Enhanced Natural Attenuation of Organic Chemical Contaminants in Groundwater

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Dedicated to

My parents for their endless love and inspiration

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Summary

This thesis presents an investigation into the enhanced natural attenuation of organic chemicals in groundwater. The focus of the research was directed towards the organic chemicals benzene, ethylbenzene, styrene, 1,2-dichloroethane and vinyl chloride, which were found to exist at a contaminated site located in the United Kingdom.

A conceptual model was developed for the study site, based on the findings of previous consultant studies and investigations which were carried out solely for the purposes of this research. Sample collection and analytical procedures were designed to yield results, which could further define contamination issues at the study site, so that remedial measures could be appropriately developed.

Lab-based microcosm treatability studies were carried out in order to determine the degradation potential of DCA, benzene and ethylbenzene under various enhanced conditions. The enhancements were selected in order to facilitate different degradation mechanisms for the organic contaminants, including aerobic respiration and reductive dechlorination. The microcosms were monitored for chemical and microbiological changes.

A hydrogen release compound (HRC®) treatment was found to effectively degrade DCA and benzene under anaerobic conditions. A microorganism containing genetic sequence similarities with *Desulfitobacterium dichloroeliminans* bacteria was detected within the HRC®-enhanced microcosms, indicating that reductive dechlorination was the likely degradation mechanism for DCA. The use of HRC® is recommended as an enhancement for the natural attenuation of organic contaminants at the study site.

Contents

Chapter 1

Introduction

1.1 Organic chemicals and land contamination

Organic chemicals are highly integrated into many aspects of our everyday lives. Naturally occurring crude oil is refined to yield a wide variety of organic chemical products that are used for energy production, transportation, packaging, medicines, cosmetics and many other industries. The conveniences which are brought about by these products are seen by many as essential for the normal function of society. Because of this, the organic chemical industry as a whole has developed to such an extent that it is often a key driver for changes in world economics and politics.

Hydrocarbons are organic compounds, and as the name suggests, their molecules are composed of carbon and hydrogen atoms (Solomons and Fryhle, 2000). For decades, oil refineries and petrochemical facilities have produced and stored hazardous organic compounds without any significant knowledge of the potential adverse effects on the environment or human health. Today, hydrocarbons account for more than one quarter of all pollution incidents, with over 5,000 such incidents in the United Kingdom (UK) each year (Environment Agency, 2005). Much of this pollution is directly related to releases from facilities which refine, process and store organic chemicals. Pollution incidents can include releases to air, land or water. The transport and fate of organic chemicals following a pollution incident are governed by a range of chemical, physical and biological parameters, which are very much site specific. The focus of this thesis is directed towards the organic chemical contamination of

land in the United Kingdom (UK). For the purposes of this thesis, the term land encompasses the whole of a soil-groundwater system.

Environment Agency (2002) suggests that there may be as many as 100,000 sites affected by contamination to some degree in England and Wales. This report continues by describing that between 5 and 20% of these may require action to ensure that unacceptable risks are minimised. Risks to human health and the environment are the primary reasons for dealing with contaminated land. However, the mechanisms for how risks are managed vary.

There are three basic approaches for dealing with contaminated land in the UK as laid out by Reynolds *et al* (2002):

1. Ensuring that existing developments and land uses are protected from existing contamination.

2. Ensuring that new developments and land uses are protected from existing contamination.

3. Ensuring that no new contamination is released by major industries.

The principle legislation which concerns the assessment and clean up of contaminated land in the UK is Part IIA of the Environmental Protection Act 1990 (Part IIA). This act defines contaminated land for the purposes of legal control and then sets out a framework under which local authorities and the UK Environment Agency will together identify contaminated land and require clean up where the requirements of the legislation are met by the service of remediation notices on 'appropriate persons' (Warren, 1996). Contaminated land is defined in the legislation as:

"Any land which appears to the local authority in whose area it is situated to be in such condition by reason of substances in, on or under the land that:

(a) Significant harm is being caused or there is a significant possibility of such harm being caused; or

(b) Pollution of controlled waters is being or is likely to be caused".

The threats posed by pollutants in contaminated ground are normally considered in relation to their impact on the health of biotic receptors and the environment (Yong and Mulligan, 2004). Reducing these impacts is the basis for the risk-based approach which is adopted for most remediation projects in the UK. Part IIA describes the need for risk to be characterised at sites based on identified linkages between pollutant sources, pathways and receptors (Warren, 1996).

Planning regulations govern the clean up of contaminated land which is chosen for redevelopment. Legislation relating to the redevelopment of brown field land was first introduced in the UK in the Town and Country Planning Act 1944. This act enabled local authorities to acquire derelict land in order to bring it back into use (Harris *et al*, 1996). This approach is still popular today, as it reduces the need for further greenfield (undeveloped land) development. The current legislation for dealing with the redevelopment of land in the UK is The Town and Country Planning Act 1990, which requires the clean up of contamination that poses risks to new developments. The Office of the Deputy Prime Minister (ODPM) has released guidance called Planning Policy Guidance 23 (PPG23), which gives further advice on the roles and responsibilities of local authorities for managing potential contamination issues at new developments. This idea of reclaiming contaminated land for new uses has a number of benefits, including:

- Financial savings can be achieved through purchasing, reclaiming and developing contaminated sites. Brownfield land is often less costly and better located for many businesses and industries than greenfield sites.

- Reclaiming brownfield land can add to the aesthetic appeal of an area. This can translate to an increase in investments to the area.

In order to prevent current and future industries from polluting the environment, Part I of the Environmental Protection Act 1990 introduced the idea of Integrated Pollution Control (IPC) in the UK. IPC is a legal system which protects the environment from any harm which is caused by the release of substances into air, water and land (Tromans and Turrall-Clarke,

1994). IPC was recently updated in the Pollution, Prevention and Control (PPC) Act 1999 as a result of the European Community (EC) Directive 96/61/EC.

Another important legislative development has significantly changed the approach to waste management in the UK. In 2001, the EC Landfill Directive was established, which set targets for member states (including the UK) for reducing the quantities and types of wastes that can be sent to landfills. Amongst other requirements, the legislation states that hazardous wastes may no longer be sent to landfill without treatment (Reynolds *et al*, 2002). This has significant implications for the approaches to dealing with contaminated land. The excavation and disposal (dig and dump) of contaminated soils has traditionally been favourable for many sites due to the speed with which the land can be treated for redevelopment purposes. The treatment of hazardous organic chemical contaminants *ex situ* may prove to be infeasible due to potential high expenses, and therefore alternative *in situ* treatments may be preferred. This thesis describes an investigation into a novel *in situ* treatment for contaminated land in the UK.

1.2 Problem definition and overview

As part of the ERDF West Wales and the Valleys Objective 1, Geoenvironmental Research Park (GRP) contract, this thesis is targeted towards researching and demonstrating a new remediation technique. Separate from the GRP contract, the owner of a former petrochemical facility is currently undergoing an extensive land regeneration program. The site owner agreed to allow access to information which relates to geoenvironmental problems in and around the site, with a view to develop an innovative remediation technique which will address a contamination issue at the site. For reasons of confidentiality, the name of the site owner and the location of the site will not be disclosed in this thesis. The former petrochemical facility described above will be identified as 'the study site' throughout this thesis.

Following a review of previous investigations of the study site, a meeting was held with the site owner to discuss potential areas of research. It was decided that this research would focus on problems related to organic chemical plumes,

which are migrating in the groundwater through oxygen depleted soils towards environmental and human health receptors in a nearby estuary. The organic contaminants considered in this thesis include benzene, ethylbenzene, styrene, 1,2-dichloroethane (DCA) and vinyl chloride (VC).

1.3 Remedial focus

The remediation technique which forms the primary focus of this research involves a biostimulation process, using microorganisms to aid in contaminant degradation. Biostimulation is a method of enhancing the natural attenuation of contaminants, and is more generally referred to as enhanced natural attenuation (ENA). The intent of biostimulation is to promote increased microbial activity with a set of stimuli to better degrade organic chemical pollutants in soil (Yong and Mulligan, 2004).

Microcosm treatability studies were undertaken in the work for this thesis to assess the potential for bioremediation using indigenous microorganisms under varied enhancement conditions. Contaminant degradation pathways are assessed through a range of chemical and biological analyses. The results of this thesis were designed to be applied in the decision making process for choosing an appropriate remediation strategy for the study site.

1.4 Objective and tasks of the investigation

The overall objective of this research is to determine the treatability of organic contaminants at the study site using an ENA treatment process.

In order to meet the above objective, the following series of tasks were undertaken:

1. Conduct a desktop investigation of the study site.
2. Carry out a sampling regime in order to collect soil and groundwater samples from the site.
3. Analyse soil and groundwater samples to characterise organic chemistry,

inorganic chemistry and physical properties.

4. Develop a conceptual model for the study site.

5. Carry out lab-based (microcosm) treatability studies using site samples in order to determine the metabolic capabilities of indigenous microorganisms for degrading/transforming the organic contaminants.

6. Monitor changes in community profiles within the microcosms using molecular microbiological methods.

1.5 Scope and limitations

The problem of organic contaminants in groundwater encompasses a large subject area, and it has not been possible to cover every aspect in this thesis. The following points define the scope and limitations of this thesis.

- The site owner made the site accessible for the acquisition of samples. However, due to financial restrictions it was not always possible to use the most appropriate sampling procedures or equipment. Wherever possible, an attempt was made to minimise any negative impacts through careful planning and good practice. It was not financially viable to employ a drilling rig and team solely for the purposes of this research. Therefore, soil samples were acquired from a rig which was commissioned by the site owner for other purposes. The soil samples were taken directly from the auger at the required depths. This exposed the deep anaerobic soils to atmospheric oxygen, thus jeopardising the integrity of samples. Exposure was minimised by quickly transferring the samples into sealed containers with minimal headspace.

- The site sampling periods were governed by the on-site availability of the drilling rig. Because of this, the samples were acquired at times which did not necessarily correspond with the scheduled analyses. This may have led to changes in the chemistry of samples during storage. During the storage period, the samples were kept at a constant temperature of 4° C in order to

reduce the metabolic rates of microorganisms within the samples.

- There are a number of organic compounds which are known to exist at the site, but which were not incorporated into the treatability studies. The compounds that are incorporated include benzene, ethylbenzene and DCA.

- Chemical standards were purchased to represent the focus organic compounds from the site. These standards were used in the microcosm studies. It is possible that pollutants at the site may have transformed chemically over the years, and so may differ slightly from the standards. No analysis was conducted to differentiate between the site and standard compounds.

- No flow through system was used in the microcosm design. Therefore, unlike the site, the microcosms received no influx of fresh nutrients or groundwater. The chosen batch design is recommended by Morse *et al* (1998). This design was also incapable of accurately quantifying degradation rates.

1.6 Thesis overview

The following sections give a brief overview of this thesis by highlighting the main issues which are contained in each chapter.

1.6.1 Natural attenuation of organic chemicals in groundwater

In Chapter 2, a literature review is presented that describes the transport and fate of organic chemical contaminants. Specific attention is given to the properties of the focus contaminants for this thesis. Natural attenuative processes for degrading organic chemicals are introduced with regards to the metabolic capabilities of microorganisms.

1.6.2 Desktop investigation of the study site

Chapter 3 gives an overview of the study site. The previous land uses, present

condition and future plans for the site are described. A detailed account of a recent site investigation by a geoenvironmental consultant, on contract from the site owner, is given.

1.6.3 Study site investigations

Chapter 4 describes the field work and sample analyses which were carried out for the purposes of this research. Details are given about the equipment and procedures which were used for sampling, handling and storage. Methods of analysis are described and the results are discussed in detail. The results of this investigation define the baseline physical and chemical parameters for the study site so that a conceptual model can be developed.

1.6.4 Microcosm Treatability Studies

Chapter 5 provides the details of microcosm studies, which were carried out in order to determine the metabolic capabilities of the indigenous soil microorganisms. These capabilities were measured in relation to a reduction in organic chemical concentrations. The microcosms were batch experiments and were analysed regularly by headspace gas chromatography in order to observe the changes in concentration for the selected compounds. Destructive sampling was also performed in order to obtain soil and porewater samples for further chemical and microbiological analysis. The results of this study are reported and discussed in detail.

1.6.5 Molecular microbiological studies

Chapter 6 describes the molecular microbiological studies which were performed on microcosm samples. The results give an indication of how the diversity of microorganisms changed throughout the course of the microcosm study. Sequencing of genomic materials was also carried out in an attempt to identify individual microorganisms.

1.6.6 Conclusions and suggestions for further research

In Chapter 7, conclusions are presented for the investigation, and recommendations are given for areas of research that relate to, but were not within the scope of this thesis.

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Chapter 2

Natural Attenuation of Organic Chemicals in Groundwater

2.1 Introduction

The aims of this literature review are to introduce the reader to the problems associated with organic pollutants in groundwater, and to describe the ways in which soil microorganisms can help to mitigate such problems.

Section 2.2 provides the scope of this review, including an introduction to the focus organic compounds for this thesis. An overview of the important issues to consider for a site contaminated with organic compounds is given in Section 2.3. Recommendations are discussed regarding the best approaches for investigating contaminated sites. The properties, which govern the transportation and transformation of organic compounds in groundwater, are introduced.

In Section 2.4, special attention is given to those issues, which relate to the transport and fate of the focus compounds for this thesis. Details such as chemical structures and properties, potential hazards and degradation pathways are discussed.

The use of natural attenuative processes for removing organic contaminants from groundwater is discussed in Section 2.5. Microbiological processes play a major role in directing the fate of organic chemicals in the soil-groundwater environment. The metabolic capabilities of different microorganisms are

introduced, along with an overview of the techniques, which are used for microbial characterisation. A distinction is made between monitored and enhanced natural attenuative processes, and each are described in detail.

Conclusions are drawn on the findings of this review in Section 2.6. The knowledge, which was acquired, on the behaviour of organic chemicals in groundwater, is set into the context of the overall thesis objectives.

2.2 Scope of the review

This literature review aims to provide the details of previous investigations into the natural attenuation of organic contaminants in groundwater. It is directed primarily towards the attenuation of those compounds, which hold direct relevance to this thesis. These compounds include benzene, ethylbenzene, styrene, 1,2-dichloroethane (DCA) and vinyl chloride (VC).

2.3 Organic contaminants in groundwater

2.3.1 Investigation of contaminated sites

This section provides an introduction to some of the main approaches for dealing with contaminated sites. Emphasis is placed on the procedures for investigating such sites.

Hydrocarbons are organic compounds and represent one of the most important groups of chemicals to mankind because of their natural abundance, their industrial importance and their extensive use as a primary energy source throughout the world (Chakraborty and Coates, 2004). Significant quantities of organic compounds have found their way into the soil-groundwater environment due to inappropriate handling and disposal, as well as through accidental spillage. Sites which contain such contamination have the potential of damaging the environment and human health, and thus need to be evaluated as to whether and what actions should be taken to control the contamination or impacts (Ma *et al*, 2002). Environment Agency (2002) suggests that there may be as many as 100,000 sites affected by contamination to some degree in

England and Wales. Of these sites, the most common contaminants include organic compounds and metals.

A number of countries have developed policies for dealing with contaminated land. Contaminated land policies are well developed in the US, including the wide acceptance of natural attenuation as a remedial technique. The study site for this thesis is based in the UK. For these reasons, this thesis will describe some of the important legislative approaches of the United States (US) and United Kingdom (UK). A more detailed introduction to contaminated land policies in the UK was given in Chapter 1.

There are various approaches taken by the policies. One approach is to restrict the development of greenfields (undeveloped land) by encouraging the re-development of brownfield land. Brownfield sites are defined in US Public Law (2002) as real property, the expansion, redevelopment or reuse of which may be complicated by the presence or potential presence of a hazardous substance, pollutant or contaminant. Brownfield redevelopment in the UK is governed by the planning system. DETR (2000) states that for new developments, it is the developer's responsibility to carry out the necessary remediation and that, in most cases, the enforcement of remediation requirements will be through planning conditions and building control rather than through a remediation notice under Part IIA.

The basic approach for dealing with brownfield redevelopment is similar to that which is taken for sites with historical contamination (refer to the discussion on Part IIA in Chapter 1). Sites are assessed according to their potential risks to the environmental or human health. Weeks *et al* (2004) lays out a framework for assessing ecological risks at contaminated sites in the UK. A series of standard investigative and analytical procedures are proposed for determination of potential ecological risks. In 2002, the UK Environment Agency (EA) and the Department for Environment, Food and Rural Affairs (DEFRA) published a series of reports which provide a framework for the assessment of human health risks from contaminated land. In addition to these reports, a Contaminated Land Exposure Assessment model (CLEA 2002 software) was developed as a risk assessment tool. Standards, which set limits on the

exposure of the focus organic compounds for this thesis, are discussed further in Chapters 3 and 4. A detailed risk assessment for the study site was not within the scope of this thesis.

As part of the United States Environmental Protection Agency's (US EPA) Brownfields Economic Redevelopment Initiative, technical guidance is given which covers the key steps to redeveloping brownfield sites for their respective industrial sector (EPA, 2000a). The main steps which are outlined for the brownfields redevelopment process are: 1) site selection, 2) phase I site investigation and due diligence, 4) phase II site investigation, 5) evaluate remedial options, 6) develop remedy implementation plan, 7) remedy implementation, and 8) begin redevelopment activities. An important point brought up in the guidance is the need for maintaining a flexible and iterative approach. In this way, unanticipated developments (such as a previously unidentified plume of contamination in groundwater) can be incorporated into the appropriate stage of the redevelopment process. During the phase I site investigation and due diligence stage, the potential health and environmental risks are assessed. If unacceptable levels of contamination exist, then further investigation and remedial action must be taken.

A risk-based approach (i.e. where action must be taken when a perceived risk is above defined acceptable levels) has also been adopted in the United Kingdom for dealing with contaminated land. The EA and DEFRA has developed a set of model procedures for the management of contaminated land (EA and DEFRA, 2004). These procedures provide an approach to investigating and remediating land from a range of different contexts (e.g. historical contamination and brownfield redevelopment). The basic model is split into three separate stages (EA and DEFRA, 2004):

1. Risk Assessment (preliminary risk assessment; generic quantitative risk assessment; detailed quantitative risk assessment)

2. Options Appraisal (Identification of feasible remediation options; detailed evaluation of options; developing the remediation strategy)

3. Implementation of the Remediation Strategy (preparation of the implementation plan; design, implementation and verification; long-term monitoring and maintenance)

Important outcomes of an effective site investigation should include details on a site's geology, hydrogeology, groundwater chemistry, geochemistry and microbiology. Once all of the investigative data has been analysed, a conceptual model for the site can be developed. Detailed conceptual models allow for potential contamination problems to be considered in the context of all of the different scientific issues as a whole. Any uncertainties or unknowns in the model can be highlighted, and re-addressed through further investigation. Once completed, the conceptual model should enable the development of the most appropriate risk assessment and if necessary, remedial measures. A principle aim of this thesis was to develop a conceptual model of the study site (discussed in Chapters 3 and 4).

2.3.2 Multi-phase transport issues

There are four distinct phases for organic compounds within a soil-groundwater system: gaseous, solid, aqueous and immiscible. The extent to which a given contaminant will exist or partition between each phase will depend on its physical and chemical properties as discussed in the following sections.

2.3.2.1 Volatisation

The focus compounds for this thesis are all classified as volatile organic compounds (VOCs). VOCs are defined by EPA (2003) as those compounds of carbon that undergo atmospheric photochemical reactions. This definition is predominantly associated with the formation of ozone in urban environments. However, other potential risks to human health can also be attributable to behaviour or hazardous VOC chemicals. USGS (2005) describes VOCs as compounds, which have high vapour pressures and are commonly found as contaminants in groundwater. The high vapour pressures of VOCs suggest that they may easily volatise from the solid or liquid phase into a vapour.

Risks can arise due to the presence of VOC contamination within the vadose zone, or near to the surface of the groundwater table. Potential problems include difficulties in predicting the transport and fate of contamination, and health risks posed to receptors at the ground surface. The partitioning of organic compounds between aqueous and gaseous phases is described by Henry's Law constants (Sander, 1999).

2.3.2.2 Sorption

Organic compounds are considered to be in a solid phase when they have been adsorbed onto the surfaces of soil particles, soil organic matter (SOM) or amorphous materials within a soil matrix. The adsorption of organic chemical plumes can retard the migration of contaminants, and in some cases decrease their bioavailability due to entrapment within small pore spaces (Noordman *et al*, 2002). Studies reported by Volkering *et al* (1993) and Noordman *et al* (2002) suggest that certain microorganisms have been shown to produce surfactants, which can facilitate the desorption and increase the bioavailability of sorbed organic chemicals.

The primary mechanism of organic adsorption is the hydrophobic bond between the synthetic chemicals and natural organic matter (Yong and Mulligan, 2004). For this reason, the partition coefficient between water and organic carbon (k_{oc}) is a key property for describing the adsorption potential of organic chemicals in soil-water systems. To fully interpret the adsorption potential at a particular site, the k_{oc} must be put in context with the amount of organic carbon content of the soil (Karickhoff, 1984).

The octanol/water partition coefficient (k_{ow}) is also sometimes used to describe the partitioning of organic chemicals between water and organic matter. Octanol is an organic solvent, which is used as a surrogate for natural organic matter (Chiou *et al*, 1982). The hydrophobic or hydrophilic nature of an organic chemical can be estimated using the octanol/water partition coefficient. Yong and Mulligan (2004) suggests that chemicals with k_{ow} values of less than 10 are considered to be relatively hydrophilic, and those with values in excess of 10^4 are considered to be very hydrophobic.

2.3.2.3 Free and dissolved phases

Organic contaminants can exist in three distinct liquid forms within a soil-groundwater system. These forms are generally categorised into Light Non-Aqueous Phase Liquids (LNAPL), Dense Non-Aqueous Phase Liquids (DNAPL) or dissolved phase. Together, LNAPL and DNAPL are known as immiscible or free-phase liquids (also termed just Non-Aqueous Phase Liquids (NAPL)). Figure 2.1 illustrates how these forms might exist at a contaminated site. The relative density (d) of a particular compound will determine whether the compound is more likely take a LNAPL (d<1) or DNAPL (d>1) in a groundwater system. Relative density is a unitless constant, which relates the density of a particular chemical to that of water.

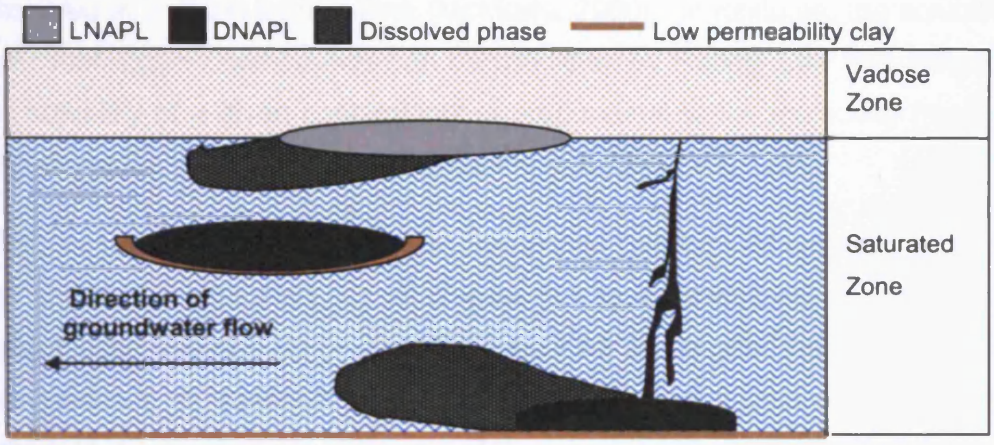

Figure 2.1 Organic pollutant phases in a soil-groundwater system

The migration of DNAPL compounds through an aquifer can occur vertically as well as laterally. Disconnected blobs and ganglia of organic liquid can form at the end of migrating DNAPL plumes (Environment Agency, 2003). This nonlinear movement is related to porescale hydrodynamic instabilities (Environment Agency, 2003), and is often referred to as contaminant 'fingering'. Although it is not within the scope of this thesis to pursue DNAPL fingering in extensive detail, it is noted that this behaviour is an important issue to consider when designing approaches to characterise and remediate DNAPL contaminated sites.

Organic chemicals, which are present as NAPL, represent a potential long-term source of contamination at a site. Therefore, the rapid identification and removal of these sources can help to reduce any potential long-term risks. On larger sites, such as the study site, it can often prove difficult to detect the presence of NAPL contamination. Cohen *et al* (1993) suggests that the presence of NAPL may be inferred if groundwater concentrations exceed 1% of the effective solubility of the NAPL chemical.

Predicting the aqueous phase of any particular contaminant in groundwater can usually be approximated using a compound's solubility limit. The solubility limit of a compound describes the maximum solute concentration that can be dissolved at a given temperature (McMurry, 2000). In mixtures, the solubility of individual organic compounds can be predicted by Raouls Law that states that the solubility of a given contaminant is proportional to the molecular fraction of the contaminant in the mixture (Domenico and Schwartz, 1998).

Sites, which are contaminated with more than one type of organic compound, can be more complicated to characterise due to the possibility of plume mixing. Lighter hydrocarbons (such as BTEX) may be transported uncharacteristically downwards in an aquifer through mixing with a plume of greater density. Other factors, such as the solubility limit of a plume, and degradation mechanisms, are likely to be altered through the mixing of different organic compounds.

2.3.3 Degradation mechanisms

There are numerous ways in which an organic contaminant can be degraded. It is possible that more than one degradation pathway may be responsible for the removal of an organic compound from a particular site. This section gives a review of some of the key degradation mechanisms for organic compounds in groundwater.

Oxidation-reduction (redox) reactions often play a direct role in the degradation of organic chemicals in the environment. Redox reactions in the environment consist of two half-reactions. For example, the redox reaction which occurs between hydrogen and chlorine can be simplified to the oxidation half-reaction:

$$H_2 \rightarrow 2H^+ + 2e^-$$

And the reduction half reaction:

$$Cl_2 + 2e^- \rightarrow 2Cl^-$$

Combining these two half-reactions yields the following balanced redox reaction:

$$2H^+ + 2Cl^- \rightarrow 2HCl$$

Determination of the activity of electrons in a sample of groundwater yields a variable known as the redox potential (Eh). The standard potential is referenced to the half-reaction between the hydrogen ion and molecular hydrogen (shown above), which has an Eh of 0 millivolts (mV).

Vance (2002) gives the chain of redox reactions, which have been shown to facilitate the oxidation of organic compounds (e.g. hydrocarbon contaminants). The following reactions are listed in order of utilisation, based on the amount energy that can be consumed by indigenous microorganisms. The values given in parentheses next to each of the 6 redox reactions above represent the corresponding Eh range, as described by Vance (2004).

1. Aerobic respiration (250mV and higher)

$$C_{org} + O_2 \leftrightarrow CO_2 + H_2O$$

2. Nitrate reduction (250mV to 100mV)

$$C_{org} + NO_3^- \leftrightarrow N_2 + HCO_3^- + CO_2 + H_2O$$

3. Manganese reduction (100mV to 0mV)

$$C_{org} + MnO_2 + H^+ \leftrightarrow Mn^{2+} + HCO_3^- + H_2O$$

4. Iron reduction (100mV to 0mV)

$$C_{org} + Fe(OH)_3 \leftrightarrow Fe^{2+} + HCO_3^- + H_2O$$

5. Sulphate reduction (0mV to –200mV)

$$C_{org} + SO_4^{2-} \leftrightarrow HS^- + HCO^{3-} + H^+$$

6. Methane fermentation (-200mV and lower)

$$C_{org} + H_2O \leftrightarrow CH_4 + HCO_3^- + H^+$$

As is shown above, knowledge of the redox potential of ground water can be used as an indicator of certain geochemical activities such as sulphate reduction. This knowledge can be useful when assessing contaminated sites by helping to characterise the state and location of a contaminant plume (plume delineation and redox measurements is discussed further in Section 2.5.3). However, for a more complete site characterisation, other water quality indicators should also be assessed (including pH, dissolved oxygen, temperature, electrical conductivity, etc).

Redox reactions can occur abiotically as well as biotically. Though, the number of functional groups of organic chemical pollutants that can be oxidised or reduced under abiotic conditions is smaller than those that can be oxidised or reduced under biotic conditions (Scharzenbach *et al*, 1993). There are other abiotic mechanisms, such as hydrolysis and elimination, which have also been shown to degrade a range of organic chemicals in the environment.

There are three known degradation pathways for chlorinated hydrocarbons, as described by Parker and Mohr (1996). The first is by use as an electron donor, or primary substrate, in redox reactions (such as those shown above). The second is through co-metabolic mechanisms, where other hydrocarbons (such as fuel hydrocarbons) act as the primary substrate, and the chlorinated compounds are indirectly transformed. The third (and most common)

mechanism for degrading chlorinated hydrocarbons is by use as an electron acceptor in a process called reductive dechlorination. During reductive dechlorination, the chlorinated hydrocarbon is not used as a carbon source for the microorganisms, which drive the reaction. The process involves the replacement of an atom of chlorine with an atom of hydrogen.

The reaction rates for the biodegradation of organic contaminants are difficult to quantify accurately due to the complexity of soil-groundwater systems (Yong and Mulligan, 2004). For example, an organic chemical may be removed from groundwater through a combination of microbial activities and partitioning. The half-life of a compound is used to describe the time required to reduce a concentration to half of its original value, and can be useful for assessing risk and designing more efficient remedial schemes. Actual half-life values are likely to vary depending on environmental conditions.

More detailed discussions regarding known degradation pathways, for the focus compounds of this thesis are given in Section 2.4.

2.4 Focus organic compounds

2.4.1 Aromatic hydrocarbons

Petroleum hydrocarbons exist naturally in the environment. Spormann and Widdel (2000) speculate that this may explain why many types of microorganisms have evolved metabolic capabilities to utilise these compounds as electron donors for aerobic or anaerobic respiration, and as carbon sources for cell synthesis.

BTEX compounds (benzene, toluene, ethylene and xylenes) are classified as aromatic hydrocarbons and are constituents of petroleum. Benzene is the parent compound of aromatic hydrocarbons, and is recognised by its special ring shape (McMurry, 2000). Figure 2.2 shows molecular and hybrid dash formulas for each of the BTEX compounds. Xylene can exist in three distinct forms, described by the position of the second methyl group on the benzene ring: ortho-, meta- and para-xylene (i.e. o-xylene, m-xylene and p-xylene).

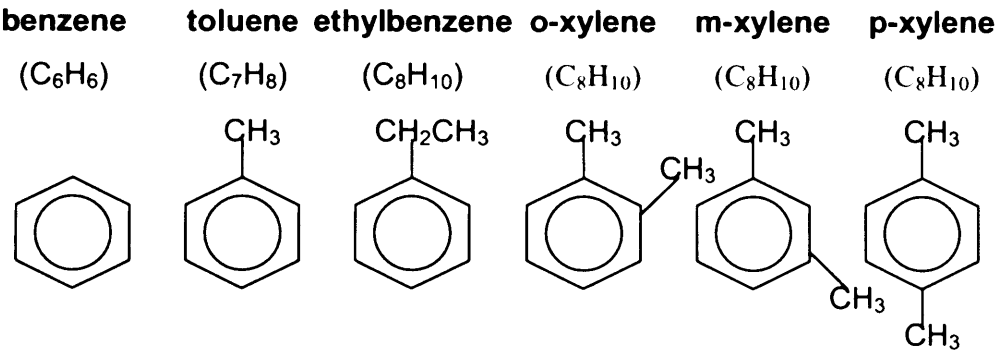

Figure 2.2 Chemical formula and structure of BTEX compounds

2.4.1.1 Benzene

Benzene is a clear, colourless and highly flammable aromatic liquid. It is a constituent of gasoline, and a base compound for the production of a range of different rubbers, plastics and other synthetic materials. The release of benzene to water and land is primarily associated with petroleum refining facilities (EPA, 1995).

Benzene has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1982). In an environmental system, the risks associated with benzene exposure (as with any other potentially hazardous materials) will be dependent upon the source-pathway-receptor linkages at the particular location. Exposure limits and action levels have been developed within UK and US legislation to control these risks. These standards will be discussed further in Chapters 3 and 4.

Once released into a soil-groundwater system, the transport and fate of benzene will be governed by the properties of the compound, and the site-specific conditions, which exist at the particular location. In many cases, benzene may be present as a mixture or fuel component at contaminated sites. Some of the important properties of benzene, which were introduced in Section 2.3.1, are given in Table 2.1.

Table 2.1 Important properties of benzene in a soil-groundwater system (EPA, 1995; EPA, 2005*)

Property	Value
Aqueous solubility (mgL^{-1})	1800
Relative density (dimensionless)	0.88
Henry's Law constant @ 25°C (dimensionless)	0.227*
Octanol/water partition coefficient, k_{ow} (dimensionless)	134
Water/organic carbon partition coefficient, k_{oc} (dimensionless)	98

Benzene is a highly volatile substance. Therefore, when released to the soil surface, it is likely to volatise very quickly or leach through to the groundwater table (EPA, 1995). The introduction of benzene directly to the subsurface via underground storage facilities is a separate issue. This thesis will concentrate solely on the behaviour of the focus compounds following a release to the surface of soil. The results of computational studies by Chiang *et al* (1989) suggest that approximately 5% of benzene mass is lost due to volatisation in a sandy aquifer. Yong and Mulligan (2004) suggest that the losses of BTEX compounds through volatisation are insignificant when compared to biodegradation in the subsurface.

Spectrum Laboratories (2005) report on the persistence of benzene in aquatic environments. Half-life values for benzene are reported as being between 3 and 23 days dependent on the temperature and available oxygen. This range of values gives a rough estimation of the persistence of benzene in an aquatic environment. Actual half-life values are likely to depend greatly on site-specific conditions.

Benzene is a soluble compound, which will rapidly dissolve into groundwater. However, if sufficient quantities are present, benzene may persist in LNAPL form due to its low relative density. The migration of benzene in groundwater is likely to be predominantly governed by its hydrophilic nature. This is assumed due to the substance's high solubility and low partition coefficients. The hydrophobic or hydrophilic tendencies of compounds and their relationships to

partition coefficients were introduced in Section 2.3.2. The mobility of benzene, however, can be retarded greatly depending on *in situ* conditions. Sorption can be significantly enhanced due to the presence of higher organic contents (Zytner, 1994), or certain mineral surfaces (Rogers *et al*, 1980).

Numerous studies have described the degradation of benzene under both aerobic and anaerobic conditions. The degradation of BTEX in groundwater has been shown to proceed rapidly in the presence of $2mgL^{-1}$ dissolved oxygen (Salanitro, 1993). If oxygen is not present, then anaerobic benzene degradation may occur, depending on the availability of electron acceptors (Anderson and Lovely, 1997). The series of electron accepting conditions (from oxygen to carbon dioxide), which are responsible for the degradation of organic chemicals, are given by the six redox reactions shown in Section 2.3.3. Each of the redox reactions proceeds in sequence until either the electron acceptor or carbon source is depleted.

Cerniglia (1984) and Gibson and Parales (2000) describe the degradation pathway for benzene under aerobic conditions. This pathway is also described by (UMBBD, 2005) as part of the beta-1,2,3,4,5,6-hexachlorocyclohexane degradation map.

Dioxygenase enzymes catalyse the initial step in benzene degradation by converting the parent compound into a dihydrodiol. Catechol is then formed by dehydrogenation. The aromatic ring is eventually split to produce either muconic acid (via an ortho-pathway) or 2-hydroxymuconic semialdehyde (via a meta-pathway) (Cerniglia, 1984). The final product of benzene mineralisation under aerobic conditions is carbon dioxide. The aerobic benzene degradation pathway is presented in Figure 2.3.

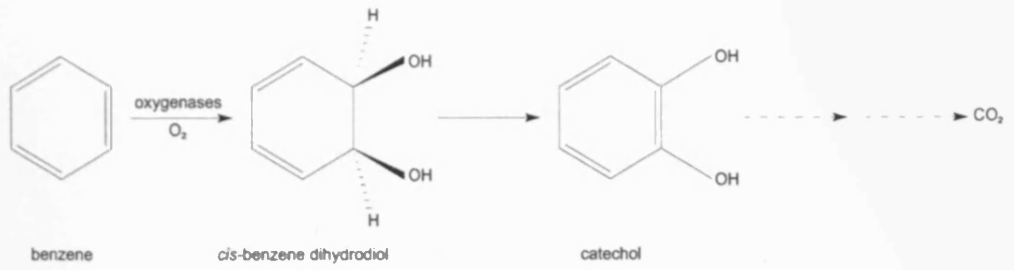

Figure 2.3 Representation of an aerobic benzene degradation pathway

Recent investigations have been carried out in order to describe the anaerobic degradation of benzene. Enrichment studies have shown the effective degradation of benzene under nitrate-reducing (Burland and Edwards, 1999), iron-reducing (Lovely *et al*, 1996), sulphate-reducing (Coates *et al*, 1997) and methanogenic (Weiner and Lovely, 1998) conditions. Until recently, no single organism had been isolated, which could degrade benzene under anaerobic conditions (Chakraborty and Coates, 2004). Coates *et al* (2001) isolated and described two bacterial strains, which were found to completely mineralise benzene to carbon dioxide under nitrate-reducing conditions.

Coates *et al* (2002) provides a review of several possible biochemical pathways for the anaerobic degradation of benzene. The initial step in the pathway is believed to involve the carboxylation (to benzoate), hydroxylation (to phenol) or methylation (to toluene) of benzene. The next step is likely to be a full conversion to benzoate (also known as benzoyl-CoA), followed by the splitting of the aromatic ring. Benzoate is known to be a central intermediate in the anaerobic degradation pathways of a variety of aromatic compounds (UMBBD, 2005). The anaerobic benzene degradation pathway is presented in Figure 2.4.

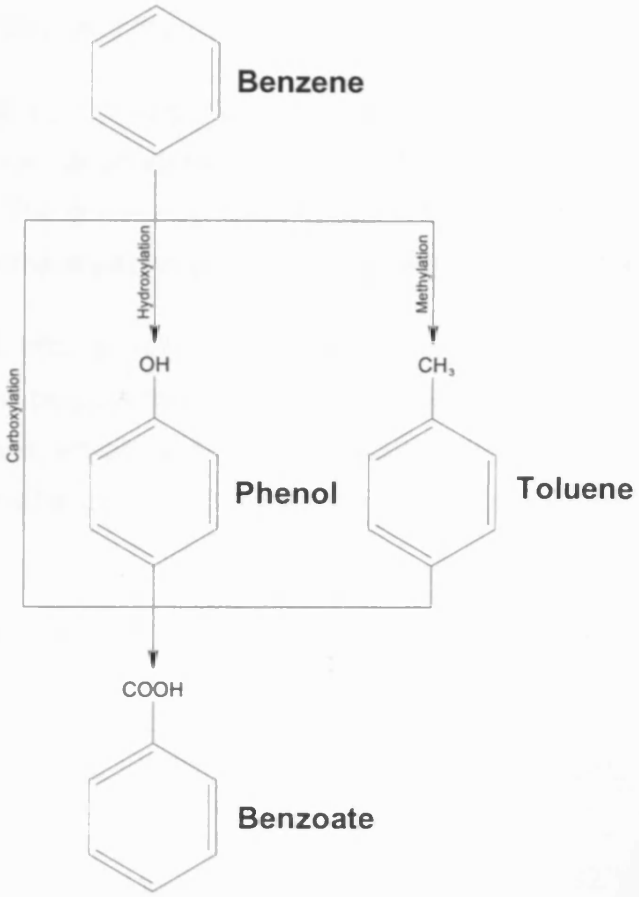

Figure 2.4 Representation of the anaerobic benzene degradation pathway

The abiotic degradation of BTEX compounds has been investigated by Angley *et al* (1992) and Larsen *et al* (1992). They conducted lab-based column experiments, and determined that insignificant losses of BTEX concentrations were attributable to abiotic mechanisms. This is further supported by the suggestions of Mcallister and Chiang (1994), who state that BTEX compounds are unlikely to undergo chemical reactions despite the high degree of unsaturation of benzene-like alkenes.

2.4.1.2 Ethylbenzene

Ethylbenzene is a colourless aromatic liquid with a sweet, gasoline-like odour. The primary use for ethylbenzene is for the production of styrene (see Section

2.4.1.3), however it is also utilised as a solvent for coatings, and in the production of rubber and plastic wraps (EPA, 1995).

Ethylbenzene is not officially classified as a human carcinogen, but has been shown to produce carcinogenic effects in laboratory tests on animals (Sigma-aldrich, 2004). The government standards, which set limits on the exposure of ethylbenzene in the environment, are discussed further in Chapters 3 and 4.

Once released into a soil-groundwater system, the transport and fate of ethylbenzene will be governed by the properties of the compound, and the site-specific conditions, which exist at the particular location. Some of the important properties of ethylbenzene, which were introduced in Section 2.3.1, are given in Table 2.2.

Table 2.2 Important properties of ethylbenzene in a soil-groundwater system (EPA, 1995; EPA, 2005*)

Property	Value
Aqueous solubility (mgL^{-1})	140
Relative density (dimensionless)	0.87
Henry's Law constant @ 25°C (dimensionless)	0.322*
Octanol-water partition coefficient, k_{ow} (dimensionless)	1413
Water-organic carbon partition coefficient (dimensionless)	164

Like benzene, ethylbenzene is a highly volatile substance, and is likely to volatise very quickly or leach through to the groundwater table (EPA, 1995). Data provided by Spectrum Laboratories (2005) suggests that the half-life of ethylbenzene in an aquatic environment is likely to be around 2 to 14 days. This is some 10 to 20 days less than that reported for benzene. The actual persistence of ethylbenzene will depend greatly on site-specific conditions.

Ethylbenzene has higher partition coefficients and lower solubility than benzene, making it more susceptible to partition into the solid phase. However, all BTEX compounds are soluble in groundwater, and sorption will be dependent upon the organic content and mineral characteristics of the soil.

A number of pathways have been investigated for the aerobic and anaerobic degradation of ethylbenzene. It can be used as a primary substrate through a number of different electron-accepting conditions. The degradation of BTEX compounds according to redox reactions was discussed in Sections 2.3.3 and 2.4.1.1.

Lee and Gibson (1996) and UMBBD (2005) discuss the aerobic degradation pathways for ethylbenzene. There are two primary metabolic pathways for aerobic ethylbenzene degradation. Both of these pathways are initiated by dioxygenation of the aromatic ring. However the types of dioxygenase enzymes vary. The first pathway is driven by an ethylbenzene dioxygenase, which leads to an extradiol ring cleavage (UMBBD, 2005). UMBBD, 2005 identifies the principle intermediates of this first pathway as cis-2,3-dihydroxy-2,3-dihydro-ethylbenzene, 2,3-dihydroxy-ethylbenzene, 2-hydroxy-6-oxo-octa-2,4-dienoate, 2-hydroxypenta-2,4-dienoate, 4-hydroxy-2-oxovalerate, propanoate, acetaldehyde and pyruvate. The other known pathway is initiated by a napthalene 1,2-dioxygenase, which degrades in turn degrades ethylbenzene to styrene and/or 2-hydroxyacetophenone (Lee and Gibson, 1996). The degradation of styrene is discussed further in Section 2.4.1.3. Information regarding the degradation of 2-hydroxyacetophenone is currently not available (UMBBD, 2005). Further investigation into the listed pathway intermediates for ethylbenzene degradation was not within scope of this thesis (with the exception of styrene). The complete mineralisation of ethylbenzene under aerobic conditions results in the production of carbon dioxide. The aerobic ethylbenzene degradation pathway is presented in Figure 2.5.

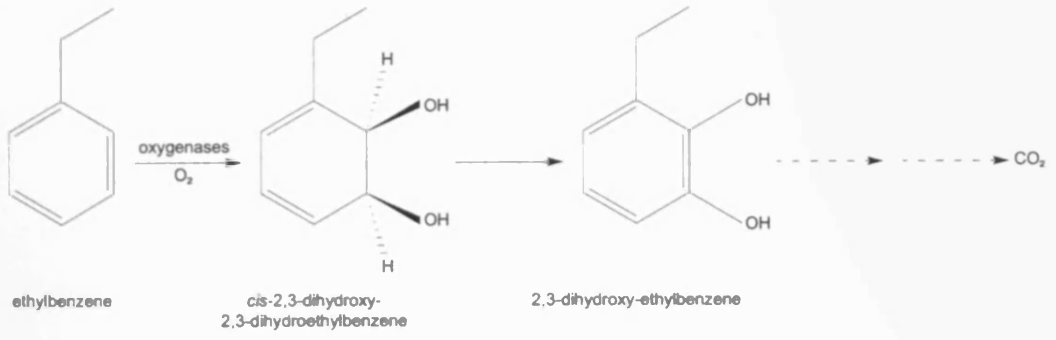

Figure 2.5 Representation of the aerobic ethylbenzene degradation pathway

The anaerobic degradation of ethylbenzene has been coupled to nitrate reduction (Ball *et al*, 1996; UMBBD, 2005) and sulphate reduction (Kniemeyer *et al*, 2003). There are likely to be other metabolic routes, however at present, relatively little is known about the anaerobic degradation of ethylbenzene (Chakraborty and Coates, 2004). Under nitrate-reducing conditions, Ball *et al* (1996) suggests that the anaerobic degradation of ethylbenzene is initiated by a dehydrogenation of ethylbenzene to 1-phenylethanol, and subsequent conversion to benzoate and acetate. The pathway under sulphate-reducing conditions is activated by the addition of fumarate, thus forming 1-phenylethylsuccinate (Kniemeyer *et al*, 2003). The anaerobic ethylbenzene degradation pathway is presented in Figure 2.6.

The abiotic transformation of ethylbenzene is unlikely based on the findings of previous investigations (refer to Section 2.4.1.2).

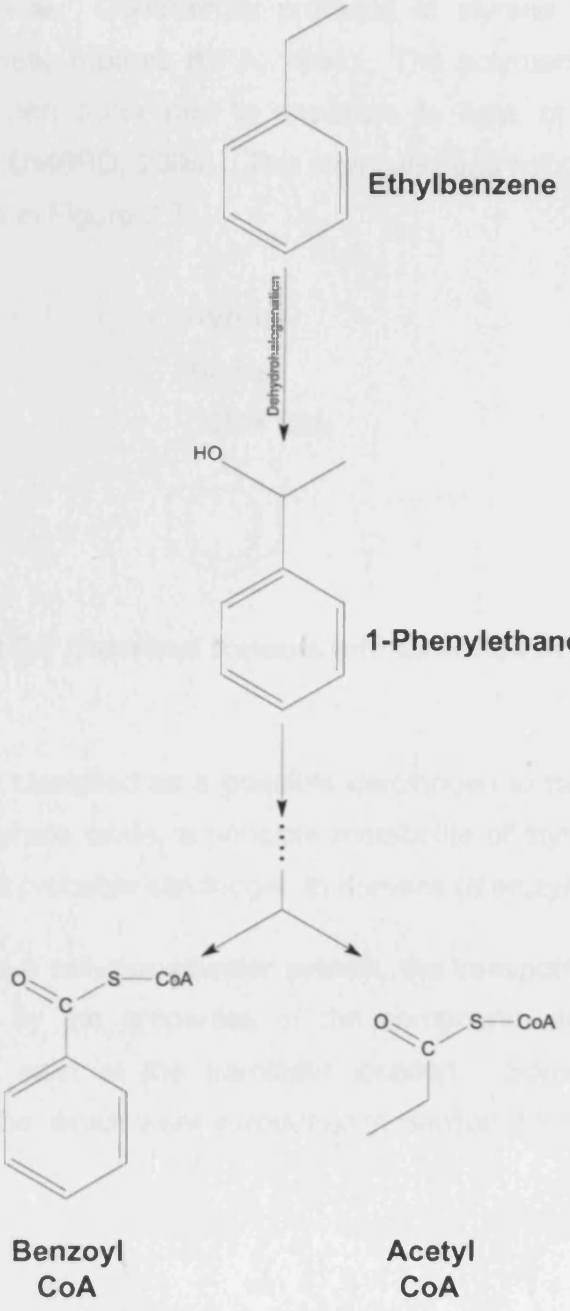

Figure 2.6 Representation of the anaerobic ethylbenzene degradation pathway

2.4.1.3 Styrene

Styrene is an aromatic liquid, which is described as having a floral odour. The production of styrene in the petrochemical industry typically starts with benzene.

Benzene can be alkylated with ethylene to give ethylbenzene, which can be converted to styrene. Commercial products of styrene include polystyrene plastics and synthetic rubbers (EPA, 1995). The polymerisation of styrene to form polystyrene can occur due to exposure to light, or the presence of a peroxide catalyst (UMBBD, 2005). The molecular and hybrid dash formulas for styrene are shown in Figure 2.7.

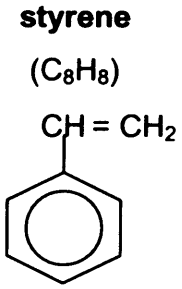

Figure 2.7 Chemical formula and structure of styrene

Styrene has been classified as a possible carcinogen to humans by the IARC (IARC, 2002). Styrene oxide, a principle metabolite of styrene degradation, is also classified as a probable carcinogen in humans (Marczynski *et al*, 2000).

Once released into a soil-groundwater system, the transport and fate of styrene will be governed by the properties of the compound, and the site-specific conditions, which exist at the particular location. Some of the important properties of styrene, which were introduced in Section 2.3.1, are given in Table 2.3.

Table 2.3 Important properties of styrene in a soil-groundwater system (EPA, 1995; EPA, 2005*)

Property	Value
Aqueous solubility (mgL^{-1})	310
Relative density (dimensionless)	0.91
Henry's Law constant @ 25 °C (dimensionless)	0.113*
Octanol-water partition coefficient, k_{ow} (dimensionless)	891
Water-organic carbon partition coefficient (dimensionless)	520

Once released to the soil surface, it is likely to volatise very quickly or leach through to the groundwater table (EPA, 1995). The migration of styrene in groundwater is comparable to that of BTEX compounds. Refer to Section 2.4.1.1 for a description of the behaviour of benzene in groundwater. Limited information was found describing the reaction kinetics for styrene degradation in aquatic environments. However, Spectrum Laboratories (2005) suggests that styrene may persist in soils for up to 2 years. The persistence of styrene is likely to less under aerobic conditions.

A number of pathways have been investigated for the aerobic and anaerobic degradation of styrene. It can be used as a primary substrate through a number of different electron-accepting conditions. The degradation of BTEX compounds according to redox reactions was discussed in Sections 2.3.3 and 2.4.1.1.

O'Leary *et al* (2002) and UMBBD (2005) discuss the known aerobic degradation pathways for styrene. There are two primary pathways for aerobic styrene degradation. The first and most studied pathway involves an initial oxidation of the vinyl side-chain of styrene (O'Conner and Dobson, 1996). Intermediates of this pathway include styrene oxide, phenylacetaldehyde (PAAL), 2-phenylethanol, phenylacetic acid (PAA), 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, fumarate and acetoacetate. As was previously stated styrene oxide is a potentially hazardous compound, which is classified as a probable carcinogen in humans (Marczynski *et al*, 2000). This first pathway

was investigated as part of the work for this thesis (results presented in Chapter 4). The second aerobic pathway for styrene degradation involves direct oxidation and ring cleavage. Studies by Warhurst *et al* (1994) revealed that styrene can be degraded by a pure culture of *Rhodococcus rhodochrous*. The studies describe the metabolism of styrene as proceeding via styrene *cis*-glycol and 3-vinylcatechol, prior to ring cleavage and subsequent conversion to acetaldehyde and pyruvate. Further discussion on the role of certain microbial species for degrading the focus compounds of this thesis is presented in Chapter 6.

The anaerobic transformation of styrene was investigated by Gbric-Gallic *et al* (1990). In contrast to the initial aerobic degradation step, which utilises oxygen, the anaerobic process derives the necessary oxygen from other sources within a soil-groundwater system (O'Leary *et al*, 2002). The actual anaerobic degradation pathway, however, is not too dissimilar to the side-chain oxidation pathway described above. Pathway intermediates again include PAAL, 2-phenylethanol and PAA. Divergence of the aerobic and anaerobic pathways occurs when PAA is converted to benzoic acid via benzyl alcohol and benzaldehyde (Gbric-Gallic *et al*, 1990).

Few studies were found, which examine abiotic transformations of styrene in groundwater. ATSDR (1992) states that there is no information suggesting that styrene is likely to undergo hydrolysis in water, nor would its chemical structure suggest such potential. Therefore, abiotic losses of styrene in the environment are assumed to be negligible based on this statement and the previous studies regarding BTEX compounds, which were discussed in Section 2.4.1.1. The transformation of styrene by polymerisation, however, may be a significant issue with regards to the transportation and fate of styrene in the environment. Styrene polymer (polystyrene) is a highly viscous substance, which may act to clog pore spaces in a soil-groundwater environment. An investigation into the potential effects of styrene polymerisation at the study site is discussed in Chapter 4.

2.4.2 Chlorinated hydrocarbons

Chlorinated hydrocarbons are, by definition, hydrocarbons that contain one or more atoms of chlorine. This thesis is concerned the fate of two chlorinated hydrocarbons at the study site. DCA and VC are both classified as chlorinated aliphatic hydrocarbons (CAHs). DCA is a chlorinated alkane and VC an alkene.

The behaviour of chlorinated hydrocarbons in the environment differs significantly from that of BTEX compounds. The immiscible nature of chlorinated organics in groundwater combined with their high relative densities often causes them to exist as DNAPLs (Sabatini *et al*, 1996). Chlorinated hydrocarbons often persist in the environment for very long periods, partly due to the fact that most contaminated sites contain insufficient electron donors (EPA, 1995; Findlay and Fogel, 2000).

The fate of chlorinated hydrocarbons (as with any other contaminants) will be related to the properties of the particular compound(s) and site-specific hydrogeological, chemical and biological conditions. The following Sections give some of the chemical properties and known degradation pathways for DCA and VC.

2.4.2.1 1,2-Dichloroethane (DCA)

DCA is a colourless liquid with a sweet odour. There are many commercial uses for DCA. These include its use in making chemicals involved in plastics, rubber and synthetic textiles (EPA, 1995). It is also known to be used directly as a solvent for resins, photography, cosmetics and drugs. The release of DCA to the environment is primarily from petrochemical production facilities, however, other contaminated sites exist, which use DCA as a cleaning solvent (EPA, 1995). The molecular and hybrid dash formulas for DCA are shown in Figure 2.8.

1,2-dichloroethane

$(C_2H_4Cl_2)$

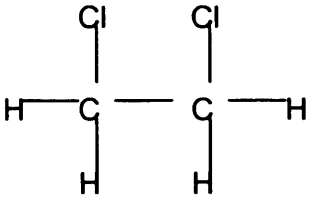

Figure 2.8 Chemical formula and structure of DCA

DCA has been classified as a possible carcinogen in humans by the IARC (IARC, 1999a). The government standards, which set limits on the exposure of DCA in the environment, are discussed further in Chapters 3 and 4. Some of the important properties of DCA, which were introduced in Section 2.3.1, are given in Table 2.4.

Table 2.4 Important properties of DCA in a soil-groundwater system (EPA, 1995; EPA, 2005*)

Property	Value
Aqueous solubility (mgL^{-1})	8700
Relative density (dimensionless)	1.24
Henry's Law constant @ 25°C (dimensionless)	0.04*
Octanol-water partition coefficient, k_{ow} (dimensionless)	30
Water-organic carbon partition coefficient (dimensionless)	33

Once released to the environment, DCA is likely to undergo volatisation before leaching through to the groundwater table (EPA, 1995). The density and immiscible nature of DCA in groundwater are significant properties, as they describe the tendency of the compound to exist as DNAPL. However, DCA also has a relatively high solubility when compared to that of the BTEX compounds. The tendency of the compound to exist as DNAPL will be dependent upon the quantities present. Larger plumes of DCA will be more likely to persist as a long-term source of dissolved-phase contamination at the plume boundaries. A zone of dissolved-phase DCA would be expected to migrate down hydraulic gradient from the DNAPL (see Figure 2.1 for an illustration of this scenario). Spectrum Laboratories (2005) state that DCA has been shown to have a half-life of 10 days in an aquatic environment.

The high solubility and low partition coefficients of DCA suggest that it is relatively hydrophilic, and therefore likely to remain in NAPL or dissolved

phases, rather than partition to solid surfaces. The hydrophobic or hydrophilic tendencies of compounds and their relationships to partition coefficients were introduced in Section 2.3.2.

DCA has been shown to be degraded via aerobic and anaerobic mechanisms. As stated in Section 2.3.3, there are three principle mechanisms for degrading chlorinated hydrocarbons. The following paragraphs describe each of these mechanisms with regards to DCA degradation.

Parker and Mohr (1996) describe how DCA can be utilised as a primary substrate under aerobic and anaerobic conditions. In these reactions, the facilitating microorganisms obtain energy and carbon from the degraded chlorinated compound. The use of DCA as a primary substrate proceeds along the series of redox reactions discussed under Section 2.3.3, with dissolved oxygen being utilised first. Anaerobic degradation mechanisms become more effective as the number of chlorine atoms increase (Yong and Mulligan, 2004). This is due to a lower oxidation state, which is associated with the presence of halogen atoms. DCA contains two atoms of chlorine, and is therefore considered to be a substrate, which can be effectively degraded aerobically.

When DCA is utilised as a primary substrate, the degradation process is often initiated through a removal of a chlorine atom and replacement of a hydroxide group, thus producing 2-chloroethanol (Yong and Mulligan, 2004). This degradation pathway proceeds through the other intermediates including chloroacetaldehyde, chloroacetic acid and glycolate (UMBBD, 2005). The final products of aerobic DCA degradation include carbon dioxide, water and chloride (Van den Wijngaard *et al*, (1992).

Cometabolism is another potential mechanism for DCA degradation. Studies by Dyer *et al* (2000) showed the effective cometabolic degradation of DCA using molasses and alcohol as the primary carbon substrates. Cometabolic mechanisms require the presence of a primary substrate for the effective degradation of the cosubstrate. Maintaining optimal quantities of cosubstrate and substrate often proves difficult due to competition for enzymes (Yong and Mulligan, 2004).

Reductive dechlorination is considered the most significant mechanism for the degradation of chlorinated hydrocarbons (Parker and Mohr, 1996). The successful reductive dechlorination of DCA has been shown through a number of investigations. The initial step in reductive dechlorination typically involves the replacement of a chlorine atom with hydrogen. The direct degradation pathway for DCA through reductive dechlorination would produce chloroethane, which is subsequently converted to ethane.

There are two other potential pathways to consider for the reductive dechlorination of DCA. The first involves the removal of a halogen atom and adjacent hydrogen atom. This reaction is called dehydrohalogenation, and is a mechanism, which converts alkanes to alkenes. In the case of DCA, the product of dehydrohalogenation is VC. Further discussion regarding the degradation mechanisms and pathways for VC are given in Section 2.4.2.2.

The other potential pathway for reductively dechlorinating DCA is dihaloelimination. Like dehydrohalogenation, this mechanism also involves the conversion of an alkane to an alkene. However, dihaloelimination simultaneously removes two atoms of chlorine in the process. The dihaloelimination of DCA yields ethene. Dyer *et al* (2000) suggests that dihaloelimination is a cometabolic process, which is typically driven by sulphate and methanogenic bacteria.

Maymó-Gatell *et al* (1999) investigated the reductive dechlorination of DCA using laboratory culture tubes. The study found that DCA was converted to 99% ethene and 1% VC under anaerobic conditions, thus indicating that dihaloelimination was occurring.

Degradation pathways for DCA are presented in Figure 2.9.

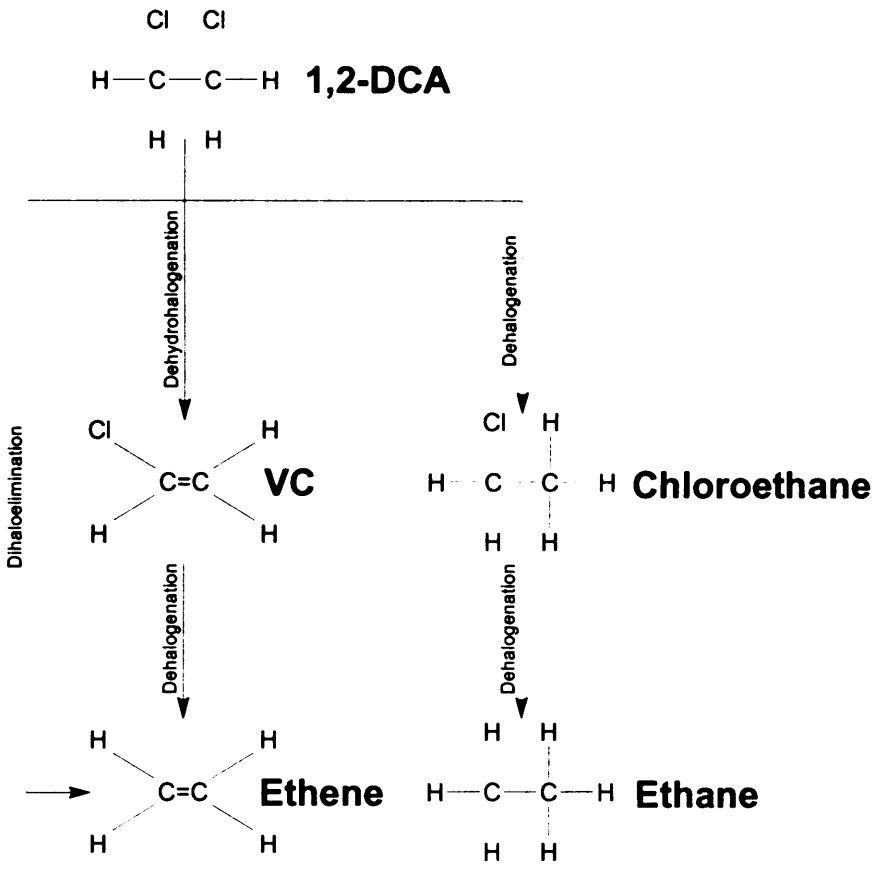

Figure 2.9 Representation of the 1,2-DCA degradation pathway

Studies conducted by Gerriste *et al* (1991) identified fermentation as another potential mechanism for the degradation of DCA. The products of this pathway include ethene and carbon dioxide. The degradation of DCA through fermentation is important to consider, especially when *in situ* conditions lack sufficient electron acceptors and organic carbon (Dyer *et al*, 2000).

EPA (2000b) gives an overview of the abiotic degradation mechanisms for CAHs. The degradation of DCA through hydrolysis was investigated by Barbash and Rienhard (1989), and was found not to be a significant removal process. The elimination process Dehydrohalogenation can also occur through abiotic mechanisms, but is more effective for more chlorinated CAH compounds (EPA, 2000b). Therefore, it is not considered to be a significant process for the removal of DCA. The reductive dechlorination of CAHs can occur in the

absence of microorganisms. An extremely strong reductant, such as zero-valent iron, is required to drive this mechanism.

2.4.2.2 Vinyl chloride (VC)

VC is a colourless gas with a sweet odour. The boiling point of VC is –13.9 degrees Celsius (NIOSH, 2004). VC is used in the manufacture of numerous products in the construction, electrical insulation, medical and automotive industries, but is perhaps most used for the production of polyvinyl chloride (PVC) (EPA, 1995). The release of VC to the environment is predominantly associated with petrochemical production facilities. However, the presence of VC in the subsurface may be the result the degradation of a parent compound such as TCE. The molecular and hybrid dash formulas for VC are shown in Figure 2.10.

Vinyl chloride

(C_2H_3Cl)

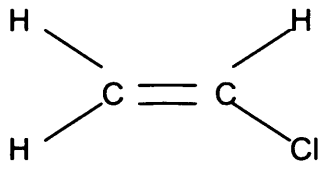

Figure 2.10 Chemical formula and structure of VC

The International Agency for Research on Cancer (IARC) suggests the possibility of carcinogenic effects on humans from exposure to VC (IARC, 1974). A more recent compilation of scientific studies by EPA (2000c) defines vinyl chloride as a known human carcinogen. The government standards, which set limits on the exposure of VC in the environment, are discussed further in Chapters 3 and 4. Some of the important properties of VC, which were introduced in Section 2.3.1, are given in Table 2.5.

Table 2.5 Important properties of vinyl chloride in a soil-groundwater system (EPA, 1995; EPA, 2005*)

Property	Value
Aqueous solubility (mgL^{-1})	2700
Relative density (dimensionless)	0.91
Henry's Law constant @ 25°C (dimensionless)	1.11*
Octanol-water partition coefficient, k_{ow} (dimensionless)	4.0
Water-organic carbon partition coefficient (dimensionless)	56

Once released to a soil-groundwater system, volatisation is likely to be the most significant transport mechanism for VC (IPOCS, 1999). VC is less dense than water, and is therefore unlikely to exist as a DNAPL. This is in contrast to many chlorinated hydrocarbons, whose relative densities are greater than one (e.g. DCA). However, if VC were released as part of a contaminant mixture, the plume behaviour may vary significantly. The high solubility and low partition coefficients of VC suggest that it is most likely to persist in groundwater as dissolved-phase contamination. VC can persist for long periods in the environment. BMZ (2000) describes VC as having a half-life of up to 5 weeks or 2 years under aerobic and anaerobic conditions respectively.

Complete degradation of VC has been shown through both aerobic and anaerobic experiments. Aerobic degradation mechanisms often prove more effective for the removal of VC from groundwater due to its high oxidation state (Semprini *et al*, 1995; Weidemeier *et al*, 1996). Under aerobic conditions, VC is degraded to produce glycolic acid or carbon dioxide (IPOCS, 1999).

The effectiveness of aerobic VC degradation is in contrast to the advantages of anaerobic degradation for other, more chlorinated alkenes, such as tetrachloroethene (PCE) (Maymó-Gatell *et al*, 1999). VC is a known metabolite of PCE degradation (Freeman and Gossett, 1989). Other compounds, which fall into the degradation pathway of PCE, include trichloroethene (TCE), dichloroethene (DCE) and ethene. DCE can take on various chemical

configurations. Figure 2.11 shows the sequence of reductive dechlorination products for PCE.

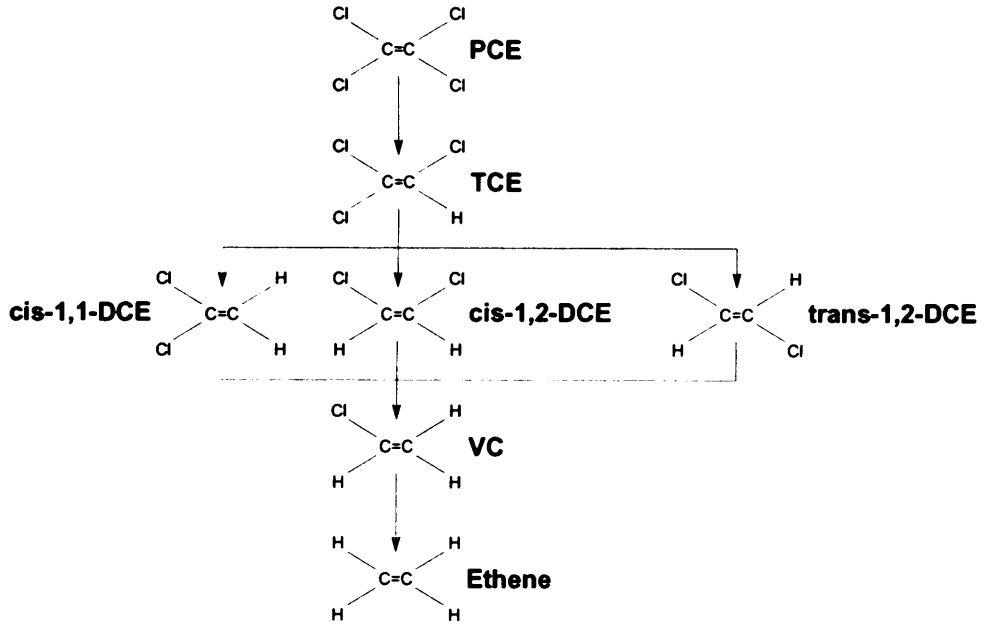

Figure 2.11 Representation of the PCE reductive dechlorination degradation pathway

As discussed in Section 2.4.2.1, VC can also be produced via the degradation of DCA. This occurs through the dehydrohalogenation of DCA, where the alkane is converted to an alkene. Interpreting investigative data from sites, which contain VC and other chlorinated solvents, can prove difficult. This is due to the uncertainties regarding whether or not the observed VC is a plume from source, or a product of other solvent degradation.

Studies by Hartmans and De Bont (1992) have identified *Mycobacterium* sp as a microorganism capable of using VC as a sole carbon source for degradation. However, the majority of investigations indicate that VC is not degraded under most conditions as the sole carbon source (IPOCS, 1999). Therefore, cometabolic mechanisms are considered to be effective for the remediation of VC.

Reductive dechlorination is likely to be the principle mechanism, which drives the degradation of VC under anaerobic conditions. Maymó-Gatell *et al* (1999)

carried out an investigation into the anaerobic degradation of chlorinated ethenes (including VC). The results of this investigation suggested that ethene was the final degradation product of VC under controlled anaerobic conditions. The degradation of VC, however, occurred at a slower rate than that of PCE and TCE. Other possible degradation products of VC under anaerobic conditions include ethane, methane and chloromethane (IPOCS, 1999).

A summary of a number of studies investigating the chemical hydrolysis (an abiotic process) of VC is presented in IPOSC (1999). It is suggested that hydrolysis has been shown to be an insignificant mechanism for the degradation of VC. The degradation of VC via other abiotic mechanisms is also considered to be insignificant, based on the discussions regarding abiotic DCA removal (refer to Section 2.4.2.1).

2.5 Natural attenuation of organic contaminants

2.5.1 Background to in situ *biological techniques*

A variety of chemical, physical and biological techniques are available for the treatment of contaminated land. These techniques are often categorised as either *in situ* or *ex situ* treatments. *Ex situ* technologies involve the extraction of contaminated soils or groundwater from the subsurface and subsequent treatment on or off site. *In situ* technologies are designed to treat the contaminants within the ground with minimal disturbance.

Reference to the occurrence of organic chemical degradation as a result of microbial metabolic activities (i.e. biodegradation) can be found from as early as the 1940s. Audus (1949) describes how a reduction in concentrations of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in soil is related to the activity of microorganisms. The implications of this were wide reaching; however the immediate effect was to limit the persistence of a potentially hazardous compound. The environmental applications of hydrocarbon biodegradation became a major focus of research in the 1960s (Atlas and Cerniglia, 1995). This research was largely driven by advances in the petroleum industry. Atlas and Cerniglia (1995) describe how these early biochemistry studies were part of

basic research on the physiology of microorganisms. Since the 1960s, research and development into bioremediation applications (including municipal sewage treatment, agrichemical industry, soil/ groundwater remediation, and dealing with oil spills) has been wide spread (Alexander, 1999). FRTR (2005) presents a comprehensive review of modern remedial technologies.

NRC (1993) reported on a recent and notable investigation into whether microorganisms were capable of degrading organic contaminants. They concluded not only that biological activities were a viable treatment option, but that passive remediation techniques should be further investigated for field application. *In situ* biological techniques are favourable, as they often make use of the natural capabilities of the indigenous soil microorganisms for degrading contaminants (Yong and Mulligan, 2004). In some cases, *in situ* biological techniques may prove to be more cost-effective than other, more aggressive approaches.

EA (2000) defines natural attenuation in groundwater as 'the effect of naturally occurring physical, chemical and biological processes, or any combination of those processes to reduce the mass, concentration, flux or toxicity of polluting substances'. EPA (1996) proposes a similar definition for natural attenuation as the 'use of natural processes to contain the spread of the contamination from chemical spills and reduce the concentration and amount of pollutants at contaminated sites'. The use of natural attenuative processes as part of a technique for remediating contaminated land can fall into two categories: monitored natural attenuation (MNA) and enhanced natural attenuation (ENA). These are discussed independently in Sections 2.5.3 and 2.5.4.

The natural processes, which allow for the remediation of contaminants through natural attenuation, are biological degradation, dilution and partitioning to soil fractions (Yong and Mulligan, 2004). This Section (2.5) will focus primarily on the natural attenuation of organic chemicals through biological processes.

2.5.2 The role of microorganisms

Knowledge of the indigenous microbial populations and their metabolic capabilities is important when considering the natural attenuation of organic

contaminants. This section provides a brief introduction to soil microbiology in the context of contaminated soil-groundwater systems.

Highly varied microbial populations exist in most soil-groundwater environments. In these environments, microorganisms most often occur as microcolonies attached to soil particles, rather than drifting free in a groundwater solution (Madigan *et al*, 2003). In the saturated subsurface, the activity of microorganisms is related to the availability of certain electron acceptors (Vance, 2002). Dissolved oxygen is the first of the electron acceptors to be consumed. This is typically carried out by aerobic microorganisms through respiration. Once the oxygen is exhausted, other electron acceptors such as nitrate, iron (III), manganese (IV), sulphate and carbon dioxide are consumed. This sequence of consumption is given as a series of redox reactions and discussed further in Section 2.3.3. Microorganisms acquire energy from the exchange of electrons in these reactions (Madigan *et al*, 2003).

The types of microorganisms, which are able to thrive in each of the redox conditions, vary. For example, the microorganisms responsible for nitrate reduction may be a completely different species to those, which facilitate methanogenesis. In the absence of fresh nutrients, each type of microorganism will undergo a series of growth phases including inoculation, exponential growth, stationary phase and death (Madigan *et al*, 2003). This growth cycle is typically characterised for a microbial population within an enclosed vessel in a laboratory. In a natural soil-groundwater system, however, the growth of microorganisms is more difficult to characterise due to the influx of fresh nutrients and other chemicals.

Micoorganisms can be classified through a variety of differing approaches. One system relies of classification according to their level of reliance (or non-reliance) on oxygen. The following bullets list these classifications (Mulligan *et al*, 2003):

- Aerobic microorganisms can utilize molecular oxygen as a final electron acceptor.

- Obligate aerobes are aerobic microorganisms that cannot live in the absence of molecular oxygen.

- Anaerobic microorganisms do not require molecular oxygen as a final electron acceptor.

- Facultative anaerobes are aerobic microorganisms that can utilize fermentation when molecular oxygen is absent, and can utilize aerobic cellular respiration when molecular oxygen is present.

- Aerotolerant anaerobes are non-facultative anaerobes that are able to grow in the presence of molecular oxygen.

- Microaerophiles are aerobic microorganisms that cannot live in the absence of molecular oxygen; however, they require limited quantities of molecular oxygen for growth.

- Obligate (strict) anaerobes are anaerobic microorganisms that cannot grow (or in some cases survive) in the presence of molecular oxygen.

Another, more basic classification for bacteria, places them into one of two major groups; gram-positive and gram-negative. These distinctions relate to the chemical structure of a cell's outer membrane; however, from a practical standpoint, this difference lends itself to a useful laboratory technique (discussed below).

The activity and numbers of microorganisms in soils depend largely upon the availability of water and nutrients. This thesis focuses primarily on microorganisms, which obtain energy and carbon from organic compounds (*chemoorganotrphs*); however, other organisms are able to obtain energy from other sources (such as light). For the purposes of this thesis, the term nutrient applies to any chemical that contributes to the overall metabolic function of a cell. There are many different chemical nutrients required by cells for metabolic function and growth (replication). Some nutrients (such as carbon, hydrogen, oxygen, nitrogen, phosphorus, etc) are, in many cases, required in relatively large amounts for cell metabolism, whereas others (such as chromium, cobalt, copper, manganese, etc) are required, but in lesser quantities. Every

microorganism requires its own individual range and quantities of nutrients for metabolic function and growth (see discussion below on culture media).

The most nutrient rich horizon of a typical soil environment is located near to the ground surface (Madigan *et al*, 2003). It is this region where microbial growth is most active. However, lower in the subsurface and within the saturated aquifer, microbial populations can also be highly active dependent on nutrient availability.

The influx of organic chemical contaminants into a soil-groundwater environment is likely to have a significant effect on microbial diversity and growth. Some organic chemicals may have a toxic effect on soil microorganisms. Further discussion regarding the toxicology of organic chemicals is not within the scope of this thesis. The types of microorganisms to be expected in soils contaminated with organic chemicals will vary with each site-specific situation. However, organic chemicals do offer a source of carbon to indigenous populations, and those microorganisms most capable of degrading a specific chemical will likely thrive. As was discussed above, the more easily utilized electron acceptors (including molecular oxygen) are typically reduced rapidly in the presence of a carbon source, leading to the formation of more reduced conditions.

The ability of microorganisms to make use of organic chemicals as a source of carbon is dependant on the presence of certain enzymes (proteins) that are produced by a cell. Particular enzymes catalyse the break down of organic substrates. The production of enzymes by microorganisms is not constant. Many enzymes are produced on an as needed basis to support metabolic function (termed adaptive enzymes) (Madigan *et al*, 2003). However, in a contaminated groundwater system, the high mobility of some organic chemicals may reduce the exposure time of the compounds and thus restrict the production of adaptive enzymes. Certain bacteria can be conditioned in the laboratory, by inducing the production of adaptive enzymes, to address this issue as a remedial treatment.

Measuring the diversity and growth of indigenous microorganisms could prove useful for determining the potential for remediation by natural attenuation.

There are a variety of techniques available for characterising microbial populations in environmental samples. Traditional methods for analysing microbial diversity and growth involve the use of various culturing, isolation and microscopy techniques.

Microbial growth can be stimulated in the laboratory through the inoculation of organisms on culture media. Different types of culture media consist of different combinations of nutrient contents; each designed to promote microbial growth. Some culture media are designed to target specific microbial species (such as *Escherichia coli*), whereas others promote growth in a wider range of microorganisms. Culture media introduced to an agar plate (and inoculated with microorganisms) can yield pure cultures (those containing only a single type of microorganism). This is achieved through a separation of the different microbial colonies based on appearance (often using a succession of plates). Each pure culture colony can be classified according to the media on which it was grown, and the full results (including all colonies observed from a single source) may be compiled to give a basic indication of microbial diversity.

Another traditional approach for characterising microorganisms (which may be used as a compliment to the above-mentioned plating methods) is through the use of microscopy. For the purposes of this thesis, only light microscopy will be introduced; however, other microscopy techniques (including electron microscopy) have been employed for the characterisation of microorganisms. Visual inspections of microorganisms, through light microscopy, can allow for assessments of cell numbers, size, structure and motion. A major limitation of standard light microscopy is insufficient contrast and resolution. The use of staining is often employed to improve the user's ability to distinguish between and characterise objects (e.g. microbial cells). A gram stain is a popular microbial stain as it allows for a differentiation in the appearance of gram-positive and gram-negative bacteria under a microscope. When counting microorganisms in a sample using light microscopy, both living and non-living microorganisms are summed. In order to obtain a count of living microorganisms, a sample may be mixed with a culture medium, incubated then the colonies formed are counted. This method assumes that each living cell in

the original sample will go on to form a colony, and is referred to as the viable count technique.

Recent advances in the field of molecular microbiology have provided new insight into the diversity of microorganisms in the environment. It is estimated that only 1% of microorganisms can be isolated in pure cultures (Amann *et al*, 1995). Molecular methods, on the other hand, can assess much of the genomic cell material contained within environmental samples. Certain limitations due exist (for example, the amount of genomic materials that can be assessed is directly related to the efficiency of the extraction process, as discussed in Chapter 6). Molecular microbiological techniques form a major part of the investigations carried out for this thesis, and are discussed in greater detail under Chapter 6.

2.5.3 Monitored natural attenuation (MNA)

MNA has only recently (within the past decade) become acceptable as a remediation technique (Mulligan, 2002). MNA is considered to be a passive remediation technique. It is designed to make use of the natural capabilities of the indigenous microorganisms in order to degrade contaminants. In 2000, the UK Environment Agency released guidance on the assessment and monitoring of natural attenuation (EA, 2000). The guidance states that a validation of the effectiveness of MNA as a remedial method is essential. The principle lines of evidence, which should be demonstrated for natural attenuation, include:

1. Documenting the loss of contaminant mass in the field over time (also referred to as a shrinking plume),

2. Determination of geochemical and biochemical indicators which demonstrate the natural attenuation process that is resulting in the reduction in contaminant concentrations, and

3. Obtaining the appropriate microbiological data to support the occurrence of biodegradation.

Determination of geochemical and biochemical parameters (second line of evidence as presented above) is helpful for plume delineation as well as

providing a line of evidence for MNA. One example of this is the monitoring of redox variations in and around a contaminant plume. A plume that is migrating with the flow of groundwater will typically develop distinct redox zones. On the outer edge of a plume, redox conditions are more likely to indicative of oxygen or nitrate-reducing conditions. Further towards the centre of a plume, more reduced conditions (e.g. sulphate-reducing and methanogenic) are likely to persist. Characterising the shape and state of the various redox zones is important for understanding MNA mechanisms within a particular plume.

The above-stated lines of evidence were first introduced in 1993 by the US National Research Council (NRC, 1993). The above lines of evidence are quoted for natural attenuation as general technique, and so encompass both MNA and ENA.

Yong and Mulligan (2004) suggest that the strict requirements of monitoring programs, which are required for determining the lines of evidence, may in some cases make other, more active techniques, favourable. This relates to the rapid removal of contaminants, which is associated with some active techniques, such as pump-and-treat.

EA (2000) and EA (2004b) give recommendations on the steps, which should be taken for the use of MNA in the UK. Prior to the implementation of a MNA scheme, a detailed monitoring scheme is required, which will determine whether or not the contaminants are being degraded naturally under the site-specific conditions.

2.5.4 Enhanced natural attenuation (ENA)

ENA is a remediation technique whereby natural conditions are enhanced in order to improve the natural attenuation capabilities of a soil. ENA can fall into two different categories: biostimulation and bioaugmentation (Yong and Mulligan, 2004).

Biostimulation involves the addition of nutrients and/or growth substrates to enhance the degradation of organic chemicals in soil (Yong and Mulligan, 2004). Amendments can include the addition of electron acceptors, electron

donors or carbon sources. The choice of nutrients to be used will depend upon the types of organic chemicals and *in situ* conditions.

The use and perception of bioaugmentation for remediating contaminated aquifers has changed recently. Previously, the technique was only considered if the indigenous microbial populations were thought to be incapable of degrading the organic contaminants at a particular site (Yong and Mulligan, 2004). The technique involves the introduction of non-indigenous microorganisms in order to enhance contaminant degradation. The technique is typically used to decrease the inoculation (or lag) phase before indigenous microorganisms can develop sufficient numbers to effectively degrade organic contaminants. Studies reported by Atlas (1991) suggested that indigenous microorganisms would eventually out-compete the introduced microorganisms. However, the growth and compatibility of the indigenous and introduced microorganisms may, in some cases, be difficult to predict.

Another approach to bioaugmentation, which may counter some of these concerns, involves the extraction, enrichment and re-introduction of indigenous microorganisms. Studies by Weber and Corseuil (1994) showed that indigenous microorganisms could be effectively enriched *ex situ* so that their ability to degrade benzene could be enhanced.

With the development of molecular methods for monitoring microbial populations in soils, the application of bioaugmentation has become more favourable. Bacterial enzymes, which are known to facilitate the degradation of certain organic compounds, can be analysed in relation to their respective encoding genes. This allows for the isolation of more relevant strains, and improved monitoring accuracy of growth. One area where this type of approach has led to significant usage in the field relates to the isolation and introduction of *Dehalococcoides* bacteria to sites contaminated with chlorinated hydrocarbons. Companies such as Regenesis Bioremediation Products Inc and SiREM have made a number of registered products (select microbial communities with enrichments) available.

Bioaugmentation is often used in combination with biostimulation to enhance contaminant degradation. As was previously stated, the lines of evidence for

natural attenuation (given in Section 2.5.3), also apply to ENA. However, unlike MNA, ENA involves the introduction of substances or microorganisms to a natural system. Thus, the demonstration of natural attenuation, prior to the implementation of an ENA scheme, cannot be determined through site monitoring only. Weidemeier *et al* (1998) describes the use of microcosm studies for physically demonstrating the occurrence of natural attenuation, and determining whether indigenous biota are capable of degrading site contaminants.

ENA is investigated in this thesis as a potential remedial measure for addressing contamination issues at the study site. Biostimulation was adopted as the preferred method. The choice of soil amendments and design of treatability studies are given in Chapter 5.

2.6 Conclusions

The contamination of groundwater with organic chemicals can pose potential risks to human health and the environment. This thesis focuses on organic chemicals, which are present at the study site, including benzene, ethylbenzene, styrene, DCA and VC.

Each of these compounds has been shown to have potential hazardous effects on human health and the environment. The actual risks, which are posed by the presence of such contamination, will be dependent upon the pollutant linkages, the properties of the compounds and a number of site-specific environmental conditions. Pollutant linkages describe the source-pathway-receptor relationships and are discussed in further detail in Chapter 3. The last two factors will govern the migration of the contaminants in soil.

To understand the potential risks, a detailed site investigation is required. The results of such an investigation should be brought together to form a conceptual model of the site, so that contamination issues can be considered in the context of other important scientific parameters (such as the geology, hydrogeology and groundwater chemistry).

Understanding the transport and fate of organic chemicals in a soil-groundwater environment is essential for the design of effective remedial measures. Each of the focus compounds for the thesis is highly volatile. It is likely that the release of the contaminant compounds to the surface of land would result in a significant loss of mass through volatisation. However, the remaining mass may leach through to the groundwater table and dissolve rapidly, due to their high aqueous solubility. However, if significant mass were present at the groundwater interface, the compounds may remain as free-product (DNAPL or LNAPL, dependent upon the relative density of the compound). Significant quantities of DCA would tend to migrate towards the bottom of the aquifer, whereas the lighter aromatic hydrocarbons are more likely to remain nearer to the top of the groundwater table. The possibility of plume mixing may have an effect on the transport of the focus compounds at the site. Lighter hydrocarbons may be transported uncharacteristically downwards in an aquifer through mixing with a plume of greater density.

The focus compounds would likely remain in the dissolved phase, rather than partition to soil fractions, due to low partitioning coefficients. This suggests the potential for high mobility in the groundwater system.

Previous studies into the degradation of the focus compounds have found that both aerobic and anaerobic pathways can be effective routes for contaminant removal. In the presence of sufficient oxygen, the contaminants would likely be consumed rapidly by indigenous microorganisms. However, the possibility exists that the compound are present lower in the aquifer under more reduced conditions. The investigations presented in Chapters 3 and 4 describe the state and location of contamination within the aquifer at the study site. In a reduced environment, the availability of electron acceptors and donors will play a large role in the degradation potential of the contaminants.

The remediation of organic chemicals in groundwater can occur through a number of different approaches. *In situ* biological techniques can be favourable, as they often make use of the natural capabilities of soil microorganisms for degrading organic contaminants. Biological techniques are

typically less aggressive than other approaches, but can result in longer removal times.

The natural attenuation of organic compounds in soils is an *in situ* biological technique, which uses natural processes in soil to contain the spread of contamination from chemical spills and reduces the concentrations and amounts of pollutants at contaminated sites. The work for this thesis was set out to investigate the potential for remediation by ENA. The specific process, which was studied, involves the biostimulation of indigenous microorganisms.

The following chapters will give the details of an extensive study site investigation, a set of microcosm treatability studies and the molecular microbiological analysis of microcosm results.

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Chapter 3

Desktop Investigation of the Study Site

3.1 Introduction

The aim of this chapter is to provide the details of a desktop investigation into the study site, including details on past uses and current contamination issues. The findings of the desktop investigation will be used in the formation of a conceptual site model of the site for this thesis. Given the scale of the site, a full detailed site investigation was not realistic within the scope of the thesis. Therefor, the conclusions of third party site investigations are brought together in order to aid in defining contamination problems, thus allowing the focus of this research to be finalised.

Section 3.2 provides an overview of the study site's local setting, including descriptions of the regional geology, nearby watercourses and residential and commercial properties in the surrounding area.

Section 3.3 describes the history of the study site. The development and subsequent use of the study site by a large petrochemical company is outlined in order to provide some background to the many contamination issues that exist there today.

The current situation and future site development plans are described in Section 3.4. Emphasis is placed on the need for remedial measures to be implemented prior to redevelopment.

Section 3.5 brings together the investigative findings of CELTIC Technologies Ltd (CELTIC). Important geological, hydrogeological, hydrological and chemical characterisation results are presented.

Section 3.6 concludes by briefly outlining the focus of this thesis. An interpretation of the CELTIC data is discussed in order to help in defining the state and mobility of contamination at the study site. A more specific problem definition and set of research tasks are then provided.

3.2 Local setting

The study site is a based at the former petrochemical factory, the history of which is described in Sections 3.3. The local topography is predominantly flat, and comprises an area of approximately 1 km^2. Figure 3.1 shows a plan view location map of the local area surrounding the study site.

The British Geological Society carried out surveys around the turn of the century, which describe the underlying solid geology of the region. The area is composed of carboniferous middle and lower coal measures from the Palaezoic era. The different components of the drift geology originate from a range of sources, including river deposits and blown sands. A more detailed description of the drift geology is provided in Section 3.5.3.

Three watercourses border the site: a brook to the Northwest, a river to the North and Northwest, and a saltwater bay to the Southwest. The bay and mouth of the river are estuarine.

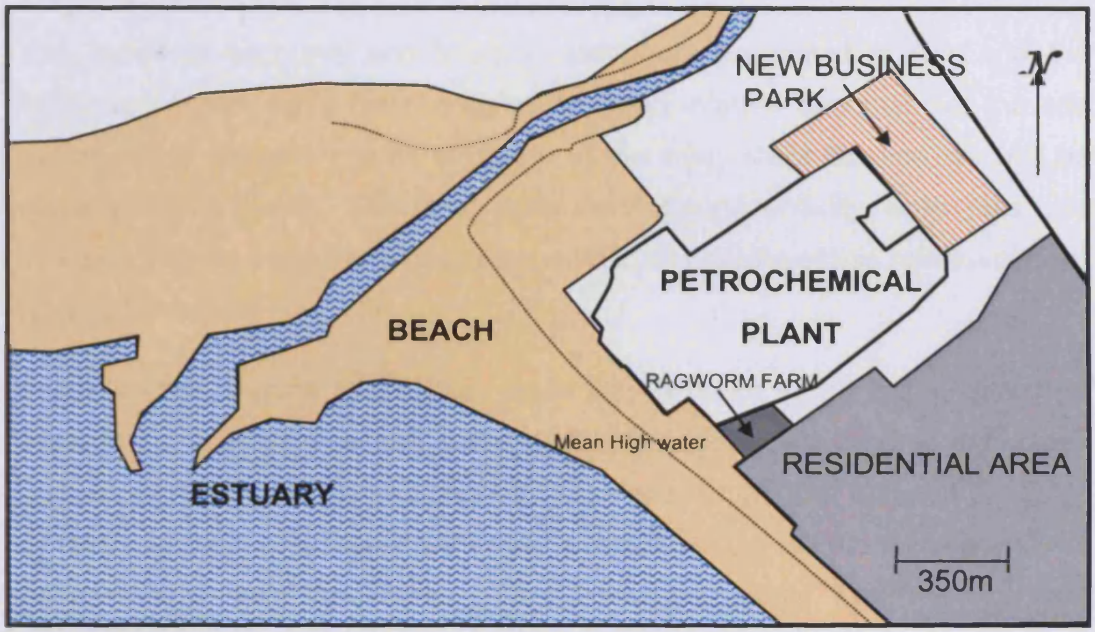

Figure 3.1 Location map of the study site and surrounding area (adapted from Otaegi, 2002)

A new business park has already been developed to the North and Northeast of the site. This land was formerly used by the petrochemical factory. The business park is stage 1 of the long-term plan to develop a larger area for businesses to set up and flourish.

A residential area borders the site to the Southeast. Over the factory's history, a number of complaints have been brought forth from residents living in this area. Such complaints regarded nuisance from noise, dust, odours and so on. To the South and Southwest of the site lies a public beach, which is frequented by many residents and visitors throughout the year.

A recent development at the site is the ragworm farm, located in the Southern corner of the plot. Ragworms are often used as bait by the fishing industry. The farm pumps water from the beach in order to supply fresh nutrients to the ragworms. The water cycles through the ragworm tanks before being released through an outlet on the beach. Although contamination issues from the site do have a potential impact on the farm, it was not the subject of this research

3.3 Site history

The historical accounts and images, which are presented in this and the following section, have been adapted from an internal document of the site owner. This document gives evidence of the study site's location, as well as naming the site owner. Due to an agreement of confidentiality, which was held between the site owner and this thesis author, the details of this reference have been withheld.

Following the Second World War, there was a major surge in the growth of Britain's petrochemical industry. By 1962, the industry had become the largest in Europe. This growth was partly due to an exemption from revenue duties on certain oils, but was more generally a result of an increased demand for petrochemical-based products. These products range from plastics and synthetic rubbers to man-made detergents.

On the 31st January 1961, an announcement was made to the media that a new petrochemical facility was to be developed on land at the study site. The site was ideally located near to both a petroleum refinery (for source materials), and an estuarine port (for shipping purposes).

Figure 3.2 Study site prior to the development of the petrochemical factory

The main challenge to site developers came from the fact that the study site, at that time, was covered in sand dunes. In places, the dunes reached over 30 metres above sea level, whereas other areas were fully covered by water. The picture in Figure 3.2 shows the study site before development began. The

dunes were levelled and compacted, and a layer of slag was laid in order to prevent wind erosion. Approximately 1600 piles were then sunk to support the many buildings, roads and rail tracks that were constructed.

In 1963, the factory was commissioned and began operations. The primary source compound, or feedstock, for the site was naptha. This fed the facility's steam cracker, which produced a number of chemicals, including ethylene. The products of the steam cracker in turn supplied the other main plants of the time, including the butadiene extraction unit, styrene monomer plant and ethylene dichloride (EDC) plant. It also produced gasoline as a by-product.

Good productivity and a greater demand for the products saw a call for further expansion of the site. Between 1968 and 1973, planning, construction and commissioning of the new expansion took place. New units included an updated steam cracker, vinyl chloride monomer (VCM) plant, EDC plant, chlorine production facility, styrene monomer plant, polyvinyl chloride (PVC) plant, vinyl acetate (VA) plant, isopropanol (IPA) plant, amongst others. A 105 MW power station was also integrated into the new expanded factory in order to cope with the increased demand for power. Figure 3.3 shows an aerial image of the site in full operation, following the expansion.

Figure 3.3 Petrochemical factory in 1977 following the expansion

3.4 Decommissioning and redevelopment

The petrochemical factory continued operations successfully for more than 3 decades. There are likely to be a number of reasons for the move towards decommissioning the site. One issue, which arose in 1985, was the closure of the nearby refinery, which was the main source of feedstock for the steam cracker. Within a few years, however, a rise in demand and favourable costs allowed the company to maintain their economic productivity, even though they had to ship in most of their raw materials.

Real problems became apparent in the early 1990's. A recession in the European economy led to a reduced demand for the factory's products. In 1994, the closure of the main steam cracker was announced. The other plants were able to remain active for a few years, but the damage to the overall factory due to the cracker's closure was beyond repair.

Today (2005), there are no active plants at the study site. As can be seen in the photograph in Figure 3.4, the site's landscape has changed significantly from the mid-1970s (Figure 3.3).

Figure 3.4 Petrochemical factory in 2000 after many of the plants had been decommissioned and demolished

Current work at the study site is focused towards demolition of previous structures and infrastructure, as well as the characterisation and subsequent clean up of any land contamination problems. This work is being carried out with a focus on the planned future land usage. The site is scheduled to be redeveloped into a new business park which will spawn new businesses and boost employment for the local community.

Due to the hazardous nature of the organic chemical compounds which were handled, stored and produced at the petrochemical factory, there was potential for pollution of the land underlying the site. In this case, contaminants refer to any foreign compounds, and pollution refers to contamination which poses a significant risk to receptors. The sources for contamination were more likely the result of regular spills and leaks, rather than mass contamination incidents.

An initial site investigation of the land at the study site was carried out by CELTIC in order to determine whether or not there was a) a risk to the surrounding environment or human health due to contaminants from the site, and b) a risk to proposed future developments.

Significant masses and concentrations of organic chemical pollutants were identified at the study site during the initial investigation. Therefore, it was recommended that further investigations and remedial measures were needed in order to remediate the land before the planned development can take place. Detailed findings of the CELTIC investigations are discussed further in Section 3.5.

3.5 Study site investigations

3.5.1 Overview

There have been a number of site investigations carried out on the study site. Many of the investigations were undertaken prior to the development of the petrochemical facilities on the site. The purposes and findings from each of the investigations vary. Many were undertaken primarily to acquire geotechnical data for the site, and others focused more on environmental issues, like the chemical analysis of groundwater. It is not relevant to cover the findings of all

previous site investigation in this thesis. This review therefore focuses on the most recent investigations, which relate to contamination issues at the site.

Most operations at the petrochemical factory were closed down by the end of the 20th century. In order to make the land suitable for redevelopment, the site owner appointed CELTIC to carry out an environmental assessment of the land.

From March 2001 until July 2001, CELTIC began an initial investigation into potential contamination issues at the study site. This first investigative phase was designed to determine the overall extent of contamination and assess the risk potential. The results of this first phase of investigation highlighted a number of different pollutant linkage scenarios where significant risk was posed to receptors.

Due to the unacceptable levels of risk which were identified, a second phase of investigation followed in March 2002 in order to further define the contamination problems so that effective remedial measures could be developed. CELTIC's main investigative tasks included the development of a conceptual model for the site's geology and hydrogeology, and a characterisation of contaminant plumes in the underlying soils and groundwater.

The site investigation details provided in this section give an overview of the site conditions and an introduction to contamination issues at the study site. The full extent of data collected from CELTIC investigations of the site is far greater than is presented here. Attempts were made to obtain extensive digital site figures/graphics; however, it is noted that no digital site map showing detailed sample locations was provided for use in this thesis. In the opinion of this thesis author, this is a weakness of the thesis.

The intention of this review is to form a conceptual model from which the focus problem for this thesis was defined. Once the problem was defined, detailed research tasks and clear objectives could then be developed. A number of different sources were used when compiling the data (CELTIC, 2001, 2002a, 2002b and Otaegi, 2002).

3.5.2 Techniques used

The section aims to provide the details of those techniques, which were used, for the collection and analysis of soil and groundwater samples from the study site. All of the techniques were carried out by, or under the instructions of CELTIC, on behalf of the site owner.

Membrane interface probes (MIPs) were used for investigating the underlying lithology and contamination across the study site. The MIP can evaluate the presence of volatile organic compounds (VOCs) in the subsurface. As the MIP devise is pushed into the subsurface, VOCs diffuse across the MIP's membrane and are absorbed into a vehicle gas where they are transported to ground level and into analysed by a mobile lab.

A large number of shallow and deep boreholes were drilled, with hollow stem augers, in order to provide key geological, hydrogeological and potential contaminant information across the site. 50mm diameter screened wells were placed in many of the boreholes for future use.

Trial pits were dug to sample and assess the shallow unsaturated ground. All boreholes, MIPs and trial pits were surveyed to metres above ordnance datum (mAOD). This was done to ensure that accurate groundwater and geological comparisons could be formed from analytical results.

Solid samples were taken from boreholes and trial pits across the site. The borehole samples were acquired straight from the spiral fluting of the augers as they were pulled from the ground. Samples were placed into 125ml glass jars, sealed with a Teflon lined cap and stored at 4 degrees Celsius. The three principle areas of focus were the unsaturated zone, smear zone/ capillary fringe and saturated zone. Onsite photoionisation detector (PID) headspace measurements were carried out on soil samples. PID measurements can determine concentrations of organic vapours, and are useful for field screening soil samples to determine which are most likely to be contaminated. Selected soil samples were sent to a lab to be further analysed.

Peristaltic pumps were used to collect groundwater from monitoring wells. Samples were analysed in-situ using handheld instruments for pH, temperature,

REDOX potential, dissolved oxygen, conductivity and alkalinity. Select groundwater samples were collected in one-litre amber glass jars, sealed with Teflon lined caps and stored at 4 degrees Celsius before being sent for laboratory analysis.

Table 3.1 lists the soil and groundwater parameters which were analysed for in the laboratory. Analytical techniques for the determination of VOC and SVOC concentrations were the only laboratory methodologies, which were made available for this thesis. Soil and groundwater samples were analysed using gas chromatography separation and mass spectroscopic interpretation (GC-MS). Methodologies for the VOC and SVOC analyses were based on guidance from the US Environmental Protection Agency's EPA 8260 and EPA 8270, respectively.

Table 3.1 Laboratory analyses of soil and groundwater samples (CELTIC, 2002a)

Soils	Groundwater
Metals (unsaturated zone): B, Cd, Cr, Cr^6, Cu, Fe, Be, Pb, Ni, Zn, V, As	Metals: B, Cd, Cr, Cr^6, Cu, Fe, Be, Pb, Mn, Ni, Zn, V
Metals (saturated zone): B, Cd, Cr, Cr^6, Cu, Pb, Ni, Zn, V, As	Total arsenic and mercury
Total mercury	Total cyanide
Total cyanide	Chemical oxygen demand (COD)
Total sulphate	Major inions and cations: Ca, Mg, Na, K, Cl, SO^4, NO^3
Elemental sulphur	Dissolved methane, ethane and nitrate
TPH (aromatic and aliphatic, 13 carbon band split)	VOC and TIC
VOC and tentative identified compounds (TIC)	SVOC and TIC
SVOC and TIC	NAPL characterisation (TPH profile, density and viscosity)
Total organic carbon (TOC)	Glycol
Polychlorinated biphenols (PCBs)	Salinity.
Asbestos	
pH	
Moisture content	
Particle size distribution (PSD)	

3.5.3 Geology

CELTIC carried out a detailed investigation of the underlying geology at the study site. The investigation failed to determine the presence of the underlying coal measures, which are shown in British Geological Society surveys of the region (BGS, 1972). Glacial till, which was identified underneath the site, is believed (by CELTIC) to be situated directly atop of the coal measures. For the purposes of this thesis, the glacial till is assumed to be the bottom of the aquifer.

Figure 3.5 shows the conceptual drift geological model of the area. This model was compiled by CELTIC, and is based on data obtained from historical sources such as BGS (1972) as well as through borehole logging. A horizontal scale is provided in the figure. A description of vertical scale is provided in the text below.

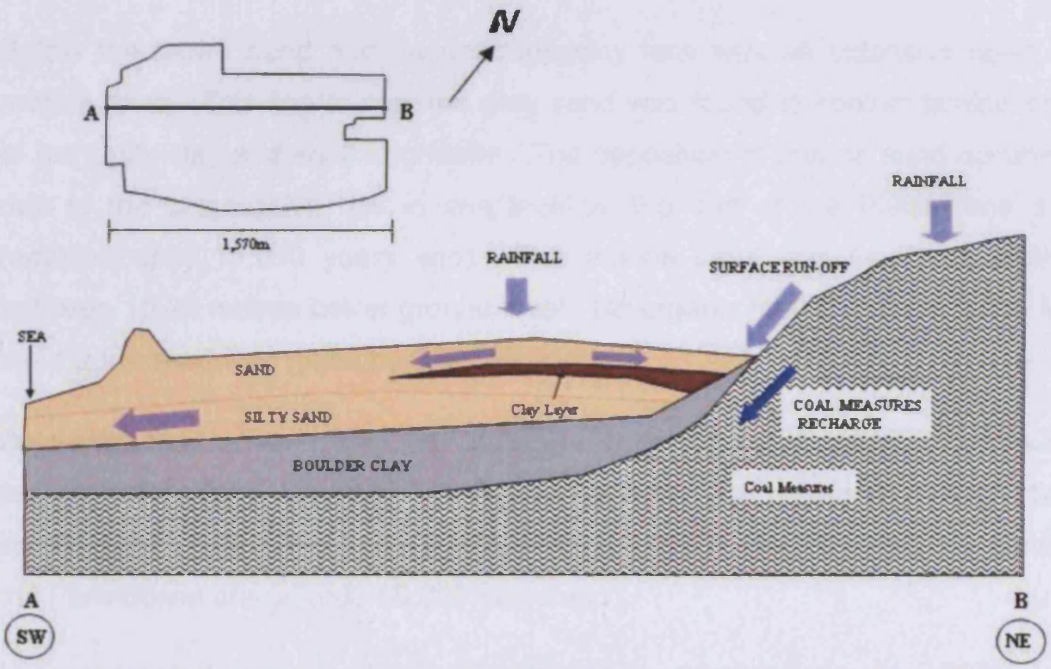

Figure 3.5 Conceptual geological cross section of the study site area (CELTIC, 2002a)

Up to around 1 metre of made-ground forms the surface layer over much of the site. This is composed mainly of slag, gravel, sand, brick and concrete, and was laid in order to prevent surface erosion.

Below the made-ground, CELTIC identified a layer of blown sand (shown as 'sand' in Figure 3.5). Prior to the development of the area back in the 1960's, these blown sands existed as crescentic dunes. The blown sands contain less silty content than the underlying marine sands, and were found between 0-15 metres below ground level. TOC determination of the upper sands gave results of less than 0.5% for all samples.

An intermediate clay lens was found to extend laterally across parts of the site. The known extent of this clay layer is illustrated in Figure 3.6. The lens lies

between the blown and marine sands, and is located 4-7 metres below ground level. The clay layer appears to gradually thin to nothing in the middle of the site. Localised areas of high clay and silt content, found throughout the marine sand, has led to some uncertainties as to the extent of the clay layer. An investigation into these uncertainties is not included within the scope of this thesis.

Below the blown sand and intermediate clay lens was an extensive layer of marine sand. This fine to medium grey sand was found to contain laminations of grey silty clay and shell fragments. The deposition of marine sand occurred due to the progressive rise in sea level at the end of the Pleistocene era (approximately 11,000 years ago). The marine sand was found at depths between 10-26 metres below ground level. No organic matter determination for the marine sand was made by CELTIC.

Below the marine sand, CELTIC expected to detect the presence of alluvium organic-rich silty. However, its presence was not detected through the investigations. The alluvium silt typically formed due to glacial melting during the Pleistocene era (25,000-50,000 years ago).

Immediately below the marine sand, CELTIC identified boulder clay. This clay is described under BS5930 (1999) as clay with angular gravels and cobbles, accumulated under glacial conditions during the Devensian ice age (50,000 – 70,000 years ago). The boulder clay gradually lowers as it approaches the estuary, where it is around 25-30 metres below ground level.

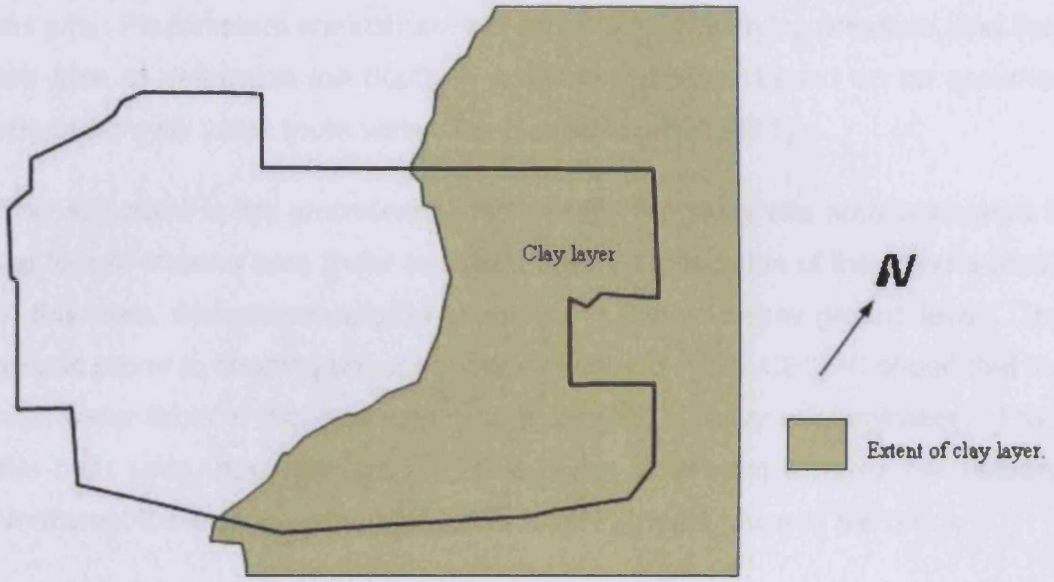

Figure 3.6 Extent of the intermediate clay layer under the study site (CELTIC, 2002a)

3.5.4 Hydrogeology and hydrology

Stand pipes and monitoring wells may be used to assess fluctuations in groundwater levels. Stand pipes are often smaller in diameter than a typical monitoring well, which is designed for pumping out samples of groundwater for chemical and/or biological analysis. By relating depth-to-water measurements (taken from top of casing) to a standard ordinance datum, groundwater elevations may be tabulated to reveal a horizontal value of hydraulic gradient. Depth to groundwater measurements were obtained from numerous well locations across the site. The results of this work were compared to surveyed top of casing (TOC) measurements. Figure 3.7 shows the groundwater elevations in the area of the study site nearest the estuary, as plotted by the Surfer software 1997. This area has been referenced to the National Grid (i.e. eastings and northings).

At the time of this thesis, no time-based groundwater level monitoring data was obtained (with the exception of high and low tide measurements). This thesis author suggests that the use of down hole piezometers and data loggers would prove useful for further interpreting the complex hydrogeological conditions at

the site. Piezometers are instruments capable of quantifying pressure, and thus are able to determine the depth in a column of water based on an assumed specific gravity value (pure water has a specific gravity of 1)

The high point in the groundwater table within the study site area is situated in the former styrene area (refer to Figure 3.8 for the location of the styrene area). In this area, the groundwater is situated 1-2 metres below ground level. The area is prone to flooding under periods of heavy rainfall. CELTIC stated that the high water table in this area might be a related to heavy contamination. From this high point, flow radiates in 3 directions: Southwest towards the estuary; Northwest towards the river; and North and Northeast towards the brook.

In Section 3.5.3, the boulder clay was described as being lower towards the estuary end of the site. The CELTIC investigations identified an apparent relationship between this slope in the boulder clay and the shape of the groundwater table. The results describe the local groundwater flow across the site as being predominantly towards the estuary.

Estuarine environments have a number of characteristics which set them apart from other coastal areas. They are generally defined as areas of water where the mouth of a river or land drainage mixes with saline water from a tidal sea (Barnes, 1997). In the case of the study site, the tidal range of the adjacent estuary can be as large as 50 feet (approx.15.4 metres), the second largest in the world. A variety of plant and animal species can live within estuarine environments. These include shellfish, molluscs, fish and worms. Most species living in estuaries often struggle to survive due to their inability to cope with variations in the saline content of water. Barnes (1997) states that high temperatures, low oxygen levels or the presence of toxic wastes may reduce the ability of certain animals to tolerate low salinities while, conversely, animals existing towards the limit of their salinity tolerance will be susceptible to pollution.

When fresh groundwater meets saline water, it is typically accelerated upwards due to the difference in densities between the two fluids. This stratification occurs mostly due to differing salt contents. Fresh water has a salt concentration of less than 1000 ppm (parts per million), whereas sea water can

range between 1000 and 35000 ppm salt (Mabey, 1983). The 'saline wedge', which is formed, is subject to changes in shape and location dependant on the hydrological and hydrogeological characteristics of the particular area.

The saline wedge at the study site could be an important factor when considering the transport of potential pollutants from the groundwater underlying the site into the estuarine environment. There are a number of effects that the saline wedge could have on the state and mobility of pollutants in the groundwater (Otaegi, 2002; Dyer, 1997; Fletcher, 1989; and Yechieli *et al.*, 2001):

- The saline wedge is subjected to large movements from tidal forces. Estuaries typically release a large volume of freshwater at low tide, and conversely hold back the flow at high tide. This varied flow pattern may allow for a significant increase in pollutant diffusion and dilution, thus reducing the potential for harm to the marine environment.

- The changes in flow brought about by the tidal movements may also act to restrict the flow of pollutants at the saline interface, thus allowing more time for attenuation of pollutant compounds.

- Pollutants being transported by the fresh groundwater may be pushed up the saline wedge as they migrate towards the saline body of water. This 'up-welling' may expose potential hazardous compounds to receptors at the ground surface.

- The increased salinity of groundwater as it approaches the salt water environment may slow down the rate of biochemical degradation of organic chemicals.

- Salinity variations bring about changes in pH and redox potential, which in turn can cause flocculation of clay minerals. Heavy metal adsorption can occur on the surfaces of such flocculates.

Groundwater chemistry analyses, which were carried out by CELTIC (discussed in more detail under Section 3.5.5), indicate that the saline intrusion into the

area beneath the site was found to be minimal. CELTIC suggested that this is likely to be a factor of the steep angle of the saline wedge.

This thesis author suggests that further investigation into the interactions between contaminant plumes and the saline wedge could provide vital information regarding the fate and transport of the potentially hazardous compounds. However, such an investigation is beyond the scope of this thesis.

A brook runs along the Northeast of the site. All runoff from the hills to the East and Northeast of the site was assumed by CELTIC to discharge to the brook. Therefore the runoff does not recharge the sand aquifers below the site. Topographically, the brook is lower than the site itself. Therefore, all recharge to the local aquifer is assumed to be from direct rainfall onto the study site.

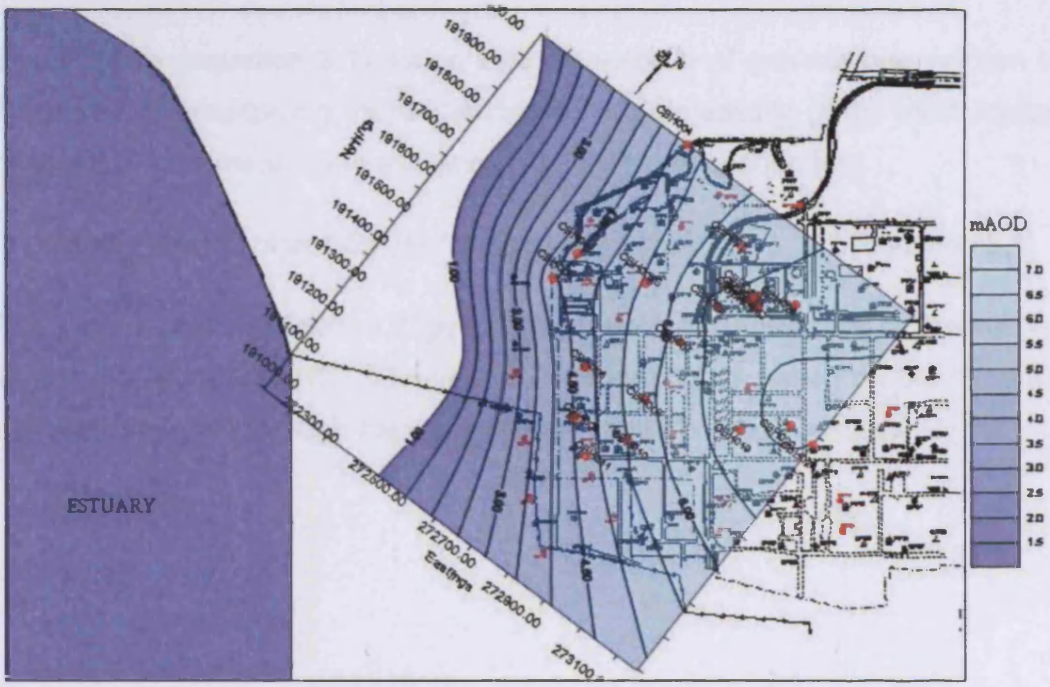

Figure 3.7 Groundwater elevations towards the Southwest side of the study site (7th June 2002) (CELTIC, 2002a)

As stated above, groundwater levels were monitored across the study site. CELTIC calculated the general hydraulic gradient (*I*) at the study site to be 0.009.

The tidal influence only reaches the Western edge of the former petrochemical site boundary, where fluctuations of 0.01-0.03 metres were measured. In the beach area tidal fluctuations ranged between 0.8-1 metres.

CELTIC carried out a pumping test in order to determine the hydraulic characteristics of the aquifer within the radius of influence of the well. The well was located towards the estuary end of the study site. Four monitoring wells were situated around the borehole. The Moench analysis for non-steady flow was the primary method, which was used to interpret the data. Moench (1996) describes this method. The Theis recovery method was then used to obtain further aquifer properties as the well recharged. This method is given by Theis (1935). Both of the methods used are designed for unconfined aquifer pumping test analysis.

Darcy's Law (equation 3.1) states that the velocity of groundwater (v) can be calculated by multiplying the underlying soil's permeability (k) by the hydraulic gradient (I). Permeability is also known as hydraulic conductivity.

$$Velocity\ (v) = Permeability\ (k) * Hydraulic\ gradient\ (I) \qquad \text{[Equation 3.1]}$$

The values given in Table 3.2 give the hydraulic characteristics of the aquifer, as calculated by CELTIC. The velocity was calculated based on the hydraulic conductivity found through the Moench analysis of pumping results.

Table 3.2 Aquifer hydraulic characteristics across the study site (CELTIC, 2002a)

Hydraulic Gradient (*I*)		0.009
Hydraulic Conductivity (*k*)	Moench	31.4 m/day
	Theis recovery	38.9 m/day
Transmissivity (*T*)	Moench	629 m^2/day
	Theis recovery	778 m^2/day
Storativity (*S*)		0.759
Groundwater Velocity (*v*)		0.2826 m/day 28.26 $x10^{-2}$ m/day

CELTIC conducted another pumping test within the styrene area. The results of this test described the permeability of the blown sands in the styrene area as being lower than that of the rest of the site. CELTIC interpreted this as being a function of contamination in this area, and not the natural permeability.

The clay layer, shown in Figure 3.6, may be a contributing to an apparent vertical hydraulic gradient in the aquifer. The clay potentially acts as an aquitard, thus restricting recharge to the lower sands. This leads to a difference in hydraulic heads between the blown and marine sands, which are separated by the clay layer. CELTIC found that the piezometric head in the lower (marine) sands was 1-2 metres lower than that of the upper sands.

As was mentioned in Section 3.3, a number of piles were sunk during the construction phase of the petrochemical factory. Some of these piles are known to pierce through the intermedisate clay layer, thus making for potential preferential groundwater pathways between the blown and marine sands.

3.5.5 Groundwater chemistry

Groundwater samples were collected and analyses under the direction of CELTIC. Over one hundred different sampling points were used for these

analyses. It is not within the scope of this thesis to present results from each of these points. Instead, this section will focus on the analytical results as a whole, in order that the reader may draw a more general picture of the groundwater conditions at the study site.

Table 3.3 gives a statistical interpretation of the groundwater analyses, which were carried out *in situ*. The values shown in the table represent groundwater samples from across the site.

Table 3.3 Statistical interpretation of groundwater analyses (CELTIC, 2002a)

Parameter	Mean	Standard deviation	Range (min/max)
Dissolved Oxygen (mgL^{-1})	2.7	2.5	0.0/10.4
Redox potential, Eh (mV)	-37.8	229.9	-1583.0/239.0
pH	8.0	1.0	6.3/11.9
Temperature (degrees C)	13.2	1.5	10.6/17.6
Conductivity (μScm^{-1})	505.5	332.3	102.0/1874.0

The results given in Table 3.3 show high variability, which is highlighted by the standard deviation and range values. This suggests that the presence of contamination is likely to be variable between sampling points. Examination of the minimum Eh (-1583.0 mV) and maximum pH (11.9) values shows the sorts of conditions, which may be associated with heavy organic chemical contamination. Microbial activity is largely responsible for the changes in groundwater chemistry, which are brought about by the influx of a fresh organic carbon source.

The concentrations of other groundwater analytes, which were determined on site samples by CELTIC, are given in Table 3.4. These values represent groundwater which was extracted from a borehole located near to the eventual focus area of the site for this thesis. A more detailed description of the focus area is provided under Section 3.6.

Table 3.4 Typical groundwater chemistry results near to the focus area (CELTIC, 2002a)

Analyte	Concentration
Nitrate (mgL^{-1})	0.6
Iron III (mgL^{-1})	42.1
Sulphate (mgL^{-1})	82
Methane (mgL^{-1})	ND

The results shown in Table 3.4 represent the findings from a single sampling point, and are therefore not representative of the overall site. Further groundwater chemistry characterisation was deemed necessary for the aims of this thesis. The results of these further studies are later compared to those shown in Table 3.4 (refer to Chapter 4).

3.5.6 Contaminant distribution and movement

CELTIC carried out an extensive investigation in order to identify and quantify contaminants at the study site. A range of contamination issues was identified at the study site. This section focuses on those issues, which fit into the focus of the research. The focus is further defined through the conclusions given in Section 3.6.

3.5.6.1 Plume characterisation

CELTIC collected a number of shallow vadose soil samples from the study site. Their analysis of these samples gave results, which indicated the presence of organic contaminants. However, the quantities detected within the soil samples were not significant. CELTIC suggested that the majority of contamination incidents were likely to have originated at the ground surface, and that the contaminants seem to have rapidly migrated through the vadose zone and into the groundwater.

Groundwater contamination was found to be widespread across the study site. Towards the estuary end of the site, a number of chlorinated and BTEX

hydrocarbons were detected in solute form. In particular, 1,2-dichloroethane (DCA), vinyl chloride (VC), benzene and ethylbenzene were identified as key contaminants. The properties and behaviour of these pollutant compounds in a soil-groundwater environment are discussed in Chapter 2. Samples analysed from a borehole near to the area of the site, which was later selected as the main focus for this thesis (Figure 3.8, Section 3.6), gave concentrations of 2,050 mgL^{-1} of DCA, 132 mgL^{-1} of VC, 4.4 mgL^{-1} of benzene and 1 mgL^{-1} of ethylbenzene. These results give an indication of the range of concentrations, for the four contaminants, which can be found at a single sampling point.

CELTIC encountered difficulties with plume delineation on the half of the site nearest the estuary. The large area and depth of the overall site meant that extrapolating data between sampling points could produce large margins for error. CELTIC interpreted the unusual plume distributions across the site as being a result of transport by preferential pathways. Plumes appear to be fingering into narrower pathways as they move towards the estuary.

DCA was found in the groundwater at concentrations exceeding 1000 mgL^{-1}. The Environmental Quality Standard (EQS) for DCA in coastal or estuarine environments is 10 μgL^{-1} (EA, 2005). Sabatini *et al* (1996) states that 1,2-DCA has an aqueous solubility of 8690 mgL^{-1}. Groundwater concentrations in excess of 1% of solubility are typically indicative of the presence of DNAPL. DCA concentrations in excess of 10% have been detected at the study site. However, no DNAPL was detected during the investigations. The presence of DNAPL would act as a secondary source for pollution, and so add to the long-term contamination problems at the site.

CELTIC has detected the presence of benzene and ethylbenzene near the bottom of the aquifer (i.e. boulder clay surface). Benzene and ethylbenzene have relative densities of 0.87 and 0.86 mgL^{-1} (ChemExper, 2005), respectively, and were therefore expected to be predominantly present towards the top of the water table. There are a number of possible explanations for the downward migration of these compounds from their sources. DCA and VC are denser liquids, and were expectedly detected towards the bottom of the aquifer. It is possible that benzene and ethylbenzene plumes may have mixed with DCA

and/or VC plumes and were thus transported together. Another possibility is that the lighter hydrocarbons may be migrating along a vertical hydraulic gradient, which is transporting the compounds downward in the aquifer. The intermediate clay lens may contribute to this unusual groundwater movement. The apparent vertical hydraulic gradient and potential effects of the clay lens on the overall groundwater flow were discussed in Section 3.5.4.

The persistence of organic pollutants is important when considering the fate of a particular compound, and will influence the potential risk that is posed to environmental or human health receptors. The recalcitrance of chlorinated hydrocarbons has been well studied, (Chapter 2). Chlorinated hydrocarbons are typically more persistent in the environment than the more basic aromatic compounds like benzene and ethylbenzene. EPA (2005a) states that DCA is likely to persist in groundwater for a very long time, and that there is little degradation by microbes. In contrast, benzene is well known to be degraded in ground waters (EPA, 2005b). However, there are many variables between the various pollutants, aqueous phase and soil fractions which can significantly affect the attenuation of compounds at any particular site (Yong and Mulligan, 2004). In other words, the attenuation of organic pollutants in a soil-groundwater system is more likely to be governed by site-specific conditions.

At the study site, benzene contamination was widespread. This is due to its regular use for a number of different processes across the former petrochemical plant. Ethylbenzene contamination follows a similar pattern to that of benzene. As stated previously, CELTIC detected these compounds towards the bottom of the aquifer, where conditions are likely to be predominantly anaerobic. The anaerobic degradation of benzene in the environment is much less studied than the well-known aerobic mechanisms. Concentrations for benzene in solute form exceeded 100 mgL^{-1} in samples taken from a number of monitoring wells across the site. At present, there are limited records for changes in plume concentrations over time. In addition, the source concentrations for most of the site's pollutants are unknown, due to accidental and undocumented spillages. For these reasons, there is no way of knowing precisely how the compounds are being degraded or persisting in the subsurface without further study.

Another contamination problem which was identified through the investigations, but is not directly related to the focus area, is that of styrene. Styrene monomer was found in soil (adsorbed), groundwater (solute), and free-phase (LNAPL) form. In the groundwater, it was detected at concentrations, which were up to maximum aqueous solubility limits (i.e. 13 mgL^{-1}). The effects of the styrene contamination on the hydrogeology of the site are not fully understood. However, it is possible that these effects may add to the possibility of a restricted recharge of groundwater to below the clay lens. The styrene monomer contamination is localised to the area directly below the former styrene units. It does not appear to be migrating laterally across the site.

3.5.6.2 Pollutant linkages

Part IIA of EA 1990 gives a definition of contaminated land. Under this definition, land is classed as contaminated if: a) a risk of harm is posed to human health or the environment, or b) controlled waters are being threatened. The need for clearly defined source, pathway and receptor relationships is also emphasised in the legislation. A summary of the pollutant linkages, which were identified by CELTIC, is given in this section.

A full risk assessment of the contamination scenarios was carried out for the study site, however the findings of this risk analysis are confidential to the site owner, and are therefore not included in this thesis.

As stated previously, the sources of contamination at the study site are very difficult to define. The predominant types of contaminant sources were from spills, leaks and accidents at the petrochemical works. Such contamination incidents are assumed to have occurred throughout the lifetime of the factory, and not all at once. The contamination of the site is also widespread. In other words, it is not localised to one particular region of the site.

The pathways for most organic pollutants at the site can be generalised as solute transport with the local groundwater through the saturated sandy aquifer. In terms of the focus area, the direction of this pathway is towards the estuary. Clarification of unknowns regarding the downward flow of groundwater and contaminants at the site would help to better characterise the pathway. Another

potential pathway is through the vadose zone. Volatile pollutants in gaseous phase could migrate upwards towards the ground surface.

Potential on-site human health receptors were identified, where exposure to VOCs could occur as gaseous hydrocarbons migrate from the shallow contaminated aquifer, through the vadose zone and into the atmosphere. The mechanism of exposure is most likely to be inhalation.

Off-site receptors were characterised as users of the estuarine beach adjacent to the site. Exposure could originate from gaseous VOC migration or soluble pollutant transport in the groundwater. The groundwater rises up to ground surface level at the beach due to the saline intrusion. The off-site mechanisms for exposure include inhalation, direct skin contact and ingestion.

The estuary itself was also considered to be a potential receptor. The potential ecological impact due the introduction of solute form organic pollutants needs to also be considered.

3.6 Conclusions and problem definition

The details of the investigations, which have been discussed in this chapter, are useful for defining the study site conceptual model for this thesis. Understanding the site's past, present and planned future uses allow for a clearer problem definition, so that the appropriate steps can be taken towards achieving clean-up goals. This research is being carried out in order to provide detailed scientific information, which will aid in the development of a full-scale remediation scheme.

Contamination incidents over the past few decades have resulted in a large range of potential contamination problems at the study site. The site is currently undergoing a period of decommissioning, deconstruction and remediation, with a view to redevelop the area. Over the past several years, CELTIC was employed as a consultant to the site owner, and has undertaken a large-scale investigation into contamination related issues at the site.

Following the acquisition of data for this desktop study, a meeting was held with the site owner to discuss potential areas of research. It was decided that there was a lack of sufficient scientific knowledge to be able to proceed immediately with remediation measures in the area of the site nearest the estuary. This research is therefore directed towards contamination issues in this area. Due to the large extent of this area of the site, a smaller focus area was chosen for detailed investigation. Figure 3.8 shows a closer view of the study site, with the focus area highlighted.

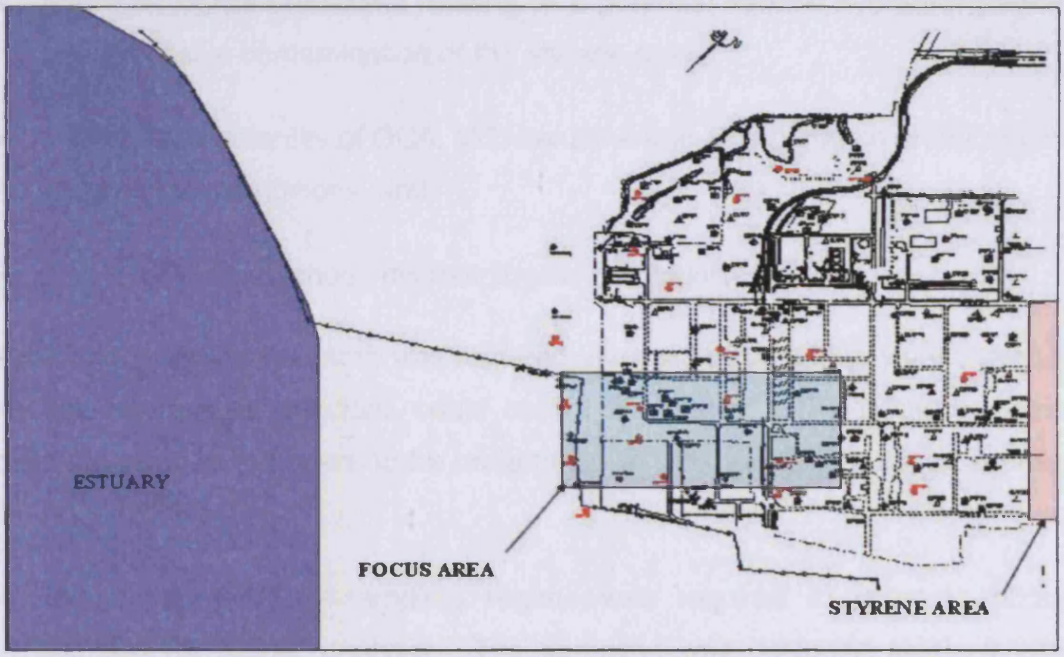

Figure 3.8 Plan view showing the focus and styrene areas (adapted from CELTIC, 2002a)

CELTIC identified a range of chlorinated and BTEX hydrocarbon plumes which are mobile in the groundwater system underlying the focus are of the study site. Four of the main contaminant compounds, which were identified, include DCA, VC, benzene and ethylbenzene. These were designated as the focus contaminants for this research. The actual location and delineation of these plumes proved to be difficult due to the extent of the site and an apparent degree of contaminant fingering. It is likely that these plumes are also mixing to form more complicated plumes containing multiple contaminant compounds.

Much of the contamination was detected in anaerobic environments towards the bottom of the aquifer. The plumes are a problem due to their movement towards an adjacent beach and estuary, where potential risks to receptors exist.

At this stage in the thesis progression, there were still a number of conceptual model uncertainties to be clarified. Such uncertainties included:

- The potential presence of harmful metabolites of styrene within the focus area,
- Hydrogeological unknowns relating to a potential vertical hydraulic gradient and excessive contamination of the styrene area,
- Degradation potential of DCA, VC, benzene and ethylbenzene under natural and enhance conditions, and
- Details of the indigenous microbiological communities.

Additional scientific research was required in order to clarify these uncertainties so that appropriate solutions could be recommended. The following points describe tasks, which were to be undertaken for this thesis in order to address the uncertainties.

- An independent site-sampling regime was required in order to obtain samples for further analysis. The sampling was designed solely for the purposes of this research. This allowed for sampling, transportation and storage procedures to be carefully planned and documented, thus providing a record of potential sources for error.
- A number of lab-based analyses were planned in order to characterise soil and groundwater samples from the site. These analyses were designed to provide a better knowledge of the hydrogeological effect which the styrene area might be having on the focus area of the site, and to fully characterise the physical, biological and chemical properties of the soils and groundwater from the focus area.

- Enhanced bioremediation was identified as a possible treatment option for the focus contaminants. In order to fully describe the potential for remediation via enhanced bioremediation, microcosm treatability studies were to be undertaken. These studies were designed to provide results under a range of different enhancement options.

- Understanding the metabolic activities of microorganisms in the environment is essential for describing the degradation pathways of organic pollutant compounds. Molecular microbial techniques were chosen for monitoring changes in microbial community profiles throughout the microcosm studies.

The tasks outlined above, form the principle focus of the work which was carried out for this thesis. The following chapters will give detailed descriptions of the analyses and discussions on the results of this work.

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Chapter 4

Study Site Investigations

4.1 Introduction

This chapter describes the study site investigations, which were designed to provide an insight into certain contamination problems at the site. Field work and laboratory analyses were coordinated for the investigations. The investigative results and conclusions will form the basis for the design of microcosm treatability studies.

Section 4.2 will set the background to the investigations by defining the purpose of the work. The investigative work was concentrated towards two separate areas: the styrene area, and the focus area of the study site.

In Section 4.3 an analysis of samples obtained from the styrene area is presented. The analyses were primarily designed to give an insight into the effects of heavy contamination within this area on the overall hydrogeology of the study site.

Contamination issues within the focus area are addressed in Section 4.4. Analyses on samples from this area were focused on describing a large range of physical and chemical properties. The analytical procedures and results of the investigative work are discussed.

Conclusions are drawn on the analytical results in Section 4.5, and help to accurately describe problems at the study site, and thus allow for the site conditions to be effectively simulated in the laboratory.

4.2 Purposes of the investigations

Following an extensive review of issues relating to contamination at the study site, a program of further site investigation was deemed necessary (refer to Section 3.6, Chapter 3). CELTIC carried out the previous investigations of the site, in accordance with their own study objectives. The further work, which is described in this chapter, was carried out under the direction of this thesis author.

The investigations were designed to give a first hand knowledge of the techniques used, and so provide a better perspective from which to interpret the results. The central focus of this research is to conduct treatability studies, based on an enhanced bioremediation technique. Proceeding with only a limited knowledge of the adopted methodologies of previous investigations might have jeopardised the integrity of the results. Proceeding otherwise could be seen as a 'black box' approach to the problem, where results are obtained without a fundamental understanding of the science involved (Yong, 2001).

The details presented in the following two Sections (4.3 and 4.4) represent two distinctive investigations, each with their own objectives and conclusions. Combining the results of these investigations with the previously reviewed site data provides a clearer conceptual site model, from which remedial treatments can be designed. The fresh soil and groundwater samples extracted during the investigations were also intended for use in the microcosm studies that followed.

4.3 Styrene area investigations

4.3.1 Overview

The former styrene production and storage area has been found, by previous investigations, to be heavily contaminated with styrene and BTEX compounds. Delineation of the BTEX plumes has shown significant migration towards potential receptors. However, styrene monomer appears to be localised to the source area. There are likely to be a number of processes restricting the movement of styrene, including polymerisation, volatilisation, chemical and

biological transformation. Understanding the physical, biological and chemical processes which affect the mobility or loss of a pollutant is key to developing appropriate remedial strategies. For instance, if much of the styrene was found to exist as a polymer, it could result in a significant blockage of soil pore spaces, which in turn would make remedial techniques such as bioventing and air sparging less effective.

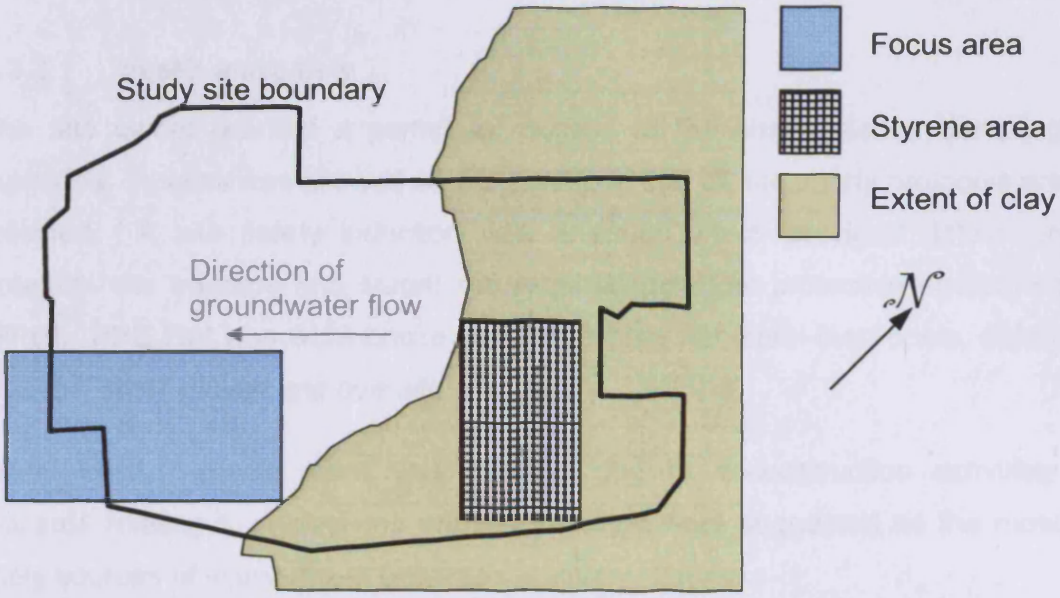


Figure 4.1 Study site plan showing the focus and styrene areas (adapted from CELTIC, 2002a)

The central focus of this research is directed towards contaminants which are migrating towards the estuary. There are potential contaminants in the focus area, which may have migrated from the styrene area of the site, and have not yet been detected in previous site investigations. Figure 4.1 shows the location of the styrene area in relation to the focus area. Such compounds might include metabolites of styrene (e.g. persistent organic acids). There is also the need for further clarification of the hydrogeological regime, due to a lack of knowledge regarding the mechanism(s) that may be causing the downward migration of light hydrocarbon compounds (discussed in Chapter 3). Potential sources for the apparent vertical migration of contaminants are discussed.

The main tasks of the styrene-area studies were:

1. To collect soil and free-phase hydrocarbon samples from the site,
2. To identify and quantify metabolites of styrene,
3. To identify and quantify styrene monomer, and
4. To identify styrene polymer.

4.3.2 Field work

4.3.2.1 Health and safety

The site owner granted a permit for access to the study site for sampling purposes. Access was allowed on the condition that all site safety protocols are followed. A site safety induction was attended which provided details on potential site hazards and stated the required personal protective equipment (PPE). PPE that was worn onsite included a hard hat, steel-toed boots, safety glasses, nitrile gloves and overalls.

When onsite, special care was needed due to deconstruction activities. Hazards relating to rubble and uneven surfaces were suggested as the most likely sources of injury.

In addition to the health and safety requirements of the site owner, Cardiff University School of Engineering requires that a risk assessment be carried out for any academic work. This risk assessment presented a review of all potential hazards, from sampling through to the analysis and safe disposal of materials. All potential risks concluded from the assessment were mitigated through safe practice and appropriate PPE.

4.3.2.2 Sample collection

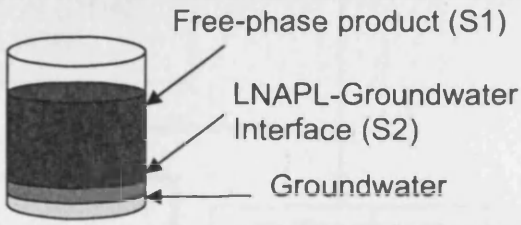
Due to certain thesis limitations, it was not possible to be present for the sampling event described in this section; nor was it within the thesis budget to hire a drilling rig for the sole purpose acquiring sample cores for this research. A consulting engineering company, working on behalf of the site owner, coordinated the field event and collected the samples described below. They had their own objects for the drilling, and sample handling techniques were planned by the consultant. It is acknowledged that more appropriate sample

collection techniques are available. Further discussion on appropriate sampling techniques is presented in Section

Soil samples were obtained from hollow stem borehole augers, using split spoon samplers. The cores acquired were approximately 50mm in diameter. The techniques may have led to the exposure of soil cores to the atmosphere. This would have potentially facilitated the volatisation of certain organic compounds. Soil samples were wrapped in parafilm and stored at 4 degrees Celsius.

Groundwater samples were collected from sampling wells using a 40mm x 1m polypropylene bailer. The liquid sample primarily consisted of free product, but did contain trace amounts of groundwater. The sample was transferred from the bailer into a 1-litre amber glass jar. The jar was filled completely and placed in a 4 degrees Celsius refrigerator for storage. In total, three types of samples were obtained from the styrene area: free-phase hydrocarbons, blown sand and clay. Table 4.1 shows the samples with a brief description of each. The numbering of sample labels is prefixed with SS, which stands for 'Styrene-area Sample'.

Table 4.1 Descriptions of samples acquired from the styrene area

Sample Label	Borehole Identification	Description
SS1	CBH 77	
SS2		
SS3	CBH 79	Free-phase product
SS4	CBH 87	Free-phase product
SS5	SP1	Blown sand taken from 4m bgl
SS6		Clay taken from 6m bgl
SS7		Clay taken from 6m bgl
SS8	SP2	Blown sand taken from 4m bgl
SS9		Clay taken from 6m bgl
SS10		Clay taken from 6m bgl

Samples S9 and S10 (originating from sampling point SP2), were duplicate samples of the intermediate clay lens, which underlies much of the East and Northeast of the study site.

4.3.3 Analytical procedures and results

The Minton, Treharne & Davies Group (MTD) carried out the analyses on samples from the styrene area. The work was done under the instruction and supervision of this thesis author. A schematic representation of the analytical work is shown in Figure 4.2.

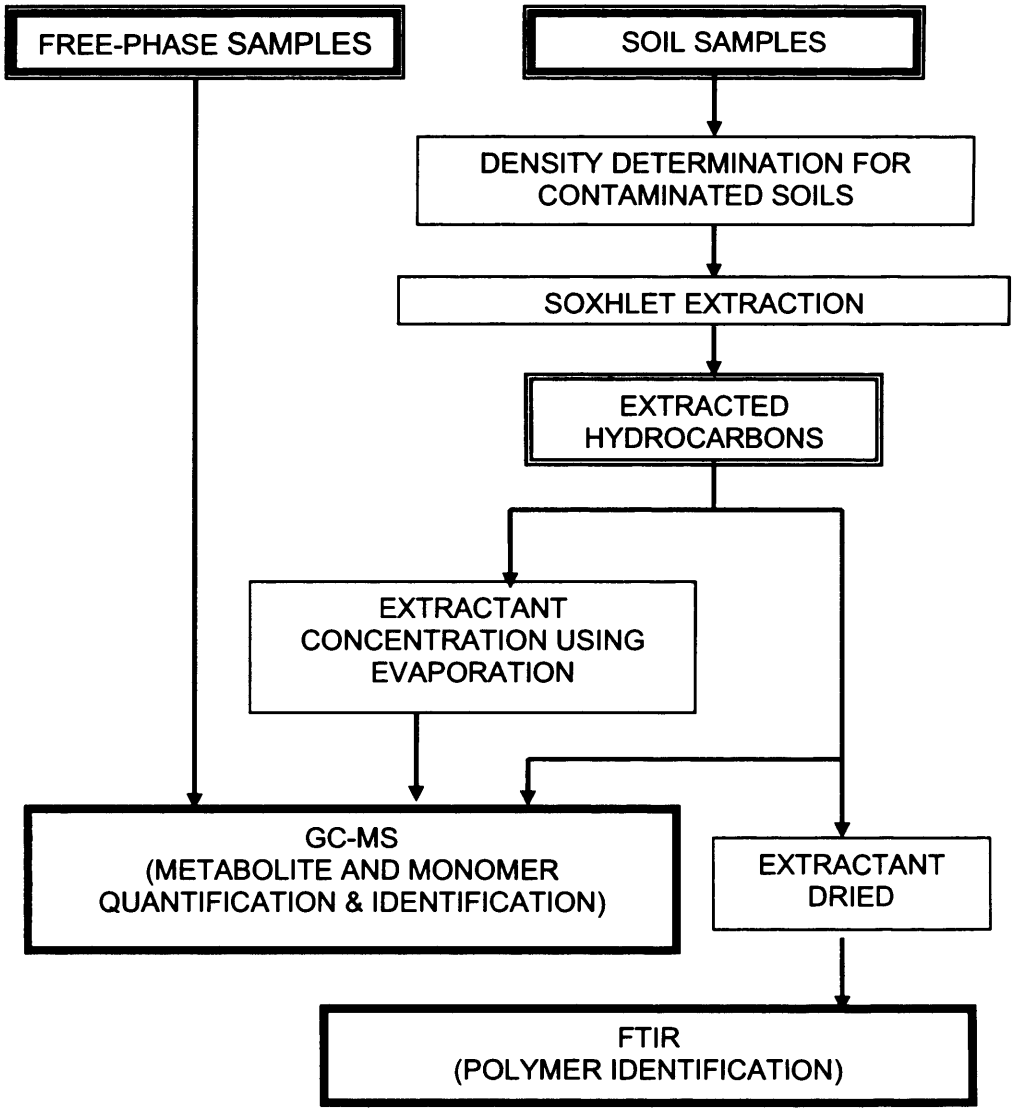


Figure 4.2 Schematic representation of the styrene area analyses

4.3.3.1 Styrene monomer and metabolite determination

Styrene monomer was presumed to be the most plentiful organic pollutant in the samples obtained from the styrene area. There are several microorganisms that are capable of initiating styrene biodegradation, and metabolism proceeds via one of two known aerobic pathways; the principal reaction may either be an ethylene side-chain monooxygenation or a 2,3-dioxygenation (Kraus *et al,* 2002). An aerobic pathway was assumed due to the detailed knowledge of metabolites, which is discussed in previous research. The known degradation pathways for styrene were discussed in more detail in Chapter 2. The styrene monomer may, however, be degrading through anaerobic mechanisms. A full investigation into the actual mechanism(s), which may be responsible for styrene degradation *in situ*, was not within the scope of this thesis. This section describes a study, which was carried out, in order to identify one potential styrene degradation pathway.

In addition to styrene monomer, five metabolites were selected as the main analytes for this stage of the organic chemical characterisation. The choice of metabolites was based upon their position within the degradation pathways (discussed in Chapter 2), and the commercial availability of the compounds as standards. The following metabolite standards were used for calibration of instrumentation, and were searched for during the analyses of the samples:

1) Styrene Oxide
2) Phenalacetic Acid
3) 2,5-Dihydroxyphenylacetic Acid
4) 2-Hydroxyphenylacetic Acid
5) Phenylacetaldehyde

Prior to extraction, solubility tests were performed to determine which solvents should be used for the extraction procedure. The metabolites were firstly dissolved into 100ml of dichloromethane (DCM). However, the 2,5-dihydroxyphenylacetic acid and 2-hydroxyphenylacetic acid were not readily dissolved in the DCM. This suggested that the DCM extraction may not be effective in removing these particular organic acids from the soil samples.

Approximately 10mL of methanol were then added to the mixture, which then dissolved the acids straight away.

Further tests were then performed in order to gain more knowledge about the role of the acids and their polarity and partitioning between different compounds. The two acids were dissolved in water, lab-standard styrene monomer and caustic (dilute potassium hydroxide - strong base). This was a non-quantitative analysis, but the amounts used were roughly 5mg of the acid in 5ml. 2-hydroxyphenylacetic acid partially dissolved in all three of the liquids. On the other hand, 2,5-dihydroxyphenylacetic acid dissolved straight away in the potassium hydroxide and turned a rusty colour. It also dissolved straight away in the water. However, it failed to dissolve in the styrene monomer. This suggested that if 2,5-dihydroxyphenylacetic acid is present at the study site, it might be highly mobile. The compound would seem to preferentially dissolve in groundwater over styrene.

A Soxhlet apparatus was used to extract the target analytes from the solid soil samples. Each of the samples was extracted separately with DCM, methanol and toluene. 10g soil samples were extracted with 150ml solvent. The extracts were later concentrated ten-fold by evaporation.

Extracts were analysed using a gas chromatograph with a mass spectrometer (GC-MS). Chapter 5 provides more extensive details of GC-MS theory (with regards to the analysis of samples from microcosm studies). Such details are not presented in this chapter since the analytical methods were predominantly selected and carried out by MTD (as stated above).

Prior to analysis, the GC-MS was first calibrated using standards of each of the target analytes. Although MTD stated that calibration results fell within appropriate quality control (QC) limits, no calibration or other QC data was provided to this thesis author by MTD.

Styrene monomer was determined on the extracts of the solid samples using butyl acetate as internal standard. Retention times for both compounds were predicted to be very similar. Samples S1 and S2 were diluted 1:1 with methanol

prior to analysis by GC-MS. The liquid extracts were injected directly into the GC-MS apparatus.

The instrument parameters were as follows:

GC Agilent 5890 with 5971 mass selective detector
Column HP5 MS 30m x 0.25

The detection limit for the soil extract analyses was 20 $mgkg^{-1}$. Only two of the soil samples (S9 and S10) gave positive results for styrene monomer. The other soil extracts gave no detection within the stated limits. The results for sample S9 showed 700 and 5,300 $mgkg^{-1}$ for the DCA and methanol extracts respectively. Sample S10 showed 30 $mgkg^{-1}$ for both the DCM and methanol extracts. All toluene extracts gave negative results. These results are given in Table 4.2. 'ND' stands for not detected.

Table 4.2 Styrene concentrations determined on soil samples (data provided by MTD)

Sample	Styrene monomer concentration ($mgkg^{-1}$)		
	DCM extracted	Methanol extracted	Toluene extracted
S5	ND	ND	ND
S6	ND	ND	ND
S7	ND	ND	ND
S8	ND	ND	ND
S9	700	5,300	ND
S10	30	30	ND

No styrene metabolites were detected in the soil extracts (i.e. samples S5, S6, S7, S8, S9 and S10). The extracts were concentrated ten-fold by evaporation, yet still no metabolites were detected with a detection level of 5mg/kg. Styrene metabolites were also not detected in the liquid samples (i.e. samples S1, S2, S3 and S4) at a detection level of $5mgL^{-1}$.

The negative results for the metabolite compounds might indicate the absence of microbial degradation processes for the styrene. However, this study only focused on the aerobic degradation of styrene monomer. Anaerobic processes may play a role in styrene degradation at the study site. Further study into this

issue was beyond the scope of this thesis. For the purposes of this research, the lack of presence of styrene metabolites is enough to proceed with the focus area field work and analyses without the need of including any previously unidentified pollutants.

4.3.3.2 Styrene polymer determination

Polymerised styrene was identified gravimetrically on the dried residue of free-phase styrene samples (samples S1, S3 and S4) and DCM and toluene extracts, then its presence confirmed by Fourier Transform Infra Red Spectroscopy (FTIR). The drying process was designed to remove any volatile components, leaving behind the more stable polymers.

There were residues observed following drying, but an accurate quantification was not possible. The residues were examined by FTIR and the spectra obtained did not resemble that of polystyrene.

The dried residues of free-phase samples S1, S3 and S4 gave results of 1.9, 4.7 and 4.1 % weight/volume respectively. Examination of these residues by FTIR gave spectra that compared well to that of a spectrum of polystyrene.

This styrene polymer study was a simple identification process. Further polymer characterisation would be required before solid conclusions could be drawn regarding the effects of such compounds on the fate of contaminants at the study site. Further polymer characterisation was beyond the scope of this thesis.

There are a number of potential issues, which may be related to the presence of styrene polymer at the study site. The availability of nutrients for the metabolic function of microorganisms may be hindered by the presence of styrene polymer. Styrene polymer is a highly viscous substance, which could potentially be restricting the flow of nutrient-rich groundwater through that area of the site. The presence of styrene polymer and potential for pore blockage may also be affecting the flow of groundwater across much of the study site.

4.4 Focus area investigations

4.4.1 Overview

Extensive contamination of the groundwater system exists underlying the focus area of the site. The principle contaminants of concern for this research are 1,2-dichloroethane (DCA), vinyl chloride (VC), benzene and ethylbenzene. The compounds were predominantly found in solute form, with high concentrations detected deep in the aquifer (15 – 25 metres bgl).

Previous investigations of the site provided little insight into the diversity of microbiological populations. Knowledge about the diversity of microorganisms within the soil-groundwater system could help to define the potential for remediation using indigenous microbial species. Characterising indigenous microorganisms at a range of depths and locations became a major focus of these investigations. However, the main aim of these investigations was to define those parameters, which would allow for an effective lab-based simulation of site conditions (i.e. microcosm studies).

Soil and groundwater sampling was planned in order to gather enough materials for a full biological, chemical and physical characterisation of conditions within the focus area. As with the styrene area work, funding restrictions meant that all samples would need to be obtained off the back of consultant drilling investigations. This made for difficulties regarding the scheduling of analytical work, due to a lack of knowledge about quantities and types of samples which were to become available.

The main tasks of the focus area investigations were:

1. To collect soil and groundwater samples from the site,
2. To carry out onsite chemical analyses on groundwater samples,
3. To determine the physical and mineralogical properties of soil samples, and
4. To chemically characterise groundwater samples.

4.4.2 Field work

Focus-area field work was designed and carried out in order to provide details on *in situ* conditions, and to collect samples for use in microcosm studies. Photographs of the sampling and on site analyses are provided in appendix I.

4.4.2.1 Health and safety

Permission for site access in order to carry out sampling activities was again granted by the site owner. A risk assessment was conducted in order to avoid potential hazards, both onsite and in the laboratories. Refer to Section 4.3.2.1 for further information on health and safety issues.

4.4.2.2 Soil sample collection

The first stage of field work involved the sinking of 3 new boreholes in the focus area of the site. This drilling work was carried out by a contractor (Drillcorp), and supervised by a consultant (CELTIC Technologies Ltd), who was investigating on behalf of the site owner. The main purposes of the drilling, from the consultant's perspective, were to inspect VOC concentrations in the soil (carried out onsite) and install monitoring wells for future use. For the purposes of this research, this stage of the field work was carried out in order to acquire soil samples at a range of depths and boreholes. Figure 4.3 shows the approximate locations of the 3 boreholes in relation to potential sources and receptors. The source area is assumed to be the former ethylene dichloride plant area, where contamination incidents for DCA were documented (CELTIC, 2002). The receptors shown in the figure are located in the adjacent estuarine environment and public beach. Refer to Chapter 3 for more details on environmental concerns in estuarine environments. The three boreholes are labelled B1, B2 and B3, as shown.

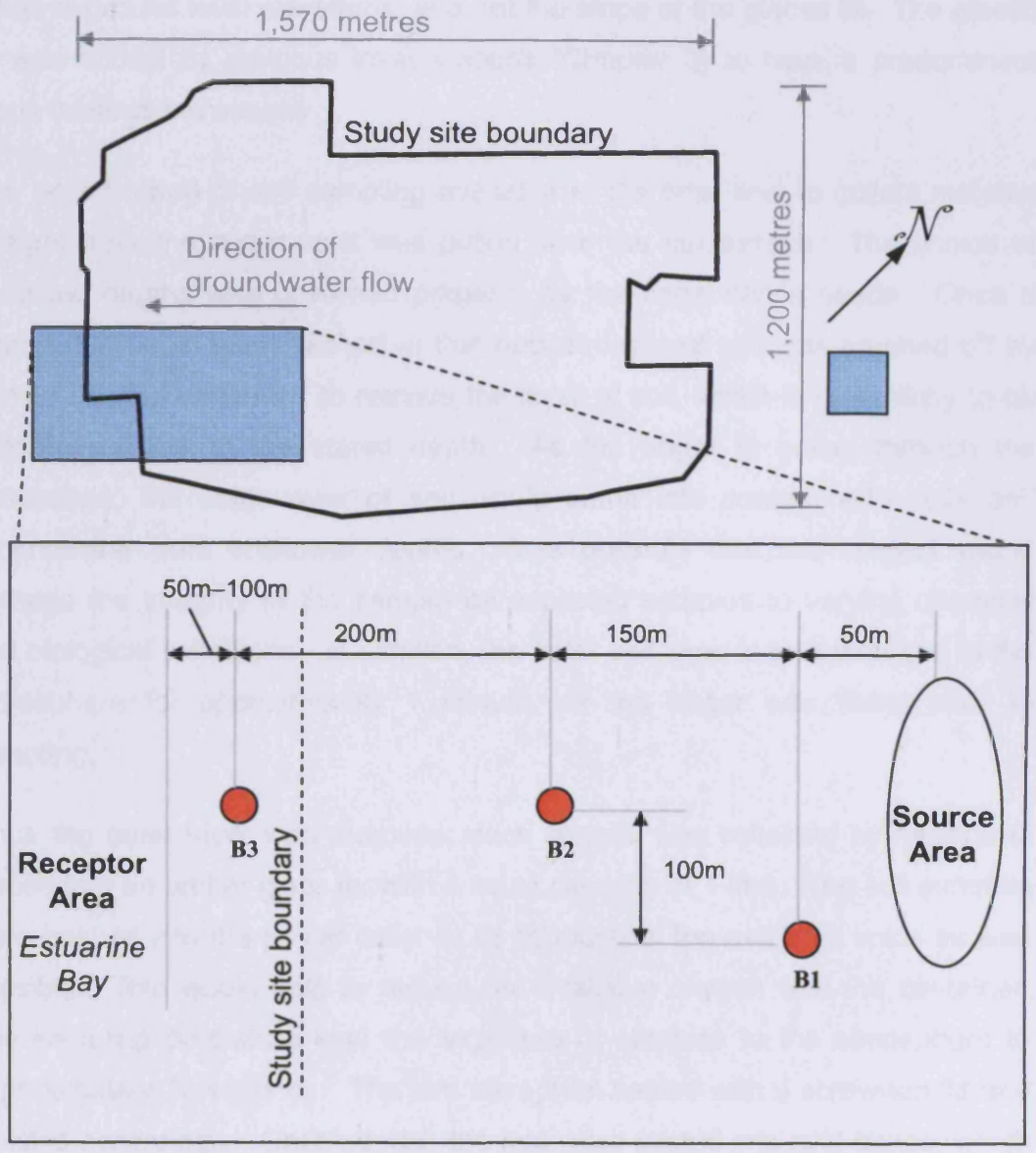


Figure 4.3 New borehole locations (B1, B2 and B3) in relation to potential source and receptor areas

The 3 boreholes were installed in August 2003. Hollow stem augers were used to allow for the 50mm diameter monitoring wells to be inserted. The last 6 metres of the inserted wells were screened/ slotted to allow for the influx of groundwater. For each of the three points, the augers were drilled down to the glacial till. The depths, from ground surface level to the glacial till for boreholes B1, B2 and B3 were 24.7, 23.5 and 22 metres respectively. Notice that the depths decrease as they approach the estuary. This is assumed to be a result

of lower ground level elevations, and not the slope of the glacial till. The glacial till was shown by previous investigations (Chapter 3) to have a predominant slope towards the estuary.

The only method of soil sampling available at the time was to collect material straight from the auger as it was pulled from the subsurface. The choice of sampling depths was governed primarily by the consultant's needs. Once a designated depth was reached, a thin outside layer of soil was brushed off by hand. This was intended to remove the layer of soil, which is most likely to be unrepresentative of the stated depth. As the auger is pulled through the subsurface, the outer layer of soil would come into contact with soils and groundwater from shallower depths. It is possible that this contact would damage the integrity of the sample by exposing samples to varying chemical and biological conditions. In addition, the outer soil layer is also exposed to the atmosphere for approximately 1 minute, as the auger was rising prior to sampling.

Once the outer layer was removed, each sample was collected by hand, and placed into an amber glass jar with a liquid capacity of 1-litre. The soil samples were pushed into the jars in order to fill as much of the available voids as was possible. This would help to reduce the available oxygen with the container. The sampling procedure kept the exposure of samples to the atmosphere to approximately 30 seconds. The jars were then sealed with a screw-top lid and labelled accordingly. Once sealed, the jars were placed into cool boxes, which maintained temperatures between 4 and 8 degrees Celsius. The ambient air temperature for the day of sampling ranged between 18 and 23 degrees Celsius. A total of 8 soil samples were obtained. Sample descriptions and sources are given in Table 4.3. The numbering of sample labels is prefixed with FS, which stands for 'Focus-area Sample'.

If additional resources had been available for this soil sampling phase, sampling procedures would have followed the recommendations set forth by the American Society for Testing and Materials (ASTM). The current ASTM guidance document, entitled Standard Guide for Sampling Waste and Soils for Volatile Organic Compounds (ASTM, 2006), suggests appropriate methods for

sampling, handling and storage of VOCs. Of particular note is the recommendation that all soil samples collected for VOC analysis be preserved with a solvent in order to maintain sample integrity prior to analysis. The addition of a solvent (such as methanol) helps to reduce the loss of VOCs to the headspace (and porespace) within a container of soil, and thus improves the accuracy of VOC assessment.

Each of the collected soil samples gave an odour of VOCs. The samples from borehole B1 produced noticeably stronger VOC odours than the samples from boreholes B2 and B3. There were no obvious distinctions between the appearances of the soil samples from each of the boreholes. Very thin pockets of silty sand were observed as the auger was pulled from the subsurface.

Table 4.3 Descriptions for soil samples from the 3 new boreholes

Borehole	Sample label	Depth (metres bgl)	Description
B1	FS1	17.0	Marine sand
	FS2	24.5	Marine sand
	FS3	25.1	Glacial till
B2	FS4	12.0	Marine sand
	FS5	18.0	Marine sand
B3	FS6	10.0	Marine sand
	FS7	16.0	Marine sand
	FS8	22.0	Marine sand + Glacial till

4.4.2.3 Groundwater sample collection

The second sampling stage was carried out in order to obtain groundwater samples from the 3 new boreholes (i.e. B1, B2 and B3). No consultants were involved during the groundwater sampling stage. Once acquired, the samples were sent to MTD for chemical analysis, and later used in microcosm studies.

The sampling techniques were intended to provide undisturbed samples. A low-flow rate peristaltic pump was used to extract groundwater. Peristaltic pumps provide relatively undisturbed samples when compared to other groundwater sampling methods (Puls and Barcelona, 1995).

Difficulties arose in locating borehole B3, which was set in the public beach area. The landscape of the beach had been altered over the winter months due to tidal and meteorological conditions. The approximate location of B3 was determined using a global positioning system (GPS). Manual digging within a 5 square metre area eventually resulted in a successful find.

The sampling took place in August 2004. Given the low flow technique, which was used, borehole purging was limited to the level of the groundwater table. Borehole B1 proved the most troublesome due to an apparent slow rate of well recharge. The peristaltic pump could only cope with heads of less than approximately 5 metres. Once a couple of litres had been sampled from the well in borehole B1, the pump was unable to hold enough suction. Five litres of groundwater were collected from each of the other two boreholes.

The analyses carried out by MTD on these samples also led to problems. Long delays and communication difficulties with the lab jeopardised the quality of the results. The analyses took approximately two months to complete. With large potential errors arising from these problems, it was deemed necessary to revisit the study site and collect another set of fresh samples.

The second round of groundwater sampling took place in March 2005. On this occasion, peristaltic sampling was not used due to the difficulties encountered during the previous site visit. Bailers were instead used to collect groundwater samples. Prior to the actual sampling, each of the boreholes was purged. This would allow for the fresh influx of groundwater through the 6-metre screen and into the monitoring wells. The water which was present in each of the wells prior to purging, may have been stagnant for a prolonged period of time, and thus it would not be representative of the natural groundwater. Three well volumes were removed from each borehole using a bailer (dimensions: 40mm diameter, 1000mm length).

Approximately one week after the purging was carried out, the sampling commenced. Care was taken to minimise the loss of VOC contamination by gently lowering the bailer into each borehole. Samples were transferred into glass jars and sealed. Separate containers were used for storing groundwater,

which would later be assessed for VOC, TOC and other chemical parameters. Containers were placed into cool boxes for transport to MTD.

The groundwater, which was extracted from borehole B1, was distinctly different from samples from the other two boreholes. B1 samples were very dark in colour, and had a light sheen of free-phase at the surface.

4.4.2.4 Onsite analyses

Onsite analyses were carried out during the groundwater sample collection stage of the focus area field work. Peristaltic pumps were used to collect groundwater from the subsurface. Measurements were taken for temperature, pH, redox potential, dissolved oxygen and electric conductivity. Handheld instruments were used to make the analyses on groundwater samples from boreholes B1, B2 and B3.

Groundwater was extracted from the monitoring wells using a low-flow rate peristaltic pump. The sampled water was then pumped into 1-litre amber glass jars with the tubing placed at the bottoms of the containers. The jars were filled and allowed to overflow until approximately 2 litres of groundwater had passed through the jar. This method was designed to act as a simple flow through cell so that the samples would have minimal disturbance through exposure to oxic conditions.

Probes were inserted into the jars, and three separate readings were taken. The values shown in Table 4.4 represent the mean of the three readings for each parameter.

Table 4.4 Results of the onsite analyses for boreholes in the focus area

Sampling point	Temperature (degrees Celsius)	pH	REDOX potential, Eh (mV)	Dissolved oxygen (mg/L)	Electrical conductivity (uS/cm)
B1	18.9	10.8	-251.5	2.8	5,100
B2	18.7	8.2	-105.0	2.1	698
B3	22.1	7.7	-28.0	1.9	14,710

The temperature in borehole B3 was measured at approximately 3 degrees Celsius higher than that found in the other two boreholes. The groundwater level in borehole 3 is nearer to the ground surface. Heat from solar radiation at the ground surface is likely to be the reason for the elevated temperature in this borehole.

Biodegradation rates influence and depend on redox potential, with lower values typically being associated with more contaminated areas (San Mateo, 1996). Because of this, redox values can give an indication of the locations of organic chemical plumes. A redox potential of -251.5 mV was measured in samples from borehole B1. This indicates reduced conditions and likely high quantities of contaminants, when compared to groundwater taken from boreholes B2 and B3. Redox conditions in a soil-groundwater environment were discussed further in Chapter 2.

The measured values of pH were inversely comparable to the redox potential values, with higher redox values being associated with a lower pH. San Mateo (1996) describes optimal pH conditions for microbial growth within the 6-8 range. However, this range is not vital for microbial growth. There are many microbial species which are known to be tolerant to, and in many cases thrive in highly acidic and alkaline conditions. With such high pH values (mean value 10.8) detected in borehole B1, it is likely that the diversity of active microbial species will differ from that of the other boreholes.

Dissolved oxygen (DO) concentrations greater than 1-2 mgL^{-1} are needed to maintain aerobic conditions at plume boundaries (San Mateo, 1996). The measured values onsite gave a range of 1.9-2.8 mgL^{-1}. It could therefore be assumed that the supply of DO in the groundwater is sufficient to facilitate aerobic biodegradation. However, oxygen is likely to be less plentiful within the centre of hydrocarbon plumes. This is due to the reduced conditions which can be brought about by the metabolic activities of indigenous microorganisms in the presence of excess organic compounds. It is also likely that the measured DO values are slight exaggerations of actual *in situ* conditions, due to exposure to atmospheric oxygen during the sampling procedures.

Electrical conductivity (EC) is a measure of a waters ability to conduct electrical current, and is related to the amount of dissolved mineral present (Mechenich and Andrews, 2004). Jackson Bottom (2005) lists some typical EC ranges for natural waters: Drinking water = 50, Streams running through granite, silicon or other igneous rock = 10-50, Streams running through limestone formations = 150-500 and ocean water = 53,000 (all units in microSiemans/cm). The highest detected EC values from Figure 4.4 were found in borehole B3. The high conductivity of B3 samples is likely to be related to the borehole's close proximity to the estuary. Elevated salt concentrations will increase EC in water. The values are not high enough, however, to indicate the intrusion of pure sea water into the borehole. Although less than borehole B3, the EC found in borehole B1 was higher than expected. B1 is located further inland on the study site is not likely to be affected by the influx of saline water from the estuary. It may be that the values are the result of a dechlorination of halogenated hydrocarbons. Though it is also possible than other dissolved compounds are responsible.

4.4.3 Analytical procedures and results

4.4.3.1 Particle size distribution

Dry sieving was used to determine the particle size distribution of the soil sample FS5. The procedure was based on BS 1377 (1990a). The sample was first dried at 60 degrees Celsius to remove excess moisture. A representative sample was obtained by using a cone and quarter technique with approximately 150g of sample. 78.73g of representative material was then placed into the sieves and shaken for ten minutes. The particle size distribution curve for this analysis is shown in Figure 4.4.

The results show that sample FS5 is predominantly fine sand. The size distribution of the sand-sized material consists of 77.3% fine sand, 9.9% medium sand and 0.9% coarse sand. 0.3% of the sample tested was too large to pass through the sieve with an aperture size of 2mm. 11.6% of the sample was found to be silt or clay-sized (i.e. <63 microns). No further characterisation

was made of the <63-micron fraction. A number of shell fragments were observed in the 1180 – 2000 microns range.

The larger granular particles which account for much of the sample contribute directly to the mechanical properties of the soil, whereas the smaller fractions are more related to the physico-chemical and chemical properties (Yong and Mulligan, 2004). This relates directly to the fact that smaller soil particles have larger specific surface areas (SSA) than larger particles. The low quantity of silt and clay-size particles found during the analysis could therefore suggest a reduced level of physical and chemical interactions between dissolved pollutants and soil surfaces.

4.4.3.2 Mineralogy

X-ray diffraction (XRD) was used to determine the mineralogy of the soil sample FS5. XRD is a method whereby crystalline compounds can be identified and quantified. Crystal and mineral substances are identified by relating the angle of incidence of the X-rays to the distance between atoms within a crystalline structure.

The soil sample was first prepared by grinding into a fine powder, before being packed inside an aluminium holder. The sample is left to run on the XRD apparatus for 25 minutes. The resulting diffractogram is shown in Figure 4.5. The unknown peaks were then identified by comparison to an integrated computer database of over 70,000 known mineral phases.

A comparison of diffractogram peak areas does not give fully quantitative results. Therefore, it is more accurate to define the technique as an identification and semi-quantitative analysis.

The most dominant mineral fraction that was found in sample FS5 is quartz, which accounted for 87.0% of the FS5 mineral composition. Other identified components, and their respective percentages include calcite (6%), albite (1.7%), illite (0.8%), kaolinite (0.5%) and hematite (0.5%). A further 3.3% was assumed for other minor mineral constituents, which remain unidentified.

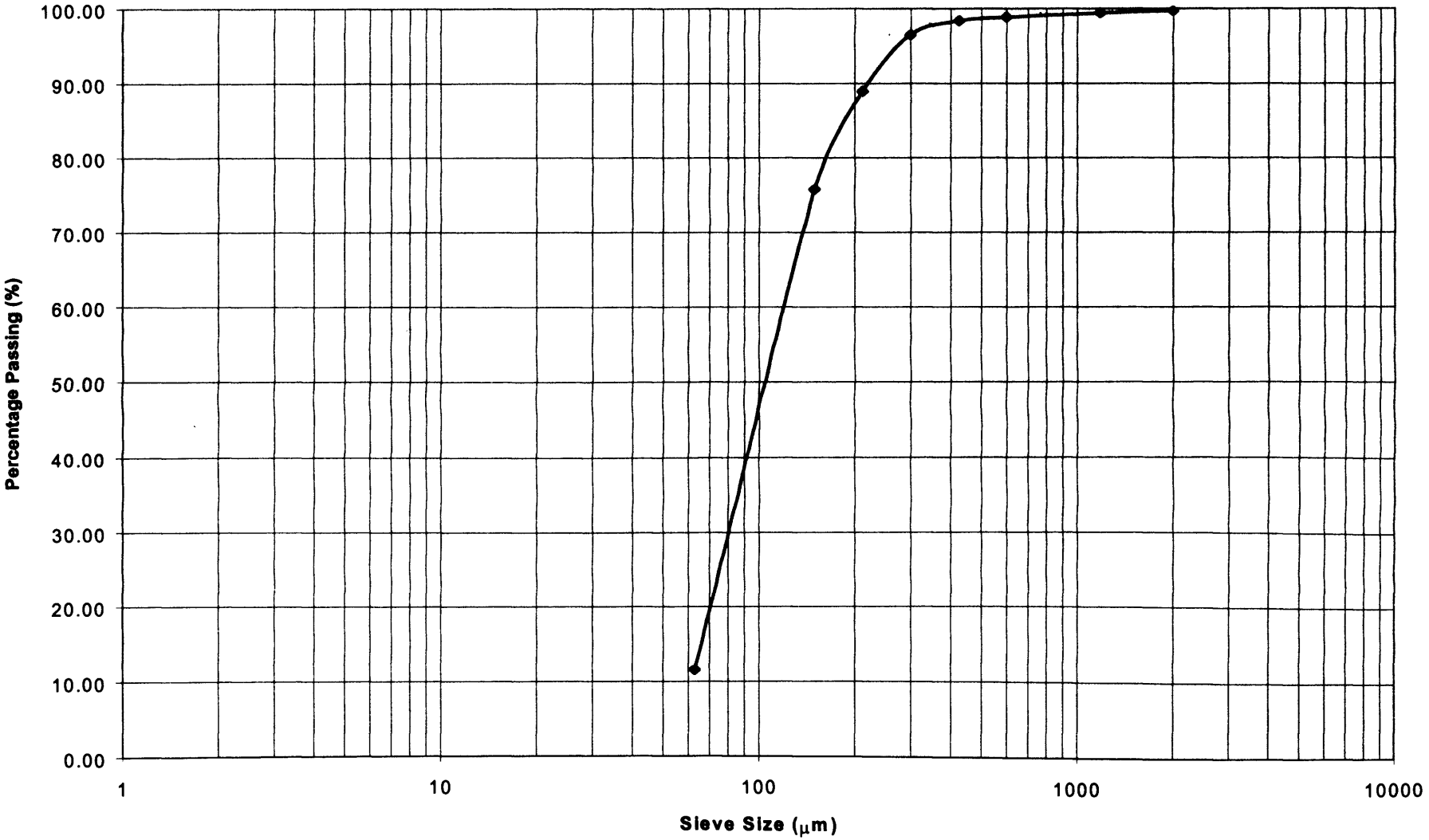


Figure 4.4 Particle size distribution curve for sample FS5

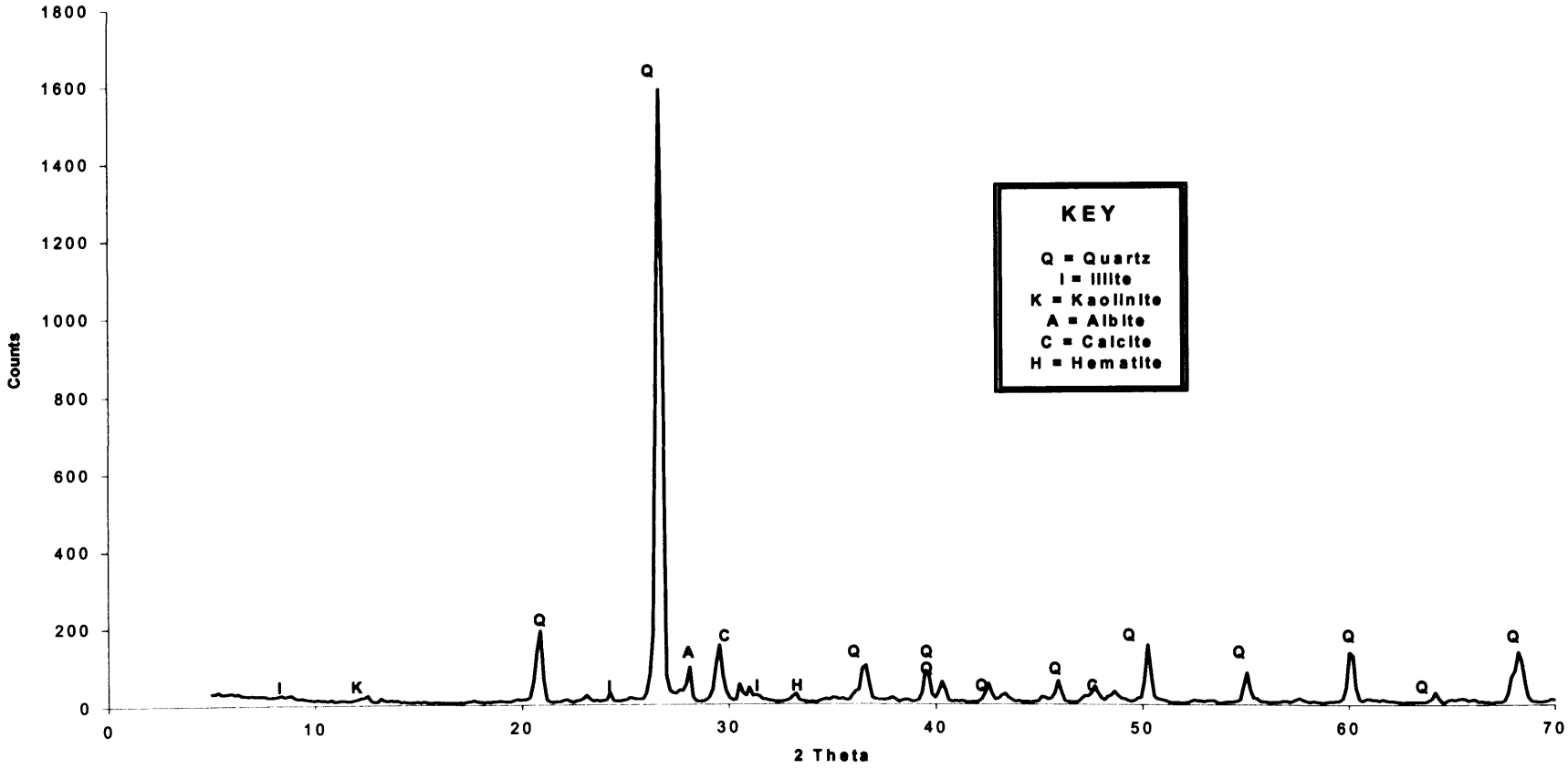


Figure 4.5 Mineralogical diffractogram results for sample FS5

4.4.3.3 Moisture content

Determination of the moisture content of sample FS5 was based on the oven-drying gravimetric method outlined in BS 1377 (1990b). Approximately 30g of bulk soil was added to a dish, then placed into an oven at 105 degrees Celsius. The sample was heated at 4-hour intervals until the weight did not exceed 0.1% of the original mass. Total drying time was approximately 16 hours. The weight of soil was measured before and after the drying process, and the moisture content was recorded as a percentage of the dry soil mass. Triplicate samples were dried, and an average value determined for percentage moisture content. Sample FS5 was found to have a moisture content of 13.3%.

These results were intended to provide a basis from which to combine soil and groundwater samples to form a slurry with a particular solid/liquid ratio. This slurry was used during the microcosm studies. Slurry mass balances and the subsequent development of the microcosm experiments are discussed in the following chapter.

4.4.3.4 Groundwater characterisation

Groundwater analysis of the samples described under Section 4.4.2.3 was carried out by MTD. As previously stated, an original round of analyses was carried out on groundwater samples by MTD starting in August 2004. The results of these original studies are not presented here due to large potential errors, and the availability of more accurate data. The following paragraphs detail the findings of work carried out by MTD in March 2005. Groundwater samples from boreholes B1, B2 and B3 were analysed, and took approximately 3 weeks to complete. The results of the groundwater analyses are displayed in Tables 4.6 and 4.7.

Groundwater samples were analysed using a gas chromatograph with a mass spectrometer (GC-MS). The instrument parameters were as follows:

GC Agilent 5890 with 5971 mass selective detector

Column HP5 MS 30m x 0.25

Chapter 5 provides more extensive details of GC-MS theory (with regards to the analysis of samples from microcosm studies). Such details are not presented in this chapter since the analytical methods were predominantly selected and carried out by MTD (as stated above).

A full suite of VOCs was selected for GC-MS analysis. Prior to analysis, the GC-MS was first calibrated using standards of each of the analytes from the target VOC list. Although MTD stated that calibration results fell within appropriate quality control (QC) limits, no calibration or other QC data was provided to this thesis author by MTD.

Groundwater samples were injected directly injected into the GC-MS inlet system. The results for the three of the focus compounds for this research are presented in Table 4.6. The other focus compound (vinyl chloride, VC) was not investigated during these analyses. Problems arose with the handling of vinyl chloride standards in the laboratory. It would have been impractical to proceed with VC as a focus, due primarily to the difficulties with introducing the compound into the microcosm experiments. The boiling point of VC is –13.9 degrees Celsius, and it is usually sold in a solution of methane. The full VOC results are given in appendix II. The detection limit for the analysis was set at 2 μgL^{-1} (ppb).

Table 4.6 Main VOC results from analysis of groundwater samples (data provided by MTD)

Analyte	Borehole B1	Borehole B2	Borehole B3
Benzene	12,900 μgL^{-1}	2 μgL^{-1}	943 μgL^{-1}
Ethylbenzene	16 μgL^{-1}	<2 μgL^{-1}	<2 μgL^{-1}
1,2-dichloroethane	879,000 μgL^{-1}	5,508 μgL^{-1}	22,600 μgL^{-1}

DCA was the most abundant pollutant compound, which was detected. The presence and high quantities of DCA correspond with the previous findings of CELTIC, which were presented in Chapter 3. 879 mgL^{-1} of DCA was measured in groundwater samples from borehole B1. The highest concentrations of pollutant compounds, for each of the 3 listed analytes, were found in borehole

B1. This was expected given the findings of onsite analyses (discussed in Section 4.4.2.4). These include a low redox potential, high pH and high EC.

The Environmental Quality Standard (EQS) for DCA and benzene in coastal or estuarine environments are 10 and 30 μgL^{-1} respectively (EA, 2005). DCA and benzene concentrations far exceeded these values. There is currently no EQS for ethylbenzene. The New Dutch List gives ethylbenzene an action level of 150 μgL^{-1}. The results of this investigation show concentrations of ethylbenzene below this level. However, the investigations of CELTIC (from Chapter 3) suggest that ethylbenzene concentrations have been detected well within the mgL^{-1} (ppm) range. For this reason, ethylbenzene remained a major focus of investigation for this thesis.

Table 4.7 Groundwater chemistry results for boreholes B1, B2 and B3 (data provided by MTD)

Analyte	Borehole B1	Borehole B2	Borehole B3
TOC	360 mg/L	<5 mg/L	22.4 mg/L
Chloride as Cl	1,270 mg/L	35 mg/L	870 mg/L
Sulphate as SO_4	96 mg/L	59 mg/L	101 mg/L
Nitrate as NO_3	<0.3 mg/L	<0.3 mg/L	<0.3mg/L
Ferrous iron as Fe_2	No result obtained	<1 mg/L	18 mg/L
Total alkalinity as $CaCO_3$	4140 mg/L	150 mg/L	250 mg/L
Methane	<5 mg/L	<5 mg/L	<5 mg/L

Total organic carbon (TOC) determination was also carried out on groundwater samples. The samples were first pre-treated with 10% HCl (hydrochloric acid) and purged with gaseous nitrogen in order to remove the inorganic carbon content. Carbonaceous components were then oxidised to carbon dioxide. The TOC content of the liberated gas was then measured using a non-dispersive infrared detector. The detection limits for this analysis were 5mg/L.

Determinations of TOC can provide valuable diagnostic evidence of the extent groundwater contamination by organic compounds (Barcelona, 1984). Higher TOC values were attributed to the presence of higher pollutant concentrations. For example, groundwater samples from borehole B1 gave the highest TOC

results (360 mg/L) as well as the highest dissolved DCA, benzene and ethylbenzene concentrations.

Chloride ion concentrations were obtained using a chloride analyser. The device makes use of a coulometric titration method, which relies on the formation of insoluble salt (silver chloride), following the introduction of an electric current to the sample.

High chloride concentrations (4140 mgL^{-1}) were found in samples from borehole B1. These results are comparable with the elevated EC values, which were obtained during the onsite analyses. This is true due to the possibility that the free chloride ions may combine with other inorganic chemicals, such as sodium, to form soluble salts (e.g. sodium chloride). The presence of such salts in the groundwater would increase the EC. A potential source of the increased chloride ion concentrations in borehole B3 is the dechlorination of DCA, or other chlorinated hydrocarbons. If this is true, then these results show that pollutant degradation is occurring, and is likely to be the result of the metabolic activities of soil microorganisms.

Ion chromatography was used to determine the quantities of sulphate and nitrate in the groundwater samples. The analytes were selected due their known relevance to the oxidative and reductive conditions within groundwater systems. No nitrate was measured at concentrations above the detection limit of 0.3 mgL^{-1}, and it was therefore not considered to have any significant influence on oxidative or reductive processes within this area of the study site. The presence of sulphate was detected in all three of the sampled boreholes (i.e. B1, B2 and B3). This gave an indication of the potential for sulphate reduction by indigenous microorganisms. Sulphate reducing bacteria may play a role in the degradation of the focus compounds. The microcosm and microbiological studies, discussed in the following chapters, were designed to identify the sorts of bacterial species, which may be responsible for such degradation.

A colorimetric technique was used to find the amount of iron II (ferrous iron) in groundwater samples. Ferrous iron is typically formed in groundwater when redox potential values are low, thus allowing for the reduction of ferric iron, or

iron III (Faulkner *et al*, 1999). The groundwater sample from borehole B3 gave the only positive result for ferrous iron. It was predicted that ferrous iron concentrations would be highest in samples from borehole B1, given the low redox potential, which were measured during the onsite analyses. Unfortunately, however, no result for this sample was obtained by MTD due to sample interference.

The alkalinity of groundwater samples was determined as a function of the activity of calcium carbonate ($CaCO_3$). The procedures used were originally developed by the University of Massachusetts' Water Resources Research Center (River Watch Network, 1992). A reagent (sulphuric acid) was combined with samples until a pre-determined pH was reached. The amount of reagent added corresponds to the total alkalinity of the sample.

Alkalinity is a measure of water's ability to neutralise acids, and so is directly related to pH (Mechenich and Andrews, 2004). This is shown through a direct comparison of alkalinity and pH values from borehole B1 samples. Both values were very high when compared with samples from the other boreholes. The high alkalinity in borehole B1 is also likely to be related to the high EC values discussed in Section 4.4.2.4.

Dissolved methane concentrations were measured using the method described by McAuliffe (1971). A headspace equilibrium technique was performed, whereby a groundwater sample was shaken vigorously in a nitrogen gas filled syringe. The gas was then analysed by GC-MS.

The presence of dissolved methane in groundwater could be an indicator for the occurrence of methanogenesis. Methanogenesis is a process whereby hydrocarbons can be degraded, with the end product being carbon dioxide and methane. It is a strictly anaerobic reaction, and is associated with redox potentials lower than -200 mV (Vance, 1998). The redox potential of groundwater from borehole B1 was around -251.5 mV, and thus it was expected that dissolved methane would be detected. However, no dissolved methane was found in any sample. It may have proved more effective to have used an onsite dissolved methane analyser, since some methane could have been lost

through handling and storage. No appropriate analyser was available at the time of sampling.

4.5 Conclusions

The results of both the styrene-area and focus-area analyses provide the required details, which allow for the next stages of this research to proceed. All analytical work was either carried out or supervised by the thesis author.

4.5.1 Styrene area

No metabolites of styrene monomer were detected in the analyses described under Section 4.3.3. This indicates the absence of any significant styrene degradation. Styrene polymer was identified in the analyses. If the styrene is not being metabolised by soil microorganisms, as this research indicates, then polymerisation may be a very influential factor. It is possible that polymerised styrene is acting to inhibit the degradation/ transformation of the styrene. The presence of styrene polymer may also hinder many traditional remedial processes.

The local and overall study site hydrogeology may have been altered due to the presence of the highly viscous styrene polymer. The results of previous site investigations found the presence of lighter compounds such as benzene and ethylbenzene towards the bottom of the aquifer. It is possible that severely clogged pore spaces in the styrene area may be a partial cause for the apparent downward flow of lighter hydrocarbons in the aquifer. However, the downward migration of benzene and ethylbenzene is more likely to be the result of the transport characteristics of a mixed plume. Benzene, ethylbenzene and DCA were all detected within the same samples of groundwater. The concept of mixed plume transport and the behaviour of DCA in groundwater were introduced in Chapter 2.

Further research into the styrene area issues would likely prove more efficient than proceeding directly with the implementation of a remedial process. However, for the purposes of this thesis, the above stated conclusions

completed the styrene area work. All further efforts were directed towards contamination issues within the focus area.

4.5.2 Focus area

The findings of the focus area investigations describe a wide range of physical and chemical properties. Bringing together the results of both onsite and laboratory-based analyses showed that borehole B1 differed significantly from the other two boreholes, which were sampled. The high pH (10.8) and low Eh (-251.5 mV) values suggested the presence of high contaminant concentrations. This was confirmed through the laboratory analyses, where DCA concentrations were found at 879 mgL^{-1}. Benzene was also a major contaminant in the B1 sample, which was measured at 12.9 mgL^{-1}. Relating these observed values with EQS limits further highlights the magnitude of contaminant concentrations at the study site. Ethylbenzene was also detected in borehole B1, but was at a concentration below the New Dutch List action level. The high TOC values of groundwater from borehole B1 were attributed to the contamination. DCA and benzene were also found in boreholes B2 and B3, though to a lesser extent than the B1 results.

Soil physical and mineralogical analyses were carried out on sample FS5, which was from borehole B2. Particle size analysis found the soil to be composed of primarily fine sand-sized particles. Less than 12% of the soil mass consisted of silt or clay-sized particles. Quartz was the dominant mineral component of the sample. Only trace amounts of clay minerals were detected. These results suggest that the soil minerals are likely not to have a significant influence on the fate of organic pollutants in the groundwater. The soluble pollutants are likely to be highly mobile, with minimal partitioning onto soil solids.

One of the aims of the focus area investigations was to collect samples for use in microcosm studies. Groundwater taken from borehole B1 showed sheen at the surface when purged into a 5-gallon bucket, indicating the presence of free phase hydrocarbon product. The aim of the planned microcosm studies was to investigate the biodegradation of solute-phase contaminants. Borehole B3 was located on a public beach. The logical follow-up approach to the planned

bench-scale investigations in this thesis is an *in situ* pilot study. Due the close proximity of the estuary (marine environment) to borehole B3, it is possible that undefined chemical interactions may occur between the groundwater and estuarine water in this area. Additionally, the site owner indicated that all attempts to leave objects (such as well locator stakes) in the public beach area resulted in damage or loss of such objects. For these reasons, it was concluded that further study in this area may not be practical. Therefore, samples from boreholes B1 and B3 were ruled out predominantly because of heavy contamination and public space issues respectively.

It was decided that soil and groundwater samples from borehole B2 were to be used for this purpose. Two sample depth intervals existed for borehole B2 (FS4 at 12ft bgl; and FS5 at 18ft bgl). Given the previously identified (by CELTIC, see Chapter 3) elevated contaminant concentrations near the bottom of the aquifer, sample FS5 was selected as the source material for further study. The next stage of the thesis research was to conduct lab-based studies into the treatability of the contaminant compounds. These studies are presented in Chapter 5.

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Chapter 5

Microcosm Treatability Studies

5.1 Introduction

This chapter gives the details of microcosm treatability studies, which were carried out on samples from the study site. These lab-based trials focused on a range of enhanced natural attenuative processes, and were designed to simulate both natural and enhanced conditions at the site.

Section 5.2 defines the purpose for the studies, and provides a background to the techniques which were employed. Batch-type microcosms were used so that destructive sampling could be carried out, thus allowing for a more accurate analysis of materials. The choice of source materials is justified, based on the required objectives of the work.

Details on the experimental set-up are outlined in Section 5.3. A scoping study was carried out in order to determine the best and most practical methods for developing the microcosm tests. All set-up procedures were carefully planned and performed with a focus on minimising unnecessary errors.

Section 5.4 describes the methods and findings for tests, which were carried out, on the microcosms. Regular monitoring was performed in order to quantify changes in pollutant concentrations, groundwater chemistry and microbial community profiles. The results of the test are discussed in detail. The findings of the microbiological analysis are provided in more detail under Chapter 6.

Conclusions for the microcosm studies are presented in Section 5.5. A particular microcosm enhancement is proposed as the most effective for facilitating the reduction of pollutant concentrations.

5.2 Purpose and background of the studies

5.2.1 Study objectives

The microcosm treatability studies were designed to provide information relating to a range of potential enhancements for the natural attenuation of organic pollutants at the study site. Enhancements were chosen with the aim of inducing both oxidative and reductive conditions within the microcosms. The main objective of these studies was to determine a best enhancement for facilitating the reduction/removal of the focus pollutant compounds. The organic pollutant compounds, which form the main analytes for this work, are 1,2-dichloroethane (DCA), benzene and ethylbenzene.

5.2.2 Microcosm techniques

It was concluded in previous chapters that the degradation of the focus pollutant compounds at the study site is occurring very slowly. The likely reason for this is an insufficient nutrient source (including electron acceptors/donors) for the indigenous microorganisms. Monitoring the slow degradation of organic pollutants in the subsurface could prove difficult and time consuming. For this reason, lab-based studies have been designed to simulate *in situ* conditions, with the aim of simplifying and speeding up the characterisation of pollutant degradation. The studies also allow for different enhancement conditions to be trialled.

There are two well-recognised microcosm designs, which were considered for this research. The first is recommended by Wiedemeier *et al* (1998), and is designed to provide an estimation of the *in situ* rate constant for pollutant degradation. Microcosm bottles are set-up to closely simulate *in situ* conditions, and so have a high ratio of solids (soil) to groundwater with no headspace. The bottles are sampled destructively at regular intervals to detect any changes in pollutant concentrations.

The second method for microcosm design is recommended by Morse *et al* (1998). The draft document describes a reductive anaerobic biological *in situ* treatment technology (RABITT) for the remediation of chloroethenes. A similar method description is also given by Findlay *et al* (1995). This technique does not aim to determine the rate constant, and is a more simplified procedure. The principle aim of the RABITT protocol is to simply determine whether natural attenuation is occurring, and to evaluate whether enhancements could be made to speed up the natural processes. The microcosms consist of slurries, made up of approximately 50% solids. A headspace of at least 60 cubic centimetres is recommended for each bottle. The headspace is used for removal of gases to be analysed so that destructive sampling is not required at shorter time intervals, making this technique typically less time or labour intensive.

The RABITT protocol was adopted as the basis for the microcosm development as part of this thesis. Some alterations were made to the recommendations of the RABITT protocol in order to accommodate the specific needs of this research. The full details of the microcosm design and set-up are presented in Section 5.3.

The testing procedures were designed so that headspace samples would be taken at regular intervals. Duplicates were also produced for each microcosm type so that destructive sampling could also be carried out. Sacrificed microcosms were then able to be analysed chemically and biologically.

5.2.3 Materials used

This section provides an overview of the materials used in the microcosms. Details on the preparation of the microcosm experiments are provided in Section 5.3.

5.2.3.1 Site samples and contaminants

Soil and groundwater samples from the study site formed the basic materials for the studies. The samples which were used originated from borehole B2 at the study site. All microcosms were spiked with laboratory standard organic compounds. The quantities and concentrations of contaminant compounds

used are shown in Table 5.1. The values shown in the figure are based on the addition of compounds into 80 mL of groundwater from the site. Residual contamination within study site samples was assumed negligible for the purposes of the microcosm studies. The concentrations of the focus organic compounds within study site samples were determined through the analyses presented in Chapter 4 (refer to Table 4.6).

Table 5.1 Quantities and concentrations in each of the test microcosms

Contaminant	Quantities added (mg)	Concentrations (mgL^{-1})
Benzene	8.0	100
Ethylbenzene	0.8	10
DCA	8.0	100

5.2.3.2 Enhancements

The choice of enhancements was based on the need to simulate a range of enrichment conditions. Pollutant compounds at the study site were found under predominantly anaerobic conditions (i.e. located deep in the aquifer). However, for full-scale remediation it would be possible to introduce oxygen into this anaerobic environment. This could be done through either pumping gaseous oxygen into the subsurface (via air sparging), or introducing an oxygen releasing chemical additive in liquid form. The review of degradation pathways, which was presented in Chapter 2, identified both aerobic and anaerobic mechanisms for the removal of the focus compounds. To determine whether aerobic or anaerobic conditions would prove more effective for the natural attenuation of the focus contaminants at the study site, both were induced and examined within the microcosms.

5.2.3.2.1 Oxidative additives

Oxygen functions as an electron acceptor in the subsurface. In relation to the degradation of organic pollutants, oxygen accepts electrons that are released from the breakdown of the organic chemicals by microorganisms. Such microorganisms obtain energy through aerobic respiration.

The introduction of gaseous oxygen into the microcosm bottles would have proven difficult in terms of quantifying the oxygen in the system and ensuring that an equal dispersion of the gas was being achieved. This approach may also act to transfer contaminant mass from the aqueous to the gaseous phase via physical sparging. For these reasons, Oxygen Release Compound (ORC®), a registered product of Regenesis Bioremediation Products (Regenesis) was chosen as the best additive for inducing aerobic conditions.

ORC® is an inorganic chemical mixture of magnesium peroxide (MgO_2), magnesium oxide (MgO) and magnesium hydroxide [$Mg(OH)_2$]. It is an insoluble white powder, and is designed to time release oxygen (O_2) when hydrated. Equation 5.1 shows this reaction.

$$MgO_2 + H_2O \rightarrow \frac{1}{2} O_2 + Mg(OH)_2 \qquad \text{[Equation 5.1]}$$

ORC® is intercalated with a food-grade phosphate ion, giving it the time-release property (Koenigsberg and Sandefur, 2001). Intercalation describes the way in which phosphates permeate into the magnesium peroxide crystal, partially inhibiting the influx of water into the structure.

ORC® was chosen as an enhancement for its proven effectiveness in case studies. The product was originally used for the remediation of BTEX and other lighter hydrocarbon contaminated sites, but has since moved on to a wide range of more complicated hydrocarbon compounds (Koenigsberg and Sandefur, 2001). Reed *et al* (2003), Mysona and Hughs (1999) and Fischer and Reed (2001) have all shown that ORC® can effectively clean up sites contaminated with BTEX compounds. Studies have been undertaken to determine the effectiveness of an aerobic treatment (ORC®) when compared to a separate anaerobic treatment for degrading chlorinated hydrocarbons, with mixed results. Pentachlorophenol, 1,1-dichloroethene and vinyl chloride were all shown to be more effectively degraded under enhanced aerobic conditions (Carson and Voegeli, 2001, Macewen *et al*, 2003). This is in contrast to the results of work carried out by Cornuet *et al* (2000), who found that cis-1,2-dichloroethene was more effectively degraded under anaerobic conditions. It is the opinion of the

thesis author that the variations between these findings result from site-dependent conditions, and therefore will not necessarily apply to every situation.

Stoichiometry was used to determine the amount of ORC® to add into microcosms. This required equation balancing and mass calculations for the assumed aerobic degradation reactions of each pollutant compound. Numerous studies have demonstrated the successful aerobic degradation of benzene and ethylbenzene in soil-groundwater systems (Wilson *et al*, 1986, Lee *et al*, 1987 and Jindrova, 2002). Lee *et al* (1987) suggests that BTEX compounds are some of the most aerobically biodegradable found in the subsurface environment. The balanced aerobic respiration reactions for benzene and ethylbenzene degradation are given in equations 5.2 and 5.3 respectively. Van den Wijngaard *et al* (1992) states that DCA can be used as a growth substrate by aerobic bacteria, such as Xanthobacter, Ancylobacter and Pseudomonas strains, which completely oxidise DCA to CO_2, H_2O and chloride. A number of other references have also described the aerobic mineralisation of DCA, including Gerritse *et al* (1999) and Dyer *et al* (2000). The reaction in equation 5.4 shows the aerobic mineralisation of DCA.

$$6C_6H_6 + 15O_2 \rightarrow 12CO_2 + 6H_2O \qquad \text{[Equation 5.2]}$$

$$2C_8H_{10} + 21O_2 \rightarrow 16CO_2 + 10H_2O \qquad \text{[Equation 5.3]}$$

$$C_2H_4Cl_2 + 3O_2 \rightarrow 2CO_2 + 2H_2O + Cl_2 \qquad \text{[Equation 5.4]}$$

10% of ORC® mass is converted to oxygen over a period of six months, so that 10g of ORC® will produce 1g of oxygen. The quantities of oxygen required to degrade each of the contaminant compounds are shown in Table 5.2. The final oxygen requirement was multiplied by a factor of 5 in order to account for other non-target organic compounds (Birnstingl, 2004). Based on these calculations, 1.75g of ORC® was added to each of the relevant microcosms.

Table 5.2 Stoichiometry for the calculating the required ORC® mass

Contaminant mass in each microcosm (mg)	Required oxygen for 1mg of contaminant	Required oxygen for the concentrations added
8.0 benzene	3.08 mg	24.64 mg
0.8 ethylbenzene	3.17 mg	2.54 mg
8.0 DCA	0.97 mg	7.76 mg
Total oxygen required = 34.94 mg		
x 5 to account for non-target compounds = 174.7 mg		
x 10 for equivalent ORC® mass required = 1747.0 mg = 1.75 g		

5.2.3.2.2 Reductive additives

Two other enhancements were introduced, which were designed to maintain anaerobic conditions within microcosms. The enhancements are known to produce hydrogen, which can act as an electron donor in the subsurface. Some soil microorganisms have been shown to make use of molecular hydrogen through a process known as reductive dechlorination. Reductive dechlorination and other degradation processes were discussed in Chapter 2. Reductive dechlorinating bacteria substitute the hydrogen for chlorine on chlorinated hydrocarbons. This process could prove effective for reducing the concentrations of DCA in the microcosms.

Once again, the stoichiometry of contaminant degradation was calculated. No additive was designed to directly enhance the anaerobic degradation of benzene or ethylbenzene. However, Lovely (1997) suggests that BTEX compounds may be degraded by anaerobic bioremediation just as effectively as aerobic bioremediation. For a more comprehensive review of anaerobic degradation mechanisms refer to Chapter 2.

The anaerobic degradation of DCA to ethene was assumed, as suggested by Gerritse *et al* (1999). The reductive dechlorination reaction for this reaction is given in equation 5.5. A more detailed assessment of DCA degradation

pathways was presented in Chapter 2 of this thesis. For the sake of the stoichiometric calculations complete mineralisation was assumed.

$$H_2 + C_2H_4Cl_2 \rightarrow C_2H_4 + 2HCl \qquad \text{[Equation 5.5]}$$

One of the anaerobic enhancements was HRC® (hydrogen release compound), which is also a registered product of Regenesis. It is a highly viscous syrupy amber liquid. As the name suggests, HRC® is also designed to release hydrogen into the subsurface to facilitate reductive dechlorination. The product consists of lactic acid, which is released when hydrated. Molecular hydrogen and carbon dioxide are products of the breakdown of lactic acid (Regenesis, 2005). Like the ORC® product, HRC® is also a slow-release compound. This is important when one considers the competition for hydrogen between reductive dechlorinators and methanogens in the subsurface. High concentrations of hydrogen favour methanogenic activity, whereas reductive dechlorinators are best supported in conditions of low hydrogen concentrations (Regenesis, 2005). The slow-release action of HRC® is designed to produce these low-hydrogen conditions.

HRC® has been shown through extensive research to be a successful treatment for a range of chlorinated hydrocarbons. Markley and Sieczkowski (2003), Murray *et al* (2001), Willet *et al* (2004), Farone *et al* (2000) and Jones (2003) have all demonstrated the successful use of the product for facilitating the reductive dechlorination of chlorinated ethanes and ethenes. Farone and Palmer (2001) compared HRC® to two other reducing agents, molasses and vegetable oil, and found the HRC® much more effective. The experiment was carried out in a flow-through cell. Both the molasses and vegetable oil were found to transport through the column before they were able to release sufficient electron donor into the system.

In calculating the amount of HRC® to add, benzene and ethylbenzene were removed from consideration. Molecular hydrogen was unlikely to have a direct effect on the oxidation of benzene and ethylbenzene. Indirectly, however, molecular hydrogen may interfere with the desired reaction by acting as a preferential electron donor for other reactions, such as sulphate reduction, over that of benzene.

Birnstingl (2004) suggested that approximately 6g HRC® would treat 4L of groundwater contaminated with 100ppm DCA. 80mL of contaminated groundwater will be used in the microcosm experiments. Therefore, 120mg of HRC® were introduced into the relevant microcosms.

Molasses was chosen as an alternative anaerobic electron donor source. It has a similar consistency to that of HRC®, but is darker brown in colour. The Molasses was chosen for its cheapness, availability and effectiveness in previous studies for facilitating reductive dechlorination.

Molasses is a complex sugar, which can be naturally fermented in the environment to produce molecular hydrogen (Chapelle, 1993). The fermentation pathway of molasses yields the intermediate pyruvic acid, which in turn can be oxidised to produce a range of organic acids (acetic, lactic and formic), alcohols (ethyl alcohol, butanol and isopropyl alcohol), hydrogen and carbon dioxide (Chapelle, 1993; Dyer *et al*, 2000). Additional carbon dioxide may result from reactions between organic acids and calcium carbonate. One source of calcium carbonate is from shell fragments, which were observed in study site samples (see Chapter 4).

The organic acids that are produced through the fermentation of molasses may act as carbon sources for the metabolic function of indigenous microorganisms. Thus, the molasses additive was designed to facilitate reductive dechlorination cometabolically. Cometabolic degradation was discussed in Chapter 2.

A number of studies have given recommendations for the amount of molasses that should be added for facilitating the reductive dechlorination of tetrachloroethylene (PCE) (Wu *et al*, 1998; Kao *et al*, 2003; Suthersan and Payne, 2003). The suggestions of these studies vary considerably. Kao *et al* (2003) suggested the addition of 1.6g of molasses for the treatment of 1g of PCE. Dyer *et al* (2000) used the suggestions of Wu *et al* (1998) to calculate that approximately 30g of molasses would be required for the treatment of 1g of DCA.

This research required the degradation of 8mg of DCA. Using the values calculated by Dyer *et al* (2000), 240mg of molasses would be required. This

equates to approximately 360mL of molasses (assuming the density of molasses is approximately 1.5gmL^{-1}). Only 80mL of groundwater were to be used for the microcosm experiments. Therefore, another amount of molasses was required.

The adopted quantity was based on the recommendations of Suthersan and Payne (2003) who suggest the use of up to 10% molasses (by volume) in solution for the treatment of PCE at concentrations exceeding 500mgL^{-1}. Therefore, 8mL of molasses were added in solution for the treatment of 80mL of groundwater from the study site.

5.3 Experimental set-up

Table 5.3 shows the labels and descriptions for each of the five different types of microcosm. Four duplicates were made of each microcosm type to allow for periodic destructive sampling. The full details of the microcosms' set-up are discussed in this Section. An abiotic microcosm was intended to provide a control for use in determining any potential abiotic degradation processes.

Table 5.3 Descriptions for the 5 different microcosm types

Labels	Enhancement added	Microcosm description
M1	Oxygen Release Compound ORC®	Enhanced microcosm for facilitating the oxidation of all introduced pollutant compounds using ORC®
M2	Molasses	Enhanced microcosm for facilitating the reductive dechlorination of DCA using molasses
M3	Hydrogen Release Compound HRC®	Enhanced microcosm for facilitating the reductive dechlorination of DCA using HRC®
M4	None (standard control)	Standard control made to represent *in situ* conditions without enhancement.
M5	None (abiotic control)	Biotic content of the slurry mix was sterilised prior to the introduction of pollutant compounds.

5.3.1 Microcosm pre-development studies

Experiments were undertaken in order to determine the best methods for use in the set-up of microcosms. Different slurry mixing and loading techniques were trialled for optimisation.

The RABITT protocol recommends a 50% solid-liquid slurry mixture (Morse *et al*, 1998). The first test aimed to examine the consistency and workability of this slurry. 1kg of a dry, uncontaminated sand was combined with 1L of tap water in a 5L graduated column. The mixture was stirred vigorously with a wooden spoon, in an attempt to homogenise. Once the stirring action had ceased, nearly all of the solids would settle within 5 seconds. This was based on visual observations. The results of this simple test suggested that it would be difficult to maintain slurry homogeneity for each microcosm if the mixing was to be carried out in bulk. Therefore, it was decided that soil and groundwater samples would be homogenised and added into microcosm bottles individually. Once inside the microcosm bottles, then slurry homogenisation would take place.

The openings and necks of microcosm bottles had diameters of 2.5cm. Different loading techniques were tested in order to determine the most efficient means of introducing soil samples into the microcosm bottles. A funnel was deemed necessary due to smallness of the opening. The fastest loading was achieved by adding approximately 10g of soil into the funnel, then pushing the soil through using a steel rod (diameter 1cm).

5.3.2 Microcosm preparation

All tools and containers used in the set-up of the microcosm experiments were sterilised by autoclaving at 121 degrees Celsius for 15 minutes prior to use.

Microcosms were prepared in 240mL amber serum bottles, with Teflon^{TM} lined septa and a polypropylene screw on cap. A 50% solid slurry was introduced to the bottles, containing 80g (dry weight) soil and 80mL groundwater, leaving a headspace of approximately 80cm^3.

3kg of bulk soil were homogenised using a stainless steel spatula. The source of the soil materials was borehole B2, sample FS5 (refer to Chapter 4 for details on this sample, and why it was selected as a source material for the microcosms). The moisture content and dry weight of the soils were taken into account when calculating the required bulk mass of soil to be introduced. The results presented in Chapter 4 state that the bulk soil sample, FS5 had a moisture content of 13.3%. This means that approximately 10g out of an 80g sub-sample would consist of porewater. To achieve the desired slurry mix, 90g of bulk soil were combined with 70mL of groundwater.

Following homogenisation, the 90g soil samples were weighed and introduced to microcosm bottles, using the techniques developed under the scoping studies. Groundwater from borehole B2 was used for the microcosms. 70mL groundwater samples were measured and introduced into microcosm bottles via a funnel.

After the slurry components were introduced to each of the microcosm bottles, they were purged with nitrogen gas (N_2) using a long (approximately 5cm) stainless steel needle. The purging process lasted approximately 2 minutes for

each microcosm bottle. This served two purposes: a) to homogenise the slurry, and b) to remove excess oxygen (O_2).

Once the slurries had been purged, pollutant compounds and enhancement additives were introduced in the quantities discussed under Section 5.2.3. The microcosms were then capped, and another round of purging commenced. This time nitrogen gas was injected via a syringe needle through the septa, and into the headspace of each microcosm. A second needle was injected into the septa to allow for the gasses to cycle through the headspace and out. The second needle was removed approximately 5 seconds before the nitrogen gas injection ceased. This allowed for a build up of positive pressure within the microcosms. The positive pressure is preferred, as it reduces the chances of contamination entering the microcosms during future sampling events (Morse *et al*, 1998).

Abiotic microcosms (M5) underwent a sterilisation stage following the addition of slurry materials. This was carried out 24 hours prior to the development of the other microcosms. Sterilisation was done by autoclaving the microcosm bottles (containing the slurry mixture) at 121 degrees Celsius for 30 minutes. This procedure was consistent with regular sterilization activities (for media and materials) conducted as part of the work for this thesis. However, it is noted that, due to the quantities of slurry materials within the abiotic microcosms, this sterilization process may not have been adequate. This is discussed later in this Chapter and in Chapter 6. Once the sterilisation process was complete, pollutant compounds were added to the abiotic microcosms using the same methods as with the other microcosm types.

All microcosm bottles were placed onto a rotational agitation plate for 30 minutes to ensure equal distribution of additives. Between sampling events, microcosm bottles were stored in a constant temperature room at 25 degrees Celsius.

It is noted that the techniques used for sample acquisition, handling and microcosm set-up were not optimal for maintaining anaerobic conditions. The techniques used were limited financially and by the lack of availability of certain appropriate equipment and facilities.

It is necessary to consider further the implications of this potential exposure to oxygen. One major consideration is the effects (both immediate and longer-term) on the indigenous micoorganisms. The microcosm studies conducted for this thesis were designed to simulate anaerobic conditions at the site. Variations in microbial diversity and predominant microbial colonies would likely occur based on an increased availability of molecular oxygen. In relation to the microcosm studies, it is likely that the microbiological communities of the introduced materials varied from that which was present under *in situ* conditions.

In Chapter 2, a classification system for microorganisms was introduced, which relates microorganisms to their level of reliance (or non-reliance) on oxygen. Obligate (or strict) anaerobes are not able to grow (or in some cases survive) in the presence of molecular oxygen. However, many anaerobic microorganisms can survive in the presence of molecular oxygen; in fact, some can utilise molecular oxygen as a terminal electron acceptor (e.g. facultative and aerotolerant anaerobes). It is possible that indigenous obligate anaerobes within the microcosm source materials did not survive the preparation process due to oxygen exposure. This is significant as it may have reduced the possibility for certain indigenous microbial populations to degrade the focus compounds.

Furthermore, residual oxygen within the microcosms may have allowed for aerobic microorganisms to be responsible for the degradation of organic chemicals. Based on the results of the microcosm studies (discussed later in this Chapter), this was the case. Over the course of the microcosm study period, it is likely (based on the results discussed later in this Chapter) that molecular oxygen was depleted with the microcosms, and that more reduced conditions became prevalent. Therefore, the results obtained from the earlier stages of the microcosm study period were not representative of *in situ* anaerobic conditions. This topic is re-addressed with regards to the results of this study later in this Chapter.

5.4 Microcosm testing procedures and results

5.4.1 Overview

Microcosm testing was conducted with the aim of characterising the degradation of organic pollutants. The results of the monitoring allowed for recommendations to be concluded regarding the effectiveness of the tested treatments. A range of tests was performed on microcosms at a number of different time intervals. The headspace of each microcosm was extracted and analysed regularly for monitoring changes in pollutant concentrations. Other analyses were carried out less frequently, and involved the destructive sampling of microcosms. This is the reason for the development of duplicate microcosms of the same type. Upon destruction, gaseous, porewater and mixed slurry samples were collected and analysed for a range of chemical and biological properties, which will be described in the following sections.

A full suite of analyses was performed at the start (day 0) of the microcosm experiments. The scheduling of all sampling events is given in Table 5.4, and photographs of the microcosm experiments are shown in appendix III. The source of microcosm materials, as discussed above, was borehole B2, sample FS5 (refer to Chapter 4, Table 4.3 for details on this sample, and why it was selected as a source material for the microcosms).

The actual microcosm experiments will be referred to as the test microcosms within this section, in order to differentiate them from the standard microcosms that were produced for calibration purposes. One microcosm was set up and fully analysed on day 0 of the experiments. This microcosm has been labelled 'baseline', and was created in order to obtain baseline data for microcosm conditions on day 0.

Headspace samples were extracted in order to monitor any changes in the chemical composition of the gases. This included an analysis of pollutant concentrations. All headspace sampling was performed using a lockable syringe, with a 500µL (equivalent to 0.5mL or 0.5cm^3) liquid capacity. A full syringe volume was extracted from the headspace and analysed for each test. Two different types of headspace tests were carried out. These are discussed

in Sections 5.4.2 and 5.4.3. A detailed discussion of the results presented in this section is provided in Section 5.5.

Table 5.4 Microcosm sampling and analysis timetable

SAMPLE	DAYS									
	0	2	7	12	15	23	34	53	59	79
Baseline	SOXG									
M1 (I)	S		OGX							
M1 (II)	S				OGX					
M1 (III)	S					O	OGX			
M1 (IV)	S	O	O	O	O	O	O	O	O	OGX
M2 (I)	S		OGX							
M2 (II)	S				OGX					
M2 (III)	S					O	OGX			
M2 (IV)	S	O	O	O	O	O	O	O	O	OXG
M3 (I)	S		GX							
M3 (II)	S				OGX					
M3 (III)	S					O	OGX			
M3 (IV)	S	O	O	O	O	O	O	O	O	OGX
M4 (I)	S		OGX							
M4 (II)	S				OGX					
M4 (III)	S					O	OGX			
M4 (IV)	S	O	O	O	O	O	O	O	O	OGX
M5 (I)	S		OGX							
M5 (II)	S				OGX					
M5 (III)	S					O	OGX			
M5 (IV)	S	O	O	O	O	O	O	O	O	OGX
M5 (V)	SX*									

KEY

M1 = ORC ENHANCED MICROCOSMS
M2 = MOLASSES ENHANCED MICROCOSMS
M3 = HRC ENHANCED MICROCOSMS
M4 = NO ENHANCEMENT CONTROL MICROCOSMS
M5 = ABIOTIC CONTROL MICROCOSMS
(I – IV) = DUPLICATE SAMPLES

S = SET-UP
O = HEADSPACE SAMPLING FOR GC-MS ANALYSIS
G = HEASPACE SAMPLING FOR NGA ANALYSIS
X = DESTRUCTIVE SAMPLING FOR:
- SOIL MICROBIAL ANALYSIS
- POREWATER CHEMISTRY ANALYSIS
X* = DESTRUCTIVE SAMPLING FOR SOIL MICROBIAL ANALYSIS OF ABIOTIC MICROCOSM

5.4.2 Regular monitoring of contaminants

The first headspace tests were carried out to determine changes in pollutant concentrations. An 8000 Series Thermal Finnigan gas chromatograph with an AS800 mass spectrometer (GC-MS) was used to regularly monitor changes in the concentrations of the focus pollutant compounds. The choice of column within the GC-MS device is crucial for obtaining the necessary range and accuracy of data. A gas chromatograph works by separating compounds relative to the polarity of a solid chemical column liner and the polarity of individual compounds within an injected chemical mixture. This relative polarity controls the speed (or retention) of compounds travelling through the device. The column liner is often referred to as the stationary phase. A Varian Factor Four VF-23MS 30m x 0.25mm capillary column with a 0.25µm stationary phase was selected, and found to be accurate for assessing the focus analytes under this section.

Gaseous samples were directly injected into the GC-MS inlet. The time for injection was approximately 1 second. The temperature for the inlet zone was 200 degrees Celsius, and the oven was maintained at a constant 30 degrees Celsius. The retention window was set at 10 minutes for each sample injection.

The obtained results were interpreted using GC-MS software MassLab Version 1.4. Chromatogram peaks were integrated and compared to the relevant calibration curves for the quantification of analytes. However, the automated integration did not always appear to give accurate peak area results. Therefore, some peak integrations were adjusted in order to obtain more accurate quantifications.

5.4.2.1 Standards and calibration

Blank GC-MS runs were conducted in order to become familiarised with the system and to check the consistency of results. During a blank run, the chromatogram results should appear relatively flat due to the absence of foreign compounds in the carrier gas. Inconsistencies were observed in the resulting chromatograms from the blank runs. The initial run was found to contain up to 30% higher intensities for the first 3 minutes of the retention window, when

compared to the last 7 minutes. This contrasted with blank runs, which were carried out following the initial run. The later runs gave flatter chromatogram results as expected.

These inconsistencies can be observed in the chromatograms shown in Figure 5.1. Chromatogram B from the figure shows a blank run, which was carried out following one blank and two standard microcosm runs, and is clearly much flatter than chromatogram A. The higher intensities shown in chromatogram A may be the result of the intrusion of laboratory air into the inlet while the system was idle. However, a detailed investigation was not carried out to determine the actual source of error. In order to maintain consistent results, one blank and two standard microcosm runs were first carried out before any of the actual test microcosms were analysed.

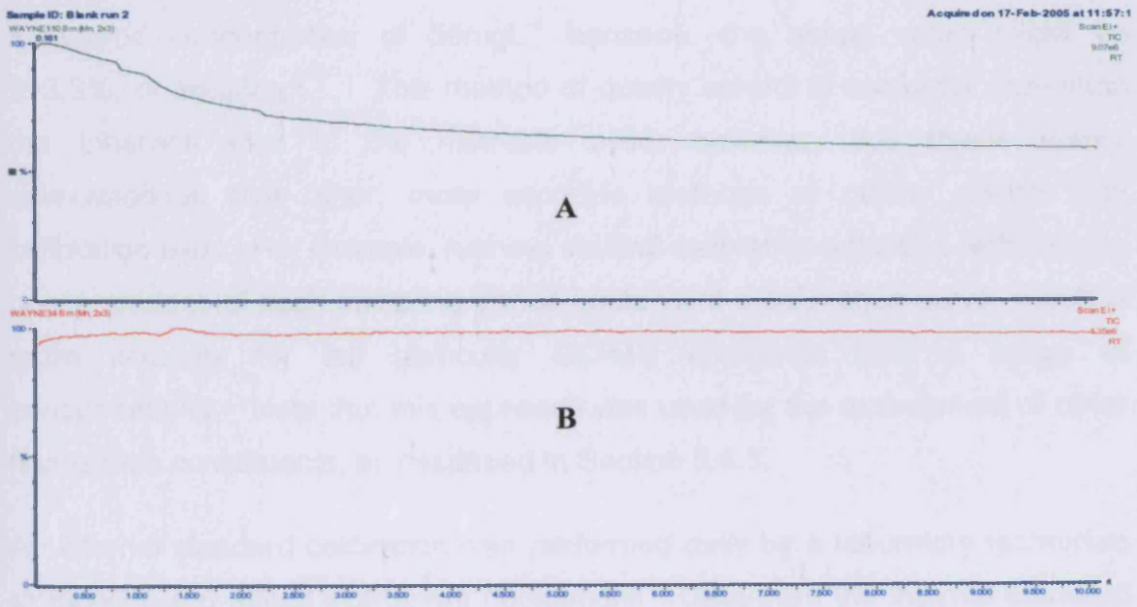

Figure 5.1 Chromatogram results, showing the difference between A) the first blank run of the day, and B) a blank run following one blank and two standard microcosm runs

Three identical standard microcosms were set up a) to ensure that the focus compounds could be detected within the stated retention window, and b) for calibration purposes. 160mL of tap water and predetermined amounts of contaminant compounds (8g benzene, 0.8g ethylbenzene, 8g DCA) were

introduced to standard microcosms. A soil-water slurry was not used for the standards due to a shortage of soil materials from the study site.

The headspaces of the standard microcosms described above were analysed at the four main destructive sampling periods (i.e. days 7, 15, 34 and 79). The resulting chromatogram peaks were integrated (using the above-stated MassLab software) to obtain peak areas. The raw QC/MS data from the analysis of standard microcosms is included in Appendix V. The results obtained were used to assess variations in method accuracy over the period of the microcosm studies. The results of the three standard microcosms were averaged. Figure 5.2 shows the percentage variation in signal magnitudes (integrated peak areas) that were observed from the standard microcosm analyses. Maximum variations in benzene, ethylbenzene and DCA were determined to be 13.3%, 32.4% and 18.5% respectively. In other words, for a measured concentration of 50mgL^{-1} benzene, the actual value might be ±13.3%, or ±6.65mgL^{-1}. This method of quality control is useful for assessing the inherent error in the methods used; however, this thesis author acknowledges that other, more accurate methods of quality control and calibration exist. For example, running several calibration solutions with varying concentrations at each sampling period could yield a calibration curve, which is more accurate for the particular GC-MS apparatus over a range of concentrations. Note that this approach was used for the assessment of other headspace constituents, as described in Section 5.4.3.

An internal standard calibration was performed daily by a laboratory technician to monitor and adjust instrument fluctuations. Data from the internal standard calibrations was not made available to this thesis author.

The possibility of investigating vinyl chloride (VC) within the microcosms was ruled out in Section 4.4.3.4 (Chapter 4). However, VC has been shown to be produced from the degradation of DCA (degradation pathways discussed in Chapter 2). The detection of VC within the test microcosms could have been used as an indicator for degradation. In order to determine whether VC could be detected within the retention window, it was included in one of the standard microcosms at a concentration of 100 mgL^{-1}. VC was observed on the resulting

chromatogram within a retention time of 1-2 minutes. Given the column and set up which was used for the GC-MS runs, this is also the retention time of many other lighter hydrocarbons. This was determined once the test microcosms had been run. A range of non-focus compounds fell within the 1-2 minute retention window, making it impossible to integrate individual peaks. Therefore, VC was not detectable throughout the GC-MS analyses. DCA, benzene and ethylbenzene were all detectable within the 10-minute retention window.

Quantification of focus compounds was determined relative to the maximum peak intensities measured for each test microcosm type over the course of the microcosm studies. The highest peak intensities for benzene, ethylbenzene and DCA were calibrated at 100, 10 and 100 mgL^{-1} respectively. Undetectable peak intensities were assumed to represent concentrations of zero mgL^{-1}.

The quantities of aqueous phase contamination vary proportionately to the amounts found in the gaseous headspace according to Henry's Law. The calibration methods described above provided enough data to be able to directly relate headspace results to solute concentrations. Therefore, Henry's Law constants were not considered during this stage of the investigation, but are addressed later in Section 5.4.2.2.1.

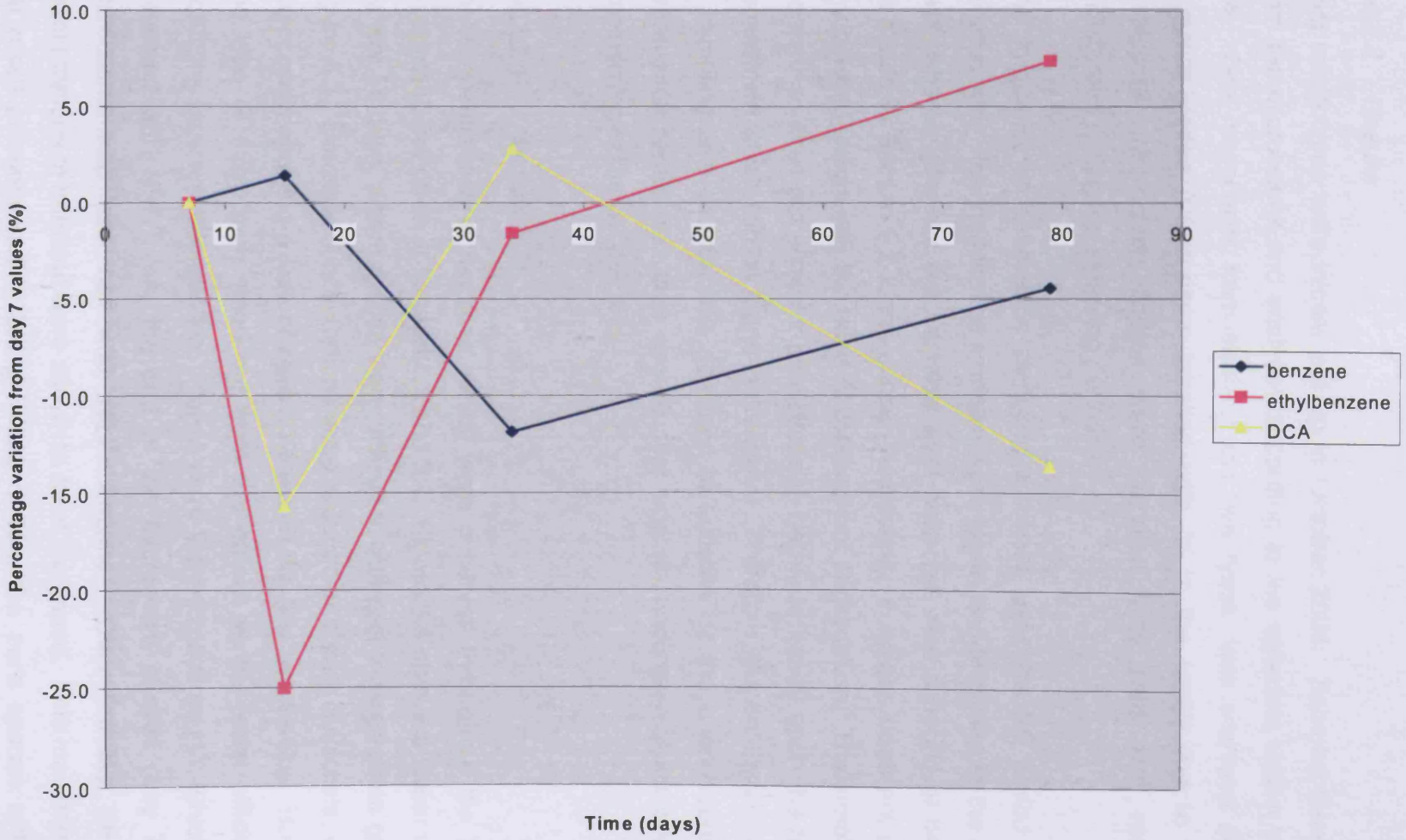

Figure 5.2 Variations in contaminant percentages within standard microcosms

5.4.2.2 Results

Test microcosms were initially set up in October 2004. Samples were taken from the microcosms and analysed according to the schedule shown in Table 5.4. One microcosm, from each of the five types, was analysed at every sampling period (microcosms labelled with IV in the table) due to budget restrictions. Microcosm bottles were handled with care and remained undisturbed during the sampling periods.

The frequency of headspace sampling lessened over the full period of the experiments. Five headspace samples were taken and analysed in the first 15 days, whereas the last five samples were collected over a period of 64 days. As stated in Section 5.2.2, the chosen microcosm technique does not provide any detailed insight into the rates of contaminant degradation. The simplicity of the method does not allow for predictions to be made based upon the rates of degradation which can be found in literature. Therefore, the continual increase in sampling time periods was designed to account for the unpredictability of degradation rates, and thus ensure that enough accurate results could be obtained regardless of this rate.

5.4.2.2.1 Benzene

The concentrations of benzene, which were observed throughout the 79-day experiment, are given in Figures 5.3 and 5.4. Figure 5.4 shows a closer view of the last 56 days, where results were difficult to interpret in large scale given in Figure 5.3. Benzene concentrations were reduced at a very quick rate, with all microcosms showing a loss of over 80% within the first two weeks. However, one type of microcosm stood out from the others as the least efficient for facilitating benzene degradation. These were the microcosms M3, which were enhanced with HRC®. At the end of the microcosm studies (day 79), all microcosms had less than 400 μgL^{-1} benzene. Over the last 46 days, concentrations of benzene were shown to have increased. This may have been the result of contaminants being released from the pore spaces within the slurries.

5.4.2.2.2 Ethylbenzene

The concentrations of ethylbenzene, which were observed throughout the 79-day microcosm experiment, are given in Figure 5.5. The general profile of ethylbenzene loss in all of the microcosm types was similar to that of the results for benzene (Figures 5.3 and 5.4). Ethylbenzene degradation occurred very quickly, with no positive results found after day 23. Over 90% of the initial ethylbenzene concentrations were lost within the first two weeks. Analysis of microcosms M4 and M5 showed reductions in ethylbenzene concentrations, following similar patterns to the other microcosm types.

5.4.2.2.3 1,2-dichloroethane

DCA concentrations throughout the microcosm studies are shown in Figures 5.5 and 5.7. DCA degradation proceeded more slowly than that of benzene and ethylbenzene. In fact, the initial week of the experiment saw an increase in DCA concentrations, with the exception of the microcosms with no enhancement (M4). This initial period was interpreted as being the result of a slow equilibrium between gaseous and liquid phases. Once in equilibrium, the highest GC-MS peak intensity for DCA was observed. This was then equated to 100 mgL^{-1} for calibration purposes.

The microcosms enhanced with HRC® (M3) proved to be the most effective for degrading DCA. Concentrations of DCA within M3 were quickly reduced to below detection limits by day 12 of the studies. The molasses enhanced microcosms (M2) also enabled for rapid DCA degradation. DCA concentrations were found to reduce within microcosms M5 (abiotic), but not as rapidly as in microcosms M2 and M3. One of the lowest apparent DCA degradation rates was observed in the ORC® enhanced microcosms (M1). M1 microcosms were designed to facilitate the aerobic degradation of DCA.

These findings alone do not define the mechanisms of degradation. To further describe the degradation within microcosms, porewater chemistry (Section 5.4.4) and microbiological results (Chapter 6) are also considered.

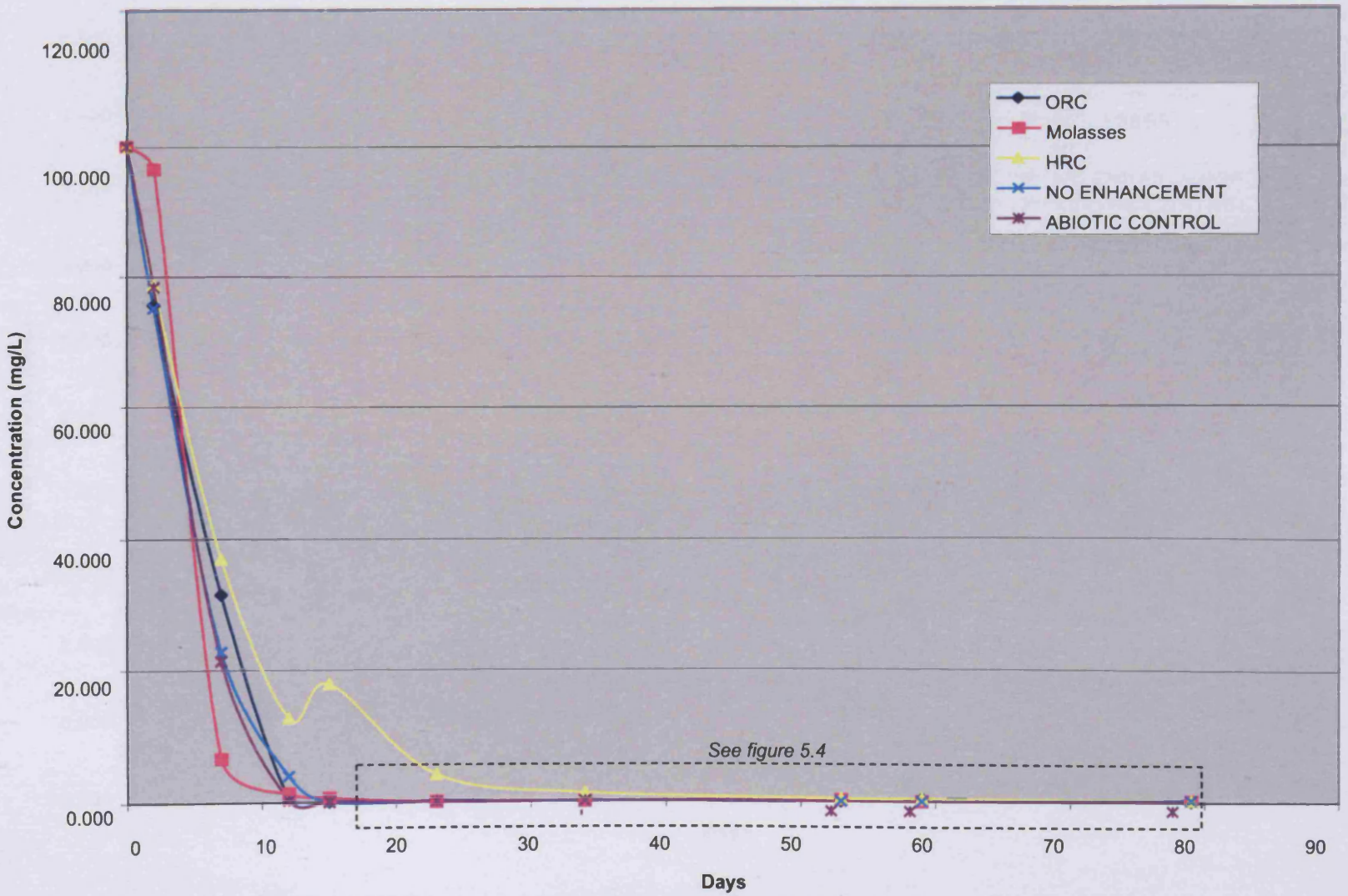

Figure 5.3 Benzene degradation in test microcosms over the 79-day test period

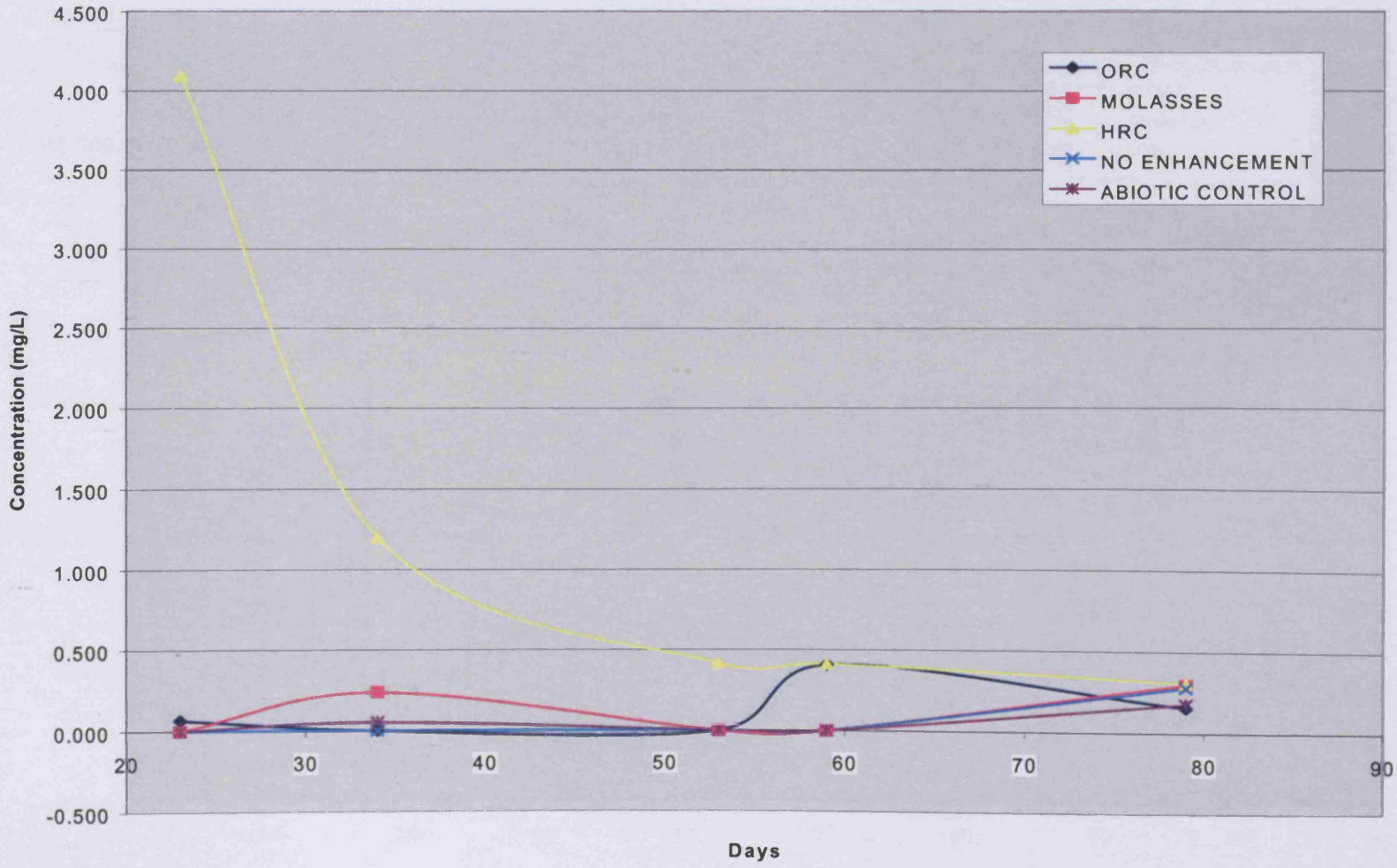

Figure 5.4 Benzene degradation in test microcosms during the final 56 days

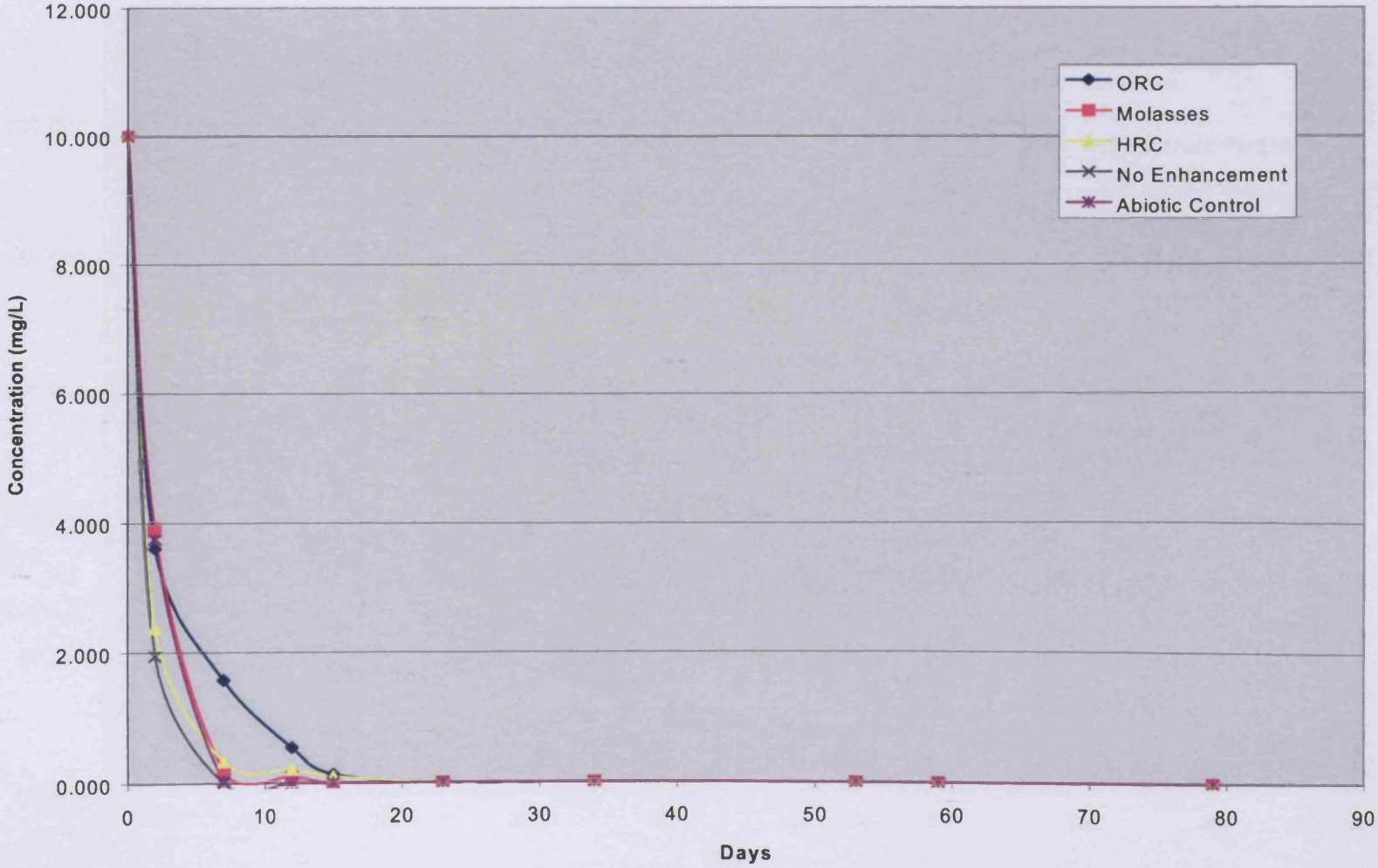

Figure 5.5 Ethylbenzene degradation in test microcosms over the 79-day test period

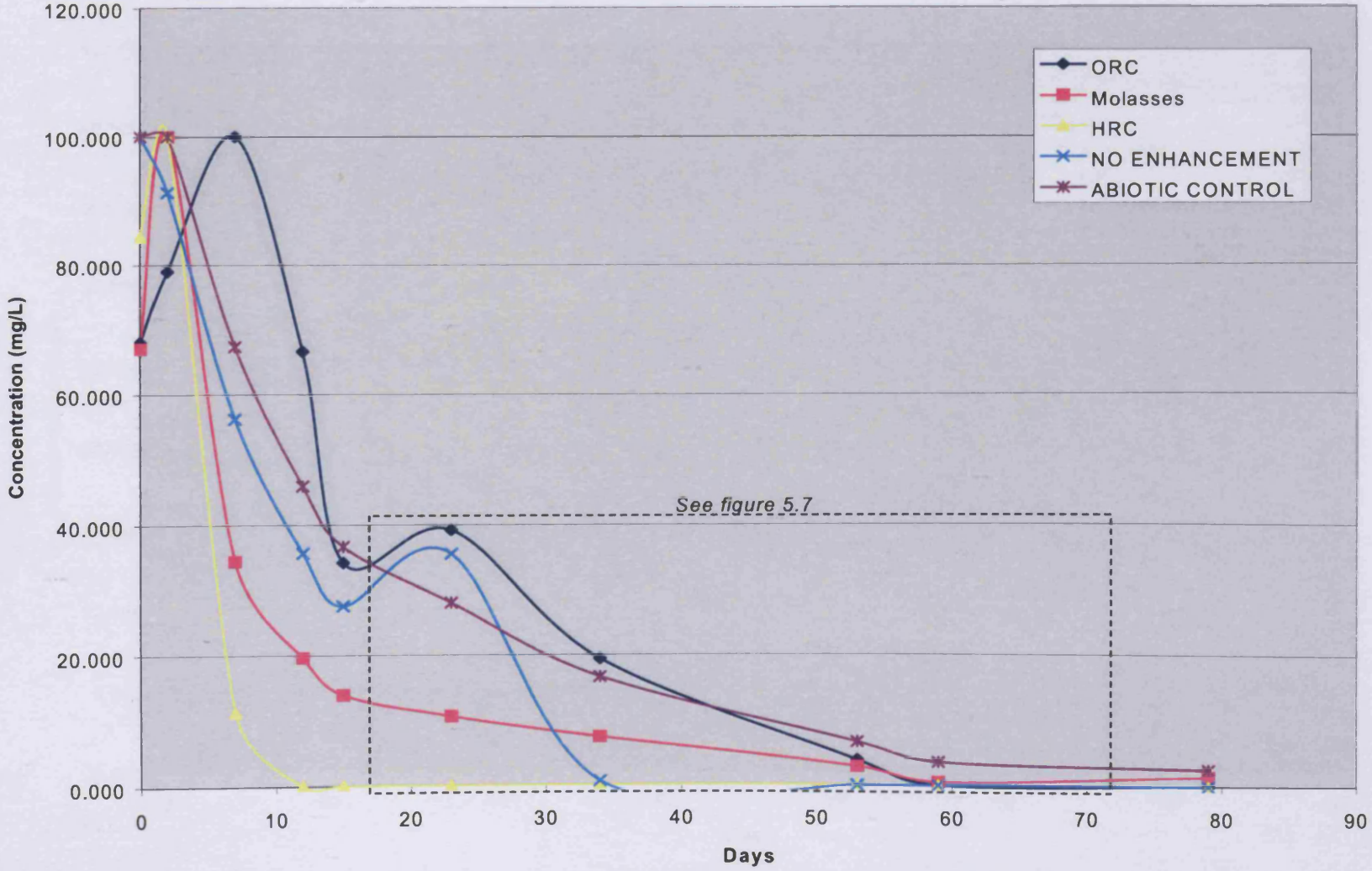

Figure 5.6 DCA degradation in test microcosms over the 79-day test period

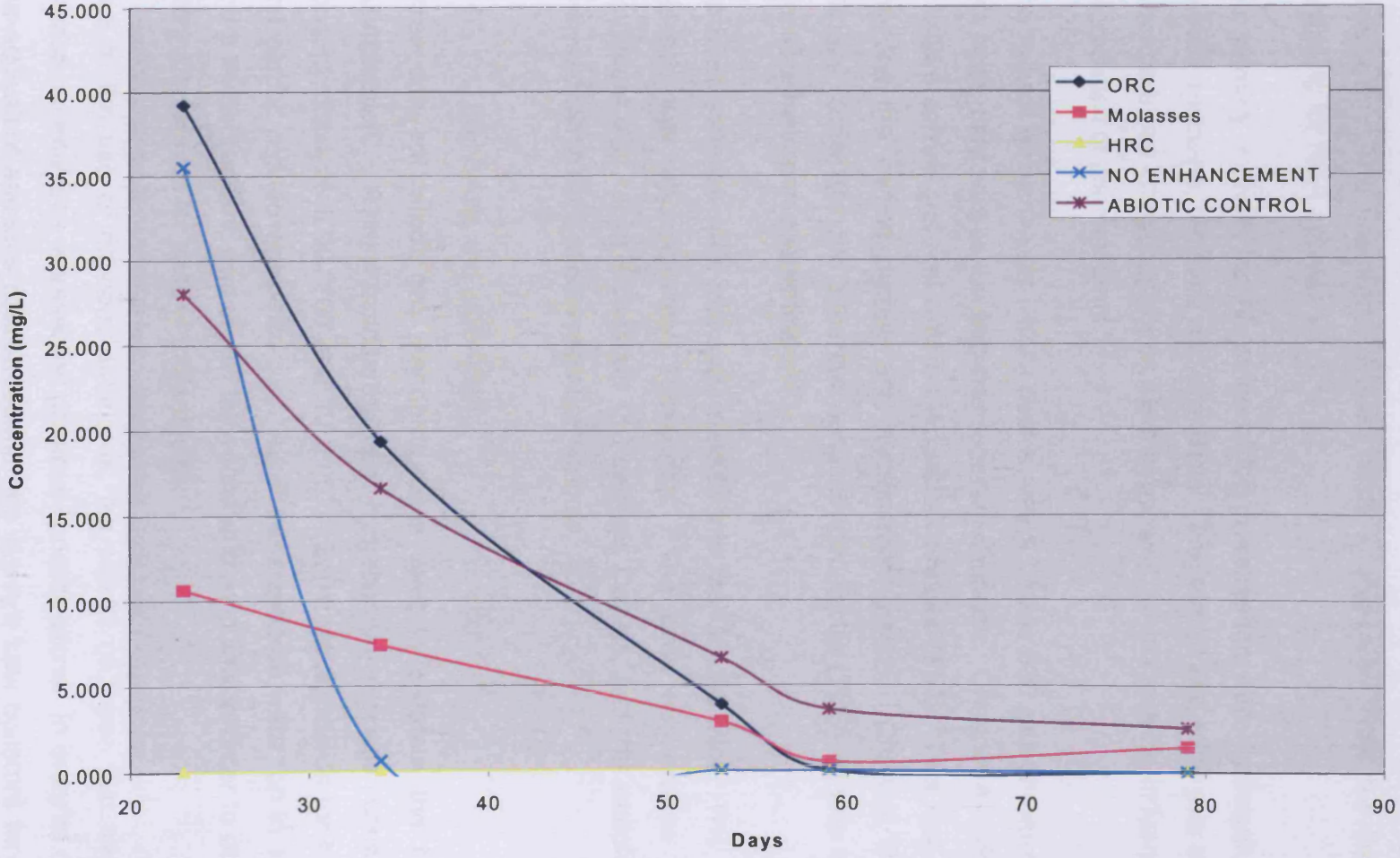

Figure 5.7 DCA degradation in test microcosms during the final 56 day

5.4.3 Additional headspace analyses

Additional headspace tests were carried out in order to monitor concentration changes of other gaseous components prior to the destruction of each test microcosm. The frequency of these tests is shown in Table 5.4 (shown as 'sampling for NGA analysis').

The primary analytes for this stage of the investigation were hydrogen, carbon dioxide, methane, ethylene and acetylene. The aim of this work was to further describe those conditions within the microcosms, which may be influencing the degradation of contaminants.

The natural gas analyser (NGA) device was a Clarus 500 gas chromatograph. The instrument was set up with two separate channels. Channel A consisted of a helium carrier gas and a flame ionisation detector (FID). This first channel provided the carbon dioxide and hydrocarbon results. Channel B had a nitrogen carrier gas and a thermal conductivity detector (TCD). It was set up to measure hydrogen concentrations.

Gaseous samples were manually injected into the GC-MS inlet. The time for injection was approximately 2 seconds. The oven temperature for the instrument was set at a constant 110 degrees Celsius, and the samples were analysed within a 20-minute retention window.

5.4.3.1 Standards and calibration

Three different mixed gas standards were used to calibrate the NGA for quantification. These standards had hydrocarbon (various constituents) concentrations of 1000, 100 and 10 mgL^{-1}. Separate standards for hydrogen and carbon dioxide were also run. All of the standards were run in triplicate, and a linear trendline was drawn between the data points in order to obtain the calibration curves for each of the analytes.

Due to the use of gaseous standards, the results of these test microcosm headspace analyses represented gaseous concentrations. In order to calculate the equivalent aqueous concentrations, the Henry's Law constant for each of the analytes was required. Henry's Law defines the relationship between the

gaseous and aqueous phase concentrations of a particular compound at equilibrium. The Henry's Law constants used for these studies were taken from Sander (1999). Once the gaseous and aqueous concentrations were obtained, the total quantities of compounds were calculated, based on the known slurry and headspace volumes.

5.4.3.2 Results

Specific results from the additional headspace analyses are described below. The more significant findings are discussed in Section 5.5.

5.4.3.2.1 Molecular hydrogen

Hydrogen determination in microcosms was conducted with the primary aim of assessing the performance of the reductive additives. These additives were originally chosen for their abilities to release hydrogen, and so facilitate reductive dechlorination (refer to Section 5.2.3.2.2).

The total quantities of hydrogen, which were detected in microcosms M2 and M3, are given in Figure 5.8. Molecular hydrogen measurements for all other microcosms were below detection limits. The quantities shown are a summation of hydrogen contained in aqueous and gaseous headspace phases. This was calculated in accordance with Henry's Law, which describes how compounds are proportioned between the aqueous and gaseous phases. Total hydrogen masses reached maximum values of 15,725 and 420 mg for microcosms M2 and M3 respectively. Significant molecular hydrogen peaks for these two microcosms were noted at day 34.

5.4.3.2.2 Carbon dioxide

Carbon dioxide (CO_2) was monitored in all microcosms. The purpose for the carbon dioxide measurements was to give an indication of microbial activities, which may be associated with contaminant degradation. The mineralisation of contaminant compounds under aerobic conditions was expected to produce carbon dioxide (refer to Section 5.2.3.2.1).

The total quantities of carbon dioxide determined within microcosms are shown in Figure 5.9. The values given are a summation of the carbon dioxide contained in aqueous and gaseous headspace phases. The highest quantities of carbon dioxide were produced in microcosms M2 and M3. The only microcosm that did not give any results for carbon dioxide (above detection limits) was microcosm M1. Significant peaks of carbon dioxide content were noted in microcosms M2 and M3 at day 20 and day 34 respectively.

5.4.3.2.3 Methane

Methane was quantified in all microcosms in order to determine any methanogenic activities. Methanogenesis in a soil-groundwater system is usually described as a reaction between an electron donor and carbon dioxide, which react to form methane and water. A typical electron donor for methanogenic reactions is molecular hydrogen. The presence of methane in a soil-groundwater environment is indicative of highly reduced conditions.

The total quantities of methane determined within microcosms are shown in Figure 5.10. The values given are a summation of the methane contained in aqueous and gaseous headspace phases. Methane production was most elevated in microcosms M1 and M3. In contrast, no significant variations in methane were observed in microcosms M2 and M5.

5.4.3.2.4 Ethylene and Acetylene

Ethylene and acetylene are known metabolites of DCA (see Chapter 2 for a description of degradation pathways). These compounds were quantified in order to confirm the degradation of DCA. Unfortunately, ethylene and acetylene were measured as a single quantity, due to the fact that their chromatogram peaks formed at the same retention time. This means that these results may give an indication of DCA degradation, but do not describe the exact pathway.

The quantities of ethylene and acetylene determined within microcosms are shown in Figures 5.11 and 5.12. Two figures were produced due to the large variation in scale between microcosms M3 and the other four microcosm types. Figure 5.12 gives the quantities found within microcosms M3. The values given

represent the masses detected within the headspace of each microcosm. A comparison has not been made with the relative aqueous concentrations due the differing Henry's Law constants of ethylene and acetylene. All microcosms showed an increase in ethylene and/or acetylene concentrations over the course of the studies. Microcosms M2 and M3 were found to contain the highest amounts of ethylene and/or acetylene. Microcosms M3 contained a maximum of 678mg of ethylene/acetylene (the highest noted). Significant peaks of ethylene/acetylene content were noted in microcosms M2 and M3 at day 10 and day 20 respectively.

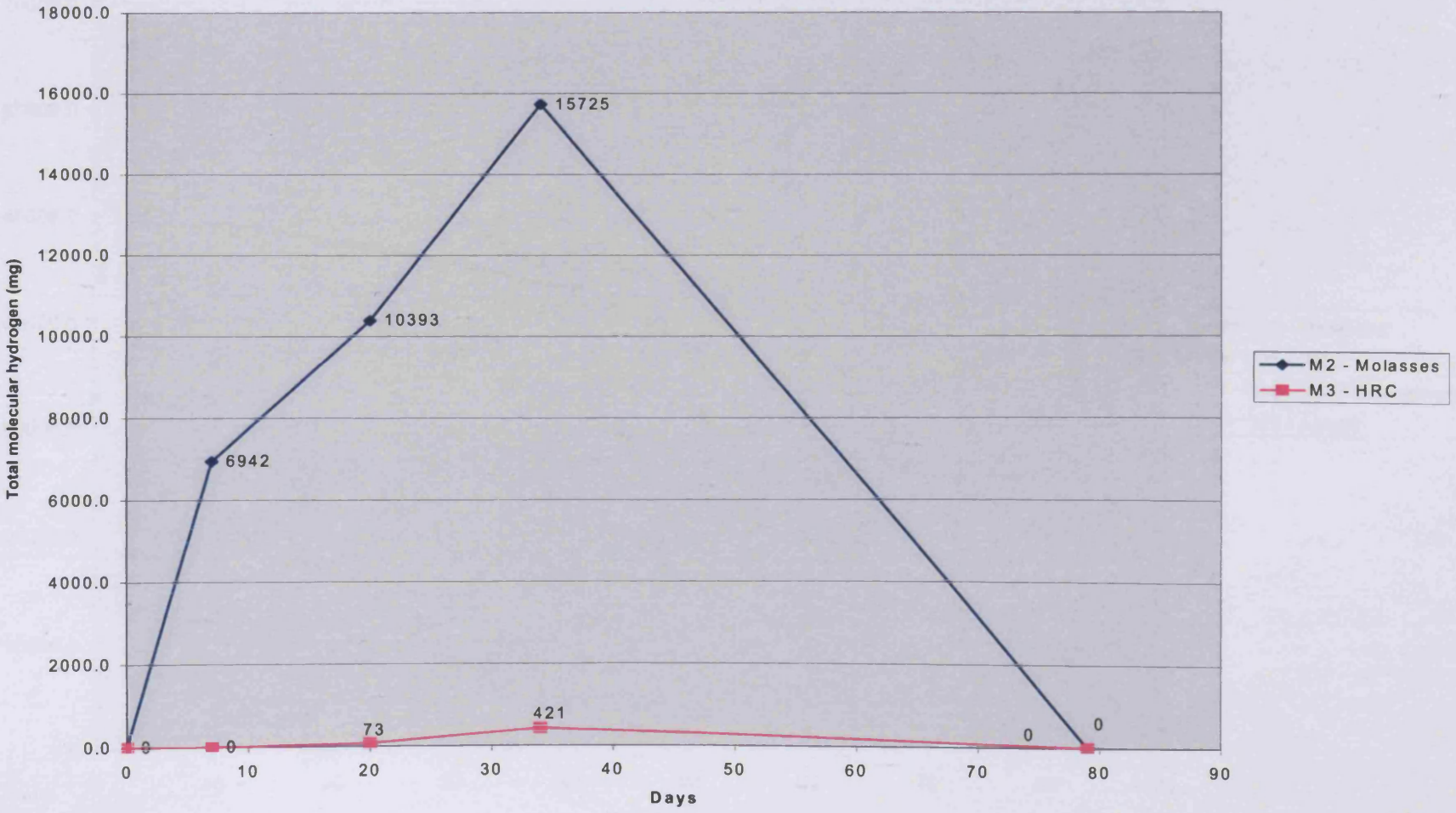

Figure 5.8 Changes in molecular hydrogen quantities within test microcosms M2 and M3

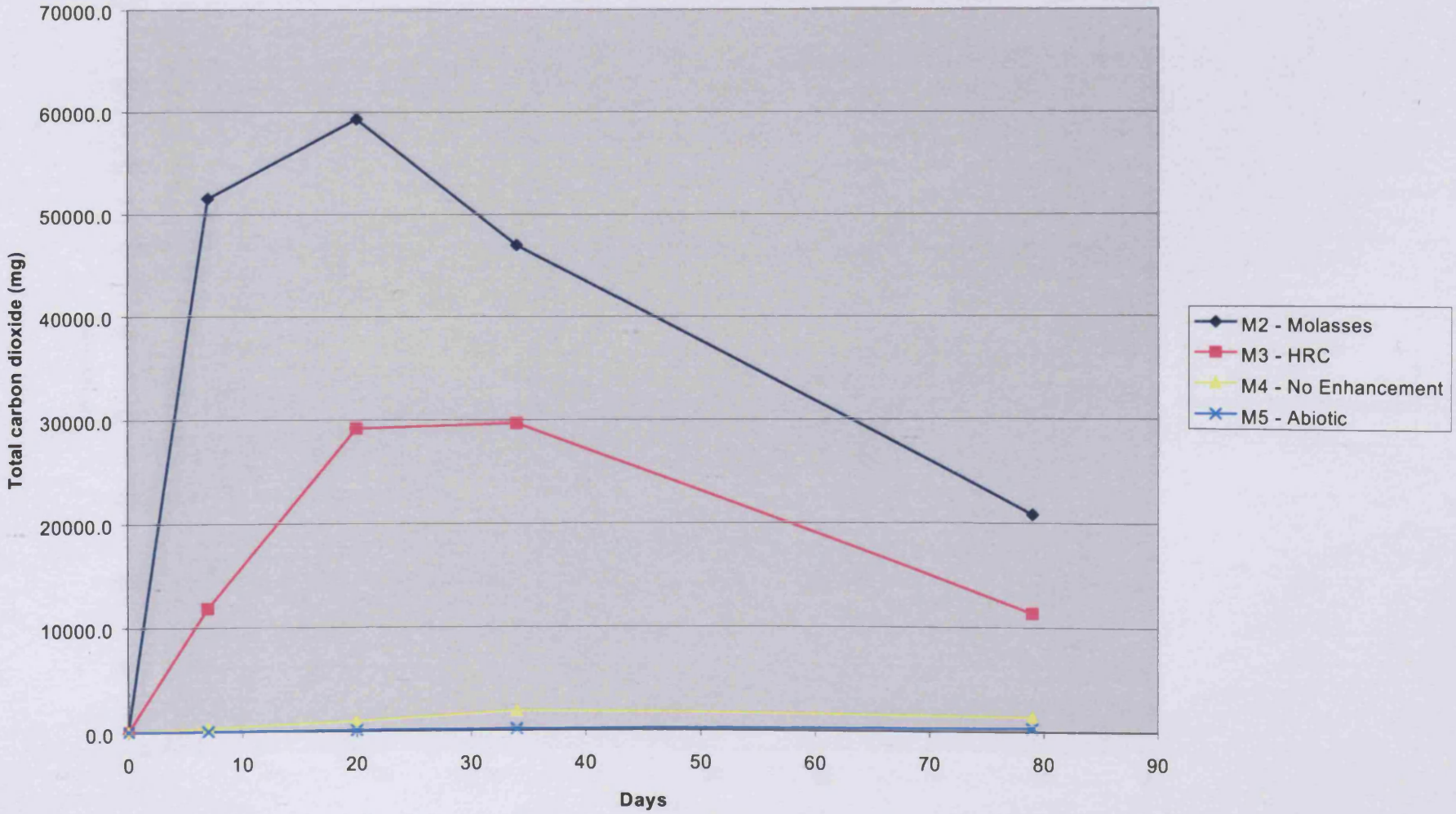

Figure 5.9 Changes in carbon dioxide quantities within test microcosms

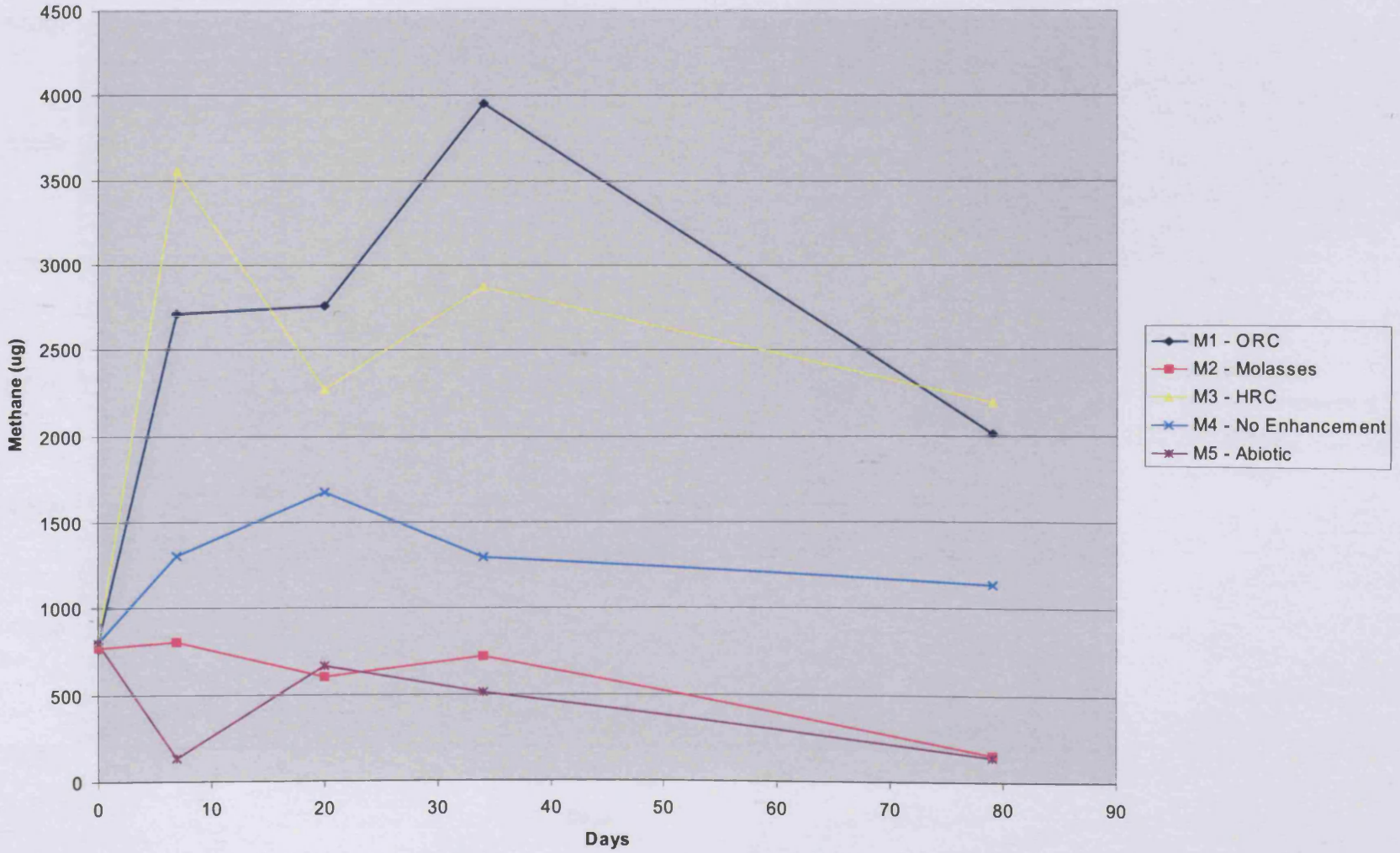

Figure 5.10 Changes in methane quantities within test microcosms

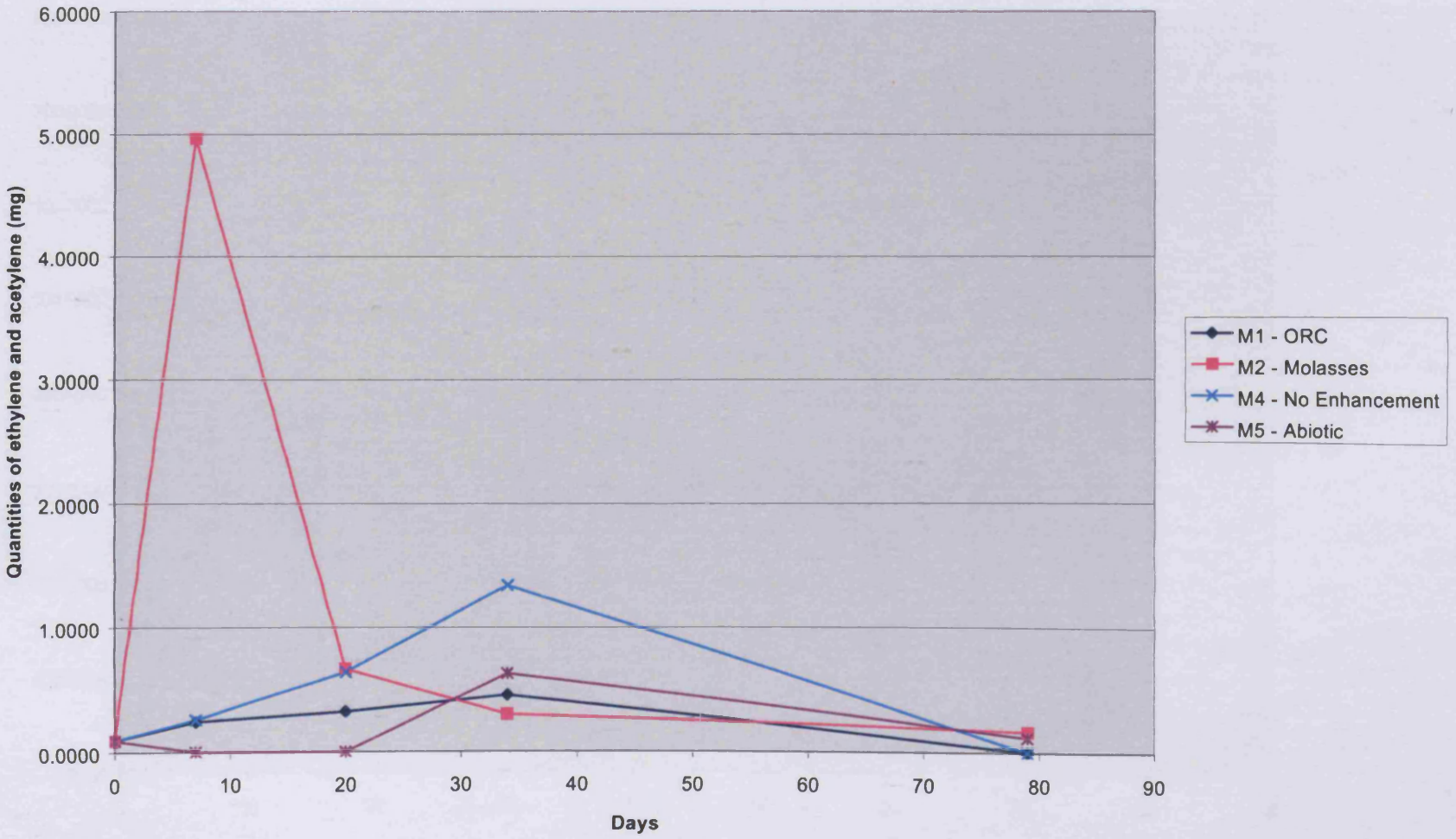

Figure 5.11 Amount of ethylene and acetylene determined from NGA analyses

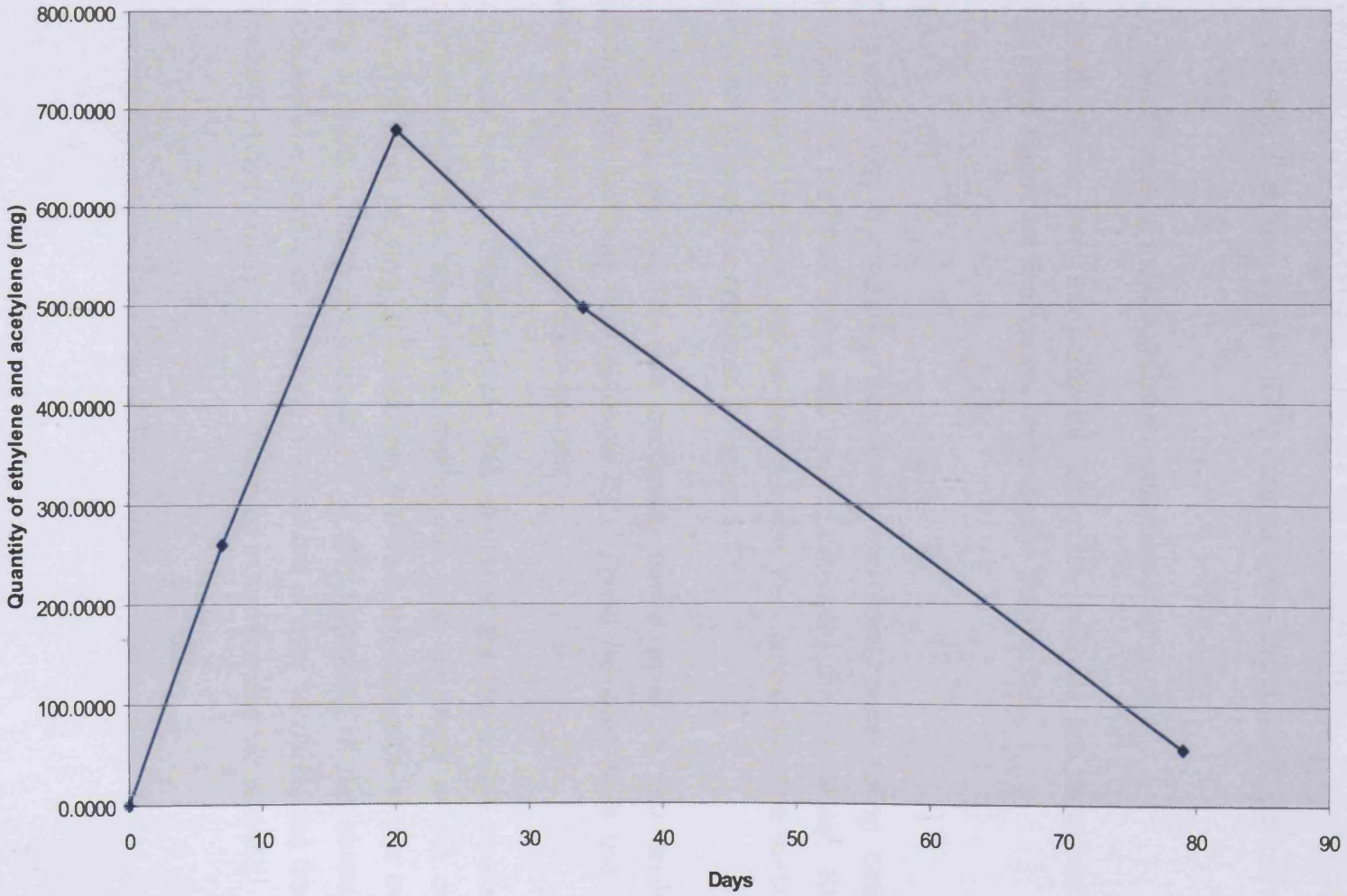

Figure 5.12 Amount of ethylene and acetylene in microcosms M3, determined from NGA analyses

5.4.4 Porewater chemistry analysis

Porewater samples were collected from microcosm slurries by destructive sampling. The frequency of the destructive sampling is shown in Table 5.4. Two main types of porewater analyses were carried out:

1. pH and dissolved oxygen (DO) determination using handheld instruments, and
2. quantification of major ions by ion chromatography.

Specific results from the porewater chemistry analysis are described below. The more significant findings are discussed in Section 5.5.

5.4.4.1 pH

Porewater was immediately transferred into 30mL vials during destructive sampling. A handheld meter was used to determine the pH values. Readings were taken in triplicate, and an average was then calculated. The variations in pH within microcosms are given in Figure 5.13.

The starting pH of 8.2 was assumed, based upon *in situ* analysis of groundwater samples from borehole B2. These samples were used in the development of the microcosm slurries.

A general trend of decreasing pH was seen over the first couple weeks of the microcosm studies, after which the values rose by about a unit over the following 60 or so days. Microcosms M1 were the exception to this trend, as they showed a sharp increase in pH at the beginning of the studies. This increase was significant, reaching a maximum of over 10 during the first week. The largest decreases in pH were observed in microcosms M2 and M3.

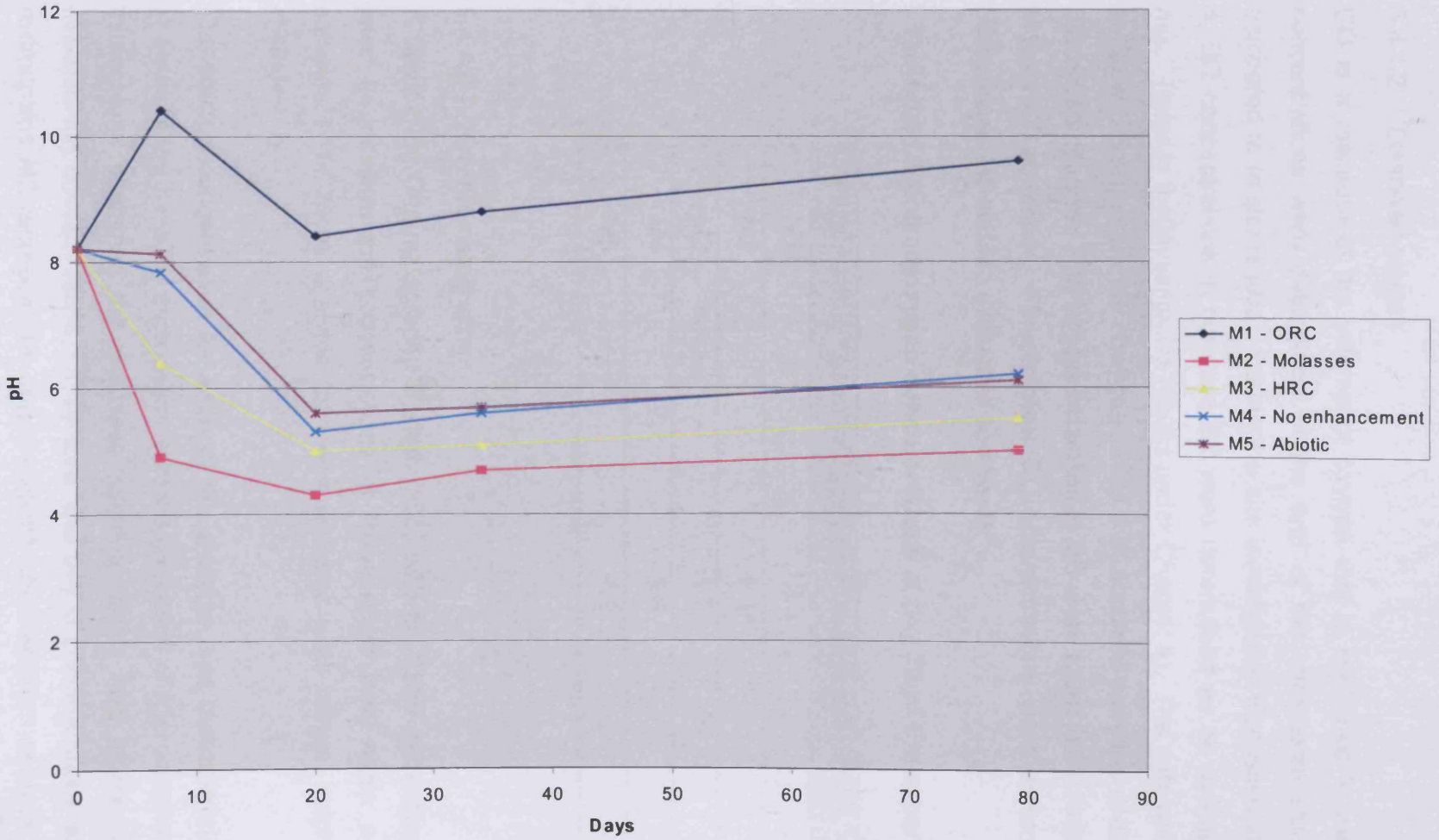

Figure 5.13 Variations in pH during the microcosm studies

5.4.4.2 Dissolved oxygen

DO is a measure of the volume of oxygen that is contained in water. DO concentrations were determined at the end of the microcosm studies, and compared to *in situ* results. During the site investigation discussed in Chapter 4, DO concentrations in borehole B2 were determined as an average of 2.1 mgL^{-1} (refer to the investigative results under Chapter 4). The data points given in Table 5.5 represent the changes in DO that occurred over the course of the microcosm studies. The largest increases in DO were found within microcosms M1 (ORC® enhanced). Much lower DO concentrations were determined for microcosms M2 and M3 (reductive additives).

Table 5.5 Dissolved oxygen concentrations at day 79 of the experiments

Microcosm	Dissolved oxygen concentration (mgL^{-1})
M1	12.5
M2	0.4
M3	1.7
M4	3.8
M5	4.2

5.4.4.3 Ion chromatography

A Dionex Ion Chromatography System (ICS) with an AS50 auto-sampler was used to measure the concentrations of three major ions within porewater samples. The focus analytes for these analyses were nitrate, sulphate and chloride.

The starting concentration for each of the analytes was determined from an analysis of the baseline microcosm, with the exception of microcosms M2. M2 microcosms contained the molasses additive, which was found to contain elevated ion concentrations. Therefore, the day 0 concentrations shown for microcosms M2 represent the concentrations obtained from analysis of a 7% solution of pure molasses.

Due to the sensitivity of the ICS to organic chemicals, porewater samples were first filtered prior to analysis. Onguard II RP cartridges were used to filter the samples. The cartridges required conditioning by purging with 5mL methanol, then 10mL of water before use.

5.4.4.3.1 Nitrate

Figure 5.14 shows the variations in nitrate during the microcosm studies. Only microcosms M2 showed significant changes in nitrate concentrations over the course of the microcosm studies. Nitrate concentrations within M2 showed a significant peak (> 100 mgL^{-1}) at day 20, after which it rapidly dropped down to zero. The quick rate of nitrate loss between days 20 and 34 may have been the result of nitrate reduction. However, the extremely high magnitude of the sudden loss may suggest an error in the analysis. Microbiological analysis of soil samples from days 20 and 34 were designed to provide details on the potential presence of nitrate reducing bacteria. These microbiological issues are addressed further in Chapter 6.

5.4.4.3.2 Sulphate

The variations in sulphate within microcosms are shown in Figure 5.15. Changes in sulphate concentrations followed much more of a pattern across all microcosms, when compared to the nitrate results. Sulphate concentrations showed significant peak in almost all microcosms at around day 20, then began to fall until day 34. The concentrations then levelled off for the remaining duration of the experiment. The drop in concentrations between day 20 and 34 could be indicative of sulphate reduction occurring to varying degrees within all microcosms.

Benzene and ethylbenzene were almost completely degraded within microcosms by day 20. In contrast the sulphate reduction does not seem to have begun until after this period. Because of this, it is likely that benzene depletion is the result of another mechanism, such as aerobic respiration. Such respiration requires the presence of oxygen.

5.4.4.3.3 Chloride

Chloride ion concentrations were determined in order to provide another reference to DCA dechlorination. Chloride ions may be presence in the porewater following the exchange of a hydrogen atom for a chlorine atom on the DCA molecule. Figure 5.16 shows the results of the chloride monitoring.

Chloride concentrations showed significant peaks in almost all microcosms at around day 20, before returning back to levels similar to the staring values. Once again the microcosms M2 showed the most significant increase in chloride concentration. The levelling off of chloride after day 34 may be the result of the ions binding with other elements, such as hydrogen to form hydrochloric acid.

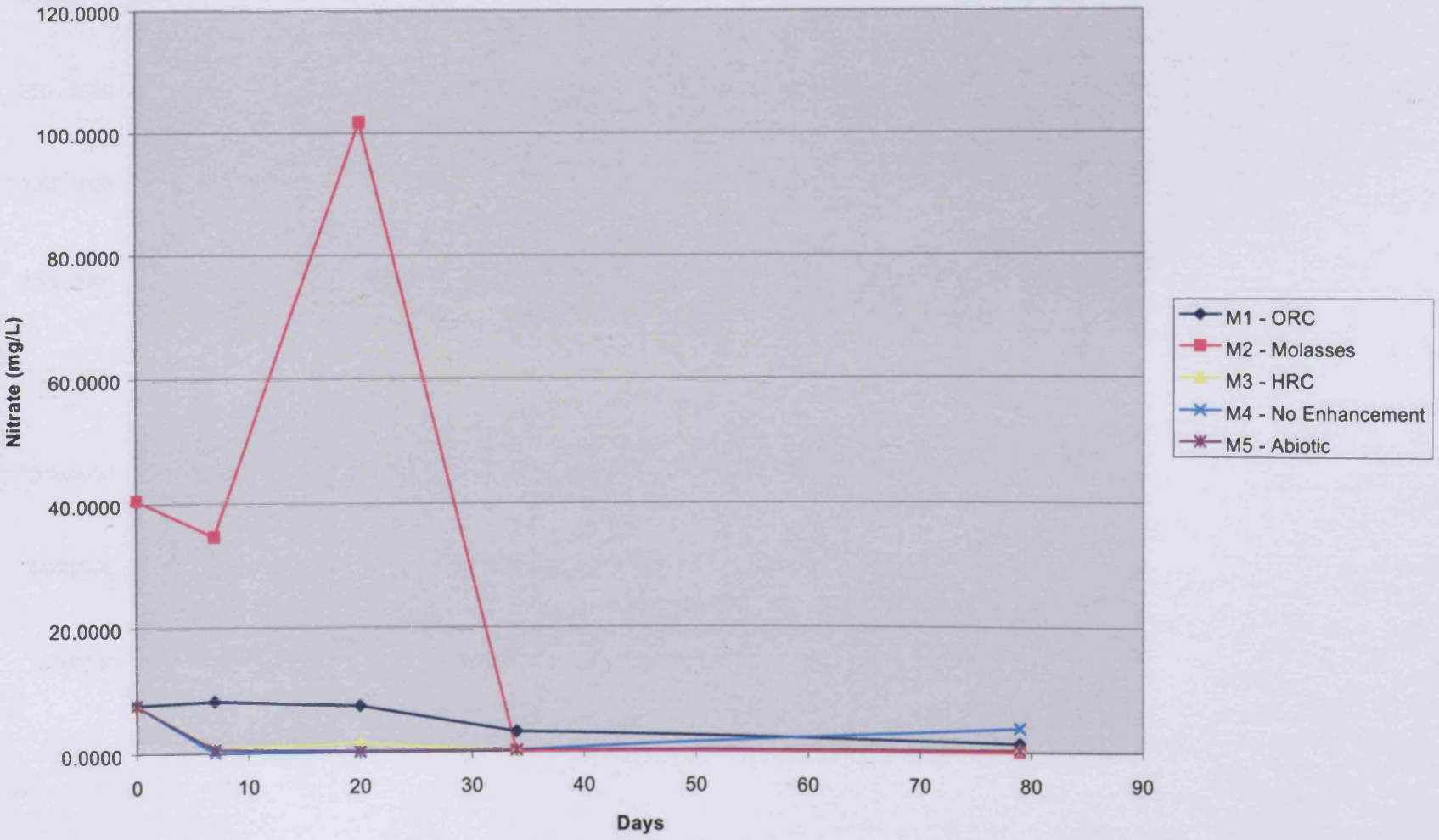

Figure 5.14 Variations in nitrate concentrations throughout the microcosm studies

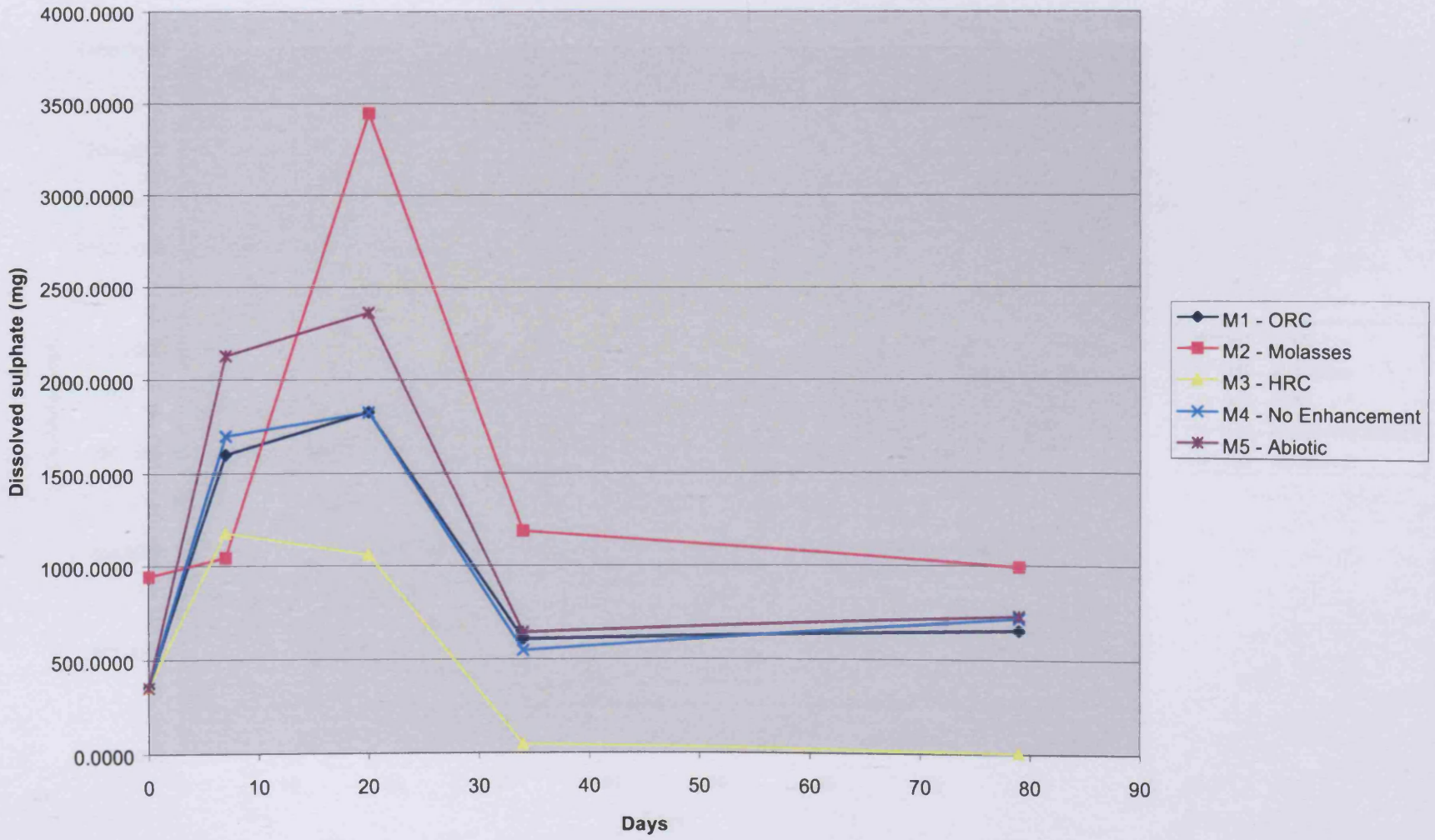

Figure 5.15 Variations in sulphate concentrations throughout the microcosm studies

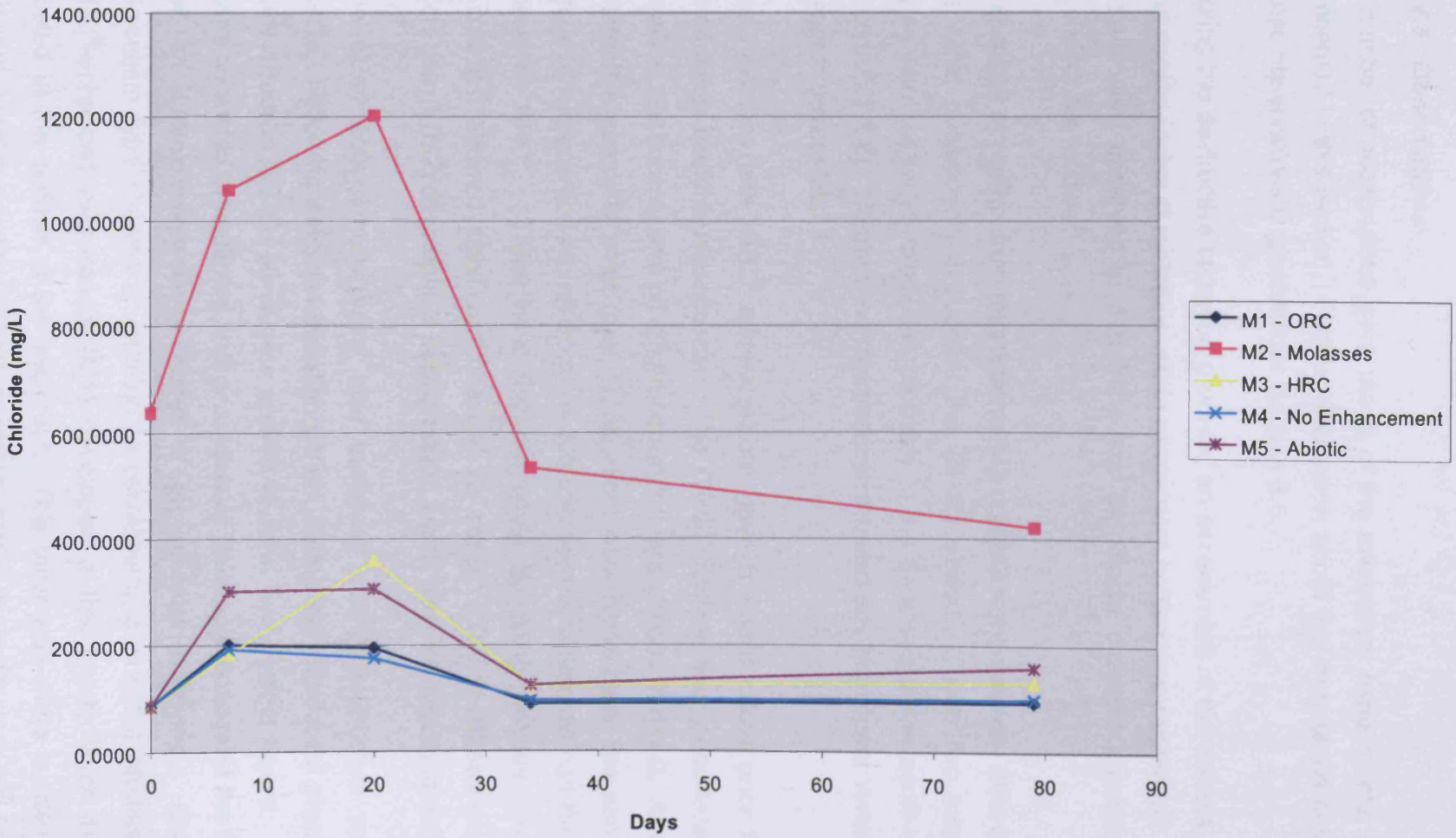

Figure 5.16 Variations in chloride concentrations throughout the microcosm studies

5.4.5 Observations

A number of observations were made of the microcosm experiments, and are presented in this section. Further discussion about the importance of some of these observations is given under Section 5.5.

During the destructive sampling process, an assessment of the colours, odours and consistencies of materials within microcosm bottles was carried out. These factors were observed to determine any significant changes throughout the course of the studies.

The colour of slurries from microcosms M2 and M3 were distinctly different from the other microcosms throughout the 79-day period. These two microcosm types were darker in colour due probably to the darkness of the additives (see Section 5.2.3.2). None of the microcosms showed any significant variations in colour in the studies.

The molasses and HRC® additives both gave a sweet odour prior to their introduction into the microcosms. The ORC® additive was odourless. The baseline microcosm gave off a light odour of hydrocarbons at day 0. At the first destructive sampling point (day 7), all microcosm types gave the same light odour of hydrocarbons apart from the M3 microcosm, which still had the sweet odour of HRC®. Throughout the remaining sampling periods, only one microcosm showed significant changes in odour. The molasses-enhanced microcosms (M2) developed a strong odour, which was not directly identifiable.

The slurry consistencies were also assessed during destructive sampling events. Following the initial agitation period, during the microcosm preparation stage (Section 5.3.2), all slurries settled to form two distinct layers. These layers consisted of sediment and groundwater filled pore spaces at the bottom, and an approximately equal depth of groundwater overlying. Only one microcosm type showed a variation in consistency during the studies. The ORC®-enhanced microcosms (M1) developed a thin crusty layer that was located at the surface of the sediment. This layer was white in colour, and required cracking before slurry samples could be extracted for microbial analysis. No further characterisation was made of this layer.

During the day 7 headspace extraction procedure for microcosm M2, very little force was required when drawing back the syringe's plunger. This was suggestive of a high positive pressure within the microcosm bottle. Subsequent headspace sampling events showed a development of greater positive pressure in microcosm types M2 and M3. Prior to the destructive sampling of sub-samples M1-M5 (III) (day 34), the pressures within all microcosms were assessed using a syringe with a loose fitting plunger. After piercing the septa, the headspace gases were extracted using only the pressures within microcosms to fill the syringe. The total volumes that extracted were then recorded. This procedure was conducted to obtain a rough approximation of the pressure development within microcosms. Approximately $30cm^3$ was removed from microcosm M2(III) and M3(III). This is equivalent to about 40% of the available volume in positive pressure. The pressures within the other microcosms were found to be relatively neutral.

5.5 Discussion

This section brings together the most significant results from the chemical analysis of microcosms. The performance of microcosm enhancements is discussed in relation to expectations. The degradation of the focus contaminants is discussed separately in subsection 5.5.3.

An increase in several parameters at the focus of the additional headspace (Section 5.4.3) and porewater chemistry (Section 5.4.4) analyses was noted. Generally speaking, these increases are most likely the result of increased microbiological activity. The gaseous headspace increases during the initial 20-day sampling period correspond with predictable release products of the enhancements (such as hydrogen, refer to Section 5.2.3.2.2) and metabolites of the focus contaminant (such as carbon dioxide and ethylene/acetylene; refer to Chapter 2, Section 2.4). The peaks in nitrate and sulphate contents (and subsequent rapid reductions in these concentrations) are most likely a result of oxidation (followed by reduction) reactions.

5.5.1 Performance of enhancements

5.5.1.1 ORC®

As stated in Section 5.2.3.2.1, the addition of ORC® (into microcosms M1) was designed to release oxygen, and thus stimulate contaminant degradation through aerobic respiration. The levels of DO within microcosms M1 increased from the *in situ* measured value of 2.1mgL^{-1} to 12.5mgL^{-1} over the course of the experiment (79-day period). The development of oxygen within these microcosms is likely to be directly related to the hydration of the ORC® compound, and is in agreement with expectations.

The pH increases within microcosms M1 were significant, reaching a maximum of over 10 during the first week. EPA (1995) states that pH values of less than 5 or greater than 10 are generally unfavourable for microbial activity. Relating these results to conditions at the study site may suggest that an initial period of efficient contaminant degradation could be followed by an inhibition of microbial activities due to high pH variations. This scenario could be improved through the introduction of additives to the groundwater system, which are designed to alter pH conditions. Such additives could include lime or hydrochloric acid for initiating an increase or decrease in pH respectively (EPA, 1995).

No carbon dioxide was detected in microcosms M1 (ORC®-enhanced) following the baseline day 0 results. The limits of detection were approximately 1 mgL^{-1}. Carbon dioxide was an expected product of focus compound degradation under aerobic conditions (see equations 5.2, 5.3 and 5.4). The lack of carbon dioxide within these microcosms suggests that other degradation mechanism may have played a role in the degradation of the focus compounds.

Methane production was observed within microcosms M1. The production of methane is associated with highly reduced conditions (discussed in Chapter 2). Such conditions were unexpected due to the oxidising conditions that were induced by the ORC® additive.

A distinct crusty layer was observed at the surface of the slurries within M1 microcosms. The effects that this layer may have had on the chemical and/or biological conditions within the microcosms are unknown. It is possible,

however, that the layer may have stratified the microcosms, with the development of more reduced conditions in the underlying slurry, and the persistence of oxidised conditions in the overlying groundwater. A higher proportion of the ORC® additive would most likely have been present in the overlying groundwater, as the substance was added as a dissolved solute. It is the opinion of this thesis author that the available oxygen in the slurry may have been consumed within the first 7 days. Following this initial aerobic period, in which benzene and ethylbenzene were rapidly lost, more reducing conditions would have persisted (including sulphate-reducing and eventually methanogenic). This theory would help to explain the presence of both methane and oxygen within the microcosms.

The above assumption is not likely to be relevant when considering the use of ORC® in the field due to the unrepresentative nature of the microcosm design, where a distinction exists between slurry and groundwater. This distinction is not present within soil-groundwater systems.

5.5.1.2 Molasses

The addition of molasses into M2 microcosms was designed to release molecular hydrogen, and so facilitate the reductive dechlorination of DCA. Stoichiometric analysis of equation 5.5 suggests that 1g of hydrogen is required for the mineralisation of 49.5g of DCA. This means that the 8mg that were added to each microcosm required 0.16mg of hydrogen. The concentrations of hydrogen that were detected in the molasses-enhanced microcosms reached a maximum of 15.7g, far exceeding the requirements for DCA degradation.

Large quantities of carbon dioxide were also found to be produced within these microcosms. The carbon dioxide was considered primarily to be a product of molasses fermentation, rather than through the aerobic degradation of the organic contaminants. Chapelle (1993) and Dyer *et al* (2000) describe the fermentation by-products of molasses. They describe that the fermentation pathway of molasses yields the intermediate pyruvic acid, which in turn can be oxidised to produce a range of organic acids (acetic, lactic and formic), alcohols (ethyl alcohol, butanol and isopropyl alcohol), hydrogen and carbon dioxide.

Additional carbon dioxide may result from reactions between organic acids and calcium carbonate. One source of calcium carbonate is from shell fragments, which were observed in study site samples (see Chapter 4).

The organic acids that are produced through the fermentation of molasses may act as carbon sources for the metabolic function of indigenous microorganisms. Thus, the molasses additive was designed to facilitate reductive dechlorination cometabolically. Cometabolic degradation was discussed in Chapter 2.

The drop in pH within the M2 microcosms is likely to be related to the production of carbon dioxide. The dissolution of carbon dioxide can yield bicarbonate (HCO_3), which in turn can act to reduce pH (Buttner *et al*, 1993). Another factor they may have contributed to the lowering of pH is an increased content of organic acids (products of molasses fermentation, as discussed above). The pH reached a minimum of 4.3, which may be low enough to inhibit microbial activity (EPA, 1995).

The production of excess hydrogen and carbon dioxide within the microcosms resulted in large positive pressures. Dyer *et al* (2000) observed a similar build up of gases during a study, which used molasses as a treatment for DCA in flow-through cells. The development of excess gases may have serious implications for the use of molasses for full-scale *in situ* treatments. Pockets of gas forming around a plug of molasses *in situ* could act to decrease the bioavailability of the molasses (carbon source) for cometabolic purposes. However, the delivery of electron donor (e.g. hydrogen) for reductive dechlorination may actually be enhanced through the production and transport of the gases (Dyer *et al*, 2000).

The results of batch studies by Wu *et al* (1998) suggest that only 0.5% of the electrons produced from the fermentation of molasses are used for dehalogenation. The remaining electrons are used for methane production (methanogenesis). Unexpectedly, significant methane production was not observed within M2 microcosms. It is the opinion of this thesis author that the excesses in hydrogen and carbon dioxide within these microcosms may have led to some inhibition for both methanogenesis and reductive dechlorination.

A sharp increase in the concentrations of ethylene and/or acetylene was observed over the first 7 days within microcosms M2. Using equation 5.4 as a guide for stoichiometry, 2.3mg of ethylene should have been produced from the mineralisation of 8mg of DCA. An increase of just under 5mg was observed between days 0 and 7. This is approximately double that of the expected quantity. The excess ethylene and/or acetylene may be from either analytical error or the degradation of non-target compounds (e.g. molasses components or other groundwater contaminants from the study site).

Ion chromatographic analysis of molasses found that electron acceptors (nitrate, sulphate and chloride) were present in higher quantities than within the study site samples (represented by the baseline microcosm). Therefore, the addition of molasses into microcosms M2 significantly altered their ion chemistry. An increase in nitrate was only observed in microcosms M2. The nitrate concentration rapidly increased by over $60mgL^{-1}$ during weeks 2-3 before reducing to zero by day 34. This decrease in concentration may give an indication of nitrate-reduction within microcosms M2 around this period.

The variations in sulphate and chloride concentrations followed a similar pattern for all microcosms, with an initial increase (starting day 0) being followed by a sudden reduction around day 34. The reductions in sulphate concentrations around this period could be indicative of sulphate-reducing conditions. The increase in chloride concentrations may be associated with the dechlorination of DCA. However, no significant differences were observed between the chloride variations within microcosms (apart from the high concentrations associated with molasses as discussed above).

The addition of extra ions into the microcosms may have acted to inhibit the preferred reaction (i.e. reductive dechlorination) due to competition between indigenous microorganisms and varied redox conditions. Further qualification of these conditions was an objective of the microbiological analyses (presented in Chapter 6).

5.5.1.3 HRC®

The addition of HRC® was, like the molasses, designed to release molecular hydrogen, and so facilitate the reductive dechlorination of DCA within microcosms M3. A peak hydrogen quantity of 420.5mg was observed at day 34. As was discussed in the previous section, 0.16mg of hydrogen was required for the complete mineralisation of 8mg DCA. The hydrogen production within HRC®-enhanced microcosms is therefore above required amounts, but not as excessively as that which was observed in molasses-enhanced microcosms. A hydrogen content above the discussed stoichiometric requirements is necessary to account for the use of hydrogen in reactions other than dehalogenation (e.g. methanogenesis) (Wu *et al*, 1998).

Like the molasses microcosms, the quantities of carbon dioxide within microcosms M3 increased significantly through the course of the experiments. The carbon dioxide increase in microcosms M3 was considered primarily to be a product of lactate degradation (refer to Section 5.2.3.2.2). As discussed previously, an increase in carbon dioxide can lead to a reduction in pH. This relationship was observed within the M3 microcosms, where the pH levels dropped to a minimum of 5. Although the contaminants were successfully degraded within the M3 microcosms (see Section 5.5.3), the pH low pH may have inhibited the degradation to a certain extent (EPA, 1995). This potential inhibition would need further consideration when designing a full-scale HRC®-enhanced remediation scheme. As was mentioned in Section 5.2.3.2, adjustments may need to be used in order to increase pH values up to levels that are preferred by indigenous microorganisms.

The production of excess hydrogen and carbon dioxide within the microcosms resulted in large positive pressures. Even though the quantities of hydrogen and carbon dioxide were less than that which was measured in microcosms M2, the positive pressure within microcosms M3 appeared to be comparable. Careful consideration of the full-scale use of HRC® *in situ* would be required due to this gas production (refer to the discussion in Section 5.5.1.2).

Methane production was observed within M3 microcosms. The largest increase in methane occurred in the first 7 days of the experiments, and is likely to be the

result methanogenic activities. It is the opinion of this thesis author that the use of hydrogen for methanogenesis was not enough to inhibit dehalogenation reactions. This is assumed due to the efficient degradation of DCA, which was observed in the HRC®-enhanced microcosms (M3).

Over 600mg of ethylene and/or acetylene were observed within microcosms M3. This quantity far exceeds the expected amounts from the mineralisation of DCA (2.3mg). The excess ethylene and/or acetylene may be from either analytical error or the degradation of non-target compounds (e.g. molasses components or other groundwater contaminants from the study site).

5.5.2 Control microcosms

Standard control microcosms (M4) were designed to represent conditions at the study site without the addition of enhancements. All three of the focus contaminants were successfully degraded in the M4 microcosms. The apparent rate of benzene degradation within the M4 microcosms resembled that which was observed in the ORC® (M1), molasses (M2) and abiotic (M5) microcosms. These similarities suggest that the addition of ORC® and molasses did not enhance the degradation of benzene. The degradation of benzene within the HRC®-enhanced microcosms is discussed further in Section 5.5.3.1.

It was expected that very little degradation, if any, would be observed within the abiotic microcosms (M5). However, the apparent rate of degradation showed no significant difference from that observed in the standard control microcosms (M4). This led to three likely conclusions:

- The focus contaminants could have been degraded through abiotic mechanisms.
- Contaminants may have escaped from the microcosms through leaks due to insufficient sealing.
- The sterilisation techniques, used for preparing the abiotic microcosms may have been ineffective, thus providing the possibility for microbial driven degradation.

Reduced concentrations were observed for each of the focus compounds within the abiotic microcosms. It is unlikely that abiotic processes would have been responsible for the effective degradation of all three focus contaminants. The insignificance of abiotic processes for degrading the focus contaminants was highlighted in Chapter 2 based on the findings of previous research.

The microbiological analysis of slurry samples was designed to identify microbial populations. As part of the studies, an assessment of microbial growth is examined. If no apparent growth were to be concluded, then the issue of potential leakage would be significant.

The loss of contaminant concentrations in the abiotic microcosms is a weakness of this thesis. Further scoping studies and additional control microcosms are highly recommended by this thesis author as approaches for limiting such errors (as well as allowing for a better assessment of the sources for such errors). These additional measures were not conducted for the work carried out for this thesis due to scope limiting factors (such as financial and material shortages). A clarification for the failing of the abiotic microcosms and the details of microbial studies are given in Chapter 6.

5.5.3 Degradation of contaminants

5.5.3.1 Benzene and ethylbenzene

The enhancements for this research were selected to target contaminants in differing ways. The HRC® and molasses additives were not expected to enhance benzene or ethylbenzene degradation directly (refer to Section 5.2.3.2.2), but rather to facilitate the dechlorination of DCA. The ORC® additive, on the other hand, was designed to facilitate aerobic degradation mechanisms (e.g. aerobic respiration), which have been shown to effectively mineralise benzene and ethylbenzene (refer to Chapter 2). The rapid degradation of benzene and ethylbenzene during the initial 7-day period may indicate the presence of residual oxygen at the start of the study (day 0) within all microcosms.

None of the enhancements were shown to degrade benzene more effectively than the standard conditions from microcosms M4. For this reason, no direct conclusions could be drawn regarding the most effective additive for enhancing benzene degradation.

The degradation of benzene in the HRC®-enhanced microcosms occurred less rapidly than in the other microcosms. However, benzene concentrations were still reduced to less than 0.5% of the initial 100mgL^{-1} over the full 79-day study period. At day 15 of the study, approximately 18mgL^{-1} of benzene existed within the HRC®-enhanced microcosms. Also around this period, methane production was observed, which suggested the prevalence of reduced conditions. Thus the degradation of benzene within the M3 microcosms may have been attributable to mechanisms such as sulphate-reduction or methanogenesis.

5.5.3.2 1,2-dichloroethane

In contrast to the benzene and ethylbenzene results, a distinction can be made between the results of the different microcosms in relation to variations in DCA concentrations. The most rapid reductions in DCA concentrations were observed in the molasses-enhanced and HRC®-enhanced microcosms (M2 and M3 respectively).

These results correspond with expectations, as HRC® and molasses have been shown to facilitate the effective reductive dechlorination of hydrocarbons such as DCA (refer to Section 5.2.3.2.2 for a review). Both enhancements were designed to facilitate dechlorination under reduced anaerobic conditions. These findings suggest that anaerobic mechanisms may have been more successful for the degradation of DCA during these studies. This statement assumes that the ORC®-enhanced microcosms were in fact completely aerobic. As was discussed in Section 5.5.1.1, this may not have been the case. Lower DO concentrations were determined for microcosms M2 and M3, which were both also found to contain high carbon dioxide concentrations. This suggests that any residual oxygen present at the start of the microcosm studies was utilised most quickly by the microcosms with reductive additives.

The HRC®-enhanced microcosm appeared to facilitate DCA degradation more efficiently than the molasses-enhanced microcosms. Both additives were shown to have released more than sufficient molecular hydrogen for the dechlorination of DCA. However, the release of molecular hydrogen from the HRC® additive (M3) occurred at a much slower rate and had a lower peak value than molasses. These results suggest that the high excesses in hydrogen and carbon dioxide within microcosms M2 may have contributed to an inhibition in DCA degradation.

5.6 Conclusions

The microcosm treatability studies were designed to describe the potential for bioremediation based on a number of different enhancements. The results of detailed headspace and porewater chemical analyses were brought together in this chapter. In the following chapter, the findings of biological studies are added in order to further define the conditions within the microcosms.

The microcosms enhanced with ORC® (M1) were expected to degrade benzene and ethylbenzene through aerobic respiration. DO concentrations within microcosms M1 reached values of over 12 mgL^{-1}, which should have been more than sufficient for facilitating aerobic respiration. Due to the rapid degradation of benzene and ethylbenzene within the M1, M2, M4 and M5 microcosms, no definitive conclusions can be made as to the effectiveness of the enhancements. This rapid degradation may be the result of effective aerobic mechanisms that were brought about by the presence of residual oxygen within all microcosms at day 0.

The molasses-enhanced microcosms (M2) were successful in rapidly degrading DCA. The molasses released more than the required amount of molecular hydrogen for facilitating reductive dechlorination. This excess in hydrogen was accompanied by elevated concentrations of ions within the molasses itself. The addition of extra ions (including nitrate and sulphate) into the microcosms may have acted to inhibit the preferred reaction (i.e. reductive dechlorination) due to competition between indigenous microorganisms and varied redox conditions.

The microcosms enhanced with HRC® (M3) reduced DCA concentrations more efficiently than the other microcosms. The production of sufficient quantities of hydrogen is likely to have facilitated the reductive dechlorination of DCA. Methane production was also observed, but it would appear that the competition for molecular hydrogen for methanogenic activities was not sufficient to inhibit dechlorination. Slower reductions in benzene concentrations were observed within the HRC®-enhanced microcosms compared to all other microcosms. This was attributed to the rapid consumption of oxygen, and inhibition of benzene degradation under reduced conditions. This said, benzene concentrations were still reduced to less than 0.5% of the initial 100mgL^{-1} over the 79-day study period. Therefore, anaerobic conditions did facilitate benzene degradation, but less rapidly than that which occurred under the initial aerobic conditions (as discussed above).

Oxygen appears to have been rapidly consumed within microcosms M2 and M3, thus allowing more reduced conditions to develop for facilitating the reductive dechlorination of DCA. The breakdown of molasses and HRC® produced excess gases (hydrogen and carbon dioxide) within the microcosms. It is recommended that future batch studies of molasses-enhanced and HRC®-enhanced degradation be carefully monitored for changes in pressure within the microcosm bottles. The high positive pressures observed during these studies were not deemed to have significantly affected the accuracy of test results. Further research is recommended in order to investigate the effects of pressure development during *in situ* applications of molasses and HRC® enhancement.

The loss of contaminants within abiotic microcosms (M5) suggests error in the analyses. The loss was not thought to be related to abiotic degradation mechanisms. It is more likely that the sterilisation procedure was unsuccessful, or that contaminants may have been lost through leaks in the microcosm bottles. The microbiological analyses described under Chapter 6 were designed to assess microbial growth within microcosm slurries, and thus further clarify this situation.

5.7 References

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Chapter 6

Molecular Microbiological Studies

6.1 Introduction

This chapter gives the details of molecular microbiological studies, which were carried out on samples of the microcosm experiments from Chapter 5. These analyses were designed to complement the results of chemical characterisation of the microcosms.

Section 6.2 provides some background to the methods adopted for assessing microorganisms within the microcosms. The ways in which the metabolic activities and growth of microorganisms are directly linked to the genetic information contained within their cells are discussed. Justifications are provided for the methods adopted for microbial assessment.

The objectives of the molecular microbiological studies are stated in Section 6.3 along with an overview of molecular methods. The techniques are related to the natural mechanisms that carry genetic information throughout a cell.

The profiling of microbial communities within microcosm samples was determined using molecular methods. The procedures and results of this work are provided in Section 6.4. Evidence of changes in microbial communities within microcosms was observed and related to the degradation of the focus organic chemical contaminants.

Section 6.5 provides the findings of an investigation into the presence of dehalogenase genes within microcosm samples. Dehalogenase genes encode

enzymes, which are known to be directly linked to the degradation of chlorinated hydrocarbons (Janssen *et al*, 1985).

The results of the microbiological analyses are concluded in Section 6.6. The more significant findings are discussed with regard to the degradation of organic chemicals within microcosms.

6.2 Background and Methodology

6.2.1 Classification of organisms

A three-domain system is used to describe the classification of all organisms according to cell structure. Humans, along with most other multi-cellular organisms, are classified as eukaryotes. The cells of eukaryotic organisms contain a distinct nuclear membrane, which contains deoxyribonucleic acid (DNA) (Madigan *et al*, 2003).

All other organisms are classified together as prokaryotes. These organisms are mostly unicellular, and are distinguished by their free-floating DNA segments and lack of nuclear membrane. Woese *et al* (1990) introduced the idea of splitting prokaryotes into two separate branches, thus forming the three-domain system. The studies by Woese *et al* (1990) involved the use of molecular analyses to describe the phylogenetic relationships between organisms. Phylogenetic relationships represent the evolutionary ties between life forms (Madigan *et al*, 2003). Prokaryotic microorganisms fall into two distinct classifications called bacteria and archaea. The basic cell structures of these two groups are very similar. However, small differences in their genetic functions and cell membranes set them apart. The genetic functions of microorganisms are discussed later in this Chapter. Microorganisms have a significant influence in ecological and environmental issues. They exist in large numbers within a soil system, and drive many of the key reactions responsible for the degradation of organic compounds. The roles of microorganisms in a soil-groundwater system were introduced in Chapter 2. Prokaryotic microorganisms (in particular bacteria) form the focus of the molecular microbiological studies described in this chapter.

6.2.2 Traditional culturing vs. molecular techniques

The work for this thesis was focused on the use of molecular methods for investigating microorganisms within the study microcosms. This Section addressed the question of why these methods were adopted over traditional techniques, which have been used satisfactorily in previous studies.

Traditional methods for assessing microbial communities typically involve the use of various culturing, isolation and microscopy techniques. Molecular biology can be defined as a study of microorganisms using techniques that analyse the biochemical components of cells (Rochelle, 2001). This definition includes investigations into the basic molecular constituents of life, such as nucleic acids and proteins.

This thesis focuses on the problem of reducing the concentrations of certain chemical constituents in groundwater at a contaminated study site (as described in Chapter 1). This situation requires an economical and practical approach (in terms of time, effort and materials). One major benefit of adopting molecular methods is the time required to complete the analyses. Traditional methods for characterising microorganisms within samples can be time consuming; often requiring culture methods to be determined for the range of potential organisms to be investigated. For example, targeting 20 different organisms in 20 different microcosm samples would require 400 separate agar plates. This approach may have proven expensive in terms of time and materials. Molecular methods allow for the rapid screening of numerous samples for relatively high numbers of organisms/genes (Hugenholtz and Goebel, 2001). Using the techniques described herein, numerous species characterisations were made during the work for this thesis. In practice, these characterisations can be completed in approximately 2 to 3 days (from extraction to sequence comparison). However, it is noted that the molecular assessments conducted by this thesis author took several weeks to complete. This was due to the learning process, in which the methods described herein were trialled in order to reduce inconsistencies and errors in the procedures used.

It has been estimated that over 99% of microorganisms in nature cannot be cultured in the laboratory (Amann *et al*, 1995). One limitation of this thesis relates to the fact that the microcosms themselves were a form of laboratory culturing; therefore, many indigenous microbial species from the study site may not have grown within the microcosms. However, the adoption of molecular methods may have allowed for a larger percentage of the dominant microorganisms within microcosms to be assessed.

The accuracy of results obtained using molecular and traditional techniques are difficult to compare due to the varied approaches. It is important to recognise the important roles, which both methods can play in environmental microbiology. Traditional methods can provide useful data about the behaviour and growth of microorganisms (thus allowing for more accurate classifications to be made). Additionally, traditional methods may have proven useful for this thesis with regards to an assessment of the efficiency of the sterilisation process used for the abiotic microcosms.

In order to relate molecular sequence results to a particular type of microorganism for classification, a database of previously sequenced microorganisms is used (e.g. Genbank). Molecular methods are relatively new in development, and as such the available data for comparison is limited. However, the quantity of sequences within the database continues to grow rapidly due to the increasing popularity of molecular microbiological methods. Species identification has historically been defined in relation to the results achievable using traditional approaches to characterising microorganisms. However, since the inception of environmental molecular microbiology, some new definitions have been proposed. Stackebrandt and Goebel (1994) suggested that organisms sharing greater than 97% similarities between their 16S rRNA gene sequences can be related on a species level. This definition of species is useful for the rapid screening of samples (as was required for this thesis – see Sections 6.4.7 to 6.4.9); however, for a more accurate definition of species using molecular methods, attention would have to be given to describing the entire DNA sequence (not just the 16S rRNA-encoding gene).

6.2.3 Linking metabolism to genetics

Microorganisms survive by carrying out various biochemical reactions, which ultimately allow for cell replication (reproduction) to occur. Collectively, the complete set of biochemical reactions, which occur within organisms, is termed metabolism (Madigan *et al*, 2003). The metabolic functions of microorganisms can alter the chemical and physical properties of their surroundings (Newman and Banfield, 2002). It is important to have a fundamental understanding of the growth and metabolism of the different microorganisms so that their effects on the environment can be accurately described. This Section will focus on metabolic function in relation to the genetic information contained within cells.

As with all organisms, the metabolic and reproductive functions of bacteria are described by genetic materials. Most of a cell's genetic information is contained within genomic DNA. DNA exists as a double helix structure (typically circular), with two sides being held together by hydrogen bonds between four main structural units called nucleotides (Madigan *et al*, 2003). These nucleotides are found in pairs (called base pairs) and consist of adenine, guanine, cytosine and thymine. Base pairs always occur as adenine with thymine and cytosine with guanine.

The information contained in genetic materials flows within a cell according to three primary mechanisms: replication, transcription and translation. Madigan *et al* (2003) gives detailed descriptions for these mechanisms and a summary is provided in the following paragraphs.

DNA replication within bacteria is an asexual process. The replication process begins at a specific location on a chromosome of DNA, and typically proceeds in both directions from this origin. The parent DNA strands are first separated (denatured) before the new complimentary strands are synthesised. The formation of a primer initiates the synthesis of new nucleotides by enzymes called DNA polymerases. Once the entire DNA genome has been replicated, the chromosomes are split into two separate cells. The replication process is illustrated in Figure 6.1.

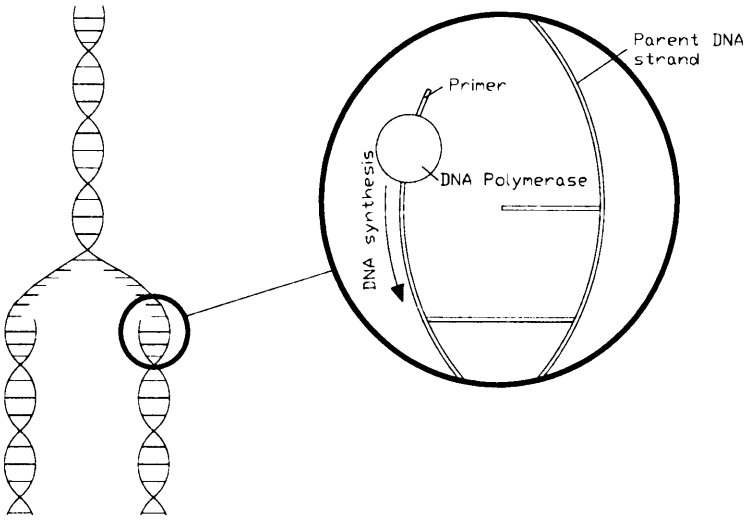


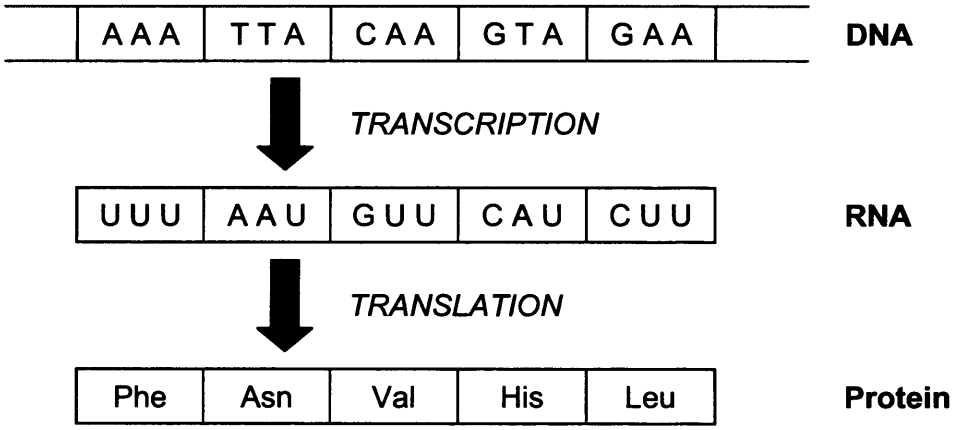
Figure 6.1 Replication and DNA synthesis

Transcription is a process whereby ribonucleic acid (RNA) is synthesised. RNA serves a variety of purposes within a cell, such as carrying genetic information from DNA and acting as a structural component within ribosomes. For the purposes of this review, transcription will be considered in conjunction with translation to describe the chain of events which leads to the expression of genes. Gene expression describes the process in which a gene's encoded information is converted into the structures that are present or operating within a cell (e.g. proteins and RNA) (Madigan *et al*, 2003).

During transcription, RNA polymerase enzymes synthesise RNA in a similar way to DNA polymerase in the production of DNA. The genetic coding of RNA also contains 4 base compounds. However, RNA replaces thymine with uracil. The synthesised messenger RNA (mRNA) then carries genetic information to the ribosome. In prokaryotes, the ribosome consists of two subunits, each containing ribosomal RNA (rRNA) and ribosomal proteins. One sub-unit consists of 16S rRNA and the other 23S rRNA.

mRNA reacts with the ribosome and transfer RNA (tRNA) in the process of translation, whereby proteins are synthesised. Figure 6.2 shows the main stages involved in the synthesis of proteins. tRNA reads the encoded mRNA sequence three bases at a time, and proceeds to attract the appropriate amino acid. The group of three mRNA bases is referred to as a codon. Each codon encodes a specific amino acid. However, some amino acids can be encoded by more than one codon. This property is referred to as degeneracy. Amino acids are chained together to form proteins, which when completed, proceed to carry out various cell functions.

Figure 6.2 The main stages involved in the synthesis of proteins from basic genetic information



The processes described in this Section form the basis from which molecular microbiological techniques have been developed. A description of different approaches to the molecular analysis of environmental samples is provided later in this chapter.

6.3 The adopted approach

6.3.1 Study objectives

Molecular microbiological studies were undertaken in order to monitor changes in microbial community profiles and attempt to identify dehalogenase-encoding genes within microcosm samples.

For the purposes of this thesis, all descriptions of molecular methods will be biased towards the analysis of DNA fragments. However, other genomic materials, which fall outside of the scope of this thesis, may also be analysed by similar techniques.

6.3.1.1 Community profiling

The term microbial diversity is often used to describe the results of molecular microbiological studies using the techniques described herein (Muyzer *et al*, 1993; Hugenholtz and Goebel, 2001; Keller and Zengler, 2004). Keller and Zengler (2004) define microbial diversity as the sum of all the different species in a given environment; however, depending on the methods used, the number of species that exist in a single sample may differ substantially. As stated previously, molecular methods allow for a larger percentage of microorganisms from a single sample to be assessed when compared to traditional methods (Amann *et al*, 1995). However, the objectives of this thesis did not include a full assessment of microbial diversity, but rather an investigation into the predominant microbial species involved in the degradation of the focus organic compounds. In order to identify the maximum number of microbial species within the microcosm samples (and thus attempt to attain a description of microbial diversity, as defined above), further DNA amplification, separation and molecular cloning would be recommended. The use of molecular cloning was not within the scope of this thesis; but, details of this technique are presented by Hugenholtz and Goebel (2001). For the purposes of this thesis the term community profiling is used to describe the assessment of dominant microbial communities within microcosm samples (Muyzer *et al*, 1993).

The changes in microbial community profiles were addressed through a combined PCR and DGGE analysis of 16S rDNA genes. Sequencing of DGGE bands and comparison with sequences from a gene database was also performed to characterise individual microbial strains. This characterisation was targeted at identifying genomic similarities between microorganisms from the microcosms and microorganisms that can be related to the degradation of the focus compounds for this thesis.

6.3.1.2 Dehalogenase gene identification

One objective of the microcosm studies was to determine the treatability of a chlorinated hydrocarbon (1,2-dichloroethane (DCA)). Studies by Olaniran *et al* (2004) have shown that organisms, which produce dehalogenase enzymes, can effectively degrade compounds such as DCA. Identifying the genes, which encode the dehalogenase enzymes, could give an indication of the potential for degradation and help to describe the active degradation pathway(s). Two dehalogenase primers were used for the PCR amplification of microcosm samples. These primers were investigated by Hill *et al* (1999).

6.3.2 Overview of molecular methods

A wide range of methods are available for the molecular analysis of environmental samples. This Section provides an overview of the various methods adopted for the assessment of microcosm samples during the work for this thesis.

6.3.2.1 Sampling and storage considerations

In order to maintain the accuracy of molecular techniques, care must be taken during the initial sample extraction and storage stages. Physical, chemical and thermal changes during sample extraction will all have potential impacts on the integrity of samples. The activity of microorganisms is directly linked to variations in temperature (Madigan *et al*, 2003). In order to maintain the integrity of environmental samples intended for molecular analysis, they are stored at approximately –20 °C. This low temperature should restrict the activity of microorganisms. In fact, many species are not able to survive at these temperatures. However, their molecular structure is likely to remain intact for future analysis.

6.3.2.2 Extraction of cell materials

The first analytical process in molecular analysis involves the extraction of materials from within cells. Numerous methods have been developed in order to extract cell materials from different environmental samples. Extraction procedures vary according to the composition of the environmental source

materials. This is due partly to the interactions that can occur between the cell materials (e.g. DNA) and other sample components such as clay fractions. Negatively charged DNA segments may bind to positively charged clay surfaces, and so inhibit the extraction process (Yong and Mulligan, 2004). Other sources of error, which arise when removing genomic materials, include the extraction of non-target substances such as humic acids (Tsai and Rochelle, 2001). Humic acids can inhibit certain molecular techniques, including the polymerase chain reaction (PCR).

Extraction methods aim to remove genomic materials through a series of physical and chemical techniques. Tsai and Rochelle (2001) describe methods for genomic extraction. In order to extract materials from a cell, the cell wall must first be broken down. This is referred to as cell lysis, and is typically initiated through a bead beating process. Once the genomic materials have been released from the cell, they are collected through filtration with a binding matrix.

6.3.2.3 Amplification of DNA targets

Culture-independent studies of microbial diversity were introduced by Olsen *et al* (1986) and Pace *et al* (1986). In order to investigate and compare the genetic signatures of large numbers of microorganisms, a DNA sequence in common with all prokaryotes is targeted. As was discussed in Section 6.2.3, rRNA is a nucleic acid that plays a direct role in the creation of proteins within all bacteria. The genes, which encode the rRNA, are contained within DNA. These genes are collectively known as rDNA. The focus of most molecular studies for profiling microbial communities is directed towards the 16S rDNA gene. Studies have shown that evolutionary changes have left some regions of the 16S rDNA gene unaltered (conserved regions), whereas other regions vary from species to species (unconserved regions) (Macnaughton and Stephen, 2001). It is this combination of conserved and unconserved regions, which make the 16S rDNA gene favourable for comparative microbial community profiling analysis.

The typical method used for selecting and replicating specific genetic sequences makes use of a polymerase chain reaction (PCR). The process of repeatedly replicating sequences to form greater quantities is referred to as amplification. The PCR technique amplifies target sequences through a similar process to replication, which occurs naturally within a cell (described in Section 6.2.3). DNA polymerase and selected primers are used in conjunction with other reagents in order to synthesise DNA fragments. The DNA polymerase, which is used for a PCR, is obtained from *Thermus aquaticus*. The resulting 'taq' polymerase is thermostable, and therefore able to withstand the high temperatures required for denaturing DNA strands during a PCR.

There are three main steps in a PCR:

1. Thermal denaturing of the target DNA,
2. Annealing of the primers to the single stranded DNA, and
3. Elongation (DNA synthesis) by the DNA polymerase.

The selection and design of primers is a crucial factor for the performance of PCR amplification. Two primers are required for PCR amplification. These are usually called the forward and reverse primers. Primer selection will depend upon a number of different factors, including the length of the target sequence and the requirements for further analysis of the amplified products. A large range of primers has been developed for the analysis of 16S rDNA, dehalogenase genes and others. Primer selection for this research is discussed later in this chapter.

6.3.2.4 DNA fingerprinting and sequence analysis

The separation of amplified PCR products can allow for a comparison to be made between different sequences. Comparing DNA sequences in this manner has been referred to as "fingerprinting" (Macnaughton and Stephen, 2001). With regards to the analysis of 16S rDNA sequences from environmental samples, the fingerprint can be used as a general gauge of microbial diversity.

There are three main methods for separating PCR products, as described by Macnaughton and Stephen (2001). These include:

1. Denaturing gradient gel electrophoresis (DGGE),
2. Temporal thermal gel electrophoresis (TTGE) and
3. Thermal gradient gel electrophoresis (TGGE).

The general concept behind each of the three techniques is the same. PCR products are run through a polyacrylamide gel and denatured by a chemical or thermal mechanism. Most PCR products will denature at different locations or times in the gel based upon nucleotide content (i.e. proportions of adenine, guanine, cytosine and thymine). At the point of denaturing, the products will become immobile in the gel. The research reported on in this thesis focused solely on the DGGE separation of DNA fragments.

Following the separation process, the individual bands of denatured product can be excised from the gel. The content of the individual bands may or may not represent the gene sequence of an individual species. However, sequences appearing within the same band are likely to have high genetic similarities (Jackson *et al*, 2000).

Excised bands can be processed and re-amplified using the PCR technique. The products of this amplification can then be sequenced using high performance liquid chromatography (HPLC). The results of sequencing are compared to other known sequences using gene sequence databases (e.g. Genbank) in order to describe the likely taxonomy of species.

6.4 Monitoring changes in community profiles

6.4.1 Personal protection and cross contamination

The molecular studies described in this chapter were conducted in a microbiological laboratory at the School of Biosciences, Cardiff University. The laboratory was qualified for the investigation of pathogenic microorganisms. Personal protective equipment was required, including sterile gloves and a long, high-fastened laboratory coat. Frequent surface and hand disinfection was

carried out. Laboratory surfaces were cleaned using a 30% ethanol to sterile distilled H_2O (dH_2O) mixture.

In addition to personal protection, these measures were also designed to minimise the biological contamination of test samples. Gloves were frequently changed and in certain circumstances fume cupboards were used, to reduce the potential for cross-contamination between samples. Sterilisation methods for utensils and containers will be discussed, where appropriate, within the following Sections.

6.4.2 Microcosm sampling

Slurry samples were acquired from all microcosm bottles so that the changes in microbial community profile could be monitored throughout the course of the treatability studies. Below is a reminder of the microcosm types, which were introduced in Chapter 5.

Microcosm M1 – *ORC® enhanced*
Microcosm M2 – *Molasses enhanced*
Microcosm M3 – *HRC® enhanced*
Microcosm M4 – *Standard control (no enhancement)*
Microcosm M5 – *Abiotic control*

Sub-labels I, II, III and IV represent the sampling periods for each of the microcosm types at days 7, 15, 34 and 79 respectively. The sub-labels B (for baseline) and V represent day 0 for microcosms M1-M4 and M5 respectively. For a better illustration of the sampling periods refer to Table 5.4, Chapter 5.

Sampling techniques were designed to minimise errors through careful handling and appropriate storage. A stainless steel spatula was used to homogenise the slurry inside microcosm bottles before extracting the materials. The spatula was heat sterilised using a bunsen burner prior to and between sampling periods. Slurry samples were transferred to 1.5 mL Epindorf tubes, and stored at –20 °C prior to analysis. The Epindorf tubes were autoclaved (121 °C for 15 minutes) and ultra violet (UV) treated (1200 μJcm^{-2} for 5 minutes) before use.

6.4.3 Gel electrophoresis checks

One frequently used technique in molecular microbiology involves the use of gel electrophoresis in order to determine the effectiveness of methods, such as DNA extraction and PCR amplification. The principle theory behind the technique is based upon the negative charge of DNA (Madigan *et al*, 2003). When placed in an electric current, the negatively charged DNA will move towards a positive node. The speed with which DNA will move within a gel will be dependent upon the size of the fragment. Smaller fragments travel more quickly.

Agarose was combined with a buffer to produce the gel, into which the DNA fragments were injected. TBE (tris, boric acid and EDTA) was used as a buffer for the analyses, and is a solution of 108g tris base, 55g boric acid and 7.4g EDTA all topped up to 10L with dH_2O. Ethidium bromide was also added to the gel as a stain, which is used to view the DNA fragments under UV light.

Gels were prepared within clean plastic trays and allowed to set. Combs of various sizes were used to insert injection wells at one end of the gel. Once the gels were set, they were lowered into the gel electrophoresis tanks, which contained TBE buffer. Positive and negative nodes were located at opposing ends of the tanks.

The DNA was injected at the end of the gel nearest to the negative node. A loading dye was injected with the DNA so that the progression of DNA within the gels could be observed visually. A ladder (or marker) was used as a gauge for each gel electrophoresis run in order to make a comparative analysis of DNA fragment sizes. Ladders consist of DNA fragments of various known sizes. The two most used ladders for this research were the 100 base pair ladder (Promega) and the Hyperladder I (Bioline). The base pair band configurations for these ladders are shown in Figures 6.3 and 6.4 respectively. Positive and negative controls were also used to further confirm the accuracy of the procedure.

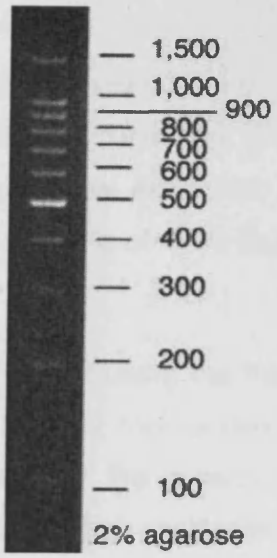


Figure 6.3 100 base pair ladder (Promega)

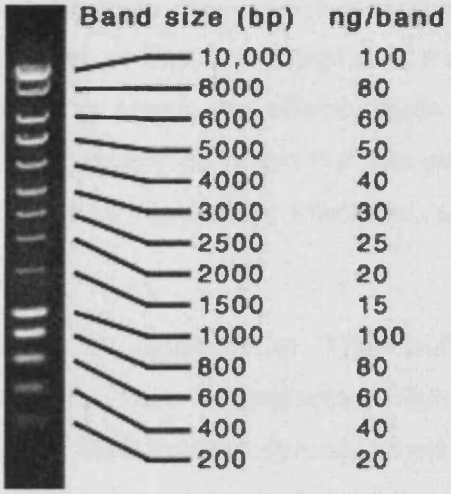


Figure 6.4 Hyperladder I ladder (Bioline)

For all electrophoresis runs, the electrodes were connected to the tanks and were set at 80V. The runs lasted for approximately 30-45 minutes, or until the loading dye neared the end of the tray. The finished gels were viewed under UV light using the software Gene Genius^{TM} Bioimaging (Synoptics).

Gel electrophoresis was performed on the products of genomic extractions and PCR runs. The specific details on the set-up of the gels vary, and will be provided in the relevant Sections below.

6.4.4 Total genomic extraction

As discussed in Section 6.3.2.2, the first step in most molecular analyses is the extraction of genomic materials. Although the focus of the research was directed towards DNA analysis, the extraction method removed all genomic materials from the cell. The targeting of DNA fragments will be discussed in the following Section.

Genomic extraction was carried out using the FastDNA® Spin Kit (for soil) that was supplied by Qbiogene. The kit makes use of a bead-beating method for cell lysis, and a binding matrix for the collection of genomic materials. The technique was extensively trialled by researchers at the School of Bioscience (Cardiff University), and has been shown to effectively extract genomic materials from a range of different soil types. Extractions were performed on approximately 0.5g of sample from each of the microcosms. It is possible that insufficient DNA was removed, or that DNA fragments may have been damaged during extraction. In order to check the effectiveness of the extractions, gel electrophoresis was used. It should be noted that gel electrophoresis checks of extraction results are limited by resolution; therefore, small quantities of cells may not be identified.

Gels were set-up with 0.7% agarose in TBE buffer, then spiked with 0.5μgmL^{-1} ethidium bromide. 10μL of extracted DNA and 1μL of λ/Hind III ladder were added with 5μL BBS loading dye (supplied with the extraction kit). Topsoil was taken from a flower garden, and used as a positive control. This soil was chosen due to its good potential for high microbial activity, and therefore high cell counts.

The ladder (λ/Hind III) proved to give irregular results in all gel electrophoresis checks on extractions. This is of little consequence to the overall results, as this stage of the analysis is simply a check to determine whether or not the extracted material is present. If the bands in the gel image were to appear lower in the gel, then it is likely that small DNA fragments were extracted. For this situation, the ladder would be of greater importance. The ladder is represented by an 'L' shown above its lane in the gel results.

Figure 6.5 Extraction results for the baseline microcosm

The gel image shown in Figure 6.5 gives the extraction results for the baseline microcosm (B). The same extraction technique was used for the baseline microcosm samples and the positive control (+). The positive control shows an apparent high magnitude of genomic materials when compared to baseline extracts. This indicates that more genomic material was likely present with the positive control sample when compared to that of the baseline microcosm (B). This was expected since the positive control was obtained from a sample of aerobic topsoil, which is likely to contain higher numbers of microorganisms. The concept of higher microbial numbers in the upper horizon of a soil system was introduced in Chapter 2. A faint signature of product is shown in lane B; thus indicating that some genomic materials were successfully extracted.

Figure 6.6 Extraction results for microcosms M1-M4

The extraction results for all sub-samples of the M1-M4 microcosms are given in the gel shown in Figure 6.6. Like the baseline microcosm results (Figure 6.5), the positive control (+) in the above Figure appears much brighter than the microcosm extractions. All microcosm extractions appear to have yielded a slight product except for that shown in lane 12 (microcosm sample M3(IV)). As discussed earlier in this Section, this is not necessarily indicative of a lack of extracted product; but rather would likely suggest that lower quantity of microorganisms was present within the sample.

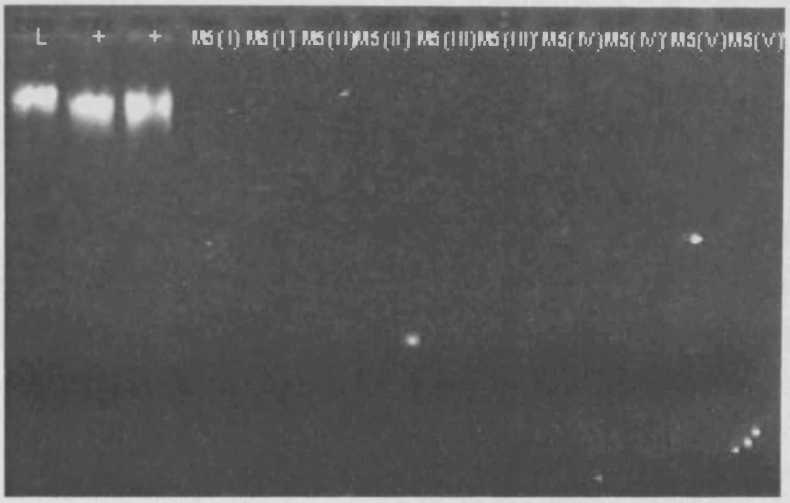


Figure 6.7 Extraction results for the abiotic microcosms

Two separate extractions were carried out on each of the abiotic microcosm sub-samples (M5 I, II, III, IV & V). Figure 6.7 gives the extraction results for the M5 samples. Two positive controls were used (lanes 2-3). The two lanes representing extracted of positive control samples gave positive results, suggesting that the extraction process was successful. However, lanes representing extracted abiotic microcosm samples appear negative. This indicates that either no cells were present, or that they were likely to have been present in lesser quantities than that shown for the other microcosms.

At this stage in the research, the abiotic microcosm extractions were progressed to PCR amplification to attempt to further confirm the absence of signs of microbial growth. Since the molecular methods used for this research were not quantitative, signs of microbial growth could not be assessed directly. Growth was therefore determined according to increases or decreases in band

magnitudes. This approach to growth assessment was used on PCR and DGGE gel results, and is discussed in the following Sections.

6.4.5 Polymerase chain reaction (PCR)

PCR amplification was used to target 16S rDNA genes from sample extractions. The term DNA template is used to identify the targeted sequence. The following sub-sections describe the methodology of the amplifications and present the gel electrophoresis results of PCR products.

6.4.5.1 Trial PCR amplification

PCR analysis was conducted in two stages. The first stage was designed to ascertain whether or not the genes could be effectively amplified. Primers were chosen for this stage, which target a large portion of the total 16S rDNA gene. The selected primers were 27F (forward primer) and 1492R (reverse primer). Primers were obtained from MWG-Biotech and diluted to 20pmolμL^{-1} prior to use.

A number of reagents were added to the reaction mixture for each sample. These included 34.75µL dH_2O, 5µL reaction buffer (X10; Bioline), 3µL magnesium chloride ($MgCl_2$), 1µL dNTPs (25mM; Bioline), 2µL forward primer, 2µL reverse primer, 1µL BSA, 0.25µL taq polymerase and 1µL DNA template. The total reaction mixture amounted to 50µL.

The reaction mixture was prepared with special care so that biological contamination was excluded. All work was performed under a fume cupboard, and all plasticware was sterilised by autoclaving and UV treatment prior to use.

A DNA Engine Dyad Thermal Cycler (MJ Research Inc) was used to perform the reaction in accordance with the following conditions:

5 minutes @ 94 °C

1 minute @ 94 °C
1 minute @ 50 °C
90 seconds @ 72 °C
} 30 cycles

10 minutes @ 72 °C

Gel electrophoresis was used to interpret the results of the PCR. Gels were set-up with 1.2% agarose in TBE buffer, then spiked with 0.5µgmL^{-1} ethidium bromide. 5µL of extracted DNA and 5µL of 100bp ladder (Promega) were added with 2µL blue/orange loading dye. DNA extracted from a pure culture of *Escherichia coli* (E.coli) was used as a positive control. A negative control was also used to determine whether any biological contamination was introduced during the set-up of the reaction mixture. In the negative control, dH_2O replaced the DNA template.

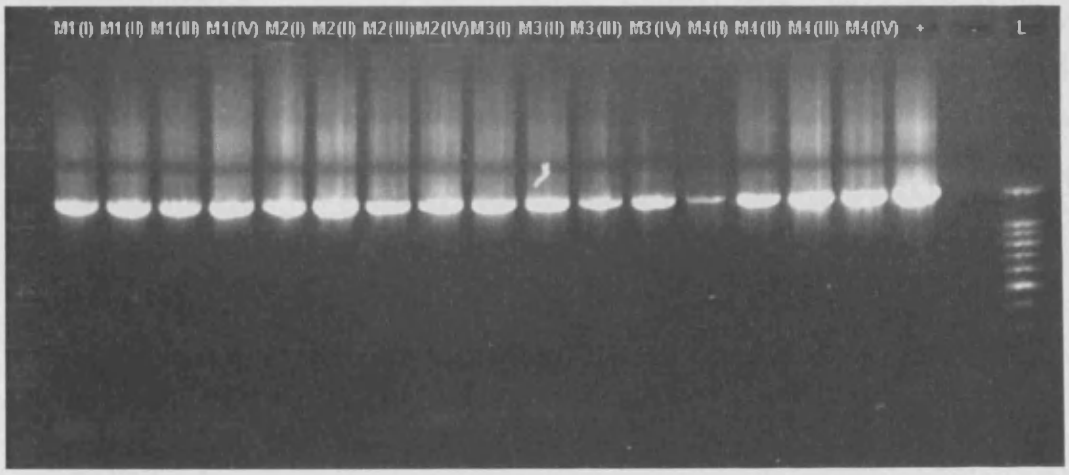


Figure 6.8 PCR products for microcosms M1-M4 using a 1465 bp primer

The gel image shown in Figure 6.8 gives the PCR results for microcosms M1-M4. All microcosm extractions show product, which appears just below the 1500 base pair (bp) band on the 100bp ladder (refer to Figure 6.3). This is consistent with the 1465bp target fragment. Both positive and negative controls

gave their respective expected results (i.e. positive = strong product, negative = no product apparent). These results show that the amplification was successful for all (M1-M4) targets.

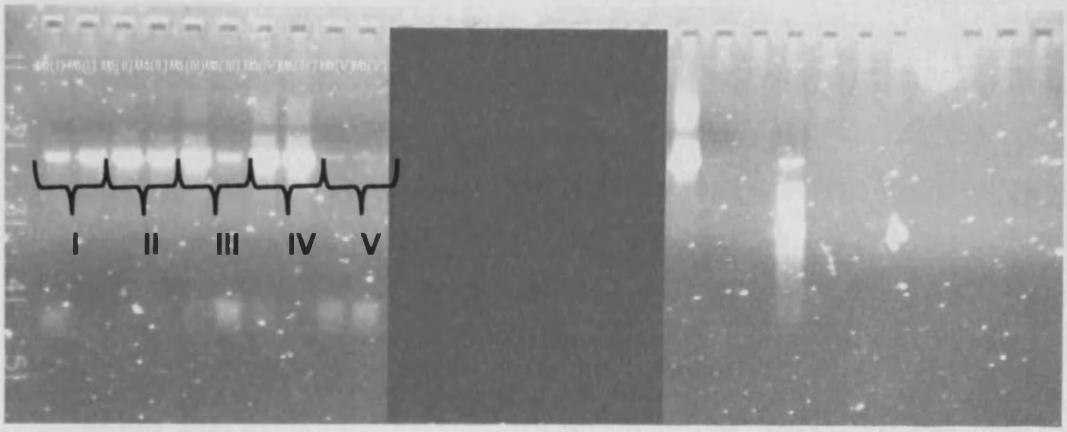


Figure 6.9 PCR product for microcosms M5 using a 1465 bp primer

Figure 6.9 shows a gel image of the PCR results for the abiotic microcosm (M5) extracts. A grey box has been placed in the centre of the image to cover the results of work not related to this research. All of the abiotic extractions (shown on the left-hand side of the grey box) showed PCR product. Like the M1-M4 results, the M5 products appear in the appropriate size range, and the positive and negative controls are in accordance with expectations. The positive, negative and ladder are all shown to the right of the grey box.

The gel shown in Figure 6.9 appears cloudy. This is likely to be due to either the addition of too much agarose, or insufficient dissolution of agarose during the gel set-up. This cloudiness should not affect the quality of the results.

The most notable observation to be made from the M5 PCR gel is the apparent increase in magnitude between M5(V) (day 0) and M5(IV) (day 79) products. This is likely to be indicative of an increase in microbial activity over this time period. This is significant, as these microcosms were designed to act as abiotic controls for the treatability experiments (refer to Chapter 5). Signs of microbial activity within the abiotic microcosms will need to be considered in relation to the variations in chemistry. This interpretation is presented later in this Chapter.

Due to the apparent activity in M5 microcosms, further molecular analysis was required in order to describe any changes in community profile.

6.4.5.2 Amplification for community fingerprinting

The second PCR stage was designed to obtain the appropriate 16S rDNA fragments for use in community fingerprinting. The fingerprinting analyses will be covered in Section 6.4.6. The target fragment for these analyses was first described by Muyzer *et al* (1993). The selected primers (357F and 518R) target a small portion of the 16S rDNA gene. The forward primer was designed with a GC-clamp. GC-clamps consist of a series of guanine and cytosine bases, which are attached to the primer sequence. The purpose of using a GC-clamp will be discussed in Section 6.4.6. Primers were obtained from MWG-Biotech, and diluted to 20pmolμL^{-1} prior to use.

Reagents were added to the reaction mixture in the same quantities as described under Section 6.4.5.1 with regards to the trial PCR amplifications. Once again the total reaction mixture amounted to 50µL. The same care was taken to minimise biological contamination during set-up.

A DNA Engine Dyad Thermal Cycler (MJ Research Inc) was used to perform the reaction in accordance with the following conditions:

5 minutes @ 95 °C

30 seconds @ 94 °C
30 seconds @ 55 °C
1 minute @ 72 °C
— 9 cycles

30 seconds @ 92 °C
30 seconds @ 52 °C
1 minute @ 72 °C
— 25 cycles

10 minutes @ 72 °C

Gel electrophoresis was used to interpret the results of the PCR. Refer to Section 6.4.5.1 for a description of the gel set-up. All materials and quantities

used during this stage were equal to that of the previous, including the ladder, loading dye and positive and negative controls.

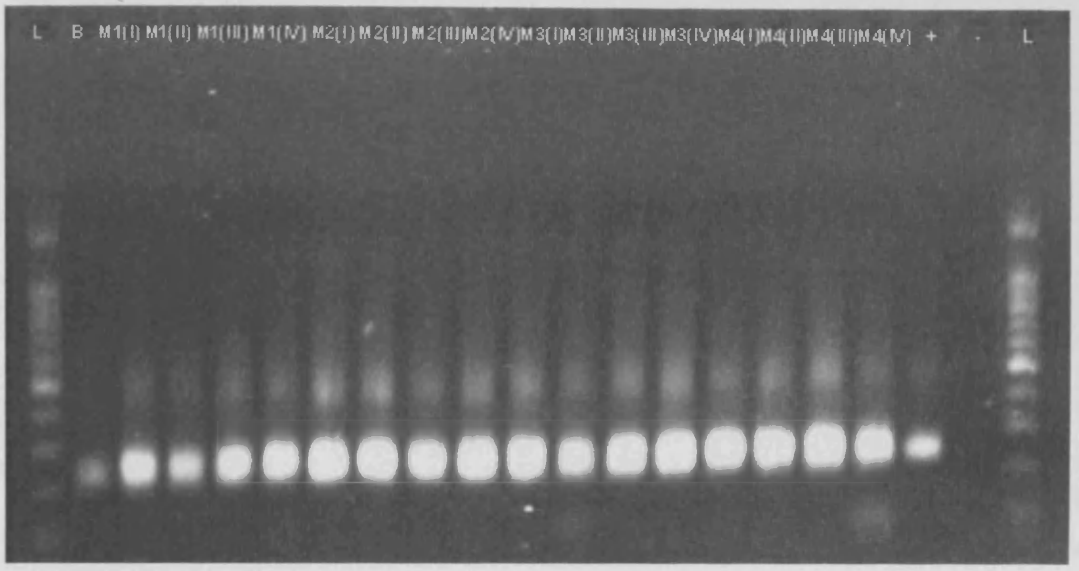


Figure 6.10 PCR products for microcosms B and M1-M4 using a 161 bp primer

The gel image shown in Figure 6.10 gives the PCR results for microcosms B and M1-M4. All microcosm extractions show product, which appears adjacent to the 200bp band on the 100bp ladder. This is consistent with the 161bp target fragment. Both positive and negative controls gave expected results.

These results show that the amplification was successful for the B and M1-M4 targets. There is a clear difference between the magnitude (brightness) of sample B compared to that of all other samples. Sample B is located next to the ladder on the left side of the gel image, and appears less bright than the M1-M4 bands. The increased brightness in the M1-M4 bands could be indicative of increased microbial activity following day 0 of the microcosm studies.

Figure 6.11 PCR products for microcosms M5 using a 161 bp primer

Figure 6.11 shows a gel image of the PCR results for the abiotic microcosm (M5) extracts. All of the abiotic extractions showed PCR product within the expected size range (i.e. 161 bp). The positive and negative controls are in accordance with expected findings.

The M5(V) (day 0) bands appear less bright than the M5(IV) (day 79) products. This is consistent with the previous results shown in Figure 6.9, and may be indicative of an increase in microbial activity during this period of the study.

All PCR products were stored at –20 °C while awaiting further analyses. The 161bp target fragments were used to construct community profiles (fingerprinting). Fragment separations are discussed in the following Section.

6.4.6 Denaturing gradient gel electrophoresis (DGGE)

DGGE was used for the separation of amplified PCR products. The technique was first used for the community profiling of environmental samples by Muyzer *et al* (1993).

DGGE uses a chemical mechanism to denature, and thus separate DNA fragments. Denaturing occurs within a polyacrylamide gel along an increasing gradient of formamide and urea. DNA fragments, which are more difficult to denature, will migrate further in the gel where formamide and urea concentrations are higher. Once denatured, the mobility of fragments with the

gel is dramatically decreased. Sheffield *et al* (1989) showed that the resolution of denatured DNA fragments could be enhanced through the addition of a GC-clamp to one of the primers used for amplification.

Three gel solutions were produced for the construction of gels: 0%, 30% and 60%. The diagram shown in Figure 6.12 gives the locations of the solutions within a gel. The 0% solution contained acrylamide, Tris-Acetate-EDTA buffer (TAE) and H_2O, but no formamide or urea. Therefore, no denaturing would occur within the areas of gel, which were produced from 0% solution. It was primarily used for the construction of injection wells. The 30% and 60% solutions contained acrylamide, buffer, H_2O, formamide and urea. The percentages stated for each solution represent their respective formamide and urea content. The 30% and 60% solutions were injected into the gel at a gradient, with 60% towards the bottom and 30% towards the top of the gel. Gels were polymerised through the addition of temed and ammonium persulphate (APS) to the gel solutions prior to injection.

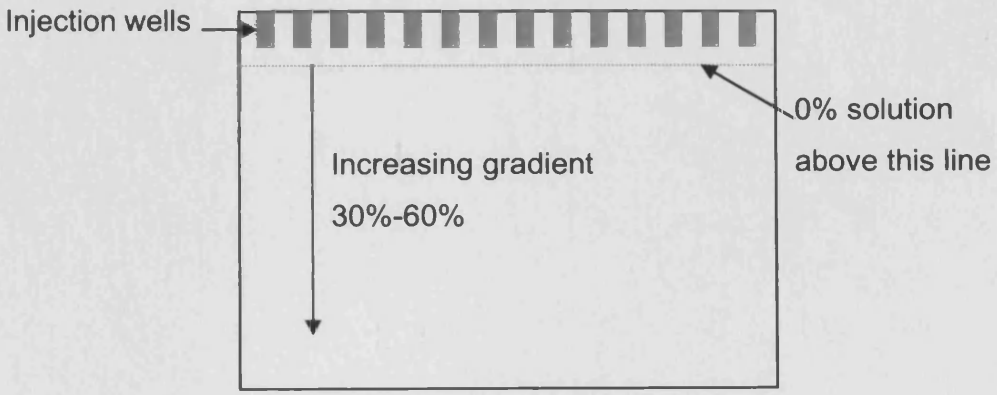


Figure 6.12 The locations of formamide and urea solutions within a polyacrylamide gel

10µL of PCR product (161bp results from Section 6.4.5.2) and 5µL of ladder were added into injection wells with 2µL blue/orange loading dye. The ladder consisted of PCR products from E.Coli and three other pure cultures. Multiple ladders were used in order to account for any gel inconsistencies.

Gels were introduced into a DGGE tank containing TAE buffer. The tank consisted of a heating element and a pump for circulation. An electric current was then sent through the gel to induce the migration of DNA fragments. Initially the gel was run at 80V for ten minutes. This low voltage is used to allow for the DNA fragments to work there way into the gel as evenly as possible. After ten minutes, the system was raised to 200V and allowed to run for 290 minutes.

Once the run was complete, the gel was removed from the tank and set on a rocking plate for 30 minutes with 25µL gold stain and 250mL TAE buffer. The stain allows for a clearer gel image when viewed under UV light.

The gel shown in Figure 6.13 gives the DGGE results for microcosm types B and M1-M4. The labeled bands in the Figure represent those, which have been cut from the gel for further analysis (discussed further in Section 6.4.7).

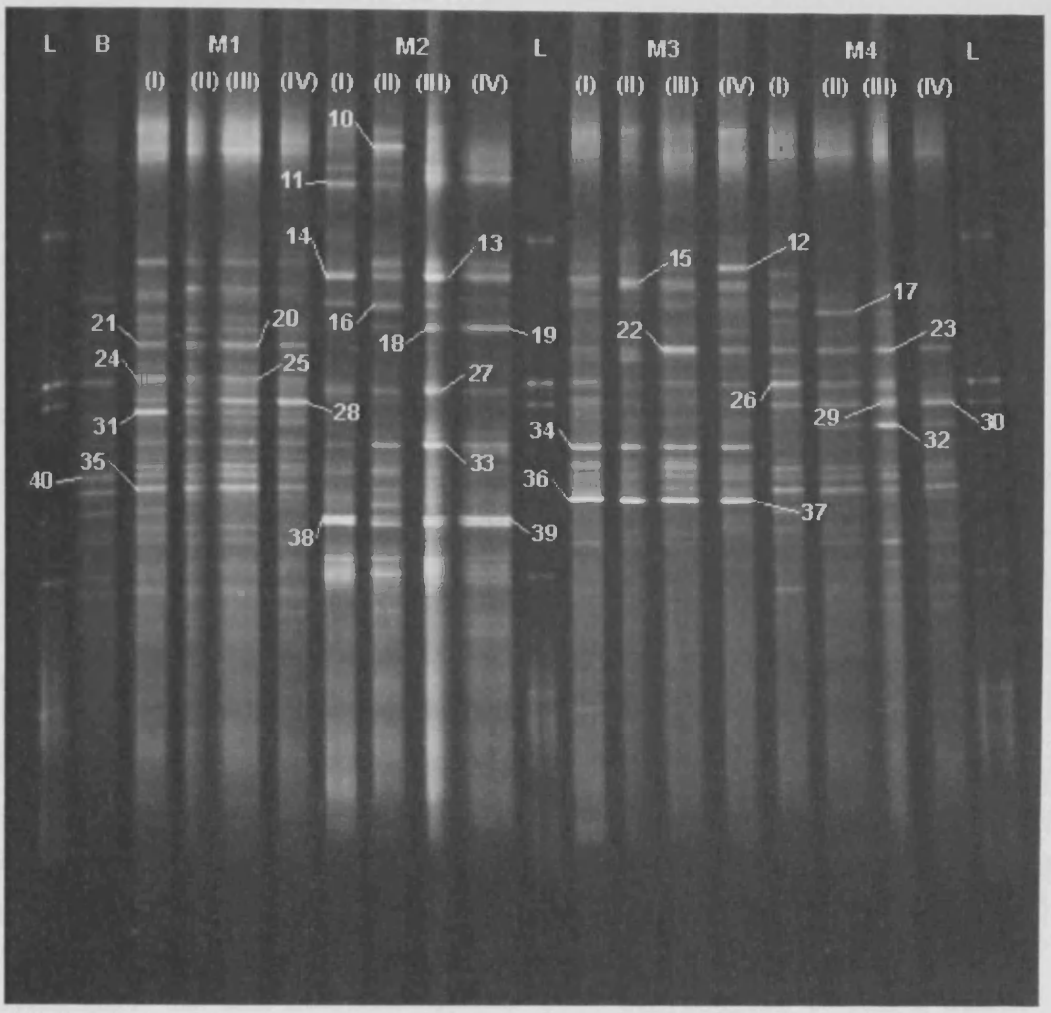


Figure 6.13 DGGE results for microcosms B and M1-M4

The large numbers of bands shown in Figure 6.13 indicate a high diversity of microorganisms within each of the slurry samples. Amplified product from B appears to be more faint than the results shown in other lanes. This is consistent with the differences in magnitude observed from PCR gels (Figure 6.10).

From these results, it appears that each microcosm type had its own diverse spread of genetic sequences. This would indicate that the variations in microcosm conditions had a direct effect on the diversity of dominant microorganisms. A horizontal band comparison would suggest that there is no single dominant band consistent for all microcosm types. However, there are a couple of microcosm types that do appear to have bands in common. An example of this is shown by the location of the bands labeled 33 and 34. Both

bands appear high in relative brightness and originate from different microcosms (M2(III) and M3(I)).

A general comparison of the M1 and M4 results suggests similar microbial diversities. A number of dominant bands appear in both microcosm types. M1 microcosms were enhanced with oxygen to facilitate the aerobic degradation of organic compounds. The microbial communities within M1 were therefore expected to be dominated by organisms, which obtain energy through aerobic respiration. However, the results of chemical analyses (discussed in Chapter 6) suggested that products of anaerobic degradation processes were present within the M1 microcosms. Due to the band similarities, and the expected anaerobic conditions within the standard microcosms (M4), it is likely that similar conditions persisted within the M1 and M4 microcosm slurries.

Another trend that requires attention is the change in a particular band's brightness between different microcosm sampling periods. A change in brightness could be indicative of microbial growth. Changes in brightness were apparent for several DNA fragments. The bands labeled 12, 19, 28 and 30 are all clearly brighter than those which precede them.

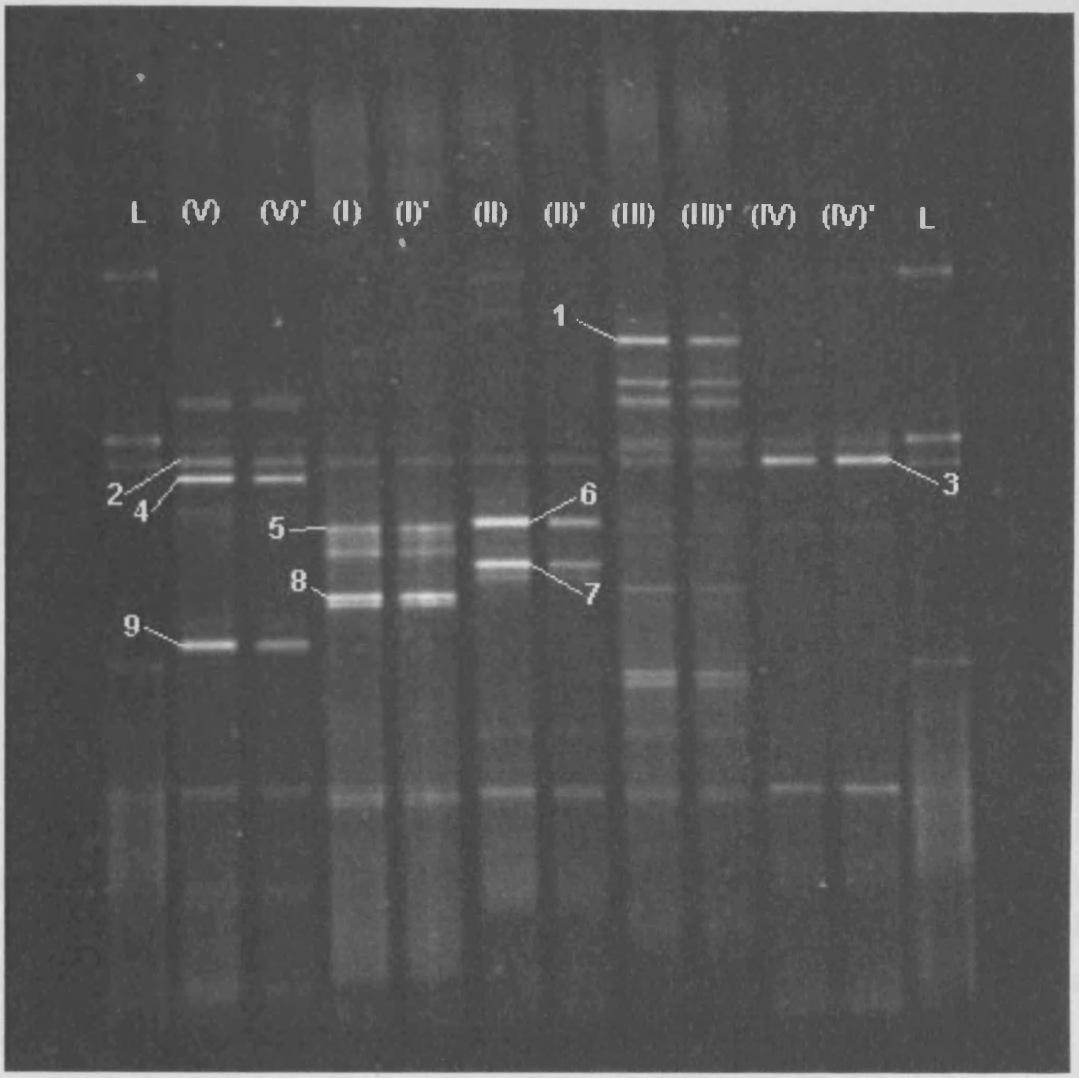


Figure 6.14 DGGE results for the abiotic microcosms M5

The gel shown in Figure 6.14 gives the DGGE results for microcosm type M5 (abiotic control). These results are provided in duplicate in order to check the consistency of the analyses. Each of the duplicate samples appears nearly identical, suggesting that consistency was achieved.

Once again, the labeled bands in the Figure represent those, which have been cut from the gel for further analysis, and are discussed further in Section 6.4.7. The presence of bands within the DGGE gel image further supports the earlier finding that biological growth within the abiotic controls has occurred. However, there is no distinguishable pattern of growth between the fragments from the different sampling periods. This is in contrast to the results of microcosms M1-

M4 discussed above. It is likely that the lack of pattern is the result of random DNA amplifications brought about by the low availability of DNA template. This is assumed based on the earlier results of the genomic extractions, where no extracted materials were observed (see Figure 6.7). With little available template, and without altering the annealling temperatures, primers may bind randomly to DNA (Sambrook and Russell, 2001).

Further analysis of DGGE bands (labeled in Figures 6.13 and 6.14) was carried out in order to characterise individual species. The methods and results of these further studies are presented in the following Section.

6.4.7 Sequencing 16S rDNA fragments

This Section describes a series of analytical procedures and results that help to describe the sequences of the various 16S rDNA fragments.

Following DGGE analysis, the polyacrylamide gel was transferred to a UV viewer in order to excise selected bands. The choice of which bands to cut out was based upon their locations within the gel and their magnitudes (brightness). DNA fragments degrade when exposed to UV light. This degradation was directly observed while the gel was placed on the viewer, and meant that the bands required rapid excision.

The excised bands were combined with 100µL dH_2O and left to sit for 10 minutes before removing the supernatant. The bands were then left to air dry, after which they were mashed with a pipette tip. Approximately 15µL dH_2O was added and the mix was placed into a freezer at –20 °C. These steps were carried out in order to release the DNA fragments from the gel bands.

PCR was then used to re-amplify the DNA fragments. The reagents for the reaction included 40.75µL dH_2O, 5µL reaction buffer (X10; Bioline), 1.5µL magnesium chloride ($MgCl_2$), 0.5µL dNTPs (25mM; Bioline), 0.5µL forward primer, 0.5µL reverse primer, 0.25µL taq polymerase and 1µL DNA template. The total reaction mixture amounted to 50µL.

Gel electrophoresis was used to interpret the results of the PCR. Refer to Section 6.4.5.1 for a description of the gel set-up. All materials and quantities

used during this stage were the same, apart from the ladder, which for these analyses was Hyperladder 1 (Bioline). The gel shown in Figure 6.15 shows the results of this PCR amplification. All results fall with the expected range (i.e. approximately 200 bp).

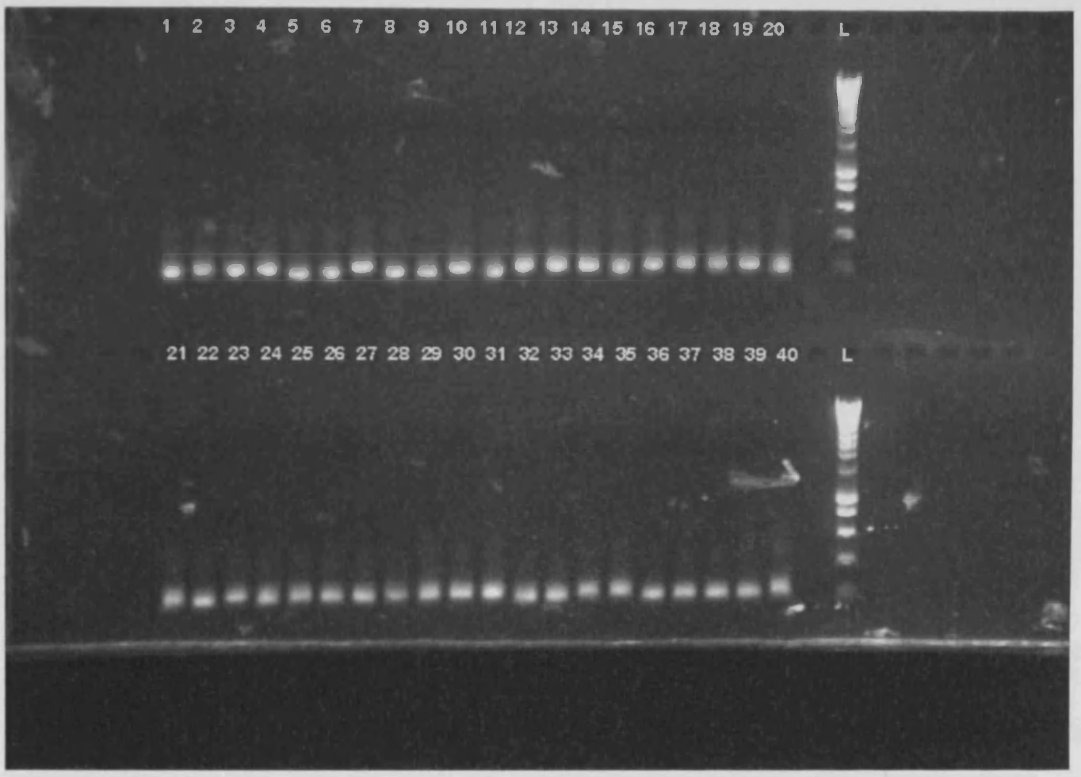


Figure 6.15 PCR products for excised DGGE bands

The PCR products were then centrifuged in Microcon YM-50 filters to remove any larger DNA fragments that may have been present. Another gel electrophoresis was then carried out using the exact same set-up as the gel shown in Figure 6.15. The gel, which followed filtration, is shown in Figure 6.16. The bands, shown in the gel image, were quantified using the GeneSnap™ programme (Synoptics).

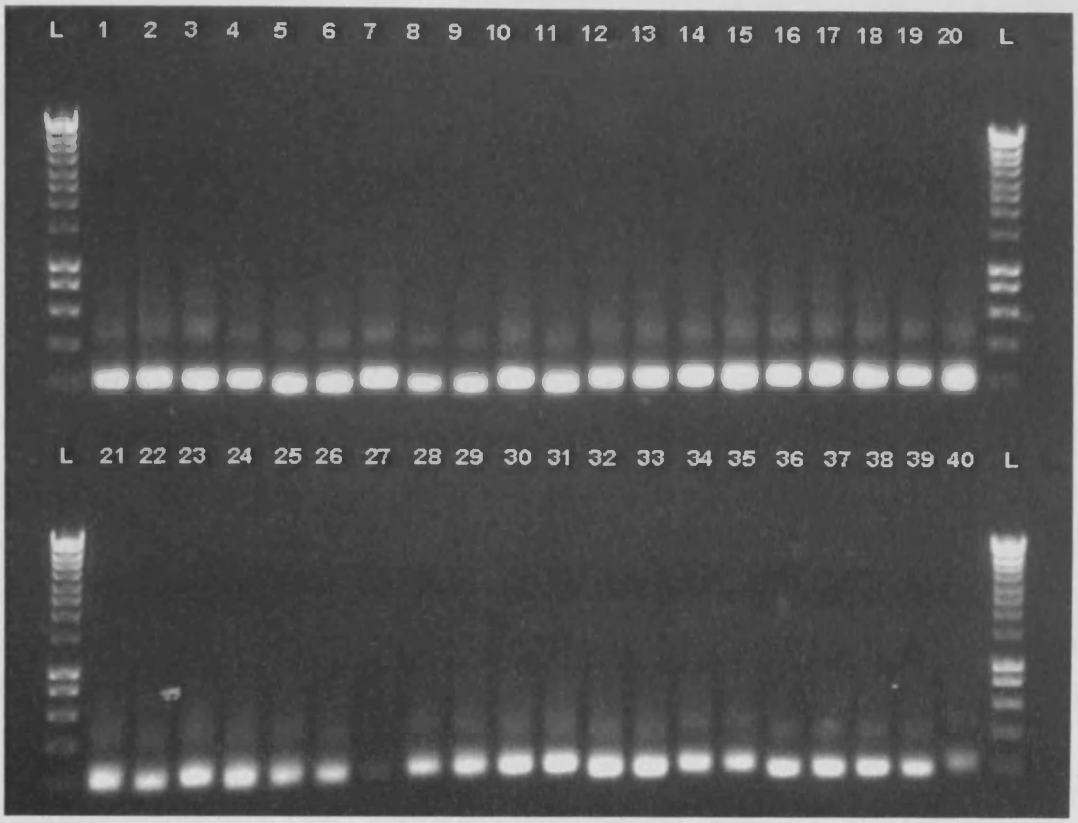


Figure 6.16 PCR products for filtered DGGE bands used for quantification

In the Figure above, band 27 appears to have very little product. If the sequencing results turned out to be inconclusive for this band, then re-amplification and filtering would be recommended.

The filtered PCR products were sequenced directly with the 518R primer using an ABI PRISM 3100-Genetic Analyzer (Applied Bio-systems). The obtained partial bacterial 16S rDNA sequences were compared to other sequence data for identification as discussed in the following Section).

6.4.8 Comparison of 16S rDNA fragments

Sequence data obtained for the excised DGGE band were run through the Basic Local Alignment Search Tool for Nucleotides (BLASTN) search (http://www.ncbi.nlm.nih.gov/blast/). The program compares nucleotide sequences to sequence databases and calculates the statistical significance of each match. The searched databases include the nucleotide sequence database GenBank. The databases contain nucleotide sequences from a

variety of sources. Many sequences originate from pure laboratory cultures of bacteria from environmental samples. By comparing sequencing results with microorganisms that have been classified using traditional techniques, more accurate species comparisons may be made.

The results of the BLASTN search are presented in Table 6.1. Two sequence matches are provided for each of the band sequences, and are identified by their sequence similarities (statistical significance) and isolation environments. The listed results represent bacterial nucleotide sequences that most closely matched those found within the microcosms. If no reference is provided for a particular isolation environment, then the date of direct submission to the database is given. The alignment length and similarity of sequence matches will affect the accuracy of the results. Some of the sequencing results (shown in Table 6.1) are inconclusive due to these inaccuracies and/or the irrelevance of matches.

Madden (2002) suggests that finding similarities between sequences using BLASTN can allow a user to infer the function of newly sequenced genes, predict new members of gene families, and explore evolutionary relationships. For the purposes of this thesis the search results were primarily used to explore relationships between microorganisms within the microcosms and previously identified microorganisms from the database. Based on these relationships conclusions are then drawn regarding the relevance of the match species to the degradation of organic compounds.

As discussed in Section 6.2.2, a basic species identification can be defined for organisms sharing greater than 97% similarities between their 16S rRNA gene sequences (Stackebrandt and Goebel, 1994). The following Section provides a discussion on the results of the sequence comparisons, with a particular focus on the relevance of these results to the degradation of the focus compounds for this thesis.

Table 6.1 BLASTN search results for excised DGGE bands

Band	Sample	Top two results of search	% Match	Alignment length (bp)	Phylogenetic affiliation	Source/ Isolation environment
1	M5(II)	*Pseudomonas putida MBR2*	99	143	Gammaproteobacteria	Rhizosphere of field crop Manua, India (2003)
		Pseudomonas putida KT2440	99	143	Gammaproteobacteria	Amplified from pure culture (2002)
2	M5(V)	*Escherichia coli HPC119*	92	148	Gammaproteobacteria	Soil contaminated with polyaromatic hydrocarbons (2005)
		Escherichia coli MBAE104	91	147	Gammaproteobacteria	Deep sea sample (2003)
3	M5(I)	*Escherichia coli MBAE104*	97	147	Gammaproteobacteria	Deep sea sample (2003)
		Escherichia coli HPC119	97	148	Gammaproteobacteria	Soil contaminated with polyaromatic hydrocarbons (2005)
4	M5(V)	*Hydrogenophaga sp MW3*	99	121	Betaproteobacteria	Nuclear power plant cooling water system (2004)
		Hydrogenophaga sp J-11	99	121	Betaproteobacteria	Natural mineral water after bottling (Loy *et al*, 2005)
5	M5(IV)	*Pseudomonas sp HV3*	97	95	Proteobacteria	Amplified from pure culture (Kilpi *et al*, 1997)
		Sphingomonas sp B26	97	94	Alphaproteobacteria	Elm tree tissues (Mocali *et al*, 2003)
6	M5(III)	*Unidentified proteobacterium*	100	80	Proteobacteria	Chlorophenol-contaminated boreal groundwater (Mannisto *et al*, 1999)
		Stenotrophomonas maltophilia strain iMTI2	98	85	Gammaproteobacteria	Amplified from pure culture (2005)

Table 6.1 BLASTN search results for excised DGGE bands (continued)

7	M5(III)	*Unidentified bacterium*	98	158	Unknown	Microaerophilic wastewater biofilms (Okabe *et al*, 2005)
		Unidentified bacterium	97	158	Unknown	Activated sludge (2004)
8	M5(IV)	*Rhodopseudomonas palustris strain N1*	98	86	Alphaproteobacteria	Amplified from pure culture (2003)
		Bradyrhizobium sp Spain-6	98	86	Alphaproteobacteria	Nodulating shrubby legumes, Spain (2001)
9	M5(V)	*Unidentified alphaproteobacterium*	88	96	Alphaproteobacteria	Wildflower Crocus Albiflorus (2005)
		Aurantimonas sp ICS20433	88	98	Alphaproteobacteria	Inertidal sediments of Incheon North Harbor (2003)
10	M2(II)	*Bacillus racemilacticus NCIMB10274*	95	108	Firmicutes	Amplification of pure culture (2003)
		Bacillus racemilalticus	95	108	Firmicutes	Amplification of pure culture (Suzuki and Yamasato, 1994)
11	M2(I)	*Eubacterium nitrogenes strain JCM6485*	95	104	Firmicutes	Amplification from pure culture (2000)
		Unidentified bacterium	96	105	Unknown	Phenol-contaminated wastewater, anaerobic conditions (2005)
12	M3(IV)	*Pseudomonas corrugata*	98	108	Gammaproteobacteria	Nitrifying biofilm (2005)
		Pseudomonas fluorescens strain iCTES96	97	104	Gammaproteobacteria	Amplification from pure culture (2005)
13	M2(III)	*Sporolactobacillus terrae strain DSM 1169T*	98	108	Firmicutes	Amplified from pure culture (2004)
		Bacillus racemilacticus NCIMB10274	96	107	Firmicutes	Amplification from pure culture (2003)

Table 6.1 BLASTN search results for excised DGGE bands (continued)

14	M2(I)	*Sporolactobacillus terrae strain DSM 1169T*	94	142	Firmicutes	Amplified from pure culture (2004)
		Bacillus racemilacticus NCIMB10274	91	142	Firmicutes	Amplification of pure culture (2003)
15	M3(II)	Incomplete sequence				
16	M2(II)	*Sporolactobacillus terrae strain DSM 1169T*	89	155	Firmicutes	Amplified from pure culture (2004)
		Bacillus racemilacticus NCIMB10274	94	103	Firmicutes	Amplification of pure culture (2003)
17	M4(II)	*Variovorax sp.*	97	85	Betaproteobacteria	Mars Odessy orbiter and encapsulation facility (La Duc *et al*, 2003)
		Variovorax paradoxus	97	85	Betaproteobacteria	Anaerobic enrichment culture, Greenland ice core (Sheridan *et al*, 2003)
18	M2(III)	Incomplete sequence				
19	M2(IV)	*Unidentified bacterium*	98	156	Unknown	Sulphate-removal reactor, China (2005)
		Serratia fonticola	98	156	Gammaproteobacteria	Cave stream sediment, Mammoth Cave, Kenucky (2002)
20	M1(III)	*Unknown bacterium clone*	86	90	Alphaproteobacteria	Oral swab from noma lesion (2002)
		Unknown bacterium clone	88	70	Alphaproteobacteria	Bulk soil sample (2005)
21	M1(I)	Incomplete sequence				
22	M3(III)	*Unidentified bacterium clone*	98	88	Unknown	Pig manure storage pit (2004)
		Fermicutes sp strain FTB41	97	89	Firmicutes	Human subgingival plaque

Table 6.1 BLASTN search results for excised DGGE bands (continued)

23	M4(III)	*Bacteroidetes bacterium*	95	143	Bacteroidetes	Marine surface water, North Sea (Sekar *et al*, 2004)
		Bacteroidetes bacterium	97	129	Bacteroidetes	Loch Fyne fish farm particulate fraction (2004)
24	M1(I)	*Unidentified bacterium clone*	94	119	Unknown	Pacific Ocean deep-sea sediment (2005)
		Unidentified bacterium clone	93	119	Alphaproteobacteria	Pacific Ocean deep-sea sediment (2003)
25	M1(III)	*Unidentified bacterium clone*	90	93	Alphaproteobacteria	Flowering plant, Thlaspi Goesingense (2004)
		Sphingomonas sp clone	93	77	Alphaproteobacteria	Subsurface water, South Africa (2005)
26	M4(I)	*Unidentified bacterium clone*	94	78	Alphaproteobacteria	Sea water sample (Frias-Lopez *et al*, 2002)
		Unidentified bacterium clone	94	77	Alphaproteobacteria	Pacific Ocean deep-sea sediment (2003)
27	M2(III)	*Unidentified bacterium clone*	85	97	Unknown	Pig manure storage pit (2004)
		Clostridium aminovalericum strain DSM 1283	98	80	Firmicutes	Amplified from pure culture (2004)
28	M1(IV)	*Bacteroidetes bacterium clone*	96	87	Baceroidetes	MTBE-contaminated shallow aquifer (2003)
		Bacterium IAFESB3	96	87	Baceroidetes	Groundwater in limestone aquifer (2004)
29	M4(III)	*Bacteroidetes bacterium clone*	91	83	Bacteroidetes	Biofilm dominated by sulphate-reducers (2004)
		Bacteroidetes bacterium clone	91	82	Baceroidetes	MTBE-contaminated shallow aquifer (2003)

Table 6.1 BLASTN search results for excised DGGE bands (continued)

30	M4(IV)	*Bacteroidetes bacterium clone*	100	139	Baceroidetes	MTBE-contaminated shallow aquifer (2003)
		Bacterium IAFESB3	100	139	Baceroidetes	Groundwater in limestone aquifer (2004)
31	M1(I)	*Unidentified betaproteobacterium*	97	165	Betaproteobacteria	Freshwater source, UK (2005)
		Hydrogenophaga sp J-11	97	165	Betaproteobacteria	Natural mineral water after bottling (Loy *et al*, 2005)
32	M4(III)	*Acetiubrio sp clone*	97	140	Firmicutes	Salt marsh, Sapelco Island (2004)
		Acetivibrio cellulolyticus ATCC33288	97	140	Firmicutes	Amplified from pure culture (1994)
33	M2(III)	*Unidentified bacterium clone*	99	119	Unknown	Fe(III)-reducing bacteria from Savannah River site (2005)
		Clostridium sp FRJ	99	119	Firmicutes	Amplified from pure culture (2005)
34	M3(I)	*Unidentified bacterium clone*	93	146	Unknown	Acidic subsurface sediment (Petrie *et al*, 2003)
		Desulfitobacterium dichloroeliminans strain LMG P-21439	97	118	Fermicutes	Amplified from pure culture (De Wildeman *et al*, 2003)
35	M1(III)	*Unidentified bacterium clone*	98	165	Unknown	PAH-contaminated soil (2005)
		Thiobacillus sp 100B-B7	96	165	Betaproteobacteria	Activated sludge (2005)

Table 6.1 BLASTN search results for excised DGGE bands (continued)

36	M3(I)	*Unidentified bacterium clone*	99	119	Unknown	TCE-dechlorinating groundwater (Macbeth *et al*, 2004)
		Clostridium cronatovorans strain RCB	98	119	Firmicutes	Anaerobic granular sludge (2005)
37	M3(IV)	*Unidentified bacterium clone*	98	128	Unknown	TCE-dechlorinating groundwater (Macbeth *et al*, 2004)
		Clostridium cronatovorans strain RCB	98	119	Firmicutes	Anaerobic granular sludge (2005)
38	M2(I)	*Clostridium sp slone*	100	119	Firmicutes	Anerobic sludge (2005)
		Clostridium sp strain VeCb10	100	119	Firmicutes	Anoxic bulk soil of a flooded rice microcosm (1998)
39	M2(IV)	*Clostridium sp slone*	100	119	Firmicutes	Anerobic sludge (2005)
		Clostridium sp strain VeCb10	100	119	Firmicutes	Anoxic bulk soil of a flooded rice microcosm (1998)
40	B	*Thiobacillus sp 100B-B7*	92	166	Betaproteobacteria	Activated sludge (2005)
		Unidentified bacterium clone	97	117	Unknown	PAH-contaminated soil (2005)

6.4.9 Discussion of community profiling results

There are two well known database tools used by molecular microbiologists for relating organisms to the substrates that they have been shown to degrade (Urbance *et al*, 2003). The University of Minnesota Biocatalysis/Biodegradation Database (UMBBD, 2005) provides chemical and degradative pathways for a wide range of organic chemicals, and the Biodegradative Strain Database (BSD) (Urbance *et al*, 2003) gives information on degradative bacteria and the hazardous substances that they degrade. Both of these databases were searched for reference to the sequence matches given above with regards to the potential for degradation of DCA. No direct matches were found in these databases; however, the databases are relatively new in development. Through the addition of increasing sources of research data (like that achieved during this thesis investigation) these databases will likely prove useful for a wide range of applications. The discussion on sequencing results (based on a review of available scientific literature) is presented below, and focuses on the microbial matches, which help to explain the degradation of focus compounds within the microcosm experiments.

Bacterial sequences matching that of E.Coli were found in bands 2 and 3. The same bands also appear to be present in all abiotic extractions. The presence of E.Coli may have been attributable to a contaminated batch of taq polymerase, which was used during the PCR amplifications (Rochelle *et al*, 1992). However, no PCR negative controls were found to contain product, and the sequences from the other microcosm extracts (B, M1-M4) did not match well with that of E.Coli. Therefore, E.Coli is likely to have been present in all abiotic samples, and had survived the sterilisation process.

Due to the apparent random amplification of the abiotic extractions (discussed in Section 6.4.6), it was difficult to draw any real conclusions from the sequencing of PCR products. No significant sequence matches were identified between the M5 bands.

As was previously mentioned (Section 6.4.6), the community profiles of the M1 (aerobically-enhanced) and M4 (no enhancement) microcosm samples appear

similar. The most obvious similarities between the two microcosm types were the locations and increasing magnitudes of the bands numbered as 28, 29 and 30 (from Figure 6.13). Sequencing results confirmed the similarities of these bands, and suggested that the DNA was representative of a bacteroidetes bacterium. The species matches show that the bacteria have been identified in hydrocarbon-contaminated groundwater and have been associated with sulphate reduction. These results confirm the earlier assumption that an increase in growth was shown for a potentially anaerobic species within the oxygen-enhanced microcosms.

The dominant bands excised from lanes representing microcosms M2 (molasses-enhanced) and M3 (HRC®-enhanced) (i.e. band 33, 34, 36, 37, 38 and 39) all produced good sequencing results (sufficient alignment length similarities). Each of these bands shows strong similarities to known anaerobic fermicutes bacteria. None of these bands appeared in the lane represented by microcosm B (day 0 of the studies). This suggests that the activities of these microorganisms may have increased soon after the start of the microcosm studies.

Strong sequence similarities between bands 33&34, 36&37 and 38&39 show the consistency of results on fragments from the same locations within the DGGE gel. Bands 36 and 37 were only found from extracts of microcosms M3 (HRC®-enhanced). The sequences for these bands show similarities to *Clostridium cronatovorans* strain RCB, and has also been found in a TCE-dechlorinating bacterial community (Macbeth *et al*, 2004).

Bands 33 and 34 were found from extracts of microcosms M2 and M3, but appear more consistently in M3 samples. The sequences for these bands show similarities to *Desulfitobacterium dichloroeliminans* strain LMG P-21439, and has been associated with iron(III)-reducing species (De Wildemans *et al*, 2003, and Petrie *et al*, 2003). Studies by De Wildemans *et al* (2003) have investigated the reductive dechlorination of dichloro-alkanes (such as DCA) by *Desulfitobacterium dichloroeliminans* through dehalorespiration.

The sequencing results suggest that the microbial communities assessed from microcosms M3 may be best suited to degrading DCA under anaerobic

conditions. This is consistent with the results of chemical analyses on microcosms (discussed in Chapter 5).

6.5 Identifying dehalogenase genes

1,2-dichloroethane was a focus compound for the microcosm studies. The degradation pathways for 1,2-dichloroethane (DCA) were discussed in Chapter 2. The degradation of DCA as a primary substrate was shown to progress through the intermediates 2-chloroethanol, chloroacetaldehyde, chloroacetic acid and glycolate (UMBBD, 2005).

Microbial enzymes are responsible for catalysing the degradation of DCA and its pathway intermediates. Studies conducted by Janssen *et al* (1985) found that α-halocarboxylic acid dehalogenase enzymes could facilitate the degradation of chloroacetic acid. The studies also suggested that the degradation of carboxylic acids, such as chloroacetic acid, was more rapid under elevated pH conditions. This is in contrast to the dehalogenation of DCA, which was determined to be more rapidly consumed under neutral pH conditions (Janssen *et al*, 1985).

Hill *et al* (1999) investigated two gene families, which encode α-halocarboxylic acid dehalogenase enzymes. The two gene families were called group I (*deh*I) and group II (*deh*II) dehalogenase genes. Degenerate primers were developed for the amplification of *deh*I and *deh*II genes. A degenerate primer is actually a set of primers, which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences (Rybicki, 2001).

The presence of *deh*I and *deh*II genes within microcosm samples would give an indication of the DCA degradation pathway. The aforementioned dehalogenase genes were used to amplify DNA extracts using PCR. The primers were diluted to 10pmolμL^{-1} prior to use.

Reaction mixtures for the *deh*I and *deh*II PCRs were produced similarly to those described under Section 6.4.5. The reagents included 29.25μL dH_2O, 5μL reaction buffer (X10; Bioline), 3μL magnesium chloride ($MgCl_2$), 0.5μL dNTPs

(25mM; Bioline), 5µL forward primer, 5µL reverse primer, 1µL BSA, 0.25µL taq polymerase and 1µL DNA template. The total reaction mixture amounted to 50µL.

A touchdown program was used for the PCR amplification of *dehI* genes. Touchdown programs are designed to by-pass spurious amplification without lengthy optimisation procedures (Don *et al*, 1991). Temperatures begin above that which is required for primer annealing. They then decrease at pre-set intervals until a final (touchdown) temperature is reached.

A DNA Engine Dyad Thermal Cycler (MJ Research Inc) was used to perform the reaction in accordance with the following conditions for the *dehI* analysis:

2 minutes @ 94 °C

20 seconds @ 92 °C
30 seconds @ 70 °C (minus 1 °C per cycle) } 20 cycles
30 seconds @ 72 °C

20 seconds @ 92 °C
30 seconds @ 51 °C } 20 cycles
30 seconds @ 72 °C

A different program was used for the amplification of *dehII* gene fragments. The differing programs are related to the required annealing conditions for the individual primers. Again, a DNA Engine Dyad Thermal Cycler (MJ Research Inc) was used to perform the reaction in accordance with the following conditions for the *dehII* analysis:

5 minutes @ 94 °C

45 seconds @ 92 °C
2 minutes @ 55 °C } 36 cycles
45 seconds @ 72 °C

5 minutes @ 72 °C

Gel electrophoresis was used to interpret the results of the PCR amplifications. Refer to Section 6.4.5.1 for a description of the gel set-up. The ladder Hyperladder 1 (Bioline) was used to compare fragment sizes. *Pseudomonas putida* PP3 contains both *dehI* and *dehII* genes (Hill *et al*, 1999), and was therefore used as a positive control for the PCR analysis.

The results of the PCR amplifications for the *dehI* and *dehII* genes are given in Figures 6.17 and 6.18 respectively. The ladders and positive controls (especially the 1/10 dilutions) appear to be of good quality. Therefore, the amplification and gel electrophoresis procedures were successful. Very little product is shown for any of the microcosm samples. However, faint product is shown in the B, M2, M3 and M4 lanes for the *dehII* results.

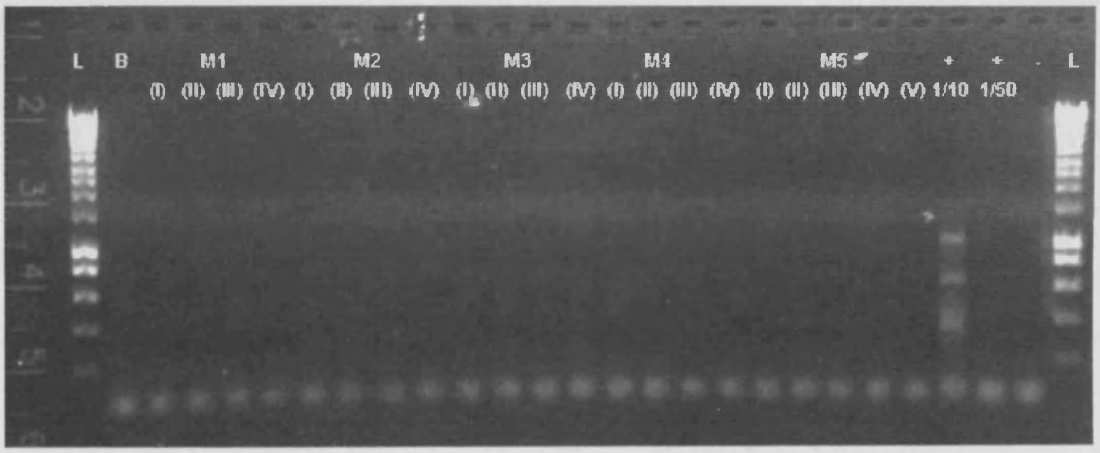


Figure 6.17 *dehI* gene amplifications of microcosm extracts

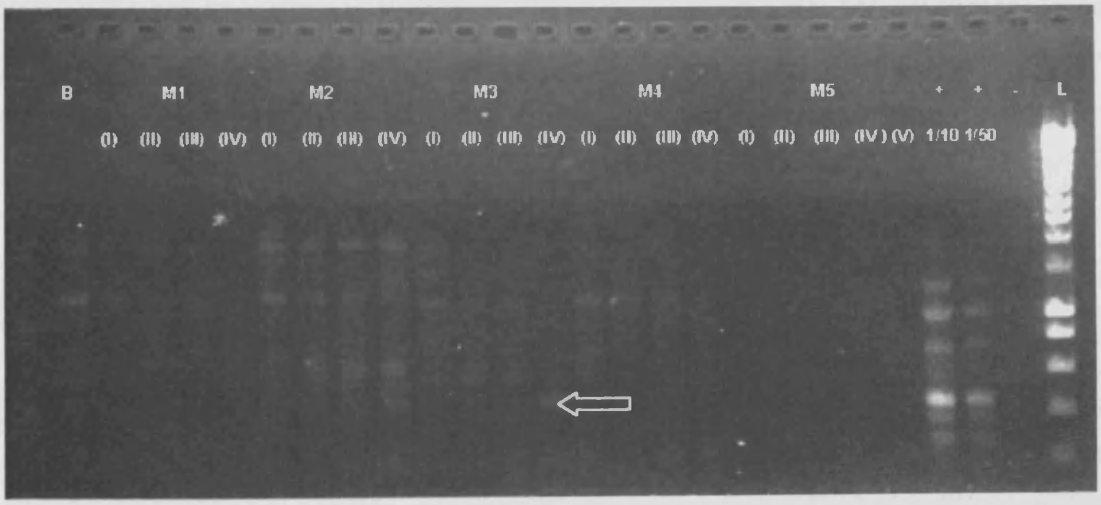


Figure 6.18 *dehII* gene amplifications of microcosm extracts

Studies into bacteria, which have been enriched and isolated on halocarboxylic acid, suggest that the diversity of *deh*II genes was greater than that of the *deh*I genes (Marchesi and Weightman, 2003). The results shown in Figures 6.17 and 6.18 are consistent with these findings. The *deh*I gene amplifications appear fainter than the respective *deh*II gene results.

The bands shown at the bottom of the gel in Figure 6.17 do not represent amplified dehalogenase genes. The fragment sizes are far too small. They are likely to be the remains of primer, which did not anneal to any DNA templates.

The targeted sequences for the *deh*I and *deh*II genes contain 230 and 422 base pairs respectively. Only one band appears to be located within the appropriate size fraction. This band is identified by an arrow in Figure 6.18, and originates from sample M3(IV).

There are also a number of other faint bands spread throughout the larger size ranges (mostly in the M2 lanes). These may have been the result of primer annealing followed by conglomeration of product to form larger DNA fragments. Due to the difficulties in defining such bands, no significant conclusions can be drawn from their presence. However, the appearance of product within any size range could be indicative of amplified dehalogenase genes.

The presence of PCR products from the amplification of *deh*I and *deh*II genes gives an indication that DCA may have been degraded according to the pathway suggested at the start of this Section. The results shown suggest that chloroacetic acid was to some extent degraded to glycolate by the *deh*I and *deh*II enzymes within the microcosms. However, the absence of any distinctive bands suggests that a separate degradation pathway (such as reductive dechlorination) may have contributed to the degradation of DCA.

6.6 Conclusions

The microbiological analyses described in this Chapter were conducted in order to further characterise conditions within the microcosm experiments (Chapter 5). Molecular methods were used, which can rapidly screen a larger proportion of microorganisms within environmental samples than that of traditional

culturing techniques. The microbial community profiles (based the 16S rRNA-encoding gene) and presence of dehalogenase genes within samples were assessed.

Abiotic microcosms were analysed in order to determine the effectiveness of the sterilisation procedure. Potential signs of growth were observed in the results of PCR gels. These signs were based upon differences in band magnitudes between the M5(V) microcosm (day 0) and the other abiotic microcosms. However, no distinct signs of growth were observed during the DGGE analysis. These results suggest the potential for microbial growth within abiotic microcosms, but are not conclusive. However, significant reductions in contaminant concentrations were noted for the M5 microcosms (as presented in Chapter 5). It is the opinion of this thesis author that the loss of contaminant concentrations is likely the result of an ineffective sterilisation procedure. It is likely that the sterilisation process failed due to the large quantity of material being sterilised. This thesis author suggests that a minimum of one additional autoclave procedure (using the same temperature and timing as discussed in Chapter 5) should have been conducted. It is also recommended that more repetitions of control microcosms should have been set up and investigated in order to minimise (and allow for a better assessment to be made of) errors.

Towards the end of the experiments (day 79) bacteria with DNA sequences resembling that of sulphate-reducers were found in microcosms M1 and M4. This suggests that anaerobic conditions may have developed within the oxygen-enhanced microcosm. This is likely to be the result of the full consumption of available oxygen within the contaminated slurries.

Anaerobic chloro-alkane degrading bacteria were identified in microcosms M2 and M3 using 16S rDNA sequence matching. These bacteria are likely to have been directly involved with the degradation of DCA within microcosms. Microcosms M3 showed the most significant results for these microorganisms, suggesting that the enhancements within the M3 experiments provided the best conditions for the growth of these species.

Further analyses were carried out to determine the presence of α-halocarboxylic acid dehalogenase genes within microcosm samples. Faint results were

achieved using the *dehII* gene primers. The genes were apparent predominantly in samples from microcosms M2 and M3. However, the faintness of the results suggests that the enzymes, which are encoded by these genes, may not be the sole driver for DCA degradation.

In the following chapter, the results of all of the analytical work for this thesis are brought together. Further interpretations are provided of the microcosm and microbiological experimental results in relation to the study site conceptual model.

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Chapter 7

Conclusions and Suggestions for Further Research

7.1 Introduction

This chapter brings together the full results of the research that was carried out for this thesis. As stated in Chapter 1, the overall objective of this research was to determine the treatability of organic contaminants from the study site through an enhanced natural attenuation (ENA) process. The conclusions of the previous chapters are discussed in order to meet this objective.

The results of desktop, field work and analytical investigations of the study site are discussed in Section 7.2. These investigations allowed for a conceptual model to be developed and refined for the study site.

In order to assess the potential for treatment using ENA, microcosm treatability studies were conducted. In Section 7.3, the results of chemical and microbiological analyses on microcosm samples are discussed, and related to the effectiveness of the various enhancements.

The objective of this thesis was designed to allow for the results of this work to be applied in the decision making process for choosing an appropriate remediation strategy. Recommendations for the use of ENA at the study site are provided in Section 7.4.

A number of important scientific uncertainties arose through the course of this research, which did not fall within the scope of this thesis. These issues are suggested as areas for further research in Section 7.5.

7.2 Conceptual model of the study site

Contamination incidents over the past few decades have resulted in a large range of potential contamination problems at the study site. The site is currently undergoing a period of decommissioning, deconstruction and remediation, with a view to redevelop the area. Over the past several years, CELTIC Technologies Ltd (CELTIC) was employed as a consultant to the site owner, and has undertaken a large-scale investigation into contamination related issues at the site. The results of CELTIC investigations were examined as part of this thesis during a desktop study of the study site (see Chapter 3). During a meeting with the site owner, it was decided that there was a lack of sufficient scientific knowledge to be able to proceed immediately with remediation measures in the area of the site nearest the estuary. This research is directed towards contamination issues for the area (shown in Figure 7.1).

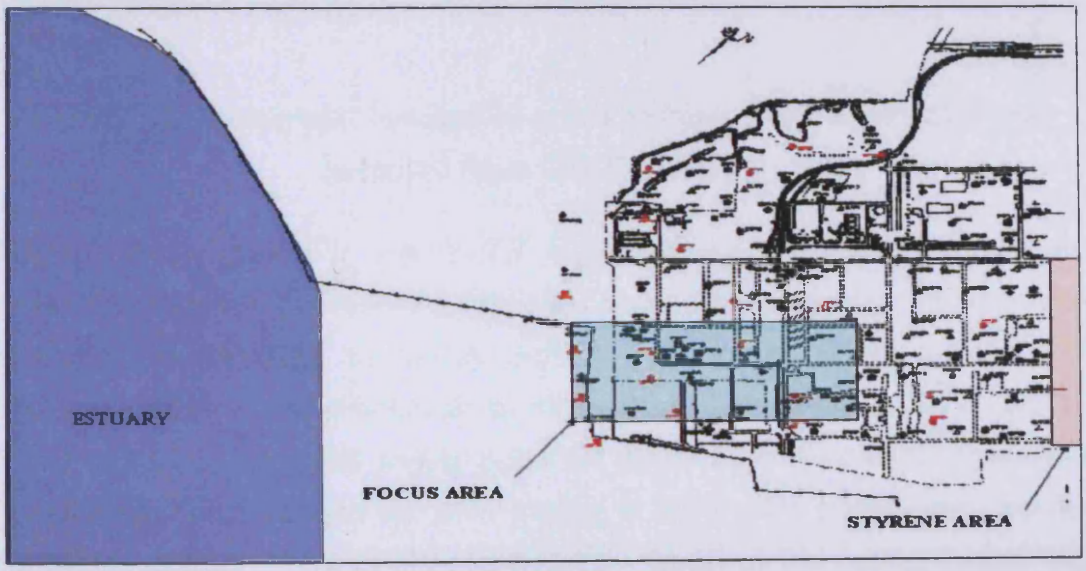

Figure 7.1 Plan view of the study site

During the desktop investigation of the focus area, contaminant compounds were identified, which were deemed to pose potential risks to environmental and human health receptors. The focus compounds for this thesis were initially defined as 1,2-dichloroethane (DCA), vinyl chloride (VC), benzene and ethylbenzene. VC was later removed from the treatability analyses due to the difficulties involved with handling a compound with such a low boiling point (-13.9°C).

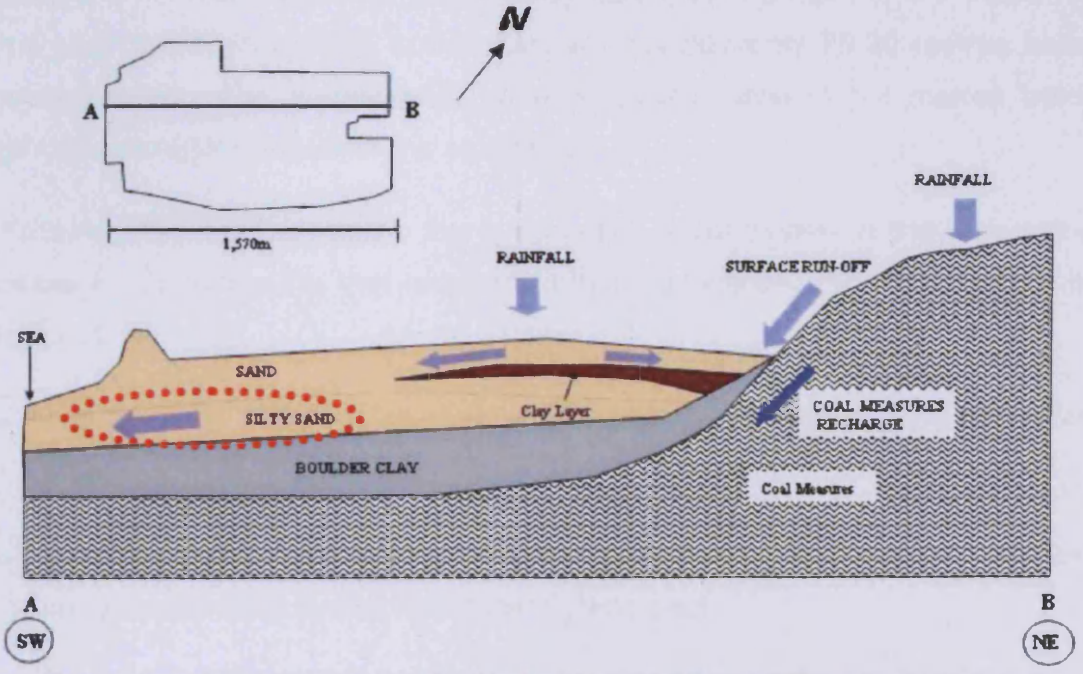

Figure 7.2 Conceptual geological cross section of the study site area (adapted from CELTIC (2002))

The red circle, shown in Figure 7.2 highlights the general location of the contaminant pathways underlying the focus area. The focus contaminants were all found predominantly as dissolved-phase compounds in the groundwater. The actual location and delineation of contaminant plumes proved to be difficult due to the extent of the site and an apparent degree of contaminant fingering. It is likely that these plumes are also mixing to form more complicated plumes containing multiple contaminant compounds. Much of the contamination was detected in anaerobic environments towards the bottom of the aquifer.

As the contaminant plumes migrate towards the estuary, they encounter the saline wedge. The transport and fate of contaminant plumes migrating through this zone are not well understood. However, it is likely that the dissolved contaminants will be transported along the saline wedge towards the ground surface (and potential pollutant receptors).

The underlying local geology consists of three main strata layers in the focus area of the study site (refer to Figure 7.2). These layers are blown sand, marine sand and boulder clay. The boulder clay acts as the bottom of the aquifer for the purposes of this study, and is located approximately 25-30 metres below ground level. The groundwater table is located around 1-2 metres below ground level across much of the study site.

Following the desktop study, there were still uncertainties in the conceptual model for the study site that required further clarification. These uncertainties included:

- The potential presence of harmful metabolites of styrene within the focus area,
- Hydrogeological unknowns relating to a potential vertical hydraulic gradient and excessive contamination of the styrene area,
- Degradation potential of DCA, VC, benzene and ethylbenzene under natural and enhance conditions, and
- Details of the indigenous microbiological communities.

In order to clarify these uncertainties, further site investigations were designed and carried out solely for the purposes of this thesis. These investigations included field work and laboratory analysis of soils and groundwater from the styrene and focus areas of study site (see Figure 7.1). In addition to the clarification of the aforementioned uncertainties, the investigations allowed for the sampling and analytical procedures to be better understood.

The results of further investigations into the styrene-area suggested that there were no significant metabolites of styrene monomer. However, the presence of

styrene polymer was detected. The local and overall study site hydrogeology may have been altered due to the presence of the highly viscous styrene polymer. The results of the desktop study found the presence of lighter compounds such as benzene and ethylbenzene towards the bottom of the aquifer. It is possible that severely clogged pore spaces in the styrene area may be a partial cause for the apparent downward flow of lighter hydrocarbons in the aquifer. However, the downward migration of benzene and ethylbenzene is more likely to be the result of mixing with a denser plume (e.g. DCA).

The findings of the focus area investigations described a wide range of physical and chemical properties. Particle size analysis found the marine sand to be composed of primarily fine sand-sized particles. Less than 12% of the soil mass consisted of silt or clay-sized particles. Quartz was the dominant mineral component of the sample. Only trace amounts of clay minerals were detected. These results suggest that the soil minerals are likely not to have a significant influence on the fate of organic pollutants in the groundwater. The soluble pollutants are likely to be highly mobile, with minimal partitioning onto soil solids.

Groundwater samples from three boreholes (B1, B2 and B3) were analysed. Refer to Chapter 4 for the locations of these boreholes. The previously identified focus contaminants were detected during the analysis of groundwater. The results from borehole B1 differed significantly from the other two boreholes, which were sampled. The high pH (10.8) and low Eh (-251.5 mV) values suggested the presence of high contaminant concentrations. This was confirmed through the laboratory analyses, where DCA concentrations were found at 879 mgL^{-1}. Benzene was also a major contaminant in the B1 sample, which was measured at 12.9 mgL^{-1}. Relating these observed values with EQS limits further highlights the magnitude of contaminant concentrations at the study site. Ethylbenzene was also detected in borehole B1, but was at a concentration below the New Dutch List action level. The high TOC values of groundwater from borehole B1 were attributed to the contamination. DCA and benzene were also found in boreholes B2 and B3, though to a lesser extent than the B1 results.

One of the aims of the focus area investigations was to collect samples for use in microcosm studies. Samples from boreholes B1 and B3 were ruled out because of heavy contamination and public space issues respectively. It was decided that soil and groundwater samples from borehole B2 were to be used for this purpose.

7.3 Microcosm treatability studies

7.3.1 Enhanced natural attenuation (ENA)

ENA is a remediation technique whereby natural conditions are enhanced in order to improve the natural attenuation capabilities of a soil. Amendments can include the addition of electron acceptors, electron donors or carbon sources. For the purposes of this thesis, a range of enhancements was chosen due to the varying properties and known degradation pathways of the focus contaminants (discussed in Chapter 2). Both oxidative and reductive conditions were investigated.

The demonstration of natural attenuation, prior to the implementation of an ENA scheme, cannot be determined through site monitoring only. Weidemeier *et al* (1998) describes the use of microcosm studies for physically demonstrating the occurrence of natural attenuation, and determining whether indigenous biota are capable of degrading site contaminants.

There are various microcosm designs that can be adopted for use in treatability studies. The development of microcosms for this thesis was carried out according to the recommendations of Morse *et al* (1998). Five types of microcosm were set-up as shown in Table 7.1.

Table 7.1 Descriptions for the 5 different microcosm types

Labels	Enhancement added	Microcosm description
M1	Oxygen Release Compound ORC®	Enhanced microcosm for facilitating the oxidation of all introduced contaminant compounds using ORC®
M2	Molasses	Enhanced microcosm for facilitating the reductive dechlorination of DCA using molasses
M3	Hydrogen Release Compound HRC®	Enhanced microcosm for facilitating the reductive dechlorination of DCA using HRC®
M4	None (standard control)	Standard control made to represent *in situ* conditions without enhancement.
M5	None (abiotic control)	Biotic content of the slurry mix was sterilised prior to the introduction of contaminant compounds.

7.3.2 Performance of enhancements

Four sub-samples of each microcosm were set-up to allow for destructive sampling. Porewater and slurry samples were taken during the destructive sampling events, and analysed for chemical and microbiological changes. Regular headspace samples were also extracted from microcosms and analysed for variations in contaminant concentrations. The results of the chemical and microbiological analyses are brought together in the following sections in order to describe the effectiveness of enhancements.

7.3.2.1 ORC®

The microcosms enhanced with ORC® (M1) were expected to degrade benzene and ethylbenzene through aerobic respiration. DO concentrations within microcosms M1 reached values of over 12 mgL^{-1}, which should have been more than sufficient for facilitating aerobic respiration. Due to the rapid degradation of benzene and ethylbenzene within the M1, M2, M4 and M5

microcosms, no definitive conclusions can be made as to the effectiveness of the enhancements. This rapid degradation may be the result of leaks in the microcosm bottles or effective aerobic mechanisms, which were brought about by the presence of residual oxygen within all microcosms at day 0.

Towards the end of the experiments (day 79) bacteria with DNA sequences resembling that of sulphate-reducers were found in microcosms M1 and M4. This suggests that anaerobic conditions may have developed within the oxygen-enhanced microcosm. A distinct crusty layer was observed at the surface of the slurries within M1 microcosms. It is possible that the layer may have stratified the microcosms, with the development of more reduced conditions in the underlying slurry, and the persistence of oxidised conditions in the overlying groundwater.

DCA was effectively degraded within the ORC®-enhanced microcosms. However, it was not possible to determine the extent to which the additive may have enhanced this degradation due to the potential development of reduced conditions.

7.3.2.2 Molasses

The molasses-enhanced microcosms (M2) were successful in rapidly degrading DCA. The molasses released more than the required amount of molecular hydrogen for facilitating reductive dechlorination. This excess in hydrogen was accompanied by elevated concentrations of ions within the molasses itself. The addition of extra ions (including nitrate and sulphate) into the microcosms may have acted to inhibit the preferred reaction (i.e. reductive dechlorination) due to competition between indigenous microorganisms and varied redox conditions.

Oxygen appears to have been rapidly consumed within the molasses enhanced microcosms, thus allowing more reduced conditions to develop for facilitating the reductive dechlorination of DCA. The breakdown of molasses produced excess gases (hydrogen and carbon dioxide) within the microcosms. The high positive pressures observed during these studies were not deemed to have significantly affected the accuracy of test results.

Anaerobic chloro-alkane degrading bacteria were identified in microcosms M2 using 16S rDNA sequence matching. These bacteria are likely to have been directly involved with the degradation of DCA within microcosms. Further analyses were carried out to determine the presence of α-halocarboxylic acid dehalogenase genes within microcosm samples. Faint results were achieved using the *dehI*I gene primers for the analysis of M2 samples. However, the faintness of the results suggests that the enzymes, which are encoded by these genes, may not be the sole drivers for DCA degradation.

As was previously mentioned, the rapid degradation of benzene and ethylbenzene may be the result of effective aerobic mechanisms that were brought about by the presence of residual oxygen within all microcosms at day 0. The degradation of benzene and ethylbenzene within the M2 microcosms closely resembled that which was observed in the standard microcosms (M4). Because of this, effectiveness of the molasses additive for enhancing benzene and ethylbenzene degradation was inconclusive.

7.3.2.3 HRC®

The microcosms enhanced with HRC® (M3) reduced DCA concentrations more efficiently than the other microcosms. The production of sufficient quantities of hydrogen is likely to have facilitated the reductive dechlorination of DCA. Methane production was also observed, but it would appear that the competition for molecular hydrogen for methanogenic activities was not sufficient to inhibit dechlorination.

As with the molasses-enhanced microcosms, oxygen appears to have been rapidly consumed within microcosms M3, thus allowing more reduced conditions to develop for facilitating the reductive dechlorination of DCA. The breakdown of HRC® also produced excess gases (hydrogen and carbon dioxide). These gases were not deemed to have significantly affected the accuracy of test results.

The HRC®-enhanced microcosms showed the most significant microbiological results, suggesting that the enhancements within the M3 experiments provided the best conditions for the growth of these species. The two most dominant

species within the M3 microcosms contained genetic sequences that resembled those of *Clostridium cronatovorans* strain RCB (part of a TCE-dechlorinating bacterial community) and *Desulfitobacterium dichloroeliminans* strain LMG P-21439 (iron(III)-reducing species with dehalorespiration capabilities) (Macbeth *et al*, 2004; De Wildemans *et al*, 2003; Petrie *et al*, 2003). These bacteria are likely to have been directly involved with the degradation of DCA within the HRC®-enhanced microcosms.

Further analyses were carried out to determine the presence of α-halocarboxylic acid dehalogenase genes within microcosm samples. The HRC®-enhanced microcosm M3(IV) gave a result that indicated the presence of the *deh*II gene. However, the faintness of the result suggests that the enzymes, which are encoded by these genes, may not be the sole drivers for DCA degradation.

Slower reductions in benzene concentrations were observed within the HRC®-enhanced microcosms compared to all other microcosms. This was attributed to the rapid consumption of oxygen, and inhibition of benzene degradation under reduced conditions. This said, benzene concentrations were still reduced to less than 0.5% of the initial $100mgL^{-1}$ over the 79-day study period. Therefore, anaerobic conditions did facilitate benzene degradation, but less rapidly than that which occurred under the initial aerobic conditions.

7.3.3 Abiotic control microcosms

The loss of contaminants within abiotic microcosms (M5) suggests error in the analyses. The loss was not thought to be related to abiotic degradation mechanisms. It is more likely that the sterilisation procedure was unsuccessful, or that contaminants may have been lost through leaks in the microcosm bottles. Potential signs of microbial growth were observed in the results of PCR gels for the abiotic samples. These signs were based upon differences in band magnitudes between the M5(V) microcosm (day 0) and the other abiotic microcosms. However, no distinct signs of growth were observed during the DGGE analysis. These results suggest the potential for microbial growth within abiotic microcosms, but are not conclusive.

7.4 Study site recommendations

The results of this thesis have been brought together to describe the use of ENA as a potential remediation technique for contaminants at the study site. The adopted approach to the treatability studies was not designed to quantify the rates of degradation, but rather to ascertain the potential for enhanced treatment using the additives shown in Table 7.1.

The focus contaminants for this thesis (DCA, benzene and ethylbenzene) exist at the study site in mixed plumes. The review given in Chapter 2 described a range of varied degradation mechanisms for the three compounds. Previous research suggested a variety of anaerobic and aerobic mechanisms for the successful degradation of the individual contaminants. However, the design of a remediation strategy for the contaminants in a mixture is more complicated, and may require the use of multiple treatments.

The results of the treatability studies for this thesis suggest that the natural attenuation of a mixed plume of DCA, benzene and ethylbenzene can be enhanced through the addition of HRC®. The microcosm studies showed that indigenous microorganisms could effectively degrade both benzene and DCA under reduced anaerobic conditions. The full degradation of ethylbenzene may have been attributable to aerobic conditions that existed at the start of the study.

If an HRC®-enhanced treatment were to be considered for full-scale implementation at the study site, further plume delineation would be essential for targeting contaminants in the focus area. Plume delineation is currently being carried out alongside other groundwater chemistry analyses by environmental consultants at the study site. The results of this work were not available for incorporation into this thesis.

Although limitations due exist (as discussed in previous Chapters), the wide range of biological, chemical and physical methods used during the work for this thesis were conducted under a limited budget, yet the achieved results provide useful data for selecting a more appropriate remedial approach. Using such cost-effective approaches to treatability assessment can save project time and

budget, as well as reducing the chances of implementing an ineffective remedial strategy. Furthermore, the recommendation of ENA through biostimulation is in itself an economic and non-intrusive remedial solution.

7.5 Suggestions for further research

During the course of this thesis, several areas of research arose that could benefit from further investigation. The following suggestions are given to allow for further scientific developments in the fields of study, which were incorporated into this research.

- Describing the transportation and fate of organic contaminants migrating from groundwater to estuarine environments could prove useful for risk determination. It is likely that interactions along the saline-freshwater interface and tidal movements would have a significant impact on the transformation and/or migration of solute contaminants. However, no literature was found that gives a comprehensive description of this scenario. Research based upon the use of numerical models or lab-scale investigations could lead to the development of more accurate risk assessments.

- Many contaminated sites (such as the study site) contain plumes of mixed contaminant compounds. Characterising the most efficient mechanism(s) of degradation for mixed plumes can prove difficult due to the differing properties of the compounds and site specific conditions. No literature was found that described the degradation potential of a mixed plume of DCA, benzene and ethylbenzene prior to this thesis. Unfortunately, the approaches to developing remediation strategies at many contaminated sites are not likely to involve the same level of detailed as is provided in this thesis. For the application of more efficient remediation techniques, it is recommended that detailed research should be carried out for sites containing previously uninvestigated mixtures.

- During the microcosm studies for this thesis, a distinct crusty layer was observed at the surface of the slurries within the ORC®-enhanced microcosms. The effects that this layer may have had on the chemical

and/or biological conditions within the microcosms were not fully characterised. The layer may have led to stratification between anaerobic and aerobic conditions, which led to difficulties in the interpretation of results. Further investigation into the composition of this layer, and into whether such a material could develop *in situ* could prove useful for future ORC® applications.

- The sterilisation procedure (heat treatment) that was used for the abiotic microcosms in this thesis may have been ineffective. In order to confirm the effectiveness of such procedures, it is recommended that future studies incorporate a check for microbial activity (e.g. viable count) prior to commencement of the microcosm experiments.

- The molecular techniques employed during the work for this thesis did not quantify microbial activity and were not fully capable of distinguishing between living and dead microbial cells. The distinction between living and dead microbial cells is especially important when heat sterilisation is to be used for abiotic control samples. Madigan *et al* (2003) states that heat treatment and microbial death may result in cell lysis. A more detailed quantification of the relationship between the heat treatment of environmental samples and cell lysis could be useful for studies that intend to analyse the treated materials by molecular microbiological methods.

- The build up of excess gases was observed within the molasses and HRC®-enhanced microcosms. The development of excess gases may have serious implications for the use of these additives for full-scale *in situ* treatments. Pockets of gas forming around an enhanced zone of molasses or HRC® could act to decrease the bioavailability of the molasses (carbon source) for cometabolic purposes. However, the delivery of electron donor (e.g. hydrogen) for reductive dechlorination may actually be enhanced through the production and transport of the gases (Dyer *et al*, 2000). Further research into the effects of such gaseous developments could prove useful for maximising the effectiveness of *in situ* applications.

- This scientific quality of this thesis was enhanced through collaborations between the Cardiff University Schools of Engineering, Biology and Earth Sciences. It is the opinion of this thesis author that the inclusion of pure sciences into engineering research is an important step towards the development of more innovative techniques, and something that should be pursued by all fields of engineering.

7.6 References

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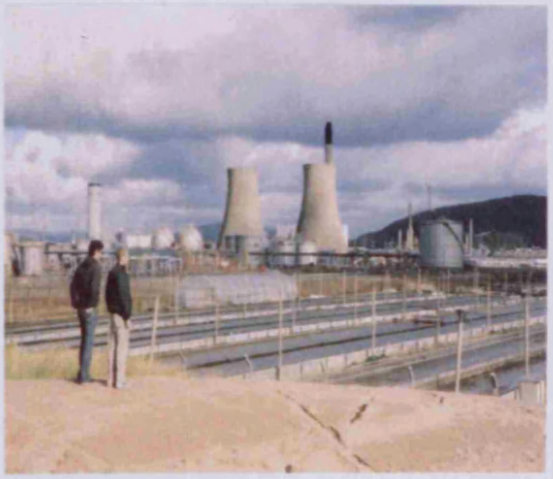
Appendix I

Photographs of the Field Work



North facing view over the study site showing the ragworm farm in the foreground and cooling towers in the background (Spring 2004)

East facing view over the study site, (Spring 2005)

View along the beach area which borders the study site (Spring 2005)

Trying to locate borehole B3 in the beach area (Spring 2005)

Extracting groundwater from borehole B3 using a bailer (Spring 2005)

Overhead view of borehole B3 sampling (Spring 2005)

Groundwater from borehole B3 with a light sheen (Spring 2005)

Extracting groundwater from borehole B2 using a bailer (Spring 2005)

Groundwater from borehole B2 (Spring 2005)

Groundwater from borehole B1, which appears dark in colour (Spring 2005)

Groundwater from borehole B1 with a light sheen (Spring 2005)

Conducting on site analyses of pH, dissolved oxygen, redox potential and temperature using hand held meters (Spring 2005)

Appendix II

VOC Results

All VOC results stated as μgL^{-1}

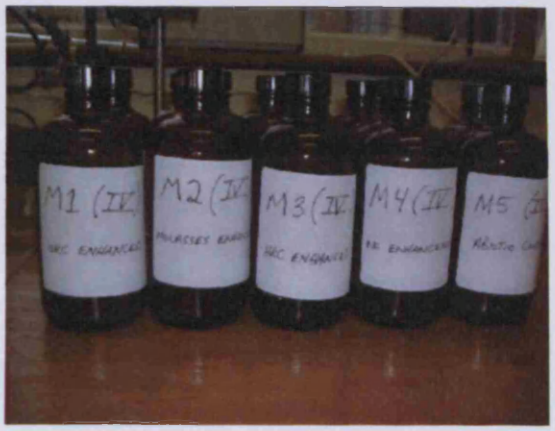
Compound	B1	B2	B3
Toluene	1110	<2	41
Styrene	2	<2	<2
Tetrachloroethene	<2	<2	<2
Bromodichloromethane	<2	<2	<2
Dibromochloroethane	<2	<2	<2
1,3-Dichlorobenzene	<2	<2	<2
Chlorobenzene	<2	<2	<2
1,2-Dichlorobenzene	<2	<2	<2
1,4-dichlorobenzene	<2	<2	<2
Trichloroethene	15	13	14
1,2-dichloroethane	879000	5580	22600
Dichloromethane	141	<20	142
1,2,3-Trichlorobenzene	<2	<2	<2
1,3,5-Trichlorobenzene	<2	<2	<2
Ethylbenzene	16	<2	<2
1,1,2-Trichloroethane	<2	<2	<2
1,2-Dibromoethane	<2	<2	<2
Benzene	12900	2	943
2-Chlorotoluene	<2	<2	<2
4-Chlorotoluene	<2	<2	<2
1,2,4-Trichlorobenzene	<2	<2	<2
1,1,1-trichloroethane	<2	<2	<2
1,1-Dichloroethane	252	2	11
Trans-1,2-Dichloroethene	34	<2	<2
2,2-Dichloropropane	<2	<2	<2
Cis-1,2-Dichloroethene	84	<2	11
Bromochloromethane	<10	<10	<10
1,1-Dichloropropene	<2	<2	155
Dibromomethane	<2	<2	<2
1,2-Dichloropropane	40	32	427000
Trichloromethane (chloroform)	<2	<2	<2
Tribromomethane (bromoform)	<2	<2	<2
Tetrachloromethane	<2	<2	<2
Trans-1,3-Dichloropropene	<2	<2	<2
Cis-1,3-Dichloropropene	<10	<10	<10
1,3-Dichloropropane	<2	<2	39
1,1,1,2-Tetrachloroethane	<2	<2	<2
1,3 & 1,4-Dimethylbenzene (m&p-Xylene)	10	<2	7
1,2-Dimethylbenzene (o-xylene)	11	<2	8
1-Methylethylbenzene	<2	<2	<2

Bromobenzene	<2	<2	<2
1,2,3-Trichoropropane	<2	4	7900
1,1,2,2-Tetrachloroethane	<10	<10	<10
n-Propylbenzene	<2	<2	<2
1,3,5-Trimethylbenzene	<2	<2	<2
1,1-Dimethylethylbenzene	<2	<2	<2
1,2,4-Trimethylbenzene	<2	<2	<2
1-Methylprpylbenzene	<2	<2	<2
4-Isopropyltoluene	<2	<2	<2
n-Butylbenzene	<2	<2	<2
1,2-Dibromo-3-Chloropropane	<10	<10	<10
Napthalene	<10	<10	<10
Hexachloro-1,3-Butadiene	<2	<2	<2
1,2-Dichloroethene	5930	297	1980

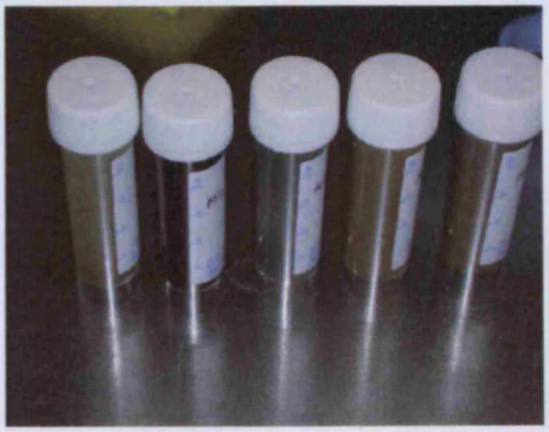
Appendix III

Photographs of the Microcosm Experiments

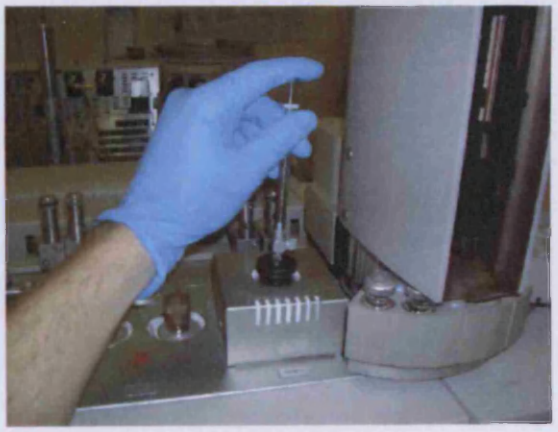
Microcosm bottles sealed with screw on lids and septa



Microcosm bottles with labels shown



Porewater samples from microcosms



Microcosm headspace samples being directly injected into the GC-MS

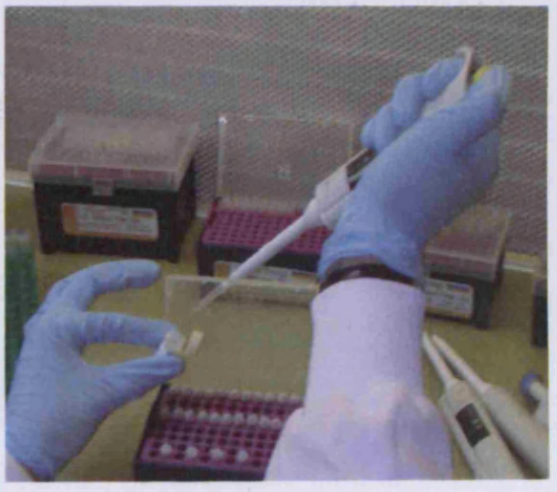
Appendix IV

Photographs of the Microbiological Analyses

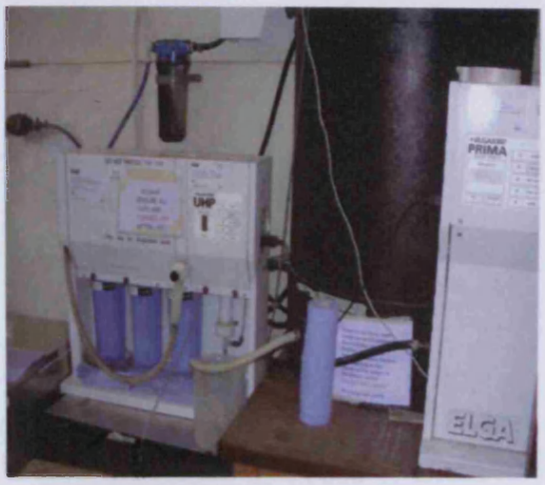
UV gel viewer

Gel electrophoresis tanks

PCR set-up was conducted underneath a fume hood



Mixing reagents during PCR set-up



Water purification system

PCR apparatus

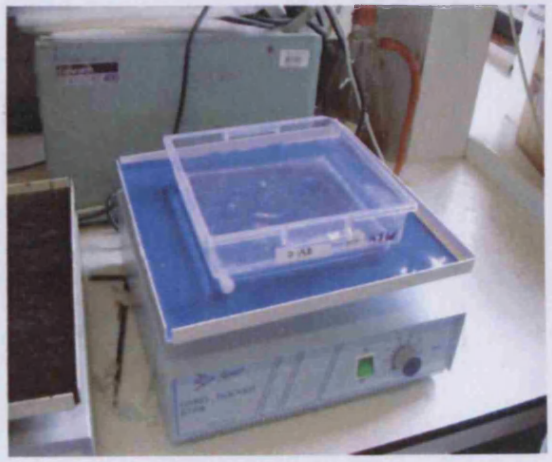
Development of the DGGE gel

Combs located within the DGGE gels for the formation of injection wells

Injection of PCR products into the DGGE wells

Insertion of the gels into the DGGE tank

The DGGE system in operation



DGGE gel on a tilt plate with gold stain