5 or 5 mM), the duration of the supply (1 or 2 weeks) or the shifts between these concentrations (midway through the 2 week period), no differences in the community structure between the different treatments were observed. This outcome refutes the hypothesis tested in this chapter that phenol concentration affects microbial community structure. A possible explanation is that the time-scale of the microcosm studies (1 or 2 weeks) was not long enough to allow changes in microbial community structure to develop but this has still to be determined in future experiments. However, there are alternative explanations. Massol-Deyá *et al.* (1997) used 3 toluene-degrading strains (*Pseudomonas putida* PaW1, *Burkholderia cepacia* G4, and *B. pickettii* PKO1) to inoculate a bed reactor treating BTX compounds (benzene, toluene and *p*-xylene) and they observed that their initial dominance within the microbial community was followed by their displacement by the indigenous bacteria when the toluene concentration fell below 0.140 mg $L^{-1}$. The reason for their displacement was attributed to the ability of the indigenous bacteria to grow on particularly low concentrations of toluene, i.e. they had very high affinity for toluene (*Ks*). In another study, Watanabe *et al.* (1996) showed that phenol-degrading bacteria isolated from a

cultures supplied with low concentrations of phenol had a higher apparent affinity for phenol (*Ks* < 0.1 μM) than isolates from cultures enriched with higher concentrations of phenol (*Ks* around 0.3 μM). As phenol was supplied continuously either at 0.5 or at 5 mM in the current study, both of which are quite high concentrations, the absence of differences in the community structure between the different treatments may have resulted from bacteria with a low affinity to phenol dominating all of the microcosms. Strains with a high affinity for phenol were either not selected in chapter 4 or were never given the chance to grow under conditions in which they could outcompete strains with a low affinity for phenol but higher growth rate. Therefore, in future experiments a wider range of phenol concentrations should be investigated, particularly low concentrations. In addition, microcosms could be established using natural inocula in which a wider range of microbes with different affinities for phenol and growth rates might be expected.

Although similar attached communities established within all microcosms of the different experimental treatments, within each microcosm distinct differences in the relative abundance of the 7 strains were observed. All microcosms were dominated by the phenol-degrading *Pseudomonas* sp. 264. The second phenol degrading isolate (*Acidovorax* sp. 284) also established in the sand microcosms but to about the same extent as the non-degrading *Acidovorax* sp. is19, *Stenotrophomonas* sp. 245, and *Rhizobium* sp. 235 strains, while establishment of *Bacillus* sp. 297 was very poor. Interestingly, the third phenol-degrading isolate (*R. erythropolis* 256), which was the only isolate that could also utilise 5 mM phenol, was under-represented in all microcosms. The relative abundance of the 7 strains was unexpected because it was predicted that phenol-degrading species would dominate the microcosm communities as stated in the hypotheses of this study (in 5.1). However, this is not necessarily the case because complex metabolic and cell-cell interactions may occur within microbial communities.

To begin with, it is well established that bacterial growth differs between pure and mixed cultures. For example, Goswami *et al.* (2005) showed that although the growth rate of pure *R. erythropolis* M1 culture on 2-chlorophenol was higher than of *Pseudomonas fluorescens* P1, in mixed culture the *P. fluorescens* P1 strain exhibited a higher growth rate. When grown on phenol, the *R. erythropolis* M1 strain exhibited higher growth in both pure and mixed cultures. Thus, in this study it is very likely that although *R.*

*erythropolis* 256 had the ability to utilise phenol when grown in pure liquid cultures, it may have been outcompeted by other phenol-degrading species (i.e. *Pseudomonas* sp. 264) in the assembled microbial microcosms.

Secondly, in mixed cultures bacteria can thrive because they can grow on metabolites of the main carbon source (phenol in this study). During this study, the ability of the isolated strains to utilise phenol metabolites (via the aerobic pathway) such as catechol, *cis,cis*-muconic acid and 2-hydroxy-*cis,cis*-muconate semialdehyde was not tested. However, it was interesting that although small concentrations of *cis,cis*-muconic acid were detected in the liquid cultures of *R. erythropolis* 256 and *Acidovorax* sp. 284 (see 4.3.1), little or no *cis,cis*-muconic acid was detected in the sand columns inoculated with the 7 strains (with the exception of one replicate of treatment I – 0.5 mM phenol for one week); this may be an indication that *cis,cis*-muconic acid was utilised by one of the other strains present.

Moreover, the production of metabolites may regulate the expression of phenol metabolic genes in the bacteria present either positively or negatively. For example, *cis,cis*-muconic acid (an aerobic metabolite of phenol) was shown to induce the synthesis of both phenol monooxygenase and catechol 1,2-dioxygenase by *Pseudomonas putida* PaW85 (Kasak *et al.*, 1993). In contrast, 4-hydroxybenzoate (a metabolite of phenol via the anaerobic degradation pathway) was shown to repress the degradation of phenol in *R. erythropolis* CCM2595 (Veselý *et al.*, 2007), and benzoate was found to completely block degradation of phenol by *Ralstonia eutropha* (Ampe *et al.*, 1998). In this study, it is not known whether gene regulation is responsible for some of the observed differences in the relative abundances of the 7 strains.

In addition to metabolic interactions, metabolically inactive bacteria may acquire the ability to degrade phenol due to cell-cell interactions. The cell-cell interactions are particularly favoured within complex biofilm communities because the proximity of different microbial strains favours exchange of mobile genetic material. In fact, horizontal gene transfer between environmental bacteria is considered to be an important adaptation mechanism to the presence of xenobiotic compounds (Marri *et al.*, 2007; Nojiri *et al.*, 2004). Previous studies have demonstrated *in situ* horizontal transfer of the C23O gene

(encoding catechol 2,3-dioxygenase) between endophytic and rhizosphere bacteria (Wang *et al.*, 2007b) and of the *ndo* gene (encoding naphthalene dioxygenase) between bacteria inhabiting Antarctic soils contaminated with polycyclic aromatic hydrocarbons (Ma *et al.*, 2006). Another study (Peters *et al.*, 1997) demonstrated that the deliberate introduction in a phenol-polluted river of a *Pseudomonas putida* strain harbouring the *phe*BA gene (which encodes both catechol 1,2-dioxygenase and phenol monooxygenase) resulted in the acquisition of the *phe*BA gene by different indigenous *Pseudomonas* species. Gene transfer between different bacterial species has also been reported at very high conjugation rates, even after 2 hours (Hausner and Wuertz, 1999). Thus, it is possible that initially metabolically inactive bacteria within the microcosms of this study may have required the ability to grow on phenol (or its metabolites) due to horizontal gene transfer. It is also worth mentioning that it has been demonstrated (Ghigo, 2001; Reisner *et al.* 2006) that conjugative plasmids can enhance biofilm development itself.

As discussed above, microbial structure in attached communities can be shaped by a range of different metabolic and cell-cell interactions. In addition, flow rates were shown to affect the structure of attached microbial communities (Rickard *et al.*, 2004b). Using similar microcosm sand columns, Mr Abdul Razak (University of Sheffield) found the oxygen availability is also a determining factor in biofilm development of a *P. putida* strain (unpublished data). Other studies showed that the mineral surface (Li and Logan, 2004), the grain size (Antizar-Ladislao *et al.*, 2000), the nutrient supply (Sgountzos *et al.*, 2006), the solution chemistry (Chen and Walker, 2007; Yang *et al.*, 2007) can affect biofilm development or amount of attached biomass. However, these factors were not varied during our microcosm studies and their effects on the structure of complex attached communities remain unexplored.

### 5.4.2 Establishment of bacteria in existing attached microbial communities

The potential of a mixture of 3 bacterial strains (*Methylibium* sp. 272, *Pseudomonas* sp. 273, *Pseudomonas* sp. 282) that independently did not have the ability to attach to quartz sand (as determined by fluorescence microscopy) to establish in already formed attached microbial communities was investigated as described in 5.2.1. As

shown previously (Fig. 4.8), in shaken liquid cultures these 3 isolates could utilise fully 0.5 mM phenol (in AB nimimal medium) but they were intolerant to 5 mM phenol (in R2A broth). Following a similar experimental approach, Li *et al.* (2008) investigated the establishment of dual-species biofilms, using a 3,5-dinitrobenzoic acid (3,5-DNBA)-degrading *Comamonas testosteroni* A3 strain with no biofilm forming ability in combination with a number of strains with high biofilm-forming abilities. They found that *C. testosteroni* was immobilised and embedded within biofilm formations in 2 occasions (with *P. putida* M9 and with *Aeromonas hydrophila* M22) and that these dual-species biofilms exhibited resistance to 3,5-DNBA shock loadings and exhibited degradation rates up to 90% within 6h. However, they were unable to explain the mechanisms involved in the immobilisation of the *C. testosteroni* A3 strain.

In this study, profiling of the attached community structure of the re-inoculated sand columns was performed by DGGE (Treatments VIII and X, in Fig. 5.6) and *Methylibium* sp. 272 was not detected, as expected according to its behaviour as a single isolate, but the 2 *Pseudomonas* strains (273 and 282) established within the existing microbial communities to a lesser or a higher extent. However, the same result was observed when the 3 isolates were used to inoculate sterile sand columns (as a negative control) which were then supplied with the same concentrations of phenol for 1 week (treatments VII and IX). This apparent contradiction may have occurred for a number of reasons. Firstly, because the assay for initial attachment to sand grains used microscopy of SYTO 9 stained grains after a 24 h period; if the *Pseudomonas* strains attached at a very low density, but grew quickly in the microcosm, they may not have been detected in the attachment assay. Alternatively, the initial attachment may have been very weak such that cells were dislodged during the assay and thus scored as non-attachers. Another possibility is that the properties of the mixed inoculum differed from that of the single inocula due to interactions such as co-aggregation between the strains. It would be possible to distinguish between these alternatives by performing additional experiments in which individual isolates were used to inoculate microcosms and then harvested after 1 week (allowing time for low numbers of cells to multiply) or by direct visualisation of the attachment process in flow cells (allowing loosely attached cells to be visualised without disturbance). Moreover, inoculation of sand columns with mixtures of both *Pseudomonas*

sp. 273 and 282 could be used to test whether interactions between these two strains can trigger bacterial attachment.

When these isolates were used to inoculate microcosms in which a microbial community was already established, the possibility of cell-cell interactions with the already established cells is enhanced. As it is illustrated in Fig. 5.10, biofilm communities do not consist exclusively by microbial cells that have the ability to attach to mineral surfaces. Initial attachment by primary colonisers (Fig. 5.10.a) that grow on the substratum forming microcolonies (Fig. 5.10.b) may be followed by secondary colonisers (Fig. 5.10.c) which although lack the ability to attach directly to the substratum, attach to the already established cells instead. Coadhesion of planktonic cells to the attached cells can occur in three different ways: i) as independent single cells, ii) as autoaggregated cells of one species or iii) as coaggregated cells of more than one bacterial species (Fig. 5.10.c; Rickard *et al.*, 2003). Following adhesion, the multi-species biofilm matures (Fig. 5.10.d), sheltered within the EPS (extracellular polymeric substances) matrix; the role of EPS in the adhesion of planktonic bacteria has not been determined yet (Rickard *et al.*, 2003). Thus, the establishment of *Pseudomonas* spp. 273 and 282 could also be attributed to their coaggregation potential with each other or with some of the isolates of inoculum 7. However, this has still to be investigated by coaggregation assays (for example as described in Adav *et al.*, 2008; Rickard *et al.*, 2004a; or Buswell *et al.*, 1997) between the 10 bacterial strains that were used in the laboratory microcosms of this study.

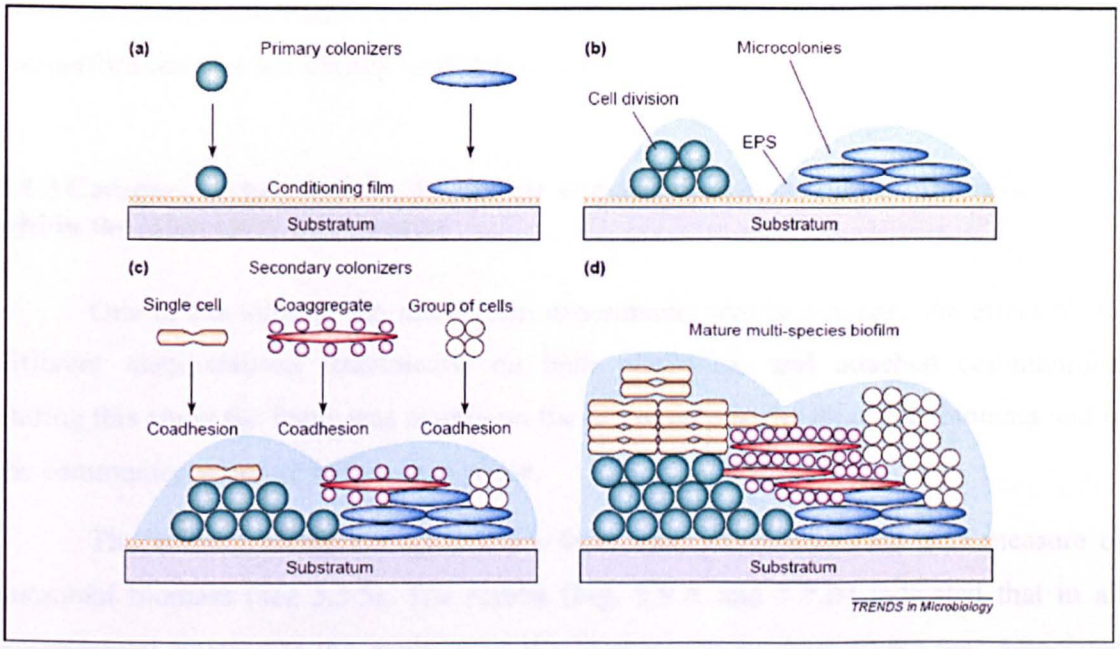



**Figure 5.10.** A diagram illustrating the stages of biofilm development of multi-species biofilms by primary and secondary colonisers and the possible roles of coaggregation in the development. Taken from Rickard *et al.* (2003)

Regarding the behaviour of the second mixture of isolates (inoculum 3) to the different concentrations of phenols supplied (0.5 or 5 mM), two observations can be made. Firstly, it is remarkable that although *Pseudomonas* spp. 273 and 282 were intolerant to 5 mM phenol even in the presence of other carbon sources (Fig. 4.8), they were able to establish in the microcosms supplied with 5 mM phenol (treatments IX and X, in Fig. 5.6). Although, it is not clear why, it is widely accepted that biofilm formation is a defensive response of microorganisms to harmful compounds (Jefferson, 2004; Hall-Stoodley *et al.*, 2004). Secondly, it was noticeable that *Pseudomonas* sp. 282 established much more strongly in the re-inoculated columns supplied with 5 mM phenol than in the re-inoculated columns supplied with 0.5 mM phenol (treatments X and VIII respectively, in Fig. 5.6). This behaviour was not phase-dependent because in the planktonic community *Pseudomonas* sp. 282 was again more abundant at 5 mM than at 0.5 mM phenol (treatments X and VIII respectively, in Fig. 5.7). Moreover, it was not due to the phenol concentration itself because *Pseudomonas* sp. 282 could utilise 0.5 mM phenol as sole carbon source (in shaken liquid cultures, Fig. 4.8) and it thrived within the 3 microcosm community (treatment VII, in Fig. 5.6). Therefore, its weaker establishment in

the re-inoculated microcosms supplied with 0.5 mM phenol is likely to have resulted from out-performance by the already established strains.

### 5.4.3 Comparison between the planktonic and attached microbial communities within the laboratory microcosms

One of the aims of the microcosm experiments was to compare the effect of the different manipulations (treatments) on both planktonic and attached communities. During this study the focus was mainly on the differences in the microbial biomass and in the community structure within each phase.

The amount of the extracted DNA from each phase was used as a measure of microbial biomass (see 5.3.5). The results (Fig. 5.9.A and 5.9.B) indicated that in all experimental treatments the majority of the biomass (more than 90 %) was associated with the attached microbial communities, in agreement with previous studies. For example, Griebler *et al.* (2002) found that the ratio of attached to planktonic bacteria was 1657:1 and 59:1 in microcosms incubated for 10 months with pristine and BTEX contaminated groundwater respectively while Lehman *et al.* (2004) also observed higher microbial biomass within the core (basalt rock) than the groundwater samples from a polluted aquifer (with chlorinated hydrocarbons, sewage, and low-level radioactive liquid waste).

The effects of the different manipulations on the structure of the attached community were discussed previously (5.4.1 and 5.4.2); with the exception of one strain of inoculum 3 (*Methylibium* sp. 272), all other strains were detected in the attached microbial communities but with different degrees of attachment. However, the planktonic communities were characterised by the presence of fewer bacterial strains (Fig. 5.7). The higher microbial diversity within the attached community (including bacteria with no obvious phenol-degrading ability) is not surprising because as Hall-Stoodley *et al.* (2004) state: "*biofilm formation represents a protected mode of growth that allows cells to survive in hostile environments*". As discussed in 5.4.2, these bacteria can exist in attached communities due to favourable conditions for metabolic or cell-cell interactions.

Nevertheless, it is not clear why from all isolates used for the inoculation of the microcosm sand columns, the 3 *Pseudomonas* strains (264, 273, and 282) dominated the planktonic communities (see 5.3.4 and Fig. 5.7). Their ability to degrade (0.5 mM phenol) is only a partial explanation because other phenol-degrading species (*Acidovorax* sp. 284 and *R. erythropolis* 256) were not detected in the effluent, although they established in the attached communities. Of course, this may be an indication that *Acidovorax* sp. 284 and *R. erythropolis* 256 exist preferentially in the attached phase, a view supported by Jefferson (2004) who stated "*the biofilm mode of growth may be the default mode of growth for at least some bacterial species suggesting that we should be questioning what triggers the planktonic mode of growth rather than what motivates the biofilm mode of growth*". Therefore, another parameter should be considered about the presence of *Pseudomonas* spp. 264, 273 and 282 in the effluent. Given the facts that *Pseudomonas* spp. 264, 273 and 282 were the most dominant strains within the corresponding attached communities and cells from biofilm communities continuously detach, what was detected in the effluent of the microcosms could have been simply cell debris derived from the biofilm communities. It is characteristic that Sgountzos *et al.* (2006) reported that in sand-packed columns supplied with phenol a range of factors such as low flow rates, oxygen availability and nutrient supply led to increased detachment of *P. fluorescens* cells.

In addition to the 3 *Pseudomonas* strains, *Stenotrophomonas* sp. 245 was also present in some planktonic communities, more evidently in the effluent of the microcosms supplied with 5 mM phenol. Its presence in the effluent of these treatments may be explained by its tolerance to phenol toxicity because, as it was shown earlier in the R2A broth cultures supplemented with 5 mM phenol, *Stenotrophomonas* sp. 245 was the most tolerant strain after the phenol degrading *R. erythropolis* 256 (Fig. 4.8.B).

### 5.4.4 Degradation of phenol by mixed microbial consortia

Degradation of phenol within the microcosm sand columns was quantified and phenol degradation per unit of biomass was calculated as described in 5.3.5 (Fig. 5.9.C and 5.9.D respectively). The results indicated that all established microbial communities

were capable of utilising phenol but differences in the amount of degraded phenol were observed between treatments.

In general, at the end of the second week phenol removal was higher in the microcosms supplied with 5 mM phenol (treatments III, V, IX and X in Fig. 5.9.C) than in the microcosms supplied with 0.5 mM phenol (II, VI, VII and VIII in Fig. 5.9.C). This trend might be explained by simple Michaelis-Menten kinetics; at a higher substrate (phenol) concentrations, metabolic reactions (phenol degradation) increase for cells which are supplied with concentrations of phenol below their $K_m$.

Nevertheless, it is noticeable that at the end of the first week phenol degradation was very limited in the microcosms inoculated with the 7 bacterial strains and supplied with 5 mM phenol (treatment IV, in Fig. 5.9.C), indicating possible phenol toxicity effects. This is supported by the fact that the microcosms supplied with 0.5 mM phenol for 1 week and then shifted to 5 mM during the second week exhibited higher phenol degradation than the microcosms supplied continuously with 5 mM phenol for 2 weeks (treatments III and V respectively, in Fig. 5.9.C). Thus, it appears that a step-wise increase in phenol concentration enhanced the adaptation of this microbial community to phenol toxicity while the supply of a high initial phenol load resulted in a longer lag phase. There are a number of studies that demonstrated longer lag phases at increased phenol concentrations, either for single bacterial strains (Kumar *et al.*, 2005; Juang and Tsai, 2006) or for mixed bacterial cultures (Tziotzios *et al.*, 2005; Saravanan *et al.*, 2008). Once the bacteria acclimatise to phenol and catabolic genes are expressed, then degradation rates increase. For example, it is characteristic that phenol degradation rates were always found to increase with consecutive/subsequent phenol re-amendments of phenol-degrading cultures (Antizar-Ladislao and Galil, 2003; Guieysse *et al.*, 2001; Tziotzios *et al.*, 2005).

In this study, the highest degradation of phenol was observed within the sand columns supplied with 0.5 mM phenol in the first week and then swapped to 5 mM or 470 mg $L^{-1}$ phenol in the second week (treatment III, in Fig. 5.9.C) at about 0.6 mM. Considering the fact that the phenol was supplied at a rate of 3 ml $h^{-1}$ and the pore volume within the sand columns was 2 ml, it means that phenol was being degraded at a rate of

0.9 mM $h^{-1}$ or 84.6 mg $L^{-1}$ $h^{-1}$ or 2.03 g $L^{-1}$ $d^{-1}$. This value is comparable with phenol degradation rates mentioned in the literature. In a packed-bed reactor inoculated with a *R. erythropolis* strain and supplied with 400 mg $L^{-1}$ or 600 mg $L^{-1}$ phenol degradation rates of 14.4 and 16.1 g $L^{-1}$ $d^{-1}$ respectively were measured (Prieto *et al.*, 2002) while in another bed reactor (with mixed microbial communities) fed with 527 mg $L^{-1}$ phenol a maximum removal value of 12.65 g $L^{-1}$ $d^{-1}$ was detected (Tziotzios *et al.*, 2005). In addition, Shimp and Pfaender (1987) reported that extended exposure of mixed aquatic bacteria to phenol resulted in increased biodegradation capacity of other aromatic compounds too (*m*-cresol, *m*-aminophenol and *p*-chlorophenol).

Another interesting question is whether the attached or the planktonic microbial community was responsible for the majority of the measured consumption of phenol within the sand microcosms. This question was not investigated directly during this study but other studies (based solely on phenol degradation data) have demonstrated that phenol removal was faster by attached microbial cells (in packed-bed reactors) than by suspended microbial cultures (Prieto *et al.*, 2002; Tziotzios *et al.*, 2005). Considering that in this study the majority of the microbial biomass was found within the attached phase, it is expected that the contribution of the planktonic community to phenol degradation was low, something which highlights again the importance of the attached microbial communities in polluted environments.

### 5.4.5 Conclusions

In this study, the effects of phenol concentration (and shifts in phenol concentration) on planktonic and attached microbial community structure were investigated by using microcosm sand columns inoculated with a mixture of 7 (functionally characterised) bacterial isolates. The results indicated that within the time scale of the experiment (2 weeks) the concentrations of phenol that were used (0.5 and 5 mM) did not influence microbial community structure within the different treatments. However, the DGGE analysis indicated that:

a) all 7 strains established within the attached microbial community (to different degrees) but fewer were present in the planktonic community

b) one phenol degrading isolate (*Pseudomonas* sp. 264) dominated the attached community but the other two phenol degrading isolates (*Acidovorax* so. 284 and *R. erythropolis* 256) were present at the same or lower densities than non-degrading isolates

Thus, these results suggest that higher species diversity can exist in attached microbial communities and their relative abundance is not related exclusively to their ability to utilise the carbon source present (phenol), as metabolic and cell-cell interactions can also shape microbial community structure.

Moreover, the ability of organisms that appeared not to attach independently to mineral surfaces (quartz sand in this study) to establish in existing attached communities was examined by re-inoculating microcosm columns with a mixture of 3 isolates (*Methilibium* sp. 272, *Pseudomonas* spp. 273 and 282). However, when this mixture was used to inoculate sterile sand columns (as a negative control), *Pseudomonas* spp. 273 and 282 attached to the sand, suggesting that the attachment assay (in Chapter 4) did not accurately assess bacterial attachment in experiments of longer duration. Nevertheless, it was noticeable that in the re-inoculated microcosms, *Pseudomonas* sp. 282 established more strongly in the microcosms supplied with 5 mM than in the microcosms supplied with 0.5 mM phenol while in the sterile microcosms *Pseudomonas* sp. 282 established to the same extent at both phenol concentrations. This result is a strong indication that establishment of introduced bacteria may be influenced by metabolic or cell-cell interactions with existing attached microbial communities.

# Chapter 6

# General discussion

Natural aquifers contain diverse microbial communities which have the potential for *in situ* bioremediation. Microorganisms in aquifers can exist either attached to mineral surfaces or free-floating (planktonic) within the groundwater flow. In many cases it has been demonstrated that more than 90 % of the microbial biomass exists within the attached phase (Holm *et al.*, 1992; Alfreider *et al.*, 1997; Griebler *et al.*, 2002). Nevertheless, most of the microbiological studies on polluted aquifers have examined the microbial diversity of either planktonic communities (in groundwater samples) or the attached communities (in core/sediment samples) but rarely both. The aim of this thesis was to investigate the effect of groundwater pollution on the diversity of planktonic and attached microbial communities and to make comparisons between the two.

The first objective of this study was to investigate how the abundance and diversity of microbial communities altered across a geochemical pollutant gradient. This objective was examined in groundwater samples (i.e. planktonic communities) from a phenol-polluted aquifer at Four Ashes. The results indicated that microbial abundance increased about 100-fold at the fringes of the contaminant plume in borehole 59 (10, 11, 12 and 30 mbgl) compared to total cell numbers in uncontaminated groundwater. In addition, the microbial community structure (as determined by DGGE analysis) varied with depth across the steep geochemical gradient present at the plume fringe.

Due to limitations in available boreholes it was not possible to determine whether the structure of the attached communities also differed with depth. However, given the marked differences in microbial community in the planktonic samples taken across the plume fringe, and the differences in planktonic and attached communities at 30 mbgl, it would be highly surprising if this was not reflected in similarly marked differences in the attached communities elsewhere in the plume. If these limitations were overcome in the future (i.e. by drilling additional boreholes), a direct comparison of the attached and planktonic microbial communities across the geochemical gradient would be possible. This would enable an investigation of which of the two phases is more stable temporally and spatially (across pollutant gradients or in different areas of the plume) and whether the species composition of the communities is most strongly governed by growth phase (planktonic vs attached) or geochemical conditions (position within the plume). Members

of planktonic communities clustered separately to those from attached communities in marine environments with samples taken from different areas (Moesender *et al.*, 2001; Ghiglione *et al.*, 2007).

The importance of *in situ* studies arises from their ability to measure the actual situation in polluted aquifer. However because of the complexity of polluted environments, the interpretation of DNA fingerprinting patterns (DGGE, T-RFLP, SSCP etc.) based on environmental factors is very challenging. In recent years, a number of studies have used statistical tools to relate differences in DNA fingerprinting profiles to environmental variables (Fromin *et al.*, 2002; Fry *et al.*, 2006; Imfeld *et al.*, 2008). For example, Fahy *et al.* (2005) using multivariate analysis of geochemical data and bacterial community structure (T-RFLP profiles) in groundwater samples from a BTEX contaminated aquifer showed that other "anoxia-related parameters" (oxygen, carbon dioxide, methane, sulphate, nitrate, dissolved iron and manganese) accounted for 60 % of the variance in bacterial community structures while benzene concentration accounted for only 7.7 %. In future studies at Four Ashes, such a multivariate approach could be used if community profiles were obtained from other regions of the plume and related to the multiple geochemical parameters such as pH, concentrations of dissolved oxygen, other electron acceptors and their reduced forms (e.g. $Fe^{3+}/Fe^{2+}$, $Mn^{4+}/Mn^{2+}$, $SO_4^{2-}/S^{2-}$) and products of methanogenic or fermentative processes (e.g. $CH_4$, $H_2$) that have been described at this site (Thornton *et al.*, 2001b). The DGGE profiles of the groundwater samples analysed in this study suggested that the pollutant load may have a determining role, because the profiles of microbial communities from spatially distant samples with high concentrations of organic pollutants (i.e from 12 and 30 mbgl) were more similar to each other than microbial communities in samples from less polluted depths (10, 11 mbgl).

The second objective of this study was to compare the microbial diversity of planktonic and attached communities under the same geochemical conditions. This objective was examined using culture-independent methods in one aquifer depth, at the bottom of borehole 59 (30 mbgl), where TPC was 1220 mg $L^{-1}$ in 2006. The DGGE analysis indicated that both communities were moderately diverse (based on the number of DGGE bands), certainly more diverse than extreme acidic environments (e.g. Tinto

river in Spain; González-Toril *et al.*, 2003) but less diverse than rhizosphere bacterial communities (e.g. Vestergård *et al.*, 2008). In addition, the limited number of common bands between these samples, coupled with cluster analysis of the DGGE profiles, indicated that the two communities differed markedly.

DGGE analysis has its limitations and in order to resolve the diversity and composition in these two microbial communities, cloning and partial sequencing of 16S rRNA gene fragments was performed. The two clone libraries that were created (one from the groundwater and one from the sand sample) demonstrated that there was limited overlap (only 5 common ribotypes) between the two microbial communities. It also indicated that the planktonic microbial community was less diverse, as it was dominated by only four bacterial phylogenetic groups ($\alpha$-/$\beta$-Proteobacteria, Bacteroidetes and Firmicutes) and 70% of the groundwater clones belonged to only 6 ribotypes. Based on the phylogenetic affiliations of the sequenced clones, bacterial species related to the geochemical environment of that plume depth (30 mbgl in borehole 59) could be identified, including likely phenol-degrading and nitrate-reducing species. Nevertheless, their phylogenetic affiliations were not able to explain why some of the environmental clones existed in only one of the two phases and others in both. For example, ribotype SP11F11 (100% sequence identity to phenol degrading *Azoarcus* sp. PbN1) was found in both clone libraries while ribotypes is14 and is19 (closely related to potentially phenol degrading *Acidovorax* strains) were found only in the attached phase.

The functional questions that arose from the *in situ* studies regarding the influence of the geochemical environment on the microbial structure of both planktonic and attached communities were addressed in this study by using laboratory microcosms that were inoculated with mixtures of characterised isolates from the Four Ashes site with different functional properties (ability to degrade or tolerate phenol and to attach to sand).

As the pollutant load may have an effect on the structure of microbial communities, the third objective of this study was to examine specifically how different phenol concentrations and shifts in phenol concentration affect microbial community composition and function in defined microcosms. During the course of the microcosm studies (2 weeks) and for the concentrations of phenol that were used (0.5 and 5 mM

phenol), no significant differences in the structure of planktonic or attached microbial communities were observed between the different treatments (although differences were observed when pre-existing communities were re-inoculated with other mixtures of isolates). However, these microcosms were inoculated with a small number of microbial strains relative to the natural microbial community composition. Natural inocula would contain more functionally diverse microbial communities including members with different affinities and tolerances to phenol. Future microcosm experiments (with natural inocula or mixtures of isolated bacteria) could examine a wider range of phenol concentrations (e.g. 0.01, 0.1, and 5 mM phenol) as well as different sampling intervals (1 day, 1 week, 1 month). In addition, it would be interesting to explore whether the availability of other carbon sources (e.g. cresols) or alternative electron acceptors (e.g. nitrate) significantly affects microbial community structure. For example microcosms could be established with different phenol concentrations, different concentrations of dissolved oxygen and nitrate and different mineral substrata (quartz sand grains coated with different iron oxides).

Some of the results obtained in microcosm experiments were in accordance with the findings of the *in situ* studies, especially with respect to the diversity and composition of planktonic communities. In Chapter 3, it was found that the attached community that developed on the sterile quartz sand incubated in the aquifer was not a subset of the planktonic community but was different and more diverse. This apparent paradox (as it was called in 3.4.2) was attributed to the fact that although the groundwater sample reflected the composition of the planktonic community at that time point, the sand sample would have "recorded" all the different microorganisms that managed to establish during its 14 month incubation in the aquifer. Despite the simplicity of the microcosm experiments, the microcosm results are consistent with the hypothesis that the attached community may be more diverse because most of the bacteria flowing through a porous medium can establish in attached communities either as primary attachers (due to cell-mineral interactions) or as secondary attachers (due to cell-cell interactions) while only a few of them will exist in both phases (e.g. *Pseudomonas* spp. 264, 273, 282) or only in the planktonic phase (e.g. *Methylibium* sp. 272, which did not attach). The extent of establishment of newly introduced bacterial species may depend on the interactions

with the pre-existing communities (e.g. *Pseudomonas* sp. 282 established less strongly in the re-inoculated microcosms supplied with 0.5 mM phenol). The compositions of the two communities may also differ because microorganisms that exist in both phases at low densities will be a numerically important component in the less diverse planktonic community but may not be detected in diverse attached communities where other species dominate.

The microcosm experiments also revealed that two of the phenol-degrading species established to the same or lower densities as isolates that did not have the ability to degrade phenol (as assessed in shaken liquid cultures). These experiments show that the ability to degrade phenol is not the major factor that determined establishment in the microcosm. The expression of phenol-degrading genes may be repressed in the complex communities or other organisms may be able to preferentially establish either as a result of horizontal gene transfer or metabolic interactions with other species (as discussed in 5.4.1).

In future experiments, the use of $^{13}C$ labelled phenol and RNA-SIP (Stable Isotope Probing) could elucidate which species actually utilise either phenol or its metabolites in these mixed communities. For example, by using a two species system inoculated with a phenol-degrading *P. putida* and a non-degrading *P. chlororaphis* strain, Manefield *et al.* (2002) showed that while $^{13}C$ was initially incorporated into the phenol degrading strain only (as determined by DGGE analysis), 72 hours later the $^{13}C$ was also incorporated into the *P. chlororaphis* strain, indicating cross-feeding between the species. Thus, by using well defined microcosm studies, it may be possible to relate the effects of different manipulations on both microbial community structure and function.

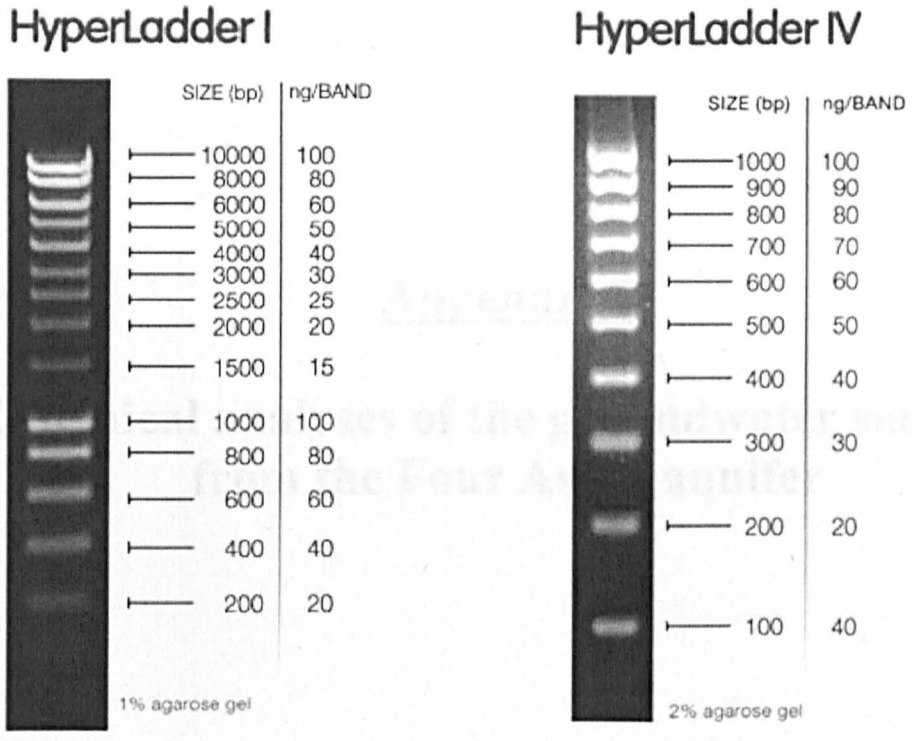
The study of the effect of different environmental conditions on microbial community composition and function *in situ* is very challenging. 16S rRNA gene cloning and sequencing allows the composition of complex microbial communities to be identified. High-throughput metagenomic approaches, i.e. random sequencing of whole community DNA, can describe more accurately (as they avoid biases introduced during PCR amplification) the composition of complex microbial communities as well as the genes that are present; thus their use in microbial ecology studies is increasing in the last

few years (see also 1.3). However, these genes may not become expressed in complex microbial communities where a range of metabolic interactions occur. Therefore in recent years, metatranscriptomics, i.e. the random sequencing of whole community mRNA, has been employed to identify the genes that are actually expressed in natural environments. By combining the two approaches (metagenome and metatranscriptome sequences) valuable information can be obtained especially regarding the responses of a given community to environmental changes. For example, Gilbert *et al.* (2008) compared the effect of elevated $CO_2$ concentrations on 4 marine mesocosms samples (taken at two time points and from induced phytoplankton mid- and post-bloom communities) and they found that the metagenomes and metatranscriptomes were different in taxonomic composition and that the majority of the highly clustered transcripts belonged to novel gene families, indicating the effectiveness of this approach in "*discovering novel genetic capacity*". In addition, when Gilbert *et al.* (2008) compared the level of diversity (assembly) they found that the number of clusters within the four metagenomes was similar in all four samples and 2x or 9x the number of clusters within the metatrancriptomes of the mid-bloom and post-bloom treatments respectively; this finding indicated that the size of the metatranscriptome was smaller and that it became more homogeneous in the post-bloom conditions. The geochemical gradients that are found in polluted aquifers (often in the sub-metre scale) offer an ideal system where the effect of different environmental conditions on the diversity (compositional and functionial) of *in situ* microbial communities (both planktonic and attached) can be investigated by combined metagenomic and metatranscriptomic approaches in the future.

This study has provided a better understanding of the microbial ecology of the phenol polluted aquifer at the Four Ashes site and it demonstrated that under the same geochemical conditions planktonic and attached microbial communities may differ significantly. The differences in community composition may also infer functional differences between the two communities. Therefore, this study highlights the need for investigating both planktonic and attached microbial communities when studying biogeochemical processes in polluted aquifers.

# Appendix 1

# Hyperladders I and IV




The size (in bp) and the DNA content (in ng) of the bands comprising hyperladders I and IV (Bioline, UK).

# Appendix 2

# Chemical analyses of the groundwater samples from the Four Ashes aquifer

## Borehole 59 – Organic compounds

**Table 1.** The concentrations (all in mg $L^{-1}$) of organic compounds measured in groundwater samples pumped from various depths in Borehole 59 in 2005 and in 2006. The concentration of xylenols was determined only in the 2005 samples. The concentration of acetate was measured only in the 2006 samples. TPC = the sum of phenol and cresols. 4-HB = 4-hydroxybenzoic. BDL = below detection limits.

| | Depth (mbgl) | TPC | phenol | *o*-cresol | *m*-/*p*-cresol | 3,4 xylenol | 3,5 xylenol | 2,3 xylenol | 2,4 & 2,5 xylenols | 2,6 xylenol | catechol | Acetate | 4-HB aldehyde | 4-HB acid | 4-HB alcohol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | 6 | 0.23 | 0.09 | 0.03 | 0.11 | 0.01 | BDL | BDL | BDL | 0.02 | BDL | | BDL | BDL | BDL |
| | 8 | 0.14 | 0.04 | 0.03 | 0.07 | 0.03 | BDL | BDL | BDL | 0.02 | BDL | | BDL | BDL | BDL |
| | 9 | 0.39 | 0.13 | 0.10 | 0.16 | BDL | 0.01 | BDL | BDL | BDL | BDL | | 0.01 | BDL | BDL |
| | 10 | 5.3 | 0.61 | 0.41 | 4.28 | 0.24 | 0.68 | 0.03 | 0.82 | 0.24 | 0.06 | | BDL | BDL | BDL |
| | 11 | 232.9 | 74.95 | 35.40 | 122.55 | 5.70 | 19.30 | 25.84 | 33.62 | 16.14 | 0.97 | | BDL | BDL | BDL |
| | 30 | 350.89 | 141.78 | 39.65 | 169.46 | 4.14 | 14.34 | 20.38 | 27.15 | 21.32 | 1.00 | | 0.09 | BDL | 0.06 |
| 2006 | 6 | BDL | BDL | BDL | BDL | | | | | | | <10.00 | BDL | BDL | BDL |
| | 8 | 1.26 | 0.65 | 0.34 | 0.27 | | | | | | | <10.00 | BDL | BDL | BDL |
| | 9 | BDL | BDL | BDL | BDL | | | | | | | <10.00 | BDL | BDL | 1.32 |
| | 10 | 0.49 | 0.29 | 0.2 | BDL | | | | | | | <10.00 | BDL | BDL | BDL |
| | 11 | 280.2 | 89.68 | 46.26 | 144.26 | | | | | | | 88.50 | 0.26 | 5.72 | 27.43 |
| | 12 | 662.73 | 243.01 | 115.67 | 303.06 | | | | | | | 157.92 | 1.11 | 9.44 | BDL |
| | 30 | 1220.36 | 488.19 | 133.3 | 598.87 | | | | | | | 64.24 | 0.34 | 3.38 | BDL |

## Borehole 59 – Organic compounds

**Table 2.** The concentrations (all in mg $L^{-1}$) of inorganic compounds measured in groundwater samples pumped from various depths in Borehole 59 in 2005 and in 2006. The concentrations of phosphate, nitrite and ammonium were below detection limits (BDL) in all depths and they are not included in the table.

| | Depth (mbgl) | Nitrate | Sulphate | Magnesium | Calcium | Sodium | Chloride | Potassium | Bromide | Fluoride |
|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | 6 | 82.30 | 22.01 | 7.21 | 106.85 | 20.95 | 24.41 | 1.77 | BDL | BDL |
| | 8 | 78.08 | 24.83 | 7.98 | 107.48 | 23.04 | 24.25 | 1.53 | BDL | BDL |
| | 9 | 81.52 | 22.41 | 7.55 | 108.57 | 20.41 | 23.94 | 1.43 | BDL | BDL |
| | 10 | 41.26 | 15.88 | 15.09 | 101.99 | 175.15 | 53.75 | 2.06 | 0.70 | BDL |
| | 11 | BDL | 108.29 | 53.80 | 72.82 | 815.65 | 162.48 | 2.60 | 3.28 | BDL |
| | 30 | 28.68 | 63.67 | 12.65 | 115.71 | 170.90 | 55.70 | 2.27 | 0.74 | BDL |
| 2006 | 6 | 114.99 | 24.87 | 9.76 | 78.12 | 20.78 | 16.85 | 1.72 | <1.00 | 0.18 |
| | 8 | 111.74 | 28.78 | 7.78 | 82.38 | 25.31 | 17.62 | 1.64 | <1.00 | 0.22 |
| | 9 | 114.32 | 24.48 | 7.25 | 82.54 | 20.22 | 17.13 | 1.52 | <1.00 | 0.23 |
| | 10 | 84.37 | 24.35 | 8.03 | 92.26 | 78.20 | 25.56 | <0.20 | <1.00 | 0.21 |
| | 11 | 0.57 | 119.99 | 42.73 | 81.49 | 633.28 | 73.64 | <0.20 | 2.64 | <0.10 |
| | 12 | <0.20 | 303.69 | 58.25 | 59.90 | 853.11 | 101.13 | <0.20 | 5.63 | <0.10 |
| | 30 | 15.46 | 110.36 | 27.16 | 97.70 | 288.20 | 66.76 | <0.20 | <1.00 | <0.10 |

**Borehole 60 – Organic compounds**

**Table 3.** The concentrations (all in mg $L^{-1}$) of organic compounds measured in groundwater samples pumped from various depths in Borehole 60 in 2005 and in 2006. The concentration of xylenols was determined only in the 2005 samples. The concentration of acetate was measured only in the 2006 samples. TPC = the sum of phenol and cresols. 4-HB = 4-hydroxybenzoic. BDL = below detection limits.

| | Depth (mbgl) | TPC | phenol | *o*-cresol | *m*-/*p*-cresol | 3,4 xylenol | 3,5 xylenol | 2,3 xylenol | 2,4 & 2,5 xylenols | 2,6 xylenol | catechol | Acetate | 4-HB aldehyde | 4-HB acid | 4-HB alcohol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | 12 | 0.23 | 0.10 | 0.00 | 0.13 | BDL | BDL | BDL | BDL | BDL | BDL | | BDL | BDL | BDL |
| | 15 | 0.02 | 0.02 | BDL | BDL | BDL | 0.03 | BDL | BDL | 0.03 | BDL | | BDL | BDL | BDL |
| | 18 | 317.17 | 141.92 | 40.70 | 135.09 | 3.23 | 6.30 | 14.83 | 20.56 | BDL | 1.60 | | 0.10 | 0.11 | BDL |
| | 19 | 407.92 | 182.33 | 53.22 | 172.37 | 4.44 | 8.43 | 20.10 | 28.78 | BDL | 3.08 | | 0.19 | BDL | BDL |
| | 20 | 831.29 | 373.81 | 109.41 | 348.07 | 9.03 | 17.37 | 46.33 | 57.61 | BDL | 6.01 | | 0.47 | BDL | BDL |
| | 45 | 5809.45 | 2253.42 | 580.26 | 2975.77 | 56.24 | 102.09 | 231.51 | 401.52 | BDL | 19.19 | | 0.98 | 1.47 | BDL |
| 2006 | 12 | BDL | BDL | BDL | BDL | | | | | | | <10.00 | BDL | BDL | 1.60 |
| | 15 | BDL | BDL | BDL | BDL | | | | | | | <10.00 | BDL | BDL | 1.02 |
| | 16 | BDL | BDL | BDL | BDL | | | | | | | <10.00 | BDL | BDL | 1.53 |
| | 17 | 0.44 | 0.33 | 0.11 | BDL | | | | | | | <10.00 | BDL | BDL | 0.78 |
| | 18 | 242.93 | 109.82 | 32.31 | 100.8 | | | | | | | 10.65 | 0.25 | 3.01 | 8.16 |
| | 45 | 5579.44 | 2106.8 | 618.3 | 2854.34 | | | | | | | 114.50 | 1.64 | 9.02 | 30.01 |

## Borehole 60 – Inorganic compounds

**Table 4.** The concentrations (all in mg $L^{-1}$) of inorganic compounds measured in groundwater samples pumped from various depths in Borehole 60 in 2005 and in 2006. The concentrations of phosphate, nitrite and ammonium were below detection limits (BDL) in all depths and they are not included in the table.

| | Sample | Depth (mbgl) | Nitrate | Sulphate | Magnesium | Calcium | Sodium | Chloride | Potassium | Bromide | Fluoride |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2005 | 60/12 | 12 | 80.11 | 28.14 | 3.69 | 97.71 | 10.95 | 26.79 | 2.04 | BDL | BDL |
| | 60/15 | 15 | 91.81 | 45.14 | 15.57 | 52.23 | 7.71 | 45.32 | 6.81 | BDL | BDL |
| | 60/18 | 18 | BDL | 197.72 | 37.49 | 181.27 | 55.74 | 66.56 | 5.40 | 0.54 | BDL |
| | 60/19 | 19 | BDL | 227.49 | 38.93 | 187.52 | 65.45 | 70.41 | 4.68 | 1.14 | BDL |
| | 60/20 | 20 | BDL | 336.74 | 54.87 | 250.10 | 111.41 | 108.77 | 5.29 | 3.63 | BDL |
| | 60/45 | 45 | 1.76 | 290.66 | 42.83 | 336.34 | 52.11 | 178.84 | 5.91 | 4.49 | BDL |
| 2006 | 60/12 | 12 | 92.75 | 38.87 | 11.33 | 49.11 | 9.66 | 26.76 | <0.20 | <1.00 | <0.10 |
| | 60/15 | 15 | 105.04 | 45.11 | 16.86 | 38.44 | 7.56 | 32.26 | <0.20 | <1.00 | <0.10 |
| | 60/16 | 16 | 102.49 | 45.74 | 16.74 | 37.94 | 6.70 | 31.86 | <0.20 | <1.00 | <0.10 |
| | 60/17 | 17 | 100.68 | 46.79 | 16.58 | 37.59 | 7.19 | 31.75 | <0.20 | <1.00 | <0.10 |
| | 60/18 | 18 | 0.43 | 136.17 | 33.69 | 85.49 | 47.61 | 36.47 | <0.20 | <1.00 | <0.10 |
| | 60/45 | 45 | 2.28 | 304.52 | 32.99 | 229.44 | 51.51 | 111.98 | <0.20 | 3.47 | <0.10 |

# Appendix 3

# Closest relatives of the sequenced isolated bacteria and environmental clones

| Ribotype ID[1] | Sequence length[2] | is G[3] | is S[4] | cl G[5] | cl S[6] | Closest relatives[7] (GenBank match & type strain from RDP) | Accession number[8] | Max ID[9] | Max score[10] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **α-Proteobacteria ( 9 / 9 / 21 )** | | | |
| ig209 | 971 | 2 | | | | *Agrobacterium tumefaciens* CCBAU 85034 (*Rhizobium radiobacter*) | EU256459 | 99 | 1718 |
| | | | | | | Rhizobium radiobacter (T) | M11223 | 0.986 | 0.954 |
| SP01C11 | 685 | | | | 2 | *Agrobacterium tumefaciens* C4 (*Rhizobium radiobacter*) (phenol-degrading, nitrate-reducing) | AF508093 | 100 | 1236 |
| | | | | | | *Rhizobium daejeonense* (T) L61T (cyanide treatment bioreactor) | AY341343 | 0.981 | 0.909 |
| SP12D05 | 711 | | | | 1 | alpha proteobacterium A0721 | AF235998 | 99 | 1274 |
| | | | | | | *Ensifer adhaerens* Sulf-1426 (rhizosphere of Lolium perenne) | AM922194 | 98 | 1232 |
| | | | | | | *Rhizobium sullae* (T) IS 123=USDA 4950=DSM 14623 | Y10170 | 0.983 | 0.911 |
| ig235 | 900 | 1 | | | | *Rhizobium huautlense* OS-49.b (barnyard dust) | AM237359 | 100 | 1624 |
| | | | | | | *Rhizobium huautlense* (T) SO2 | AF025852 | 0.969 | 0.87 |
| S23 | 695 | | | | 1 | *Pseudodevosia insulae* DS-56 (soil) | EF012357 | 99 | 1249 |
| | | | | | | *Devosia neptuniae* (T) J1 | AF469072 | 0.96 | 0.864 |
| SP12C05 | 668 | | | | 1 | uncultured bacterium KuyAS25 (glacier) | EU263680 | 100 | 1205 |
| | | | | | | *Bradyrhizobium* sp. BTA-1 (T) | AJ558025 | 0.985 | 0.929 |
| S41 | 426 | | | | 1 | uncultured α-Proteobacterium AKYH1214 (farm soil) | AY921890 | 100 | 769 |
| | | | | | | *Blastochloris viridis* (T) ATCC19567 | D25314 | 0.953 | 0.764 |
| SP12C06 | 726 | | | | 1 | *Methylocystis* sp. m261 (methanotrophic) | DQ852351 | 99 | 1299 |
| | | | | | | *Methylocystis* sp. SV97 (T) (methanotrophic, Arctic wetland soil) | AJ414656 | 0.985 | 0.930 |
| ig260 | 971 | 1 | | | | *Rhodobacter ovatus*, type strain JA234T (industrially polluted fresh water pond) | AM690348 | 97 | 1638 |
| | | | | | | *Rhodobacter sphaeroides* (T) 2.4.1 | X53853 | 0.965 | 0.816 |
| S30 | 655 | | | | 1 | *Phenylobacterium lituiforme* (T) FaiI3 (thermal aquifer) | AY534887 | 0.963 | 0.841 |
| S48 | 646 | | | | 1 | uncultured bacterium clone ORSFC2_d03 (river sediments) | EF393423 | 99 | 1155 |
| | | | | | | *Brevundimonas* sp. FWC04 | AJ227793 | 99 | 1155 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP12A07 | 721 | | | 1 | 3 | uncultured *Caulobacter* sp. D-1 (natural mineral water) | AF523036 | 99 | 1279 |
| | | | | | | *Caulobacter* sp. ECN-2008 | AM940947 | 98 | 1245 |
| | | | | | | *Caulobacter fusiformis* (T) ATCC 15257 | AJ227759 | 0.981 | 0.899 |
| is12 | 983 | | 1 | | | *Sphingomonas* sp. KT-1 (freshwater) | AB022601 | 99 | 1743 |
| | | | | | | *Sphingopyxis chilensis* (T) S37 (chlorophenol-degrading) | AF367204 | 0.991 | 0.949 |
| SP01A08 | 704 | | | | 5 | *Sphingomonas* sp. CFO6 (Carbofuran-contaminated soil) | U52146 | 97 | 1202 |
| | | | | | | *Sphingobium cloacae* (T); S-3 (nonylphenol-degrading bacterium) | AB040739 | 0.970 | 0.870 |
| ig217 | 838 | 2 | | | | *Sphingomonas* sp. LP7A (mine water) | AB434710 | 98 | 1454 |
| | | | | | | *Sphingomonas aquatilis* (T) JSS-7 (natural mineral water) | AF131295 | 0.983 | 0.921 |
| ig220 | 977 | 2 | | | | *Sphingomonas* sp. TSBY-34 (frozen soil) | DQ166180 | 99 | 1730 |
| | | | | | | *Sphingomonas faeni* (T); MA-olki (air-borne, Antarctica) | AJ429239 | 0.992 | 0.974 |
| GP01H11 | 684 | | | 4 | | *Magnetospirillum* sp. CF19 (freshwater microcosm) | AJ863152 | 99 | 1207 |
| | | | | | | *Magnetospirillum magnetotacticum* (T) DSM 3856 | Y10110 | 0.971 | 0.891 |
| GP01F09 | 705 | | | 2 | | *Magnetospirillum gryphiswaldense* (T) MSR-1/DSM 6361 | Y10109 | 0.977 | 0.899 |
| SP12C09 | 739 | | | | 3 | uncultured bacterium Amb_16S_1475 (trembling aspen rhizosphere) | EF018961 | 98 | 1287 |
| | | | | | | Bacterium Ellin328 | AF498710 | 95 | 1110 |
| | | | | | | *Azospirillum brasilense* (T) ATCC 49958 | AY150046 | 0.902 | 0.653 |
| SP11G10 | 619 | | | | 1 | *Azospirillum brasilense* ISSDS-858 (agricultural soil) | EF634031 | 97 | 1052 |
| | | | | | | *Azospirillum brasilense* (T) ATCC 49958 | AY150046 | 0.969 | 0.841 |
| GP01B07 | 657 | | | 2 | | uncultured bacterium EXP.1-16S-13C-light-Clone_38 (wetland rice soil) | AB273827 | 99 | 1122 |
| | | | | | | *Azospirillum brasilense* (T) ATCC 49958 | AY150046 | 0.981 | 0.872 |
| | | | | | | **β-Proteobacteria ( 27 / 43 / 56 )** | | | |
| is19 | 960 | | 4 | | 3 | Beta proteobacterium NOS3 (activated sludge) | AB076845 | 98 | 1682 |
| | | | | | | *Acidovorax defluvii* (T) BSB411 (activated sludge) | Y18616 | 0.984 | 0.913 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| is14 | 937 | | 5 | | 17 | *Variovorax* sp. ANRB-Zg (acetate-utilizing anaerobes from freshwater sediment) | AJ276398 | 99 | 1667 |
| | | | | | | *Acidovorax defluvii* (T) BSB411 (activated sludge) | Y18616 | 0.987 | 0.880 |
| is08 | 947 | | 5 | | | Beta proteobacterium NOS8 (activated sludge) | AB076846 | 100 | 1703 |
| | | | | | | *Simplicispira psychrophila* (T) LMG 5408 | AF078755 | 0.982 | 0.907 |
| ig240 | 664 | 2 | | | | *Xylophilus ampelinus* (T) ATCC 33914 | AF078758 | 0.987 | 0.927 |
| ig227 | 972 | 4 | | | | *Variovorax* sp. 44/31 (hydrocarbon-contaminated Antarctic soil) | AY571831 | 99 | 1728 |
| | | | | | | *Variovorax paradoxus* (T) DSM 66 | AJ420329 | 0.980 | 0.859 |
| ig207 | 1016 | 4 | | | | *Variovorax paradoxus* CAI-26 (commercial airliner cabin air) | DQ257419 | 99 | 1820 |
| | | | | | | *Variovorax paradoxus* (T) DSM 66 | AJ420329 | 0.983 | 0.833 |
| SP12A05 | 666 | | | | 1 | *Simplicispira* sp. R-23033 (commercial nitrifying inoculum) | AM236310 | 97 | 1088 |
| | | | | | | *Simplicispira psychrophila* (T) LMG 5408 | AF078755 | 0.963 | 0.826 |
| ig229 | 934 | 1 | | | | *Rhodoferax* sp. KAR67 (Arctic permafrost soil) | EF451697 | 98 | 1613 |
| | | | | | | *Rhodoferax ferrireducens* (T) T118 (psychrotolerant, oxidises acetate with the reduction of iron) | AF435948 | 0.981 | 0.904 |
| SP12A10 | 653 | | | | 4 | uncultured bacterium UASB1 (anaerobic sludge reactor) | AB329645 | 99 | 1173 |
| | | | | | | Bacterium BH3O1 (aquifer) | AY928221 | 98 | 1128 |
| | | | | | | *Rhodoferax ferrireducens* (T) T118 (psychrotolerant, oxidises acetate with the reduction of iron) | AF435948 | 0.984 | 0.887 |
| SP12D01 | 589 | | | | 2 | uncultured beta proteobacterium 52GS2 (lake water) | EF203832 | 99 | 1041 |
| | | | | | | *Rhodoferax ferrireducens* (T) T118 (psychrotolerant, oxidises acetate with the reduction of iron) | AF435948 | 0.989 | 0.933 |
| ig272 | 667 | 1 | | | | uncultured bacterium BF0001C036 (indoor dust) | AM697195 | 99 | 1195 |
| | | | | | | Denitrifying bacterium W125 (upland soil) | AB162105 | 97 | 1135 |
| | | | | | | *Methylibium petroleiphilum* (T) PM1 (methylotroph, isolated from compost biofilter) | AF176594 | 0.969 | 0.864 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP12C07 | 665 | | | | 2 | uncultured bacterium MBR283-100 (H2-fed fixed-film bioreactor) | AY913832 | 95 | 1054 |
| | | | | | | Beta proteobacterium NOS8 (activated sludge) | AB076846 | 94 | 1041 |
| | | | | | | *Acidovorax defluvii* (T) BSB411 (activated sludge) | Y18616 | 0.936 | 0.780 |
| S42 | 461 | | | | 1 | uncultured bacterium M14C24 (radionuclide contaminated sediment) | EU331388 | 99 | 821 |
| | | | | | | *Janthinobacterium agaricidamnosum* (T) W1r3T | Y08845 | 0.972 | 0.85 |
| SP12C02 | 663 | | | | 1 | *Ultramicrobacterium* str. HI-G4 (cave rock surface) | DQ205303 | 96 | 1088 |
| | | | | | | *Herbaspirillum huttiense* (T) ATCC 14670T | AB021366 | 0.938 | 0.824 |
| SP01D11 | 583 | | | | 1 | uncultured soil bacterium L1A.13E10 | AY989480 | 99 | 1018 |
| | | | | | | *Undibacterium pigrum* CCUG 49009 (drinking water) | AM397630 | 98 | 991 |
| | | | | | | *Duganella zoogloeoides* (T) IAM12670 | D14256 | 0.937 | 0.746 |
| ig262 | 970 | 1 | | | | *Pigmentiphaga* sp. JSM 061001 | EU583723 | 99 | 1721 |
| | | | | | | *Pigmentiphaga kullae* (T) K24 | AF282916 | 0.970 | 0.879 |
| SP11F12 | 659 | | | | 1 | uncultured bacterium LWS-T4972 (lake sediment) | EU546289 | 96 | 1085 |
| | | | | | | *Methylophilus* sp. ECd5 (methylotroph) | AY436794 | 94 | 1009 |
| | | | | | | *Herbaspirillum lusitanum* (T) P6-12 | AF543312 | 0.905 | 0.681 |
| SP01B12 | 654 | | | | 1 | uncultured bacterium clone NGD19 (paddy soil) | EF614037 | 97 | 1097 |
| | | | | | | *Siderooxidans ghiorsii* strain LD-1 (wetland plant rhizosphere) | DQ386859 | 92 | 973 |
| | | | | | | *Gallionella ferruginea* (T) (iron-oxidizing) | L07897 | 0.928 | 0.750 |
| S01 | 509 | | | | 1 | uncultured bacterium TA3_54 (drinking water) | EU746716 | 99 | 901 |
| | | | | | | *Gallionella ferruginea* (T) (iron-oxidizing) | L07897 | 0.954 | 0.843 |
| GP12F06 | 377 | | | 1 | | uncultured bacterium UASB1 (anaerobic sludge reactor) | AB329645 | 99 | 726 |
| | | | | | | Bacterium RSD-1-10 | AY822520 | 98 | 693 |
| | | | | | | *Gallionella ferruginea* (T) (iron-oxidizing) | L07897 | 0.927 | 0.728 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP01A06 | 669 | | | | 1 | *Sterolibacterium* sp. TKU1 (industrial water cooling system) | AM990454 | 97 | 1121 |
| | | | | | | *Thiobacter subterraneus* (T) C55 (subsurface hot aquifer) | AB180657 | 0.895 | 0.697 |
| SP11F11 | 657 | | | 16 | 7 | uncultured soil bacterium F45_Pitesti (oil polluted soil) | DQ378207 | 100 | 1186 |
| | | | | | | *Azoarcus* sp. PbN1 (anaerobic degradation of hydrocarbons by denitrifying bacteria) | X83532 | 100 | 1186 |
| | | | | | | *Azoarcus buckelii* (T) U120 = DSM 14744 | AJ315676 | 0.998 | 0.979 |
| SP01B06 | 632 | | | | 1 | uncultured beta proteobacterium ADK-MOh02-42 (acid-impacted lake) | EF520482 | 99 | 1126 |
| | | | | | | *Azoarcus* sp. T3 (fuel contaminated aquifer) | Y11041 | 89 | 829 |
| | | | | | | *Azoarcus indigens* (T) VB32 | AF011345 | 0.991 | 0.676 |
| SP11F04 | 658 | | | | 2 | uncultured *Hydrogenophilaceae* bacterium D25_43 | EU266916 | 100 | 1187 |
| | | | | | | *Thiobacillus denitrificans* (T) NCIMB 9548 | AJ243144 | 0.963 | 0.809 |
| GP12E12 | 619 | | | 1 | | Beta proteobacterium 5Z-C1 (rice field soil) | AJ224618 | 93 | 930 |
| | | | | | | *Azoarcus buckelii* (T) U120 = DSM 14744 | AJ315676 | 0.924 | 0.738 |
| SP11E12 | 638 | | | | 1 | uncultured beta proteobacterium A22 (coal mining-associated lake sediments) | AM713395 | 92 | 933 |
| | | | | | | Denitrifying bacterium 72Chol | Y09967 | 91 | 883 |
| | | | | | | *Sterolibacterium denitrificans* (T) Chol-1S | AJ306683 | 0.904 | 0.693 |
| SP11E09 | 633 | | | | 1 | uncultured bacterium LS4-252 | AB234251 | 95 | 1002 |
| | | | | | | *Sterolibacterium denitrificans* (T) Chol-1S | AJ306683 | 0.920 | 0.747 |
| GP02A11 | 687 | | | 1 | | uncultured bacterium Pav-006 | DQ642320 | 99 | 1214 |
| | | | | | | *Sterolibacterium denitrificans* (T) Chol-1S | AJ306683 | 0.917 | 0.711 |
| GP01E09 | 666 | | | 1 | | Beta proteobacterium G5G6 (anaerobic toluene-degrading) | EF219370 | 96 | 1081 |
| | | | | | | *Sterolibacterium denitrificans* (T) Chol-1S | AJ306683 | 0.914 | 0.677 |
| GP12E08 | 656 | | | 9 | | uncultured beta proteobacterium 055T7 (freshwater sediment) | DQ110036 | 95 | 1058 |
| | | | | | | Beta proteobacterium KF034 (freshwater river) | AB376632 | 93 | 984 |
| | | | | | | *Denitratisoma oestradiolicum* (T) AcBE2-1 | AY879297 | 0.943 | 0.67 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP12B04 | 722 | | | 14 | 8 | uncultured bacterium clone 1013-28-CG13 (uranium-contaminated aquifer) | AY532566 | 98 | 1261 |
| | | | | | | *Denitratisoma oestradiolicum* (T) AcBE2-1 | AY879297 | 0.938 | 0.635 |
| | | | | | | **γ-Proteobacteria ( 23 / 2 / 7 )** | | | |
| ig59/30-4 | 1032 | 4 | | | | *Pseudomonas* sp. BFXJ-8 (phenol-degrading) | EU013945 | 99 | 1849 |
| | | | | | | *Pseudomonas veronii* (T) CIP 104663 (natural mineral water) | AF064460 | 0.995 | 0.974 |
| ig264 | 951 | 2 | | | | *Pseudomonas putida* strain PC36 (phenol degrading *Pseudomonas* species) | DQ178233 | 99 | 1701 |
| | | | | | | *Pseudomonas lini* (T) CFBP 5737 (rhizospheric soil) | AY035996 | 0.986 | 0.954 |
| is282 | 723 | | 1 | | 1 | *Pseudomonas putida* strain IA2YCDA (BTEX contaminated industrial site) | AY512612 | 99 | 1292 |
| | | | | | | *Pseudomonas umsongensis* (T) Ps 3-10 (farm soil) | AF468450 | 1.000 | 0.988 |
| is17 | 944 | | 9 | | 1 | *Pseudomonas* sp. 'ARDRA PS2' (BTEX contaminated soil) | AY364086 | 100 | 1685 |
| | | | | | | *Pseudomonas frederiksbergensis* (T) JAJ28 (soil at a coal gasification site) | AJ249382 | 0.989 | 0.953 |
| is290 | 666 | | 1 | | 1 | *Pseudomonas* sp. JN18_A60_A4 (PCB-dechlorinating enrichment culture) | DQ168654 | 99 | 1187 |
| | | | | | | *Pseudomonas monteilii* (T) CIP 104883 (clinical specimens) | AF064458 | 0.964 | 0.861 |
| G34 | 637 | | | 1 | | uncultured bacterium 1013-28-CG9 (uranium contaminated aquifer) | AY532564 | 91 | 902 |
| | | | | | | *Pseudomonas* sp. TSBY-66 | DQ173022 | 90 | 865 |
| | | | | | | *Pseudomonas frederiksbergensis* (T) JAJ28 (soil at a coal gasification site) | AJ249382 | 0.902 | 0.675 |
| S13 | 665 | | | | 1 | uncultured γ-Proteobacterium AKYG1642 (farm soil) | AY921834 | 99 | 1189 |
| | | | | | | *Dokdonella koreensis* (T) DS-123 (soil) | AY987368 | 0.936 | 0.800 |
| ig245 | 705 | 6 | | | | *Stenotrophomonas* sp. DM1-41 (hydrocarbon contaminated Antarctic soil) | DQ109991 | 100 | 1267 |
| | | | | | | *Stenotrophomonas rhizophila* (T) e-p10 (rhizosphere of oilseed rape) | AJ293463 | 1.000 | 0.999 |
| SP01C12 | 665 | | | 1 | 3 | *Methylobacter tundripaludum* (T) SV96 (methane-oxidising, from Arctic wetland soil) | AJ414655 | 1.000 | 0.987 |
| | | | | | | **Actinobacteria ( 44 / - / 2 )** | | | |
| is292 | 705 | 2 | 9 | | | *Rhodococcus* sp. AP17 (hydrocarbon polluted sand) | EU374915 | 99 | 1260 |
| | | | | | | *Rhodococcus fascians* (T) ATCC 12974T | X81930 | 0.996 | 0.971 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ig214 | 1025 | 1 | | | | *Rhodococcus* sp. P11-B-8<br>(Arctic deep-sea sediment) | EU016150 | 100 | 1844 |
| | | | | | | *Rhodococcus yunnanensis* (T) YIM 70056<br>(forest soil) | AY602219 | 0.989 | 0.967 |
| ig256 | 993 | 1 | | | | *Rhodococcus erythropolis* IAM 1503 | AB429544 | 99 | 1777 |
| | | | | | | *Rhodococcus baikonurensis* (T) GTC1041<br>(Russian space laboratory) | AB071951 | 0.997 | 0.975 |
| ig242 | 625 | 1 | | | | *Rhodococcus erythropolis* Ri81<br>(BTEX contaminated aquifer) | AM905948 | 100 | 1128 |
| | | | | | | *Rhodococcus erythropolis* (T) ATCC 4277T | X81929 | 1.000 | 0.987 |
| is291 | 704 | | 3 | | | *Nocardia* sp. FXJ2.005<br>(meadow soil) | EU677781 | 99 | 1256 |
| | | | | | | *Nocardia ignorata* (T) IMMIB R-1434 =<br>DSM 44496 = NRRL B-24141 | AJ303008 | 0.990 | 0.955 |
| ig250 | 973 | 1 | | | | *Marmoricola aurantiacus* (T) BC361<br>(marble statue) | Y18629 | 0.990 | 0.960 |
| ig202 | 1027 | 1 | | | | *Aeromicrobium* sp. MOLA 34<br>(North Western Mediterranean Sea) | AM990810 | 97 | 1739 |
| | | | | | | *Aeromicrobium fastidiosum* (T) DSM 10552T<br>(Antarctic sandstone) | Z78209 | 0.985 | 0.920 |
| ig269 | 698 | 4 | | | | *Nocardioides* sp. IMCC1764<br>(freshwater pond) | DQ664208 | 98 | 1187 |
| | | | | | | *Nocardioides ganghwensis* (T) JC2055<br>(tidal flat sediment) | AY423718 | 0.967 | 0.875 |
| ig213 | 898 | 2 | | | | *Actinobacterium* 4c-1<br>(Nuclear Waste-Contaminated sediments) | AY561534 | 99 | 1593 |
| | | | | | | *Nocardioides plantarum* (T) DSM 11054T<br>(Antarctic sandstone) | Z78211 | 0.980 | 0.923 |
| ig218 | 717 | 1 | | | | *Nocardioides plantarum* (T) DSM 11054T<br>(Antarctic sandstone) | Z78211 | 0.994 | 0.964 |
| ig270 | 705 | 1 | | | | *Actinotelluria brasiliensis* Tu6233 | DQ029102 | 98 | 1213 |
| | | | | | | *Blastococcus saxobsidens* (T) BC448<br>(marble) | AJ316571 | 0.941 | 0.792 |
| ig253 | 384 | 1 | | | | uncultured organism MC061014<br>(hospital therapy pool) | AY898007 | 99 | 675 |
| | | | | | | *Microsphaera* sp. G-96<br>(garden soil) | EF600014 | 96 | 616 |
| | | | | | | *Nakamurella* □*ultipartite* (T) JCM 9543T | Y08541 | 0.961 | 0.81 |
| SP12A04 | 646 | | | | 2 | uncultured bacterium D25_13<br>(tar-oil contaminated aquifer sediments) | EU266892 | 97 | 1090 |
| | | | | | | *Terracoccus luteus* (T) DSM 44267 | Y11928 | 0.883 | 0.617 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ig216 | 823 | 1 | | | | Cellulomonas composti, TR7-06<br>(cattle farm compost) | AB166887 | 98 | 1427 |
| | | | | | | *Cellulomonas terrae* (T) DB5<br>(cellulolytic and xylanolytic, from soil) | AY884570 | 0.981 | 0.871 |
| ig261 | 943 | 1 | | | | *Micrococcus luteus* EHFS1_S01Hd<br>(clean room) | EU071591 | 99 | 1689 |
| | | | | | | *Micrococcus luteus* (T) ATCC 4698 | AF542073 | 0.996 | 0.971 |
| ig201 | 1047 | 2 | | | | *Arthrobacter* sp. HX2<br>(alpine grassland soil) | EF601814 | 99 | 1867 |
| | | | | | | *Arthrobacter oxydans* (T) DSM 20119 | X83408 | 0.987 | 0.948 |
| ig257 | 1004 | 1 | | | | Glacial ice bacterium G50-PD1<br>(glacial ice, China) | AF479348 | 99 | 1792 |
| | | | | | | *Clavibacter michiganensis* (T) ATCC 33566 | U30254 | 0.996 | 0.971 |
| ig251 | 1009 | 1 | | | | *Rathayibacter caricis* (T) VKM Ac-1799<br>(phyllosphere of *Carex* sp.) | AF159364 | 0.997 | 0.980 |
| ig233 | 995 | 1 | | | | uncultured bacterium, BF0001C062<br>(indoor dust) | AM697221 | 98 | 1712 |
| | | | | | | *Frigoribacterium* sp. OS-12A<br>(lead-zinc mine tailing site) | EF612311 | 97 | 1691 |
| | | | | | | *Frigoribacterium faeni* (T) 801<br>(airborne dust, phychrophilic) | Y18807 | 0.976 | 0.904 |
| ig258 | 952 | 9 | | | | *Frigoribacterium* sp. GIC6<br>(glacial ice core) | AY439262 | 99 | 1694 |
| | | | | | | *Frigoribacterium faeni* (T) 801<br>(airborne dust, phychrophilic) | Y18807 | 0.986 | 0.948 |
| | | | | | | **Bacteriodetes ( 1 / 36 / 10 )** | | | |
| SP01C03 | 664 | | | | 1 | uncultured bacterium LWS-T4850<br>(Lake sediment) | EU546334 | 99 | 1180 |
| | | | | | | Bacterium DG890 | AY258122 | 87 | 773 |
| | | | | | | *Fluviicola taffensis* (T) RW262<br>(river epilithon) | AF493694 | 0.833 | 0.474 |
| SP11E06 | 639 | | | | 1 | uncultured gold mine bacterium D28 | AF337883 | 98 | 1092 |
| | | | | | | Marine *Bacteroidetes* bacterium RS.Sph.009<br>(mucus of coral *Fungia granulosa*) | DQ097283 | 85 | 728 |
| | | | | | | *Flavobacterium psychrolimnae* (T) LMG 22018<br>(microbial mats in Antarctic lakes) | AJ585428 | 0.84 | 0.528 |
| SP11H03 | 637 | | | | 1 | uncultured eubacterium WCHB1-69<br>(chlorinated-solvent-contaminated aquifer) | AF050545 | 99 | 1139 |
| | | | | | | *Fluviicola taffensis* (T) RW262<br>(River epilithon) | AF493694 | 0.848 | 0.467 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP11F10 | 684 | | | | 1 | uncultured *Bacteroidetes* bacterium AS28 (activated sludge of domestic wastewater) | EU283360 | 97 | 1157 |
| | | | | | | *Cytophaga* sp. Dex80-37 (East Pacific) | AJ431253 | 86 | 735 |
| | | | | | | *Arcicella aquatica* (T) NO-502 (freshwater lake) | AJ535729 | 0.822 | 0.422 |
| SP11G05 | | | | | 2 | uncultured *Flavobacteria* bacterium AUVE_02G10 (cropland) | EF650994 | 94 | 1021 |
| | | | | | | *Fluviicola taffensis* (T) RW262 (River epilithon) | AF493694 | 0.873 | 0.559 |
| G46 | 592 | | | 1 | | uncultured *Bacteriodetes* bacterium 44a-B1-42 (subsurface acid mine drainage system) | AY082469 | 99 | 1058 |
| | | | | | | *Sphingobacteriales* bacterium Kimo37 (Black rust formation) | AB260041 | 87 | 706 |
| | | | | | | *Alkaliflexus imshenetskii* (T) Z-7010 (soda lake) | AJ784993 | 0.836 | 0.478 |
| GP01A08-2 | 625 | | | 2 | | uncultured bacterium MBR283-102 ($H_2$-fed fixed-film bioreactor) | AY913837 | 95 | 1002 |
| | | | | | | *Paludibacter propionicigenes* (T) WB4 (rice-field soil) | AB078842 | 0.92 | 0.717 |
| G16 | 672 | | | 4 | | uncultured bacterium TBM.20DEC-31 (industrial wastewater treatment) | EF608389 | 98 | 1148 |
| | | | | | | *Proteiniphilum acetatigenes* (T) TB107 | AY742226 | 0.961 | 0.773 |
| GP12A09 | 670 | | | 3 | | uncultured *Bacteroidetes/Chlorobi* group bacterium D25_22 (tar-oil contaminated aquifer sediment) | EU266899 | 99 | 1195 |
| | | | | | | *Proteiniphilum acetatigenes* (T) TB107 | AY742226 | 0.955 | 0.757 |
| GP01H05 | 715 | | | 25 | | uncultured *Bacteroidetes/Chlorobi* group bacterium D25_27 (tar-oil contaminated aquifer sediment) | EU266902 | 100 | 1290 |
| | | | | | | uncultured Iron-reducing enrichment clone Cl-A4 (polluted esturary sediments) | DQ676996 | 94 | 1110 |
| | | | | | | *Bacteroidales genomosp.* P1 clone P4PB_6 (subgingival plaque) | AY341819 | 84 | 767 |
| | | | | | | *Lutibacter litoralis* (T) CL-TF09 (tidal flat sediment) | AY962293 | 0.801 | 0.455 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GP12B04 | | | | 1 | | uncultured *Bacteroidetes/Chlorobi* group bacterium D25_27 (tar-oil contaminated aquifer sediment) | EU266902 | 92 | 937 |
| | | | | | | *Azospira oryzae* MFC-EB31 (microbial fuel cell) | AJ630299 | 82 | 625 |
| | | | | | | *Lutibacter litoralis* (T) CL-TF09 (tidal flat sediment) | AY962293 | 0.805 | 0.446 |
| ig263 | 933 | 1 | | | | *Taxeobacter gelupurpurascens* (T) Txg1 (Antarctic soils and sandstone) | Y18836 | 0.978 | 0.951 |
| S38 | 423 | | | | 1 | unidentified eubacterium | AF010027 | 98 | 688 |
| | | | | | | *Saprospiraceae* bacterium MS-Wolf2-H (lake freshwater) | AJ786323 | 88 | 461 |
| | | | | | | *Haliscomenobacter hydrossis* (T) | M58790 | 0.826 | 0.428 |
| SP11H01 | 699 | | | | 3 | uncultured *Bacteroidetes* bacterium CC_04 (paper pulp column) | EF562555 | 98 | 1220 |
| | | | | | | *Flavobacteria* bacterium KF030 (freshwater lake) | AB269814 | 96 | 1146 |
| | | | | | | *Terrimonas ferruginea* (T) | M62798 | 0.907 | 0.581 |
| | | | | | | **FIRMICUTES ( 8 / 27 / - )** | | | |
| GP01C04 | 683 | | | 9 | | uncultured bacterium clone GIF4 (monochlorobenzene contaminated groundwater) | AF407196 | 99 | 1209 |
| | | | | | | *Desulfosporosinus* sp. STP12 (sulfate-reducing, pristine freshwater lake sediments) | AJ582757 | 97 | 1155 |
| | | | | | | *Desulfosporosinus meridiei* (T) S10 (aquifer contaminated with gasoline) | AF076527 | 0.959 | 0.819 |
| GP12E10 | 619 | | | 3 | | uncultured bacterium P3IB-23 (Uranium mine sediment) | AF414571 | 99 | 1119 |
| | | | | | | *Sedimentibacter* sp. IMPC3 (ferric-reducing, corroded oil station tank) | EF189918 | 96 | 1029 |
| | | | | | | *Desulfosporosinus meridiei* (T) S10 (aquifer contaminated with gasoline) | AF076527 | 0.855 | 0.648 |
| GP02B05 | 663 | | | 1 | | uncultured bacterium d162 (trichloroethene-contaminated groundwater) | AF422686 | 99 | 1168 |
| | | | | | | *Clostridium* sp. PPf35E6 (sulfate reducing reactor treating metal-containing wastewater) | AY548783 | 96 | 1079 |
| | | | | | | *Anaerovorax odorimutans* (T) NorPut | AJ251215 | 0.961 | 0.778 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GP01D11 | 710 | | | 12 | | uncultured bacterium HDBW-WB46 (deep subsurface groundwater) | AB237709 | 98 | 1227 |
| | | | | | | *Acetobacterium* sp. R6T (psychrophilic acetogenic, wetland soil) | EU443246 | 96 | 1191 |
| | | | | | | *Acetobacterium carbinolicum* (T) DSM 2925 | X96956 | 0.969 | 0.863 |
| ig211 | 542 | 1 | | | | *Paenibacillus* sp. Enf35 (alpine subnival plants) | DQ339607 | 99 | 962 |
| | | | | | | *Paenibacillus amylolyticus* (T) NRRL NRS-290T | D85396 | 0.994 | 0.954 |
| is297 | 678 | 1 | 5 | | | *Bacillus* sp. MHS035 | DQ993300 | 100 | 1223 |
| | | | | | | *Bacillus muralis* (T) LMG 20238 (mural painting biofilm) | AJ628748 | 0.998 | 0.970 |
| ig59/30-2 | 968 | 1 | | | | *Sporosarcina* sp. NP23 (cold saline sulfidic spring, Canadian High Arctic) | EU196333 | 99 | 1734 |
| | | | | | | *Sporosarcina macmurdoensis* (T) CMS 21w (cyanobacterial mat, Antarctica) | AJ514408 | 0.997 | 0.962 |
| GP12F05 | 648 | | | 1 | | uncultured bacterium R1Cb8 (anaerobic denitrifying reactor) | EF063632 | 96 | 1059 |
| | | | | | | rumen bacterium R-7 (sheep rumen) | AB239481 | 92 | 884 |
| | | | | | | *Clostridium saccharolyticum* (T) DSM 2544 | Y18185 | 0.804 | 0.462 |
| GP01H03 | | | | 1 | | uncultured bacterium clone AME E27 | DQ191697 | 94 | 1032 |
| | | | | | | *Bacillus* sp. BA315 | AY836770 | 80 | 562 |
| | | | | | | *Acholeplasma vituli* (T) FC-097 (but Phylum: Tenericutes) | AF031479 | 0.805 | 0.434 |
| | | | | | | **Other – Unclassified ( 2 / 5 / 40 )** | | | |
| ig232 | 530 | 1 | | | | *Deinococcus radiopugnans* (T) ATCC 19172T | Y11334 | 0.996 | 0.962 |
| ig252 | 995 | 1 | | | | Bacterium Ellin510 | AY960773 | 99 | 1779 |
| | | | | | | *Deinococcus radiomollis* PO-04-20-144 (alpine environment) | EF635405 | 97 | 1683 |
| | | | | | | *Deinococcus deserti* (T) VCD115 (Sahara desert) | AY876378 | 0.915 | 0.709 |
| SP01C08 | 489 | | | | 2 | uncultured gamma proteobacterium 1B_26 (fuel-contaminated laboratory microcosms) | AY251214 | 99 | 758 |
| | | | | | | *Azotobacter vinelandii* | AB175657 | 86 | 581 |
| | | | | | | *Pseudomonas indica* (T) | AF302795 | 0.867 | 0.620 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP12C08 | 621 | | | | 2 | uncultured bacterium CV90<br>(cave wall biofilms) | DQ499320 | 89 | 821 |
| | | | | | | Delta proteobacterium 102<br>(iron-reducing, from Arctic sediments) | | | |
| | | | | | | *Pelobacter acetylenicus* (T) WoAcyl<br>DSM2348 | X70955 | 0.817 | 0.459 |
| SP12B02 | 651 | | | | 1 | uncultured bacterium R1-15<br>(Methane-consuming sludge) | AB280279 | 85 | 729 |
| | | | | | | Bacterium Ellin5294 | AY234645 | 87 | 574 |
| | | | | | | *Okibacterium fritillariae* (T) VKM Ac-2059 | AB042094 | 0.770 | 0.389 |
| SP11H12 | 434 | | | | 1 | uncultured bacterium HDSP-88<br>(surface pond water) | EU283166 | 91 | 621 |
| | | | | | | Bacterium Ellin504 | AY960767 | 85 | 480 |
| | | | | | | *Patulibacter minatonensis* (T) KV-614 | AB193261 | 0.832 | 0.459 |
| S08 | 604 | | | | 1 | uncultured bacterium clone 2N1-43<br>(rhizosphere soil) | EU160021 | 96 | 976 |
| | | | | | | *Phaeospirillum* sp. MPA1 | AF487433 | 80 | 540 |
| | | | | | | *Byssovorax cruenta* (T) By c2 = DSM 14553<br>(cellulose-degrading) | AJ833647 | 0.835 | 0.486 |
| SP12D04 | 548 | | | | 1 | uncultured bacterium LWS-T4601<br>(lake sediment) | EU546359 | 99 | 971 |
| | | | | | | *Fervidomicrobium thiophilum* SR | EF554597 | 82 | 493 |
| | | | | | | *Thermoanaerobacter kivui* (T) | L09160 | 0.836 | 0.511 |
| SP11F09 | 653 | | | | 1 | uncultured bacterium FukuN97<br>(lake bacterioplankton) | AJ290002 | 98 | 1142 |
| | | | | | | *Bacteriovorax* sp. PNEc1 | AY294221 | 97 | 1106 |
| | | | | | | *Bacteriovorax stolpii* (T) uki-2 | M34125 | 0.954 | 0.777 |
| S20 | 465 | | | | 1 | uncultured delta proteobacterium D12_01<br>(tar-oil contaminated aquifer sediments) | EU266811 | 100 | 839 |
| | | | | | | Sulfate-reducing bacterium STP23<br>(oligotrophic lake sediment) | AJ006620 | 96 | 764 |
| | | | | | | *Desulfobacterium catecholicum* (T) DSM<br>3882 | AJ237602 | 0.906 | 0.692 |
| S12 | 696 | | | | 1 | uncultured bacterium clone GOUTB20<br>(monochlorobenzene cont. groundwater) | AY050605 | 99 | 1229 |
| | | | | | | *Algidimarina propionica* AK-P<br>(cold marine sediments, Alaska) | AY851291 | 90 | 964 |
| | | | | | | *Desulfobacterium indolicum* (T) DSM 3383<br>(marine mud) | AJ237607 | 0.911 | 0.627 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP01A03 | 685 | | | | 1 | uncultured bacterium 656043 (wastewater treatment plant) | DQ404599 | 97 | 1151 |
| | | | | | | *Solibacter usitatus* Ellin6076 | CP000473 | 92 | 996 |
| | | | | | | *Caloramator indicus* (T) IndiB4 (non-volcanically heated aquifer waters) | X75788 | 0.796 | 0.467 |
| SP11E11 | 616 | | | | 1 | uncultured bacterium clone M14C12 (radionucleotide contaminated sediments) | EU331377 | 98 | 1079 |
| | | | | | | *Geothrix fermentans* H5 | U41563 | 98 | 1052 |
| SP01B05 | 701 | | | | 1 | *Leptospira biflexa* (T) patoc Patoc 1 | Z12821 | 0.907 | 0.726 |
| SP01C05 | 674 | | | | 1 | *Leptospira borgpetersenii* L0101 | EF537004 | 85 | 756 |
| | | | | | | *Leptospira borgpetersenii* (T) JAVANICA VELDRAT BATAVIA 46 | Z21630 | 0.860 | 0.582 |
| SP12A02 | 684 | | | | 1 | uncultured bacterium YCB65 (geothermal spring mat) | EF205465 | 83 | 636 |
| | | | | | | *Leptospira fainei* serovar Hurstbridge BKID6 | AY996789 | 83 | 616 |
| | | | | | | *Leptospira broomii* (T) 5399; ATCC BAA-1107 (humans) | AY796065 | 0.890 | 0.487 |
| SP11H09 | 598 | | | | 1 | uncultured bacterium CS2_38 (oil palm empty fruit bunches compost) | EF221926 | 96 | 852 |
| | | | | | | Agricultural soil bacterium SC-I-55 | AJ252641 | 82 | 558 |
| | | | | | | *Chondromyces apiculatus* (T) Cm a14 | AJ233938 | 0.810 | 0.415 |
| S25 | 694 | | | | 2 | uncultured bacterium clone: CMBR-1 (municipal wastewater bioreactor) | AB305029 | 99 | 1225 |
| | | | | | | Filamentous symbiotic bacterium of *Methylobacterium* sp. strain TFF | AB112774 | 93 | 886 |
| | | | | | | anaerobic filamentous bacterium KIBI-1 (T) (Levilinea saccharolytica) (methanogenic sludge granules) | AB109439 | 0.873 | 0.514 |
| S37 | 648 | | | | 1 | uncultured bacterium DOK_BIODYN_clone616 (agricultural soil) | DQ828254 | 98 | 791 |
| | | | | | | *Bellilinea caldifistulae* (thermophilic digester sludge) | AB243672 | 82 | 601 |
| | | | | | | anaerobic filamentous bacterium KIBI-1 (T) (Levilinea saccharolytica) (methanogenic sludge granules) | AB109439 | 0.802 | 0.411 |
| S21 | 695 | | | | 1 | uncultured bacterium LaP15L89 (anoxic river sediment) | EF667686 | 96 | 1110 |
| | | | | | | *Caldilinea aerophila* (thermophile) | AB067647 | 87 | 838 |
| | | | | | | *Heliobacterium chlorum* (T) | M11212 | 0.782 | 0.377 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP01C04 | 589 | | | | 1 | uncultured *Nitrospirae* bacterium 406 (iron-oxidation biofilm) | AB252944 | 100 | 1063 |
| | | | | | | Candidatus *Nitrospira defluvii* Contig5882 (wastewater treatment plant) | EU559167 | 99 | 1058 |
| | | | | | | *Nitrospira moscoviensis* (T) (nitrite-oxidizing) | X82558 | 0.914 | 0.674 |
| SP12B06 | 717 | | | | 1 | uncultured bacterium RRA044 (Urban Creek Sediment) | EU284500 | 99 | 1164 |
| | | | | | | *Verrucomicrobiae* bacterium pACH90 (waterlogged archaeological wood) | AY297806 | 95 | 1148 |
| | | | | | | *Opitutus terrae* (T) PB90-1 (anoxic flooded rice field) | AJ229235 | 0.930 | 0.735 |
| SP11F01 | 678 | | | | 1 | uncultured bacterium clone lka36 (sulfidic cave stream biofilm) | EF467542 | 99 | 1200 |
| | | | | | | Bacterium Ellin514 | AY960777 | 90 | 921 |
| | | | | | | *Verrucomicrobium spinosum* (T) DSM 4136T | X90515 | 0.788 | 0.437 |
| GP12E06 | 650 | | | 2 | 1 | uncultured epsilon proteobacterium CC1_CL33 (microbial mat from sulfidic cave spring) | DQ295573 | 99 | 1159 |
| | | | | | | *Sulfurimonas denitrificans* (T) DSM 1251 (deep-sea hydrothermal vent) | L40808 | 0.92 | 0.721 |
| S07 | 713 | | | | 3 | uncultured bacterium IC-33 (sewer system) | AB255061 | 90 | 957 |
| | | | | | | *Microbulbifer maritimus* (T) TF-17 (intertidal sediment) | AY377986 | 0.770 | 0.344 |
| SP01D08 | 629 | | | | 2 | uncultured bacterium RRB046 (urban creek sediments) | EU284552 | 99 | 1124 |
| | | | | | | *Acidimicrobium ferrooxidans* TH3 (iron-oxidizing, acidophilic) | EF621760 | 85 | 520 |
| | | | | | | *Thermodesulfobium narugense* (T) Na82 (sulfate-reducing, hot spring) | AB077817 | 0.788 | 0.452 |
| SP12A06 | 662 | | | | 3 | uncultured bacterium clone oc57 (microbial fuel cell) | AY491598 | 99 | 1175 |
| | | | | | | *Desulfobacterium corrodens* (marine enrichment culture) | AY274450 | 78 | 522 |
| | | | | | | *Desulfonatronum cooperativum* (T) Z-7999 (anoxic soda lake) | AY725424 | 0.773 | 0.370 |
| G41 | 406 | | | 1 | | uncultured bacterium ADK-WSe02-91 (acid-impacted lake) | EF520621 | 98 | 697 |
| | | | | | | *Pelodictyon luteolum* E1P1 | AM050131 | 87 | 390 |
| | | | | | | *Pelobacter acidigallici* (T) MaGal12T (DSM 2377T) | X77216 | 0.757 | 0.404 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SP11E03 | 664 | | | | 1 | uncultured bacterium Pia-s-69<br>(freshwater sediment) | EF632929 | 98 | 1137 |
| | | | | | | *Desulforegula conservatrix* (T)<br>(freshwater lake sediments) | AF243334 | 0.780 | 0.364 |
| SP01C07 | 666 | | | | 1 | uncultured green sulfur bacterium GR-296.II.73<br>(uranium mining waste piles) | AJ301570 | 89 | 807 |
| | | | | | | *Clostridium* sp. FCB45<br>(anoxic bulk soil) | AJ229248 | 78 | 517 |
| | | | | | | *Thermoanaerobacterium aotearoense* (T) JW/SL-NZ613T<br>(hot spring, New Zealand) | X93359 | 0.782 | 0.359 |
| SP11H08 | 714 | | | | 1 | uncultured bacterium 656054<br>(contaminated sediment) | DQ404610 | 97 | 1216 |
| | | | | | | candidate division OP11; LGd12<br>(Lake sediment) | AF047565 | 95 | 1151 |
| | | | | | | *Marinobacter lutaoensis* (T) T5054; CCRC 17087; JCM 11179T<br>(coastal hot spring) | AF288157 | 0.691 | 0.253 |
| GP01E12 | 669 | | | 2 | | uncultured bacterium XJ109<br>(aerobic activated sludge) | EF648155 | 92 | 962 |
| | | | | | | *Spirochaetes* bacterium SA-10 | AY695841 | 89 | 892 |
| | | | | | | *Spirochaeta stenostrepta* (T) | M88724 | 0.876 | 0.573 |
| SP11F05 | 634 | | | | 1 | uncultured bacterium clone LWS-T4855<br>(Lake sediment) | EU546329 | 92 | 919 |
| | | | | | | planctomycete A-2<br>(wastewater treatment plant) | AM056027 | 91 | 883 |
| | | | | | | *Isosphaera pallida* (T) DSM 9630T | AJ231195 | 0.724 | 0.336 |
| SP01A02 | 636 | | | | 2 | uncultured bacterium Hg92H6<br>(from sponge in the Pacific ocean) | EU236426 | 89 | 838 |
| | | | | | | *Planctomycete* GMD14H10<br>(Sargasso Sea bacterioplankton) | AY162122 | 79 | 524 |
| | | | | | | *Aquabacterium parvum* (T) B6<br>(Berlin drinking water system) | AF035052 | 0.717 | 0.330 |
| SP11F07 | 730 | | | | 1 | uncultured planctomycete DEL13<br>(river biofilm) | AJ616264 | 98 | 1000 |
| | | | | | | *Pirellula staleyi* ATCC 35122 | AF399914 | 88 | 720 |
| | | | | | | *Pirellula staleyi* (T) | M34126 | 0.827 | 0.485 |

[1] Ribotype ID — Identical sequences over their overlapping length (i.e. more than 99% sequence similarity) were grouped under the same ribotype ID. The ribotype ID corresponds to the ID of the sequence that was used for phylogenetic analysis (normally the longest sequence).

[2] Sequence length — The length (bp) of the sequence that was used for phylogenetic analysis.

[3] is G — The number of groundwater isolates that belong to each ribotype.
[4] is S — The number of sand isolates that belong to each ribotype.
[5] cl G — The number of groundwater clones that belong to each ribotype.
[6] cl S — The number of sand clones that belong to each ribotype.

[7] Closest relative — For each ribotype, the closest type strain relative was identified using RDP II (sequences of good quality and >1200 bp long); type strains are indicated by an uppercase T enclosed in parenthesis next to the species name. The closest GenBank matches were also included when there was a cultured organism with highest ID sequence similarity. In case an environmental clone had even higher sequence similarity, this was also included and it is clearly stated as 'uncultured'. Information on the environments that these organisms have been detected/cultured from was based on their GenBank record.

[8] Accession number — The sequence accession numbers of the closest relatives

[9] Max ID — The max ID of the RDP matches refers to "similarity score", i.e. the percent sequence identity over all pairwise comparable positions when run with aligned sequences and they are shown in the table below with 3 decimal points (for example 0.986, 0.981 etc). The max ID of the GenBank matches are shown as % (for example 99, 98, etc.).

[10] Max score — The Max score of the RDP searches refers to "seqmatch or S_ab score", i.e. the number of (unique) 7-base oligomers shared between the sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences; this score is shown with 3 decimal points (e.g. 0.954, 1.000 etc.). The max score of the NCBI Blastn search is calculated from the sum of the match rewards and the mismatch, gap open and extend penalties independently for each segment. For this search the following settings were used: blastn algorithm, nr database, expect threshold = 10, word size = 11, match/mismatch scores = 2/-3, gap costs = existence 5/extension 2.

# References

**Aamand, J., Jørgensen, C., Arvin, E. & Jensen, B.K.** (1989) Microbial adaptation to degradation of hydrocarbons in polluted and unpolluted groundwater. *Journal of Contaminant Hydrology* 4(4), 299-312.

**Abd-El-Haleem, D., Moawad, H., Zaki, E.A. & Zaki, S.** (2002) Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. *Microbial Ecology* **43**(2), 217-224.

**Abraham, W.R., Strömpl, C., Meyer, H., Lindholst, S., Moore, E.R.B., Christ, R., Vancanneyt, M., Tindall, B.J., Bennasar, A., Smit, J. & Tesar, M.** (1999) Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov with *Maricaulis maris* (Poindexter) comb. nov as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. *International Journal of Systematic Bacteriology* **49**, 1053-1073.

**Abulencia, C.B., Wyborski, D.L., Garcia, J.A., Podar, M., Chen, W.Q., Chang, S.H., Chang, H.W., Watson, D., Brodie, E.L., Hazen, T.C. & Keller, M.** (2006) Environmental whole-genome amplification to access microbial populations in contaminated sediments. *Applied and Environmental Microbiology* **72**(5), 3291-3301.

**Achour, A.R., Bauda, P. & Billard, P.** (2007) Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. *Research in Microbiology* **158**(2), 128-137.

**Acinas, S.G., Antón, J. & Rodríguez-Valera, F.** (1999) Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **65**(2), 514-522.

**Adav, S.S., Lee, D.J. & Lai, J.Y.** (2008) Intergeneric coaggregation of strains isolated from phenol-degrading aerobic granules. *Applied Microbiology and Biotechnology* **79**(4), 657-661.

**Alexander, M.** (1999) *Biodegradation and Bioremediation.* Academic Press. San Diego, California, USA.

**Alfreider, A., Krössbacher, M. & Psenner, R.** (1997) Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Research* **31**(4), 832-840.

**Alfreider, A. & Vogt, C.** (2007) Bacterial diversity and aerobic biodegradation potential in a BTEX-contaminated aquifer. *Water Air and Soil Pollution* **183**(1-4), 415-426.

**Alfreider, A., Vogt, C. & Babel, W.** (2002) Microbial diversity in an *in situ* reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis. *Systematic and Applied Microbiology* **25**(2), 232-240.

**Amann, R.I., Ludwig, W. & Schleifer, K.H.** (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(1), 143-169.

**Ampe, F., Léonard, D. & Lindley, N.D.** (1998) Repression of phenol catabolism by organic acids in *Ralstonia eutropha*. *Applied and Environmental Microbiology* **64**(1), 1-6.

**Andersen, S.M., Johnsen, K., Sørensen, J., Nielsen, P. & Jacobsen, C.S.** (2000) *Pseudomonas frederiksbergensis* sp nov., isolated from soil at a coal gasification site. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1957-1964.

**Andersson, A.F., Lindberg, M., Jakobsson, H., Bäckhed, F., Nyrén, P. & Engstrand, L.** (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**(7), e2836.

**Anneser, B., Einsiedl, F., Meckenstock, R.U., Richters, L., Wisotzky, F. & Griebler, C.** (2008) High-resolution monitoring of biogeochemical gradients in a tar oil-contaminated aquifer. *Applied Geochemistry* **23**(6), 1715-1730.

**Antizar-Ladislao, B. & Galil, N.I.** (2003) Kinetics of biodegradation during remediation of consecutive accidental spills of chlorophenols in a sandy aquifer. *Water Science and Technology* **47**(9), 157-164.

**Antizar-Ladislao, B., Katz, S. & Galil, N.I.** (2000) Phenol remediation by biofilm developed in sand soil media. *Water Science and Technology* **42**(1-2), 99-104.

**Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H. & Oyaizu, H.** (2000) Phylogenetic affiliation of the Pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1563-1589.

**Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J. & Weightman, A.J.** (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Applied and Environmental Microbiology* **71**(12), 7724-7736.

**Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J. & Weightman, A.J.** (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology* **72**(9), 5734-5741.

**Atagana, H.I.** (2004) Biodegradation of phenol, *o*-cresol, *m*-cresol and *p*-cresol by indigenous soil fungi in soil contaminated with creosote. *World Journal of Microbiology & Biotechnology* **20**(8), 851-858.

**Baek, S.H., Kim, K.H., Yin, C.R., Jeon, C.O., Im, W.T., Kim, K.K. & Lee, S.T.** (2003) Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions. *Current Microbiology* **47**(6), 462-466.

**Bak, F. & Widdel, F.** (1986) Anaerobic degradation of phenol and phenol derivatives by *Desulfobacterium phenolicum* sp. nov. *Archives of Microbiology* **146**(2), 177-180.

**Bakermans, C. & Madsen, E.L.** (2002) Diversity of 16S rDNA and naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters. *Microbial Ecology* **44**(2), 95-106.

**Bastos, A.E.R., Moon, D.H., Rossi, A., Trevors, J.T. & Tsai, S.M.** (2000) Salt-tolerant phenol-degrading microorganisms isolated from Amazonian soil samples. *Archives of Microbiology* **174**(5), 346-352.

**Bauer, R.D., Maloszewski, P., Zhang, Y.C., Meckenstock, R.U. & Griebler, C.** (2008) Mixing-controlled biodegradation in a toluene plume - Results from two-dimensional laboratory experiments. *Journal of Contaminant Hydrology* **96**(1-4), 150-168.

**Bazylinski, D.A. & Frankel, R.B.** (2004) Magnetosome formation in prokaryotes. *Nature Reviews Microbiology* **2**(3), 217-230.

**Bedard, D.L., Bailey, J.J., Reiss, B.L. & Jerzak, G.V.** (2006) Development and characterization of stable sediment-free anaerobic bacterial enrichment cultures

that dechlorinate Aroclor 1260. *Applied and Environmental Microbiology* **72**(4), 2460-2470.

**Bekins, B.A., Cozzarelli, I.M., Godsy, E.M., Warren, E., Essaid, H.I. & Tuccillo, M.E.** (2001) Progression of natural attenuation processes at a crude oil spill site: II. Controls on spatial distribution of microbial populations. *Journal of Contaminant Hydrology* **53**(3-4), 387-406.

**Bekins, B.A., Godsy, E.M. & Warren, E.** (1999) Distribution of microbial physiologic types in an aquifer contaminated by crude oil. *Microbial Ecology* **37**(4), 263-275.

**Bergauer, P., Fonteyne, P.A., Nolard, N., Schinner, F. & Margesin, R.** (2005) Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts. *Chemosphere* **59**(7), 909-918.

**Besemer, K., Moeseneder, M.M., Arrieta, J.M., Herndl, G.J. & Peduzzi, P.** (2005) Complexity of bacterial communities in a river-floodplain system (Danube, Austria). *Applied and Environmental Microbiology* **71**(2), 609-620.

**Bharathkumar, S., RameshKumar, N., Paul, D., Prabavathy, V.R. & Nair, S.** (2008) Characterization of the predominant bacterial population of different mangrove rhizosphere soils using 16S rRNA gene-based single-strand conformation polymorphism (SSCP). *World Journal of Microbiology & Biotechnology* **24**(3), 387-394.

**Blaire, S., Briggs, G., McKay, C.P., Pollard, W.H., Greer, C.W. & Whyte, L.G.** (2007) Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. *Fems Microbiology Ecology* **59**(2), 513-523.

**Boll, M., Fuchs, G. & Heider, J.** (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. *Current Opinion in Chemical Biology* **6**(5), 604-611.

**Bollmann, A., Lewis, K. & Epstein, S.S.** (2007) Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Applied and Environmental Microbiology* **73**(20), 6386-6390.

**Bos, R., van der Mei, H.C. & Busscher, H.J.** (1999) Physico-chemistry of initial microbial adhesive interactions - its mechanisms and methods for study. *Fems Microbiology Reviews* **23**(2), 179-230.

**Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., Moletta, R. & Wagner, M.** (2000) Ecological study of a bioaugmentation failure. *Environmental Microbiology* **2**(2), 179-190.

**Brambilla, E., Hippe, H., Hagelstein, A., Tindall, B.J. & Stackebrandt, E.** (2001) 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. *Extremophiles* **5**(1), 23-33.

**Breinig, S., Schiltz, E. & Fuchs, G.** (2000) Genes involved in anaerobic metabolism of phenol in the bacterium *Thauera aromatica*. *Journal of Bacteriology* **182**(20), 5849-5863.

**Broholm, M.M. & Arvin, E.** (2000) Biodegradation of phenols in a sandstone aquifer under aerobic conditions and mixed nitrate and iron reducing conditions. *Journal of Contaminant Hydrology* **44**(3-4), 239-273.

**Brown, G.R., Sutcliffe, I.C., Bendell, D. & Cummings, S.P.** (2000) The modification of the membrane of *Oceanomonas baumannii*$^{T}$ when subjected to both osmotic and organic solvent stress. *Fems Microbiology Letters* **189**(2), 149-154.

**Bürgmann, H., Pesaro, M., Widmer, F. & Zeyer, J.** (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *Journal of Microbiological Methods* **45**(1), 7-20.

**Burkert, U., Warnecke, F., Babenzien, D., Zwirnmann, E. & Pernthaler, J.** (2003) Members of a readily enriched beta-proteobacterial clade are common in surface waters of a humic lake. *Applied and Environmental Microbiology* **69**(11), 6550-6559.

**Busse, H.J., Denner, E.B.M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A. & Kampfer, P.** (2003) *Sphingomonas aurantiaca* sp. nov., *Sphingomonas aerolata* sp. nov. and *Sphingomonas faeni* sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas*. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1253-1260.

**Buswell, C.M., Herlihy, Y.M., Marsh, P.D., Keevil, C.W. & Leach, S.A.** (1997) Coaggregation amongst aquatic biofilm bacteria. *Journal of Applied Microbiology* **83**(4), 477-484.

**Caldeira, M., Heald, S.C., Carvalho, M.F., Vasconcelos, I., Bull, A.T. & Castro, P.M.L.** (1999) 4-Chlorophenol degradation by a bacterial consortium: development of a granular activated carbon biofilm reactor. *Applied Microbiology and Biotechnology* **52**(5), 722-729.

**Callegan, R.P., Nobre, M.F., McTernan, P.M., Battista, J.R., Navarro-González, R., McKay, C.P., da Costa, M.S. & Rainey, F.A.** (2008) Description of four novel psychrophilic, ionizing radiation-sensitive *Deinococcus* species from alpine environments. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1252-1258.

**Cariello, N.F., Thilly, W.G., Swenberg, J.A. & Skopek, T.R.** (1991) Deletion mutagenesis during polymerase chain reaction: dependence on DNA polymerase. *Gene* **99**(1), 105-108.

**Carvalho, M.F., Vasconcelos, I., Bull, A.T. & Castro, P.M.L.** (2001) A GAC biofilm reactor for the continuous degradation of 4-chlorophenol: treatment efficiency and microbial analysis. *Applied Microbiology and Biotechnology* **57**(3), 419-426.

**Cases, I. & de Lorenzo, V.** (2001) The black cat/white cat principle of signal integration in bacterial promoters. *Embo Journal* **20**(1-2), 1-11.

**Cases, I., de Lorenzo, V. & Pérez-Martín, J.** (1996) Involvement of sigma$^{54}$ in exponential silencing of the *Pseudomonas putida* TOL plasmid *Pu* promoter. *Molecular Microbiology* **19**(1), 7-17.

**Chapelle, F.H.** (2001) *Groundwater microbiology and geochemistry.* John Wiley & Sons. New York, USA.

**Chen, C.L., Wu, J.H. & Liu, W.T.** (2008) Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. *Water Research* **42**(8-9), 1963-1976.

**Chen, G.X. & Walker, S.L.** (2007) Role of solution chemistry and ion valence on the adhesion kinetics of groundwater and marine bacteria. *Langmuir* **23**(13), 7162-7169.

**Chen, S.Y. & Dong, X.Z.** (2005) *Proteiniphilum acetatigenes* gen. nov., sp nov., from a UASB reactor treating brewery wastewater. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2257-2261.

**Chen, W., Brühlmann, F., Richins, R.D. & Mulchandani, A.** (1999) Engineering of improved microbes and enzymes for bioremediation. *Current Opinion in Biotechnology* **10**(2), 137-141.

**Cho, H.B., Lee, J.K. & Choi, Y.K.** (2003) The genetic diversity analysis of the bacterial community in groundwater by denaturing gradient gel electrophoresis (DGGE). *Journal of Microbiology* **41**(4), 327-334.

**Christensen, B.B., Haagensen, J.A.J., Heydorn, A. & Molin, S.** (2002) Metabolic commensalism and competition in a two-species microbial consortium. *Applied and Environmental Microbiology* **68**(5), 2495-2502.

**Christensen, T.H., Bjerg, P.L., Banwart, S.A., Jakobsen, R., Heron, G. & Albrechtsen, H.J.** (2000) Characterization of redox conditions in groundwater contaminant plumes. *Journal of Contaminant Hydrology* **45**(3-4), 165-241.

**Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M. & Tiedje, J.M.** (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Research* **35**, D169-D172.

**Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M. & Tiedje, J.M.** (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* **31**(1), 442-443.

**Connon, S.A. & Giovannoni, S.J.** (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Applied and Environmental Microbiology* **68**(8), 3878-3885.

**Cortés-Lorenzo, C., Molina-Muñoz, M.L., Gómez-Villalba, B., Vilchez, R., Ramos, A., Rodelas, B., Hontoria, E. & González-López, J.** (2006) Analysis of community composition of biofilms in a submerged filter system for the removal of ammonia and phenol from industrial wastewater. *Biochemical Society Transactions* **34**, 165-168.

**Costerton, J.W., Stewart, P.S. & Greenberg, E.P.** (1999) Bacterial biofilms: A common cause of persistent infections. *Science* **284**(5418), 1318-1322.

**Costerton, J.W. & Wilson, M.** (2004) Introducing Biofilms. *Biofilms* **1**, 1-4.

**Cozzarelli, I.M., Bekins, B.A., Baedecker, M.J., Aiken, G.R., Eganhouse, R.P. & Tuccillo, M.E.** (2001) Progression of natural attenuation processes at a crude-oil spill site: 1. Geochemical evolution of the plume. *Journal of Contaminant Hydrology* **53**(3-4), 369-385.

**Cozzarelli, I.M., Herman, J.S., Baedecker, M.J. & Fischer, J.M.** (1999) Geochemical heterogeneity of a gasoline-contaminated aquifer. *Journal of Contaminant Hydrology* **40**(3), 261-284.

**Crosby, L.D. & Criddle, C.S.** (2003) Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. *Biotechniques* **34**(4), 790-802.

**Crump, B.C., Armbrust, E.V. & Baross, J.A.** (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. *Applied and Environmental Microbiology* **65**(7), 3192-3204.

**Cummings, D.E., Snoeyenbos-West, O.L., Newby, D.T., Niggemyer, A.M., Lovley, D.R., Achenbach, L.A. & Rosenzweig, R.F.** (2003) Diversity of *Geobacteraceae* species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microbial Ecology* **46**(2), 257-269.

**Cunningham, J.A., Rahme, H., Hopkins, G.D., Lebron, C. & Reinhard, M.** (2001) Enhanced in situ bioremediation of BTEX-contaminated groundwater by combined injection of nitrate and sulfate. *Environmental Science & Technology* **35**(8), 1663-1670.

**Darzynkiewicz, Z., Evenson, D., Staiano-Coico, L., Sharpless, T. & Melamed, M.R.** (1979) Relationship between RNA content and progression of lymphocytes through S phase of cell cycle. *Proceedings of the National Academy of Sciences of the United States of America* **76**(1), 358-362.

**Davey, M.E. & O'Toole, G.A.** (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **64**(4), 847-867.

**Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. & Greenberg, E.P.** (1998) The involvement of cell-to-cell signals in the

development of a bacterial biofilm. *Science* **280**(5361), 295-298.

**Davis, J.W., Odom, J.M., DeWeerd, K.A., Stahl, D.A., Fishbain, S.S., West, R.J., Klecka, G.M. & DeCarolis, J.G.** (2002) Natural attenuation of chlorinated solvents at Area 6, Dover Air Force Base: characterization of microbial community structure. *Journal of Contaminant Hydrology* **57**(1-2), 41-59.

**Delong, E.F., Franks, D.G. & Alldredge, A.L.** (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnology and Oceanography* **38**(5), 924-934.

**DeRito, C.M., Pumphrey, G.M. & Madsen, E.L.** (2005) Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. *Applied and Environmental Microbiology* **71**(12), 7858-7865.

**Díaz, E.** (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *International Microbiology* **7**(3), 173-180.

**Dinsdale, E.A., Pantos, O., Smriga, S., Edwards, R.A., Angly, F., Wegley, L., Hatay, M., Hall, D., Brown, E., Haynes, M., Krause, L., Sala, E., Sandin, S.A., Thurber, R.V., Willis, B.L., Azam, F., Knowlton, N. & Rohwer, F.** (2008) Microbial ecology of four coral atolls in the Northern Line Islands. *PLoS ONE* **3**(2), e1584.

**Dowd, S.E., Sun, Y., Secor, P.R., Rhoads, D.D., Wolcott, B.M., James, G.A. & Wolcott, R.D.** (2008) Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *Bmc Microbiology* **8**. Article no.: 43.

**Dryden, S.C. & Kaplan, S.** (1990) Localization and structural analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides*. *Nucleic Acids Research* **18**(24), 7267-7277.

**Eganhouse, R.P., Cozzarelli, I.M., Scholl, M.A. & Matthews, L.L.** (2001) Natural attenuation of volatile organic compounds (VOCs) in the leachate plume of a municipal landfill: Using alkylbenzenes as process probes. *Ground Water* **39**(2), 192-202.

**Eguchi, M., Kitagawa, M., Suzuki, Y., Nakamuara, M., Kawai, T., Okamura, K.,**

**Sasaki, S. & Miyake, Y.** (2001) A field evaluation of *in situ* biodegradation of trichloroethylene through methane injection. *Water Research* **35**(9), 2145-2152.

**Ehrt, S., Schirmer, F. & Hillen, W.** (1995) Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. *Molecular Microbiology* **18**(1), 13-20.

**Fahrbach, M., Kuever, J., Meinke, R., Kämpfer, P. & Hollender, J.** (2006) *Denitratisoma oestradiolicum* gen. nov., sp nov., a 17β-oestradiol-degrading, denitrifying betaproteobacterium. *International Journal of Systematic and Evolutionary Microbiology* **56**, 1547-1552.

**Fahy, A., Ball, A.S., Lethbridge, G., Timmis, K.N. & McGenity, T.J.** (2008) Isolation of alkali-tolerant benzene-degrading bacteria from a contaminated aquifer. *Letters in Applied Microbiology* **47**(1), 60-66.

**Fahy, A., Lethbridge, G., Earle, R., Ball, A.S., Timmis, K.N. & McGenity, T.J.** (2005) Effects of long-term benzene pollution on bacterial diversity and community structure in groundwater. *Environmental Microbiology* **7**(8), 1192-1199.

**Farhadian, M., Vachelard, C., Duchez, D. & Larroche, C.** (2008) In situ bioremediation of monoaromatic pollutants in groundwater: A review. *Bioresource Technology* **99**(13), 5296-5308.

**Farrelly, V., Rainey, F.A. & Stackebrandt, E.** (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S ribosomal RNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* **61**(7), 2798-2801.

**Felsenstein, J.** (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**(4), 783-791.

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

**Felske, A., Wolterink, A., van Lis, R., de Vos, W.M. & Akkermans, A.D.L.** (1999) Searching for predominant soil bacteria: 16S rDNA cloning versus strain

cultivation. *Fems Microbiology Ecology* **30**(2), 137-145.

**Ferguson, A.S., Huang, W.E., Lawson, K.A., Doherty, R., Gibert, O., Dickson, K.W., Whiteley, A.S., Kulakov, L.A., Thompson, I.P., Kalin, R.M. & Larkin, M.J.** (2007) Microbial analysis of soil and groundwater from a gasworks site and comparison with a sequenced biological reactive barrier remediation process. *Journal of Applied Microbiology* **102**(5), 1227-1238.

**Feris, K.P., Hristova, K., Gebreyesus, B., Mackay, D. & Scow, K.M.** (2004) A shallow BTEX and MTBE contaminated aquifer supports a diverse microbial community. *Microbial Ecology* **48**(4), 589-600.

**Fetter, C.W.** (1993) *Contaminant hydrogeology.* Prentice-Hall, New Jersey, USA.

**Finneran, K.T., Johnsen, C.V. & Lovley, D.R.** (2003) *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *International Journal of Systematic and Evolutionary Microbiology* **53**, 669-673.

**Franzmann, P.D., Robertson, W.J., Zappia, L.R. & Davis, G.B.** (2002) The role of microbial populations in the containment of aromatic hydrocarbons in the subsurface. *Biodegradation* **13**(1), 65-78.

**Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M. & Rossi, P.** (2002) Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environmental Microbiology* **4**(11), 634-643.

**Fry, J.C.** (1990) Direct methods and biomass estimation. *Methods in Microbiology* **22**, 41-85.

**Fry, J.C., Webster, G., Cragg, B.A., Weightman, A.J. & Parkes, R.J.** (2006) Analysis of DGGE profiles to explore the relationship between prokaryotic community composition and biogeochemical processes in deep subseafloor sediments from the Peru Margin. *Fems Microbiology Ecology* **58**(1), 86-98.

**Fujii, K., Urano, N., Ushio, H., Satomi, M. & Kimura, S.** (2001) *Sphingomonas cloacae* sp. nov., a nonylphenol-degrading bacterium isolated from wastewater of a sewage-treatment plant in Tokyo. *International Journal of Systematic and Evolutionary Microbiology* **51**, 603-610.

**Gafan, G.P. & Spratt, D.A.** (2005) Denaturing gradient gel electrophoresis gel expansion (DGGEGE) - An attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *Fems Microbiology Letters* **253**(2), 303-307.

**Garcia-Junco, M., Gomez-Lahoz, C., Niqui-Arroyo, J.L. & Ortega-Calvo, J.J.** (2003) Biosurfactant- and biodegradation-enhanced partitioning of polycyclic aromatic hydrocarbons from nonaqueous-phase liquids. *Environmental Science & Technology* **37**(13), 2988-2996.

**Garrity, G.M., Bell, J.A. & Lilburn, T.G.** (2004) *Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology, Second Edition. Release 5.0.* (available at: http://141.150.157.80/bergeysoutline/main.htm).

**Geng, A.L., Soh, A.E.W., Lim, C.J. & Loke, L.C.T.** (2006) Isolation and characterization of a phenol-degrading bacterium from an industrial activated sludge. *Applied Microbiology and Biotechnology* **71**(5), 728-735.

**Ghiglione, J.F., Mevel, G., Pujo-Pay, M., Mousseau, L., Lebaron, P. & Goutx, M.** (2007) Diel and seasonal variations in abundance, activity, and community structure of particle-attached and free-living bacteria in NW Mediterranean Sea. *Microbial Ecology* **54**(2), 217-231.

**Ghigo, J.M.** (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* **412**(6845), 442-445.

**Gilbert, J.A., Field, D., Huang, Y., Edwards, R., Li, W., Gilna, P. & Joint, I.** (2008) Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities. *PLoS ONE* **3**(8), e3042.

**Godoy, F., Vancanneyt, M., Martinez, M., Steinbüchel, A., Swings, J. & Rehm, B.H.A.** (2003) *Sphingopyxis chilensis* sp. nov., a chlorophenol-degrading bacterium that accumulates polyhydroxyalkanoate, and transfer of *Sphingomonas alaskensis* to *Sphingopyxis alaskensis* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* **53**, 473-477.

**Gomez-Alvarez, V., King, G.M. & Nüsslein, K.** (2007) Comparative bacterial diversity in recent Hawaiian volcanic deposits of different ages. *Fems Microbiology Ecology* **60**(1), 60-73.

**González-Toril, E., Llobet-Brossa, E., Casamayor, E.O., Amann, R. & Amils, R.** (2003) Microbial ecology of an extreme acidic environment, the Tinto river. *Applied and Environmental Microbiology* **69**(8), 4853-4865.

**Gordon, R.Y., Bocharova, L.S., Kruman, II, Popov, V.I., Kazantsev, A.P., Khutzian, S.S. & Karnaukhov, V.N.** (1997) Acridine orange as an indicator of the cytoplasmic ribosome state. *Cytometry* **29**(3), 215-221.

**Görke, B. & Stülke, J.** (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**(8), 613-624.

**Goswami, M., Shivaraman, N. & Singh, R.P.** (2005) Microbial metabolism of 2-chlorophenol, phenol and *p*-cresol by *Rhodococcus erythropolis* M1 in coculture with *Pseudomonas fluorescens* P1. *Microbiological Research* **160**(2), 101-109.

**Griebler, C., Mindl, B., Slezak, D. & Geiger-Kaiser, M.** (2002) Distribution patterns of attached and suspended bacteria in pristine and contaminated shallow aquifers studied with an in situ sediment exposure microcosm. *Aquatic Microbial Ecology* **28**(2), 117-129.

**Guieysse, B., Wikström, P., Forsman, M. & Mattiasson, B.** (2001) Biomonitoring of continuous microbial community adaptation towards more efficient phenol-degradation in a fed-batch bioreactor. *Applied Microbiology and Biotechnology* **56**(5-6), 780-787.

**Haack, S.K., Fogarty, L.R., West, T.G., Alm, E.W., McGuire, J.T., Long, D.T., Hyndman, D.W. & Forney, L.J.** (2004) Spatial and temporal changes in microbial community structure associated with recharge-influenced chemical gradients in a contaminated aquifer. *Environmental Microbiology* **6**(5), 438-448.

**Hallbeck, L., Stahl, F. & Pedersen, K.** (1993) Phylogeny and phenotypic characterization of the ctalk-forming and iron-oxidizing bacterium *Gallionella ferruginea*. *Journal of General Microbiology* **139**, 1531-1535.

**Hall-Stoodley, L., Costerton, J.W. & Stoodley, P.** (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* **2**(2), 95-108.

**Hall-Stoodley, L. & Stoodley, P.** (2002) Developmental regulation of microbial biofilms. *Current Opinion in Biotechnology* **13**(3), 228-233.

**Hamzah, R.Y. & Al-Baharna, B.S.** (1994) Catechol ring-cleavage in *Pseudomonas cepacia*: the simultaneous induction of *ortho* and *meta* pathways. *Applied Microbiology and Biotechnology* **41**(2), 250-256.

**Handelsman, J.** (2004) Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews* **68**(4), 669-685.

**Harrison, I., Williams, G.M., Higgo, J.J.W., Leader, R.U., Kim, A.W. & Noy, D.J.** (2001) Microcosm studies of microbial degradation in a coal tar distillate plume. *Journal of Contaminant Hydrology* **53**(3-4), 319-340.

**Hausner, M. & Wuertz, S.** (1999) High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology* **65**(8), 3710-3713.

**Hazen, T.C., Jiménez, L., de Victoria, G.L. & Fliermans, C.B.** (1991) Comparison of bacteria from deep subsurface sediment and adjacent groundwater. *Microbial Ecology* **22**(3), 293-304.

**Heinaru, E., Truu, J., Stottmeister, U. & Heinaru, A.** (2000) Three types of phenol and *p*-cresol catabolism in phenol- and *p*-cresol-degrading bacteria isolated from river water continuously polluted with phenolic compounds. *Fems Microbiology Ecology* **31**(3), 195-205.

**Heipieper, H.J., Diefenbach, R. & Keweloh, H.** (1992) Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Applied and Environmental Microbiology* **58**(6), 1847-1852.

**Heipieper, H.J., Keweloh, H. & Rehm, H.J.** (1991) Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. *Applied and Environmental Microbiology* **57**(4), 1213-1217.

**Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H. & de Bont, J.A.M.** (1994) Mechanisms of resistance of whole cells to toxic organic solvents. *Trends in Biotechnology* **12**(10), 409-415.

**Helton, R.R., Liu, L. & Wommack, K.E.** (2006) Assessment of factors influencing direct enumeration of viruses within estuarine sediments. *Applied and Environmental Microbiology* **72**(7), 4767-4774.

**Hendrickx, B., Dejonghe, W., Boënne, W., Brennerova, M., Cernik, M., Lederer, T., Bucheli-Witschel, M., Bastiaens, L., Verstraete, W., Top, E.M., Diels, L. & Springael, D.** (2005) Dynamics of an oligotrophic bacterial aquifer community during contact with a groundwater plume contaminated with benzene toluene, ethylbenzene, and xylenes: an *in situ* mesocosm study. *Applied and Environmental Microbiology* **71**(7), 3815-3825.

**Hendrickx, B., Dejonghe, W., Faber, F., Boënne, W., Bastiaens, L., Verstraete, W., Top, E.M. & Springael, D.** (2006a) PCR-DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites. *Fems Microbiology Ecology* **55**(2), 262-273.

**Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Rüegg, I., Bucheli-Witschel, M., Faber, F., Egli, T., Mau, M., Schlomann, M., Brennerova, M., Brenner, V., Pieper, D.H., Top, E.M., Dejonghe, W., Bastiaens, L. & Springael, D.** (2006b) Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: Distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. *Journal of Microbiological Methods* **64**(2), 250-265.

**Hentzer, M., Teitzel, G.M., Balzer, G.J., Heydorn, A., Molin, S., Givskov, M. & Parsek, M.R.** (2001) Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *Journal of Bacteriology* **183**(18), 5395-5401.

**Herrmann, H., Müller, C., Schmidt, I., Mahnke, J., Petruschka, L. & Hahnke, K.** (1995) Localization and organization of phenol degradation genes of *Pseudomonas putida* strain H. *Molecular & General Genetics* **247**(2), 240-246.

**Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K. & Molin, S.** (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology-Uk* **146**, 2395-2407.

**Hinteregger, C., Leitner, R., Loidl, M., Ferschl, A. & Streichsbier, F.** (1992) Degradation of phenol and phenolic compounds by *Pseudomonas putida* EKII. *Applied Microbiology and Biotechnology* **37**(2), 252-259.

**Hiraishi, A.** (1997) Transfer of the bacteriochlorophyll b-containing phototrophic bacteria *Rhodopseudomonas viridis* and *Rhodopseudomonas sulfoviridis* to the

genus *Blastochloris* gen. nov. *International Journal of Systematic Bacteriology* **47**(1), 217-219.

**Hirsch, P. & Rades-Rohkohl, E.** (1990) Microbial colonization of aquifer sediment exposed in a groundwater well in Northern Germany. *Applied and Environmental Microbiology* **56**(10), 2963-2966.

**Hogan, D. & Kolter, R.** (2002) Why are bacteria refractory to antimicrobials? *Current Opinion in Microbiology* **5**(5), 472-477.

**Hohnstock-Ashe, A.M., Plummer, S.M., Yager, R.M., Baveye, P. & Madsen, E.L.** (2001) Further biogeochemical characterization of a trichloroethene-contaminated fractured dolomite aquifer: Electron source and microbial communities involved in reductive dechlorination. *Environmental Science & Technology* **35**(22), 4449-4456.

**Holm, P.E., Nielsen, P.H., Albrechtsen, H.J. & Christensen, T.H.** (1992) Importance of unattached bacteria and bacteria attached to sediment in determining potentials for degradation of xenobiotic organic contaminants in an aerobic aquifer. *Applied and Environmental Microbiology* **58**(9), 3020-3026.

**Holmes, D.E., Finneran, K.T., O'Neil, R.A. & Lovley, D.R.** (2002) Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Applied and Environmental Microbiology* **68**(5), 2300-2306.

**Hopkins, G.D. & McCarty, P.L.** (1995) Field evaluation of *in-situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. *Environmental Science & Technology* **29**(6), 1628-1637.

**Huber, T., Faulkner, G. & Hugenholtz, P.** (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**(14), 2317-2319.

**Hugenholtz, P.** (2002) Exploring prokaryotic diversity in the genomic era. *Genome Biology* **3**(2), REVIEWS0003.

**Hugenholtz, P., Goebel, B.M. & Pace, N.R.** (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of*

*Bacteriology* **180**(18), 4765-4774.

**Humphry, D.R., Black, G.W. & Cummings, S.P.** (2003) Reclassification of '*Pseudomonas fluorescens* subsp. *cellulosa*' NCIMB 10462 (Ueda *et al.* 1952) as *Cellvibrio japonicus* sp. nov. and revival of *Cellvibrio vulgaris* sp. nov., nom. rev. and *Cellvibrio fulvus* sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology* **53**, 393-400.

**Hunkeler, D., Höhener, P. & Zeyer, J.** (2002) Engineered and subsequent intrinsic *in situ* bioremediation of a diesel fuel contaminated aquifer. *Journal of Contaminant Hydrology* **59**(3-4), 231-245.

**Huson, D.H., Auch, A.F., Qi, J. & Schuster, S.C.** (2007) MEGAN analysis of metagenomic data. *Genome Research* **17**(3), 377-386.

**Imfeld, G., Nijenhuis, I., Nikolausz, M., Zeiger, S., Paschke, H., Drangmeister, J., Grossmann, J., Richnow, H.H. & Weber, S.** (2008) Assessment of *in situ* degradation of chlorinated ethenes and bacterial community structure in a complex contaminated groundwater system. *Water Research* **42**(4-5), 871-882.

**Iribar, A., Sánchez-Pérez, J.M., Lyautey, E. & Garabétian, F.** (2008) Differentiated free-living and sediment-attached bacterial community structure inside and outside denitrification hotspots in the river-groundwater interface. *Hydrobiologia* **598**, 109-121.

**Isken, S. & de Bont, J.A.M.** (1998) Bacteria tolerant to organic solvents. *Extremophiles* **2**(3), 229-238.

**Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M. & Nasu, M.** (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. *Fems Microbiology Ecology* **32**(2), 129-141.

**Jarabo-Lorenzo, A., Pérez-Galdona, R., Donate-Correa, J., Rivas, R., Velázquez, E., Hernández, M., Temprano, F., Martínez-Molina, E., Ruiz-Argüeso, T. & Léon-Barrios, M.** (2003) Genetic diversity of bradyrhizobial populations from diverse geographic origins that nodulate *Lupinus* spp. and *Ornithopus* spp. *Systematic and Applied Microbiology* **26**(4), 611-623.

**Jefferson, K.K.** (2004) What drives bacteria to produce a biofilm? *Fems Microbiology Letters* **236**(2), 163-173.

**Jiang, Y., Wen, J.P., Li, H.M., Yang, S.L. & Hu, Z.D.** (2005) The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*. *Biochemical Engineering Journal* **24**(3), 243-247.

**Juang, R.S. & Tsai, S.Y.** (2006) Growth kinetics of *Pseudomonas putida* in the biodegradation of single and mixed phenol and sodium salicylate. *Biochemical Engineering Journal* **31**(2), 133-140.

**Juck, D., Charles, T., Whyte, L.G. & Greer, C.W.** (2000) Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. *Fems Microbiology Ecology* **33**(3), 241-249.

**Jukes, T.H. & Cantor, C.R.** (1969) Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21-132. Edited by H.N. Munro. Academic Press, New York, USA.

**Junca, H. & Pieper, D.H.** (2004) Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. *Environmental Microbiology* **6**(2), 95-110.

**Kämpfer, P., Rainey, F.A., Andersson, M.A., Lassila, E.L.N., Ulrych, U., Busse, H.J., Weiss, N., Mikkola, R. & Salkinoja-Salonen, M.** (2000) *Frigoribacterium faeni* gen. nov., sp nov., a novel psychrophilic genus of the family *Microbacteriaceae*. *International Journal of Systematic and Evolutionary Microbiology* **50**, 355-363.

**Kane, S.R., Chakicherla, A.Y., Chain, P.S.G., Schmidt, R., Shin, M.W., Legler, T.C., Scow, K.M., Larimer, F.W., Lucas, S.M., Richardson, P.M. & Hristova, K.R.** (2007) Whole-genome analysis of the methyl *tert*-butyl ether-degrading beta-proteobacterium *Methylibium petroleiphilum* PM1. *Journal of Bacteriology* **189**(5), 1931-1945.

**Kanso, S. & Patel, B.K.C.** (2004) *Phenylobacterium lituiforme* sp. nov., a moderately thermophilic bacterium from a subsurface aquifer, and emended description of the genus *Phenylobacterium*. *International Journal of Systematic and Evolutionary Microbiology* **54**, 2141-2146.

**Kappler, A., Emerson, D., Edwards, K., Amend, J.P., Gralnick, J.A., Grathwohl, P., Hoehler, T. & Straub, K.L.** (2005) Microbial activity in biogeochemical

gradients - new aspects of research. *Geobiology* **3**(3), 229-233.

**Kasak, L., Hôrak, R., Nurk, A., Talvik, K. & Kivisaar, M.** (1993) Regulation of the catechol 1,2-dioxygenase- and phenol monooxygenase-encoding *pheBA* operon in *Pseudomonas putida* Paw85. *Journal of Bacteriology* **175**(24), 8038-8042.

**Kelly, D.P. & Wood, A.P.** (2000) Confirmation of *Thiobacillus denitrificans* as a species of the genus *Thiobacillus*, in the β-subclass of the *Proteobacteria*, with strain NCIMB 9548 as the type strain. *International Journal of Systematic and Evolutionary Microbiology* **50**, 547-550.

**Kepner, R.L. & Pratt, J.R.** (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiological Reviews* **58**(4), 603-615.

**Keweloh, H., Diefenbach, R. & Rehm, H.J.** (1991) Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. *Archives of Microbiology* **157**(1), 49-53.

**Khan, S.T., Horiba, Y., Yamamoto, M. & Hiraishi, A.** (2002) Members of the family *Comamonadaceae* as primary poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. *Applied and Environmental Microbiology* **68**(7), 3206-3214.

**Kisand, V. & Wikner, J.** (2003) Combining culture-dependent and -independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter. *Applied and Environmental Microbiology* **69**(6), 3607-3616.

**Kivisaar, M., Kasak, L. & Nurk, A.** (1991) Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. *Gene* **98**(1), 15-20.

**Kjelleberg, S. & Molin, S.** (2002) Is there a role for quorum sensing signals in bacterial biofilms? *Current Opinion in Microbiology* **5**(3), 254-258.

**Klappenbach, J.A., Saxman, P.R., Cole, J.R. & Schmidt, T.M.** (2001) rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Research* **29**(1), 181-184.

**Koizumi, Y., Kojima, H. & Fukui, M.** (2003) Characterization of depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments. *Fems Microbiology Ecology* **46**(2), 147-157.

**Kukor, J.J. & Olsen, R.H.** (1990) Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c. *Journal of Bacteriology* **172**(8), 4624-4630.

**Kukor, J.J. & Olsen, R.H.** (1991) Genetic organization and regulation of a *meta* cleavage pathway for catechols produced from catabolism of toluene, benzene, phenol, and cresols by *Pseudomonas pickettii* PKO1. *Journal of Bacteriology* **173**(15), 4587-4594.

**Kukor, J.J. & Olsen, R.H.** (1992) Complete nucleotide sequence of *tbuD*, the gene encoding phenol cresol hydroxylase from *Pseudomonas pickettii* PKO1, and functional analysis of the encoded enzyme. *Journal of Bacteriology* **174**(20), 6518-6526.

**Kukor, J.J. & Olsen, R.H.** (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Applied and Environmental Microbiology* **62**(5), 1728-1740.

**Kumar, A., Kumar, S. & Kumar, S.** (2005) Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194. *Biochemical Engineering Journal* **22**(2), 151-159.

**LaMontagne, M.G. & Holden, P.A.** (2003) Comparison of free-living and particle-associated bacterial communities in a coastal lagoon. *Microbial Ecology* **46**(2), 228-237.

**Lane, D.J.** (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in bacterial Systematics*, pp. 115 - 175. Edited by E. Stackebrandt & M. Goodfellow. John Wiley & Sons, Chichester, UK.

**Lee, D.H., Zo, Y.G. & Kim, S.J.** (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Applied and Environmental Microbiology* **62**(9), 3112-3120.

**Lee, J.S., Shin, Y.K., Yoon, J.H., Takeuchi, M., Pyun, Y.R. & Park, Y.H.** (2001) *Sphingomonas aquatilis* sp. nov., *Sphingomonas koreensis* sp. nov. and *Sphingomonas taejonensis* sp. nov., yellow-pigmented bacteria isolated from natural mineral water. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1491-1498.

**Lehman, R.M. & O'Connell, S.P.** (2002) Comparison of extracellular enzyme activities and community composition of attached and free-living bacteria in porous medium columns. *Applied and Environmental Microbiology* **68**(4), 1569-1575.

**Lehman, R.M., O'Connell, S.P., Banta, A., Fredrickson, J.K., Reysenbach, A.L., Kieft, T.L. & Colwell, F.S.** (2004) Microbiological comparison of core and groundwater samples collected from a fractured basalt aquifer with that of dialysis chambers incubated in situ. *Geomicrobiology Journal* **21**(3), 169-182.

**Lehours, A.C., Evans, P., Bardot, C., Joblin, K. & Gérard, F.** (2007) Phylogenetic diversity of Archaea and Bacteria in the anoxic zone of a meromictic lake (Lake Pavin, France). *Applied and Environmental Microbiology* **73**(6), 2016-2019.

**Leitão, A.L., Duarte, M.P. & Oliveira, J.S.** (2007) Degradation of phenol by a halotolerant strain of *Penicillium chrysogenum*. *International Biodeterioration & Biodegradation* **59**(3), 220-225.

**Lejeune, P.** (2003) Contamination of abiotic surfaces: what a colonizing bacterium sees and how to blur it. *Trends in Microbiology* **11**(4), 179-184.

**Lendvay, J.M., Sauck, W.A., McCormick, M.L., Barcelona, M.J., Kampbell, D.H., Wilson, J.T. & Adriaens, P.** (1998) Geophysical characterization, redox zonation, and contaminant distribution at a groundwater/surface water interface. *Water Resources Research* **34**(12), 3545-3559.

**Lerner, D.N., Thornton, S.F., Spence, M.J., Banwart, S.A., Bottrell, S.H., Higgo, J.J., Mallinson, H.E.H., Pickup, R.W. & Williams, G.M.** (2000) Ineffective natural attenuation of degradable organic compounds in a phenol-contaminated aquifer. *Ground Water* **38**(6), 922-928.

**Leys, N., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E.M. & Springael, D.** (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. *Applied and Environmental*

*Microbiology* **70**(4), 1944-1955.

**Li, B.K. & Logan, B.E.** (2004) Bacterial adhesion to glass and metal-oxide surfaces. *Colloids and Surfaces B-Biointerfaces* **36**(2), 81-90.

**Li, M., Peng, L., Ji, Z., Xu, J. & Li, S.** (2008) Establishment and characterization of dual-species biofilms formed from a 3,5-dinitrobenzoic-degrading strain and bacteria with high biofilm-forming capabilities. *Fems Microbiology Letters* **278**(1), 15-21.

**Liesack, W., Weyland, H. & Stackebrandt, E.** (1991) Potential risks of gene amplification by PCR as determined by 16s rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microbial Ecology* **21**(3), 191-198.

**Lin, C.W., Lai, C.Y., Chen, L.H. & Chiang, W.F.** (2007) Microbial community structure during oxygen-stimulated bioremediation in phenol-contaminated groundwater. *Journal of Hazardous Materials* **140**(1-2), 221-229.

**Liu, W.T., Marsh, T.L., Cheng, H. & Forney, L.J.** (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**(11), 4516-4522.

**Lonergan, D.J., Jenter, H.L., Coates, J.D., Phillips, E.J.P., Schmidt, T.M. & Lovley, D.R.** (1996) Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. *Journal of Bacteriology* **178**(8), 2402-2408.

**Lovley, D.R.** (2003) Cleaning up with genomics: Applying molecular biology to bioremediation. *Nature Reviews Microbiology* **1**(1), 35-44.

**Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A. & Goodwin, S.** (1993) *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Archives of Microbiology* **159**(4), 336-344.

**Lowe, M., Madsen, E.L., Schindler, K., Smith, C., Emrich, S., Robb, F. & Halden, R.U.** (2002) Geochemistry and microbial diversity of a trichloroethene-contaminated Superfund site undergoing intrinsic in situ reductive dechlorination. *Fems Microbiology Ecology* **40**(2), 123-134.

**Lucchini, J.J., Corre, J. & Cremieux, A.** (1990) Antibacterial activity of phenolic compounds and aromatic alcohols. *Research in Microbiology* **141**(4), 499-510.

**Luna, G.M., Manini, E. & Danovaro, R.** (2002) Large fraction of dead and inactive bacteria in coastal marine sediments: Comparison of protocols for determination and ecological significance. *Applied and Environmental Microbiology* **68**(7), 3509-3513.

**Lundberg, K.S., Shoemaker, D.D., Adams, M.W.W., Short, J.M., Sorge, J.A. & Mathur, E.J.** (1991) High-Fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. *Gene* **108**(1), 1-6.

**Lupa, B., Lyon, D., Gibbs, M.D., Reeves, R.A. & Wiegel, J.** (2005) Distribution of genes encoding the microbial non-oxidative reversible hydroxyarylic acid decarboxylases/phenol carboxylases. *Genomics* **86**(3), 342-351.

**Ma, Y.F., Wang, L. & Shao, Z.Z.** (2006) *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. *Environmental Microbiology* **8**(3), 455-465.

**Macbeth, T.W., Cummings, D.E., Spring, S., Petzke, L.M. & Sorenson, K.S.** (2004) Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture. *Applied and Environmental Microbiology* **70**(12), 7329-7341.

**Mah, T.F.C. & O'Toole, G.A.** (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9**(1), 34-39.

**Maixner, F., Wagner, M., Lücker, S., Pelletier, E., Schmitz-Esser, S., Hace, K., Spieck, E., Konrat, R., Le Paslier, D. & Daims, H.** (2008) Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium '*Candidatus* Nitrospira defluvii'. *Environmental Microbiology* **10**(11), 3043 - 3056.

**Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J.A. & Buonamici, L.W.** (2002) Field demonstration of successful bioaugmentation to achieve dechlorination of

tetrachloroethene to ethene. *Environmental Science & Technology* **36**(23), 5106-5116.

**Manefield, M., Griffiths, R.I., Leigh, M.B., Fisher, R. & Whiteley, A.S.** (2005) Functional and compositional comparison of two activated sludge communities remediating coking effluent. *Environmental Microbiology* **7**(5), 715-722.

**Manefield, M., Whiteley, A.S., Griffiths, R.I. & Bailey, M.J.** (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* **68**(11), 5367-5373.

**Margesin, R., Bergauer, P. & Gander, S.** (2004) Degradation of phenol and toxicity of phenolic compounds: a comparison of cold-tolerant *Arthrobacter* sp. and mesophilic *Pseudomonas putida*. *Extremophiles* **8**(3), 201-207.

**Markowitz, V.M., Ivanova, N.N., Szeto, E., Palaniappan, K., Chu, K., Dalevi, D., Chen, I.M.A., Grechkin, Y., Dubchak, I., Anderson, I., Lykidis, A., Mavromatis, K., Hugenholtz, P. & Kyrpides, N.C.** (2008a) IMG/M: a data management and analysis system for metagenomes. *Nucleic Acids Research* **36**, D534-D538.

**Markowitz, V.M., Szeto, E., Palaniappan, K., Grechkin, Y., Chu, K., Chen, I.M.A., Dubchak, I., Anderson, I., Lykidis, A., Mavromatis, K., Ivanova, N.N. & Kyrpides, N.C.** (2008b) The integrated microbial genomes (IMG) system in 2007: data content and analysis tool extensions. *Nucleic Acids Research* **36**, D528-D533.

**Marri, P.R., Hao, W. & Golding, G.B.** (2007) The role of laterally transferred genes in adaptive evolution. *Bmc Evolutionary Biology* **7**.

**Massol- Deyá, A., Weller, R., Ríos-Hernández, L., Zhou, J.Z., Hickey, R.F. & Tiedje, J.M.** (1997) Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. *Applied and Environmental Microbiology* **63**(1), 270-276.

**Massol-Deyá, A.A., Whallon, J., Hickey, R.F. & Tiedje, J.M.** (1995) Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. *Applied and Environmental Microbiology* **61**(2), 769-777.

**Mathieu-Daudé, F., Welsh, J., Vogt, T. & McClelland, M.** (1996) DNA

rehybridization during PCR: The 'C($_o$)t effect' and its consequences. *Nucleic Acids Research* **24**(11), 2080-2086.

**Matthies, C., Evers, S., Ludwig, W. & Schink, B.** (2000) *Anaerovorax odorimutans* gen. nov., sp. nov., a putrescine-fermenting, strictly anaerobic bacterium. *International Journal of Systematic and Evolutionary Microbiology* **50**, 1591-1594.

**Mazzoli, R., Pessione, E., Giuffrida, M.G., Fattori, P., Barello, C., Giunta, C. & Lindley, N.D.** (2007) Degradation of aromatic compounds by *Acinetobacter radioresistens* S13: growth characteristics on single substrates and mixtures. *Archives of Microbiology* **188**(1), 55-68.

**McDonnell, G. & Russell, A.D.** (1999) Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews* **12**(1), 147-179.

**Mechichi, T., Stackebrandt, E., Gad'on, N. & Fuchs, G.** (2002) Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. *Archives of Microbiology* **178**(1), 26-35.

**Meckenstock, R.U., Morasch, B., Griebler, C. & Richnow, H.H.** (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. *Journal of Contaminant Hydrology* **75**(3-4), 215-255.

**Merimaa, M., Heinaru, E., Liivak, M., Vedler, E. & Heinaru, A.** (2006) Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol-degrading *Pseudomonas* species and biotypes. *Archives of Microbiology* **186**(4), 287-296.

**Meyer, S., Moser, R., Neef, A., Stahl, U. & Kämpfer, P.** (1999) Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. *Microbiology-Uk* **145**, 1731-1741.

**Miteva, V.I., Sheridan, P.P. & Brenchley, J.E.** (2004) Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. *Applied and Environmental Microbiology* **70**(1), 202-213.

**Moeseneder, M.M., Winter, C. & Herndl, G.J.** (2001) Horizontal and vertical

complexity of attached and free-living bacteria of the eastern Mediterranean Sea, determined by 16S rDNA and 16S rRNA fingerprints. *Limnology and Oceanography* **46**(1), 95-107.

**Muckian, L., Grant, R., Doyle, E. & Clipson, N.** (2007) Bacterial community structure in soils contaminated by polycyclic aromatic hydrocarbons. *Chemosphere* **68**(8), 1535-1541.

**Müller, C., Petruschka, L., Cuypers, H., Burchhardt, G. & Herrmann, H.** (1996) Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. *Journal of Bacteriology* **178**(7), 2030-2036.

**Muyzer, G., Dewaal, E.C. & Uitterlinden, A.G.** (1993) Profiling of complex microbial populations by Denaturing Gradient Gel Electrophoresis analysis of Polymerase Chain Reaction-amplified genes coding for 16S ribosomal RNA. *Applied and Environmental Microbiology* **59**(3), 695-700.

**Muyzer, G., Teske, A., Wirsen, C.O. & Jannasch, H.W.** (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* **164**(3), 165-172.

**Nakatsu, C.H., Hristova, K., Hanada, S., Meng, X.Y., Hanson, J.R., Scow, K.M. & Kamagata, Y.** (2006) *Methylibium petrolelphilum* gen. nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the *Betaproteobacteria*. *International Journal of Systematic and Evolutionary Microbiology* **56**, 983-989.

**National research council** (2000) *Natural attenuation for groundwater remediation.* National Academy Press, Washington DC, USA.

**Nojiri, H., Shintani, M. & Omori, T.** (2004) Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. *Applied Microbiology and Biotechnology* **64**(2), 154-174.

**North, N.N., Dollhopf, S.L., Petrie, L., Istok, J.D., Balkwill, D.L. & Kostka, J.E.** (2004) Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Applied and Environmental Microbiology* **70**(8), 4911-4920.

**Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W. & Backhaus, H.** (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* **178**(19), 5636-5643.

**Nurk, A., Kasak, L. & Kivisaar, M.** (1991) Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: expression in *Escherichia coli* and *Pseudomonas putida*. *Gene* **102**(1), 13-18.

**Osborn, A.M., Moore, E.R.B. & Timmis, K.N.** (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* **2**(1), 39-50.

**Osborn, A.M. & Smith, C.J.** (2005) *Molecular Microbial Ecology.* Taylor & Francis. Abingdon, UK.

**Paarup, M., Friedrich, M.W., Tindall, B.J. & Finster, K.** (2006) Characterization of the psychrotolerant acetogen strain SyrA5 and the emended description of the species *Acetobacterium carbinolicum*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **89**(1), 55-69.

**Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R., Tsai, C.S., Park, W., Jeon, C. & Madsen, E.L.** (2003) Respiration of $^{13}$C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of $^{13}$C-labeled soil DNA. *Applied and Environmental Microbiology* **69**(3), 1614-1622.

**Palmer, J., Flint, S. & Brooks, J.** (2007) Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology & Biotechnology* **34**(9), 577-588.

**Pardieck, D.L., Bouwer, E.J. & Stone, A.T.** (1992) Hydrogen peroxide use to increase oxidant capacity for in situ bioremediation of contaminated soils and aquifers: a review. *Journal of Contaminant Hydrology* **9**(3), 221-242.

**Paster, B.J., Dewhirst, F.E., Weisburg, W.G., Tordoff, L.A., Fraser, G.J., Hespell, R.B., Stanton, T.B., Zablen, L., Mandelco, L. & Woese, C.R.** (1991) Phylogenetic analysis of the Spirochetes. *Journal of Bacteriology* **173**(19), 6101-6109.

**Paul, D., Pandey, G., Pandey, J. & Jain, R.K.** (2005) Accessing microbial diversity for bioremediation and environmental restoration. *Trends in Biotechnology* **23**(3), 135-142.

**Pearce, D.A., van der Gast, C.J., Lawley, B. & Ellis-Evans, J.C.** (2003) Bacterioplankton community diversity in a maritime Antarctic lake, determined by culture-dependent and culture-independent techniques. *Fems Microbiology Ecology* **45**(1), 59-70.

**Percent, S.F., Frischer, M.E., Vescio, P.A., Duffy, E.B., Milano, V., McLellan, M., Stevens, B.M., Boylen, C.W. & Nierzwicki-Bauer, S.A.** (2008) Bacterial community structure of acid-impacted lakes: What controls diversity? *Applied and Environmental Microbiology* **74**(6), 1856-1868.

**Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A. & Nurk, A.** (1997) Acquisition of a deliberately introduced phenol degradation operon, *pheBA*, by different indigenous *Pseudomonas* species. *Applied and Environmental Microbiology* **63**(12), 4899-4906.

**Pickup, R.W., Rhodes, G., Alamillo, M.L., Mallinson, H.E.H., Thornton, S.F. & Lerner, D.N.** (2001) Microbiological analysis of multi-level borehole samples from a contaminated groundwater system. *Journal of Contaminant Hydrology* **53**(3-4), 269-284.

**Pieper, D.H. & Reineke, W.** (2000) Engineering bacteria for bioremediation. *Current Opinion in Biotechnology* **11**(3), 262-270.

**Pombo, S.A., Kleikemper, J., Schroth, M.H. & Zeyer, J.** (2005) Field-scale isotopic labeling of phospholipid fatty acids from acetate-degrading sulfate-reducing bacteria. *Fems Microbiology Ecology* **51**(2), 197-207.

**Popp, N., Schlömann, M. & Mau, M.** (2006) Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. *Microbiology-Sgm* **152**, 3291-3304.

**Powell, S.M., Riddle, M.J., Snape, I. & Stark, J.S.** (2005) Location and DGGE methodology can influence interpretation of field experimental studies on the response to hydrocarbons by Antarctic benthic microbial community. *Antarctic Science* **17**(3), 353-360.

**Pratt, L.A. & Kolter, R.** (1999) Genetic analyses of bacterial biofilm formation. *Current Opinion in Microbiology* **2**(6), 598-603.

**Price, M.** (1996) *Introducing groundwater.* Stanley Thornes Ltd., Cheltenham, UK.

**Prieto, M.B., Hidalgo, A., Rodríguez-Fernández, C., Serra, J.L. & Llama, M.J.** (2002) Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropolis* UPV-1 immobilized in an air-stirred reactor with clarifier. *Applied Microbiology and Biotechnology* **58**(6), 853-859.

**Prommer, H., Tuxen, N. & Bjerg, P.L.** (2006) Fringe-controlled natural attenuation of phenoxy acids in a landfill plume: Integration of field-scale processes by reactive transport modeling. *Environmental Science & Technology* **40**(15), 4732-4738.

**Puhakka, J.A., Melin, E.S., Järvinen, K.T., Koro, P.M., Rintala, J.A., Hartikainen, P., Shieh, W.K. & Ferguson, J.F.** (1995) Fluidized-bed biofilms for chlorophenol mineralization. *Water Science and Technology* **31**(1), 227-235.

**Puig-Grajales, L., Rodríguez-Nava, O. & Razo-Flores, E.** (2003) Simultaneous biodegradation of a phenol and 3,4-dimethylphenol mixture under denitrifying conditions. *Water Science and Technology* **48**(6), 171-178.

**Rabus, R., Kube, M., Heider, J., Beck, A., Heitmann, K., Widdel, F. & Reinhardt, R.** (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Archives of Microbiology* **183**(1), 27-36.

**Rabus, R. & Widdel, F.** (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Archives of Microbiology* **163**(2), 96-103.

**Raes, J., Foerstner, K.U. & Bork, P.** (2007) Get the most out of your metagenome: computational analysis of environmental sequence data. *Current Opinion in Microbiology* **10**(5), 490-498.

**Rainey, F.A., Nobre, M.F., Schumann, P., Stackebrandt, E. & da Costa, M.S.** (1997) Phylogenetic diversity of the *Deinococci* as determined by 16S ribosomal DNA sequence comparison. *International Journal of Systematic Bacteriology* **47**(2), 510-514.

**Ramos, J.L., Duque, E., Gallegos, M.T., Godoy, P., Ramos-González, M.I., Rojas, A., Terán, W. & Segura, A.** (2002) Mechanisms of solvent tolerance in Gram-

negative bacteria. *Annual Review of Microbiology* **56**, 743-768.

**Reardon, C.L., Cummings, D.E., Petzke, L.M., Kinsall, B.L., Watson, D.B., Peyton, B.M. & Geesey, G.G.** (2004) Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. *Applied and Environmental Microbiology* **70**(10), 6037-6046.

**Reasoner, D.J. & Geldreich, E.E.** (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* **49**(1), 1-7.

**Reddy, G.S.N., Matsumoto, G.I. & Shivaji, S.** (2003) *Sporosarcina macmurdoensis* sp. nov., from a cyanobacterial mat sample from a pond in the McMurdo Dry Valleys, Antarctica. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1363-1367.

**Reisner, A., Höller, B.M., Molin, S. & Zechner, E.L.** (2006) Synergistic effects in mixed *Escherichia coli* biofilms: Conjugative plasmid transfer drives biofilm expansion. *Journal of Bacteriology* **188**(10), 3582-3588.

**Reysenbach, A.L., Giver, L.J., Wickham, G.S. & Pace, N.R.** (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology* **58**(10), 3417-3418.

**Rickard, A.H., Gilbert, P. & Handley, P.S.** (2004a) Influence of growth environment on coaggregation between freshwater biofilm bacteria. *Journal of Applied Microbiology* **96**(6), 1367-1373.

**Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E. & Handley, P.S.** (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology* **11**(2), 94-100.

**Rickard, A.H., McBain, A.J., Stead, A.T. & Gilbert, P.** (2004b) Shear rate moderates community diversity in freshwater biofilms. *Applied and Environmental Microbiology* **70**(12), 7426-7435.

**Rivas, R., Velázquez, E., Willems, A., Vizcaíno, N., Subba-Rao, N.S., Mateos, P.F., Gillis, M., Dazzo, F.B. & Martínez-Molina, E.** (2002) A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume

*Neptunia natans* (L.f.) Druce. *Applied and Environmental Microbiology* **68**(11), 5217-5222.

**Robertson, W.J., Franzmann, P.D. & Mee, B.J.** (2000) Spore-forming, *Desulfosporosinus*-like sulphate-reducing bacteria from a shallow aquifer contaminated with gasolene. *Journal of Applied Microbiology* **88**(2), 248-259.

**Roesch, L.F., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G. & Triplett, E.W.** (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. *Isme Journal* **1**(4), 283-290.

**Röling, W.F.M., van Breukelen, B.M., Braster, M., Lin, B. & van Verseveld, H.W.** (2001) Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Applied and Environmental Microbiology* **67**(10), 4619-4629.

**Ron, E.Z. & Rosenberg, E.** (2002) Biosurfactants and oil bioremediation. *Current Opinion in Biotechnology* **13**(3), 249-252.

**Rooney-Varga, J.N., Anderson, R.T., Fraga, J.L., Ringelberg, D. & Lovley, D.R.** (1999) Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. *Applied and Environmental Microbiology* **65**(7), 3056-3063.

**Rosenbaum, V. & Riesner, D.** (1987) Temperature-gradient gel electrophoresis - Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. *Biophysical Chemistry* **26**(2-3), 235-246.

**Rudolphi, A., Tschech, A. & Fuchs, G.** (1991) Anaerobic degradation of cresols by denitrifying bacteria. *Archives of Microbiology* **155**(3), 238-248.

**Ruimy, R., Riegel, P., Boiron, P., Monteil, H. & Christen, R.** (1995) Phylogeny of the genus *Corynebacterium* deduced from analyses of small-subunit ribosomal DNA sequences. *International Journal of Systematic Bacteriology* **45**(4), 740-746.

**Rusch, D.B., Halpern, A.L., Sutton, G., Heidelberg, K.B., Williamson, S., Yooseph, S., Wu, D.Y., Eisen, J.A., Hoffman, J.M., Remington, K., Beeson, K., Tran, B., Smith, H., Baden-Tillson, H., Stewart, C., Thorpe, J., Freeman, J., Andrews-Pfannkoch, C., Venter, J.E., Li, K., Kravitz, S., Heidelberg, J.F.,**

**Utterback, T., Rogers, Y.H., Falcón, L.I., Souza, V., Bonilla-Rosso, G., Eguiarte, L.E., Karl, D.M., Sathyendranath, S., Platt, T., Bermingham, E., Gallardo, V., Tamayo-Castillo, G., Ferrari, M.R., Strausberg, R.L., Nealson, K., Friedman, R., Frazier, M. & Venter, J.C.** (2007) The Sorcerer II global ocean sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *Plos Biology* **5**(3), 398-431.

**Ryan, D., Leukes, W. & Burton, S.** (2007) Improving the bioremediation of phenolic wastewaters by *Trametes versicolor*. *Bioresource Technology* **98**(3), 579-587.

**Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A.** (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**(4839), 487-491.

**Sait, M., Hugenholtz, P. & Janssen, P.H.** (2002) Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environmental Microbiology* **4**(11), 654-666.

**Saitou, N. & Nei, M.** (1987) The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**(4), 406-425.

**Santos, V.L. & Linardi, V.R.** (2004) Biodegradation of phenol by a filamentous fungi isolated from industrial effluents - identification and degradation potential. *Process Biochemistry* **39**(8), 1001-1006.

**Saravanan, P., Pakshirajan, K. & Saha, P.** (2008) Growth kinetics of an indigenous mixed microbial consortium during phenol degradation in a batch reactor. *Bioresource Technology* **99**(1), 205-209.

**Saul, D.J., Aislabie, J.M., Brown, C.E., Harris, L. & Foght, J.M.** (2005) Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. *Fems Microbiology Ecology* **53**(1), 141-155.

**Schink, B., Philipp, B. & Müller, J.** (2000) Anaerobic degradation of phenolic compounds. *Naturwissenschaften* **87**(1), 12-23.

**Schleifer, K.H., Schüler, D., Spring, S., Weizenegger, M., Amann, R., Ludwig, W. & Kohler, M.** (1991) The genus *Magnetospirillum* gen. nov. - Description of *Magnetospirillum gryphiswaldense* sp. nov. and transfer of *Aquaspirillum magnetotacticum* to *Magnetospirillum magnetotacticum* comb. nov. *Systematic*

*and Applied Microbiology* **14**(4), 379-385.

**Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.-H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P. & Goesmann, A.** (2008) The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *J Biotechnol* **136**(1-2), 77-90.

**Schmeling, S., Narmandakh, A., Schmitt, O., Gad'on, N., Schühle, K. & Fuchs, G.** (2004) Phenylphosphate synthase: a new phosphotransferase catalyzing the first step in anaerobic phenol metabolism in *Thauera aromatica*. *Journal of Bacteriology* **186**(23), 8044-8057.

**Schreiber, M.E. & Bahr, J.M.** (2002) Nitrate-enhanced bioremediation of BTEX-contaminated groundwater: parameter estimation from natural-gradient tracer experiments. *Journal of Contaminant Hydrology* **55**(1-2), 29-56.

**Schühle, K. & Fuchs, G.** (2004) Phenylphosphate carboxylase: a new C-C lyase involved in anaerobic in phenol metabolism in *Thauera aromatica*. *Journal of Bacteriology* **186**(14), 4556-4567.

**Schulze, R., Spring, S., Amann, R., Huber, I., Ludwig, W., Schleifer, K.H. & Kämpfer, P.** (1999) Genotypic diversity of *Acidovorax* strains isolated from activated sludge and description of *Acidovorax defluvii* sp. nov. *Systematic and Applied Microbiology* **22**(2), 205-214.

**Schumann, P., Prauser, H., Rainey, F.A., Stackebrandt, E. & Hirsch, P.** (1997) *Friedmanniella antarctica* gen. nov., sp. nov, an LL-diaminopimelic acid-containing actinomycete from antarctic sandstone. *International Journal of Systematic Bacteriology* **47**(2), 278-283.

**Schwieger, F. & Tebbe, C.C.** (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Applied and Environmental Microbiology* **64**(12), 4870-4876.

**Scow, K.M. & Hicks, K.A.** (2005) Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Current Opinion in Biotechnology* **16**(3), 246-253.

**Semprini, L.** (1997) Strategies for the aerobic co-metabolism of chlorinated solvents. *Current Opinion in Biotechnology* **8**(3), 296-308.

**Setlow, P.** (2007) I will survive: DNA protection in bacterial spores. *Trends in Microbiology* **15**(4), 172-180.

**Sgountzos, I.N., Pavlou, S., Paraskeva, C.A. & Payatakes, A.C.** (2006) Growth kinetics of *Pseudomonas fluorescens* in sand beds during biodegradation of phenol. *Biochemical Engineering Journal* **30**(2), 164-173.

**Shida, O., Takagi, H., Kadowaki, K., Nakamura, L.K. & Komagata, K.** (1997) Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp. nov. and *Paenibacillus chibensis* sp. nov. *International Journal of Systematic Bacteriology* **47**(2), 299-306.

**Shimp, R.J. & Pfaender, F.K.** (1987) Effect of adaptation to phenol on biodegradation of monosubstituted phenols by aquatic microbial communities. *Applied and Environmental Microbiology* **53**(7), 1496-1499.

**Shingler, V., Powlowski, J. & Marklund, U.** (1992) Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *Journal of Bacteriology* **174**(3), 711-724.

**Sikkema, J., de Bont, J.A.M. & Poolman, B.** (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews* **59**(2), 201-222.

**Singh, R., Paul, D. & Jain, R.K.** (2006) Biofilms: implications in bioremediation. *Trends in Microbiology* **14**(9), 389-397.

**Smith, R.L., Harvey, R.W. & LeBlanc, D.R.** (1991) Importance of closely spaced vertical sampling in delineating chemical and microbiological gradients in groundwater studies. *Journal of Contaminant Hydrology* **7**(3), 285-300.

**Spence, M.J., Bottrell, S.H., Higgo, J.J.W., Harrison, I. & Fallick, A.E.** (2001) Denitrification and phenol degradation in a contaminated aquifer. *Journal of Contaminant Hydrology* **53**(3-4), 305-318.

**Srinivas, T.N.R., Kumar, P.A., Sasikala, C., Sproer, C. & Ramana, C.V.** (2008) *Rhodobacter ovatus* sp. nov., a phototrophic alphaproteobacterium isolated from a polluted pond. *International Journal of Systematic and Evolutionary Microbiology* **58**, 1379-1383.

**Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kämpfer, P., Maiden, M.C.J., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C. & Whitman, W.B.** (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1043-1047.

**Staley, J.T. & Konopka, A.** (1985) Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Review of Microbiology* **39**, 321-346.

**Stephenson, D. & Stephenson, T.** (1992) Bioaugmentation for enhancing biological wastewater treatment. *Biotechnology Advances* **10**(4), 549-559.

**Stevens, H., Brinkhoff, T. & Simon, M.** (2005) Composition of free-living, aggregate-associated and sediment surface-associated bacterial communities in the German Wadden Sea. *Aquatic Microbial Ecology* **38**(1), 15-30.

**Stewart, P.S. & Franklin, M.J.** (2008) Physiological heterogeneity in biofilms. *Nature Reviews Microbiology* **6**(3), 199-210.

**Stickler, D.** (1999) Biofilms. *Current Opinion in Microbiology* **2**(3), 270-275.

**Stoodley, P., Sauer, K., Davies, D.G. & Costerton, J.W.** (2002) Biofilms as complex differentiated communities. *Annual Review of Microbiology* **56**, 187-209.

**Stülke, J. & Hillen, W.** (1999) Carbon catabolite repression in bacteria. *Current Opinion in Microbiology* **2**(2), 195-201.

**Su, J.J. & Kafkewitz, D.** (1994) Utilization of toluene and xylenes by a nitrate-reducing strain of *Pseudomonas maltophilia* under low oxygen and anoxic conditions. *Fems Microbiology Ecology* **15**(3-4), 249-257.

**Sutherland, I.W.** (2001) The biofilm matrix - an immobilized but dynamic microbial environment. *Trends in Microbiology* **9**(5), 222-227.

**Suzuki, M.T. & Giovannoni, S.J.** (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* **62**(2), 625-630.

**Szczepanowski, R., Bekel, T., Goesmann, A., Krause, L., Krömeke, H., Kaiser, O., Eichler, W., Pühler, A. & Schlüter, A.** (2008) Insight into the plasmid

metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. *J Biotechnol* **136**(1-2), 54-64.

**Sze, C.C., Moore, T. & Shingler, V.** (1996) Growth phase-dependent transcription of the $\sigma^{54}$-dependent Po promoter controlling the *Pseudomonas*-derived (methyl)phenol *dmp* operon of pVI150. *Journal of Bacteriology* **178**(13), 3727-3735.

**Takahata, Y., Kasai, Y., Hoaki, T. & Watanabe, K.** (2006) Rapid intrinsic biodegradation of benzene, toluene, and xylenes at the boundary of a gasoline-contaminated plume under natural attenuation. *Applied Microbiology and Biotechnology* **73**(3), 713-722.

**Takeuchi, M., Hamana, K. & Hiraishi, A.** (2001) Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *International Journal of Systematic and Evolutionary Microbiology* **51**, 1405-1417.

**Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M. & Kamagata, Y.** (2005) Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Applied and Environmental Microbiology* **71**(4), 2162-2169.

**Tamura, K., Dudley, J., Nei, M. & Kumar, S.** (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**(8), 1596-1599.

**Tarlera, S. & Denner, E.B.M.** (2003) *Sterolibacterium denitrificans* gen. nov., sp. nov., a novel cholesterol-oxidizing, denitrifying member of the *β-Proteobacteria*. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1085-1091.

**Thompson, I.P., van der Gast, C.J., Ciric, L. & Singer, A.C.** (2005) Bioaugmentation for bioremediation: the challenge of strain selection. *Environmental Microbiology* **7**(7), 909-915.

**Thompson, J.D., Higgins, D.G. & Gibson, T.J.** (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**(22), 4673-4680.

**Thornton, S.F., Lerner, D.N. & Banwart, S.A.** (2001a) Assessing the natural attenuation of organic contaminants in aquifers using plume-scale electron and carbon balances: model development with analysis of uncertainty and parameter sensitivity. *Journal of Contaminant Hydrology* **53**(3-4), 199-232.

**Thornton, S.F., Quigley, S., Spence, M.J., Banwart, S.A., Bottrell, S. & Lerner, D.N.** (2001b) Processes controlling the distribution and natural attenuation of dissolved phenolic compounds in a deep sandstone aquifer. *Journal of Contaminant Hydrology* **53**(3-4), 233-267.

**Tindall, B.J., Kämpfer, P., Euzéby, J.P. & Oren, A.** (2006) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. *International Journal of Systematic and Evolutionary Microbiology* **56**, 2715-2720.

**Top, E.M., Springael, D. & Boon, N.** (2002) Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *Fems Microbiology Ecology* **42**(2), 199-208.

**Tor, J.M. & Lovley, D.R.** (2001) Anaerobic degradation of aromatic compounds coupled to Fe(III) reduction by *Ferroglobus placidus*. *Environmental Microbiology* **3**(4), 281-287.

**Tringe, S.G. & Rubin, E.M.** (2005) Metagenomics: DNA sequencing of environmental samples. *Nature Reviews Genetics* **6**(11), 805-814.

**Tziotzios, G., Teliou, M., Kaltsouni, V., Lyberatos, G. & Vayenas, D.V.** (2005) Biological phenol removal using suspended growth and packed bed reactors. *Biochemical Engineering Journal* **26**(1), 65-71.

**Ueki, A., Akasaka, H., Suzuki, D. & Ueki, K.** (2006) *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *International Journal of Systematic and Evolutionary Microbiology* **56**, 39-44.

**Ugland, K.I., Gray, J.S. & Ellingsen, K.E.** (2003) The species-accumulation curve and estimation of species richness. *Journal of Animal Ecology* **72**(5), 888-897.

**Urich, T., Lanzén, A., Qi, J., Huson, D.H., Schleper, C. & Schuster, S.C.** (2008) Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS ONE* **3**(6), e2527.

**Van Limbergen, H., Top, E.M. & Verstraete, W.** (1998) Bioaugmentation in activated sludge: current features and future perspectives. *Applied Microbiology and Biotechnology* **50**(1), 16-23.

**van Schie, P.M. & Young, L.Y.** (1998) Isolation and characterization of phenol-degrading denitrifying bacteria. *Applied and Environmental Microbiology* **64**(7), 2432-2438.

**Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D.Y., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.H. & Smith, H.O.** (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**(5667), 66-74.

**Veselý, M., Knoppová, M., Nešvera, J. & Pátek, M.** (2007) Analysis of *catRABC* operon for catechol degradation from phenol-degrading *Rhodococcus erythropolis*. *Applied Microbiology and Biotechnology* **76**(1), 159-168.

**Vestergård, M., Henry, F., Rangel-Castro, J.I., Michelsen, A., Prosser, J.I. & Christensen, S.** (2008) Rhizosphere bacterial community composition responds to arbuscular mycorrhiza, but not to reductions in microbial activity induced by foliar cutting. *Fems Microbiology Ecology* **64**(1), 78-89.

**Viggor, S., Heinaru, E., Künnapas, A. & Heinaru, A.** (2008) Evaluation of different phenol hydroxylase-possessing phenol-degrading pseudomonads by kinetic parameters. *Biodegradation* **19**(5), 759-769.

**Vrionis, H.A., Anderson, R.T., Ortiz-Bernad, I., O'Neill, K.R., Resch, C.T., Peacock, A.D., Dayvault, R., White, D.C., Long, P.E. & Lovley, D.R.** (2005) Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. *Applied and Environmental Microbiology* **71**(10), 6308-6318.

**Wang, E.T., van Berkum, P., Beyene, D., Sui, X.H., Dorado, O., Chen, W.X. &**

**Martínez-Romero, E.** (1998) *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *International Journal of Systematic Bacteriology* **48**, 687-699.

**Wang, G.C.Y. & Wang, Y.** (1997) Frequency of formation of chimeric molecules is a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. *Applied and Environmental Microbiology* **63**(12), 4645-4650.

**Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R.** (2007a) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**(16), 5261-5267.

**Wang, Y.J., Xiao, M., Geng, X.L., Liu, J.Y. & Chen, J.** (2007b) Horizontal transfer of genetic determinants for degradation of phenol between the bacteria living in plant and its rhizosphere. *Applied Microbiology and Biotechnology* **77**(3), 733-739.

**Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T.H., Stege, J.T., Cayouette, M., McHardy, A.C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S.G., Podar, M., Martin, H.G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N.C., Matson, E.G., Ottesen, E.A., Zhang, X.N., Hernández, M., Murillo, C., Acosta, L.G., Rigoutsos, I., Tamayo, G., Green, B.D., Chang, C., Rubin, E.M., Mathur, E.J., Robertson, D.E., Hugenholtz, P. & Leadbetter, J.R.** (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**(7169), 560-U17.

**Wartiainen, I., Hestnes, A.G., McDonald, I.R. & Svenning, M.M.** (2006a) *Methylobacter tundripaludum* sp. nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78°N). *International Journal of Systematic and Evolutionary Microbiology* **56**, 109-113.

**Wartiainen, I., Hestnes, A.G., McDonald, I.R. & Svenning, M.M.** (2006b) *Methylocystis rosea* sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78°N). *International Journal of Systematic and Evolutionary Microbiology* **56**, 541-547.

**Watanabe, K., Hino, S., Onodera, K., Kajie, S. & Takahashi, N.** (1996) Diversity in kinetics of bacterial phenol-oxygenating activity. *Journal of Fermentation and*

*Bioengineering* **81**(6), 560-563.

**Watanabe, K., Kodama, Y., Hamamura, N. & Kaku, N.** (2002) Diversity, abundance, and activity of archaeal populations in oil-contaminated groundwater accumulated at the bottom of an underground crude oil storage cavity. *Applied and Environmental Microbiology* **68**(8), 3899-3907.

**Watanabe, K., Teramoto, M., Futamata, H. & Harayama, S.** (1998) Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Applied and Environmental Microbiology* **64**(11), 4396-4402.

**Watanabe, K., Teramoto, M. & Harayama, S.** (1999) An outbreak of nonflocculating catabolic populations caused the breakdown of a phenol-digesting activated-sludge process. *Applied and Environmental Microbiology* **65**(7), 2813-2819.

**Watanabe, K., Watanabe, K., Kodama, Y., Syutsubo, K. & Harayama, S.** (2000) Molecular characterization of bacterial populations in petroleum-contaminated groundwater discharged from underground crude oil storage cavities. *Applied and Environmental Microbiology* **66**(11), 4803-4809.

**Webb, J.S., Givskov, M. & Kjelleberg, S.** (2003) Bacterial biofilms: prokaryotic adventures in multicellularity. *Current Opinion in Microbiology* **6**(6), 578-585.

**Weber, F.J. & de Bont, J.A.M.** (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochimica Et Biophysica Acta-Reviews on Biomembranes* **1286**(3), 225-245.

**Weber, K.A., Urrutia, M.M., Churchill, P.F., Kukkadapu, R.K. & Roden, E.E.** (2006) Anaerobic redox cycling of iron by freshwater sediment microorganisms. *Environmental Microbiology* **8**(1), 100-113.

**Weinbauer, M.G., Beckmann, C. & Höfle, M.G.** (1998) Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. *Applied and Environmental Microbiology* **64**(12), 5000-5003.

**Weiss, J.V. & Cozzarelli, I.M.** (2008) Biodegradation in contaminated aquifers: Incorporating microbial/molecular methods. *Ground Water* **46**(2), 305-322.

**Whiteley, A.S. & Bailey, M.J.** (2000) Bacterial community structure and physiological

state within an industrial phenol bioremediation system. *Applied and Environmental Microbiology* **66**(6), 2400-2407.

**Whiteley, A.S., Wiles, S., Lilley, A.K., Philp, J. & Bailey, M.J.** (2001) Ecological and physiological analyses of Pseudomonad species within a phenol remediation system. *Journal of Microbiological Methods* **44**(1), 79-88.

**Wikström, P., Hägglund, L. & Forsman, M.** (2000) Structure of a natural microbial community in a nitroaromatic contaminated groundwater is altered during biodegradation of extrinsic, but not intrinsic substrates. *Microbial Ecology* **39**(3), 203-210.

**Willems, A. & Collins, M.D.** (1996) Phylogenetic relationships of the genera *Acetobacterium* and *Eubacterium* sensu stricto and reclassification of *Eubacterium alactolyticum* as *Pseudoramibacter alactolyticus* gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **46**(4), 1083-1087.

**Williams, G.M., Pickup, R.W., Thornton, S.F., Lerner, D.N., Mallinson, H.E.H., Moore, Y. & White, C.** (2001) Biogeochemical characterisation of a coal tar distillate plume. *Journal of Contaminant Hydrology* **53**(3-4), 175-197.

**Wilson, R.D., Mackay, D.M. & Scow, K.M.** (2002) In situ MTBE biodegradation supported by diffusive oxygen release. *Environmental Science & Technology* **36**(2), 190-199.

**Wilson, R.D., Thornton, S.F. & Mackay, D.M.** (2004) Challenges in monitoring the natural attenuation of spatially variable plumes. *Biodegradation* **15**(6), 359-369.

**Winderl, C., Anneser, B., Griebler, C., Meckenstock, R.U. & Lueders, T.** (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. *Applied and Environmental Microbiology* **74**(3), 792-801.

**Wintzingerode, F.V., Goebel, U.B. & Stackebrandt, E.** (1997) Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews* **21**(3), 213-229.

**Wobus, A., Bleul, C., Maassen, S., Scheerer, C., Schuppler, M., Jacobs, E. & Röske, I.** (2003) Microbial diversity and functional characterization of sediments from reservoirs of different trophic state. *Fems Microbiology Ecology* **46**(3), 331-347.

**Woese, C.R.** (1987) Bacterial Evolution. *Microbiological Reviews* **51**(2), 221-271.

**Woese, C.R. & Fox, G.E.** (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* **74**(11), 5088-5090.

**Wuertz, S., Okabe, S. & Hausner, M.** (2004) Microbial communities and their interactions in biofilm systems: an overview. *Water Science and Technology* **49**(11-12), 327-336.

**Xie, C.H. & Yokota, A.** (2005) Reclassification of *Alcaligenes latus* strains IAM 12599$^T$ and IAM 12664 and *Pseudomonas saccharophila* as *Azohydromonas lata* gen. nov., comb. nov., *Azohydromonas australica* sp. nov and *Pelomonas saccharophila* gen. nov., comb. nov., respectively. *International Journal of Systematic and Evolutionary Microbiology* **55**, 2419-2425.

**Yang, D., Oyaizu, Y., Oyaizu, H., Olsen, G.J. & Woese, C.R.** (1985) Mitochondrial origins. *Proceedings of the National Academy of Sciences of the United States of America* **82**(13), 4443-4447.

**Yang, L., Barken, K.B., Skindersoe, M.E., Christensen, A.B., Givskov, M. & Tolker-Nielsen, T.** (2007) Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology-Sgm* **153**, 1318-1328.

**Yap, L.F., Lee, Y.K. & Poh, C.L.** (1999) Mechanism for phenol tolerance in phenol-degrading *Comamonas testosteroni* strain. *Applied Microbiology and Biotechnology* **51**(6), 833-840.

**Yassin, A.F., Rainey, F.A. & Steiner, U.** (2001) *Nocardia ignorata* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **51**, 2127-2131.

**Yi, H. & Chun, J.** (2004) *Nocardioides ganghwensis* sp. nov. isolated from tidal flat sediment. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1295-1299.

**Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A. & Sawada, H.** (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R.*

*rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *International Journal of Systematic and Evolutionary Microbiology* **51**, 89-103.

**Yu, W., Dodds, W.K., Banks, M.K., Skalsky, J. & Strauss, E.A.** (1995) Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Applied and Environmental Microbiology* **61**(9), 3367-3372.

**Zhang, R., Liu, B.Z., Lau, S.C.K., Ki, J.S. & Qian, P.Y.** (2007) Particle-attached and free-living bacterial communities in a contrasting marine environment: Victoria Harbor, Hong Kong. *Fems Microbiology Ecology* **61**(3), 496-508.

**Zhang, X.M. & Wiegel, J.** (1994) Reversible conversion of 4-hydroxybenzoate and phenol by *Clostridium hydroxybenzoicum*. *Applied and Environmental Microbiology* **60**(11), 4182-4185.

**Zou, C.S., Mo, M.H., Gu, Y.Q., Zhou, J.P. & Zhang, K.Q.** (2007) Possible contributions of volatile-producing bacteria to soil fungistasis. *Soil Biology & Biochemistry* **39**(9), 2371-2379.