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BioTran

Microbial transport and microbial indicators of mass transport through geological media – A literature survey

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Microbial transport and microbial indicators of mass transport through geological media – A literature survey

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Foreword

As part of the Chemical and Biological Hazards Programme, a project to examine microbial effects on transport processes has been initiated. The specific objectives of the project are:

- To undertake a literature survey to review existing work and to assist in scoping the experimental programme
- To carry out an experimental programme to evaluate the impacts of microbes on transport processes and to address some of the knowledge gaps prior to developing a coupled biological, chemical transport model
- To perform laboratory tracer tests to validate the use of phage against other conventional tracers for palaeohydrogeological and hydrogeological tests
- To develop model(s) to be used as a predictive tool for the assessment of transport in a given site
- To test the use of phage and other biomarkers as a means of evaluating palaeohydrogeological evolution

This report details the literature search undertaken for the first part of the project.

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Landfill and radioactive waste disposal risk assessments are principally concerned with understanding the movement of gas, water and solutes and transport of contaminants through engineered barriers and natural groundwater systems, within the concept of 'Sources', 'Pathways' and 'Receptors'. Microbes can have an important impact on transport but at present, little effort is made to model the movement of microorganisms (including pathogens), or the impact of microbes on transport properties. Therefore, there is a need for research to understand the effects of microbes on transport processes, especially on containment properties of host rocks. Consequently, as part of the Chemical and Biological Hazards Programme, a project to examine microbial affects on transport processes has been initiated.

This literature review forms the start of the project and reviews existing work on microbial transport in the broadest terms and includes:

- A range of subsurface microbes - protozoa, bacteria, viruses and prions
- An overview of microbial activity in the subsurface (Section 2)
- A review of the processes influencing transport of microbes (Section 3)
- A review of the role of microbes in subsurface transport processes (Section 4)
- A review of modelling of microbial transport in the subsurface (Section 5)
- A review of possible biomarker tracers for use in subsurface environments and methodologies for their use (Section 6 and 7)
- An evaluation of the potential use of biomarkers for palaeohydrogeology applications (Section 8)

The review shows that many subsurface environments (particularly aquifers) have the capacity to support an indigenous microbial population and their activity and interactions can have both physical and chemical effects.

Processes influencing transport of microbes in the subsurface

The transport of microorganisms through the subsurface is governed by a number of factors which fall into two main categories; firstly, hydrogeological or abiotic factors and secondly, characteristics of the microbes which influence their potential for activity and survival. It is the interaction between these sets of factors, which will determine the extent of the transport process. Principal factors affecting transport are:

- Size and electrostatic properties of the microbe
- Groundwater flow velocity
- Particle size and pore space of substratum
- Survival stress
- Growth rates of microbes

Other factors, which potentially affect microbe fate and transport such as pressure, predation and the presence of nutrients, are less significant.

The role of microbes in subsurface transport processes

Microbes can mediate the production of new minerals either by direct adsorption, co-precipitation or encasement or indirectly as a result of redox reactions. Some are capable of growth in oxic and anoxic environments and can switch target ions in growth limiting conditions. Microbes are also able to solubilise metal sulphides creating environmentally undesirable acidic and often toxic outflows from disused industrial sites. Some microbes preferentially grow biofilms of exopolysaccarides and are able to create microenvironments within their voids and channels to promote growth and increase corrosion rates when grown on iron-containing substrates. Biofilms can also trap particles and even dead and dying bacterial biofilms are capable of creating redox barriers by consuming available oxygen and maintaining low redox potentials.

Use of biomarkers as hydrogeological tracers

Most previous work has focussed on using bacteriophage (viruses that prey on bacteria) or bacteria in laboratory experiments involving column studies using both flow-through and static columns. However, column studies are often unable to give accurate information on the combined effects that control mobility *in situ*. The ideal solution is to combine laboratory studies, which can look at individual parameters, with in situ studies, using tracer tests, which consider hydrogeological characteristics of the aquifer. Much of the literature review seems to suggest the use of the phage PRD-1 and MS-2 as recommended tracers in combination with conservative tracers in transport studies. Studies have generally been concerned with bacteriophage transport through sand and gravel. However, there has been little work to investigate bacteriophage transport through fractured rocks or the effect of rapidly changing ionic pore water composition.

In situ studies would complement laboratory experiments but are more complicated to conduct and interpret. Conservative (non-reactive) tracers are typically used to monitor velocity and direction or path of groundwater flow. The characteristics of the breakthrough curve can then be used to determine major transport parameters. Retardation of microorganisms and reversible interactions with the matrix can be determined by comparing velocities of peak concentration of the conservative tracer with introduced microorganisms. Physical heterogeneity of the system increases the differences between the breakthrough curves for conservative and microbial

tracers. Microspheres as tracers are used to study the effect of cell size in sandy aquifer sediments but can differ when introduced to porous media.

Previous studies have used the following methods:

- Forced-gradient tests. These are applicable to granular aquifers to study microbial transport. Their disadvantages are that the flow field is non-uniform and therefore more difficult to model.
- Divergent tracer tests. These involve the addition of a known quantity of microorganisms and conservative tracer into a continuous stream of groundwater being injected back into the aquifer. Because of the high degree of forced dispersion, the distance over which tracer can be monitored is limited.
- Convergent tests. These involve continuous withdrawal at the sampling well. These have the advantage in that the entire mass of conservative tracer can be withdrawn at the sampling well allowing the true mass balance calculation to be carried out on the conservative tracer and microorganisms.
- Natural gradient tests. Here, the microbial tracer is slowly added to the aquifer and groundwater advection transports the plume past rows of multilevel samplers. These are best suited to sandy aquifers where flow paths can be more easily predicted.

The quantity of microorganisms needed for tracer tests depends upon the type of injection test to be run, travel distance, type of microorganism and aquifer characteristics.

Use of biomarkers as palaeohydrogeological tools

Biomarkers can potentially be used as tracers in palaeohydrogeological investigations of groundwater systems. To date, very little use of biomarkers has been made in this field, although a limited study was made during the recent PADAMOT Project, and demonstrated that biomarkers could be preserved in fracture calcite in present-day groundwater systems. However, the analysis of very low concentrations in fracture minerals proved problematic for the use of biomarkers in this application.

A more promising approach, in the first instance, would be to look for the presence of biomarkers in groundwaters rather than in the minerals precipitated from the groundwater. This has a number of advantages:

- It is easier to collect large volume samples of water;
- Water samples and can be relatively easily processed to concentrate biomarkers for analysis.
- Water samples are easier to analyse, compared with the careful separation and processing of individual mineral generations from complex fracture mineralisation.

However, this has to be offset by shortcomings in the analysis of groundwaters including:

- Groundwater geochemistry may represent a transient state or ‘snapshot’ of an evolving system.
- Evidence of earlier groundwater events may be lost as groundwaters are flushed or displaced by successively younger groundwaters.
- Interpretation of groundwater chemistry may be complicated by the mixing of groundwaters from different sources and/or of different ages.

Recommendations for further work

Recommendations are made in two overall areas:

1. Identification of gaps in the required data for microbial transport models and a recommendation for an experimental programme to address these shortfalls.
2. Determination of a range of potential biomarkers and methodologies which can be further developed in the laboratory and tested in field situations.

There have been numerous attempts to model microbial growth in subsurface environments and its effects on contaminant transport in groundwater. These models are grouped in this report into a number of categories depending upon sophistication and the nature of the processes that they try to represent. However, it is not possible within the scope of this project to identify any single 'microbial model' for development. Rather, it is important to develop experiments to examine a wide range of parameters.

Modelling and laboratory studies of the mechanisms of microbially mediated redox reactions have focussed largely on single strains of microbes and pure minerals. The effects of rapid change of pH or ionic strength and valency on established biofilms are, however, less well understood and this report recommends that future work should develop ideas from the REX project particularly by growing biofilms of indigenous bacteria in suspensions of natural groundwater on a specific substrate e.g. diorite. This would expand current knowledge of the effects of rapid changes in pH and ionic concentration on the biofilm and a wide range of parameters could to be examined. The four main factors requiring further investigation are:

- Microbial growth on mixed naturally occurring substrates
- Effects of changing pH and ionic strengths in groundwaters
- Effects of mixed microbial strains on biofilm formation
- Limiting growth parameters

Future studies looking at biofilm development on mineral surfaces should examine physico-chemical processes and attempt to determine biofilm structure and chemical interaction with mineral surfaces. The recommended work can be achieved by the use of a flow cell similar to that used in the recent Natural Environment Research Council 'Micro to Macro' research programme. The effects on a mineral substrate and biofilm development can be monitored using a confocal scanning laser microscope. This flow cell will also enable the monitoring of effects of changing parameters such as pH and microbial strains on biofilm integrity and the possible impacts on transport processes. Additionally, it is recommended that methodologies are developed to detect and study bacteriophage in subsurface environments, to evaluate the transport of bacteriophage in groundwater systems, and to examine the impact of bacteriophage activity on biofilms.

It is recommended that the concept of using organic biomarkers as palaeohydrogeological indicators is tested on site with well characterised groundwater bodies and where there is already a good understanding of past palaeoclimatic impacts. SKB's Äspö Underground Research Laboratory (URL) site in south east Sweden provides an ideal candidate for this study. Here, groundwaters have been recharged under a range of Quatenary climatic conditions ranging from glacial to peri-glacial to marine and temperate freshwater environments, and are preserved in strongly compartmentalised fault bounded rock blocks. The URL provides access to sample these different groundwater bodies.

1 Introduction

Landfill and radioactive waste disposal risk assessments are primarily based on the precepts of contaminant transport and are principally concerned with understanding the movement of gas, water and solutes through engineered barriers and natural groundwater systems, within the concept of 'Sources', 'Pathways' and 'Receptors'. The emphasis on solute migration for landfill investigations is reflected in the theoretical development used during numerical simulation.

However, microbes can have an impact on transport processes (West 1997). For example, work undertaken as part of the REX (Redox experiment in detailed scale) project at Äspö in Sweden (Banwart 1995) demonstrated that microbes can create microenvironments conducive to mineral formation within the flow system, in this particular case clay precipitation, by changing pH or the surface charges. Additionally, the microbes also produced bio-filaments, which caused the trapping of fine-grained mineral particulates moving in the flowing porewaters (West *et al*. 1997, and Hama *et al*. 2001). Both these processes could thus impact on fluid flow through porous media e.g. by blocking of constrictions in fracture flow pathways and pore throats. Additionally, microbes, in themselves, can also be used to investigate and trace flow (West *et al*.1996; Harvey and Ryan 2004; Taylor, Cronin *et al*. 2004).

As a result, there is a need for research on the effects of microbes on transport processes especially on containment properties of host rocks. At present, little effort is made to model the movement of microorganisms (including pathogens), or the impact of microbes on transport properties. If these microbial effects and processes are moved beyond the confines of a waste disposal facility, they could present a significant risk to public safety and detrimentally affect water quality, or they may retard or enhance the migration of contaminants (Stroes-Gascoyne and West 1997; West and McKinley 2002). Additionally, these microbial processes will also impinge on e.g. aquifer recharge (Kinniburgh and Gale 1994), pathogen survival (West *et al*. 1996) and, ultimately, on groundwater protection (Pedley *et al*. 2005).

Consequently, as part of the Chemical and Biological Hazards Programme, a project to examine microbial affects on transport processes has been initiated. The specific objectives of the project are:

- To undertake a literature survey to review existing work and to assist in scoping the experimental programme
- To carry out an experimental programme to evaluate the impacts of microbes on transport processes and to address some of the knowledge gaps prior to developing a coupled biological, chemical transport model
- To perform laboratory tracer tests to validate the use of phage against other conventional tracers for palaeohydrogeological and hydrogeological tests
- To develop model(s) to be used as a predictive tool for the assessment of transport in a given site
- To test the use of phage and other biomarkers as a means of evaluating palaeohydrogeological evolution

This report details the literature search undertaken for the project.

1.1 SCOPE OF REVIEW

This literature review forms the start of the project and reviews existing work on microbial transport in the broadest terms. Specifically it:

- Includes a range of subsurface microbes - protozoa, bacteria, viruses and prions
- Gives an overview of microbial activity in the subsurface (Section 2)
- Reviews the processes influencing transport of microbes (Section 3)
- Reviews the role of microbes in subsurface transport processes (Section 4)
- Reviews modelling of microbial transport in the subsurface (Section 5)
- Reviews possible biomarker tracers for use in subsurface environments and methodologies for their use (Section 6 and 7)
- Evaluates the use of biomarkers for palaeohydrogeology applications (Section 8)

These reviews are then evaluated and recommendations made in two overall areas:

- Identifying gaps in the required data for microbial transport models and detailing a recommended experimental programme.
- Determination of the range of potential biomarkers and methodologies available to be further developed in the laboratory and which can be tested in field situations.

2 Overview of microbiological activity in geological formations

2.1 INTRODUCTION

To many biologists and geologists the idea of the biosphere extending more than few metres below the soil is a strange concept. 'Rock' is perceived as incapable of supporting life as it is thought to be dense, fairly dry and low in nutrients with a harsh environment more ideally suited to the preservation and fossilisation of biological materials than to providing a habitat for life. However, examining the subsurface in terms of the potential to support life can dispel this perception.

2.2 THE SUBSURFACE AS AN ENVIRONMENT FOR MICROBIAL GROWTH

2.2.1 Microbial growth requirements

Certain conditions for the synthesis of protoplasmic constituents and for the liberation of energy necessary for life processes must exist for an active microbial population to develop. Microbial growth requires a carbon source (organic or inorganic), nitrogen, phosphorus and sulphur, certain minerals (trace elements), water and an energy source. The biochemical liberation of energy in the absence of light requires:

- The presence of an electron donor such as oxidisable organic compounds, or, in the case of chemolithotrophs (organisms obtaining energy from oxidation of inorganic compounds), oxidisable inorganic substances such as molecular hydrogen, ammonia, sulphide or ferrous ions.
- The presence of an electron acceptor such as molecular oxygen, sulphate, nitrate, ferric compounds, carbon dioxide or simple organic compounds.

Qualitatively, by this approach, most aquifers and many other subsurface environments have a capacity to support at least a limited microbial population due to the presence of:

- Carbon sources – dissolved organic matter, carbonates, dissolved CO_2
- Electron donors – dissolved H_2 and CH_4, Fe^{2+}
- Electron acceptors – dissolved O_2, SO_4^{2-}, NO_3^-
- Water supply

However, the availability of nutrients and energy sources will control microbial growth and activity (McNabb and Dunlap 1975). While aquifers might be considered as oligotrophic, supporting organisms which require only low levels of nutrients, most groups of bacteria distinguished by their nutritional requirements may find conditions suitable. These would include denitrifiers (which use nitrate as their terminal electron acceptor), sulphate reducers (which use sulphate as their terminal electron acceptor) and 'iron' bacteria (which use oxygen as their terminal electron acceptor). Most would be attached to surfaces in a gelatinous matrix, a biofilm. Biofilms are composed of monolayers or multilayers of bacterial cells that are bound to surfaces by extracellular polysaccharides (EPS) secreted by the cells. Organisms within the biofilm create microenvironments which, under low nutrient and hostile conditions, may provide some protection from environmental extremes.

2.3 THE ROLE OF ENVIRONMENTAL CONDITIONS

Environmental conditions such as pH, temperature, pressure and redox conditions all influence microbial growth and activity (West *et al*. 1991 and West and Chilton 1997).

Each microorganism has an optimum pH requirement. Most natural groundwaters have pH values in a relatively narrow range, from about 6.0 to 8.5 and microorganisms with optima in this range are common. Lower pH values (2-5) are found in humid tropical environments, mining areas, peat bogs and geothermal areas, although the latter are not usually important for water supply because of high levels of mineralisation. Acid pH is important in areas where acid mine water (acid mine drainage, or AMD) comes into contact with water supplies, as may occur in abandoned metal or coal mining areas (e.g. South Wales, Yorkshire, Devon and Cornwall etc). In contrast, high pH values (above 9) are also found in soda lakes and high carbonate environments, or in groundwater systems affected by the porewaters leaching from large concrete masses. Some organisms can grow at these extreme pHs. Acidophilic organisms include species of *Thiobacillus*, *Sulfolobus* and *Thermoplasma*. Both *Thiobacillus* and *Sulfolobus* can oxidise sulphide minerals and produce sulphuric acid. Alkaliphilic organisms (many are *Bacillus sp*.) can have pH optima as high as 10-11.

In subsurface environments, temperature is an important factor in controlling microbial activity. Three groups of microorganisms are recognised based on their preferred temperature ranges (Table 2.1). Microbes are also tolerant of extreme pressures, up to 150 Mpa, (West *et al*. 1991) and hydrostatic pressure is not likely to prevent microbial activity in subsurface environments which are otherwise suitable as habitats. Thus microbial populations will adapt to the temperature and pressure range of a particular subsurface environment (Ehrlich 1990).

Table 2.1 Temperature Ranges for Microorganisms Group, after Ehrlich (1990)

Group	Temperature range (^{0}C)	Optimum temperature (^{0}C)
Psychrophiles	<10 to 20	15
Mesophiles	10 to 45	25 to 30
Thermophiles	42 to 99 (or higher)	Depends on organisms and normal habitat

One of the most important features of the subsurface as an environment for microbial activity is the presence or absence of oxygen. Above the water table, conditions are largely aerobic; although moisture is not necessarily uniformly distributed, so localised anaerobic conditions may exist, producing microenvironments in the smaller, discontinuous pore spaces. Moreover, seasonal variations in the elevation of the water table may produce highly variable conditions in the zone of fluctuation. The concentration of dissolved oxygen in the infiltrating water will be close to saturation near the surface, i.e. about 10 mg/l. Below the water table, the oxygen supplied from infiltrating recharge reacts with oxidisable material encountered along the flow path. If little such material is available, water containing measurable amounts of dissolved oxygen (2-5 mg/l) may persist well into the aquifer. If oxidisable material is plentiful, either naturally or because of pollution of groundwater, then anaerobic conditions may be established more quickly. Oxidation and reduction processes play an important role in controlling the chemistry of many elements, including C, N, O, S, Mn and Fe. In most groundwaters, the reaction sequence follows a predicted thermodynamic sequence (Stumm and Morgan, 1981). In a closed system, such as along a flow line in a confined aquifer, dissolved oxygen will be utilised first by reaction with organic compounds. Denitrification will follow, with reduction of MnO_2 then occurring. Reduction of FeOOH to Fe^{+2} should follow nitrate ammonification. When Eh values are sufficiently low, CO_2 and SO_4 reduction and fermentation reactions may occur almost

simultaneously. Many of these reactions are microbially mediated. In most reactions, molecules of organic carbon represent the electron donor, whereas compounds containing N, S, Mn, and Fe act in general as electron acceptors. Thus, a succession of microbes will catalyse the reactions in many subsurface systems.

2.4 SUMMARY

Many subsurface environments (particularly aquifers) have the capacity to support an indigenous microbial population, despite some extreme environmental conditions, and their activity and interactions can have both physical and chemical effects. These effects can profoundly influence not only transport of the organisms themselves in the subsurface but their metabolic activity can also alter the overall environment for all transport processes, including contaminant transport in the subsurface. These effects are reviewed in Sections 3 and 4. A review of modelling of microbial growth and effects on contaminant transport is then given in Section 5.

3 Review of processes influencing transport of microbes in the subsurface

3.1 INTRODUCTION

This section aims to consider the factors which control the fate of microbes in the subsurface. It will identify and describe the physico-chemical properties of the microbes and the subsurface which control the survival, distribution, and movement of microbes (Table 3.1). The review focuses on microbes as contaminants within the subsurface as this is where most of the research has been undertaken. The transport of microorganisms through the subsurface is governed by a number of factors which fall into two main categories: firstly, hydrogeological or abiotic factors and secondly, characteristics of the microbes which influence their potential for activity and survival (Maier *et al*. 2000). A summary of the factors affecting microbe survival is shown in Figure 3.1. It is the interaction between these sets of factors, which will determine the extent of the transport process. Much of the information available on the transport of bacteria through geological media has resulted from numerous column studies with pathogenic organisms (Lawrence *et al.* 1996). As microbes are living organisms, their transport processes are more complex than those demonstrated in studies using abiotic colloids (Ginn *et al*. 2002).

Within the subsurface, water is the primary solvent. Water, infiltrating the surface from rainfall, rivers or lakes, moves through the soil and bedrock, generally downward, to the water table. Between the surface and the water table is a zone of variable thickness called the unsaturated (or vadose) zone. Microbiological contaminants, where they enter the subsurface, are generally transported along with natural or artificial recharge, e.g. rainfall or wastewater discharge or leakage.

Table 3.1 Factors affecting Fate and Transport

Characteristics of the Microorganisms	Substratum Environment Factors
Size	Groundwater flow velocity
Half-life	Particle size
Surface electrostatic properties	Pore space
Shape	Temperature
Cell surface properties	pH and temperature
	Organic carbon
	Pressure
	Moisture
	Predation

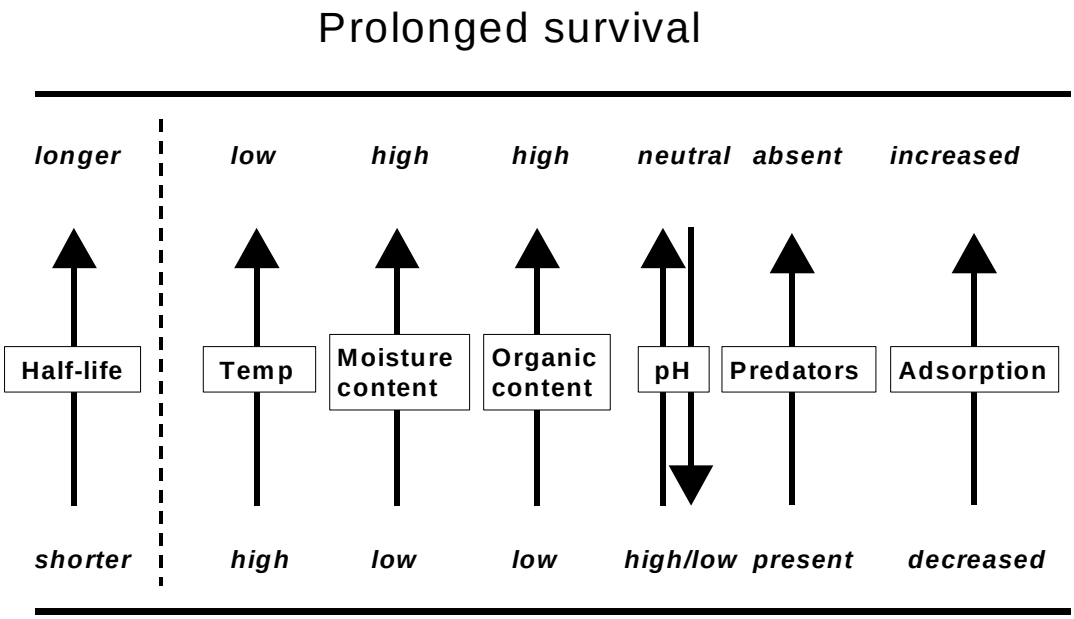

Figure 3.1 Factors affecting microbe survival and half-life

3.2 HYDROGEOLOGICAL FACTORS INFLUENCING TRANSPORT OF MICROBES

The main abiotic factors (Maier *et al*. 2000) influencing transport processes are:

- Groundwater flow velocity
- Physical characteristics of soil and aquifer material
- Solid organic carbon content
- Temperature and pH
- Water chemistry
- Moisture content

3.2.1 Groundwater flow velocity

Suspended contaminants tend to move according to the velocity of the carrying waters, the groundwater flow velocity. This flow is influenced by hydraulic conductivity (permeability), hydraulic gradient and dynamic (or effective) porosity. Soil and the unsaturated zone act as attenuating layers affecting transport through the subsurface; these mechanisms include filtration and adhesion. Interaction between cell and particle surfaces can occur in three ways:

Diffusion – as a result of Brownian motion which allows random interaction with the cells

Convection (or Advection) - this is responsible for water movement and can be orders of magnitude faster than diffusion

Active movement - cells come into contact with a surface in response to a chemotactic chemical gradient or by chance by active movement. Once contact is made between the cell and particle

surface, adhesion can take place. This can be reversible (controlled primarily by electrostatic, hydrophobic interactions or van der Waals forces) or irreversible (interaction of cell surface with solid surfaces, exopolymers cement cell to surfaces).

3.2.2 Physical characteristics

One of the controlling mechanisms for transport of microbes is the physical straining or filtration by pores in the matrix. This process is a major mechanism for removal in clay substrata where particles typically range from 0.2 to 50 μm but will have a relatively small effect in sandy substrata with particle size 0.05 to 2.0 mm. In some cases, microbes may be excluded from porous media due to micropore or size exclusion. Here the pore throats within the structured media are small and the microbes become physically excluded. This can result in a variation in microbial activity within a relatively small area. Microbes that are able to penetrate the matrix may have a high rate of activity whilst in the adjacent matrix that excludes the microbes there is no activity (Maier *et al*. 2000).

Studies looking at short-pulse inputs of bacteria through porous media, quartz and hematite-quartz showed that the bacterial attachment was reversible (McCaulou *et al*. 1994). Hydrophilic bacteria had slower attachment and detachment rates and were able to travel further before attaching, which meant detachment rates were in the order of days or weeks. They concluded that detachment was important when estimating transport for long-term systems, and in oligotrophic environments this may be the most important transport process. Data collected from column experiments (Wan *et al*. 1994) looked at the influence of the gas-water interface on transport through unsaturated media. This showed that the retention rate of bacteria is proportional to the gas saturation in porous media because of preferential sorption of bacteria onto the gas-water interface over the solid-water interface. Their conclusion was that the sorption onto the gas-water interface is essentially irreversible due to capillary forces and this sorption at the gas-water interface will strongly influence movement and distribution of microorganisms.

3.2.3 Solid organic carbon content

As detailed in Chapter 2.2, microbes require both nutrients and energy sources for their metabolic activities. The assessment of nutrient and energy supplies in the three prominent British aquifers (Table 3.2) indicates that carbon is the likely control on growth. They are thus carbon-limited aquifers (Chapelle and Bradley 1997). Electron acceptor limited aquifers are those where there is an excess of electron donors (usually carbon), so microbial metabolism is limited by the availability of electron acceptors such as dissolved oxygen, sulphate and nitrate. Examples include most natural petroleum reservoirs and aquifers that have been chemically contaminated. A well-documented example of this type of aquifer is given by Baedecker and co-workers for a site of a crude oil spill in Minnesota, USA (Baedecker *et al*. 1993). A series of zones were identified ranging from methanogenesis near the crude oil, followed by Fe (III) reduction 10 to 20 m down the hydraulic gradient followed by oxygen reduction 100 m down the hydraulic gradient. Sulphate reduction was not possible as sulphate was not available in sufficient quantities. Many contaminants that are considered virtually immobile in aqueous systems can interact with dissolved organic carbon or colloidal organic matter, resulting in migration of hydrophobic chemicals far beyond distances predicted by structure/activity relationships. Although organic matter is often present in low concentrations in subsurface systems, this organic matter can exhibit significant reactivity with contaminants (Aiken 2002).

Table 3.2 Range of concentrations of chemical constituents of groundwater in the principal British aquifers, summarised from data in Edmunds *et al.* (1989)

Parameter	Cretaceous Chalk (Berkshire)			Jurassic Limestone (Lincolnshire)			Triassic Sandstone (Shropshire)		
	Low	Average	High	Low	Average	High	Low	Average	High
Temp °C	10.1	11.0	12.5	9.0	10.5	12.0	8.5	10.3	11.5
Ph	6.8	7.2	7.5	7.0	8.0	9.5	6.5	7.0	7.3
Eh (mV)	-50	+120	+400	-300	-	+400	+250	+350	+500
Na	7	16	110	12	30	800	8	12	50
K	0.8	2	10	2	3	4	105	205	405
Ca	40	85	140	3	30	750	45	75	110
Mg	1.5	9	20	3	7	16	2.5	13	23
SO_4	6	18	55	7	65	150	14	24	65
Cl	12	17	95	22	35	950	16	35	50
NO_3N	<0.5	<1	7	<0.5	<1	5	1.0	7	9
HCO_3	250	300	350	260	320	520	150	210	320
Fe	<0.001	0.1	0.7	0.01	0.1	1.0	0.0005	0.001	0.06
TOC	0.05 - 0.2			0.02 - 0.05			0.2 - 2		

All concentrations in mg/l
Low = ten percentile, Average = fifty percentile and High = ninety percentile
TOC = Total organic carbon % weight

3.2.4 Temperature and pH

Survival rates are significantly influenced by temperature and tend to be longer at lower temperatures. Viruses have been observed to survive for up to 170 days in soil at temperatures of up to 10 ˚C (Bitton and Gerba 1984) and below 4 ˚C survival of microorganisms can extend to years (Gerba 1985). As temperature increases, inactivation is rapid with half-lives halved for every 10˚C rise in temperature between 5 and 30 ˚C (Reddy *et al*. 1981). Where the microbes have been adsorbed however, there is less temperature sensitivity (Liew and Gerba 1980). Indeed, Hurst *et al*. (1980) suggested that soils and rocks with a low temperature, moist conditions and high adsorption capacity may favour virus survival. Survival rates of microbes are significantly influenced by temperature and tend to be longer at lower temperatures. Where the microbes have been adsorbed, however, there is less temperature sensitivity (Newby *et al*. 2001).

The pH of the matrix solution does not appear to have a great effect on the transport process. However, the overall charge on the surfaces of bacteria can be affected by changes in matrix pH. Where the microbe surface becomes positively charged it will reduce the transport potential due to an increase in adsorption (Camper *et al*. 1993).

3.2.5 Water chemistry

Viable indigenous microbes are adapted to the oligotrophic nature of aquifers and they have developed various survival mechanisms (e.g. adhesion to surfaces, biofilm, etc.) that enable them to tolerate extremely low nutrient conditions. In contrast, many microbial contaminants such as enteric pathogens will be used to a rich supply of nutrients at a warm temperature of 37 ˚C. When they are present within other environments such as aquifers they will starve and die unless they can adapt to the changing conditions.

3.2.6 Moisture content and other factors

The moisture content of the subsurface is also important in transport processes; it will not only impact on survival rates but provide a fluid for transport. Where there is high moisture content and saturated conditions the microorganisms will be subject to diffusion and advection processes (Fontes *et al*. 1991).

Low nutrient conditions in an aquifer mean that indigenous microbes are well adapted to oligotrophic conditions and have various mechanisms to survive these conditions, e.g. biofilms. Studies looking at the growth rate effects on fundamental transport properties showed that bacteria growing in suboptimal conditions were able to migrate towards more favourable conditions using an enhanced chemotactic response (Mercer *et al*. 1993).

The survival of organisms in the subsurface can be influenced by the presence of antagonistic organisms. Many protozoa have been shown to actively feed upon bacterial populations (Chapelle 1992). This predation by one population on another will influence survival rates. This is an important factor in the soil zone where biological activity is greatest.

3.3 CHARACTERISTICS OF MICROORGANISMS INFLUENCING TRANSPORT PROCESSES

3.3.1 Size and Shape of Microorganisms

One of the most important factors in the transport process is the size of the microorganism. Most are 0.5 – 1 μm in diameter and 1 - 2 μm long, but they can be as small as 0.3 μm or up to 5 μm in size, usually existing as colonies. Cells <1 μm are more actively transported through porous media than those >1 μm. The small size of viruses makes their shape less critical in transport terms.

Experiments studying transport of 14 strains of bacteria through quartz sand showed smaller rounder organisms more easily transported, with spherical shapes being the most favoured (Weiss *et al* 1995). Under starvation conditions microbes typically decrease in size, which in turn can increase their transport potential. A laboratory study of the effects of starvation on bacterial cells looking at the penetration of *Klebsiella* through artificial cores demonstrated that the starved cells were able to penetrate deeper into the cores than vegetative cultures (MacLeod *et al*. 1988). The vegetative cell cultures produced 'skin' plugs and large amounts of glycocalyx, which clogged the inlet face of the core. Other studies with *Arthrobacter sp.* (Baveye 1992) showed that clogging was due, not to EPS (Extracellular polysaccharide), but to biomass accumulation. The conclusion drawn from this study was that clogging is dependant upon the bacterial strain, the supply of nutrients and the mobility of the cells.

There is also an advantage in a smaller ratio of surface area of cell to volume, which gives a more efficient exchange of nutrients from the surface. The composition of the cell surfaces, specific proteins, appendages and EPS can also play a role in transport potential. Since microbes are composed of organic materials, they tend to adhere to other organic matter when suspended in water. Many viruses, parasites and bacteria exhibit hydrophobicity and as a result of this, they are likely to adsorb to organic material in the soil/rock matrix. Viruses in the environment, because of their association with particulate matter, are often more likely to persist and be transported in the subsurface (Gerba 1984)

Studies with a motile *Bacillus* (Reynolds *et al*. 1989) have shown motility was an important mechanism in studies with Berea sandstone, although other characteristics such as random or non-random movement were also important. Non-motile bacteria also penetrated the sandstone although at a slower rate than was found with motile bacteria. Most other literature, however, seems to regard the influence of motility in transport processes as minimal (Maier *et al*. 2000, Camper *et al*. 1993) with the primary mechanism being advection, which is several orders of

magnitude higher than that of microbial movement. The Reynolds study also showed that strains of bacteria with the fastest growth rates were also the strains with the fastest penetration rates. The growth phase of the microbe can affect surface characteristics and attachment behaviour in granular media (Harvey and Harms 2001). Experiments with *Pseudomonas aeruginosa* showed increased attachment to dolomite in the stationary phase when compared to behaviour during decay phases (Grasso *et al*. 1996).

The shape of microorganisms can also be extremely variable, e.g. cocci, spiral (helix), rod-shaped, whilst some are without a well defined shape (poleomorphic). Rod-shaped organisms often have cellular appendages, flagella that aid motility usually as a result of chemotaxsis (towards beneficial substances and away from inhibitory substances) but this occurs only over short distances.

The transport of microspheres and indigenous bacteria through a sandy aquifer was investigated in forced gradient and natural gradient tracer tests in the USA (Harvey and George 1989). In natural gradient tests the surface characteristics and the size of microspheres affected attenuation; surface characteristics appeared to have the greatest affect upon retardation.

3.3.2 Half-Life

An important factor in microbial fate is the inactivation or 'die-off' rate of the organism. The maximum distances that they will move will be determined by the groundwater velocity and their survival time which can be expressed in terms of their half-life (the time taken for a 50% reduction in population)

$$C_t = C_0 e^{\left(-\left(\frac{\ln 2}{t_{1/2}}\right)\left(\frac{x}{v}\right)\right)}$$

where C_t is the concentration at time t, C_0 the initial concentration at source, $t_{½}$ the half life of the microbe, x the distance travelled and v the average groundwater velocity. A variety of factors influence half-life, including temperature and the type of organism. The most critical factors are temperature and moisture. No general rules can be applied to survival rates of non-indigenous microbes in the subsurface because there is considerable variation in both microbe type and environmental conditions. Microorganisms that are introduced to the subsurface will be transported in a viable form only as far as their life span will allow. The range of half-life amongst microorganisms varies considerably from ~500 hours for poliovirus in groundwater (Bitton *et al*. 1983) to 18 hours for *Escherichia coli* (Gerba 1985) (Table 3.3).

3.3.3 Surface electrostatic properties

As porous medium and microbial cell surfaces tend to be negatively charged, the dominant electrostatic interaction between the two is repulsion. The surface charge on a microbe varies between species and this may be affected by the pH of the matrix solution. At near neutral pH conditions and in typical natural groundwaters most microbes have a net negative surface charge, as do most small-grained mineral surfaces, e.g. clays, silts and sands. Microbes therefore are slightly repelled electrostatically in many groundwater environments and are less likely to be adsorbed. However, if attractive electrostatic potentials exist due to varying water chemistry or mineral characteristics microbes tend to adsorb.

Changes in pH will affect the electrostatic surface property of the microbe and hence its potential for sorbing. The point at which the surface charge of the microbe changes from positive to negative (and vice versa) is called the isoelectric point. This property, which can be measured in the laboratory, may be a very important parameter in controlling adsorption. Dowd and Pillai (1997) and Gerba (1984) found that bacteria and viruses with different isoelectric points displayed different sorption rates under the same environmental conditions. However, Sobsey *et*

al. (1986) found that *Poliovirus* and *Reovirus* exhibited very similar sorptive behaviour in a range of soil types even though they have different isoelectric points. A possible reason for the discrepancy in findings may be the size of microbe population. The charge strength (potential for sorbing) was found by Jansons *et al*. (1989) to be related to population size. It is possible that different populations of the same microbe may display different sorption characteristics. In most (chemically) uncontaminated aquifers in the UK, the pH of groundwaters is generally very stable due to the buffering capacity of the rock matrix and so significant changes in pH are unlikely.

Table 3.3 Examples of half-lives of pathogenic microbes derived from experimentation. (Environmental conditions not specified).

Microbe	Decay constant (hr^{-1})	Half-life (hr)	Reference
Viruses			
Poliovirus (in groundwater)	0.0013	533.2	Bitton *et al*. (1983)
	0.0088	78.8	Keswick *et al*. (1982).
Viruses (in well water)	0.0004 - 0.0037	1732.9-187.34	Bitton and Harvey (1985)
Enteroviruses	0.004	173.3	Gerba (1985)
PSD-2 and MS-2 (*E. coli*)	0.033	21.0	Dowd and Pillai (1997)
Bacteriophage (in groundwater)			
Bacteria			
Salmonella spp.	0.0078	88.9	Dowd and Pillai (1997)
(in groundwater)	0.055	12.6	Gerba (1985)
Klebsiella spp.	0.0013	533.2	Dowd and Pillai (1997)
(in groundwater)			
Escherichia coli	0.038	18.2	Gerba (1985)
	0.013	53.3	Keswick *et al*. (1982)
Streptococcus spp.	0.015	46.2	Gerba (1985)
	0.0096	72.2	Keswick *et al*. (1982)
Shigella spp.	0.028	24.8	Gerba (1985)
Faecal coliforms	0.064	10.8	Gerba (1985)

4 Review of the role of microbes in subsurface transport processes

4.1 INTRODUCTION

The origins of this section lie in work undertaken for the REX (Redox experiment in detailed scale) project (Banwart 1995). This project, undertaken at Äspö in Sweden, demonstrated that microbes can create microenvironments conducive to clay precipitation by changing pH or the surface charges. Additionally, the microbes also produced bio-filaments, which allowed the precipitation of clay minerals (West *et al*. 1997, and Hama *et al*. 2001). Both these processes could thus impact on fluid flow through porous media, e.g. by blocking of constrictions in fracture flow pathways and pore throats. It is further described below.

This section therefore examines the literature relating to the role of microbial processes in transport processes, which have been published principally since 2000, although some earlier work had been reviewed to place the current project within context.

The areas of particular interest include:

- Chemical effects (redox, pH)
- Physical effects (biofilm production)
- Biomineralisation and mineral degradation

4.2 CHEMICAL EFFECTS - REDOX REACTIONS

Complex systems exist in the natural environment, to understand the reactions taking place; the active components need to be identified. The progression of redox reactions depends on the following:

a) The ions present in the groundwater, their ionic state and valency

b) The presence of redox-sensitive minerals in the substrate

c) The microbial strains present and their growth requirements

d) The presence or absence of oxygen

Microbially mediated reactions are generally considered to be sequential and determined by redox potentials (Stumm and Morgan 1981). A typical redox sequence at circumneutral pH would begin with the removal of oxygen followed by denitrification. Depending on the metal ions present, the sequence could continue with the reduction of manganese, uranium/technetium and iron with sulphate reduction generally considered as the final phase. The presence of oxygen and redox sensitive minerals often results in one or more stages taking place simultaneously. Microbes are capable of catalysing specific redox with some thriving in both oxic and anoxic environments. Some commonly occurring bacteria and their role in redox reactions are shown in Table 4.1. *Shewanella putrifaciens* for example, can thrive in both aerobic and anaerobic environments, (Hama *et al*. 2001). The in-situ microbial remediation of toxic environments may require the indigenous bacteria to be activated by introducing growth promoters. For example, Abdelouas *et al*. (1998a) used ethanol, while Laxman and More (2002) experimented with glucose and sodium acetate. It is the presence of dissolved organic carbon and nutrients such as phosphates that stimulate the growth of denitrifying bacteria such as *Pseudomonas putrifaciens* and *Pseudomonas stutzei.*

Table 4.1 The role of microbes in redox processes

Redox Process	Mediating microbe	Reference
Denitrifying	*Pseudomonas aeruginosa* *Pseudomonas stutzei*	Abdelouas *et al*. (1998a)
Sulphate reducing	*Shewanella putrifaciens* (aerobic/anaerobic) *Desulfovibrio desulfuricans* *Desulfovibrio aespoeensis*.	West *et al*. (1997) Sani *et al*. (2004) Abdelouas *et al*. (1998b)
Iron reducing	*Shewanella putrifaciens* (aerobic/anaerobic) *Geobacter metallireducens* *Geobacter pelophilus*	West *et al*. (1997) Ehrlich (1999) Neal *et al*. (2004)
Iron oxidizing/ Sulphide oxidising	(Acidi)*Thiobacillus ferrooxidans* *Leptospirillum ferrooxidans*	Lee *et al*. (2005) Crundwell (1996)
Chromium reducing	*Pseudomonas fluorescens* (aerobic) *Bacillus sp.(*aerobic) *Enterobacter cloacae* (anaerobic) *Streptomyces griseus*	Kalinowski *et al*. (2004) Ehrlich (1999)

Pseudomonas sp. produce dense biofilms, which reduce nitrate to nitrite and subsequently nitrite to N_2 and N_2O. The intermediate formation of nitrite should produce a transient increase in H^+ ions and a corresponding fall in pH. This does not necessarily occur and it has been suggested that the reaction is buffered by the exchange of H^+ ions with K^+ and Ca^{2+}. It was also reported by Abdelouas *et al*. (1998a), that sulphate concentration remains constant throughout the denitrification stage.

As low redox potentials are reached e.g. <-100 mV, sulphate reducing bacteria such as *Shewanella putrifaciens* couple the oxidation of organic compounds and the reduction of sulphate ions to produce H_2S. Microbially generated H_2S further dissociates on dissolution in water to produce HS^- and sulphide ions, S^{2-}. Additional dissolved carbon may be required at this stage of the redox process but the residual carbon from the breakdown of dead denitrifiers may be sufficient to sustain microbial growth. This anaerobic environment allows radioactive metals such as uranium to be reduced and immobilised. In the presence of sulphate reducing bacteria, aqueous U(VI) is biomineralised into insoluble forms of U(IV) such as uraninite, UO_2 or coffinite $USiO_2$. It has been suggested by several authors that uranium reduction probably occurs as a result of enzymatic reduction by sulphide and not by the catalytic activity of microbes.

The presence or absence of redox sensitive minerals and growth limiting compounds complicate the redox process. Sani *et al*. (2004) studied the reduction of uranium in the presence of Fe(III) minerals. They found that *Desulfovibrio desulfuricans* grew significantly in sulphate limited media in the presence of Fe(III)-(hyd)oxide minerals. Sulphate was reduced and soluble U(VI) was immobilised. In lactate limited media, U(IV) was remobilised by the presence of hematite and to a lesser extent by goethite, however this was not the case with ferrihydrite. They concluded that microbially mediated reduction of soluble U(VI) to insoluble U(IV) proceeded as long as a suitable electron donor was present. Depletion of the electron donor resulted in the partial reoxidation of U(IV) when the surfaces of Fe(III)-(hyd)oxides substrates were incompletely reduced. Neal *et al*. (2001) found that hematite (α-Fe_2O_3) in the presence of *Desulfovibrio desulfuricans* undergoes dissolution by a combination of enzymatic reduction and hydrogen sulphide oxidation. The ferrous ions released are then free to react with excess H_2S to form ferrous sulphides. Abdelouas *et al*. (1999) used sandstone columns and groundwater

contaminated with uranium, sulphate and nitrate collected from a uranium mill tailings site to investigate microbially mediated reactions. They found that U(VI) was reduced along with Fe(III) from the sandstone and sulphate from the groundwater. Mackinawite, $FeS_{(0.9)}$ was formed, characterised by the appearance of black spots in the substrate along with uraninite (UO_2). It was suggested that mackinawite, in conjunction with the biomass of dead and dying bacteria, formed a redox barrier and could consume available oxygen, protecting the biogenically precipated uraninite from oxidation and dissolution. The death of microbial cells and subsequent decay may also create a redox barrier. Biogenic U(IV) occurring as uraninite (UO_2) can be protected from oxidation and dissolution by the biomass of residual dead denitrifying bacteria which consumes the available oxygen and maintains a low redox potential.

Observations made at a uranium deposit in sandstone at Oklo showed buffering by two iron minerals, siderite ($FeCO_3$) and ferrihydrite ($Fe(OH)_3$). These minerals controlled the Fe(II)/Fe(III) ratio and maintained reducing conditions even when oxygenated water passed through the substrate. Aerobic conditions also allow bacteria such as *Thiobacillus ferrooxidans* to fix inorganic carbon and convert ferrous Fe and sulphides into ferric ions and sulphuric acid, (Douglas and Beveridge, 1998). It is this process that is believed to be responsible for the creation of highly acidic tailings ponds at disused mine workings.

The redox buffering capacity of a system is enhanced by Mn (III/IV) and Fe(III) ions since both are capable of oxidising biologically reduced metals. A combination of both Mn(III)/IV) and ferrihydrites were shown by Fredrickson *et al*. (2002), to significantly retard the microbial reduction of biogenically formed U(IV) via competition as terminal electron acceptors. Solid phase biogenic Fe(II) was shown to directly reduce U(VI) but Mn(III/IV) was found to be capable of rapidly oxidising Fe(II). Some microbes are multifunctional in terms of their redox capability. For instance, *Shewanella putrifaciens* and *Geobacter metallireducens* are generally considered to be Fe(III) reducing species as they are able to utilise U(VI) in the absence of iron. Experiments undertaken by Fredrickson *et al*. (2004) using *S. putrifaciens* have shown that the Fe(II) generated as a result of the microbial reduction Fe(III) was capable of directly reducing Tc(VII). In manganese-free conditions, Burke *et al*. (2005) reported that Tc(VII) was reduced before the reduction of sulphate was complete, the controlling factor being the abiotic reduction of Tc(VII) onto solid Fe(II) phases formed by the reduction of Fe(III).

4.3 BIOFILM FORMATION

Biofilms have a widespread development and occurrence within the fracture and matrix pore spaces of soils, sediments, and rocks at, or close to, the Earth's surface. They can also be found at significant depth in some groundwater systems (as for example to depths of 500 m in the Äspö Underground Rock Laboratory (URL) in south-eastern Sweden (SKB 2003 and references therein). They can be regarded as an agglomeration of microbial cells and their excreted products attached to, or coating, mineral surfaces or other substrates (Taylor and Jaffe, 1990a). In natural environments, they are mixed microbial cultures surrounded by secreted EPS, within which cell lysis may also release a 'cocktail' of enzymes and other proteins. The presence of a biofilm changes the physical and chemical characteristics of mineral surfaces and pore systems, and may therefore influence reactions between these surfaces and dissolved aqueous chemical species, as well as affect groundwater flow.

4.3.1 Influence of biofilms on geochemical processes

Work has been undertaken on biofilm formation in a variety of environments including the subsurface. Early work undertaken by Crundwell (1996) showed that iron oxidising bacteria readily form biofilms on pyrite grains, which consist of clusters of bacteria and extracellular polymeric substances separated by water channels or voids. Crundwell also showed that direct attachment was not necessary for the bacteria to use the pyrite as an energy source; the bacteria

were seen to form at the interfaces of the solution rather than by direct attachment to the mineral surface. Studies by De Beer *et al.* (1994) found that there was a large depletion of oxygen within the bacterial clusters, within which the voids acted as conduits for the oxygen supply to the cells. *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are iron reducing bacteria, which are capable of oxidising sulphide minerals directly by attachment to the mineral surface. They may also oxidise ferrous ions Fe(II) to produce ferric ions Fe(III) which are strong oxidising agents also capable of (indirectly) oxidising sulphate minerals. Crundwell's results suggest that unreacted pyrite is covered by a corrosion product of ferric oxide or hydroxide, which is then coated by a bacterial biofilm. Consequentially, the biofilm supports a microenvironment of both ferric and ferrous ions in which bacterial growth and pyrite dissolution is enhanced.

The REX Experiment

The REX (Redox experiment in detailed scale) project at Äspö in Sweden (Banwart 1995) demonstrated that microbes could create microenvironments conducive to mineral formation within the flow system, in this particular case clay precipitation, by changing pH or the surface charges. Experiments were devised using iron reducing bacteria (IRB) and sulphate reducing bacteria (SRB) suspended in Äspö groundwater flowing through crushed Äspö diorite rock. The laboratory experiments were designed to closely resemble the field-scale *in-situ* experiments undertaken at Äspö, which evaluated the ground redox buffering capacity of the rock within the Underground Research Laboratory (URL). The laboratory experiment comprised of a series of eight columns containing IRB, closely related to *Shewanella putrifaciens* (which is known to utilise dissolved oxygen when available but is able switch to anaerobic respiration if O_2 is absent) and SRB (*Desulfovibrio aespoeensis)*. In addition to the column experiments, anaerobic, continuously stirred tank reactor (CSTR) experiments were set up containing the Äspö groundwater, with two tanks containing IRB and SRB and two abiotic tanks used as controls.

The columns containing IRB and SRB blocked after two days and all column experiments were terminated after nine days. No significant changes were seen in the 120-250 μm grain bulk material of the biotic or abiotic columns. However, in one column containing bacteria, the <2 μm mineral debris had been 'lost' or immobilised and filamentous organic meshworks appeared to have trapped fine-grained particulates within the intragranular pores. These observations were not seen in the abiotic columns. In the separated <5 μm fraction, X-ray diffraction revealed the formation of a mixed layer of chlorite-smectite, not seen in the original bulk material. Blocking of the columns occurred as a result of the liberation of 'fines' from mineral surfaces and from the neoformation of secondary clay minerals. The column experiments showed that the presence of bacteria caused mobilisation of fines, which accumulated in pore throats. Filamentous organic meshworks were formed together with a secondary mixed layer chlorite-smectite clay.

In the CSTR experiments, the 120-250 μm fraction remained unchanged but it was shown that fine mineral debris (abundant in the starting material) was reduced, possibly due to the mechanical stirring action in the vessel. In the <5 μm material, mineralogical changes were observed in both biotic and abiotic experiments including the formation of smectite which was not seen in the original bulk material. However, the degree of reaction was noticeably greater in the biotic experiments. Reactions in the vessels supported the suggestion that the fines were the most reactive material, being susceptible to dissolution and alteration.

All the results suggested that the interaction of Äspö groundwater with the starting material caused the reaction of fines to form smectite. This reaction appeared to be strongly enhanced by bacteria under both anaerobic or aerobic/anaerobic conditions, but it appeared that smectite was most likely to be formed under anaerobic conditions.

The conclusions of the REX project suggest that microbes may have created a microenvironment conducive to clay precipitation by changing pH or the surface charges. Bio-filaments may have been produced by the microbes (or the surface of microbes), which either created a chemical environment encouraging the precipitation of clay minerals, or provided suitable nucleation sites

for clay mineral formation. The charged surfaces of microbes may also have allowed clay minerals to accumulate on their surfaces.

Given these findings, a further review of recent research into the role of microbes in subsurface processes was undertaken.

The growth and impact of biofilms in geological systems was studied by Brydie *et al*. (2005) as part of the recent Natural Environment Research Council's 'Micro-to-Macro' Special Topic Research Programme. These authors grew biofilms of the common pathogenic soil bacteria *Pseudomonas aeruginosa* between quartz plates with an artificial groundwater to simulate a fracture flow system. They also introduced granular material between the quartz plates to simulate flow in a granular porous aquifer. Typical biofilms produced were highly porous, comprising umbrella-like canopies of cells and EPS, covering an interconnected network of fluid channels, anchored to the mineral/rock surfaces by cells and EPS. Bacteriogenic iron oxides (BIOS) are similarly highly microporous biofilms, produced by iron oxidizing bacteria from dissolved ferrous iron in groundwater (Ferris *et al*. 1999; 2000). Detailed studies have been made of BIOS from the Äspö URL and Stråssa Iron Ore Mine in Sweden. The BIOS has a complex structure, mainly comprising 'interwoven' twisted stalks of *Gallionella ferruginea* and filamentous sheaths of *Leptothrix sp*., with a micro- to nanoporous interstitial matrix of EPS and nanoparticulate (~20 nm diameter) granular hydrous iron oxide precipitate (Ferris *et al*. 1999; 2000; SKB 2003). The biofilm community also includes other stalked bacteria resembling *Hyphomicrobium* and *Caulobacter* species (Ferris *et al*. 1999). Within the BIOS, the microbe structures and EPS disperse and increase the volume of the iron oxides from densely packed inorganic oxide to a fluffy, 'rust-like' material with water contents of up to 99 % (SKB 2003).

The highly microporous to nanoporous structure of BIOS and other biofilms presents a very high surface area of oxide material that can interact with dissolved chemical species of the groundwater. The organic biological material also provides additional strong metal retention capacity. Overall, this can lead to very high affinities for metal sorption, much greater than that observed in simple inorganic systems. Studies from the Äspö URL, for example, have shown that metal sorption on BIOS can produce between 10^2 to 10^6-fold increased concentrations of metals such as Sr, Cs, Co, Cr, Cu, Mn, Zn, Pb, U and lanthanides compared to the groundwater (Table 4.2) (Ferris *et al*. 1999; 2000; SKB 2003). It has also been shown that BIOS has excellent adsorption and retention for lanthanides over time: 10-12 weeks old BIOS concentrated lanthanides by three orders of magnitude: 3 - 4 year old BIOS had lanthanide concentrations about one million times higher than in the Äspö groundwaters, and 10 to 100 times higher than in the granitic host rocks at Äspö.

The range of distribution coefficient (K_d) values observed for each metal reflects the organic matter content of the BIOS. Metals uptake by BIOS increases with increasing bacterial organic matter content, indicating the importance of the organic component of the biofilm on the adsorption processes. The incorporation of organic matter into particulate inorganic or mineral phases is known to dramatically affect metal partitioning, and can produce large increases in K_d (Zachara *et al*. 1994; Payne *et al*. 1996). With iron oxides, this produces a lowering of the isoelectric point of composite organic-oxide solid (Day *et al*. 1994). This can dramatically encourage increased partitioning of trace metals into the solid-phase at a constant pH, and contributes to the observed phenomenon of enhancement of metal uptake and partitioning into BIOS with increasing bacterial biomass (Zachara *et al*. 1994; Tessier *et al*. 1996; Payne *et al*. 1996; Ferris *et al*. 1999; 2000). In addition, enhanced uptake of dissolved metals by organic-rich BIOS corresponds to a higher surface density of reactive sorption sites associated with the presence of amphoteric surface functional groups (e.g. carboxyl, phosphoryl, and amino groups) that are associated with bacterial cells and EPS, compared to hydroxyl groups only on hydrous iron oxide alone (Fein *et al*. 1997; Warren and Ferris 1998). The differences in affinity of metal sorption between constituent organic and inorganic sorbants may also account for enhanced sorption of some chemical species by BIOS. Some metals, such as Cu and Zn, commonly may

display a greater affinity for the organic components in groundwater systems (Ferris *et al*. 1999). In this respect, the conclusions reached in the recent NERC 'Micro-to-Macro' Programme by Brydie *et al*. (2005) may be misleading. Based on microscopic morphological observations these authors showed that only a small proportion of the mineral surfaces in simulated fractures and porous media may be in direct contact with biofilm, concluding that the mineral surface reactivity will dominate the mineral chemistry. However, this is an over simplistic view of the system, and takes no account of the chemical properties of the biofilm itself, which may be more significant than those of the mineral surface. The application of the conclusions reached by Brydie *et al*. (2005) will therefore need to be considered with caution.

Table 4.2 Ranges of selected metal distribution coefficient (K_d) values between BIOS and Äspö groundwater from the Äspö URL and Stråssa Mine (estimates from data presented in Ferris *et al*. (1999; 2000)

Metal	K_d (minimum)	K_d (maximum)	pH	Site
Na	0.4	1.6	7-8	Äspö URL
Co	22500	77500	7-8	Äspö URL
Cr	500	1250	7-8	Äspö URL
Mn	1750	6700	7-8	Äspö URL
Cu	110	800	7-8	Äspö URL
Zn	210	1400	7-8	Äspö URL
Sr	1000	1250	8.3	Stråssa Mine
Cs	1120	11220	8.3	Stråssa Mine
Pb	1910	52480	8.3	Stråssa Mine
U	1780	5620	8.3	Stråssa Mine

Ageing of BIOS and biofilms may also be important in the fixation and re-release of metals in aqueous systems. Electron diffraction and X-ray diffraction studies show that the iron oxides in BIOS are typically poorly-ordered or amorphous, with little long-range crystalline structure (Ferris *et al*. 1999, 2000). As the biofilm ages, the hydrous iron oxide crystallites will grow, producing progressively more crystalline ferric oxyhydroxides such as ferrihydrite and eventually goethite or lepidochrocite (e.g. Kemp and Pearce 1999). This process can result in the re-release of some sorbed trace metals to the water. However, some metals such as Co and As may be incorporated and firmly bound within in the oxide crystal structure during crystallization (Fuller *et al*. 1993; Ainsworth *et al*. 1994).

The ability of sulphate reducing bacteria (SRB) biofilms to accumulate copper was investigated by White and Gadd (2000). Free-living and biofilm SRB are able to interact with metals by precipitating metal sulphides. Soluble metals and particles may also undergo biosorption at cell surfaces and combine with EPS composed of polysaccharides, mucosaccharides and proteins. Results showed that copper was accumulated by the SRB biofilms as solid copper sulphides. In addition, the biofilm also trapped precipitated copper sulphides and responded to their presence by accumulation of additional EPS. The higher accumulation of carbohydrate rather than protein suggests that a secretion of EPS may be a response to the presence of solid metal sulphides in the biofilm. These results show that sulphide precipitation is an efficient means of separating toxic metals from solution while entrapment in a biofilm reduces the mobility of the metal, a useful process in bioremediation.

This work was extended to the immobilisation of lead by SRB biofilms in a study undertaken by Beyenal and Lewandowski (2004). Biofilms of *Desulfovibrio desulfuricans* were grown anaerobically in reactors, one filled with quartz and one with hematite with lead ions, Pb^{2+} as the target material. It was thought that Fe(II) and Fe(III) ions released from the hematite would react and precipitate with the microbially produced H_2S so affecting lead precipitation. Quartz was used to represent biofilms growing on inert surfaces and, unlike White and Gadd's study, the

effects of biosorption by extracellular biopolymers was disregarded, as Beyenal's team believed that the binding capacity of biopolymers would be quickly exhausted, making their contribution negligible. Experiments took place in reactors and the concentrations of lead and iron were measured using ICP-AES. H_2S levels were monitored by microelectrode and biofilm structure was visualised by staining with acridine orange and staging on a confocal scanning laser microscope. Results showed that while the biofilms grown on quartz and hematite were heterogeneous, composed of cell clusters and voids, the biofilms grown on hematite were more porous. The average H_2S concentration was higher in the biofilm grown on quartz and lead was precipitated more readily than on hematite. When the biofilms were exposed to air, their density decreased and they became more porous. During the 18 weeks of operation, iron was being released continuously from the hematite. The study concluded that the metals which were released from redox-sensitive minerals precipitated with microbially generated H_2S and also decreased the overall metal binding capacity of the contaminant metal.

4.3.2 Biofilms and Corrosion

Biofilms associated with SRB are also known to be responsible for initiating corrosion processes by transforming sulphate into sulphide, thereby causing deterioration of metal surfaces. Keresztes *et al*. (2001) studied bacterial attachment and changes in surface chemical composition of iron with respect to the H_2S produced by bacteria. Iron coupons were placed into cultures of *Desulfovibrio desulfuricans* (a SRB) and analysed by X-ray fluorescence spectroscopy. Results showed that FeS was precipitated, causing corrosion of the iron substrate. The biofilm formed was found to contain both organic and inorganic sulphides, prompting experiments to investigate the effects of these sulphide mixes on the corrosion rate of iron. Electrochemical experiments were set up using de-aerated sodium borate buffer solutions and combinations of H_2S and Na_2S as inorganic sulphides with L-cysteine as a source of organic sulphide. A three-electrode cell was used with Armco iron rod as a working electrode, a platinum sheet as a counter electrode and a calomel electrode used as a reference. The observed differences between the corrosion rates were significant. Dissolution of the surface iron oxide layer in the buffered solutions containing pure sulphide components was explained by a reductive process whereby complexes formed at the surface result in the reduction of ferric ions to soluble ferrous ions. Where the buffered solutions contained a mixture of inorganic and organic sulphides, mixed ligand complexes were formed which enhanced the redox activity of the corrosion layer accelerating the deterioration of the surface. This implies that the surface layer plays an important role in the metabolic processes of bacteria which produce biofilms containing mixtures of inorganic and organic sulphides.

The study by Keresztes *et al*. (2001) suggested two possible mechanisms:

1. Sulphate reducing bacteria are able to utilise hydrogen as an energy source. The corrosion of the surface layer can result in an increased supply of hydrogen, producing conditions suitable for accelerated bacterial growth.
2. Alternatively, SRB cells are immobilised on an inert electrode in the presence of a suitable mediator. The corrosion potential of the iron electrode under the anaerobic biofilm would be close to the redox potential of the bacterial ferrodoxin protein or hydrogenase enzyme. The surface layer would then act as a mediator between the electrode and the bacterial enzymatic process.

The Swedish Nuclear Waste and Fuel Management Company (SKB) has investigated the influence of SRB on the corrosion of copper canisters that are planned to be used in the geological disposal and storage of spent nuclear fuel (SKB 2003). Copper is used in the canister design because of its stability and corrosion resistance under the hydrochemical conditions envisaged in the granitic environment of the Swedish disposal concept (Werme 1998). However, the presence of dissolved sulphides in groundwater, or the formation of H_2S or sulphides through the activity of SRB, may compromise the long-term stability of copper by enhancing corrosion. Results of experiments carried out in the Äspö URL, on the bacterial corrosion of copper

encased in bentonite backfill material, demonstrate that H_2S can be produced by SRB during the early stages of bentonite-swelling, and can react with the copper to form copper sulphide (SKB 2003). The environment will be extreme for microbial survival in the real disposal scenario, and high temperature, high radiation levels, high pH, and low water activity in the swelling bentonite, and low levels of organic carbon, will impact on SRB activity. However, microenvironments within SRB biofilms may possibly play an important role in mediating and facilitating SRB activity and corrosion.

Rapid corrosion of metals is often initiated by intense alteration in localised pits that coalesce as corrosion proceeds. This process is characteristic of electrochemical anodic pitting, which is caused by the formation of electrochemical cells that are set up by differential environmental conditions on the metal surface (Shreir *et al*. 1994; Kruger 2001). This is influenced by many factors, including; variations in oxygen availability, chloride distribution and surface contamination. The development of biofilms could potentially create localised microenvironments which would give rise to localised electrochemical cells and result in corrosion of metals by anodic pitting. Such processes could have important impacts on the stability of metals used underground in, for example, borehole and pipeline infrastructure for the water industry, hydrocarbon production, enhanced oil recovery and CO_2 sequestration processes, engineered structures, and barrier materials for radioactive waste disposal.

4.3.3 Influence of biofilms on physical properties

Biofilms can have a major impact on the porosity and permeability of fractures and porous media. Extensive formation of biofilm reduces pore space, which can lead to eventual blocking of the pore system (Taylor and Jaffé 1990a;b; Taylor *et al*. 1990) referred to as 'bioclogging' (Brydie *et al*. 2005). Brydie *et al*. (2005) observed a 70 % reduction in the permeability of sand due to bioclogging. Even greater permeability reductions (three orders of magnitude) were observed in earlier studies by Taylor and Jaffé (1990a). This phenomena is a well documented problem in soil science, water treatment and the petroleum industry (see Taylor and Jaffé 1990a, and references therein for further details): for example, the injection of water into hydrocarbon reservoirs to improve oil recovery is often hampered by bacterial clogging near the injection well. This may require the use of antibacterial treatments (e.g. acidification, chlorination), which themselves could inadvertently cause formation damage.

Even the development of volumetrically small amounts of biofilm can have a significant effect on the transport properties of the rock mass. Many biofilms are filamentous or bridge across pores (West *et al*. 1997; Hama *et al*. 2001; Brydie *et al*. 2005), thereby narrowing the pore throats or dividing larger pore spaces into smaller pores separated by biofilm. This reduced pore throat size and the increase pore flow-path tortuosity both result in a dramatic decrease in permeability. This effect is analogous to the significant reduction of permeability observed in sandstone reservoirs and aquifers, associated with the growth of very small amounts of authigenic fibrous illite clay (McHardy *et al*. 1982; Milodowski *et al*. 1987).

Biofilms may further reduce permeability and reduce transport by acting as a filter, trapping fine-grained mineral particles and colloidal material moving through the pore system. In addition, biofilms may cause minerals to precipitate in the pore spaces, further reducing porosity and permeability. Experimental studies have shown that even in groundwater systems with a low nutrient supply, small amounts of biofilm can form that trap particulate material and effectively prevent fluid flow after a few hours (West *et al*. 1997; Hama *et al*. 2001). Such biogenic mineral precipitates and trapped mineral matter is much more chemically and physically stable than the biofilm, and can persist in the pore system long after the biofilm has decayed or been removed (Brydie *et al*. 2005).

The development, morphology and physical stability of biofilm can be influenced by hydrodynamic conditions, chemical conditions, nature of the substrate and nutrient supply.

Changes in fluid chemistry can destabilise the biofilm, causing it to break up. High flow rates can cause shear within the biofilm, also causing it to break up (Taylor and Jaffé 1990c; Brydie *et al.* 2005). Both of these process could result in the mobilisation of microbial and biofilm material through the pore system, and could result in clogging elsewhere in the system. In a study by Leon-Morales *et al*. (2004), quartz sand cells and sand-filled microscope flow cells were used to investigate the transport characteristics of the clay colloid laponite and a biofilm forming bacterium *Pseudomonas aeruginosa*. Separate experiments were performed with each particle to determine their individual transport characteristics in clean sand columns. Additionally, a bacterial biofilm was formed prior to the introduction of the clay colloids. For flow cell experiments, cells were stained with the nucleic acid–specific fluorochrome SYTO 9. Glass chromatography columns 1cm diameter and 10cm long were used for the column experiments and stainless steel flow cells normally used for growing biofilms on the surfaces of glass slides were adapted for porous medium experiments by packing with quartz sand. Confocal microscopy was considered a suitable method for monitoring the fluorescently labelled microorganisms and laponite in the sand packed flow cells. The results showed that in clean bed columns, the laponite was strongly affected by ionic strength, the introduction of NaCl at concentrations of 1×10^{-2} M resulted in almost complete retention of laponite within the sand columns. The mobility of *Pseudomonas aeruginosa* was reduced by NaCl concentrations of up to 1 M but the effect was not as pronounced as for the laponite. At relatively high ionic strengths (7×10^{-2} M), the introduction of laponite into a sand column containing a biofilm resulted in remobilisation of a portion of the attached cells and low ionic strength solutions (6.2×10^{-4} M) caused the biofilm cells to become detached, altering the laponite elution profile.

4.4 BIOMINERALISATION

Carbonate biominerals form in alkaline environments and the most likely mechanism involves oxygenic photosynthetic microorganisms such as cyanobacteria, which use bicarbonate ions as an energy source to release hydroxyl. The structure of these biominerals depends on whether the bacteria are filamentous (stomatolites) or unicellular (thrombolitic). Silicate biominerals can also form around hot springs when the surfaces of bacterial mats become encased in amorphous silica, (Douglas and Beveridge 1998 and Castanier *et al*. 1999,). A study by Phoenix *et al*. (2000) of the silica-encapsulated cyanobacterium, *Calothrix sp*. isolated from a hot spring in Iceland, found that the mineralised cells were not only intact but also viable and photosynthetically active.

Abdelouas *et al*. (1998a) suggested that uranium (IV) produced by microbially mediated redox reactions could form biominerals either by co-precipitation with carbonates or by adsorption onto the surface of a biomass. Analysis of dark spots on the surface of bacteria showed them to be a poorly crystallised solid-solution of Ca-bearing uraninite (U, Ca)O_2. Min *et al*. (2005) considered historical evidence and deemed it likely that microbes were responsible for the production of uraninite in the uranium roll-front deposits found in North West China.

5 Review of modelling of microbial transport

There have been numerous attempts to model microbial growth in sub-surface environments and its effects on contaminant transport in groundwater. These models may be grouped into a number of categories depending upon sophistication and the nature of the processes that they try to represent.

5.1 MICROBE MASS BALANCE MODELS

This class of model is perhaps the simplest in concept since it just attempts to calculate the limits to growth from the available supplies of nutrients and energy provided by the flow of groundwater and the leaching of the solid phase. This style of model was first proposed by Grogan and McKinley (1990) and similar models were used by Baker *et al*. (1998) and Jolley *et al*. (2003)

5.2 COUPLED MICROBE GROWTH AND MASS TRANSPORT MODELS

Numerous models of this type have been reported in the literature over the past 30 or more years. Examples include Sykes *et al*. (1982), Corapcioglu and Haridas (1984), Borden and Bedient (1986), Celia *et al*. (1989), MacQuarrie *et al*. (1990), Chen *et al*. (1992), Wood *et al*. (1994), Ginn *et al*. (1995), Hunter *et al*. (1998), Schafer *et al*. (1998), Tebes-Stevens *et al*. (1998), Schirmer *et al*. (2000), Wang and Papenguth (2001), Phanikumar *et al*. (2002), Gandhi *et al*. (2002), Samper *et al*. (2003), Thullner *et al*. (2004), Phanikumar *et al*. (2005) and Hammond *et al*. (2005). These models generally include advection, dispersion, sorptive retardation, and chemical or biological reactions, the biological reactions being based on single or multiple substrates which may in some cases also interact or may include components that inhibit microbial growth. See Table 5.1 for a summary of the processes involved. The microbial growth in these models is generally treated using Monod kinetic formulations, though simpler cases may adopt first or zero order growth functions. Various reviews of these models have been made over the years including Baveye and Valocchi (1989), Essaid *et al*. (1995), Rittmann and Van Briesen (1996), and Van Cappellen and Gaillard (1996).

The review of these models by Baveye and Valocchi (1989) divided them into three groups according to the treatment of the attached bacteria. The first group (I) consisted of those models that neglected pore scale processes and assumed that the bacteria respond directly to the macroscopic bulk fluid composition. The other two groups were based upon the assumptions of the bacteria forming microcolonies (II) or biofilms (III). Examples of the first group of models may be found in Corapcioglu and Haridas (1984), Corapcioglu and Haridas (1985), and Kindred and Celia (1989). Examples of the microcolonies approach are found in Molz *et al*. (1986) and Widdowson *et al*. (1988) whilst the biofilm approach was used, for example, in Rittmann *et al*. (1980) and Bouwer and McCarty (1984). The review noted the formal similarities in all the mathematical models with the differences arising in the detailed implementation of particular terms.

Widdowson (1991), in commenting on this review, noted the particularly close association of model types I and II, but demonstrated that the differences in detail could result in noticeable differences in the calculated concentrations. He also questioned the applicability of the biofilm concept to subsurface environments.

Table 5.1 Summary of processes considered in models of microbial growth and transport

Physical / Chemical		**Comments**
	Advection	Often calculated separately using a different code.
	Dispersion/Diffusion	Often difficult to establish a suitable field scale value. May be affected by biological processes.
	Sorption	May be to host rocks or microbes in aqueous or attached phases. Functional dependence on chemical conditions generally poorly known.
	Dissolution/precipitation of minerals	Rates generally poorly known.
	Aqueous reactions	Dependent upon a suitable database of thermodynamic constants. Redox reactions are particularly important but often poorly understood.
Microbiological		
	Biofilm/microcolony growth	Generally use arbitrary assumptions about size and shape of film or colonies.
	Equilibrium growth	Suitable only in special cases.
	First order growth kinetics	Suitable only in special cases.
	Monod growth kinetics - Simple Monod - Multiple Monod - Modified Monod	These processes require the specification of many parameters which are poorly known or used as fitting coefficients.
	Inhibition/Competition	Requires many parameter values similar to Monod growth functions.
	Cometabolism	Relatively recent topic of interest
	Attachment/Detachment	Poorly understood process generally represented as first-order.
	Filtration	Usually used as a fitting parameter. Dependence on physical and chemical conditions generally not known.
Coupled		
	Permeability/porosity modification and feedback	Functional dependencies not well known.

5.3 MICROBIAL TRANSPORT AND CLOGGING MODELS

These models are really a sub-set of those described in the previous section. They are distinguished by the fact that they attempt to model the changes in the hydraulic properties of the medium as well as the microbial growth and consequent changes of porewater chemistry. Examples of such models may be found in Corapcioglu and Haridas (1984), Corapcioglu and Haridas (1985), Vandevivere *et al*. (1995), Clement *et al*. (1996), and Thullner *et al*. (2004).

Another sub-set of models which may be considered here are those concerned with the transport of viruses. These generally employ simple first order terms for growth and decay and are mainly concerned with the transport of the organism through the porous medium, treating the organism largely as a colloid. Such models are described for example in Bhattacharjee *et al*. (2002), Faulkner *et al*. (2002), and Lyon *et al*. (2002). Also related, but with a slightly different emphasis, are models that considered how contaminant transport might be enhanced by sorption to mobile bacteria (Corapcioglu and Kim 1995).

5.4 AVAILABLE CODES

Whilst numerous codes have been described in the literature, relatively few are readily available for general use. One of the most widely used codes is RT3D described in Clement (1997). This is a generalised, reactive multi-species version of the solute transport code MT3D of Zheng (1990) which includes a number of pre-programmed reaction modules, but also has a user-defined option in which the user may program a small number of well-defined subroutines to simulate the problem of interest. In its currently distributed form, it uses a groundwater flow field defined by a run of the widely used code MODFLOW, of Harbaugh and McDonald (1996). This means that one of its main limitations is that there is no feedback of the influence of say microbial growth on the flow field. Recent applications of the code have been given in Sun *et al*. (1998), Clement *et al*. (2000), and Huang *et al*. (2003).

The three-dimensional finite element code TBC, has been described in Schafer *et al*. (1998). Although use of the code appears to have been more limited than RT3D, it does incorporate a wide range of processes of interest in modelling microbial growth and transport. The main limitations appear to be the restriction to saturated conditions and the lack of any feedback from transport and growth to the flow solution. More recently, Thullner *et al*. (2004) modified this code to allow the modelling of pore clogging by bacteria, but this modified version does not appear to have been made available.

Two and three dimensional versions of the code FATMIC were described in Yeh *et al*. (1997a) and Yeh *et al*. (1997b). This (with full source code) is available from the U.S. Environmental Protection Agency website (http://www.epa.gov/ada/csmos/models/3dfatmic.html - 30 March 2006). No other reports of the use of these codes have been found and it is likely that modification to simulate reactions other than those built in will be more complex than for RT3D. However, the codes address coupled flow and transport, and also handle unsaturated flow, and so could be applicable to a wider range of problems.

Two codes, BIOMOC (Essaid and Bekins 1997), and BIOPLUME III (Rafai *et al*. 1998), are based upon the USGS Method of Characteristics (MOC) transport model described in Konikow and Bredehoeft (1978) and Goode and Konikow (1989). The BIOMOC source code is available whereas BIOPLUME III is distributed as an executable that incorporates a user-friendly interface. Both are two-dimensional codes which assume steady flow conditions.

Two codes which are more general reactive transport codes may also be considered. PHREEQC, (Parkhurst and Appelo 1999), is widely used for many geochemical modelling problems and includes a user programmable reaction kinetics package, which could be used to model microbially mediated reactions. PRECIP, (Noy 1998), is a British Geological Survey in-house developed code, which already has some support for Monod based reaction kinetics and could be easily extended to treat microbial growth. PRECIP also supports coupling between flow and transport, whereas PHREEQC requires a steady flow field. Both codes are limited to one-dimensional problems.

5.5 RECOMMENDATIONS FOR THE DESIGN OF EXPERIMENTAL PROGRAMS FOR INPUT TO MODELS

The current literature provides numerous examples of biochemical processes involved in solute transport and the numerical models that can be used to simulate them. The number of potential processes involved is large and the parameters required to simulate them are generally either uncertain or site specific in value. In order to effectively use the models it will be important to identify the particular processes (physical, chemical and biological) of importance in the specific application/end-use of interest, and design experimental work to evaluate the parameters independently, before combining them into an overall transport and reaction experiment to be modelled. An example of this approach has been given in Huang *et al.* (2003).

Thus, for example, for a column experiment it might be useful to include:

- Use of inert tracer experiments to determine porosity and dispersivity
- Use of batch experiments to determine Monod growth rate parameters, dissolution/ precipitation rates, and sorption parameters
- Monitoring of flow rate and pressure gradient, if changes of permeability are expected, for example, as a result of biofilm formation. If possible, a periodically changing concentration of inert tracer (such as Cl) should be used in order to assess any changes in porosity and dispersivity as the experiment progresses
- Use of inert beads or particles to evaluate purely physical filtration effects, as opposed to biological effects

It is important to try to provide independent estimates of as many parameters as possible to avoid ending up with many parameters that are just 'adjusted to fit the data'.

Given these findings, it is not possible within the scope of this project to identify any single 'microbial model' for development. Rather, it is important to develop experiments to examine a wide range of parameters and processes.

6 Review of possible biomarker tracers for use in subsurface environments and methodologies

6.1 TRACERS

Tracers can be used to estimate microbial transport potential and are informative in regard to biotic processes that influence microbial transport. They were first introduced in the late 19th century to investigate flow paths in aquifers when chromomeric bacteria, which formed red or yellow pigments, were used. Microbial indicators, coliform bacteria and their coliphage were first used in the 1930s to determine pathogen transport in groundwaters. Further historical information can be found in a comprehensive review of microorganisms as tracers in injection and recovery experiments by Harvey (1997).

There are a number of different tracers available for transport studies, e.g. microspheres, dyes, but the most appropriate choice of tracer will depend upon a number of factors. Ideally, a tracer

- should have no effect on aquifer properties
- will move at the same speed as the water it is tracing
- will not be absorbed or adsorbed
- should be chemically and biologically stable.

Most groundwater tracers used for geological and hydrogeological studies are substances used to determine direction and groundwater flow velocities (see Table 6.1).

Table 6.1 Hydrogeological properties, which can be measured using tracer tests (adapted from Morris *et al*. 1999)

Properties	**Examples of uses**
Flow paths	Direction of flow Connection between points
Velocities	Average water velocity, Contaminant migration
Aquifers properties	Porosity, heterogeneity, matrix diffusion
Contaminant/solute transport	Dispersion, sorption, dilution
Recharge	
Groundwater age	

In the case of transport studies, a tracer should follow the same pathway as the subject of the investigation whilst also being non-toxic, inexpensive and easy to detect.

Table 6.2 shows the variety of tracers that are available and the properties that need to be considered when assessing their suitability for a particular test.

Table 6.2 Summary of tracer types and properties, adapted from Hobson (1993)

Property	**Tracer Type**				
	Bacteria	Phage	Fluorescent Sphere	Colloid	Radio Isotopes
Cost	Medium cost	Can be expensive	Low cost	Can be expensive	Can be expensive
Toxicity	Need careful selection	OK	OK	OK	Possible concern
Ease of Use	Medium	Easy	Easy	Easy	Difficult
Detectability	Can be low	Medium/ High	Medium	High	High

A comparison of the size range of different microbial tracers is shown in Table 6.3.

Table 6.3 The size range of different microbial tracers

Tracer	**Size Range**
Viruses	0.025 – 0.1 μm
Bacteriophage	0.0045 – 0.24 μm
Bacteria	1-10 μm
Protozoa	0.5 – 100 μm

6.2 BACTERIA

Bacteria are still most common biological tracers as they are easy both to culture in large numbers and to detect with microscopy or selective media. Early studies used *E. coli* with solute tracers, which were readily identifiable, but concerns about groundwater quality required a switch to labelled indigenous groundwater bacteria, more suitable for survival in the aquifer (Harvey 1997). Labelled with stable isotopes or genetic markers means that they can survive in aquifers, be detected in very low numbers and are easily distinguishable from other indigenous microorganisms. Tracer studies with species of bacteria *E. coli* (Mallen *et al*. 2005) indicated transport was predominantly controlled by moderate reversible sorbtion and other factors such as mechanical filtration and diffusion into the matrix.

Other bacteria extensively used in tracer tests include *Pseudomonas fluorescens* (Grasso *et al.* 1996, Camesano 1998, Rijnaarts *et al.* 1993), *Bacillus* strains (Lindqvist *et al.* 1994, Harvey 1997) and *Arthrobacter* sp., (Vandvivere 1992). Some examples of distances travelled by bacterial tracers are shown in Table 6.4.

The desirable properties of a microbial tracer are highlighted below (adapted from Keswick *et al*. 1982)

The tracer should:

- Be non-pathogenic
- Not be found normally in groundwater or be easily distinguishable from indigenous microorganisms
- Be stable in the environment for a suitable length of time
- Not affect the flow of groundwater
- Move with the flow and not be filtered or absorbed
- Be readily assayable in low concentrations

- Not interact with other microorganisms that will produce changes
- Be distinguishable from other tracers in multiple tests.

Table 6.4 Examples of distances travelled by microbial tracers, adapted from Harvey (1997)

Formation	Microorganism	Type of test	Distance	Reference
Clay-rich till	*MS-2 and PRD-1*	Natural gradient	4m	McKay (1993)
Granite	*Escherichia coli*	Forced gradient	13m	Champ and Schroeter (1988)
Alluvial gravel	*Escherichia coli (rifampicin resistant)*	Natural gradient	42m	Sinton (1986)
Sand and gravel (well sorted)	Indigenous bacterial community	Natural gradient	6m	Harvey and Garabedian (1991)
Bassenden Sand	Vaccine-type Polio virus	Artificial recharge	2m	Jansons *et al.* (1989)

6.3 PROTOZOA

Protozoa are single-celled organisms which are extremely diverse in species. Their size ranges from 2 μm to several centimetres and their diverse structures mean many are motile, having flagella, e.g. *Leishmania* and *Giardia* and are able to feed in a range of environments. Their survival requires water, which is essential for normal metabolic functions. As a group they can withstand a wide range of environments, from salinity >10 % and pH <3 to pH 10. The transport behaviours of protozoa in sandy aquifers (Harvey *et al*. 1995) were studied using a natural gradient tracer test in Cape Cod over a 1 – 4 m travel distance. The results indicated that immobilisation occurred within the first metre of travel and that field and laboratory experiments to investigate abiotic aspects of flagellate transport would be better carried out using carboxylated microspheres.

The use of protozoa as tracers is limited due to their size and survival considerations. Compared with studies carried out on bacteria and viruses, there appears to be little information on protozoan transport in the literature.

6.4 VIRUSES

The survival of viruses in the subsurface depends upon similar factors as those for bacteria, e.g. temperature and pH. Their size ranges from 18 μm to several hundred nanometres and their use as tracers started in the early 1970s (Harvey 1997). Virus survival and movement in the subsurface has been the subject of a number of studies summarised by Azadpour-Keeley *et al*. (2003) which have shown that they are able to survive and travel great distances. This survival is influenced by virus type, the nature of the subsurface, temperature and moisture content of the environment with temperature being the most well defined parameter (Yates and Gerba 1984). The inactivation rate of viruses appears to be the most important factor controlling transport and survival in the subsurface; viruses absorbed on to solid surfaces are able to be active longer than those suspended in solution (Sakoda *et al*. 1997).

6.5 BACTERIOPHAGE

As animal viruses may be pathogenic to humans, the preferred choice is bacteriophage. Bacteriophages are viruses whose hosts are specific bacteria and are found in natural

environments such as rivers, lakes, marine waters and soils. Phage distribution varies with conditions and because they are able to adsorb to sediments this seems to prolong virus survival. Waters with high numbers of particles that sorb viruses protect them from inactivation, and sediment dwelling organisms may also act as viral hosts.

The advantages of bacteriophage as tracers are:

- Their small size (20 - 100 nm); advantageous, good for looking at flow paths over large distances
- Their negligible effect on groundwater quality
- Their well defined surface characteristics
- That they can be added in large concentrations
- That they can be detected in low numbers, e.g. movement of phage has been determined at less than 1 plaque forming unit per millilitre of groundwater.

The disadvantage of bacteriophage is that they undergo adsorption, being affected by electrostatic attraction/repulsion and hydrophobic sorption.

There are a number of studies of bacteriophage as groundwater tracers. One study investigating decay rate and adsorption (Rossi 1992), showed that the decay rate of phage is influenced mainly by temperature and that the adsorbtion onto a specific substrate varies with the type of phage used. Tracer experiments using three bacteriophage were carried out in Beverley, UK., (Skilton 1998) to investigate phage recovery from an aquifer. Results showed that once the phage reached the groundwater they were drawn into the fastest part of the moving water, i.e. the centre of one or more fissures, and had little contact with the rock matrix. Phage which reached there more slowly appeared to have been affected by dispersion currents within the fissures, delaying their transit and exposing them to larger areas of the rock matrix where adsorption was more likely to occur. Depending upon the media, they were able to travel large distances without suffering absorption. Phage transport was traced over a distance of 680 m in studies carried out by Martin and Thomas (1974). Results from experiments with sandy soils and fractured tuff in laboratory column experiments (Bales *et al*. 1989) showed that bacteriophage are used to best effect when estimating the maximum surface rate of transport of colloidal contaminants through a porous medium.

An extensive review of the use of bacteriophage PRD-1 was carried out by Harvey and Ryan (2004), which looked at groundwater transport, inactivation and attachment studies. They concluded that the use of PRD-1 was an important model virus for transport studies due to its relative stability over a range of temperatures, and low degree of attachment in aquifer sediments. Field experiments using PRD-1 and MS-2 coliphage (McKay and Cherry, 1993) showed PRD-1 sorbing to aquifer sediments to a lesser degree than MS-2 although this may have been due to sediment mineralogy and organic content - factors to be considered when selecting suitable phage as tracers. Studies with a conservative tracer (potassium bromide) and phage MS-2 and PRD-1 (Powelson *et al*. 1993) showed the tracers arriving at the sampling points in irregular patterns, indicating preferential flow. This study demonstrated the variability of relative transport rates that can exist between microbe and tracer.

Temperature and pH are key considerations when selecting the suitability of phage as tracers (McKay 1993). The bacteriophage PRD-1 is commonly used in transport experiments (Harvey and Ryan, 2004) as it is relatively stable over a range of temperatures and has a low degree of attachment in aquifer sediments. These studies showed the effect that temperature can have on die-off rates of the bacteriophage PRD-1. Table 6.5 shows that there is no predetermined half-life for phage and they are affected, just as bacteria are, by characteristics of the substratum such as temperature and pH.

The decay rates of phage in groundwaters vary depending upon conditions prevailing in the aquifer (Rossi 1992). Decay rate calculations must be carried out to determine the number of phage required for testing, to estimate the time it will take phage to disappear completely from the aquifer, and to obtain an estimate of the number of virulent phages remaining at the aquifer extraction point. In water the decay rate follows an exponentially decreasing function:

$$C_t = C_o\, e^{-kt}$$

where C_o is initial concentration of phage

C_t the concentration of phage at time t (in hours)

k the hourly rate of decrease

The main factors influencing the rate of decrease are temperature, pH and ion concentrations. Experiments by Rossi (1992) subjected phage, suspended in water, to a range of temperatures and showed that a decrease from 5 to 12 ºC strongly increased the decay rate. Where temperatures are sufficiently low, bacteriophage can be ideal tracers over periods of several days. They can be detected by simple and inexpensive techniques, e.g. by plating; agar overlay method, and most probable number method. A description of bacteriophage plaque assay methodology is given in Appendix I. Appendix II gives further examples of phage methodologies. The cultivation method specific to particular host bacterium will depend upon the microorganism of choice.

Table 6.5 Inactivation rates of the bacteriophage PRD-1 suspended in groundwater, adapted from Harvey and Ryan (2004)

Decay Rate ($log_{10}d^{-1}$)	Temp (˚C)	pH	Duration (days)	Reference
0.052-012	23	N/a	36-75	Yahya and Gerba (1993)
0.038	7	N/a	55	Yahya and Gerba (1993)
0	7	N/a	80	Yahya and Gerba (1993)
0.026	12	7.0	120	Schijven *et al.* (2000)
0.017-0.021	5	7.5 – 8.0	21	Schijven *et al.* (2002)
0.002±0.004	5	5 – 6.5	30 *	Ryan *et al.* (2002)
* Groundwater amended with anionic surfactants				

Harvey and Harms (2001), in their summary of column studies delineating factors controlling transport behaviour, provide a useful source of information to aid the selection of trace organisms for transport studies. Table 6.6 gives examples of some of the range of microorganisms used in column studies looking at transport behaviour.

A detailed account of methods for injecting and recovering microbial tracers are detailed by Harvey (1997) and are briefly summarised at the end of this chapter.

Table 6.6 Examples of selected column studies delineating factors controlling microbial transport behaviour, adapted from Harvey (2001)

Factor Studied	Medium	Method	Conditions	Microorganism	Reference
Attachment kinetics	Clean sand Subsurface sand	Up flow Flow through	Saturated, aerobic Saturated, aerobic	*Ps. Fluorescens* *Bacillus* sp.	Grasso *et al.* (1996) Lindqvist *et al.* (1994)
Cell surface properties, hydrophobicity and charge	Glass and Teflon	Down flow	Saturated, aerobic	Pseudomonads, coryneforms	Rijnaarts *et al.* (1993)
Fluid flow, cell density and fluid velocity	Sandy soils	Flow through	Saturated, aerobic	*Ps. Fluorescens*	Camesano and Logan (1998)
Ionic strength	Aquifer sand	Up flow	Saturated, aerobic	Pseuedomonas strain KL2	Gannon *et al.* (1991)
Pore clogging, aggregation	Fine sand	Down flow	Saturated, aerobic	*Arthobacter* sp.	Vandevivere (1992)
Viruses, electrostatic interactions	Quartz sand	Down flow	Saturated, aerobic	MS-2	Badawy *et al.* (1990)
Viruses, effect of pH	3 sandy soils	Flow through	Saturated, aerobic	PRD-1, MS-2	Kinoshita *et al.* (1993)
pH and attachment reversibility	Quartz/Fe-quartz	Static	Saturated, aerobic	PRD-1	Bales *et al.* (1993)
Taxis and chemotaxis, permeability and motility	Berea sandstone	Static	Saturated, anaerobic	*Bacillus, Enterobacter aerogenes*	Jenneman *et al.* (1985)
Protozoa, field v column transport behaviour	Aquifer sand	Up flow	Saturated, aerobic	*Spumella guttula*	Harvey *et al.* (1995)

6.6 FACTORS AFFECTING TRANSPORT RATES OF BACTERIOPHAGE AND VIRUSES IN GROUNDWATER

6.6.1 Electrostatic forces and the effects of ionic strength on bacteriophage mobility

Viruses and bacteriophage in groundwater are retarded by reversible attachment to the surfaces of mineral substrates and complete inactivation occurs when the virus particle becomes irreversibly attached. Electrostatic forces play a dominant role in viral and bacteriophage adsorption onto mineral grains. The surfaces of most minerals and viruses are negatively charged but adsorbed organic material or iron oxide surface precipitates can create positively charged 'zones' within the substrate. Electrostatic attractions between the virus particles and these positively charged regions create strong attractive forces resulting in inactivation.

Electrostatic forces can be altered by changes in ionic strength. Bales *et al*. (1993) found that lowering the ionic strength increased electrostatic repulsion and enhanced the release of MS-2 bacteriophage from silica columns. Zhuang and Yin (2003) studied the transport of ϕX174 virus and MS-2 bacteriophage suspended in buffered saline though columns of positively charged, aluminium-coated sand. They reported a reduction in electrostatic attraction with increasing salt concentration, resulting in a decrease in MS-2 sorption. The presence of bivalent cations such as Ca^{2+} and Mg^{2+} also appeared to protect MS-2 from inactivation by irreversible sorption. They suggested that a partial screening by the bivalent cations decreased electrostatic attraction between the negatively charged virus particles and the overall positively charged Al-coated sand

grains. Ionic composition was also of significance: virus transport was enhanced in the presence of phosphate (HPO_4^{2-}) compared to bicarbonate (HCO_3^-), with HPO_4^{2-} affecting MS2 more noticeably than ϕX174.

This, and similar studies by other authors e.g. Redman *et al*. (1999) suggest that small changes in hardness and total dissolved solids can affect the filtration and mobilisation of filamentous bacteriophage in subsurface systems. This is of significance when considering the filtration and mobilisation of phage following rainfall events when solids may be removed by increased groundwater flow allowing phage transport.

6.6.2 Effect of pH on bacteriophage mobility

In a study of a model aquifer comprised of quartz, feldspar and igneous rock fragments, Schulze-Makuch *et al*. (2003) linked differing transport rates of the bacteriophage MS-2 to changes in the pH of groundwater. At slightly alkaline pH, MS-2 transport rates were greater than a conservative tracer (bromide). This was accounted for by the larger size of MS-2 with pore size exclusion considered to be the contributing factor. In a neutral environment, MS-2 and bromide exhibited similar transport rates, but at slightly acidic pHs, the breakthrough concentration of MS-2 trailed behind bromide. The study suggested that as pH decreases, MS-2 becomes reversibly attached to quartz while irreversible attachment to feldspar significantly increases. This observation can be explained by the Derjaguin-Landau-Verwey-Overbeek (DVLO) potential energy profile (Schulze-Makuch *et al*. 2003). Irreversible adsorption corresponds to the primary energy minimum of MS-2-feldspar attraction while reversible adsorption corresponds to the secondary minimum of MS-2-quartz attraction, which is overcome by Brownian motion.

6.6.3 Bacteriophage size and pore size exclusion

The faster travel rate of microbes compared to chemical tracers has been widely recognised. The common explanation is pore size exclusion, whereby bacteria are only able to pass through larger pore spaces where groundwater velocity is high, and only dissolved chemicals permeate through smaller pore spaces where the velocity is reduced. However, a study by Sinton *et al*. (1997) using *Escherichia coli* and the F-RNA MS-2 bacteriophage appeared to contradict the pore size exclusion theory. F-RNA phage cells (22-30 nm diameter) are smaller than *E. coli* cells (c.1500 nm), yet the bacteria exhibited a slightly slower transport rate than the phage. The authors suggested that phage might be adsorbed onto colloidal particles leaching into the groundwater thus increasing their size with respect to bacteria and causing them to be transported at a faster rate. In a later experiment by Sinton *et al*. (2000), the transport rates of *Escherichia coli*, *Bacillus subtilis*, MS-2 and Rhodamine WT were compared. Overall, the results fitted with pore size exclusion. In terms of velocity, the transport rate of *Escherichia coli* was greater than that of *Bacillus subtilis* and Rhodamine WT. *Escherichia coli* velocity was found to be greater than MS-2 when compared with Rhodamine WT.

6.7 COMPARISON OF BIOMARKERS AND RECOMMENDATIONS FOR EXPERIMENTAL PROGRAMME

The review shows that microbial tracers are subject to environmental stresses but that these are minimised in groundwater. It is important to establish the half-life for any chosen microbial tracer and to select one appropriate to the test undertaken. In the case of pathogen transport studies the suitability of any particular microbial tracer will depend upon its surface chemistry, physiological and physical characteristics, which should be similar to the pathogen of interest. Microbial tracers must also be easily distinguishable from indigenous populations. The advantages and disadvantages of selection of a suitable microbiological tracer are summarized in Table 6.7.

Table 6.7 Summary of factors influencing choice of microbial tracer

Microbiological Tracer	Advantages	Disadvantages
Bacteria	Can culture in large quantities Easily detectable using selective media or microscopy	Do not adequately mimic transport of smaller microorganisms e.g. viruses May sorb to substrate May grow in aquifer giving spurious results
Protozoa	Diverse in size and shape	Require water for survival Too large for some substrates Susceptible to environmental stresses
Viruses	Small size Able to survive and travel great distances	Many pathogenic May sorb to substrate
Bacteriophage	Can be cultured in large numbers Small size (20 - 100 nm); advantageous for investigating flow paths over large distances Negligible effect on groundwater quality Well-defined surface characteristics Can be added in large concentrations Can be detected in low numbers	Undergo adsorption - They are affected by electrostatic attraction/repulsion and hydrophobic sorption.

The review has shown that bacteriophage make ideal tracers and so the aim of an experimental programme should be to develop methodologies to enable the use of bacteriophage in future transport studies. In summary, the aims and objectives of this proposed work should be:

- to develop methodologies for the cultivation of bacteriophage
- to enable them to be grown in sufficient quantities to use as tracers for laboratory and field experiments
- to be able to recover them from environmental samples.

The experimental work should focus on

- growth of bacteriophage
- the separation of host cell debris from phage by centrifugation
- phage enumeration.

A comparative study of methods should be made for both the production of bacteriophages in liquid media and Petri dishes and for single-agar-layer and double-agar-layer methods for plaque assay. At the end of the experimental programme, procedures should be in place to enable the cultivation and detection of bacteriophage as tracers where required in future transport studies.

7 Review and methodologies of molecular biomarker tracers for use in subsurface environments

7.1 INTRODUCTION - MOLECULAR BIOMARKER TRACERS

Biological markers (biomarkers) are organic molecules whose structure is identical to, or directly derived from, a precursor compound that was incorporated into the geological environment during normal sedimentary or hydrogeological processes, and which can be related to specific types or groups of organisms from which these compounds are derived. In contrast, compounds such as methane and graphite are not considered biomarkers because almost any organic compound can yield these products under the correct geological conditions. To date, biomarker studies have been largely applied to sediments, where these chemical fossils can provide information about the environment of deposition, as well as conditions and influences that have affected the sediments since their deposition. Biomarkers may also provide information about biochemical and plant evolution, which in turn can be used to infer the palaeoclimatic history of the depositional basin and its sedimentary source area (e.g. Schwark *et al*. 2002). Very little use has been made of biomarkers, either as tracers to study groundwater flow paths, or in the palaeohydrogeological analysis of groundwater systems.

Principal or potential biomarkers are discussed in more detail in the following sections.

7.2 PROTOZOA

7.2.1 Tetrahymanol

Tetrahymanol (gammaceran-21α-ol) is a neutral lipid that replaces steroids in the membranes of specific protozoa, functioning to stabilize phospholipid-bilayers in plasma membranes. The gammacerane type skeleton of tetrahymanol shown in Figure 7.1 differs from that of a regular hopane in that the former has a six-membered ring – whereas the latter contains a five-membered ring. Tetrahymanol was first isolated from the freshwater ciliate protozoan *Tetrahymena pyriformis* which is associated with anaerobic bacteria upon which it feeds (Peters *et al*. 2004). However, the compound has been discovered in eukaryotes, e.g. ferns and anaerobic rumen fungus, as well as other ciliates and the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (Bravo *et al*. 2001). Nevertheless, the main source of tetrahymanol in sediments is generally thought to be from bactiverous ciliates, which are found at the interface between oxic and anoxic zones in stratified water columns. Tetrahymanol is the only known precursor of the important biomarker gammacerane, which is utilised by organic petroleum geochemists as an indicator of a stratified water column in marine and non-marine source rock depositional environments. Compounds such as tetrahymanol are identified in sediments by solvent extraction, chromatographic separation (TLC or column chromatography) of the aliphatic compounds and subsequent analysis of the biomarkers by GC/MS operated in selected ion monitoring mode or GC/MS/MS (Peters *et al*. 2004).

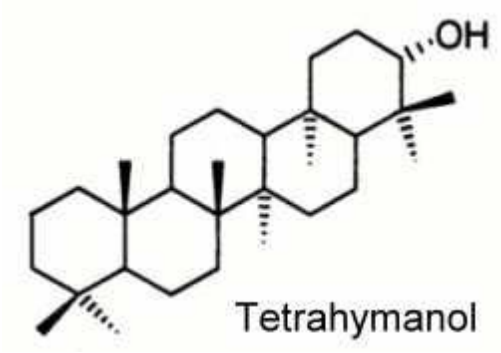

Figure 7.1 Tetrahymanol carbon skeleton - a biomarker for protozoa

7.3 BACTERIAL FINGERPRINTING

7.3.1 Fatty acid profiles

Fatty acid profiling is one approach to distinguish populations of anaerobic bacteria within a sediment, soil or water sample. Two different methods are employed. In the Sherlock system (MIDI Inc.), samples are first cultured, then saponified, methylated, extracted and analysed by GC. The distribution and relative intensities of separate fatty acids are matched to a library of over 700 bacterial species using multivariate statistics. However, as the British Geological Survey does not have the necessary equipment to perform such identifications, this would have to be carried out by a commercial, contract laboratory. The primary limitation of this method is that only living bacteria can be cultured and considerable time and effort is involved in sample preparation (Buyer 2003). An alternative fatty acid based bacterial profiling method is to use in-situ methylation using a reagent such as tetramethylammonium hydroxide (TMAH) during Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC-MS) of bacterial biomasses (Poerschmann *et al*., 2005; Voorhees *et al*. 1997). This second approach is advantageous over the Sherlock commercial system in that the sample preparation is straightforward. However, only a small number of Gram-positive and Gram-negative bacteria have been studied, therefore there are no libraries available for comparison.

7.3.2 Bacterial Hopanoids

Hopanoids are an abundant group of biomarker molecules derived in the main from bacteriahopanepolyols, which are produced in the membrane lipids of certain eubacteria. Their main function is to moderate membrane fluidity.

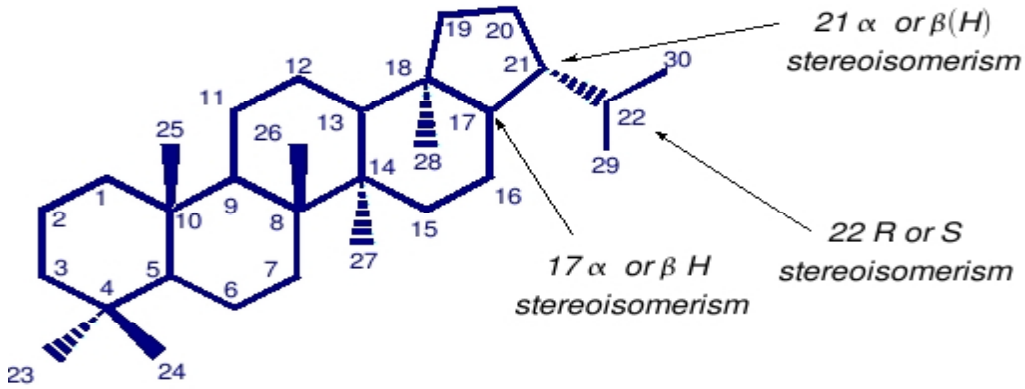

Figure 7.2 Hopanoid carbon skeleton

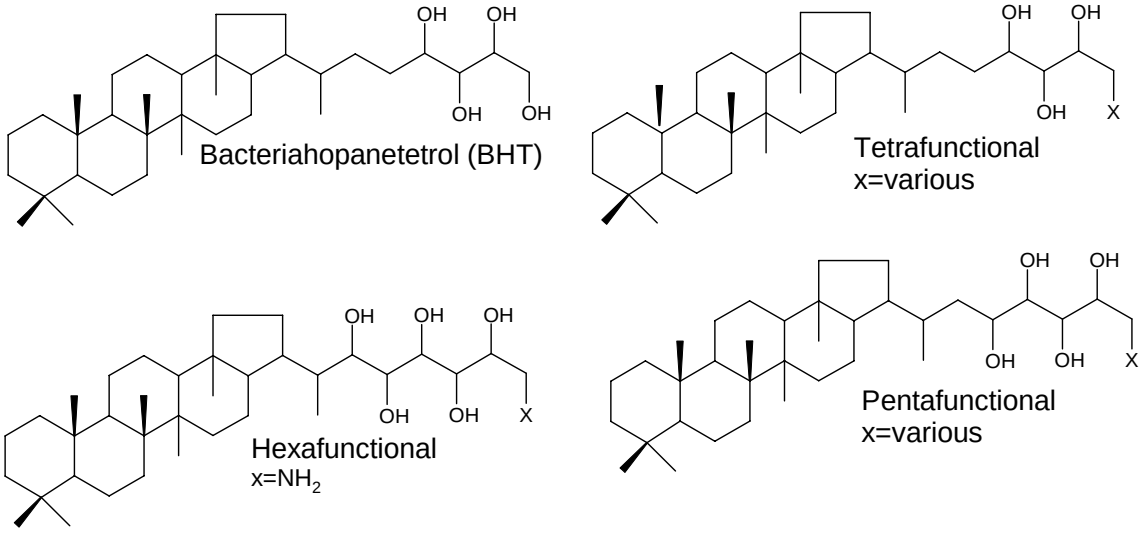

Figure 7.3 Biohopanoids

Different bacteria have distinct bacteriahopanepolyol compositions, affording these compounds the potential to act as indicators of past bacterial populations. Although the hydrocarbon skeleton of the biohopanoids are stable, sedimentary diagenesis converts the precursor compounds to geohopanoids, e.g. hopanols, hopanoic acids and hopanes as well as hopane biomarkers which are widely employed in petroleum studies (Figure 7.2). A summary of a few selected hopanoid biomarkers routinely analysed for in sediments is provided in Table 7.1. The analysis of biohopanoids is complex since hopanoid compounds with a greater degree of functionality than bacteriahopantetrol (BHT) are not amenable to analysis by GC. Thus, composite hopanoids with penta- and hexa-functionalised side chains are treated with acid and sodium borohydride to cleave the side chain and facilitate GC/MS analysis (Figure 7.3). In contrast, the generally smaller geohopanoids are readily extracted from sediments with organic solvents and compound class separation achieved by column chromatography with analysis of the saturate fraction by GC/MS in SIM mode. Such an approach may be useful in defining discrete populations of bacteria in bodies of groundwater, provided a method can be developed for extraction of target compounds from groundwater.

Table 7.1 Summary of routinely analysed hopanoids

Hopanoid	**Interpretation**
$C_{30\text{-hopanes}}$	diverse bacterial lineages
C_{31}-C_{35} (homohopanes)	diagnostic for bacteria
C_{32}-C_{36} (2α-methylhopanes)	diagnostic for cyanobacteria
28, 30-dinorhopane 25, 28, 30-trinorhopane	bacterial markers associated with anoxic depositional environments

Overall, the biomarker approach to understanding is probably not feasible within this project. The problems are manifold:

- Multiple sources of indicator compounds such as fatty acids and *n*-alkanes causing false positives
- Many of the techniques require large libraries of bacterial profiles to be in place before characterisation and identification can be performed
- Diagnostic bacterial biomarkers such as hopanoids have only been detected in about 30% of the examined eubacteria, thus representing a limited window on bacterial transport

- Diagnostic bacterial biomarkers such as C32-C36 (2α-methylhopanes) are only useful as indicators of limited environments unlikely to be relevant to nuclear repository storage issues.

7.4 NATURAL ORGANIC MATTER

A vast array of 'biomarkers' derived from soils, and the plant and microbial remains contained therein can be used as indicators of past climate and palaeovegetation. These include long chain *n*-alkanoic acids, such as C24, C26, C28 that are important components of epicuticular waxes of land plants, and true biomarkers such diterpenoids, characteristic of resins in the Pinaceae (Peters *et al*. 2004).

At the British Geological Survey, organic geochemists currently apply a variety of isotopic and molecular proxies to archaeological and geological soils/sediments to aid environmental reconstructions. These include the application of bulk measurements, Rock-eval pyrolysis, bulk ^{13}C isotopes, and atomic C/N ratios, as well as specific proxies such as *n*-alkane terrestrial to aquatic plant proxy (Lamb *et al*. 2005; Vane *et al*. 2005b). In sediments, the chemical characterisation of macromolecular plant molecules such as lignin and tannin can be used to indicate the vegetation source and microbial oxidation state of the plant derived polymer (Vane *et al*. 2005a). Although this work has been performed on particulate organic matter in soils and sediments, lignin-derived compounds have been recovered in dissolved form from natural waters using tangential-flow ultrafiltration, solid phase extraction and GC/MS analysis (Louchouarn *et al*. 2000).

7.5 TANGENTIAL-FLOW ULTRAFILTRATION AND CHEMICAL CHARACTERISATION OF DISSOLVED ORGANIC MATTER (DOM)

Dissolved organic matter (DOM) in both seawater and freshwater is comprised of a complex mixture which is a source of nutrients and energy for heterotrophic bacteria and an important part of the global carbon cycle (Benner 1991). One approach to the assessment of groundwater recharge is to characterise the DOM component of the waters. Bulk and molecular level characterisation of DOM can provide important information on specific sources of pools of organic matter, as well as pathways and mechanisms of transportation (Table 7.1) (Benner and Opsahl 2001; Raymond and Bauer 2001). Table 7.2 shows some of the analytical approaches for characterising DOM.

Recently, tangential-flow ultrafiltration has been successfully used to isolate DOM derived from seawater, river estuaries, surface water bodies as well as stream waters, prior to chemical characterisation by solid-state ^{13}C NMR, analytical pyrolysis and thermochemolysis (Frazier *et al*. 2005; Templier *et al*. 2005; van Heemst *et al*. 1999). Tangential-flow ultrafiltration concentrates organic molecules on the basis of size and does not require pH adjustments, which could modify chemical associations (Benner *et al*. 1997). Typically, 50 litre water samples are concentrated by a factor of 30-50 by passing the water through a membrane with nominal molecular weight cut-off of 1000 Daltons (pore size ~1 nm). The permeate (the <1 nm solution passing through the membrane) is classed as low-molecular weight DOM, whereas the 1-2 litres of retentate contains the high molecular weight DOM.

Characterisation of DOM from marine environments has shown that polysaccharides are the main component. Recent bulk (C/N; $\delta^{13}C$) and molecular level characterisation of DOM from a river-dominated estuary in South Carolina, USA, showed (on the basis of a variety of biologically derived compound groups, including lignin phenol distributions) that most of the material was derived from angiosperm and gymnosperm plant sources rather than salt marsh grasses (Goni *et al*. 2003). Molecular characterisation of DOM from streamwaters using plug-flow biofilm reactors and ^{13}C-labelled TMAH revealed ~100 compounds and bacterial demethylation of the lignin products (Frazier *et al*. 2005).

Given that techniques such as analytical pyrolysis and thermochemolysis with TMAH can be used to differentiate organic matter sources, it is clear that a combined tangential-flow ultrafiltration concentration method, followed by analytical pyrolysis or analytical pyrolysis with TMAH, may be appropriate to identifying the vegetation source and potentially elucidate palaeoenvironment/palaeoclimatic conditions using the recalcitrant pool(s) of organic carbon. Furthermore, the different sources of organic matter will give the latter differing chemical characteristics and, as a result, varying degrees of suitability for bacterial utilisation. If the majority of DOM is derived from surface sources and has not undergone extensive bacterial decay, ^{14}C dating will give a valid age for the water from which it was extracted (Raymond and Bauer 2001).

Table 7.2 Analytical approaches for characterising dissolved organic matter

Method	Technique	Information level	Analytical window
Elemental C/N	Destructive	General bulk	Wide
$\delta^{13}C$	Destructive	General bulk	Wide
Compound specific $\delta^{13}C$ GC-IR-MS	Destructive	Specific molecular	Limited
CuO oxidation GC-MS	Destructive	Specific molecular	Limited
Analytical pyrolysis GC-MS	Destructive	Specific molecular	Limited
TMAH thermochemolysis GC-MS	Destructive	Specific molecular	Limited
^{13}C NMR spectroscopy	Non-destructive	General molecular	Wide
Infrared spectroscopy	Non-destructive	General	Wide but insensitive

7.6 COMPARISONS WITH OTHER NON-BIOLOGICAL MARKERS AND TRACERS

Many inorganic substances have been used as water tracers but they must have high solubility at groundwater temperatures to be chemically stable for the duration of the tests. The most suitable conservative solutes are the halogen anions of I^-, Cl^- and Br^- which are often used in conjunction with particulate tracers (Behrens 1986). Chemical tracers alone may not reflect the movement of microorganisms. Conservative (non-reactive) tracers such as the halides can be used to monitor velocity, direction and dispersion of groundwater flow. Fluorescent and non-fluorescent dyes can also be useful in solute transport studies where they can be used to mimic sorbing chemicals (Sabatini and Austin 1991). However, dyes are affected by temperature, pH, salinity and turbidity, and the degree of fluorescence may vary depending upon the dye used (Ward *et al*. 1998). Microspheres as tracers are physically and chemically well defined and provide useful data on effects of cell size. Studies by Harvey and George (1989) showed that although transport behaviour of spheres differed from that of bacteria, they could nevertheless be useful in identifying preferential pathways in transport studies. Dissolved stable gases can also be used as tracers in transport studies. Experiments by Wilson and MacKay (1996) with suphur hexafluoride (SF_6), showed it behaved similarly to bromide but without problems of density changes associated with the gas dissolving; also, it did not react with aquifer material. However, because of gas volatility there may be problems with the preparation and injection processes in field studies.

8 Application of biomarkers to palaeohydrogeological investigations

8.1 INTRODUCTION

One major potential application of biomarker studies is in the field of palaeohydrogeology, which has been defined as:

".....a combination of observations on hydrochemical and isotopic differences in various groundwater zones or bodies, mineralogical data on the rock formations and the hydraulic properties of the same formations, which are then compiled to allow interpretations of the evolution of the rock-water system over long time intervals in the past"

(NEA, 1993; Bath *et al*. 2000a).

Palaeohydrogeology is of particular importance to safety assessment for the geological disposal of radioactive wastes (NEA, 2000; Bath and Degnan, 2005). It is one of two main sources of evidence (the other being natural analogues) that can be used to test or moderate confidence in hydrogeological and geochemical aspects of a long-term safety case for a geological repository. Whilst the time intervals of interest in palaeohydrogeology can range from the short timescales of anthropogenic influence to the very long timescales of geological processes, safety assessments of proposed repositories for the long-term storage or disposal of radioactive wastes must take into account scenarios for environmental change over the long period of time during which the waste will be a hazard, typically up to one million years into the future.

The scientific consensus in a number of countries is that disposing of long-lived and/or higher activity radioactive wastes and spent nuclear fuel deep underground in a 'geological repository' is the preferred option for long-term radioactive waste management. The reasons for preferring this option are (a) that the host rock for a deep repository ought to provide stable conditions for the proper performance of the engineered barrier system, and (b) the rock mass separating a repository from the surface environment acts as a further barrier to radionuclide migration. However, during the last two million years (the Quaternary Period), global climate has fluctuated between extremes of ice ages and warmer conditions than at present. During the Quaternary, large areas of northern Europe were periodically covered by ice sheets or experienced extensive permafrost in areas marginal to the ice sheets, whilst southern Europe was sometimes more pluvial (i.e. wetter). Consequently, the present-day climate is not representative of the climate that has existed for much of the Quaternary. This natural pattern of climatic fluctuation is expected to continue into the future, albeit modified by the impacts of anthropogenic greenhouse gas emissions. Variations in climate and in other environmental factors may affect future movements and compositions of groundwaters in the vicinity of a repository (e.g. by changing flow gradients, recharge, permeability, salinity, redox, organic flux) and thus affect the mobility of radionuclides and the rate of their migration back to the surface. It could be argued, therefore, that present-day groundwater conditions may not be an adequate basis for assessing long-term repository safety. However, if it can be demonstrated that, despite significant environmental change at the surface, groundwater flows and compositions at depth remain stable or change in a way that does not impact significantly on safety, then confidence in repository concepts for disposal will be increased. Palaeohydrogeological studies are therefore very valuable in understanding how groundwater systems have responded to major climatic changes during the Quaternary, and therefore, how they might be expected to respond to future climatic change.

8.2 PALAEOCLIMATIC RECONSTRUCTION

Although little direct use of biomarkers has been made in palaeohydrogeological studies, they have found indirect application by contributing to palaeoclimatic interpretations, which have subsequently fed into estimates of water balance (e.g. recharge, discharge) used in the modelling of past groundwater flow regimes. For example, biomarker information has been used in palaeohydrogeological investigations undertaken on behalf of the Spanish radioactive waste management company, Empresa Nacional de Residuous Radioactivos SA (ENRESA), through their participation in the European Union 4th Framework and 5th Framework projects, EQUIP (Bath *et al*. 2000b) and PADAMOT (Bath and Degnan 2005; Milodowski *et al*. 2005). These two projects made a detailed study (carried out by the Madrid School of Mines, Universidad Politécnica de Madrid) of biomarkers in the Padul Peat Bog, located 20 km south of Granada city, in Andalusia, southern Spain.

Padul Peat Bog is an intermontaine basinal discharge area at the foot of the Sierra Nevada. It preserves a continuous Quaternary sequence (approximately 100 m thick) of alluvial (gravels), lacustrine (marls), fluvial (sands and gravels) and palustrine (massive peats) sediments that have accumulated over the past 10,000 years. The object of the study was to use biomarkers to elucidate palaeoenvironmental information (in terms of vegetation changes and variations in hydrological inputs) which could be applied as boundary conditions in modelling the palaeohydrogeological behaviour of the deep groundwater system at the Los Ratones Uranium Mine study site in the southwest of Spain.

The Padul Peat Bog study utilised a range of fossil pollen, biomarkers and other organic geochemical markers as proxies for climate change (see also Ortiz *et al*. 2004). In particular, the concentration of total organic carbon, H/C and C/N ratios of organic matter, $\delta^{13}C$ stable isotope characteristics or organic matter, carbon preference index (CPI) and the *n*-alkane predominance were utilised as detailed below.

8.2.1 Total organic carbon (TOC)

The concentration of total organic carbon (TOC) represents the proportion of organic matter that escaped mineralisation during sedimentation, and is influenced by both the initial production of biomass and subsequent degree of degradation. TOC therefore integrates organic matter of different origins. The variation in TOC, in conjunction with sedimentological information, can be interpreted in terms of basinal palaeohydrology: low TOC corresponding to deep water (lacustrine) conditions; high TOC corresponding to palustrine (peat bog) conditions.

8.2.2 Organic matter H/C ratios

The H/C ratio of organic matter was used to provide information on the origin of the organic matter, and this in turn was used to infer changes in lake level and water inputs to lacustrine environments from the type of organic matter accumulated (Talbot and Livingstone, 1989). Algal- and amorphous-derived organic material have H/C > 1.7, and sediments with such H/C ratios reflect organic inputs dominated by organic production within a lake environment. In contrast, herbaceous remains have H/C values between 1.3 and 1.7, whilst woody material is dominated by polycyclic aromatic compounds with even lower H/C ratios of between 0.8 and 1.3. Sediments with these H/C signatures would reflect significant organic input brought into the lacustrine environment from terrestrial sources (i.e. river input or terrestrial run-off).

The C/N ratio provides an indicator of the protein content in the organic matter:

- Benthic organisms and bacteria have C/N ratios around 4.2 and 4.1, whereas in planktonic organisms the C/N ratio can vary between 4 and 7
- Fresh organic matter from lake algae has C/N values ranging between 4 and 10, whereas vascular land plants usually have C/N ratios of 20 and greater (Hedges *et al*. 1986)

- C/N ratios between 30 and 40 are characteristic of herbaceous plants, whereas C/N values of 12-17 suggest a mixture of algal and vascular plants
- Together with the carbon isotope composition, the C/N values can be used as an indicator of the sources and nature of organic matter
- $\delta^{13}C$ values between -20 and -30 ‰ can be indicative of freshwater phytoplankton (Galimov 1985)
- C3 land-plants (trees, shrubs and cold climate grasses) have a common range of $\delta^{13}C$ values from -23 to -31 ‰ (O´Leary 1981)
- Land plants that use the C4 (Hatch-Slack) photosynthetic pathway (i.e. grasses and sedges) have $\delta^{13}C$ values between -9 and -17 ‰.

In the Padul Peat Bog study, four categories of organic matter could be distinguished on the basis of carbon isotope composition and the C/N ratio, and these could be interpreted in terms of their palaeoenvironmental setting as

- Group 1 (algae-increasing water input)
- Group 2 (mixed origin: algae and C3 plants-less wet conditions)
- Group 3 (algae, and C3 and C4 land plants- warm and dry conditions)
- Group 4 (C3 land plants).

This enabled periods of wetter or drier climate to be inferred from the Padul sedimentary sequence.

8.2.3 Biomarker molecules

The variations in the *n*-alkane distribution were studied as part of the evaluation of the principal sources of biological organic matter in the Padul Peat Bog. Three main sources of biological organic matter contribute *n*-alkanes to the lacustrine environments, and are characterised by different *n*-alkane compositions (Eglinton and Hamilton 1963; Gelpi *et al*. 1970; Cranwell 1984, 1973; Cranwell *et al*. 1987; Schwark *et al*. 2002):

- Algae. These are dominated by low molecular weight *n*-alkanes, with a maximum at C17
- Aquatic macrophytes
- Submerged/floating macrophytes with *n*-alkane maxima at C21, C23 and C25
- Emergent macrophytes with *n*-alkane distributions similar to that of terrestrial plants, with maxima at C27 and C29
- Vascular plants around the lake margin. These are characterized by higher molecular weight *n*-alkanes, with maxima at C27, C29 and C31.

Together with other palaeoenvironmental proxies, the variation of the *n*-alkane predominance over time through the Padul sequence was used to infer the origin of the organic matter and in turn, as a proxy indicator of climatic variations (Ortiz *et al*. 2004).

8.3 BIOMARKERS IN DEEP GROUNDWATER SYSTEMS

The PADAMOT study (Milodowski *et al*. 2005) also undertook a very limited investigation of the potential for biomarkers within deep groundwater systems. These authors did not look at the groundwaters directly but sought evidence for the presence of biomarkers preserved within late-stage calcite fracture mineralisation. Samples of calcite mineralisation that was closely associated with the present-day deep fracture-controlled groundwater systems (up to 2000 m

deep) were examined (a) from granitic rocks from the Äspö-Laxemar area (southeast Sweden), (b) from Triassic sedimentary and Palaeozoic low-grade metamorphic volcaniclastic strata from Sellafield (northwest England), (c) from Devonian sedimentary and gneissic basement rocks from Dounreay (northern Scotland), and (d) from a shallow fresh groundwater system in Carboniferous Limestone in the English Midlands.

The calcite mineralisation was hand-separated from high-quality drillcore samples, and carefully cleaned by etching with hydrochloric acid to remove any potential surface organic contamination (e.g. from drilling fluids and subsequent sample handling). Biomarker compounds were extracted by acid dissolution of the calcite and analysis by GC-MS. Although the calcites furnished traces of organic compounds, only a few such compounds were found, and in many cases, they were present in only very low concentrations that were close to the detection limits of the analytical technique. However, the compounds identified from the calcites included octadecanoic acid, hexadecanoic acid, cyclopentane, isoquinoline and 1,2-benzenedicarboxilic acid and pyrrolo[3,2-a]dibenzo-furan. Many of these compounds cannot be regarded as definitive biomarkers since they may also be produced by abiological processes as follows:

- Hexadecanoic and octadecanoic acid (and other carboxylic acids) can be derived from plants and animals, and their presence could indicate that the calcite grew from groundwater that had organic matter derived from plant origin (i.e. picked-up if the groundwater had re-charged through a soil cover). However, these compounds may also have been produced *in-situ* through microbiological degradation of organics and decomposition of microbial cell walls.
- Quinoline and isoquinoline are alkaloids which are found in plants from a wide group of genera.
- 1,2 benzenedicarboxilic acid (phthalic acid) is a carboxylic acid, and its origin in these calcites is uncertain. Carboxylic acids can be derived by the complete oxidation of primary alcohols, but this type of 'biomarker' has also been found in Pleistocene and Holocene speleothems from the north of Spain (Prof. T. de Torres, personal communication, 2005).
- Cyclopentane belongs to the cyclo-alkane group of hydrocarbons, the most common of which are the cyclohexane and cyclopentane series. No more specific details have been found about the origin of these compounds, but they are usually involved in the structure of plant material.
- Dibenzofuran is a three-ring polycyclic aromatic furan. It is released to the environment in atmospheric emissions involved with the combustion of coal, biomass, refuse, and diesel fuel. Wastewater emissions can occur from coal tar, coal gasification, and oil operations. Potentially this could have been derived from anthropogenic contamination or hydrocarbon cracking.

Despite the non-specificity of most of these compounds, the PADAMOT investigations did successfully demonstrate the potential for biomarker preservation in late-stage calcite mineralisation associated with modern groundwater systems. One of the major limiting factors in this study was the amount of calcite available for analysis. In most cases, the late-stage fracture-lining calcites occurred only as thin film-like overgrowths on older calcite mineralisation, or as sparsely distributed, scattered crystals on fracture surfaces. This meant that it was either extremely difficult to separate physically from the older calcite substrates, or only very small amounts of material could be collected from the limited disseminated material presented in core samples.

In addition to organic compounds, the PADAMOT study also identified (in the late-stage fracture calcite) the rare presence of grass pollen grains in fracture calcites from shallow depths in the Laxemar site (Figure 8.1). This clearly demonstrates that material of biological origin can be transported by groundwater in the fracture system and can be incorporated in fracture

mineralisation, at least at shallow depths, and that the groundwater (from which the calcite grew) must have been recharged through the soil zone. Other studies of fracture calcites from the Äspö-Laxemar area have found evidence for the preservation of bacteria and bacterial biofilms preserved beneath the calcite mineralisation (Pedersen *et al*. 1997; Tullborg *et al*. 1999). Again, this suggests that it may be possible to find evidence for biomarkers and biofilm preserved in fracture mineralisation in groundwater systems.

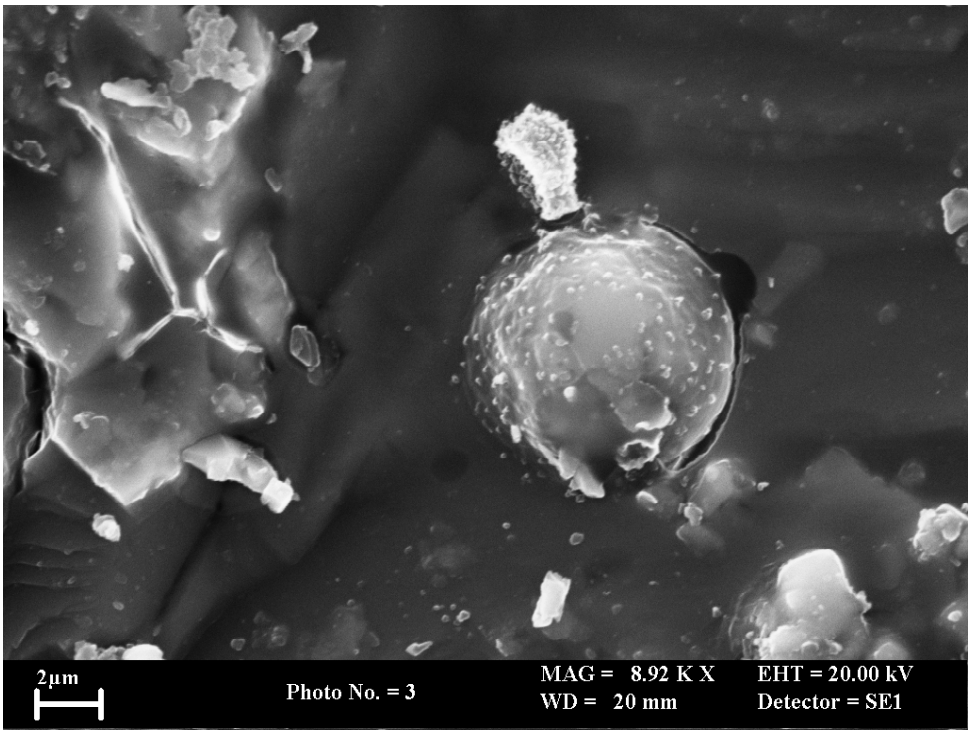

Figure 8.1 SEM image showing a grass pollen grain (ca 5 µm in diameter) enclosed within late-stage fracture calcite, Laxemar, Sweden (from Milodowski *et al*. 2005).

9 Summary and Recommendations

Many subsurface environments (particularly aquifers) have the capacity to support an indigenous microbial population and their activity and interactions can have both physical and chemical effects. The results and recommendations of the reviews in this document can be summarised in two overall areas:

- Transport processes
- Biomarkers

9.1 PROCESSES INFLUENCING TRANSPORT OF MICROBES IN THE SUBSURFACE

The transport of microorganisms through the subsurface is governed by a number of factors which fall into two main categories; firstly, hydrogeological or abiotic factors and secondly, characteristics of the microbes which influence their potential for activity and survival (Maier *et al.* 2000). It is the interaction between these sets of factors, summarised in Section 3 of this review, which will determine the extent of the transport process.

This review has shown that the factors, which appear to have most effect on transport, are:

- Size and electrostatic properties of the microbe
- Groundwater flow velocity
- Particle size and pore space of substratum
- Survival stress
- Growth rates of microbes

Other factors, which potentially affect microbe fate and transport such as pressure, predation and the presence of nutrients, are less significant.

9.2 THE ROLE OF MICROBES IN SUBSURFACE TRANSPORT PROCESSES

Microbes can mediate the production of new minerals either by direct adsorption, co-precipitation or encasement or indirectly as a result of redox reactions. Some are capable of growth in oxic and anoxic environments and can switch target ions in growth limiting conditions. Microbes are also able to solubilise metal sulphides creating environmentally undesirable acidic and often toxic outflows from disused industrial sites. Some microbes preferentially grow biofilms and are able to create microenvironments within their voids and channels to promote growth and increase corrosion rates when grown on iron-containing substrates. Biofilms can also trap particles and even dead and dying bacterial biofilms are capable of creating redox barriers by consuming available oxygen and maintaining low redox potentials.

9.2.1 Recommendations for further work

There have been numerous attempts to model microbial growth in subsurface environments and its effects on contaminant transport in groundwater. These models are grouped in this report into a number of categories depending upon sophistication and the nature of the processes that they try to represent. However, it is not possible within the scope of this project to identify any single

'microbial model' for development. Rather, it is important to develop experiments to examine a wide range of parameters.

Modelling of the mechanisms of microbially mediated redox reactions has been researched extensively, and for simplicity, laboratory based experimental work have used single strains of microbes and pure minerals. The effects of a rapid change pH or ionic strength and valency on established biofilms are, however, less well understood. It is therefore recommended that future work should develop ideas from the REX project particularly by growing biofilms of indigenous bacteria in suspensions of natural groundwater on a specific substrate e.g. diorite. This would expand current knowledge of the effects of rapid changes in pH and ionic concentration on the biofilm and a wide range of parameters would need to be examined. The four main factors requiring further investigation are:

- Microbial growth on mixed naturally occurring substrates
- Effects of changing pH and ionic strengths in groundwaters
- Effects of mixed microbial strains on biofilm formation
- Limiting growth parameters

Future studies looking at biofilm development on mineral surfaces should examine physico-chemical processes and attempt to determine biofilm structure and chemical interaction with mineral surfaces. The recommended work can be achieved by the use of a flow cell similar to that used in the recent Natural Environment Research Council 'Micro to Macro' studies (Brydie *et al.* 2005). The effects on a mineral substrate and biofilm development can be monitored using a confocal scanning laser microscope. This flow cell will also enable the monitoring of effects of changing parameters such as pH and microbial strains on biofilm integrity and the possible impacts on transport processes.

9.3 BIOMARKERS AND RECOMMENDATIONS

9.3.1 Use of biomarkers

The review has shown that there are numerous studies of the transport of microorganisms in the subsurface, both in field studies and in the laboratory, which contribute to a better understanding of transport behaviour. Most of the laboratory work using bacteriophage or bacteria has involved column studies, both flow-through and static column, which give a greater degree of control and can provide detailed information on transport mechanisms. However, column studies are often unable to give accurate information on the combined effects that control mobility *in situ.* The ideal solution is to combine laboratory studies, which can look at individual parameters, with *in situ* studies, using tracer tests, which consider hydrogeological characteristics of the aquifer. Much of the literature review seems to suggest the use of the phage PRD-1 and MS-2 as recommended tracers in combination with conservative tracers in transport studies.

9.3.2 Laboratory methods and recommendations

The design of laboratory studies on transport processes using columns will need to consider:

- Column material
- Column length and diameter
- The selection of matrix material
- Packing (carefully and uniformly to minimise macropores)
- Pump flow (Saturation of matrix and flow rate)

- Sampling (destructive/non destructive)
- Selection of microbe/tracer (conservative + non-conservative)
- Fluid composition
- Temperature
- Atmosphere

Studies have generally been concerned with bacteriophage transport through sand and gravel. Thus further work to investigate bacteriophage transport through fractured rocks is recommended particularly under conditions of rapidly changing ionic pore water composition.

9.3.3 Field methods

In situ studies would complement any laboratory experiments although these are more complicated, both technically, and in the interpretation of results. Conservative (non-reactive) tracers are typically used to monitor velocity and direction or path of groundwater flow. The characteristics of the breakthrough curve can then be used to determine major transport parameters. Retardation of microorganisms and reversible interactions with the matrix can be determined by comparing velocities of peak concentration of the conservative tracer with introduced microorganisms. Physical heterogeneity of the system increases the differences between the breakthrough curves for conservative and microbial tracers. Microspheres as tracers are used to study the effect of cell size in sandy aquifer sediments.

Some of the tests that can be used for in situ studies include:

- Forced-gradient tests – high-volume pumping at point of injection, the point of withdrawal, or both: controls flow field. These have been used in granular aquifers to study the transport of bacteria, viruses and yeasts. The disadvantage of these tests is that the flow field is non-uniform and therefore more difficult to model (Harvey and Harms 2001).
- Divergent tracer tests – the addition of a known quantity of microorganisms and conservative tracer into a continuous stream of groundwater being injected back into the aquifer. Because of the high degree of forced dispersion, the distance over which tracer can be monitored is limited.
- Convergent tests – continuous withdrawal at the sampling well. These have the advantage in that the entire mass of conservative tracer can be withdrawn at a sampling well allowing true mass balance calculations to be carried out on conservative tracer and microorganisms.
- Natural gradient tests – a tracer slowly added to aquifer and natural flow of groundwater advects this past rows of multilevel samplers. These are best suited to sandy aquifers where flow paths can be more easily predicted. (Pickens *et al*. 1978 and Smith *et al.* 1991).

In summary, the quantity of microorganisms needed for tracer tests depends upon the type of injection test to be run, travel distance, type of microorganism and aquifer characteristics. Generally, divergent tests require the largest number of microorganisms and natural gradient experiments the smallest number. Initial tests run with conservative tracers can provide flow and dispersivity information which can help in calculating volumes of microorganisms and tracers for subsequent tests.

9.4 APPLICATION OF ORGANIC BIOMARKERS IN PALAEOHYDROGEOLOGY

9.4.1 General concepts

Biomarker studies potentially could find major applications, as tracers, in the study of groundwater flow paths and in palaeohydrogeological investigations of groundwater systems. To

date, very little use of biomarkers has been made in this field. Their potential use in palaeohydrogeological studies was investigated, to a very limited extent, during the recent PADAMOT Project (Milodowski *et al*. 2005). This demonstrated that potential biomarker compounds could be preserved and analysed for in late-stage fracture calcite in present-day groundwater systems. However, the analysis of late stage calcite mineralisation for biomarkers presented a number of significant technical difficulties:

- Late-stage calcite is often present only in very small quantities, and consequently it is difficult to obtain sufficient material for analysis
- Late stage calcite may occur as fine overgrowths or coatings on older mineralisation, making it difficult to separate and analyse in sufficient quantity
- The concentration of biomarker compounds in calcite is very low, and a relatively large sample size must be processed to overcome analytical detection limits.

A more promising approach, in the first instance, would be to look for the presence of biomarkers in groundwaters rather than in the minerals precipitated from the groundwater. This has a number of advantages:

- It is easier to collect large volume samples of water
- Water samples can be relatively easily processed to concentrate biomarkers for analysis
- Water samples are easier to analyse, compared with the careful separation and processing of individual mineral generations from complex fracture mineralisation.

However, there are also shortcomings that must be taken into account in the elucidation of palaeohydrogeological information directly from groundwaters as compared to mineralogical observations:

- Groundwater geochemistry may represent a transient state or 'snapshot' of an evolving system. Whereas, minerals precipitating from the groundwater may preserve evidence of the changing geochemical environment as variations in the chemical and isotopic composition of their crystal growth zones (analogous to growth rings in trees), or may preserve microscopic samples of the groundwater as fluid inclusions trapped in the growing crystals.
- Evidence of earlier groundwater events may be lost as groundwaters are flushed or displaced by successively younger groundwaters. In contrast, information recorded by minerals precipitated from the groundwater can preserve a more complete record of palaeohydrogeological events, since once precipitated it is generally more difficult to remove minerals by dissolution.
- Present groundwater chemistry may be complicated and difficult to interpret because it may result from mixing of groundwaters from different sources and/or of different ages. In contrast, mineralisation may record these changes in different stratigraphic layers, alteration zones, or corrosion bands, as minerals precipitate or dissolve in response to changing water chemistry.

Therefore, the ideal goal would be to try to obtain biomarker information from mineral precipitates, so that a more continuous palaeohydrogeological evolution can be followed. However, this is considered to be impractical with the detection limits of the present technology, except in unusual circumstances where a large mass of mineralisation may be available for analysis. Currently, the most practical and promising option is to analyse groundwaters for the presence of biomarkers. This potentially could provide tracer information to evaluate flow paths, mixing and compartmentalisation of water masses that would have more general applications in groundwater studies, as well as providing insights into the palaeohydrogeology of groundwater systems.

9.4.2 Potential study area

Testing the concept of using biomarkers as palaeohydrogeological indicators will require a well-characterised site with two or more compartmentalised groundwater bodies, and/or a palaeohydrogeological history constrained by independent methods, involving groundwaters of different origins. In addition, for the purposes of this study, it will be necessary to be able to have easy access to sample the groundwaters.

The Äspö-Laxemar area on the Simpvarp Peninsula of the Baltic coast of south-eastern Sweden provides a potentially suitable area for this study. This area has experienced major changes in climatic state during the Quaternary, during which groundwaters of markedly different origin were potentially recharged (Milodowski *et al*. 2005). In 1994, the Swedish Nuclear Fuel and Waste Management Company (SKB) completed the construction of an underground rock laboratory (URL) facility to a depth of 500 m in Precambrian fractured granitic basement beneath the island of Äspö (SKB 2003). The Simpevarp peninsula is also currently being studied by SKB as part of their on-going site investigation programme for a potential geological repository for radioactive wastes. This involves the drilling of boreholes to a maximum depth of 1700 m within the area which has enabled both rock and groundwater samples to be taken. Within the URL and the Simpvarp site investigation area, hydrogeological zones with different groundwater types can be identified, indicating localised or compartmentalised variations in the groundwater system.

Hydrochemical interpretations have distinguished several different groundwater components. Modelling and interpretation of the groundwater chemistry has shown that several events have contributed to the distribution and composition of the present groundwater:

- Meteoric water of present climate and cool climate (probably glacial melt water)
- Baltic Sea water; present and probably also ancient
- Brine type water with very long residence time

These studies have also shown that mixing of these waters, bacterially mediated reactions (e.g. sulphur, iron and manganese reduction), ion-exchange, and calcite precipitation/dissolution have taken place in the Äspö groundwater. The palaeohydrogeological evolution of the groundwater system has been interpreted as follows:

- Deep recharge by glacial melt water, possibly from several glaciations, (earlier than 14 000 years BP) due to the high hydraulic heads caused by the ice cap, and mixing at considerable depth with saline, brine-type groundwater.
- Marine waters were introduced when the area was inundated by the Yoldia Sea (palaeo-Baltic Sea) by density turnover (inversion) of the water column(11 500 to 10 800 years BP).
- Uplift and establishment of freshwater recharge conditions during the Ancylus Lake stage of the Baltic region. During this stage a mixture of glacial meltwater and fresh meteoric water mixed with and displaced saline and brackish groundwaters (10 800 to 9 500 years BP).
- Subsequently, Baltic Sea water was introduced by density turnover during the Littorina Stage of the Baltic region (9 500 years BP to present).
- Subsequently, when Äspö rose above sea level, due to isostatic recovery following de-glaciation, meteoric water was recharged and mixed with the earlier Baltic Sea- and glacial-brine mixed waters.

9.4.3 Recommendations for further studies

The Äspö URL facility provides access to completed boreholes within different hydrogeological/fracture flow zones from which groundwater samples can be readily taken. In addition, it may be possible to sample waters from the wider region in the site investigation programme boreholes. However, in the latter case, this may prove more difficult due to strict site investigation scheduling and quality control constraints on sample collection.

It is proposed for the BIOTRAN project that 3 to 4 sites be sampled from the Äspö URL. These locations would ideally be taken from potentially compartmentalised flow zones of different groundwater types. The location and identity of these zones would be defined on the basis of information available from, and in liaison with SKB. The objective would be to analyse these waters for biomarker compounds, and to try to determine whether:

- the different groundwater types can be differentiated on the basis of different biomarker compounds
- the presence of biomarker compounds can be correlated with different climatic state or recharge origins.

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Appendix 1

DESCRIPTION OF BACTERIOPHAGE PLAQUE ASSAY METHODOLOGY

Techniques for the detection of bacteriophage in groundwaters are well documented and are by plaque, areas of cell death of host bacterium, formation technique. Where soils or solid subsurface samples are used, bacteriophage can be eluted from matrix particles and the eluate can then be assayed (Hurst 1997). The following summarises the basic methods used for groundwaters.

There are two general types of assay methods; firstly a traditional double agar layer (DAL) technique (USEPA 2001 Method 1601, Hersey *et al.* 1943) and secondly a single-agar-layer (SAL) technique (USEPA 2001 Method 1602. Eaton *et al*. 1995).

A brief summary of the basic double agar layer procedure is as follows:

1. Prepare tubes of melted low concentration top agar (e.g. 5-8 g/litre Tryptone) and place in 44.5˚C water bath.
2. Label pre-poured Petri plate containing lower layer of high concentration selective agar (10 to 15g/l) at room temperature.
3. Add 0.1ml of 4hour culture of host bacterium to top agar tube Add 1.0 ml of test sample to tube and mix.
4. Pour tube onto bottom agar plate.
5. Invert and place in incubator at ~36.5˚C for 16-24 hours*.
6. Examine for plaques and record results.

A brief summary of basic single agar layer procedure is as follows:

1. Place flasks with 100 ml sample and two 12.5 ml volumes of sterile water into 44.5˚C water bath.
2. After 3 minutes add calcium chloride solution (to negative control add 1ml tryptone broth, for positive control and 1 ml of coliphage preparation).
3. Add 5 ml of 4-hour host bacterium followed by agar. Swirl to mix.
4. Pour into 8 large Petri dishes; pour 1 dish for each control.
5. Invert and place in incubator ~36.5˚C for 16-24 hours[1].
6. Examine for plaques and record results as plaque forming units (PFU)[2].

Stock bacteriophage suspensions can be prepared by following the method summarised by Hurst (1997), adapted from Hersey *et al*. (1943). In this method a DAL assay is performed, a broth medium is then flooded onto the dishes and after an incubation period the medium containing the suspended phage is filtered to remove contaminating bacteria. The bacteriophage stock solution can then be stored under refrigeration; this stock solution should not contain tetrazolium dye.

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[1]The incubation temperature used for bacteriophage plague formation assay will depend upon the bacterial strain used and the bacteriophage being sought.

[2]The addition of tetrazolium dyes (>150µg/ml) to the agar can aid visualisation of the phage plaques, live cells in the bacterial lawn will appear coloured whilst plaque areas contain oxidised dye and will appear relatively colourless.

Appendix 2

FURTHER PHAGE METHODOLOGIES

Blandford *et al.* (2005)

PRD-1 by plaque counting technique, described in Bales *et al.* (1991)

Deborde *et al.* (1999)

Bromide by Dionex IC using AS4A column according to method by Pfaff (1993)

MS-2, PRD-1, Ø174 by host bacteria specific to the virus, single layer plaque method as described in Deborde *et al.* (1998)

Gitis *et al.* (2002)

MS-2 using *E.coli* as host bacterium and double layer plaque assay.

MS-2 labelled with fluorescein and DMF as described in Banks and Paquette (1995) or fluorescent labelling with Rhodamine B and sodium fluorescein with D.E.C., both methods purify with membrane dialysis.

Harden *et al.* (2003)

Rhodamine WT and fluorescent dyes by fluorimeter.

PRD-1 using *Salmonella typhimurium* as the host bacterium and soft agar overlay technique. Plaque forming units were calculated using the Standards Methods for Examination of Waste and Water, (Greenberg *et al.* 1992).

Sinton *et al.* (2000)

Rhodamine WT by fluorospectrophotometer.

Bacillus subtilis assay by membrane filtration (Sartorius cellulose nitrate, 0.45µm) on tryptone, glucose and rifampicin, Houston *et al* (1989).

E.coli 2690 by membrane filtration, (Sartorius cellulose nitrate, 0.45µm) on Difco mFC agar.

E.coli J6-2 by membrane filtration (Millipore HA, 0.45 µm) on macConkey agar, Sinton (1980).

F-RNA phage MS-2 using *Salmonella typhimurium* and double layer overlay method, Adams (1959). Plaque counts made against diffuse white light.

Glossary

Abiotic	Refers to nonliving basic elements and compounds of the environment
Acidophilic	Organisms tolerating low-pH environments
Aerobic action	A biological process promoted by action of bacteria in the presence of dissolved oxygen
Aerobe	An organism which grows in the presence of oxygen; may be facultative or obligate.
Alkaliphilic	Organisms tolerating high-pH environments
Alluvial	The product of sedimentation by bodies of fresh water
Ammonification	Nitrogen, in organic form, is converted by microorganisms into ammonium (NH_4^+)
Anaerobic action	A biological process promoted by action of bacteria in the absence of dissolved oxygen
Anabolism	The biochemical processes involved in the synthesis of cell constituents from simpler molecules, usually requiring energy
Anaerobe	An organism which grows in the absence of oxygen
Anthropogenic	Pertaining to the effect of human beings on the natural world
Aquifer	A formation capable of storing or transmitting water in significant or economic quantities.
Autotroph	Organisms able to utilise carbon dioxide as a sole source of carbon
Bacteriophage	Viruses of bacteria
Biodegradable	Capable of being broken-down by living organisms
Biofilms	Monolayers or multilayers of bacterial cells that are bound to surfaces by extracellular polysaccharides (EPS).
Biogenic	Material produced by the action of living organisms
Biomarker	Biological markers are organic molecules whose structure is identical to or directly derived from a precursor compound that was incorporated into the geological environment during normal sedimentary or hydrogeological processes.
Bioremediation.	Use of biological organisms to remove or detoxify pollutants from a contaminated area.
Biosphere	That part of the earth which is inhabited by living things
Brownian motion	The random movement of microscopic particles suspended in a fluid
Catabolism	The biochemical processes involved in the breakdown of organic compounds, usually leading to the generation of energy
Chemoautotroph	An autotrophic organism obtaining energy from the oxidation of inorganic compounds
Chemolithotroph	An organism that uses an inorganic compound as an energy source
Chemotactic response	Movement of microbes towards beneficial substances or away from inhibitory substances
Coliforms	Gram-negative, nonsporing facultative rods that ferment lactose with gas formation within 48 hours
Cyanobacteria	Blue-green bacteria which perform oxygenic photosynthesis

Cyst	A resting stage formed by some bacteria and protozoa in which the whole cell is surrounded by a protective layer
Denitrification	Conversion of nitrate to nitrogen gases under anaerobic conditions, resulting in loss of nitrogen from ecosystems
Denitrifiers	Microorganisms which use nitrate as their terminal electron acceptor
DOM	Dissolved organic matter
Electron acceptor	A substance which accepts electrons in an oxidation-reduction reaction.
Electron donor	A substance which donates electrons in an oxidation-reduction reaction.
Endotoxin	A toxin released from the cell
Enteric	Intestinal
EPS	Extracellular polysaccharide
Eucaryote	A cell or organism having a true nucleus
Eutrophication	Nutrient enrichment of natural waters, usually from artificial sources.
Exotoxin	A toxin released extracellularly
Facultative	A qualifying adjective indicating that an organism is capable of growth either in the presence or absence of an environmental factor
Filter medium	The material of which the biological filter is formed and on which a biological film containing bacteria and fungi develops
Gram negative	A prokaryotic cell whose cell wall contains an outer membrane composed of lipopolysaccharides, lipoprotein and other complex macromolecules
Gram positive	A prokaryotic cell whose cell wall consists chiefly of peptidoglycan and lacks the outer membrane of gram negative cells
Half-life	The half-life of a microorganism is the time required for the population to decrease to half of its initial value.
Heterotroph	Organism obtaining carbon from organic compounds
Hopanoids	A class of pentacyclic triterpenoid lipid biomarkers
Host	An organism capable of supporting the growth of a virus or parasite
Hydrostatic pressure	Force which acts upon a rock due to the mass of superincumbent material
Isoelectric point	The pH at which a molecule carries no net electrical charge.
Lanthanides	A group of fifteen closely related elements and are known as the rare earth or "inner transition" elements
Lysis	Destruction of cells through damage to or rupture of plasma membrane, allowing escape of cell contents
Methanogenesis	The formation of methane by microorganisms or natural volcanic processes
Mixed liquor	A mixture of sewage and activated sludge undergoing circulation and aeration in the aeration tank or channel of an activated sludge plant
Mixotroph	An organism able to assimilate organic compounds as carbon sources while using inorganic compounds as electron acceptors
MPN	Most Probable Number - A statistical expression providing a measure of cell number in a population
Nitrification	The conversion of ammonium to nitrate
Nitrogen fixing	Reduction of nitrogen gas to ammonium
Nutrient	A substance taken into a cell from its environment and used in catabolic and anabolic reactions

Obligate	A qualifying adjective referring to an environmental factor always required for growth
Oligotrophic	Describing a body of water in which nutrients are in low supply
Paleohydrogeological	The study of groundwater during past geological time
Pathogen	An organism capable of inflicting damage on a host it infects.
pH	An expression indicating the hydrogen-ion concentration of a solution
Phage	Viruses of bacteria
Plaque	A localised area of virus lysis on a lawn of bacteria
Protoplasmic	Substance within and including plasma membrane of a cell
Protozoa	Eukaryotic microorganisms
Prions	Proteinaceous infectious particles that infect animals
Py-GC-MS	Pyrolysis-Gas Chromatography-Mass Spectrometry
Redox	Any chemical reaction which involves oxidation and reduction.
Sulphate reducers	Microrganisms which use sulphate as their terminal electron acceptor
Species	A collection of closely related strains
Spore	A general term for resistant resting structures formed by many bacteria and fungi
TMAH	Tetramethylammonium hydroxide
TOC	Total organic carbon
Toxigenicity	The degree to which an organism is able to elicit toxic symptoms
Toxin	A microbial produced substance capable of inducing damage to a host
Tracer	An identifiable substance, such as a dye or a radioactive isotope, that is introduced into a biological or mechanical system and can be followed through the course of a process, providing information on the pattern of events in the process or on the redistribution of the parts or elements involved.
Van der Waals forces	Intermolecular attractions between one molecule and a neighbouring molecule.
Vector	An agent, usually an insect or other animal, able to carry pathogens from one host to another
Viable count	Measurement of the concentration of live cells in a microbial population
Virus	A genetic element that is able to alternate between intracellular and extracellular states, the latter being the infective state
Water table	The level below which the ground is saturated with water.

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