The impact of hyper-alkaline fluids from a geological radioactive waste repository on the biological and physical characteristics of the host rock environment

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List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ADZ	Alkali-disturbed zone
ATP	Adenosine triphosphate
BFA	Biological flow apparatus
C(A)SH	Calcium-(aluminium)-silicate-hydrate
CLSM	Confocal laser scanning microscope
ConA	Concanavalin A
CSH	Calcium-silicate-hydrate
DIW	Deionised water
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
eDNA	Extracellular Deoxyribonucleic acid
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive X-ray
EPS	Extracellular polymeric substances
ESEM	Environmental scanning electron microscope
FITC	Fluorescein isothiocyanate
GDF	Geological disposal facility
HLW	High level waste
IC	Ion chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ILW	Intermediate level waste
ISA	Isosaccharinic acid
LLW	Low level waste
MID	Multiplex identifier
MPN	Most probable number

MRD	Modified Robbins Device
MXIF	Manchester X-ray Imaging Facility
NRVB	Nirex reference vault backfill
NTP	Normal temperature and pressure
ORP	Oxidation-reduction potential
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEEK	Polyether ether ketone
PTFE	Polytetrafluoroethylene
RDP	Ribosomal database project
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
SEM	Scanning electron microscope
TAE	Tris-acetate buffer with Ethylenediaminetetraacetic acid
TE	Tris Ethylenediaminetetraacetic acid
TEAP	Terminal electron accepting process
XRD	X-ray diffraction

Abstract

The University of Manchester

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The UK's intermediate level radioactive waste (ILW) will be disposed of in a deep geological disposal facility (GDF), with extensive use of cementitious materials. Re-saturation of the GDF will generate a hyper-alkaline plume, which will migrate into the host rock, forming an alkali-disturbed zone. The physical impacts of plume - host rock interactions are relatively well understood, but microbial processes that could impact on GDF performance are not yet well characterised under these high pH conditions. Subsurface microbial processes have the potential to impact on GDF performance and ultimately radionuclide migration, for example by altering physical characteristics of the host rock via biofilm formation or mineral precipitation, or processes that can directly or indirectly influence radionuclide speciation, and hence solubility. This thesis explored some of these processes under conditions representative of a cementitious GDF for ILW. Microbial ecological studies of a hyper-alkaline field site revealed diverse bacterial communities were capable of tolerating high pH conditions representative of aspects of a GDF for ILW. Sandstone batch and flow-through experiments were established with sediments and fluids from this field site to investigate potential microbial impacts on the transmissive properties of the host rock, along with biogeochemical processes that could impact on radionuclide migration. Some systems were amended with acetate and lactate (proxies for cellulose degradation products that will be generated in a GDF for ILW), whereas others were unamended controls. Microbial processes were found to impact on the transmissive properties of the sandstone. Microbial colonisation of grain surfaces was observed, and in other column experiments, sustained injection pressure increases were observed with the addition of organic substrates. The transport of ^{99m}Tc through these columns was visualised using a gamma camera, and it was revealed that migration was much slower through the carbon-amended columns. A clogging effect was observed, and X-ray radiography revealed that this was likely a result of the generation of gas bubbles within the columns that may have formed during microbial utilisation of acetate and lactate. Microbial Fe(III)-reduction occurred in carbon-amended experiments under hyper-alkaline conditions, although spatial variation in sediment Fe(II) concentrations suggests distinct zonation of biogeochemical processes within the columns. Organic acids were utilised extensively at pH 9-10; above this pH utilisation significantly declined. When organic acid utilisation was highest, bacterial communities were diverse and non-alkaliphilic, but when the pH increased and organic acid utilisation declined, communities were dominated by obligately alkaliphilic H_2-utilising bacteria (*Serpentinomonas* sp.), suggesting rapid community adaptation. Results from this thesis highlight some of the microbial processes which could impact on GDF performance.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Chapter 1

Introduction and Thesis Structure

1.1 Introduction

Decades of nuclear power production in the UK have resulted in a legacy of 4.7 million cubic metres of radioactive waste requiring permanent disposal (NDA, 2011). A disposal facility is required to contain the radioactive waste and prevent the transport of radionuclides into the biosphere. As a result of the high toxicity of the waste and its potential to have detrimental effects on human health, it has long been recognised that the safest approach is disposal in a deep geological disposal facility (GDF) (Kim *et al*. 2011). This approach has been adopted by the UK and several other countries, with the concepts in varying stages of development. The deep geological disposal concept involves the construction of several engineered barriers and the use of a natural barrier (the host rock) to contain the waste until levels of radioactivity have decayed to less than that of background levels (Kraft, 2000). The multi-barrier concept firstly involves solidification of the radioactive waste with materials such as cement or borosililicate glass, depending on the waste type (Donald *et al*. 1997; Eskander *et al*. 2011). The solidified waste will then be encapsulated in a metal canister and then placed in the repository. A backfill material, potentially consisting of cement, bentonite clay, or other materials will then be used to fill in the repository, again depending on the waste type (Baldwin *et al*. 2008; Hicks *et al*. 2008). The host rock will then act as the final barrier, aiming to prevent the migration of radionuclides into the biosphere.

This thesis will focus on the UK's concept for intermediate level waste (ILW) disposal which will potentially utilise extensive quantities of cementitious materials, as a repository backfill material, for example. In this concept, after repository closure, re-saturation of the GDF will generate a hyper-alkaline plume. Initially, pore water pH is predicted to be around 13, forming an alkali disturbed zone (ADZ) where the physical, chemical and biological characteristics of the host rock will be significantly impacted.

The performance of the host rock as a barrier will be influenced by the presence of microorganisms. Diverse microbial populations are expected to be present in a GDF; contamination of a GDF during excavation and the operational period will inevitably lead to a range of microorganisms inhabiting the site. The deep geosphere contains an array of indigenous species, and microorganisms will also be associated with the waste itself. The presence of hyper-alkaline pore waters may initially inhibit those microorganisms not able to tolerate the extreme conditions, although some may be able to survive and adapt, and others may actually require high pH conditions for survival and growth. Microbial processes may impact on the physical characteristics of the host rock, perhaps by facilitating dissolution or precipitation of minerals in the host rock, clogging

pore spaces (Coombs *et al*. 2010), and may directly influence the solubility of key radionuclides, for example, by altering their oxidation state (Lloyd, 2003).

The main aims of this project were:

- To gain an understanding of alterations to the transport properties of sandstone by hyper-alkaline pore waters, and the biogeochemical processes occurring within these systems.
- To characterise the composition and function of microbial populations involved in the alteration of transport properties.

The objectives of this project were to:

- Characterise the bacterial population present over a range of pH values at an anthropogenic hyper-alkaline spring.
- Determine the upper pH limit of biogeochemical processes carried out by microorganisms collected from the field site in microcosm experiments.
- Undertake column experiments using crushed sandstone to determine the impact of microbial processes on transport properties, investigate the biogeochemical processes that occur within the columns to determine potential impacts on radionuclide transport, and to characterise the microbial communities and their potential roles in these processes.
- To investigate these processes further using intact sandstone to provide a system more representative of aspects of a host rock environment (e.g. in terms of porosity/ permeability).
- Comment on the potential implications for GDF processes.

1.2 Thesis structure

This thesis is comprised of a review of the relevant literature, a description of the methodologies used and 3 research papers, followed by concluding remarks and a discussion of future directions. As the thesis is presented in the alternative format style, there are instances where repetition occurs between some of the chapters.

1.3 Manuscript status and author contributions

Chapter 4 is comprised of the paper "The microbial ecology of a hyper-alkaline spring, and impacts of an alkali-tolerant community during sandstone batch and column experiments representative of a geological disposal facility for intermediate level radioactive waste."

Smith SL, Rizoulis A, West JM, Lloyd JR

Status: Manuscript has been accepted in Geomicrobiology Journal.

Author contributions: Smith SL – principal author, field sampling, microcosm and column experiments, DNA extraction , RISA, UV- vis, CLSM and cell counts ; Rizoulis A – pyrosequencing PCR and processing of pyrosequencing data; West JM (co-supervisor) – concept development and manuscript review; Lloyd JR (principal supervisor) – concept development and manuscript review.

Chapter 5 is comprised of the paper "Microbial impacts on ^{99m}Tc migration through sandstone columns under highly alkaline conditions relevant to radioactive waste disposal."

Smith SL, Boothman C, Williams HA, Ellis BL, Wragg J, West JM, Lloyd JR

Status: Manuscript prepared for submission to PLOS One.

Author contributions: Smith SL – principal author, experimental work, DNA extraction, RISA, cell counts, UV-vis; Boothman C – pyrosequencing PCR and processing of pyrosequencing data; Williams HA – experiments with gamma camera imaging of ^{99m}Tc; Ellis BL – experiments with gamma camera imaging of ^{99m}Tc; Wragg J (co-supervisor) – manuscript review; West JM (co-supervisor) – concept development and manuscript review; Lloyd JR (principal supervisor) – concept development and manuscript review.

Chapter 6 is comprised of the paper "The use of a biological flow apparatus (BFA) to quantify the impact of microbial processes; relevance to intermediate level radioactive waste geodisposal."

Smith SL, Wragg J, West JM, Lloyd JR

Status: Manuscript in preparation (target journal to be determined)

Author contributions: Smith SL – principal author, experimental work, UV-vis, cell counts, MPN, DNA extraction; Wragg J (co-supervisor) - concept development and manuscript review; West JM (co-supervisor) – concept development and manuscript review; Lloyd JR (principal supervisor) – concept development and manuscript review.

Chapter 2

Literature Review

2.1 The UK's nuclear legacy

Production of radioactive waste from the UK's nuclear power industry has been occurring for approximately 60 years, since operations began at a plant at Sellafield, UK (Gray *et al*. 1995). Significant amounts of waste with varying levels of radioactivity are also produced during decommissioning of nuclear power stations (Iguchi and Kaot, 2010). Other sources of radioactive waste include other industrial operations, decommissioning of nuclear weapons (Stefanovsky *et al*. 2004), and from hospitals as a result of the use of radioisotopes for diagnostic and therapeutic applications (Khan *et al*. 2010).

Currently, the total volume of radioactive waste in the UK is 4,720,000 m^3; this includes potential waste from future decommissioning of nuclear power stations (NDA, 2011). Radioactivity levels in the UK's waste vary significantly, and can be categorised depending on activity. Approximately 90% of the UK's total radioactive waste can be classed as low level waste (LLW), and includes items such as paper and clothes from plant operations, and also large amounts of soil and rubble from decommissioning (NDA, 2009). Intermediate level waste (ILW) can be defined as having alpha emissions of greater than 4GBq/tonne and beta/gamma emissions of more than 12 GBq/tonne (NDA, 2008). Although there is only approximately 1000 m^3 high level waste (HLW), it contains around 95% of the total radioactivity of radioactive wastes combined (NDA, 2011). HLW can be classified as waste which may significantly increase in temperature over time (NDA, 2012), and includes the liquid waste that occurs as a result of spent nuclear fuel reprocessing (Ewing *et al*. 1995).

Radioactive waste is defined as material that is contaminated with radionuclides. Radionuclides are capable of decaying via several mechanisms, including alpha, beta and gamma radiation, with varying impacts on human health (Ojovan and Lee, 2005). Several radionuclides are recognised as a priority in the UK's radioactive waste inventory (Table 1), and are included in radiological assessments (Walke *et al*. 2012), although it is recognised that U and Tc remain of the highest priority with regards to ILW and LLW (Prakash *et al*. 2013).

Table 1 Key radionuclides present in ILW, HLW and spent fuel. Half-lives are shown in years (Walke *et al*. 2012).

^{14}C (5700)
^{36}Cl (3.01×10^5)
^{79}Se (2.95×10^5)
^{93}Zr (1.53×10^6) → ^{93m}Nb (16.1)
^{94}Nb (2.03×10^4)
^{99}Tc (2.11×10^5)
^{126}Sn (2.3×10^5)
^{129}I (1.57×10^7)
^{135}Cs (2.3×10^6)
^{239}Pu (2.41×10^4) → ^{235}U (7.04×10^8) → ^{231}Pa (3.28×10^4) → ^{227}Ac (21.8)
^{240}Pu (6560) → ^{236}U (2.34×10^7) → ^{232}Th (1.41×10^{10}) → ^{228}Ra (5.75) → ^{228}Th (1.91)
^{237}Np (2.14×10^6) → ^{223}U (1.59×10^5) → ^{229}Th (7340)
^{242}Pu (3.57×10^5) → ^{238}U (4.47×10^9) → ^{234}U (2.46×10^5) → ^{230}Th (7.54×10^4) → ^{226}Ra (1600) → ^{210}Pb (22.2) → ^{210}Po (0.379)

Radioactive waste may pose a significant threat to the environment if sufficient safety measures are not in place. For example, if discharges to the marine environment occur, the effects may be fatal for some marine organisms. Contamination of marine species that are consumed by humans could also potentially have disastrous effects (Woodhead, 1971). Ionising radiation emitted from radioactive waste can also directly have fatal effects on humans, for example by causing the alteration of genetic material (Cooper *et al*. 2003); previous studies have shown the risk of cancer to increase even with low doses of ionizing radiation exposure (Wilson *et al*. 2010). Because of the harmful effects of radioactive waste on both humans and the environment, an effective method of disposal is required to contain the radioactivity, and to ensure it doesn't interact with the biosphere. As a result of the long-lived nature of some radioisotopes

that comprise radioactive waste (e.g. ^{129}I with a half-life of 16 million years (Lee *et al*. 2006)), a disposal method is needed that sufficiently isolates the waste until it is considered safe (safety must be considered over a period of up to 1 million years; NDA, 2010).

2.2 Geological disposal

2.2.1 UK geological disposal concept

The most appropriate method for the disposal of radioactive waste is considered to be geological disposal, involving several engineered barriers to prevent transport of radionuclides into the biosphere (Arter *et al*. 1991). A geological disposal facility will be located in a carefully selected host rock, up to 1000 m underground (NDA, 2010), although currently, there is no proposed host rock for a GDF in the UK (Vines and Beard, 2012). The composition of the host rock will have significant impacts on its waste containment ability (Poulain *et al*. 2008), as will the tectonic and chemical stability of the rock and surrounding geosphere (Ericsson, 1999). The host rock will be selected with particular consideration of its containment abilities to ensure minimum radionuclide migration over 100,000's years (Anderson *et al*. 2006). Broadly, the most suitable rock types are crystalline or clay rich, and salt (Gens *et al*. 2002). Clay and granite have the advantage of restricting the movement of water (Stroes-Gascoyne *et al*. 2007; Anderson *et al*. 2011), although fractures will significantly reduce this benefit. The UK's geological disposal concept considers a variety of rock types as suitable for a deep geological repository for radioactive waste, and the following rock types have been suggested as being suitable by the Nuclear Decommissioning Authority: higher strength rocks such as granite, lower strength sedimentary rocks such as clay, and evaporites including halite (NDA, 2010). The selected host rock environment will be required to have conditions that are chemically reducing at repository depth (approx. 500m; Bachu and McEwen, 2011), as several key radionuclides are less soluble in their reduced state, potentially inhibiting their migration through the geosphere (Brown and Sherrief, 1999).

2.2.2 Engineered Barrier System

The construction of multiple barriers in the UK's geological disposal concept will ensure maximum containment of radioactivity within a GDF, (West *et al*. 2011). Initially, the radioactive waste will be stabilised by solidifying with materials such as cement (Chen *et al*. 2009), borosilicate glass (Kanwar and Kaushik, 2009), or bitumen (Arter *et al*. 1991) depending on the waste type. The solidified waste will then be encased in a metal canister (*Arter et al*. 1991), which may be surrounded by an overpack, depending on the waste type (Geesey, 1993), and then transferred to the repository (Nair and Krishnamoorthy, 1999). Once the repository has been filled with canisters it will be backfilled; in some cases a natural bentonite buffer will be used, in other cases the backfill will be cementitious (Chapman and Hooper, 2012), again, depending on the waste type.

The different classifications of radioactive waste have varying requirements for their disposal. For example, some LLW may have such a low activity that it does not require deep geological disposal, so disposal in a landfill is considered perfectly safe (Environment Agency, 2010). Higher activity LLW waste requires disposal in a LLW repository. In the UK, this involves deposition into near surface concrete vaults which are backfilled and then capped. Water drainage systems and gas vents are engineered to prevent leaching and a build-up of gases respectively (Beadle *et al*. 2001). A LLW repository is in operation in the UK near Drigg in West Cumbria, where wastes are encased in concrete and disposed of in vaults. Before construction of this site, LLW waste was simply buried in trenches 8 m underground (Daish and Schwemlein, 1996).

ILW and the LLW that is not suitable for near surface disposal are to be disposed of in a deep geological repository. The UK's generic concept involves solidification of the waste with cement encapsulated in a steel canister, with disposal in caverns deep underground in a hard rock (Hicks *et al*. 2008). Because of the potential for heat generation with HLW disposal, solidification of the waste with cement would not be appropriate. Vitrification of the waste is thought to be a more effective way of solidifying the waste, to minimise leaching of radionuclides. The waste will be encased in a stainless steel canister, which will be surrounded by a thick overpack material. In this case, bentonite clay would be a more suitable buffer material because of its low permeability (Baldwin *et al*. 2008).

2.2.3 Disposal concepts outside the UK

Although geological disposal of radioactive waste using a range of engineered barriers is widely accepted as the most appropriate method of disposal in many countries, the concepts vary worldwide, with variations in the most appropriate host rock, canister materials, and backfill materials for example, and also with regards to the actual disposal process. For example, the Swedish concept for HLW involves disposal in crystalline rocks at a depth of approximately 500 m, with the use of copper cannisters surrounded by a bentonite backfill (Ericsson, 1999). The Swiss concept involves the selection of one or two sites for the disposal of HLW and ILW/LLW. The potential sites for HLW disposal all have an Opalinus clay host rock, whereas the potential host rock for ILW/LLW disposal include Opalinus clay, the Effingen Beds and the Brauner Dogger (Nagra, 2014). The Canadian concept will involve an extensive period of monitoring after the waste has been placed in the host rock (Ramana, 2013), whereas concepts from other countries such as the Netherlands, require the waste to be retrievable (Flüeler, 2001). An outline of various worldwide concepts is provided in Table 2.

Table 2 Details of international geological disposal concepts (Blue: HLW, Grey ILW).

UK (NDA, 2010; DECC, 2014)	**Canada** (NWMO.ca/siting process; opgdgr.com)	**Sweden** (SKB.se; wmsym.org)	**France** (Andra.fr)	**Finland** (Posiva.fi)
• Potential host rock- granite, clay, evaporites • Solid glass waste form surrounded by a metal container • Clay backfill	• Potential host rock- unknown • Corrosion resistant containers will be placed at a depth of 500m • Bentonite clay backfill	• Crystalline basement host rock- depth of 500m • Copper canisters • Bentonite buffer	• Host rock- Callovo-oxfordian argillaceous rock • Glass waste form • Stainless steel container	• Olkiluoto bedrock • Ceramic state waste form • Copper and cast iron canisters • Bentonite buffer
• Potential host rock- granite, clay, evaporites • Wasteform solidified with cementitious materials surrounded by stainless steel container • Cementitios backfill	• Host rock- sedimentary. Waste will be placed at a depth of 680m. • Metal/ concrete drums • Backfill- bentonite/ sand mix	• Short-lived ILW: disposed of at Forsmark at depth of 60m • Concrete containers- backfill materials include sand, crushed rock, bentonite. • Long lived ILW- disposed of with HLW	• Waste is compacted • Contained in metal/ concrete packages • Will be disposed of in HLW repository	• Repositories at Olkiluoto and Louisa • 60-100m in bedrock • Resins solidified with cement/ bitumen contained in steel canisters • Canisters contained in steel/ concrete boxes

Netherlands (Covra.nl)	**Belgium** (Ondraf.be)	**Japan** (numo.or.jp)	**Switzerland** (Nagra.ch)
• Boom clay/ salt host rock at a depth of 500m • Glass waste form • Backfill material- ground/ crushed salt/ bentonite	• Deep clay host rock • Vitrified waste form • Carbon steel over pack	• Potential host rock- unknown. Depth will be greater than 300m • Vitrified waste form • Stainless steel canister • Clay buffer	• Opalinus clay host rock • Glass waste form • Metal container • Bentonite backfill
• Boom clay/ salt host rock • Waste solidified with concrete • Concrete/ steel overpack material	• Deep clay host rock • Concrete containers • Cementitious backfill material	• Potential host rock- unknown • Long lived ILW- placed at a depth of 50-100m • Concrete backfill	• Potential host rocks- Opalinus clay, Braunder Dogger, Effingen beds, Marl formations of Helveticum • Solidified waste form contained in metal drums • Concrete conteiners • Concrete backfill

2.2.4 Cement based backfill

The UK's concept for ILW disposal involves extensive use of cementitious materials, in particular as a repository backfill material. Hydraulic cement formulations vary, but are generally comprised of calcium silicates, along with aluminium oxide and iron oxide. Several formulations have been proposed for cement backfills for a GDF, including the Nirex Reference Vault backfill (NRVB), which is composed of Portland cement along with hydrated lime and limestone flour (Butcher *et al*. 2012). Requirements for cement backfills include pH buffering capacity (Serco, 2012), and sufficient permeability to prevent build-up of gases (Iriya *et al*. 1991). A good sorption capacity is also a requirement of a cement backfill, to maximise radionuclide retention (Crossland Consulting, 2007).

2.2.5 Post closure environment

As a result of the extensive use of cementitious materials in a ILW GDF, potentially as a backfill material to surround waste packages, and to backfill access tunnels and other structures, saturation of the GDF with groundwater will lead to the formation of a hyper-alkaline plume with a pH of up to 13.5 (van Aardt and Visser, 1977, Savage *et al*. 2002), initially dominated by sodium and potassium hydroxides, and then over time will become dominated by calcium hydroxide resulting in decreasing pH (Savage and Rochelle, 1993). At this point, dissolution of Ca-Si phases will begin to occur (van Loon and Glaus, 1997), resulting in further pH decreases. Figure 1 describes the evolution of the hyper-alkaline plume over time.

The migration of a hyper-alkaline plume through the surrounding host rock will lead to the formation of an alkaline disturbed zone (Bateman *et al*. 1999), potentially altering the radionuclide retention capacity of the rock. Dissolution of primary minerals such as K-feldspar by high-pH pore waters, coupled with the precipitation of secondary phases such as calcium silicate hydrate (Braney *et al*. 1993) will alter physical characteristics (e.g. porosity and permeability) of the host rock. Steefel and Lichtner (1994) suggest that dissolution of silicate minerals by hyper-alkaline pore waters may lead to porosity increases, although some studies suggest that interactions between high pH pore water and the host rock may in some cases increase containment potential. If the hyper-alkaline pore fluid comes into contact with unaltered groundwater, minerals such as carbonate and sulfate may begin to precipitate (Savage and Rochelle, 1993); this may

lead to sealing of fractures, preventing the transport of radionuclides through the rock (Montori *et al*. 2008).

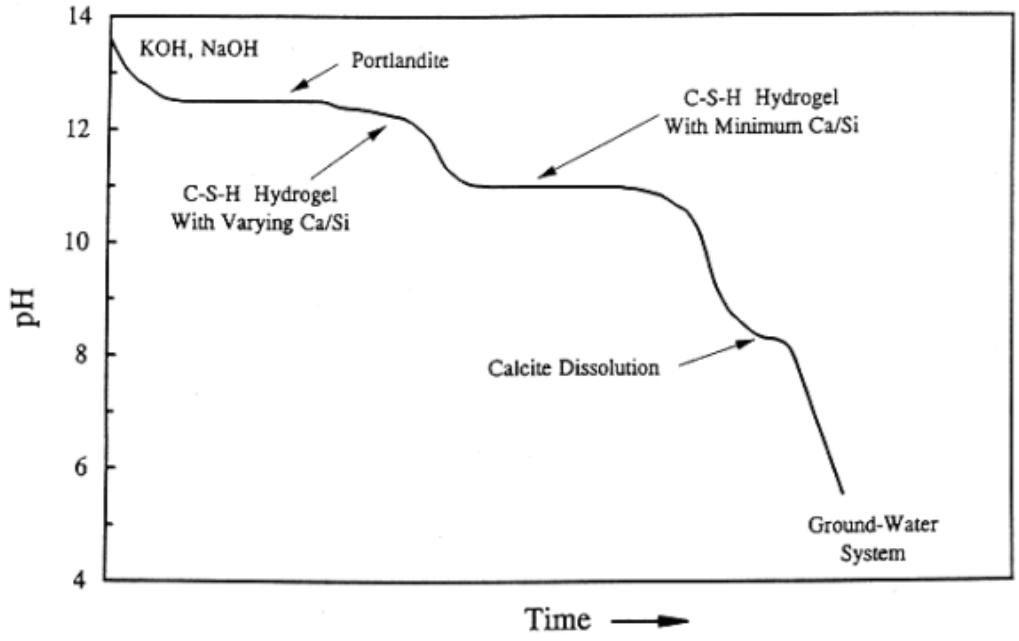

Figure 1 Pore fluid pH evolution over time (Wmsym, 2015)

Results from experiments carried out by Bateman *et al*. (1999) suggest that interactions between calcite and hyper-alkaline fluid resulted in the formation of portlandite, and interactions with quartz led to the formation of calcium-silicate-hydrate (CSH) phases. Some studies have found that the impacts of high pH pore fluid on mineralogy will be minimal, for example Soler and Mäder (2007) carried out experiments investigating impacts of hyper-alkaline fluids on granite and found no alteration occurred with regards to mineralogy and porosity. The point at which these processes occur during the lifetime of a GDF will obviously significantly impact on the extent of potential mineralogical alterations.

Any clay used in a geological repository, whether as a natural or engineered barrier, will be affected by the formation of hyper-alkaline pore waters; for example the high pH will lead to dissolution of smectite (Bauer and Velde, 1999). These reactions have the potential to reduce the capacity of clays to act as a barrier to radionuclide transport (Gaucher *et al.* 2004). Techer *et al* (2006) found that at a natural analogue site in Jordan, hyper-alkaline fluids had caused the precipitation of secondary minerals including gypsum in clays. A potential backfill clay is bentonite; the radionuclide containment ability of bentonite has been shown to be altered by interactions with hyper-alkaline pore waters. For example, the pore fluid may cause chemical and physical alterations such as swelling, and reaction products may include calcium

(aluminium) silicate hydrate (C(A)SH) phases and feldspars (Savage *et al*. 2007; Savage, 2011). High pH pore water interactions with bentonite clay may also facilitate the formation of colloids, potentially increasing the movement of radionuclides in a repository environment (Missana *et al*. 2011). The rate of groundwater flow in a repository environment will to a certain extent determine the potential changes that will occur (Savage and Rochelle, 1993).

Hyper-alkaline pore waters may also interact with repository construction materials such as copper which may be used to produce canisters to encase the waste. Chloride and hydroxide ions in the pore water will compete to complex with copper; if complexation occurs with chloride ions, the formation of a protective Cu(II) coating on the canister may be prevented, leaving the canister exposed (King *et al*. 2010). If steel canisters are to be used, the anaerobic corrosion of the steel by the hyper-alkaline pore waters will lead to generation of H_2 gas, which could have a number of impacts on the properties of a GDF (Environment Agency, 2008).

Initially, after sealing of vaults and access tunnels and closure of the GDF, dissolved oxygen will be present in GDF construction materials and wasteforms, which will be consumed early in the lifetime of a GDF by processes such as copper corrosion, aerobic microbial processes, and perhaps oxidation of minerals such as pyrite for example (Yang *et al*. 2007). Over time, conditions in a GDF will eventually become reducing as oxygen is consumed by these processes. The hyper-alkaline pore fluids will facilitate degradation of cellulosic materials, providing substrates for microbial metabolism, and corrosion of wastes and canisters along with microbial processes will begin to generate gases (NDA, 2010).

Low and intermediate level radioactive wastes often contain large amounts of cellulose in the form of items such as waste tissue paper and clothing (Glaus *et al*. 1999). The hyper-alkaline conditions in a repository environment will lead to degradation of cellulose because of its instability at high pH (van Loon and Glaus, 1997). The alkaline degradation of cellulose produces acidic degradation products including isosaccharinic acid, formic acid, acetic acid and lactic acid (Glaus *et al*. 1999). The formation of such products has the potential to increase the mobilisation of radionuclides through the formation of complexes (Knill and Kennedy, 2003). These processes are thought to occur in the early stages of evolution of a GDF for ILW, as a result of ingress of groundwater generating a hyper-alkaline plume. Because of the hyper-alkaline conditions in the near-field of a GDF, microorganisms will potentially be using cellulose degradation products as a source of energy in the far-field initially, where the pH will have dropped as a result of the loss of Na, K and Ca from solution.

Cellulolytic microorganisms are capable of degrading cellulose under aerobic and anaerobic conditions. Anaerobic cellulose degradation firstly involves cellulolytic and saccharolytic microorganisms producing enzymes capable of depolymerizing cellulose (Leschine, 1995), leading to the production of glucose, which is then fermented to produce H_2 and CO_2, along with a range of organic compounds, which are then converted to acetate and CO_2 (Béguin and Aubert, 1993); the microbial oxidation of a range of these organic compounds can be coupled to the reduction of a range of metals to conserve energy for growth (Lloyd, 2003). Although only a small percentage of microorganisms are capable of degrading cellulose, those that can are extremely diverse (Wilson, 2011), and have complex hydrolytic enzyme systems; many of the cellulases contain catalytic and carbohydrate binding modules that allow the insoluble cellulose substrate to become close in proximity to the catalytic domain, allowing its degradation (Lynd *et al*. 2002). The cellulose degradation products that form under alkaline conditions include potential electron donors for microbial processes (Glaus and van Loon, 2008). Isosaccharinic acid (ISA) is known to be one of the main products resulting from alkaline degradation of cellulose; recently, it was shown that microorganisms could degrade ISA under alkaline conditions, and couple this process to the reduction of Fe(III) and nitrate (Bassil *et al*. 2014).

2.2.6 Development of microbiological studies in the context of radioactive waste disposal

It has long been recognised that microorganisms will impact on processes that will occur in a GDF for radioactive waste, although their potential role in the alteration of radionuclide migration through the geosphere was initially ignored (Bachofen, 1990), and subsurface environments were considered to be sterile. Early studies concerning the geomicrobiology of radioactive waste disposal, for example that of West and McKinley (1985) recognised that although conditions in a GDF environment will be extreme, microbial processes could potentially impact on engineered barriers and the host rock, which could subsequently impact on radionuclide migration through the geosphere. Early studies of subsurface environments of relevance to radioactive waste disposal indicated that microbial processes could have significant impacts on radionuclide transport. For example Pedersen and Ekendahl (1990) provided evidence that microorganisms present in deep groundwater from granitic bedrock were capable of utilising a range of organic substrates to gain energy. Other studies examined other subsurface environments including mines to investigate some of the potential microbial processes that could occur in a GDF environment. Results indicated that in a highly

alkaline groundwater sample from Mol (pH 12.7), no culturable microorganisms were detected (Christofi *et al*. 1985). Results also suggested that potential repository backfill materials may stimulate microbial populations. Once it became clear that subsurface environments were in fact host to diverse microbial populations, the potential constraints on microbial growth in a GDF could be investigated.

A modelling approach was undertaken by Grogan and McKinley (1990) and considered a range of microbial mechanisms that could impact on radionuclide mobility, including the chemical environment, alterations to physical properties of engineered and natural barriers, and microbial radionuclide uptake. Other studies, for example that of Stroes-Gascoyne (1989) made predictions of microbial populations in a Canadian repository concept using nutrient and energy budget calculations, and found that disposal vaults would be capable of supporting microbial growth. Natural analogue studies then began to provide further details on microbial processes that could potentially occur under conditions of relevance to geological disposal of radioactive waste (e.g. West *et al*. 1992) although these early approaches generally used culturing techniques along with enumeration methods.

Experimental programmes carrying out research projects simulating potential repository conditions were also undertaken, along with investigations into some of the fundamental microbial interactions with radionuclides (e.g. Lovely *et al*. 1991). Results from many of these studies will be discussed later in the chapter, along with some of the more recent developments in the field of the geomicrobiology of radioactive waste disposal.

2.3 Microbial ecology of terrestrial subsurface environments relevant to ILW disposal

A GDF will likely be host to a diversity of microorganisms from a variety of sources, including contamination from the surface during the operational period of the repository. Microorganisms will also be associated with the radioactive waste itself; for example a range of studies have demonstrated a microbial presence in spent fuel ponds, with bacterial genera including *Ralstonia* (Sarro *et al*. 2005) and *Burkholderia* (Chicote *et al*. 2005) identified. Extensive studies have also been carried out characterising microbial populations indigenous to subsurface environments, which are discussed in detail throughout this chapter. According to Ghiorse and Wilson (1988), studies of subsurface microbial processes started to occur in the mid-1980's, before which, microbial

populations in the terrestrial subsurface had never really been considered. A range of studies have been carried out since, investigating the microbial populations that occur in subsurface environments, including those discussed below.

2.3.1 Limits to microbial life in the deep geosphere

The extreme conditions in the deep geosphere may limit the types of microorganisms that can survive there, and the processes that they may carry out. Sufficient sources of energy and water must be present in the deep geosphere if microbial life is to occur. Gold (1992) suggests that chemical energy must be available, but must occur in a form that enables liberation by microorganisms. Microbial adaptations, including the ability to harness energy produced by interactions between groundwater and rock, are advantageous to microorganisms living at depth (Krumholz, 2000). Water availability is a significant factor in determining the presence of microbial life in the geosphere; many environmental conditions, such as solute concentrations can impact upon the amount of water available (Colasanti *et al*. 1991). The presence of several inorganic elements including carbon, nitrogen, phosphorus and sulfur are a requirement for microbial life, along with a range of inorganic ions such as Fe and Mn. Organic matter availability and the presence of gases such as H_2 and CH_4 will also impact on the ability of a subsurface environment to sustain microbial life (Fredrickson and Balkwill, 2006).

A range of environmental conditions are required for microbial life to occur in the geosphere. These include sufficient pore space for the microbes to be transported through (Gold, 1992). It has previously been found that microbial metabolic activity and reproduction are limited when pore sizes become too small (Krumholz *et al*. 1997). Other limitations to life at depth include high temperatures, as generally a temperature increase of 3°C occurs every 100m (Fredrickson and Balkwill, 2006). High pressure may also become a limiting factor for microbial life in the deep geosphere, although at the potential depths for a geological disposal facility, this will likely not be a problem.

In some rock types such as mudstone, the low porosity may be limiting to microbes, although the potential for a large organic content within the rock could support a range of microorganisms; previously sulfate reducers and anaerobic lithotrophs have been found in mudstone. Several characteristics of sedimentary rock are also thought to be beneficial to microbial populations, for example a high Fe content (Humphreys *et al*. 2010); previously investigations of sandstone formations have revealed the presence of

sulfite reducing bacterial populations (Powell *et al*. 2003), along with sulfate reducing bacteria (Krumholz *et al*. 1997).

Because of the unfavourable environmental conditions in the deep subsurface, bacterial cells implement several mechanisms to enable survival, including sporulation and dormancy (Poulain *et al*. 2008). Under nutrient poor conditions, many species may have growth rates up to 1000 times slower than those which occur in surface environments (Geesey, 1993). It has been estimated that buried organic carbon is responsible for an energy flux which is less than 1% of that provided by surface carbon resulting from photosynthetic fixation (Whitman *et al*. 1998). Other estimates of subsurface microbial cell turnover have been made using a number of approaches, including the use of amino acid stereo-isomers to calculate turnover times; estimates range from 200-2000 years (Lomstein *et al*. 2012). The limiting conditions may initially only support certain microbial species, although these species may alter the local conditions to such an extent that development of less specialised communities may occur (McCabe, 1990).

2.3.2 Microbial presence in relevant formations

Studies investigating microbial populations in the deep subsurface have revealed diverse communities that are metabolically active, and are capable of influencing their surrounding environmental conditions. Total cell numbers in subsurface environments are variable, for example Pedersen *et al*. (2008) counted 5.7×10^4 cells mL^{-1} in a deep groundwater environment; Whitman *et al*. (1998) calculated the average number of cells in groundwater from a range of studies carried out on deep subsurface samples and suggest 1.54×10^5 cells mL^{-1} as an estimate. As these studies focus on unattached cells in groundwater, actual cell numbers in the subsurface may be much higher because of the presence of attached cells which may be difficult to estimate as the ratio of attached: unattached cells can vary between environments (Whitman *et al*. 1998).

The presence of microbial communities in a GDF could potentially impact on radionuclide migration via several mechanisms, therefore, characterisation of subsurface microbial communities is necessary to fully appreciate how the biogeochemical conditions in a GDF may develop over time. Microbial communities from a variety of rock types potentially suited to the geological disposal of radioactive waste have previously been investigated; results from several of these studies are discussed below.

Diverse bacterial communities were identified in groundwater associated with the Äspö Underground research Laboratory, including members of the genera *Acinetobacter*, *Desulfovibrio* and *Thiomicrospira*, along with clones demonstrating sequence similarity to bacteria previously identified in a hydrothermal vent (Pedersen *et al*. 1996). In Fennoscandian Shield reducing groundwaters, iron reducing bacteria, sulfate reducing bacteria and acetogenic bacteria were identified at a depth of 304-309 m (Haveman and Pedersen, 2002). Studies investigating microbial populations in bentonite clay used in sealing experiments in underground laboratories have identified culturable sulfate and nitrate reducing bacteria (Stroes-Gascoyne *et al*. 2007), and direct DNA extraction from bentonite identified members of the genera *Bacillus* and *Paenbacillus* (Fru and Athar, 2008). Although these DNA extractions from bentonite clay proved successful, other studies have found difficulties in extracting microbial DNA from opalinus clay; Poulain *et al*. (2008) carried out enrichment cultures to investigate microbial populations in opalinus clay and isolated a member of the genus S*phinogomonas,* and an isolate closely related to members of the genera *Bacillus* and *Alicyclobacillus*. Microbial populations at the Grimsel Test Site in Switzerland (450 m depth) in granitic formations have been investigated, where from enrichment cultures, bacteria belonging to the genera *Acetobacterium*, *Haloanaerobium*, *Bacillus* and *Pseudomonas* were identified (Gillow *et al*. 2000).

Investigations of archaeal populations in terrestrial subsurface environments have revealed diverse populations at significant depths. At depths of up to 190 m in Cretaceous shale/ sandstone formations, archaeal families related to *Methanobacteriaceae* and *Methanosarcinaceae* (Takai *et al*. 2003) were observed. At similar depths in a Paleosol environment, archaeal clones were identified exhibiting close associations with clones obtained from a Yellowstone National Park thermal spring (Chandler *et al*. 1998). Members of the genera *Methanohalophilus* and *Methanobacteria* were found to be associated with groundwaters collected from the granitic Äspö Underground research Laboratory.

Terrestrial subsurface viral populations are known to have significant roles in biogeochemical cycling in deep terrestrial environments (Anderson *et al*. 2013), as a result of their ability to release nutrients and compounds that can be utilised by bacterial and archaeal populations, and their impacts on bacterial and archaeal population dynamics. Predation of bacterial and archaeal populations in the subsurface by viral populations may play a role in keeping these populations in a steady state (Kyle *et al*. 2008); viruses may do this by carrying out processes such as lysing cells during biofilm development (Resch *et al*. 2005). Viruses are known to be able to penetrate the

extracellular matrix of biofilm structures (Anderson *et al*. 2013). Investigations of subsurface viral populations (granitic environment at a depth of 447 m) have revealed the presence of viral families including *Siphoviridae*, *Podoviridae*, and *Myoviridae*.

2.3.3 Subsurface microbial processes

Diverse microbial populations are capable of living in anoxic environments. Some of these may be facultative anaerobes, which can survive in the absence of oxygen, but flourish in its presence; obligate anaerobes cannot survive in the presence of oxygen because of an inability to detoxify oxygen radicals (Heritage *et al*. 1996). During the construction and operational period of a geological disposal facility, oxygen will become trapped in the pore space of rocks, so microbial activities will initially involve some aerobic processes (Yang *et al*. 2007). As the oxygen is used up by aerobes, conditions will become anoxic, unless the groundwater is delivering extremely small concentrations of oxygen (Arter *et al*. 1991). Microorganisms are known to catalyse a range of subsurface redox processes to gain energy for growth. Several terminal electron accepting processes (TEAPs) have been identified as important in geological environments. Microorganisms are capable of coupling the oxidation of organic matter present in subsurface environments to the reduction of a range of terminal electron acceptors including nitrate, Fe(III), sulfate, and Mn(IV) (Lovley and Chapelle, 1995). Mechanisms include the use of an electron shuttle, or direct transfer at the cell surface (Lloyd, 2003). Several bacterial species may be able to gain significant amounts of energy from these processes (Dong, 2008). Microbial Fe (III) reductase activity is thought to occur predominantly in the cell membrane (Gaspard *et al*. 1998; DiChristina *et al*. 2002). Several microbial species, including *Shewanella putrefaciens* possess both constitutive and inducible Fe(III) reductases; in the presence of substantial quantities of Fe(III), this microbial species is able to increase the rate at which it reduces Fe(III) (Johnson and Bridge, 2002).

Other mechanisms of microbial metal reduction include electron transfer along nanowires (Clarke *et al*. 2011). *S. oneidensis* cells possess multihaem c-type cytochromes on their outer membrane, which can form complexes and form nanowires to transfer electrons from the quinine/quinol pool in the cells inner membrane (Shi *et al*. 2012) to the outer membrane (Shi *et al*. 2009). In *Geobacter* species it has been suggested that cells use chemotaxis to locate Fe(III) oxides, and they use pili to interact with the mineral surface (Mehta *et al*. 2005).

Figure 2 outlines these process and other organic matter decomposition pathways that may occur in subsurface environments. Reduction of such electron acceptors produces a gradient of H^+ or Na^+ which is used to synthesize ATP (Ljungdahl *et al*. 2003).

Acetogenic microorganisms are capable of producing acetate from one carbon compounds such as CO_2. The reduction of these carbon compounds leads to acetate generation, and several acetogens are capable of growing with H_2/CO_2 as their only source of energy and carbon (Ljungdahl, 1986). Some microorganisms are also capable of reducing organic acids under anoxic conditions. These fermentative processes occur when the organic substrate is oxidised, coupled to the reduction of another organic substrate (or an intermediate product that has been derived from the oxidation of the original substrate; Müller, 2001). Microorganisms are also capable of utilising a number of pathways to generate methane, using substrates such as CO_2 and acetate as the terminal electron acceptor; methanogens are not capable of producing methane from the degradation of some complex carbon containing molecules (Zinder, 1993).

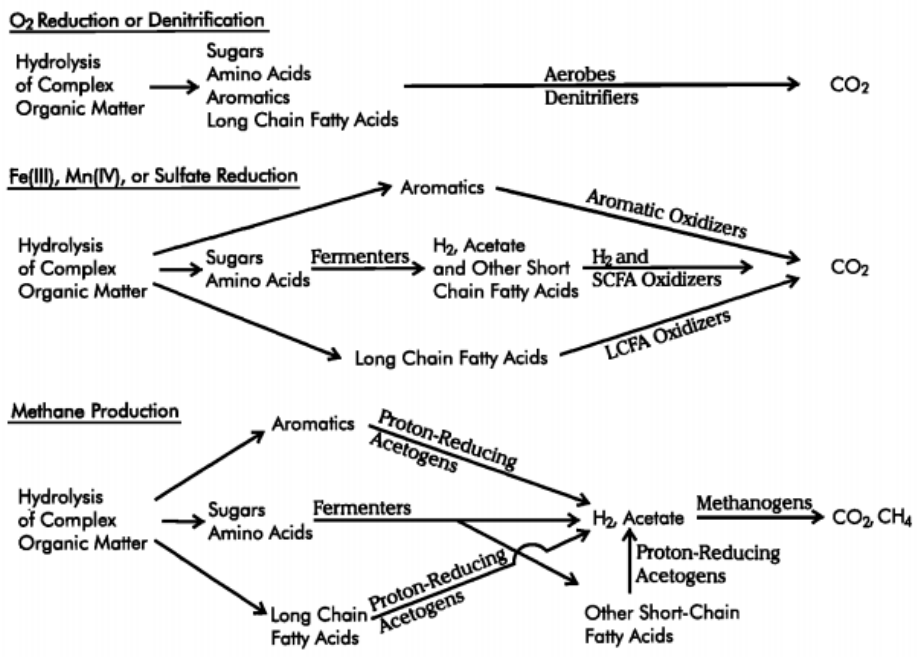

Figure 2 Subsurface organic matter decomposition processes (from Lovley and Chapelle, 1995).

2.3.4 Microbial adaptations to extreme environmental conditions

In a cementitious GDF for ILW, a range of extreme environmental conditions will occur, potentially impacting on the composition of microbial communities, and their potential function.

2.3.4.1 Ionizing radiation

Several bacterial species have been isolated that can withstand exceptionally high doses of radiation, including *Deinococcus radiodurans* which is able to withstand 5000 Gy γ-irradiation (Confalonieri and Sommer, 2011). It has been shown that even radiation sensitive species can adapt over time to become significantly radiation resistant. Harris *et al* (2009) demonstrated that by exposure to ionizing radiation over time they were able to direct *Escherichia coli* evolution to become resistant to high doses of radiation. At high levels of radiation exposure and high temperatures, DNA damage including double strand breaks and the removal of bases such as cytosine may occur (Confalonieri and Sommer, 2011), and may cause DNA lesions triggering apoptosis (Roos and Kaina, 2006). Many DNA repair mechanisms have been identified, enabling a certain degree of resistance to environmental factors damaging to DNA (Macaskie and Lloyd, 2002).

Several types of DNA damage may be reversed through repair mechanisms, including the removal of the damaged section of DNA and insertion of a new sequence (Friedberg, 2003). Bacterial species with very high resistance to ionising radiation, such as *D. radiodurans* have particularly efficient DNA repair mechanisms; this species can reassemble its genome after being shattered by exposure to radiation (Zahradka *et al*. 2006). It has been found that this species has significantly large number of genes involved in processes such as the regulation of transcription (Blasius *et al*. 2008). *D. radiodurans* cells are capable of protecting their intracellular proteins by accumulating manganese ions; the ions help to decrease amounts of radioactive oxygen species within the cell (Daly, 2009). Previous studies have shown that cells resistant to radiation contained manganese concentrations 300 times that of cells sensitive to radiation (Gross, 2007).

2.3.4.2 Heavy metal tolerance

Microorganisms inhabiting a deep geological disposal site for radioactive waste will have to be extremely robust and possess resistance mechanisms to enable them to tolerate exposure to a range of heavy metals. Several bacterial species, including *Bacillus subtilis* are resistant to heavy metals such as uranium, and may sequester them in their cells. Resistance mechanisms include an enhanced ability to bind metals in the cell wall (Suzuki and Banfield, 2004).

2.3.4.3 Hyper-alkaline environments

The hyper-alkaline nature of the pore fluids will affect the composition of microbial communities in a GDF for ILW. Some of the microorganisms indigenous to the host rock environment may not be able to tolerate the high pH environment which will form, for example the bacterium *Caloramator fervidus* is only capable of growth in the pH range 6.3-7.7 because of its inability to extrude or uptake H^+ ions (Padan *et al*. 2005). Other microbes may be able to adapt to the conditions, and some microorganisms may require a hyper-alkaline environment to thrive. Padan *et al* (2005) suggest that the distinction between growth and survival at high pH is an important one, as microorganisms exhibiting the ability to grow at high pH may exploit different mechanisms of pH tolerance to those which are able to simply survive.

Microorganisms that are capable of growing within a wide pH range are able to do so because of various cellular adaptations. Alkaliphilic microorganisms grow in an optimum pH range of between 9 and 12 (Horikoshi, 1999); other microbes may be tolerant of the high pH conditions but experience significantly reduced growth rates (Ulukanli and Diğrak, 2002). Neutralophilic bacteria may experience an inhibition in respiration at high pH (Krulwich, 1995) although some may be able to grow up to a pH of around 9 by maintaining their cytoplasmic pH at around 7.5 (Krulwich *et al*. 2011). Alkaliphilic microbial species are diverse and include several haloalkaliphilic archaebacteria, many of which have been isolated from naturally occurring high pH environments such as soda lakes (Grant *et al*. 1990). These environments are often very diverse because of factors such as the constant supply of CO_2 (Duckworth *et al*. 1996).

Microbial species that are obligate alkaliphiles are often unable to grow below pH values of around 9; it is thought that this is a result of the lipid composition of their cell membranes (Krulwich and Guffanti, 1992). Microbial growth is inhibited at hyper-

alkaline pH values, and the upper limit for growth is thought to be pH 12 (Sorokin, 2005). Adaptations of alkaliphilic bacteria allow maintenance of their optimum cytoplasmic pH (Padan *et al*. 2005). These adaptations include an increase in the activity of cation/proton anitporters (Padan *et al*. 2005), where proton movements are coupled to exchange with cations such as K^+ which are energetically beneficial to the cell (Slonczewski *et al*. 2009). Potassium removal may be necessary to increase the acidity inside the cell compared to outside (Pitryuk *et al*. 2002). Alkaliphilic bacteria possess mechanisms of creating ionic gradients, which increases their ability to adapt their internal ion concentrations, allowing them to better cope with high pH conditions (Pitryuk *et al*. 2002). In the cytoplasm of a cell, the presence of proteins that contain ionisable groups help to buffer the internal pH (Slonczewski *et al*. 2009). In some species such as *B. lentus*, when grown under high pH conditions, acidic polymers such as teichuronic acid are produced at a greater rate than when at neutral pH (Aono *et al*. 1995).

Changes in cell surface layers may be of importance in regulating internal pH under alkaline conditions. The fatty acid composition of the cell membrane may have influence over how permeable the membrane is to protons (Hall *et al*. 1995). The presence of bases such as triethanolamine permanently contained within the cell membrane also help to decrease the internal pH of the cell (Slonczewski *et al*. 2009).

Diverse microbial communities have previously been identified in subsurface and surface hyper-alkaline environments, and employ a range of metabolic processes. For example Brazelton *et al* (2012) identified bacteria living in hyper-alkaline environments (a carbonate chimney and springs that have formed in a travertine deposit) that were capable of utilizing H_2. Methylotrophic bacteria have been identified in Lonar Lake (pH 9.5-10), and have been shown to influence carbon cycling under alkaline conditions (Antony *et al*. 2010). West *et al*. (1994) identified the presence of sulfate reducing bacteria in the highly alkaline waters (pH ≤12.9) at Maqarin, Jordan, and were thought to significantly influence the geochemical processes occurring at the site. In microcosm experiments, Rizoulis *et al*. (2012) investigated electron acceptor utilization under a range of alkaline pH values using sediment collected from Harpur Hill, an anthropogenic hyper-alkaline spring. Results from these experiments indicate that microorganisms indigenous to the site were capable of reducing a variety of electron acceptors including nitrate, sulfate and Fe(III)-citrate at pH 11, with significant inhibition of these processes at pH 12.

Initially, the hyper-alkaline plume which will develop in a cementitious GDF for ILW will likely limit microbial activity to the far-field (the pH of the plume will decrease with

increasing distance from the engineered barriers), as here the pH will be more favourable to microbial activity because of the loss of Ca, K, and Na from solution with increasing distance from the engineered system. These processes will also occur in the near-field over 1000's to 10,000's years, decreasing the pH of pore waters over time, and here conditions will eventually become more favourable for microbial activity. The time that this will take is dependent on factors including host-rock type, and groundwater velocity.

2.4 Impacts of microbial processes on subsurface transport

2.4.1 Biofilm formation

The transport properties of a subsurface environment may be significantly altered by biofilm formation within pore spaces or on fracture surfaces (Humphreys *et al*. 2010). Microbial cells can exist in either a planktonic state, or as part of a multicellular biofilm (Dunne, 2002). Microbial cells have a tendency to form biofilms, perhaps as a result of nutrient concentration on surfaces (Baty *et al*. 2000), and biofilm formation has been shown to be induced when environmental conditions become stressful. As an example, some species of *Myxococcus* form biofilms in response to nutrient limitations (Davey and O'toole, 2000). Biofilm formation is advantageous to individual cells as protection is provided against extreme and fluctuating environmental conditions including pH and temperature; the close proximity of cells within a biofilm structure allows the development of a homeostatic environment (Hall-Stoodley *et al*. 2004).

Prior to biofilm formation, both organic and inorganic molecules are carried towards the surface and may accumulate; these molecules (which can include proteins) may alter the physical and chemical properties of the surface. Microbial cells that are present in the bulk liquid may come into contact with a surface by a variety of mechanisms, including convective mass transport and Brownian motion (Palmer *et al*. 2007). The surface charge of bacterial cells has been shown to be influenced by pH (Husmark and Rönner, 1990), potentially impacting the attraction of cells to surfaces. Other cell wall properties have been shown to influence attraction to surfaces, including hydrophobicity. The roughness of a surface will also influence how easily cells may attach to it (Palmer *et al*. 2007).

Once bacterial cells have attached to a surface, the expression of several biofilm specific genes will be promoted (Costerton *et al*. 1994), allowing for biofilm development and structure maturation (Davey and O'toole, 2000). For example in

Pseudomonas aeruginosa, once the bacterial cells have attached to a surface the *psl* operon is expressed increasing adhesion of the cells to the surface and each other (Flemming *et al*. 2007).

Biofilms consist of bacterial cells encased in extracellular polymeric substances (EPS) (Flemming *et al*. 2007), consisting of proteins, polysaccharides and extracellular DNA (eDNA) (Sutherland, 2001). The formation of the EPS is controlled in some species by quorum sensing; a method of bacterial cell communication involving the secretion and detection of signal molecules (Hammer and Bassler, 2003). The timing of the production of EPS components is controlled in this way, for example in some species such as *P. aeruginosa*, large amounts of eDNA are produced early on in biofilm development, enabling the strain to become established (Böckelmann *et al*. 2006). Quorum sensing is also known to regulate physiological processes in bacteria, allowing for the transition from a planktonic to a sessile state (Cvitkovitch *et al*. 2003), and also cell attachment and separation from the biofilm (Donlan, 2002). The nature of the environmental pressures selects for phenotypes better able to tolerate the conditions that are present (Kirisits *et al*, 2005). Viruses have also been shown to exist in a biofilm like structure, and may release extracellular carbohydrates to encase themselves (Thoulouze and Alcover, 2011).

2.4.2 Biofilm architecture

Biofilms can be comprised of single or multiple species, and the cells that are contained within the structure behave in a cooperative manner (Davey and O'toole, 2000). Conditions within the biofilm can vary dramatically across the structure, including variations in pH, nutrient availability and composition of the microbial community (Davey and O'toole, 2000). The composition of the EPS also determines environmental factors such as porosity and water content of the structure (Flemming *et al*. 2007). These variations can lead to niche differentiation, increasing survival ability of the individual cells (Beyenal *et al*. 2004). Changes in chemical gradients and differences in gene expression between cells can lead to an extremely heterogeneous structure (Stewart and Franklin, 2008). The EPS also provides a significant amount of protection for bacterial cells, for example by preventing antimicrobial agents diffusing into the biofilm (Davey and O'toole, 2000; Kaplan, 2010). Because of the close proximity of the individual cells within a biofilm, horizontal gene transfer can occur (Davey and O'toole, 2000); this process can increase the survival ability of the cells if they acquire genes advantageous for life in a biofilm (Kirisits *et al*, 2005), such as genes that code for the

production of the EPS (Fux *et al*. 2005). Bacterial cells may also increase the affinity of binding proteins to utilize nutrients more successfully (Roszak and Colwell, 1987). Individual cells tend to be more successful as part of a biofilm; the high cell densities and biofilm structure are advantageous, and often support symbiotic relationships (Dunne, 2002). This has been demonstrated by Hansen *et al* (2007), who found that the presence of *Acinetobacter* sp. enabled the survival of *P. putida* by providing a waste product (benzoate) that *P. putida* could use as a carbon source.

Bacterial biofilms are complex structures, and must ensure that the necessary nutrients can be transported to all cells within the structure and that waste products can be removed (Beyenal *et al*. 2004). Within the EPS, fluid filled channels are present (Kaplan, 2010); it has been demonstrated that within these channels there are connective flow patterns, facilitating a more homogenised environment for the individual cells (Costerton *et al*, 1994). As the EPS is comprised mainly of water, this is an effective mechanism for transporting substances around the structure.

2.4.3 Biofilm dispersal

Under certain circumstances it may no longer be beneficial for a bacterial cell to be a member of a biofilm community. Severe competition for nutrients can cause cells to leave the biofilm (Dunne, 2002), as can a build-up of metabolic by-products that may prove toxic (Kirisits *et al*. 2005). Cells may leave the structure individually, or as clumps depending on properties of fluid surrounding the biofilm structure (Donlan, 2002). Biofilms can actively disperse when conditions are not favourable, for example through the release of enzymes to degrade the EPS (Kaplan, 2010). Under nutrient poor conditions, bacterial cells may become motile and successfully move to an area richer in nutrients. In this case, the cells which are loosely involved in a biofilm structure will be the most successful, as they can easily move when nutrients become limiting. Passive dispersal may occur as a result of physical impacts on the biofilm, including shear as a result of movement of liquid over the biofilm (Sauer *et al*. 2004).

2.4.4 Biofilm impacts on transport in porous media

Microbial processes in the deep geosphere can have significant impacts upon the chemical and physical characteristics of the surrounding rock (Stroes-Gascoyne *et al*. 2007). Many bacterial species present in deep geological environments will form

biofilms, the presence of which can have profound impacts on the transport properties of the geosphere (Humphreys *et al*. 2010). Local geochemical conditions impact upon the composition and processes which occur within the biofilms (Anderson *et al*. 2007). Because of the extreme conditions that bacterial cells will encounter in the deep geosphere, biofilms tend to form very slowly; studies have shown that development of a biofilm on rock can take a few months, once exposed to groundwater (Anderson *et al*. 2007), and when they do form in geological substrates they can trap particulate matter (Coombs *et al*. 2008). The potential impact of a biofilm on a rock surface is dependent on several factors including biofilm thickness and rock type (Coombs *et al*. 2010), and pore connectivity, shape and size distribution (Brovelli *et al*. 2009).

Fracture pathways in the geosphere allow the transport of fluids, are pathways for the movement of microbes (Humphreys *et al*. 2010) and provide an ideal surface for the growth of biofilms (Anderson *et al*. 2006). The interface between the rock surface and fluid can be altered by the formation of a biofilm on the surface (Pedersen, 2005). The rock fractures provide a surface for processes such as ion exchange and metal adsorption (Anderson *et al*. 2007). These surfaces are particularly important in crystalline rocks, because of the lack of interconnectivity in pore space (Fredrickson and Balkwill, 2006).

If a porous biofilm forms on a rock surface, its effect on fluid transport may be minimal (West *et al*, 2002), however in many cases the presence of a biofilm may block pore channels, for example through the release of extracellular polysaccharides that close pore spaces (Arter *et al*, 1991). As a result of the porous nature of biofilms, transport through a biofilm structure may occur via convection (De Beer and Scramm, 1999). When fluid flows through a pore channel containing a biofilm, the extracellular matrix becomes saturated and trapped particulate matter block pores, restricting fluid flow (Coombs *et al*, 2008). This has been demonstrated by Harrison *et al* (2011), who found that in flow experiments, increases in injection pressure into mudstone occurred because pore spaces become blocked by biofilms. Other hydrodynamic properties of the pore space may be altered through biofilm formation, including the frictional resistance of the pores (Cunningham *et al*. 1991). Predictive models showing the accumulation of biomass demonstrate the development of preferential flow paths within the porous media (Thullner *et al*. 2002). Microbes may induce several other types of changes in the hydraulic properties of a geological environment, for example some studies have shown that secretions of bacterial compounds including polypeptides may cause decreases in surface tension (Rockhold *et al*. 2002).

2.4.5 Gas production in a GDF and its impacts on repository performance

A range of processes may potentially occur in a GDF environment that result in production of gases which may lead to a pressure build-up, potentially impacting the transport properties of the host rock. For example hydrogen gas will be produced as a result of steel (a potential canister material) corrosion (Grauer *et al*. 1990). Several metals have been proposed for the production of canisters to be used for radioactive waste disposal, including copper, carbon steel and nickel alloys (King and Padovani, 2011). One of the factors determining which metal will eventually be used for the canister material is the corrosion performance, which will be affected by repository properties such as groundwater composition (King and Padovani, 2011), composition of cement pore solution and temperature (Kursten *et al*. 2011).

Gas generation may be potentially hazardous in the case of geological disposal, as the gases may be corrosive and may eventually lead to corrosion of radioactive waste containers (Geesey, 1993). Microbial activity may affect the corrosion performance of the canister material in several ways, either by direct contact in the case of microbes using the canister as a source of energy (West and McKinley, 2003), through alteration of the groundwater chemistry that will come into contact with the canister (Ericsson, 1999), or through the alteration of electrochemical processes (Humphreys *et al*. 2010). For example in the presence of sulfate reducing bacteria, the production of hydrogen sulfide would lead to corrosion of a copper canister (Pedersen, 2002). Microbial activity may also be responsible for the production of other gases such as ammonia or carbon dioxide, which would lead to corrosion of the canister material (Geesey, 1993).

Microbial processes may be able to both stimulate and mitigate these processes. Microbial processes, including organic matter oxidation or fermentation, that lead to gas production, may cause pressure increases, and allow the formation of channels enabling increased fluid flow within the host rock (West *et al*, 2002; Humphreys *et al*, 2010). Other microbial processes may be able to mitigate pressure increases that occur as a result of gas production in a GDF, for example a number of microorganisms are capable of utilising H_2 as an electron donor (Liu and Conrad, 2011).

2.5 Microbial impacts on mineralogy

2.5.1 Microbe-mineral interactions

The porosity of a geological material is thought to have the greatest influence on fluid transport through the substrate (Neretnieks, 1980); the degree of interconnectivity of the pore space is one of the determining factors in predicting flow velocity (Walsh and Brace, 1984). Although the presence of biofilms can physically block pore spaces, they can also alter the porosity of the rock in other ways, including chemically altering the surfaces of the rock (Coombs *et al*. 2010, Wagner *et al*. 2013). This may result in changes to pore water chemistry (Charbonneau *et al*, 2006).

Microbial alteration of minerals may cause changes in the physico-chemical properties of the host rock. For example, the microbial production of organic acids may lead to mineral dissolution (Barker and Banfield, 1998). Microbial reduction of metals may lead to changes in the physical characteristics of the host rock; for example dissimilatory reduction of Fe(III) to the more soluble Fe(II) (Lovley, 1997).

Biofilms may alter the porosity and permeability of rock as a result of their ability to aggregate with colloidal materials (Gadd, 1996). Bacterial cells may release adhesions which bind to specific sites on particular surfaces (Ubbink and Schär-Zammaretti, 2007). If bacterial cells aggregate with particles such as clay, pore spaces may become blocked, decreasing the permeability of the rock through the formation of clay minerals (Behrends *et al*. 2012). If fluid flow through a fracture is reduced significantly, processes including precipitate formation and metal adsorption cannot occur on the rock surface (Anderson *et al.* 2007).

2.5.2 Microbial mineralization

Microbial cells are capable of inducing, and controlling mineralization processes. Microbial cells may act as nucleation and growth sites for mineralization. They are also capable of precipitating many minerals extracellularly, for example sulfide minerals such as pyrite, iron oxides, and manganese oxide by a variety of mechanisms (Ehrlich, 1999). Extracellular mineralization by microorganisms occurs initially by the production of a matrix outside of the cell comprising proteins, polysaccharides or glycoproteins. Cells may then transfer cations out of the cell to the matrix which acts as a nucleation and growth site for minerals (Weiner and Dove, 2003).

As biofilms contain such high densities of bacterial cells, rates of mineral precipitation may be high (Douglas and Beveridge, 1998, Ehrlich, 1999) where biofilms are present. Carbonate formation can occur when bacterial cells alter their microenvironments to become alkaline (Douglas and Beveridge, 1998). Induction of carbonate precipitation by bacterial cells has been shown to have the potential to increase concrete durability (De Muynck *et al*. 2008, Cuthbert *et al*. 2013); this process could potentially impact on the transport characteristics of a GDF. Microbially induced calcium carbonate precipitation may be of particular importance in the ADZ of a cementitious repository, because of the calcium rich nature of the pore fluids; it is thought that most bacterial species are capable of inducing the precipitation of calcium carbonate under favourable conditions (Boquet *et al*. 1973). More recent studies have shown that this process is capable of removing calcium from calcium rich waters (Hammes *et al*. 2002). Microorganisms are capable of both directly and indirectly causing the precipitation of calcium carbonate; the rate at which this process occurs is known to be influenced by a range of environmental conditions including the concentration of Ca^{2+}, inorganic carbon concentration, pore water pH, and the presence of nucleation sites for crystal growth (Hammes and Verstraete, 2002).

Microbially induced calcite precipitation has previously been linked to the fermentation of organic acids, which may be of importance during ILW disposal because of the predicted extensive organic acid production as a result of the degradation of cellulosic materials. The fermentation of organic acids including propionate, acetate and butyrate leads to the production of methane and H_2CO_3; the production of CO_3^{2-} during this process has been shown to be responsible for the precipitation of calcium carbonate (VanGulck *et al*. 2003).

Microorganisms are also capable of facilitating the precipitation of numerous other minerals, including sulfides such as Pyrite and Sphalerite, oxides such as Magnetite, sulfates such as Gypsum and Barite, and phosphates such as Vivianite (Weiner and Dove, 2003).

2.6 Microbial interactions with radionuclides

Radioactive waste is a heterogeneous substance, containing many sources of radiation including elements such as U and Np, and also fission products such as ^{90}Sr (Ewing *et al*. 1995). The waste type will have a significant impact upon microbial action (McCabe,

1990), as speciation has implications for bioavailability and toxicity (Florence, 1986, Renshaw *et al*. 2011).

Bacterial cells have an electronegative cell wall at circumneutral pH because of the presence of polymers containing carboxylic acids and phosphate esters (Shephard *et al*. 2008), enabling ion exchange processes to occur on the surface (Milodowski *et al*. 1990). Some bacterial species possessing particularly efficient cation exchange mechanisms have additional cell wall features, for example *P. aeruginosa* have further charged groups in side chains of the polymers making up its cell wall (Shephard *et al*. 2008). Within bacterial cell walls, there are selective ion transporters to facilitate the movement of ions into and out of the cell (Silver and Walderhaug, 1992). These transporters are controlled by regulators that detect the presence of ions (Silver and Walderhaug, 1992), and they differ depending on factors such as the bacterial species and the metal ion; the affinity for ions may be induced if the starvation of a particular ion occurs (Dietrich and Silver, 1994).

Microorganisms are capable of respiring a range of electron acceptors as a source of energy, including some radionuclides, leading to alteration of their speciation and affecting their mobility (Gadd, 1996). The reduction of toxic radioactive metals such as U(VI) and Tc(VII) present in radioactive waste can be accomplished by bacterial cells, although the potential for this to occur in deep subsurface environments needs to be further studied (Nazina *et al*. 2004). As metal ions in a radioactive waste repository may consist largely of toxic ions, bacterial species may act synergistically, where a particular species may metabolise the by-products of another, leading to an overall gain of energy by all cells involved (Hanselmann, 1991).

Microbial reduction of radionuclides to less soluble states offers a potential mechanism for inhibiting the transport of radioactive elements (Gadd, 2002; Lloyd, 2003). It has been found that uranium is likely reduced by some bacterial cells by firstly reducing U(VI) to U(V), after which a disproportionation step occurs to produce U(IV) (Renshaw *et al*. 2005). Several bacteria which are capable of reducing Fe(III) are also able to reduce U(VI), including *Alteromonas putrefaciens* (Lovley *et al*. 1991). Under the reducing conditions that will likely be present in a GDF, uranium is likely to be insoluble (Anderson and Lovley, 2002). This suggests that transport times of reduced radionuclides throughout the environment are likely to be significantly longer (Hu *et al*, 2008).

The presence of bacterial species in a microbial community capable of reducing U(VI) is thought to be dependent on the presence of other electron acceptors, rather than just U(VI) (Cardenas *et al*. 2010). As an example, microorganisms capable of reducing

sulfate and iron are capable of reducing U(VI) and has been shown to occur under iron and sulfate reducing conditions (Boonchayaanant *et al*. 2009). It has been demonstrated that the addition of electron acceptors known to stimulate reduction of metals promotes microbial U(VI) reduction (Moon *et al*. 2010).

Wilkins *et al*. (2007) found that some species of microorganisms that are capable of reducing U(VI) are also capable of reducing Tc(VII). Some bacteria, including *E. coli*, *Desulfovibrio desulfuricans* and *Geobacter metallireducens* are able to reduce Tc(VII) enzymatically using hydrogenase (Lloyd *et al*. 1999). In the presence of Fe(III) citrate *G. sulfurreducens* is able to reduce Fe(III) to Fe(II), the Fe(II) is then able to abiotically reduce Tc(VII) to Tc(IV) (Lloyd *et al*, 2000). Some bacterial species are capable of reducing Np(V), although because of its extremely high toxicity, this may often happen at a slow rate. Species including *G. metallireducens* are able to reduce Np(V) to Np (IV), decreasing its solubility, leading to the precipitation of $Np(OH)_4$ or NpO_2 (Icopini *et al*, 2007). Surface properties of microbial cells (for example the presence of EPS components including polysaccharides (Behrends *et al*. 2012), or ligands including phosphate or sulfide (Pedersen, 2005)) may facilitate the biosorption of radionuclides, potentially immobilizing them (Gavrilescu, 2004). Because of the capabilities of bacterial cells to biosorb metals, they are often effective in removing metals from solution. The cells may utilize several mechanisms of biosorption, including the transport of metals across cell membranes (Veglio and Beolchini, 1997). As the surfaces of biofilms are negatively charged, and they have a large surface area to volume ratio, biofilms are effective at biosorption of metals (Brown and Sherrief, 1999). Whether this process will be beneficial in a GDF is uncertain though, as a study carried out by Anderson *et al*. (2006) suggests that the presence of a biofilm on a rock surface may actually be able to sorb smaller radionuclide concentrations compared to a rock-fluid interface that contains no biofilm.

Bacterial cells are capable of producing a number of metabolites which can act as chelating agents (Gadd, 2002), especially for essential metals. For example, cells within a biofilm may release siderophores or hemophores into the EPS to enable them to acquire iron (Wandersman and Delepelaire, 2004). Bacterial cells can produce metallothionein which binds to heavy metals; the expression of metallothionein is regulated by metal concentrations (Silver and Ji, 1994). Several bacterial species, including *Pseudomonas* sp. produce other proteins that are capable of binding metals, for example cysteine rich proteins which have a high affinity for cadmium (Bruins *et al*. 2000). In some cases heavy metals may bind with functional groups that are necessary for bacterial processes (Anderson *et al*. 2011) and may displace metal ions which are

essential (Hassen *et al*. 1998), in which case they become toxic as the necessary processes will be inhibited (Baveye and Valocchi, 1989).

The process of biomethylation may alter the movement of radionuclides such as polonium (Humphreys *et al*. 2010), because of the possibility of volatilisation (Gadd, 2002) and therefore may have important implications for radioactive waste disposal. For example, species such as *E.coli* and *Chromobacterium violaceum* are capable of causing the volatilisation of polonium, with the process being enhanced at higher temperatures (Momoshima *et al*. 2007).

The presence of dissolved organic compounds in a GDF may increase the transport of radionuclides through the geosphere via the formation of complexes (Courdouan *et al*. 2007). Radionuclide complexation with cellulose degradation products increases the solubility of the radionuclides (Read *et al*. 1998), and decreases their ability to sorb to surfaces (Glaus and van Loon, 2008). Read *et al*. (1998) demonstrated a reduction in sorption of uranium to mineral surfaces in the presence of organic complexants. McCarthy (1998) found that natural organic matter in formations at Oak Ridge, a storage facility for transuranic waste, significantly increased the transport of radionuclides in groundwater.

2.7 Methodologies to investigate microbial impacts on transport characteristics in rock under hyper-alkaline conditions relevant to a GDF for ILW.

2.7.1 Natural analogues

Because of the extensive timescales involved when considering the safety of a GDF for radioactive waste, natural analogue systems for certain GDF processes allow observations to be made regarding biogeochemical processes that may occur, over much longer timescales than can be provided by laboratory experiments (Hayeman and Pedersen, 2002).

2.7.1.1 Analogues for radionuclide behaviour

Information regarding the stability of immobile uranium species over geological timescales can be gained from observing the behaviour of uranium at sites such as Ruprechtov in the Czech Republic, where uranium has been immobilised in a sedimentary layer (Noseck *et al*. 2012). Uranium deposits, such as Cigar Lake in

Canada, demonstrate that radioactive materials can be contained effectively for millions of years (Rempe, 2007). Studies of a natural nuclear fission reactor in Gabon indicate that the indigenous microbes at this site are effective at decreasing the solubility of uranium in groundwater (Haveman and Pedersen, 2002). Other natural analogues exist for many other aspects of radioactive waste disposal. For example, liquid nuclear waste may be solidified with borosilicate glass; studies on the effectiveness of this material as the first barrier to radionuclide transport have been carried out using meteorites. They demonstrate long term corrosion processes at the glass-metal interface, and transport of substances throughout the structure (Libourel *et al*. 2011).

2.7.1.2 Analogues for a cementitious GDF

Repository construction materials, such as the concrete backfill material, will have significant effects on the containment of radionuclides. Naturally occurring hyper-alkaline fluids at Maqarin, Jordan (produced from interactions with naturally occurring cement minerals (Khoury *et al*. 1992)) demonstrate impacts of high pH on mineralogy over a period of around 100,000 years (Gaucher and Blanc, 2006), and help characterise processes that will likely occur in a repository, for example fracture sealing by secondary minerals such as calcite, aragonite and gypsum (Watson *et al*. 2011). Other studies carried out at this site have investigated factors controlling the solubility of trace elements in a hyper-alkaline system (Linklater *et al*. 1996). It has also provided information on microbial processes that are capable of being carried out under hyper-alkaline conditions; results of studies by Pedersen *et al*. (2004) may suggest that although microorganisms are capable of surviving under hyper-alkaline conditions, microbial activity may be relatively low in a cementitious repository. Studies on subsurface alkaline environments, including that by Fry *et al*. (1997) state that diverse microbial communities are present and metabolically active under alkaline conditions at depths of up to 1270m, but suggest that variations in the abundance and diversity of microorganisms occur within the same rock formation. These results could have potential implications for a GDF environment, suggesting there will be spatial variation in biogeochemical cycling in a GDF host rock.

Anthropogenic deposition of lime kiln waste has occurred at Harpur Hill, near Buxton, Derbyshire. Percolation of both groundwater and rainwater through the waste pile, causing the water to become dominated by calcium hydroxide, has led to the formation of a hyper-alkaline spring. Investigations of the site may determine some of the impacts of hyper-alkaline fluid on mineralogy, and also the impacts on microbial populations and

the processes they are capable of carrying out under such conditions. Burke *et al*. (2012) found that microorganisms living in hyper-alkaline conditions at the site were capable of carrying out similar biogeochemical processes to those seen in circum-neutral soils.

Studies of serpentinising environments (characterised by highly alkaline pore waters, and H_2 generation as a result of interactions between water, and olivine and pyroxene (Schrenk *et al*. 2013)) have revealed diverse microbial communities well adapted to the extreme conditions. Hydrogen utilizing microorganisms are often found to be associated with these types of systems. For example, Tiago and Veríssimo (2013) observed members of the genus *Hydrogenophaga* in groundwater collected from a deep serpentinising environment at Cabeço de Vide, Portugal. Members of this genus were also identified in surface springs originating from a deep serpentinising system (Brazelton *et al*. 2013). Members of the genus *Serpentinomonas* have been identified in serpentinising environments, including *S. mccroryi* and *S. raichei*, which are obligately alkaliphilic hydrogen utilizing bacteria (Suzuki *et al*. 2014).

Although natural analogues may provide invaluable information on processes that may occur over the lifetime of a GDF, they certainly have their limitations which must be considered when they are to be used to make predictions on GDF evolution. For example, reaction rates may vary significantly between natural analogues and GDF environments, or processes of interest may not be able to be fully described by a single natural analogue site because of site specific processes such as groundwater flow (Chapman *et al*. 1984).

2.7.2 Experimental methodologies

A range of laboratory techniques have been developed that allow the investigation of the microbial impacts of transport characteristics of rock. For example Coombs *et al* (2008) investigated the transport of *P. aeruginosa* through rock by packing borosilicate glass columns with crushed diorite, and flowing groundwater containing *P. aeruginosa* through them. Imaging techniques such as SEM were used to observe biofilm formation and mineralogical changes. Other experiments have been carried out using pressurised flow through experiments with constant fluid flow, to study microbial interactions in intact rock (Harrison *et al*. 2011). Previous studies have investigated the impacts of bioclogging by monitoring "evolution of bulk hydraulic conductivity," and through these studies data has been gained on how to better manage field scale systems (Brovelli *et*

al. 2009). Laboratory studies to investigate transport properties have been combined with geochemical information and information from specific geological sites to provide models capable of predicting the evolution of systems over long time scales to a certain extent (Berkowitz, 2002).

Various analytical techniques have been used to investigate the microbial populations that occur in the rock, and imaging techniques such as SEM have been used to observe biofilm formation (Arnon *et al*. 2005). Other methods, including electrical induced polarization have been utilised to determine zones of iron and sulfate reduction in column experiments (Williams *et al*. 2009). Analytical methods such as X-ray diffraction and X-ray emission spectroscopy have been used to observe mineralogical changes in column experiments (Berkowitz, 2002). Previously, during flow experiments, a range of tracers have been utilised. Arnon *et al* (2005), including fluorobenzoic acid to spatially visualise flow; a similar method was used by Oswald *et al* (1997) to quantify fluid flow velocities. Other imaging techniques capable of revealing information on flow transport include magnetic resonance imaging, which has the advantage of being non-destructive (Oswald *et al*. 1997).

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Chapter 3

Methodologies

This chapter describes the experimental approaches taken to fulfil the aims of this study, along with analytical methods and data processing techniques. The limitations of the methodologies are described. The basic theories behind analytical approaches are reported; descriptions of instruments used are included, along with the quality control procedures utilised.

3.1 Field sampling

3.1.1 Site description

Fluid and sediment samples were collected from Brook Bottom Spring, a hyper-alkaline spring near Harpur Hill, Buxton, UK. Figure 1 shows the outline of the site at Harpur Hill, with detailed inserts and identification of the sampling points used for this study. A hyper-alkaline spring has developed at the site as a result of extensive deposition of lime burning waste. Lime burning at Harpur Hill spanned approximately 70 years (Anon, 2008), producing extensive quantities of waste comprising "partially calcined limestone, fused (partially melted) glassy lime clinker, lime, part-burnt coal, coal ash and coal clinker," (Milodowski *et al*. In preparation) which was deposited into the valley at Brook Bottom.

Leaching of groundwater and rainwater though the waste pile, which discharges at the base of the waste pile has allowed the formation of a hyper-alkaline spring, often reaching over pH 13. The spring water is dominated by calcium (Rizoulis *et al*. 2012), which, when it comes into contact with atmospheric CO_2, precipitates as calcium carbonate. Freshwater inputs to the site occur via rainwater and through a culvert (HH 7.5, Figure 1), which after periods of heaving rainfall is of circum-neutral pH. Various mixing zones are present across the site where the hyper-alkaline fluid interacts with freshwater; at the time of sampling for microcosm experiments, five sites were identified across the site with pH values of: 7.5, 8.9, 11.4, 12 and 13. The pH across the site is influenced by the influx of fresh water from a culvert, and by rainfall.

Fluid and slurry samples were collected from Harpur Hill as a source of alkaliphilic and alkalitolerant microorganisms at the sampling points indicated in Figure 1b.

Figure 1 a:OS map showing the location of Brook Bottom Springs at Harpur Hill b: Aerial photograph of the hyper-alkaline spring at Harpur Hill. Labels show sampling points and pH values on the first sampling date, c and d: Photographs of the field site.

3.1.2 Sample collection

Prior to field sampling, polypropylene fluid collection vessels were autoclaved (121°C for 20 minutes). pH and Eh measurements were collected in the field. Surface waters were collected by first disturbing the sediment with the sterile collection vessel slightly to maximise the chances of microorganism collection. Sample collection vessels were filled to the top and then sealed to minimise interactions between CO_2 and the fluid. Fluids were stored in a cool box packed with freezer blocks whilst in the field, and were then stored in the dark at 4°C until needed.

3.2 Geological substrate

Sherwood sandstone (from Hollington Sandstone Quarry) was used as the geological substrates in experiments carried during this study; both intact and crushed material was utilised. The crushed material was of grain size <500 µm. The sandstone is dominated by Quartz, with Microcline, Muscovite and Kaolinite.

3.3 Microcosm experiments

A number of microcosm experiments were carried out in this study. These include:

- Microcosms containing crushed sandstone and synthetic groundwater posited at pH 10 under both aerobic and anaerobic conditions.

- Microcosms containing crushed sandstone and synthetic groundwater with a circum-neutral pH that were sampled both weekly and sacrificially.

- Microcosms containing crushed sandstone and synthetic groundwater posited at pH 13.

- Microcosms assembled with fluids and sediments collected sampling points at Harpur Hill with pH values ranging from circum-neutral to hyper-alkaline (Chapter 4).

Details of these microcosm experiments and controls are outlined in Table 2.

3.3.1 Microcosm Assembly

Details of the media used in microcosm experiments are shown in Table 1. Media were prepared and autoclaved (121°C for 20 minutes). Aerobic microcosms were assembled in a laminar flow hood and sealed with a cotton wool bung. Prior to assembly of anaerobic microcosms, media were purged with N_2 for 20 minutes (this process was carried out aseptically: a 0.2 µm nylon syringe filter was attached to the N_2 gas line, to which a sterile hypodermic syringe needle was fitted). The microcosms were assembled in an anaerobic cabinet (95% N_2/ 5% H_2) and sealed with a sterile butyl septa held in place with an aluminium crimp cap. All microcosms were incubated in the dark at 21°C for four weeks.

3.3.2 Microcosm sampling

Aerobic microcosms were sampled in a laminar flow hood by first shaking the bottles to create a homogenised slurry, and then removing the cotton wool bung. A sterile syringe was used to remove a slurry sample.

Anaerobic microcosms were sampled by first agitating to ensure a homogenised slurry was created. A 5 ml syringe with a hypodermic syringe needle attached was aseptically filled with N_2 gas; the gas was then transferred to a microcosm via the septa, and a slurry sample removed using the syringe.

Sacrificial microcosms were sampled as follows: on a weekly basis one set of microcosms (three replicate experimental microcosms along with one of each control microcosm) were sacrificed. Samples were then collected in the same way as other anaerobic microcosms.

Table 1 Composition of media used in microcosm experiments. pH 10 medium was adjusted with concentrated NaOH solution

	pH 7.1	**pH 10**	**pH 13**
KCl	6.6 mgL^{-1}	6.6 mgL^{-1}	
$MgSO_4.7H_2O$	97.6 mgL^{-1}	0.966 gL^{-1}	
$MgCl_2.6H_2O$	81 mgL^{-1}	0.0810 gL^{-1}	
NaCl	9.5 mgL^{-1}	9.4 mgL^{-1}	
$Ca(OH)_2$		0.15 gL^{-1}	0.01 gL^{-1}
Na_2SiO_3			
$CaCO_3$	0.85 mgL^{-1}		
$NaHCO_3$	1.26 mgL^{-1}		
KOH			5.2 gL^{-1}
NaOH			3.8 gL^{-1}
Yeast extract	50 mgL^{-1}	50 mgL^{-1}	50 mgL^{-1}
Na-lactate	5 mM	5 mM	5 mM
Na-acetate	5 mM	5 mM	5 mM

Table 2 Microcosm experimental design. Blue: composition of experimental microcosms; orange: design of control microcosms; grey: atmosphere details; purple: method of microcosm sealing.

pH 10 aerobic	**pH 10 anaerobic**	**pH 7.1 sampled weekly**	**pH 7.1 sampled sacrificially**	**pH 13.1**	**Harpur Hill microcosms at pH 7.5, 8.9, 11.4, 12, 13**
3 replicates containing 20 g crushed sandstone and 40 ml synthetic groundwater	3 replicates containing 20 g crushed sandstone and 40 ml synthetic groundwater	3 replicates containing 20 g crushed sandstone and 40 ml synthetic groundwater	15 replicates containing 20 g crushed sandstone and 40 ml synthetic groundwater	3 replicates containing 20 g crushed sandstone and 40 ml synthetic groundwater	For each pH value: 3 replicates containing 30 g crushed sandstone, 60 ml of surface water and 5 g sediment collected from the field with 5 mM acetate, 5 mM lactate and 50 mgL^{-1}
1 autoclaved control	1 autoclaved control	1 autoclaved control	5 autoclaved controls	1 autoclaved control	1 autoclaved control
1 microcosm containing sterile synthetic groundwater only	1 microcosm containing sterile synthetic groundwater only	1 microcosm containing sterile synthetic groundwater only	5 microcosms containing sterile synthetic groundwater only	1 microcosm containing sterile synthetic groundwater only	1 microcosm containing 30 g crushed sandstone, 60 ml of surface water and 5 g sediment collected from the field. No electron donor or yeast extract added
All microcosms assembled aerobically	Media purged with N_2 gas for 20 minutes	Media purged with N_2 gas for 20 minutes	Media purged with N_2 gas for 20 minutes	Media purged with N_2 gas for 20 minutes	1 microcosm containing autoclaved surface water from the field site
Microcosms sealed with a cotton wool bung	Microcosms assembled under a 95% N_2/ 5% H_2 atmosphere	Microcosms assembled under a 95% N_2/ 5% H_2 atmosphere	Microcosms assembled under a 95% N_2/ 5% H_2 atmosphere	Microcosms assembled under a 95% N_2/ 5% H_2 atmosphere	1 microcosm containing 60 ml surface water and 5 g sediment collected from the field, with 5 mM acetate, 5 mM lactate and 50 mgL^{-1} yeast extract
	Microcosms sealed with a butyl rubber septa and aluminium crimp cap	Microcosms sealed with a butyl rubber septa and aluminium crimp cap	Microcosms sealed with a butyl rubber septa and aluminium crimp cap	Microcosms sealed with a butyl rubber septa and aluminium crimp cap	Groundwater purged with N_2 gas for 20 minutes
					Microcosms assembled under a 95% N_2/ 5% H_2 atmosphere
					Microcosms sealed with a butyl rubber septa and aluminium crimp cap

3.4 Short term column experiments with crushed sandstone

3.4.1 Experimental design

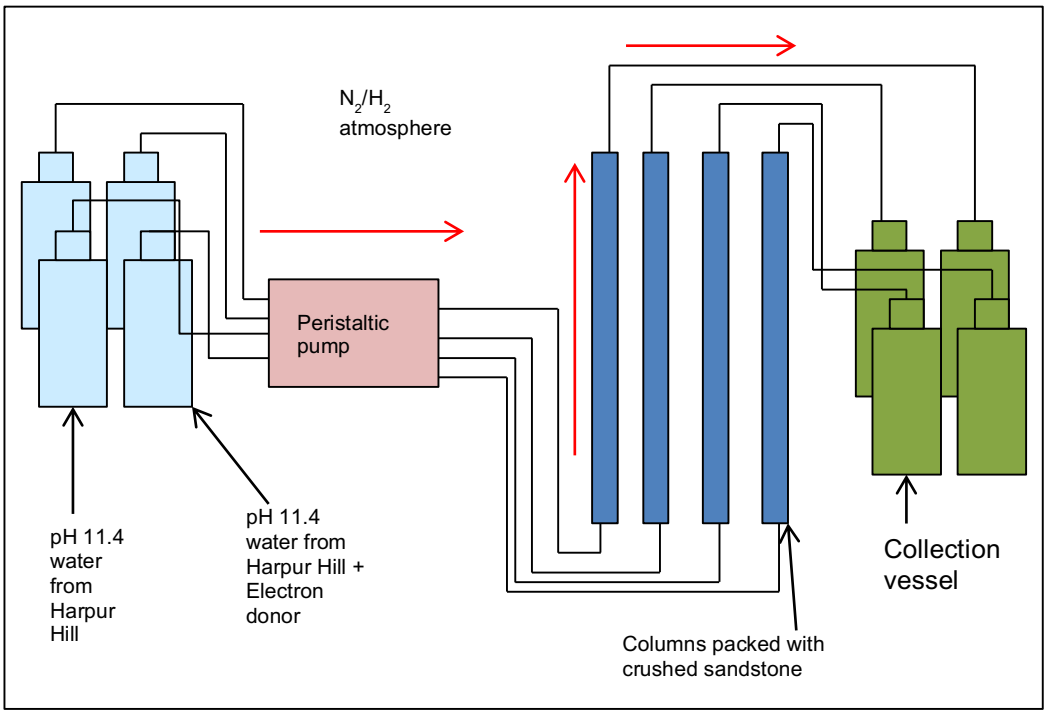

Figure 2 Schematic diagram showing the experimental setup of short term column experiments. Experiments were carried out in an anaerobic cabinet with a 95% N_2 5% H_2 atmosphere.

PEEK (polyether ether ketone) columns (ID: 7.5 mm, length 150 mm; Applied Research Europe) were used in these experiments that are further described in Chapter 4, along with PEEK fittings, as PEEK is biologically and chemically inert (Abu Bakar *et al*. 1999). Collection vessels were autoclaved, and PEEK columns, fittings and tubing were sterilised using by soaking in a Virkon (1 % solution; DuPont, Delaware, US) for 1h, and then thoroughly rinsed with sterile deionised water (DIW).

Fluid was collected from sample site HH 11.4 (Figure 1; pH 11.4 at the time of sampling) as described in section 3.1.2 and stored at 4 °C until needed. Fluid collected from the site was homogenised in a sterile polypropylene vessel; a total of 4 L of fluid was used in this experiment. This fluid was subsampled into 500 mL polypropylene bottles. To one batch of fluid, 5 mM of both acetate and lactate were added, along with 50 mg L^{-1} yeast extract (carbon amended fluid). The other batch was left unamended (control). Samples were collected from the starting fluid under anaerobic conditions so as to prevent exposing the fluid to air.

A peristaltic pump (ICP 8 Microprocessor controlled dispensing pump; Ismatec, Germany) was used to pump the fluid through the columns at a constant rate. Peristaltic pump tubing (Orange-Green, ID 0.38 mm) was used to provide an initial flow rate of 1 mLh^{-1}. Polytetrafluoroethylene (PTFE) tubing (OD 1/16 inch, was superglued into either end of the peristaltic pump tubing (ensuring that the tubing did not become blocked). PEEK fingertight fittings with ferrules were attached to the PTFE tubing, enabling the tubing to be screwed into either end of the PEEK columns. PTFE tubing was used because it is biologically (Hopp *et al*, 2003) and chemically inert (Grover *et al*, 2008). Autoclavable Polypropylene bottles were used to store the inlet fluid and collect the outlet fluid. Bottles were sealed with Parafilm®, which the PTFE tubing was pushed through.

PEEK columns were packed with crushed Hollington sandstone (grain size <500 µm; previously homogenised in a rotary blender; Milodowski 2012, personal communication). Columns were packed with the sandstone; agitation of the columns (by tapping them on a surface) during packing allowed the sediment to settle faster and ensured consolidation of the sandstone. Care was taken to ensure the threads at either end of the columns remained free of sand, to ensure that when the ends were replaced, a good seal was achieved. After sterilization of collection vessels and tubing, the experimental components were transferred to an anaerobic cabinet, and the experiment was assembled (Figure 2) under conditions that were as aseptic as possible.

A flow rate of approximately 1 mLh^{-1} was selected for these short term pilot column experiments, which ran for 6 weeks with weekly sample collection. Because the experiments were to run for such a short period of time (6 weeks) a relatively quick flow rate was selected to maximise the amount of fluid that could interact with the sandstone. A quick flow rate was also selected to ensure sufficient outlet fluid was collected for weekly analysis. Column experiments were assembled so that fluid was flowing upwards through the columns, and the presence of a frit at either end of the columns (PAT- PEEK alloyed with PTFE; 5 µm pore size) ensured the crushed sandstone was kept in place and allowed even distribution of the fluid through the sediment column. Sample collection bottles were weighed before and after fluid collection, to enable flow velocity calculations. The peristaltic pump tubing was checked on a regular basis to ensure it was not becoming worn out during the course of the experiment. The PTFE tubing was checked regularly to ensure that blockages did not occur.

At the end of the experiments, columns were removed from the anaerobic cabinet and cut into 5 x 3 cm sections. Sections were stored in ziplock bags and transferred to a biological safety cabinet. Before samples were collected for analyses, using a sterile spatula, the exposed sample at the ends of the sections was removed and disposed of due to contact with un-sterilized cutting tools. Uncontaminated sample was transferred to sterile 2 mL centrifuge tubes prior to being subject to analytical approaches.

3.5 Long-term column experiments with crushed sandstone

3.5.1 Experimental design

Further PEEK column experiments (Chapter 5) were carried out to investigate how biological processes can lead to pressure changes in a geological substrate under hyper-alkaline conditions. A significantly slower flow rate was selected to carry out these experiments to represent a more realistic flow rate for a deep geological environment, particularly because microbial community structure and development has been shown to be heavily influenced by flow rate due to differences in shear stress for example (Moreira *et al*, 2013). Groundwater flow through rocks can vary widely, and can be as fast as 1m per day (Waltham, 2009) and can be influenced by features such as fractures. Some of the potential host rocks for a GDF include granite (with a typical groundwater velocity of 0.1 m per year; Chopin *et al*. 2013), clay (with a typical groundwater velocity of 0.0003 m per year to 0.15 m per year; Harter, 2008), or salt, where no groundwater flow occurs.

For these experiments, fluid was collected from the same sample site used to collect fluid for the pilot PEEK column experiments (HH 11.4, figure 1), although at the time of sampling the fluid at this site had a slightly higher pH due to the lack of recent rainfall. Fluid was not collected from a different sample site as this was the lowest pH found on the sampling day. Components were sterilised in the same manner as the short term PEEK column experiments. Pressure transducers were sterilised by soaking in virkon and rinsing thoroughly with sterile DIW.

Experiments were assembled in a similar manner to the pilot PEEK column experiments, with several modifications. The flow rate was set at approximately 0.1 mLh^{-1}. During the first few weeks, several of the columns encountered leaks which may have slightly impacted results. Two columns encountered no flow during the first week of the experiment, due to sand grains becoming trapped in the thread of the inlet column ends, preventing a watertight seal from being achieved. Once the sand grains were

removed, a watertight seal was achieved with the help of PTFE tape on the threads. Because of blockages occurring in the PTFE tubing in the pilot PEEK column experiments from small amounts of sediment in the fluid, care was taken to ensure fluid was sediment free prior to starting the experiments. The addition of 5 mM acetate, 5 mM lactate and 50 mgL^{-1} yeast extract to stimulate biological activity was found to be sufficient in the pilot experiments, and the use of an unamended control provided a comparison to enable the impacts of biological activity to be understood, so these conditions were repeated in these longer term experiments.

These experiments aimed to investigate how biological processes (and also chemical/mineralogical changes) influenced the injection pressure into the columns; to study the microbial communities that became established within the columns, and how this differed to communities in the outlet fluid, and the function of the communities within the columns. Pressure transmitters (Danfoss MBS3000 0-4 bar 4-20 mA transmitter, M&M controls, Manchester, UK) were attached to inlet and outlet ends of the columns to measure the pressure of fluid being pumped into and coming out of the columns. Transmitters were attached to columns and PTFE tubing with a stainless steel pressure gauge tee for 1/4" NTP pressure gauge (VWR International). PTFE tubing was connected to the pressure gauge tee using PEEK 1/16" fingertight nuts and ferrules. Pressure transducer threads were wrapped with PTFE tape before connecting to the pressure gauge tees to ensure a watertight seal was achieved. A pressure transmitter which measures in the range 0-4 bar was selected due to the low pressures expected in these experiments. Pressure transmitters were wired into a National Instruments Integrated connector block. Pressure was logged in mA every two minutes over the course of the experiment.

3.5.2 Accuracy of pressure measurements

As the typical accuracy of these pressure transmitters is +/- 0.5% (Technical data sheet for Pressure transmitter for industrial applications, Type MBS 3000), the selection of a pressure transducer with a smaller range increases the overall accuracy of data collected. The electrical interface of the pressure transducers were connected to the data logger using electrical cable (Alpha Wire- 1172C SL002, Premier Farnell UK Limited). Because the distance between the pressure transmitters and the data logger was several meters, pressure transmitters were used rather than pressure transducers, as transducers should only be used if the electrical connections being used are short. Pressure transmitters contain components which amplify the signal, so are more

appropriate for running the output signal over longer distances (ESI technology Ltd, 2013). Pressure transmitters work by converting a pressure into an electrical signal which can be measured (SensorsONE, 2013). The pressure transmitters used in these experiments use "piezoresistive semi-conductive technology," (Danfoss, 2013). The movement of the membrane indicates "traction or compression on the piezoresistors," and the change in resistance is measured (Singh *et al*, 2002). Calibration curves were prepared to convert mA to bar, by plotting the pressure range of the transducers against the mA range.

The accuracy of measurements made by pressure transmitters are affected by several factors, including temperature and humidity (Hashemian and Jiang, 2009). As these experiments were carried out in a temperature regulated anaerobic cabinet, temperature fluctuations will have had minimal impact on the pressure data.

3.6 Modified Robbins Device experiments

3.6.1 Experimental design

Experiments were carried out using a modified Robbins Device (MRD) to investigate biofilm development over time on a sandstone surface. An MRD was utilised, as samples could be removed over a period of time, enabling investigation of the stages of biofilm development under highly alkaline conditions. It also has the advantage that different types of surface can be investigated (Jass and Lappin-Scott, 1992). These experiments aimed to monitor the extent of biofilm development on sandstone over time under pH conditions relevant to a geological repository environment, and to also investigate whether microorganisms preferentially attach to particular mineral surfaces. A pilot study was carried out to define the most appropriate parameters under which to carry out these experiments, including the most appropriate flow rate, and the length of time in between sample collection for example.

The fluid utilised in these experiments was collected from Harpur Hill, and at the time of sampling, had a pH of 12. Acetate, lactate and yeast extract were added to stimulate microbial activity one week before the fluid was to be used in the MRD experiments, as cell numbers in the starting fluid were found to be very low. Intact sandstone was utilised in these experiments. A fresh face was exposed on a block of Hollington sandstone using a hammer and chisel. A Dremel 800 electric hand tool fitted with a diamond cutting wheel was utilised to remove sandstone chips parallel to the fresh face. 20 sandstone chips were cut approximately 5 mm x 5 mm x 2 mm; the base of the chips

were filed down to create a flat surface that would stick to the MRD plugs. The chips were attached to the plugs using silicon sealant (containing no anti-microbial compounds), ensuring that no sealant come into contact with the exposed surface (the fresh face) of the chips. Prior to starting the experiment, a small amount of silicon sealant was left to solidify overnight and then submerged in the hyper-alkaline fluid that was to be utilised in these experiments for approximately two weeks, to ensure its integrity throughout the course of the experiment. The Modified Robbins Device contains 25 plugs; during this experiment, 20 of the plugs were fitted with sandstone chips, whilst the other five remained empty as negative controls.

A fluid re-circulation approach was adopted because of the low starting cell numbers, and the limited amount of sample fluid available. A peristaltic pump was used to pump the fluid through the MRD at a rate of 90 mLh^{-1}. Peristaltic pump tubing was connected to PTFE tubing, with one end being attached to the outlet of the fluid vessel to pump fluid into the MRD. Samples were collected from the fluid vessel periodically to monitor total cell numbers and electron donor utilisation.

Prior to experiment assembly, 1% Virkon was pumped through the MRD for approximately 24 h to sterilize it. Spare MRD plugs were soaked in Virkon for 24 h before rinsing with sterile DIW. The fluid vessel and fittings were autoclaved prior to use. After removal of the Virkon, the MRD and all tubing was then flushed through several times with sterile DIW to remove any residue left from the Virkon. Once all components had been sterilised, the MRD was transferred to a laminar flow hood where the experiment was assembled. Plugs containing the sandstone chips were placed in the MRD, along with five sterile empty plugs (two at either end, and one in the middle of the MRD). 500 mL of Harpur Hill fluid was added to the fluid vessel, along with 5 mM of both acetate and lactate, and 50 mgL^{-1} yeast extract. Once the experiment was fully assembled, it was removed from the laminar flow cabinet and the experiment was started. Plugs were removed from the MRD periodically, under conditions that were as aseptic as possible, and plugs were replaced with sterile empty plugs. Plugs were removed in triplicate (and replaced with sterile empty plugs), for imaging under the ESEM.

Further experiments were carried out with an MRD at a significantly slower flow rate than the previous experiments. A slower flow rate was selected to ensure flow conditions are closer to that which would occur in natural groundwaters. The design of these experiments was altered slightly compared to the previous set up: the MRD was set up as a flow through experiment rather than re-circulating. This was to give a better indication of the processes that were occurring within the MRD, for example electron

donor utilisation, and cell numbers throughout the course of the experiment. This experimental design also has the advantage of decreased risk of contamination when sampling the fluid. The following experiments were also carried out in the dark. The experiment was run twice under these conditions: once to view plugs under the confocal laser scanning microscope to visualise biofilm development, and once to view plugs under the ESEM to investigate microbe-mineral interactions.

For viewing plugs under the CLSM, plugs were removed from the MRD in triplicate (and were replaced with sterile empty plugs) and quickly immersed in sterile DIW. Both of the stains from the LIVE/DEAD® *Bac*Light™ cell staining kit were added (after diluting 1:200 with sterile DIW) to sterile DIW, and left for a few minutes for the stains to penetrate the cells before imaging with the CLSM. This method is discussed in greater detail later in this chapter.

3.7 Long-term experiments with Biological Flow Apparatus

Long term pressurised experiments were carried out using intact Hollington sandstone core (Chapter 6), which aimed to investigate the impact of biological activity on the transport characteristics of the sandstone, along with impacts cause by the interactions of hyper-alkaline fluids with the sandstone. These experiments were carried out using a Biological Flow Apparatus (BFA) as previously described by Wragg *et al*. (2012). The experimental rig consisted of a pressure vessel and a syringe pump Teledyne Isco, 1000D pump, Lincoln, USA); a schematic of experimental design is shown in figure 3. Initially a pilot experiment was carried out using the BFA to gain a better understanding of how best to set up subsequent experiments. For example the selection of an appropriate experimental design for the control experiment proved to be difficult. Due to the long term nature of these experiments, and limitations in numbers of pressure vessels and pumps, experiments could not be carried out with replicates, therefore it was important to carefully define the parameters of both the control and biotic experiments to ensure that the maximum amount of useful data could be obtained.

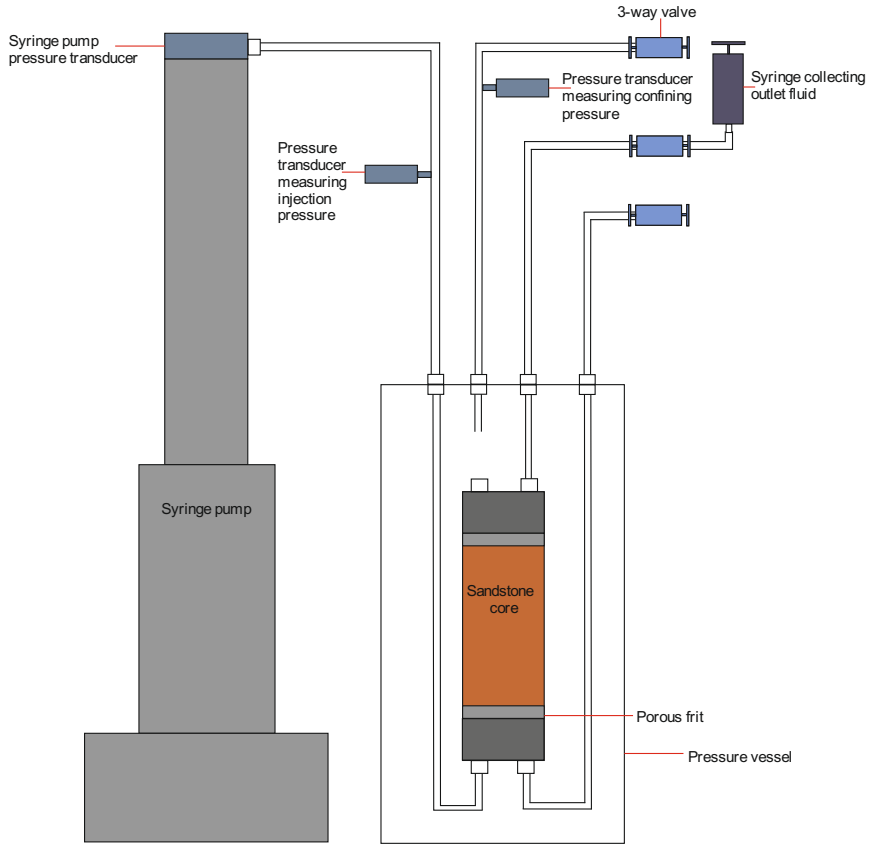

Figure 3 Schematic of experimental setup of BFA experiments

3.7.1 Pilot experiment

The pilot experiment was carried out initially as a control experiment; prior to pumping any fluid into the core, a confining pressure was applied to the core within the pressure vessel. Once this pressure had been achieved and remained constant for an extended period of time (any decreases in confining pressure were adjusted by introducing more fluid into the pressure vessel to increase the pressure), fluid was then introduced into the core. Once the core had become fully saturated and fluid had been flowing through the core for several weeks, a carbon source (5 mM of both acetate and lactate) was introduced into the fluid to stimulate biological activity. A 50 mL syringe was attached to the outlet, to collect the fluid that had been pumped through the core. The syringe was changed on a weekly basis under conditions that were as aseptic as possible.

3.7.2 Experimental aims

The pilot experiment aimed to define the parameters to be utilised in further biotic and control experiments. A confining pressure was applied to the core aiming to be representative of the conditions that will be present in a geological repository environment. Further BFA experiments aimed to investigate the impact of microbial processes under high pH conditions on transport in intact sandstone. One control (no added carbon) and one biotic (carbon stimulated) experiment were assembled.

3.7.3 Experimental design and assembly

From a block of Sherwood sandstone measuring approximately 30 x 15 x 15 cm, 7 cores were taken with a diameter of 3.7 cm and a length of 10 cm (all cores were taken from the same orientation, which was noted). Cores were stored at 4°C until needed. Frits and platens were soaked in 70% methanol in a laminar flow hood. All tubing was rinsed with 70% methanol. Cores were stored at 4°C until utilised. The core was assembled in the laminar flow hood. Porous frits (pore size approximately 10 µm, diameter 3.7 cm) were placed at both ends of the core. A heat-shrink Teflon sleeve was cut to cover the length of the core, along with the frits and plattens, and was placed on the core. Plattens were then placed on the frits, and the heat-shrink Teflon was heated with a heat gun until it shrunk to fit the core and sealed the frits and plattens to the ends of the core. A collar was then placed over the O-rings to ensure the formation of a leak-tight seal. Figure 4 shows how the cores were assembled prior to being placed in pressure vessels.

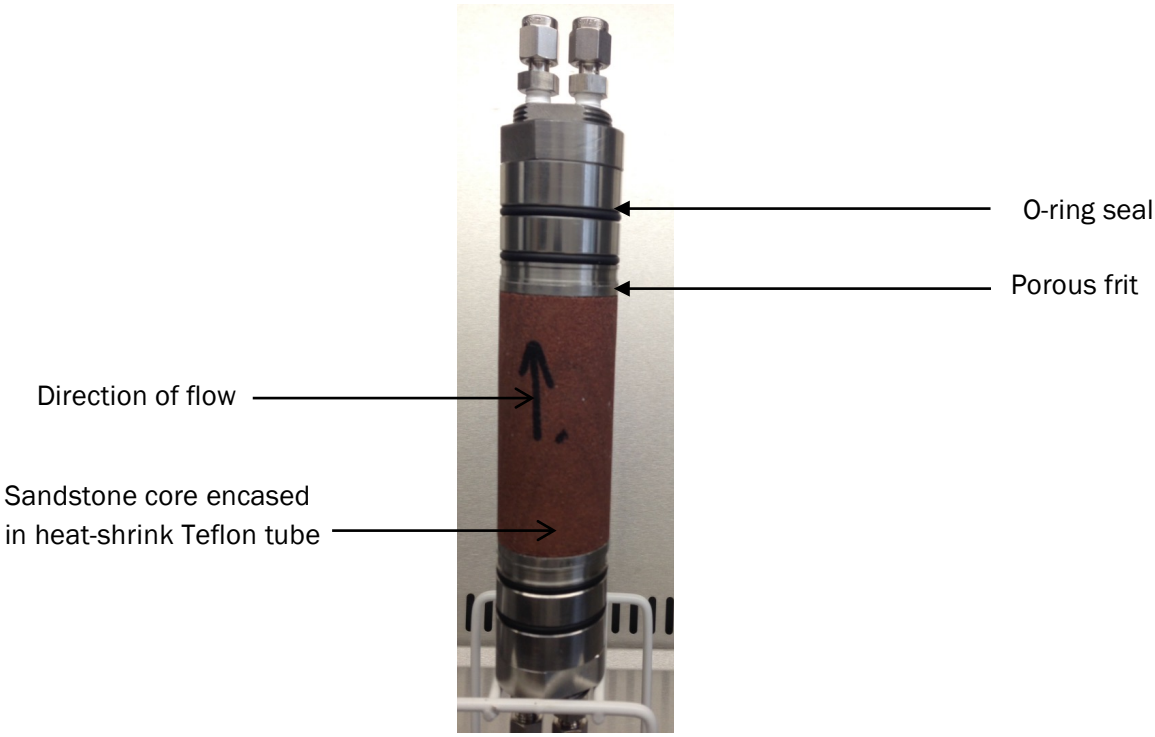

Figure 4 Sandstone core with sintered discs on either end with plattens allowing fluid in and out of the core prior to experiment assembly.

The tubing used to build BFA experiments was Swagelok 1/8 inch stainless steel tubing with an internal diameter of 0.028 inches. Stainless steel Swagelok tube fittings (Male connectors- 1/8 inch tube NTP) were used to attach tubing to the pressure vessel ports, and to connect tubing to the inlet and outlet ports on the platens. Pressure transducers (with a limit of either 40 bar or 100 bar; Gems Sensors and Controls, Plainville, US) were attached to inlet tubing to measure the inflow pressure, and to the confining pressure tubing. The valves used to control flow throughout the system were Top Industrie 1/8 inch pneumatic valves (types 1 and 5). The pumps used in these experiments were Teledyne Isco (Lincoln, USA) 1000D syringe pumps. Thermocouples were attached to the pressure vessel and pump barrel, and were also used to measure ambient temperature. Thermocouples were wired into an 8-channel thermocouple input module (National Instruments, Newbury, UK) and temperature data was logged every two minutes.

After experimental assembly, the sandstone core was placed in the pressure vessel and plumbed in. The pressure vessel was then sealed with bolts. A confining pressure (40 bar) was applied to the sandstone core by pumping water into the pressure vessel, which was then isolated once the desired pressure had been reached. The pump barrel

was then sterilised by transferring approximately 100 mL 70% methanol to the pump, and then running the pump back and forth several times at a slow rate to ensure that all surfaces came into prolonged contact with the methanol. The methanol was then removed from the pump.

3.7.4 Preparation of experimental fluids

To ensure that no scratching occurred on the inside of the pump barrel, sediment was removed from the experimental fluid. Fluid was shaken vigorously to allow for detachment of microbial cells from sediments, and was then vacuum filtered to approximately 10 µm. After filtration, samples were taken from the starting fluid, and was then quickly transferred to the pumps to minimise calcium carbonate precipitation. The valve to the core inlet was then opened and fluid was pumped through at a rate of 200 μLh^{-1}. Initially the fluid was pumped through the inlet platen and then removed to ensure that the porous frits at the inlet ends of the core became saturated with experimental fluid (ensuring the removal of air bubbles). This was carried out for four days, before then allowing the fluid to be pumped through the core and collected in a 50 mL syringe. Fluid was pumped through the core under constant flow conditions at a rate of 200 μLh^{-1}. Both the control and carbon amended experiments were firstly run as controls (to ensure stabilisation of parameters such as pH and Eh). Once conditions had stabilised, the experimental fluid was removed from one of the pumps, 5 mM of both lactate and acetate and 50 mgL^{-1} yeast extract were added to the fluid, shaken, and then returned to the pump (carbon amended experiment). Syringes were changed on a weekly basis, and weighed. Samples were collected under aseptic conditions and stored accordingly.

3.8 Analytical methods

3.8.1 Fluid pH and Eh (redox potential) measurements (used in Chapters 4, 5 and 6)

3.8.1.1 Background

The acidity or alkalinity of a solution can be described by the pH scale. The pH scale is an inversely logarithmic scale of hydrogen ion concentration (Olmsted and Williams, 1997). Eh is the potential for a system to gain (reduction) or lose electrons (oxidation) (Yu and Ji, 1993).

3.8.1.2 Method

pH and Eh measurements of surface waters (at the sampling locations identified in Figure 1) were collected in the field using a HANNA HI 9828 multiprobe calibrated to pH 7 and 10 prior to use. The Eh probe was checked prior to use by taking a reading of Zobell's solution. The probe was rinsed with DIW and dried between each measurement.

pH measurements of fluid samples collected in the lab were collected with a Mettler Toledo pH probe calibrated to pH 7 and pH 10 prior to use. Eh measurements were collected with a HANNA ORP probe after testing with ZoBell's solution (231 mV at 25°C). Both measurements were taken once values had stabilised.

3.8.2 Ion Chromatography (IC)

3.8.2.1 Background

IC methods used in chapters 4, 5 and 6 involve the use of a chromatographic column to separate ions present in a mobile phase based on their affinity to the stationary phase present within the column (comprised of a resin charged with ion-exchange groups). A detector is then used to quantify analytes; a conductivity detector was used for this project, where the sample ions are converted into a more conductive form. The concentration of analytes can then be quantified by comparison of chromatographic signals to standards of known concentration (Weiss, 1995).

3.8.2.2 Method

IC was used to measure concentrations of sulfate, nitrate, nitrite, phosphate, chloride and organic acids. Analyses were carried out by Alastair Bewsher on a Dionex ICS5000 Dual Channel Ion Chromatograph. Details of the channels are outlined in table 3.

Table 3 Details of ion chromatography equipment

	Channel 1	**Channel 2**
System type	Microbore system	Capillary system
Columns	Dionex AG18 Guard Column (50 x 2 mm) Dionex AS18 Analytical column (250 x 2 mm)	DionexAG11-HC 4u (50 x 0.4 mm) DionexAS11-HC 4u (250 x 0.4 mm)
Mobile phase	Isocratic 25 mM KOH	Electronically generated gradient of KOH
Flow rate	0.25 ml min^{-1}	15 µl min^{-1}
Typical back pressure	2,100 psi	3,200 psi
Detector	Conductivity	Conductivity
Suppressor	Electronic (16mA)	Electronic (13mA)
Injection Loop size	10 µl	0.4 µl

3.8.3 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

3.8.3.1 Background

ICP-MS is used to quantify major and trace elements in aqueous samples; the main components of an ICP-MS instrument are shown in Figure 5. The method involves nebulising a sample, converting it to a fine aerosol which is injected into the chamber along with Ar gas. In the spray chamber, the finest particles are swept towards a plasma torch, and larger particles settle as waste. The plasma ionizes the gas, and the ions are transported to an interface region where they are guided to a vacuum chamber, focussing the ion beam towards a mass separation device. After the ions have been converted to an electrical signal, this is then detected (Thomas, 2013).

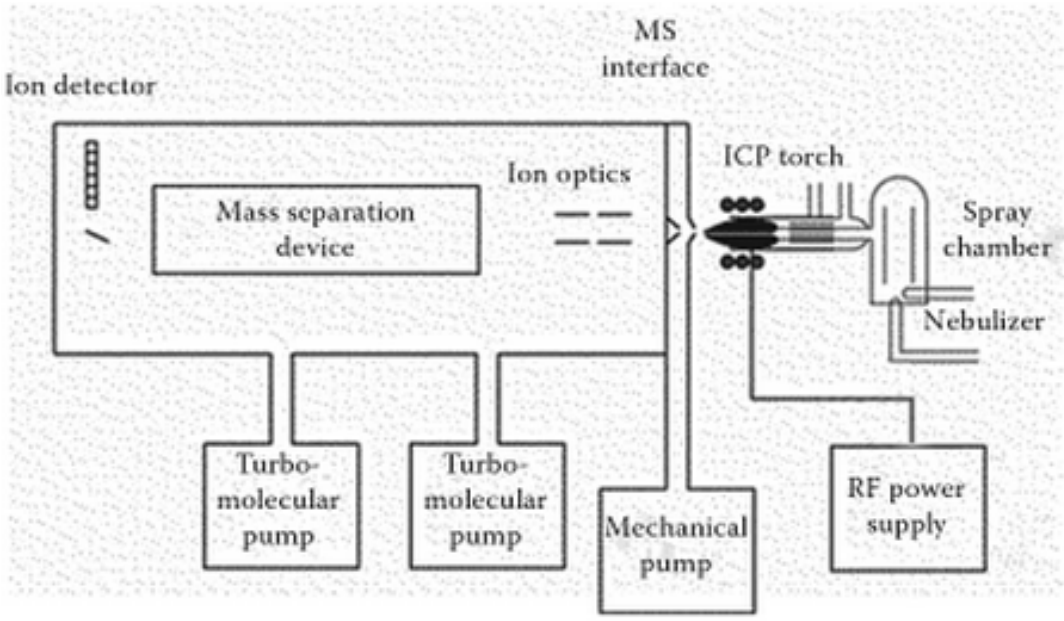

Figure 5 A schematic diagram showing the components of an ICP-MS (Thomas, 2013).

3.8.3.2 Method

Prior to analysis, samples (from chapter 4)were filtered to 0.2 µm using nylon syringe filter and acidified to 5% v/v HNO_3. Analyses were carried out on an Agilent 7500CX ICP-MS (CA, USA), which had been calibrated with Spex CertiPrep Inc standards (Middlesex, UK), with regular analyses of quality control standards throughout the analyses of samples.

3.8.4 Determination of 0.5M HCl extractable Fe(II) and Fe(III) in the sediment phase

3.8.4.1 Background

0.5M HCl extractable Fe

In the method described by Lovley and Phillips (1986), sediments are digested for 1 h in 0.5 M HCl, and considered to be a good proxy for biologically available Fe(III). This method has been used extensively in studies of microbial Fe(III) reduction (e.g. Heron *et al*. 1994; Weber *et* al. 2006; Williamson *et al*. 2013).

Ferrozine assay

A colorimetric ferrozine based assay was used to determine Fe(II) concentrations in sediment microcosm and flow through experiments (Lovley and Phillips, 1986). This

method uses the Ferrozine molecule, 3-(pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt, which forms a purple complex with Fe(II). This complex, when in solution, absorbs light at 562 nm (Stookey, 1970).

UV-Vis spectroscopy

To quantify Fe(II) using the ferrozine assay, absorbance of the Fe(II) ferrozine complex was measured at 562 nm using a UV-Vis spectrophotometer. Firstly, the absorption of Ferrozine solution (blank) at 562 nm was measured to enable correction for background absorption. The absorption of standards and samples complexed with ferrozine was then measured. UV-Vis spectrophotometry relies on the Beer-Lambert Law (Equation 1) where ε = absorptivity of substance, c = concentration and l =path length, which states that there is a linear relationship between concentration and absorbance if the path length is constant (Upstone, 2000). Given this law, the production of a calibration curve with absorbance against known concentration will result in a straight line, to which samples can be compared.

$$A = \varepsilon c l \qquad \text{Equation1}$$

To measure the absorbance of a solution, light is passed through the sample; some light may pass through the solution and some may be absorbed. The transmittance of the solution is defined as the ratio of light entering: light exiting the sample, which can be used to calculate absorbance (negative logarithm of the transmittance) (Upstone, 2000).

3.8.4.2 Method

Reagents were prepared as follows:

Table 4 Reagents used to carry out the ferrozine assay

Reagent	**Method**
Ferrozine solution	To 100 ml DIW, add 0.1 g Ferrozine and 1.196 g HEPES, pH adjusted to 7
Hydroxylamine hydrochloride (6.25M)	To 100 ml DIW, add 43.43 g hydroxylamine hydrochloride
Fe(II) standards	Prepare standards of concentrations 1, 5, 10, 20 and 50 mM Fe(II) with $FeSO_4.7H_2O$

This protocol was applied in chapters 4, 5 and 6 of this thesis. Sediments were weighed (approximately 0.1g sediment was used for the assay) and added to 5 ml 0.5M HCl. Sediments and standards (100 µl) were digested in the same way, and were left to digest for 1 h at room temperature. To quantify Fe(II) in duplicate, 50 µl of the digested material was transferred to a cuvette, to which 2.45 ml ferrozine solution was added. The colour was left to develop for 1 min before measuring absorbance at 562 nm. Fe(II) concentrations in sediments were determined by comparison with the calibration curve produced from Fe(II) standard solutions.

Total bioavailable Fe was quantified by adding 200 µl 6.25M Hydroxylamine hydrochloride solution to 5 ml of digested sample, which was left for 1 h at room temperature. In duplicate, 50 µl of digest was transferred to a cuvette, to which 2.45 ml ferrozine was added. After 1 minute, absorbance was measured at 562 nm.

2.8.5 Determination of total cell numbers

3.8.5.1 Background

Acridine orange staining was used to enable the enumeration of total cell numbers in fluid samples in chapters 4, 5 and 6, by allowing visualisation using an epifluorescence microscope. Samples were fixed before staining with a glutaraldehyde fixative. A fixative is used to enable visualisation of the cells in as realistic state as possible (Günter et al, 2008), and to inhibit cellular activity and to preserve morphology (Chao and Zhang, 2011). Glutaraldehyde fixes cell by cross-linking, and can create bonds between proteins (Chao and Zhang, 2011). This method of cell fixation has several limitations including reactions that may lead to the formation of precipitates within the cell, the fact that fixation doesn't immediately stop enzyme activity, and may cause changes to cell staining properties such as the affinity for certain stains (Hopwood, 1972). The benefit of using glutaraldehyde as a cell fixative includes the excellent preservation of fine cell structures (Melan, 1995).

Acridine orange is a DNA and RNA stain (Kepner and Pratt, 1994), and is able to distinguish between living and dead cells due to different staining mechanisms occurring in living and dead cells; penetration of the stain into dead cells causes the dissociation of acridine orange molecules which “polymerize into red fluorescent cation chains,” which does not occur in living cells, causing green fluorescence (von Bertalanffy, 1963). Acridine orange has an excitation maximum of 500 nm and an

emission maximum of 526 nm when bound to DNA, and an excitation maximum of 460 nm and emission maximum of 650 nm when bound to RNA (USF, 2007).

Cell visualisation was carried out under an epifluorescence microscope with a HBO mercury vapour arc lamp. This light source is focussed through a collector lens system, and then through a diffuser. Filters are used to select wavelengths specific to the fluorophore being used, and then a dichronic mirror is used to reflect the light towards the sample, where the flurophores become excited and fluoresce (Webb and Brown, 2013).

The acridine orange staining method was chosen because of its extensive previous use, low cost and ease of use. The method also allows for the use of a low magnification, which allows for large areas to be rapidly viewed (Htut et al. 2002). Because of the bright fluorescence of acridine orange, this allows the background fluorescence to be reduced by using a "narrow band excitation," (Bergström *et al*. 1986). The stain does have several limitations which may impact on reliability of results. For example, acridine orange is not completely DNA specific, therefore it may be difficult to distinguish which particles are microbial cells and which are not (Bergström *et al*. 1986). Acridine orange is also known to interact with humic material, potentially making it difficult to identify microbial cells (Bergström *et al*. 1986). There is also the potential to over or under stain samples, which could cause difficulty in viewing cells (Htut *et al*. 2002). Limitations are also associated with the use of the staining of sediment samples with acridine orange, particularly the obscuration of stained cells by sedimentary material under an epifluorescent microscope thus inhibiting fluorescence detection. Methods were applied throughout the course of these experiments to try and overcome this problem (e.g. using lower concentrations of sample), although the reliability of these methods is not known, which may impact on cell count reliability. Consistency was applied throughout the course of each experiment to ensure that although cell counts in sediment samples may not have been reliable, the trends in cell numbers could be observed.

3.8.5.2 Method

Fluid samples were fixed in glutaraldehyde for at least 24 h before staining with acridine orange. 1 ml of sample was fixed in 10 ml glutaraldehyde. Sediment samples were weighed under conditions that were kept as aseptic as possible. Either 1 g or 0.1 g of sample (weighed prior to analyses) was fixed in 10 ml of sample, and was then shaken and fixed for at least 24 h prior to staining.

Table 5 Reagents used to carry out total cell counts

Reagent	**Method**
1% glutaraldehyde fixative	Dissolve 16 g sodium cocodylate in 1000 ml DIW. Adjust pH to 7.4 with concentrated HCl. Add 10 ml Glutaraldehyde solution (50% solution). Vacuum filter to sterilise
Acridine orange solution	Firstly, prepare di-potassium hydrogen phosphate solution by dissolving 4.35 g K_2HPO_4 in 500 ml DIW. Then prepare a potassium di-hydrogen solution by dissolving 3.40 g KH_2PO_4 in 500 ml DIW. Combine 42 ml of the di-potassium hydrogen phosphate solution with 8 ml potassium di-hydrogen phosphate solution, adjust the pH to 7.5 using the potassium di-hydrogen phosphate solution, and add 5 ml acridine orange (BDH chemicals Ltd), and filter sterilise

Slides were prepared by submerging a filter (Isopore membrane filter, pore size 0.2 µm, Merck Millipore, MA, USA) in sterile DIW and placing on to an acetate filter that had been inserted into a Büchner flask. A flame sterilised glass filter chimney was fixed to the acetate filter. 5ml of sample fixed in glutaraldehyde was vacuum filtered onto the membrane filter. The filter was then stained with approximately 0.5 ml acridine orange for two minutes, and then rinsed twice with isopropyl alcohol. The filters were left to dry, and then cut in half. The halves were placed on two separate microscope slides, and one drop of immersion oil (Type F immersion media, Leica, Wetzlar, Germany) was placed on the filter, before placing a cover slip on the filter. Slides were viewed using a Zeiss universal microscope with a Zeiss III RS epi-fluorescence head, filter set 09 (40-490 nm). Total cells ml^{-1} were calculated using equation 2 (where C = counts in field of view, M = magnification, S= volume of sample (ml), F= volume of fixative (ml), Fi= volume of fixed sample (ml)).

$$\text{Cells ml}^{-1} = C \times M \times ((S + F)/ (Fi \times S)) \qquad \text{Equation 2}$$

3.8.6 Most probable number counts

3.8.6.1 Background

Most probable number counts are used to estimate numbers of culturable microorganisms. The methodology involves quantifying results of serial dilutions

(Beliaeff and Mary, 1993), with the use of media selective for the types of microorganism you wish to enumerate. The method assumes that microorganisms are distributed throughout the sample randomly, and that growth will occur in the culture medium if at least one microorganism is present (Cochran, 1950). Calculations of most probable numbers are based on the frequency of occurrence of a sequence of positive results that are most likely to occur when a specific number of microorganisms are present in a sample (Oblinger and Koburger, 1975). Most probable number tables are used to calculate most probable numbers in a given sample, and can be calculated from a Poisson or binomial probability model (Beliaeff and Mary, 1993).

3.8.6.2 Method

Most probable number counts were utilised in several of the experiments carried out to quantify culturable microbial populations, and some of the results are detailed in chapter 6. The Media were prepared and autoclaved as described in Table 6.

900 µl of each sterile medium was aliquoted into 10 ml autoclaved serum bottles under anaerobic conditions. For each sample, a decimal dilution series was created, from 1:10 (sample: media) down to 1:1000 and triplicates of each dilution series were prepared. Serum bottles were incubated in the dark at 21°C for two weeks, after which, a positive or negative result was noted for each serum bottle as follows: Sulfate-reducing bacteria- the production of a black precipitate; iron-reducing bacteria- loss of brown colouration; nitrate-reducing bacteria- biomass build-up. Most probable number counts were calculated using the MPN tables published in de Man (1983).

Table 6 Composition of media used during MPN analyses

Reagent	**Method (all values are in gL^{-1} unless otherwise stated)**
Trace Mineral Mix (Balch *et al*. 1979)	Nitrilotriacetic acid (1.5), $MgSO_4.7H_2O$ (3.0), $MnSO_4.2H_2O$ (0.5), NaCl (1.0), $FeSO_4.7H_2O$ (0.1), $CoSO_4$ (0.1), $CaCl_2.2H_2O$ (0.1), $ZnSO_4$ (0.1), $CuSO_4$. $5H_2O$ (0.01), $AlK(SO_4)_2$ (0.01), H_3BO_3 (0.01), $Na_2MoO_4.2H_2$ (0.01)
Trace Vitamin Mix (Balch *et al*. 1979; values in mgL^{-1})	Biotin (2), Folic acid (2), Pyridoxine hydrochloride (10), Thiamine hydrochloride (5), Riboflavin (5), Nicotinic acid (5), DL-calcium-pantothenate (5), Vitamin B_{12} (0.1)
Postgate B (sulfate-reducing microorganisms)	To tap water, add KH_2PO_4 (0.5), $CaSO_4$ (1.0), NH_4Cl (1.0), $MgSO_4.7H_2O$ (2.0), sodium lactate (3.5), yeast extract (1.0), $FeSO_4.7H_2O$ (0.5), ascorbic acid (0.1), sodium thioglycollate (0.1). Adjust pH to 7-7.5 with concentrated sodium hydroxide solution
Medium for Fe(III)-reducing microorganisms	Ferric citrate (13.7), Vitamin mix (10 ml), Mineral Mix (10 ml), $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.6), KCl (0.1), Sodium acetate (0.82). Adjust pH to 6.8 with concentrated sodium hydroxide solution
Medium for nitrate-reducing microorganisms	$NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.6), KCl (0.1), Mineral Mix (10 ml), Vitamin Mix (10 ml), Sodium acetate (0.82), KNO_3

3.8.7 Confocal Laser Scanning Microscopy

3.8.7.1 Background

Confocal laser scanning microscopy (CLSM) is a commonly used technique for biological applications, including for the imaging of living specimens, and was used in chapter 4 of this study. This microscopy technique relies on a point light source (multiple laser excitation sources; Claxton *et al*. 2005) directed onto a sample. The Emitted light from the sample is then imaged onto a point detector via a pinhole. As the pinhole blocks fluorescence that is travelling to the detector from an out of focus position, only focused fluorescence reaches the detector (Bhushan, 2012). A wide range of fluorochromes are utilised for CLSM due to the ability of certain fluorescent probes to target, for example, specific biological components, processes, and environmental variables (Claxton *et al*. 2005).

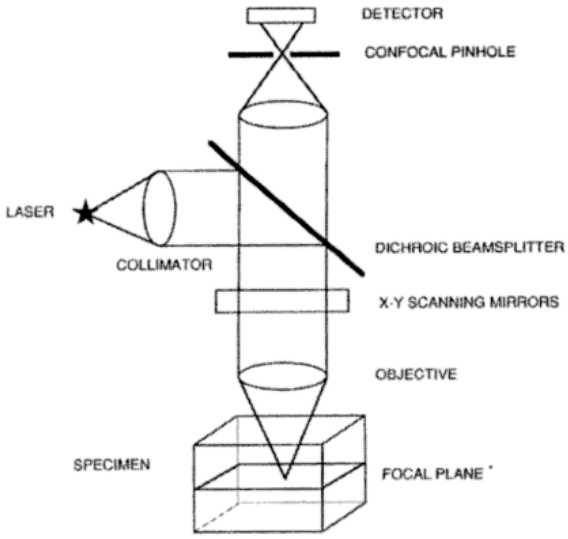

Figure 6 Principle of confocal laser scanning microscope (Masters, 2004).

The fluorochromes utilised in these experiments for imaging with a CLSM were part of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen): Propidium Iodide and Syto9. Propidium iodide is a DNA stain and is used to visualise dead cells.

3.8.7.2 Method

CLSM was carried out using a Leica TCS SP5 AOBS upright confocal using a 63x/ 0.90 HCX PL Apo objective. The confocal settings were as follows: pinhole *1 airy unit*, format *1024 x 1024*. Images were collected with the following settings: FITC 494-530 nm and Texas red 602-665 nm, using the 488 nm (25-30%) and 594 nm (100%) laser lines respectively. Sediment samples were transferred to a petri dish and immersed in sterile DIW. A combination of propidium iodide and Syto9 (from the LIVE/DEAD® BacLight™ Bacterial viability kit, Life Technologies, CA, US) were used to visualise live and dead cells. 1 µl of each stain (undiluted) was added directly to the sample and each sample was agitated for approximately two minutes before viewing under the CLSM. The use of undiluted stains proved unsuccessful as the images became completely saturated. A 1:200 dilution was prepared with sterile DIW which proved much more successful.

A negative control was prepared to investigate interactions between grain surfaces and the cell stains. Negative controls were comprised of crushed sandstone samples that had been subjected to autoclaving at 121°C for 20 minutes and were stained and

viewed under the same conditions as the experimental samples. These scans revealed that some fluorescence on the grain surfaces was clearly not a result of the presence of cell structures.

3.8.7.3 Image processing

Images were processed in ImageJ (Abramoff *et al*. 2004). The "make composite" tool was used to merge the red and the green channels. Scale bars were added to images by firstly calculating actual pixel size (equation 3) and setting the length of the pixel in ImageJ.

$$\text{Actual pixel size (nm)} = \frac{\text{(CCD pixel x binning)}}{\text{(Lens magnification x objective)}} \qquad \text{Equation 3}$$

3.8.8 Scanning Electron Microscopy (SEM)

Electron microscopy techniques were used as part of this thesis (in chapters 5 and 6) to investigate mineralogical changes that occurred over the course of experiments.

3.8.8.1 Background

SEM uses an electron gun to provide and accelerate electrons (Figure 7), the cross section of the beam is then reduced by the condenser lenses and then electrons interact with the surface of the sample (Figure 8). Electrons are scattered from various depths, with some escaping the surface of the sample. Secondary electrons are those that escape from close to the surface of the sample, with backscattered electrons scattered from deeper in the sample. These two types of electrons are used to generate an SEM image. Various deflectors are used to scan across the sample to construct an image of an area of the sample using various detectors. In this study, secondary electrons were detected to generate an image of the surface of the sample. Secondary electrons are detected by firstly accelerating them with a voltage on the scintillator. The accelerated secondary electrons interact with the scintillator material, producing light which travels to a photomultiplier tube, transforming the light into electrical signals (Khursheed, 2011).

When the primary beam interacts with the sample, photons are also generated, allowing identification of elements, enabling mineralogical characterisation of geological samples. Energy dispersive spectroscopy (EDS) involves the detection of X-rays that are generated by the beam interacting with the sample; when X-ray photons enter the detector a photoelectron is generated which interacts with atoms in the lithium silicon crystal, ionizing atoms and generating electrons (Russ, 1984; Williams *et al*. 1986). Amplification of the charge allows a signal relating to the energy to be processed, generating an EDS spectra. When the ionized atoms return to the ground state, the characteristic X-rays that are emitted allow interpretation of the elemental composition of a sample (Williams *et al*. 1986).

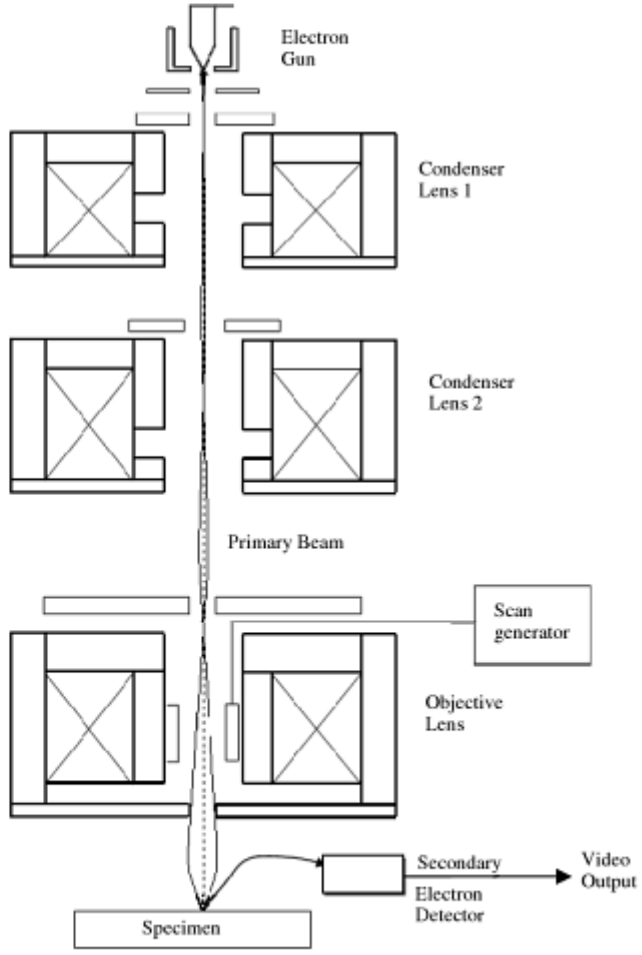

Figure 7 Schematic diagram of a Scanning Electron Microscope (Khursheed, 2011)

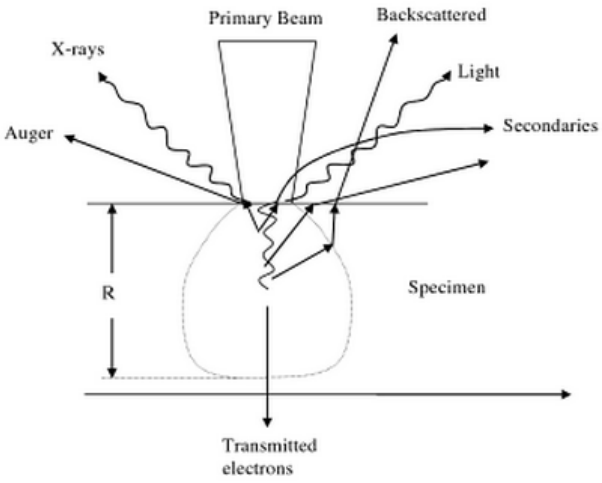

Figure 8 Beam interactions with the sample (Khursheed, 2011)

To investigate biological features of geological materials used in this project, environmental scanning electron microscopy (ESEM) was utilised. This instrument is similar to a conventional SEM, but is able to operate at low pressures, preventing drying out of biological specimens which are largely comprised of water. Secondary electron detection cannot take place in the same manner as conventional SEM, so a gas-phase detector is used. Secondary electrons are accelerated, and collisions with gas molecules present in the specimen chamber result in ionization of the electrons, causing an electron cascade to occur, amplifying the signal prior to reaching the detector (Stokes, 2003).

3.8.8.2 Method

Prior to viewing samples under conventional SEM, sediment samples were rinsed with isopropyl alcohol under anaerobic conditions, and then dried. Samples were then fixed to an SEM stub and carbon coated. An FEI Quanta 600 ESEM was used to view samples in high vacuum mode. For viewing in ESEM mode (again on an FEI Quanta 600 ESEM), samples were kept saturated prior to placing in the specimen chamber.

3.8.9 X-ray imaging

3.8.9.1 Background

Radiography and X-ray microtomography were used to image features such as pore networks in flow through experiments non-destructively. Radiography is based on the principle that the extent to which an X-ray beam will be absorbed by an object is related its density. High density objects absorb more of the X-ray beam (Erkonen and Smith, 2010). The X-rays that are passed through are then detected on a film; the image that is captured represents the contrast between densities of the different materials present in the sample (Moo-Young Jr and Laplante, 1999). The internal structure and composition of a material can be visualised by the use of X-ray computed tomography. This technique involves the collection of a series of radiographs through the depth of a sample, which can then be reconstructed into a 3D image, allowing features such as pore networks to be visualised.

3.8.9.2 Methods

X-Ray radiography

Columns and sandstone cores (from chapters 5 and 6 respectively) were imaged using a GE Isovolt 320kV unit (Brisbane, Australia). Images were collected at 0° and 90° from 0°. Image settings were as follows:

PEEK columns:

- 120kV
- 4Ma
- 1200mm (distance from focal spot in detector head to imaging plate)
- 45 seconds

Sandstone cores:

- 80kV
- 9mA
- 1200mm (distance from focal spot in detector head to imaging plate)

- 1 minute

X-Ray microtomography

Columns from chapter 4 were imaged using a Nikon XTH 225 ST system at the University of Manchester X-ray imaging facility (MXIF) Research Complex at Harwell, Rutherford Appleton Laboratories. A total of 2001 projections were collected each scan, using the following settings:

- 40kV
- 290μA

Images were processed by firstly creating a 3D stack in MATLAB (MATLAB 2012), and then filtered and segmented using AVIZO standard (Visualization Sciences Group (VSG), Bordeaux, France).

3.8.10 Gamma Camera Imaging

3.8.10.1 Background

Technetium-99m is a metastable nuclear isomer of technetium-99, containing the same numbers of protons and neutrons, but with differing energies. ^{99m}Tc is a γ-emitter with a half-life of six hours (Walker and Carroll, 2005), which decays to ^{99}Tc, a β-emitter with a half-life of 2.15 x 10^5 y (Mattsson, 1978). A scintillation (gamma) camera is used to detect γ-ray emissions; the camera contains NaI(Tl) crystals, which on interactions with γ-rays produce a flash of light which is detected by photomultiplier tubes (Gottschalk, 1966). ^{99m}Tc γ-camera imaging is common in medical applications, although a range of studies have demonstrated applications for biogeochemical studies (e.g. Lear *et al*. 2010). This technique was utilized in chapter 5 of this study.

3.8.10.2 Method

After the long term PEEK column experiments (see section 3.5) had run for approximately 8 months, columns were transferred to Manchester Royal Infirmary. The experiment was then re-assembled in front of a γ-camera (Figure 9). Three way taps were fitted at the column inlets, through which ^{99m}Tc (25-40 mBq; as pertechnetate) was

injected. The ^{99m}Tc was then flushed through the columns with the same fluids used during the long term experiment (sparged for 20 minutes with N_2), for 12 h using a peristaltic pump at a flow rate of 6 ml h^{-1}. A dual-headed Siemens Symbia T6 gamma camera was used to image the location of the radiotracer every 15 minutes over the 12 h period.

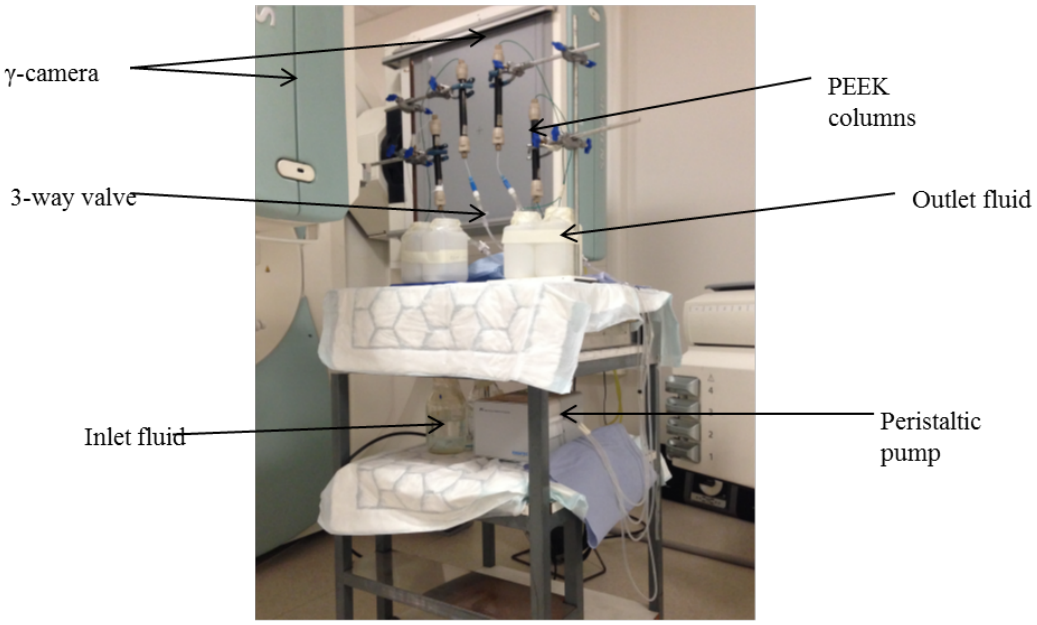

Figure 9 Experimental setup of Tc^{99m} tracer experiment

3.8.10.3 Data processing

Images were processed in Xeleris (GE healthcare). To analyse the images, the column images were divided into 3 equal sections (column inlet, mid-section, and outlet). Data collected from both gamma camera heads were used to calculate geometric means of gamma photon counts, and were normalised to take into account the variation in activity that was injected into each of the columns.

3.8.11 X-ray Diffraction

3.8.11.1 Background

X-ray diffraction is a technique used to identify particular phases within crystalline materials. The technique is based on the principle that Cu Kα X-rays will be diffracted the lattice planes of a crystalline structure (Figure 10). The incident X-rays (at an angle

of θ labelled 1 and 2 on Figure 10) interacts with the sample, either by scattering, or by passing through the sample (A) to the next lattice (B). The X-rays that are scattered by different atoms at different positions have differing phase shifts, which are detected (Dinnebier and Billinge, 2008). The diffraction patterns can be interpreted using the Bragg Equation (Equation 4, where λ = wavelength of rays, θ = angle between incident rays and surface, d = spacing between layers, n = an integer). Most materials have differing diffraction patterns; identification of sample composition takes place by comparing diffraction patterns from the sample to a database of known materials.

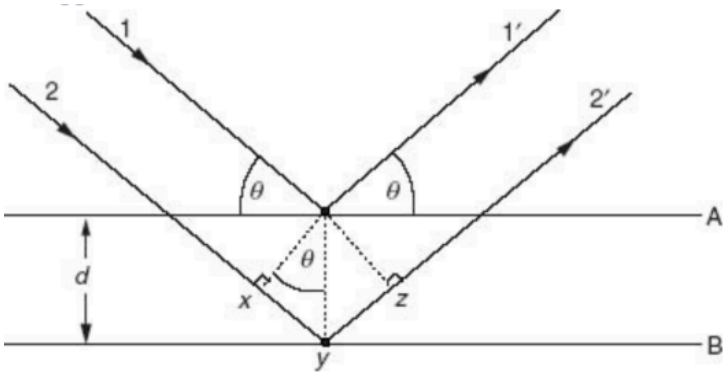

Figure 10 Diagram showing diffraction from a set of planes (West, 2014)

$$n\lambda = 2d\sin(\theta) \qquad \text{Equation 4}$$

3.8.11.2 Method

Quantitative XRD was carried out on sediment samples (starting materials and sediments used in chapter 4) using a Bruker D8Advance X-Ray Diffractometer. Samples were ground to a fine powder, and amyl acetate added. The sample was mounted on a microscope slide and then analysed.

3.8.12 DNA Extraction

3.8.12.1 Background

To investigate microbial communities in sediments and fluids, DNA was first extracted from the samples using a PowerSoil® DNA isolation kit (Mo Bio Laboratories Inc,

Carlsbad CA, USA). This kit relies on a bead-based homogenizing process to lyse cells, releasing the DNA. This method has proven to yield greater concentrations of DNA compared to other methods, but does also result in larger concentrations of contaminants including humics (Miller *et al*. 1999). Several methods are employed to separate DNA from the contaminants, whilst ensuring the integrity of DNA. These methods involve a series of centrifugation steps, with the addition of a number of reagents to precipitate contaminants. The DNA is then washed on a spin filter, before elution of the DNA from the filter. This technique was used in chapters 4, 5 and 6.

3.8.12.2 Method

DNA was extracted from sediments and fluids with a PowerSoil® DNA isolation kit (Mo Bio Laboratories Inc, Carlsbad, CA USA), following the manufacturer's instructions. The kit contains six solutions named C1-C6. 200 µl of fluid sample or 0.2 g sediment sample was transferred to a Bead Solution tube (provided in the kit) and inverted several times to mix. 60 µl of solution C1 was then added to the tube and transferred to the Powerlyser (Bench top bead-based homogenizer, Mo Bio Laboratories Inc, Carlsbad, CA USA), for 45 seconds at 2000 rpm. The tubes were then centrifuged for 1 minute at 14,000 rpm. The supernatant was then transferred to a clean microcentrifuge tube. 250 µl of solution C2 was added to the tube, vortexed, and then incubated at 4°C for 5 minutes. The tube was then centrifuged for 1 minute at 14,000 rpm. 600 µl of the supernatant was transferred to a clean microcentrifuge tube, along with 200 µl solution C3, and then vortexed. A 5 minute incubation at 4°C then took place, after which the tube was centrifuged for 1 minute at 14,000 rpm. 750 µl supernatant was transferred to a clean microcentrifuge tube, to which 1200 µl solution C4 was added. The supernatant was transferred to a spin filter and centrifuged for 1 minute at 14,000 rpm. 500 µl solution C5 was transferred to the spin filter, centrifuged for 30 seconds at 14,000 rpm. This step was then repeated. The spin filter was transferred to a clean microcentrifuge tube, and transferred to a DNA speedvac for 5 minutes to dry. 100 µl of solution C6 was added to the spin filter, and after 1 minute, was centrifuged at 14,000 rpm for a further minute. The DNA extract was stored at -20°C.

3.8.13 *Agarose Gel Electrophoresis*

3.8.13.1 Background

Agarose gel electrophoresis has been used extensively during this project, to analyse the quality of polymerase chain reaction (PCR) products, and as a means of visualising

microbial community diversity after carrying out ribosomal intergenic spacer analysis (RISA). The method is based on the migration of nucleic acids through an agarose gel when an electric field is applied. The molecules migrate through the gel at a rate determined by their size, with smaller molecules migrating further through the gel (Smith, 1996). The location of the nucleic acids within the gel after electrophoresis can be visualised by staining the gel with a fluorescent nucleic acid stain and viewing under UV light. A molecular-weight size marker can also be run on the gel at the same time as samples to enable analysis of PCR product size by comparison.

3.8.13.2 Method

Agarose gels were prepared by combining agarose (1 g to detect 16S rRNA PCR products, 3 g to detect RISA PCR products) with 100 ml 1% Tris-acetate buffer with EDTA (TAE) buffer and microwaving until boiling. Either ethidium bromide or SYBR® Safe DNA gel stain (Life Technologies, UK) was added to the gel before pouring. Once set, gels were transferred to an electrophoresis tank and were run at 100 mV for approximately 45 minutes (RISA gels were run for slightly longer to ensure sufficient separation of the bands). This technique was used in chapters 4, 5 and 6 of this thesis.

3.8.14 DNA Quantification with Nanodrop

3.8.14.1 Method

Several techniques used during this project (including PCR and 16S rRNA gene pyrosequencing) required known DNA concentrations within a DNA extract. DNA was quantified in chapters 4 and 5 of this study using a NanoDrop 1000 (Thermoscientific, US), which quantifies nucleic acids spectrophotometrically. Sterilized DIW was used as a blank, and 1 µl of DNA extract was used to quantify nucleic acid concentrations by comparison to calibration curves provided with the software.

3.8.15 16S rRNA gene polymerase chain reaction (PCR)

3.8.15.1 Background

As a result of the difficulties associated with culturing based methods to investigate microbial communities, culture- independent techniques have proven to be invaluable.

The most commonly used gene for identification of prokaryotic organisms in the 16S rRNA gene (Clarridge III, 2004). This gene is widely conserved, and the rates of sequence change between species are very different which allows measurement of phylogenetic relationships (Woese, 1987).

3.8.15.2 Method

A 16S rRNA PCR was carried to ensure DNA was successfully extracted from samples in chapters 4, 5 and 6. The primer set was comprised of 8F (5'-AGAGTTTGATCCTGGCTCAG) (Eden *et al*. 1991) and 1492R (5'-GGTTACCTTGTTACGACTT) (Lane *et al*. 1985). The following 50 µl reaction was prepared in a PCR hood sterilised by UV:

- 36.7 µl sterile DIW
- 5 µl 10 x Ex buffer (Takara Bio Europe, France)
- 4 µl dNTP mix (Takara Bio Europe, France)
- 1µl forward primer
- 1µl reverse primer
- 0.3µl ExTakara Taq (Takara Bio Europe, France)

Reaction conditions were as follows:

- 94°C for 4 minutes
- 35 cycles of:
  - 94°C for 30 seconds
  - 57°C for 30 seconds
  - 72°C for 1 minute
- Final extension at 72°C for 10 minutes

Prior to pyrosequencing, a 16S rRNA PCR was carried out targeting a 311 bp region of the gene. The primer set was comprised of 27F (5'-AGAGTTTGATCMTGGCTCAG) (Lane,

1991) and 338R (5'-GCWGCCTCCCGTAGGAT) (Daims *et al*. 1999). 454 adaptors were included in the forward primer, along with a 10 bp multiplex identifier. In a UV sterilised PCR hood, the following 50 µl reaction was prepared with sterile DIW:

- 0.4 µM forward primer
- 0.4 µM reverse primer
- 0.5 mM L^{-1} of each dNTP
- 1.8 mM L^{-1} $MgCl_2$
- 5 µl of 10 x PCR buffer
- 2 U Fast start high fidelity enzyme blend (Roche diagnostics Ltd, Burgess Hill, UK)

The PCR conditions were as follows:

- 95°C for 2 minutes
- Then 35 cycles of:
    - 95°C for 30s
    - 55°C for 30s
    - 72°C for 45
- Final extension at 72°C for 5 minutes

3.8.16 Ribosomal Intergenic Spacer Analysis (RISA)

3.8.16.1 Background

To gain an initial idea of bacterial diversity in samples collected at varying distances along columns, Ribosomal Intergenic Spacer Analysis (RISA) was carried out on some DNA extracts collected as part of chapters 4 and 5 of this project (where a crude indicator of changes in microbial community diversity was required). This method involved the use of primers that amplify the region between the 16S and 23S rRNA genes. This region varies in nucleotide sequence and length between bacterial species,

so that when the RISA PCR products are subjected to gel electrophoresis the bands (representing bacterial species) separate at varying positions on the gel, giving an indication of community diversity (Fisher and Triplett, 1999).

3.8.16.2 Method

Prior to preparation of the reaction mixture, the PCR hood was subjected to UV light for 5 minutes. The following 50 µl reaction mixture was then prepared in the PCR hood:

- 36.6 µl sterile DIW
- 5 µl 10x Ex buffer (Takara Bio Europe, France)
- 4 µl dNTP mix (2.5mM each nucleotide; Takara Bio Europe, France)
- 1 µl 25µM ITSF primer
- 1 µl 25µM ITSReub primer
- 0.4 µl Ex Takara Taq (Takara Bio Europe, France)
- 2 µl of DNA extract was added outside of the PCR hood, to prevent contaminating the PCR hood with DNA.
- In the case of negative controls, 2µl sterile DIW was added in place of DNA extract.

The primers utilised in these analyses were ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al*. 2004). The PCR reaction was carried out under the following conditions:

- Initial denaturation at 94°C for 3 minutes

Then 35 cycles of:

- Denaturation at 94°C for 45 seconds
- Annealing at 55°C for 1 minute
- Extension at 72°C for 2 minutes
- Then a final extension step at 72°C for 7 minutes

The PCR products were then run on a 3% agarose gel to ensure adequate separation of bands of similar sizes (at 100V for approximately 45 minutes).

3.8.17 16S rRNA gene pyrosequencing

3.8.17.1 Background

16S rRNA gene sequencing was carried out using a 454 pyrosequencing procedure, outlined in Figure 11, on samples collected as part of chapters 4 and 5 of this thesis. Firstly, an emulsion PCR is carried out to generate sequence clones. This protocol involves the use of a water/ oil emulsion to compartmentalise genes during amplification to minimise the generation of chimeric sequences (Williams *et al*. 2006). Amplicons that are generated during this PCR then become attached to beads, after which a primer hybridizes with the adaptor sequence (Shendure and Hanlee, 2008). Complementary strands of DNA are synthesised, with the introduction of one nucleotide type each cycle; each time a nucleotide is incorporated, pyrophosphate is released. The addition of adenosine triphosphate (ATP) sulfurylase converts this pyrophosphate to ATP, which provides the energy for luciferase to oxidise luciferin, generating light (Ronaghi, 2001) which is then detected. Many cycles (each with a different nucleotide type) means that the pattern of light detected can be converted into a sequence (Shendure and Hanlee, 2008).

3.8.17.2 Method

After carrying out a 16S rRNA PCR targeting a 311 bp region of the gene (above), the PCR products were subjected to agarose gel electrophoresis, and then purified using a QIAquick gel extraction kit (Qiagen, Crawley, UK). PCR products were sequenced on a GS Junior Platform (454 Life Sciences, Roche Diagnostics, UK).

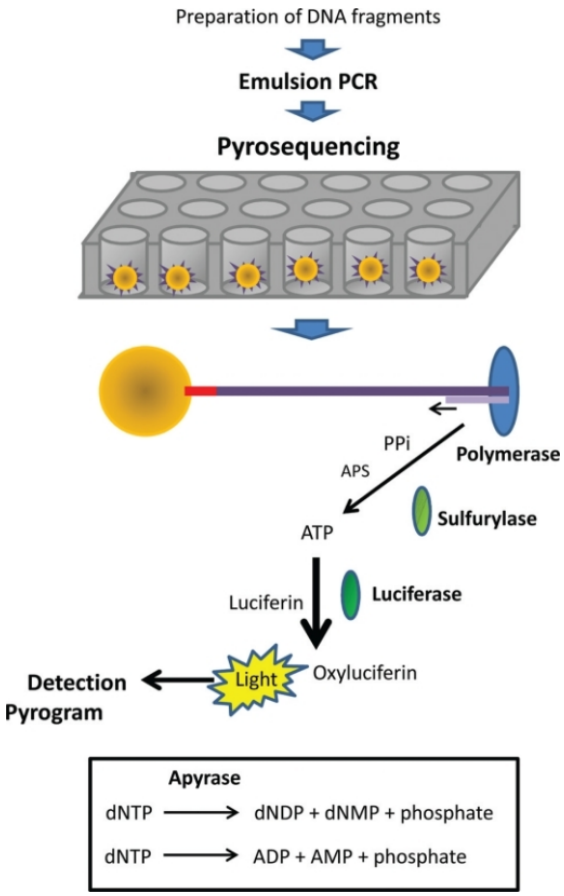

Figure 11 454 pyrosequencing procedure (Siqueira *et al*. 2012)

3.8.17.3 Data processing

Pyrosequencing data sets were processed using the QIIME pipeline (Caporaso *et al*. 2010). This pipeline was used to assign multiplexed reads to each sample. Sequences were then subjected to primer removal and quality control. Sequences shorter than 300 bp and longer than 400 bp were removed. Operational taxonomic units (OTU's) were picked using USEARCH (Edgar, 2010). Representative sequences for each OTU were selected, and taxonomy was assigned using the Ribosomal Database Project (Cole *et al*. 2009). An OTU table was produced, and then sequences were aligned. Alpha and beta diversity were plotted.

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Chapter 4

Research paper

The microbial ecology of a hyper-alkaline spring, and impacts of an alkali-tolerant community during sandstone batch and column experiments representative of a geological disposal facility for intermediate level radioactive waste.

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4.1 Abstract

Naturally occurring hyper-alkaline springs and associated hyper-alkaline environments may have components that are analogous to a cement-based deep geological disposal facility (GDF) for intermediate level radioactive waste (ILW). Such high pH environments could give insights into the biogeochemical processes that could occur in the region of a GDF environment after the ingress of GDF-derived groundwater leads to the formation of a hyper-alkaline plume in the surrounding rock mass. This study focuses on the microbial community composition found at a highly alkaline spring near Buxton, Derbyshire, England, and the variation in community structure across spatially separated sample points of contrasting pH values (ranging from pH 7.5-13). Communities containing alkaliphilic and alkalitolerant bacteria were observed across the site by PCR amplification and 16S rRNA gene pyrosequencing and included members of the families *Comamonadaceae* and *Xanthomonadaceae*. At pH 13, the sequence library was dominated by *Gammaproteobacteria* of the families *Pseudomonadaceae* and *Enterobacteriaceae*. Bacterial communities from the site demonstrated the ability to reduce Fe(III) in microcosm experiments up to pH 11.5, suggesting the potential to reduce other metals and radionuclides of relevance to cement-encapsulated intermediate level radioactive waste (ILW) disposal. In laboratory column flow-through experiments, microbial communities present at the field site were also able to colonise crushed sandstone. Bacterial community composition varied between columns that had been supplied with alkali surface waters from the site amended with carbon (lactate and acetate, as proxies for products of cellulose degradation from ILW), and control columns that were not supplied with added carbon. Members of the family *Clostridiaceae* dominated the sequence library obtained from the carbon amended column inlet (45.8% of library), but became less dominant at the outlet (20.8%). Members of the family *Sphingomonadaceae* comprised 11.8% of the sequence library obtained from the control column inlet, but were not present in sediments collected from the column outlet, whereas the relative abundance of members of the family *Comamonadaceae* increased from the column inlet (35.2%) to the column outlet (57.2%). The spatial variation in community composition within the columns is indicative of discrete biogeochemical zonation in these flow-through systems.

4.2 Introduction

The potential role of microorganisms on the containment of radioactive waste has long been recognised (e.g. Pedersen, 1996; Stroes-Gascoyne and West, 1996; West and

McKinley, 2002). Microbes will be present in the host rock of a geological disposal facility (GDF), repository structural materials and in the waste forms themselves, and contamination from the surface will occur during the operational period.

The transport properties of a GDF host rock may be altered by microbial processes, for example by the formation of biofilm across pore throats, leading to porosity and permeability alteration (Coombs *et al*. 2010). The temporal and spatial extent to which these processes will occur in the host rock of a GDF are uncertain, and is dependent on numerous factors including flow velocity and grain size (Surasani *et al*. 2013), angularity and surface roughness (Crawford *et al*. 2012), the hydraulic pressure gradient (Ginn *et al*. 2005), and biological factors including the metabolic state of cells, the isoelectric point of cell surface polymers, and processes such as filtration and dispersion (Ginn *et al*. 2005). Microbial growth in the geosphere may alter the size and shape of pore spaces, the roughness of grain surfaces, and may even cause aggregation of sediments (Atekwana *et al*. 2006). Microorganisms may also directly influence radionuclide migration through the geosphere, for example by redox state alteration (Lloyd, 2003), or interactions between extracellular biofilm components (e.g. polysaccharides) and radionuclides (Kazy *et al*. 2008).

The UK's intermediate level radioactive wastes will be disposed of in a deep (up to 1000m) GDF, which utilises a multi-barrier concept that could involve significant quantities of cementitious materials (Chapman and Hooper, 2012). In a cementitious GDF, groundwater flow through the repository will generate a hyper-alkaline plume which will interact with the host rock (Savage, 2011), forming an alkali disturbed zone (ADZ), potentially impacting on radionuclide transport processes. Alteration of host rock porosity as a result of the increased solubility of silicates and aluminosilicates under high pH conditions may occur in the early stages of hyper-alkaline plume development (Savage *et al*. 1992), although this process will be counteracted by the precipitation of calcium-silicate-hydrate minerals (Hodgkinson and Hughes, 1999; Savage, 2011). These processes are thought to occur to the greatest extent in the near-field of a GDF (Savage, 2011).

During the initial stages of repository evolution, pore fluid pH (pH 12.5-13; Bateman *et al*. 1999) may be too high to facilitate microbial activity (Rizoulis *et al*. 2012); decreases in pH over time as a result of $Ca(OH)_2$ removal will however, provide conditions more favourable for microbial growth. Natural analogue sites are potentially very useful to help understand the biogeochemical processes that may develop within the ADZ. Naturally occurring highly alkaline environments, such as soda lakes, have been reported to be highly productive and contain a wide diversity of microorganisms (Dimitriu

et al. 2008). For example, VanEngelen *et al*. (2008) isolated alkaliphilic microorganisms that were capable of Cr(VI) reduction at pH 9. Pollock *et al.* (2007) demonstrated the ability of soda lake microorganisms to reduce Fe(III) up to a pH of 10.4. This latter process has been found to be significant in soda lake environments, as it may influence sediment geochemistry, for example by acting as an electron acceptor during the oxidation of organic contaminants, and by facilitating the release of toxic trace metals into surrounding waters (Lovley, 1991). Studies of other naturally occurring alkaline environments, including saline-alkaline soils, have also identified several dominant phyla such as *Actinobacteria* and *Proteobacteria*, similar to those found in a range of other extreme environments (Keshri *et al*. 2013). Identification of several functional genes from these types of environment suggest that the microbes present could potentially utilise sulfur compounds for respiration, and catalyse a range of other biogeochemical reactions (Keshri *et al*. 2013), although the range of coupled processes that can proceed may be constrained by high pH (Burke *et al*. 2012).

Other naturally occurring highly alkaline environments include highly alkaline springs such as those that have formed at Maqarin, Jordan, as a result of groundwater interaction with naturally-occurring cement materials (Khoury *et al*. 1992). The bacterial community in the surface waters at Maqarin is thought to have adapted to the highly alkaline conditions relatively quickly, as indicated by the lack of deeply branching 16S rRNA species (Pedersen *et al*. 2004). Naturally occurring alkaline springs are also present in northern Oman, where serpentinisation causes the formation of highly alkaline waters populated with microorganisms including *Clostridia* and sulfate-reducing bacteria (Bath *et al*. 1987). In other serpentinising systems present in ophiolites, members of the genus *Hydrogenophaga* have been identified (Brazelton *et al*. 2013, Rizoulis *et al*. 2014), under highly alkaline conditions; members of this genus are capable of oxidising hydrogen, and their presence in these systems may be attributed to the generation of H_2 gas during the serpentinisation process.

Several studies have demonstrated tufa deposits (occurring where aqueous environments are supersaturated with calcium carbonate (Perri *et al*. 2012)) and their associated bodies of water contain diverse microbial communities, including members of the phylum *Cyanobacteria* (Ng *et al*. 2006), alkaliphilic members of the genus *Bacillus* and bacterial sequences exhibiting similarities to non-alkaliphilic bacteria, including *Planctomycetes* and *Pirellula* species (Stougaard *et al*. 2002). The formation of other types of highly alkaline environments may occur as a result of the influence of anthropogenic activities. For example at Brook Bottom spring, at Harpur Hill near Buxton, UK, the extensive deposition of lime burning waste has led to the formation of a

hyper-alkaline spring; soils affected by the spring have been shown to contain microbial communities that have evolved relatively quickly to the high pH conditions, and are carrying out processes commonly seen in circum-neutral soils (Burke *et al*. 2012). Rizoulis *et al*. (2012) demonstrated that microorganisms from this field site were capable of nitrate and Fe(III) reduction in microcosm experiments up to pH 11. This study aimed to investigate microbial community composition of shallow surface sediments taken from a hyper-alkaline spring at Harpur Hill near Buxton, to give insights into bacterial community changes under varying pH conditions, and to help understand the range of biogeochemical processes that are potentially carried out at high pH in such an environment. This study also investigates the impact of alkaliphilic and alkalitolerant microorganisms on transport in crushed sandstone, in a laboratory analogue intended to mimic the alkali disturbed zone that could surround a cement-based GDF for ILW. Extensive studies have been carried out investigating microbial impacts on the transmissive properties of rock (e.g. Coombs *et al*. 2010), although little is known about these processes under hyper-alkaline conditions, even though they could potentially play a role in controlling radionuclide transport in the geosphere surrounding a GDF.

4.3 Methods

4.3.1 Site description

Samples for microbial community analysis and experimental investigations were collected from Brook Bottom Springs near Harpur Hill, 2km south of Buxton (Ford and Pedley, 1996). A Hoffman lime kiln was formally in operation at the site, producing extensive quantities of lime from the lime roasting process. The waste was deposited in a valley at the site, resulting in saturation of the groundwater with calcium hydroxide, which discharges from the waste pile causing significant increases in pH. Interactions between this calcium rich fluid and atmospheric CO_2 leads to the precipitation of tufa. In some areas of the site, particularly at the base of the waste pile where a hyper-alkaline spring has formed, the pH of the water is above 13, although the pH of the fluids at the site vary drastically, and is influenced by factors such as rainfall. After periods of heavy rainfall, streamwater flowing through a culvert (HH 7.5, figure 1) is of circumneutral pH, creating mixing zones across the site facilitating calcium carbonate precipitation.

Samples were collected from five areas around the site varying in pH from 7.5-13, in April 2012. At each sampling point, pH and Eh values were measured in the field using a

HANNA HI 9828 multiprobe calibrated to pH 7 and pH 10 prior to use. Surface sediment and fluid were collected in sterile containers. Samples were stored in airtight bottles in the dark at 4° C until needed.

Figure 1 Aerial photography of Brook Bottom Spring at Harpur Hill in Buxton. Sampling points, and corresponding pH values at the time of sampling are also shown (Ordnance Survey data 2013; Grid reference at centre: SK 056 717).

4.3.2 Microcosm experimental design

Microcosm experiments were assembled with sediment and fluid collected from the variable pH sampling points to investigate microbial activity across the pH ranges found at the Harpur Hill site. Sandstone from the Hollington Sandstone Quarry (Sherwood Sandstone group) was used in these experiments as it contains a range of minerals, some of which may be found in a host rock for a GDF including quartz, feldspar, and micas. The friable nature of the sandstone also means that when the material is crushed, the creation of fresh (highly reactive) mineral surfaces is kept to a minimum, so reactions taking place within experiments occur with aged surfaces (AE Milodowski,

personal communication, July 2014). Serum bottles (100 mL) contained 30 g crushed sandstone (Particle size <500 µm), 5 g of Harpur Hill sediment, and 60 mL of fluid from the corresponding sampling point. Acetate (5 mM) and lactate (5 mM) were added to each microcosm bottle as electron donors, along with low amounts of yeast extract (50 mgL^{-1}; DIFCO, UK). Electron acceptors included Fe(III) in the sandstone, along with nitrate (concentrations ranging from around 0-4 mgL^{-1} in Harpur Hill fluids) and sulfate (concentrations varying from 3-15 mgL^{-1} in Harpur Hill fluids) in the Harpur Hill groundwater. Microcosms were assembled under anaerobic conditions under a headspace of 95% N_2/5% H_2 and sealed with a grey butyl rubber septa, held in place with an aluminium crimp cap. A range of control experiments were assembled ((i) autoclaved microcosm, (ii) surface water with no sediment, (iii) no electron donor control, and (iv) a sandstone free control) with fluid from each of the Harpur Hill sample sites. Microcosms were sampled aseptically under anaerobic conditions on a weekly basis for 4 weeks.

4.3.3 Column experimental design

Fluid samples were collected from the pH 11.4 sampling point in June 2012, and stored at 4° C for two months until needed. PEEK columns (15 cm long, 0.75 cm internal diameter; Applied Research Europe, Berlin, Germany) were packed with crushed sandstone (Grain size<500 µm; approximately 10 g). Columns were assembled in duplicate, with two receiving fluid with the addition of 50 mgL^{-1} yeast extract, along with 5 mM of both sodium acetate and sodium lactate as electron donors. Two columns received unamended fluid (controls). Under anaerobic conditions (95% N_2 / 5% H_2), fluid was pumped through the columns by a peristaltic pump at an initial rate of approximately 1 mLh^{-1}, for 46 days. The outlet fluid was sampled on a weekly basis; total cell counts were made and the fluid chemistry was analysed; the flow rate for each of the columns was calculated.

At the end of the experiments, the columns were cut in to 5 x 3cm sections, the exposed sample at the ends of the sections was removed aseptically, and sediment samples were taken from each section and preserved for analyses as described below.

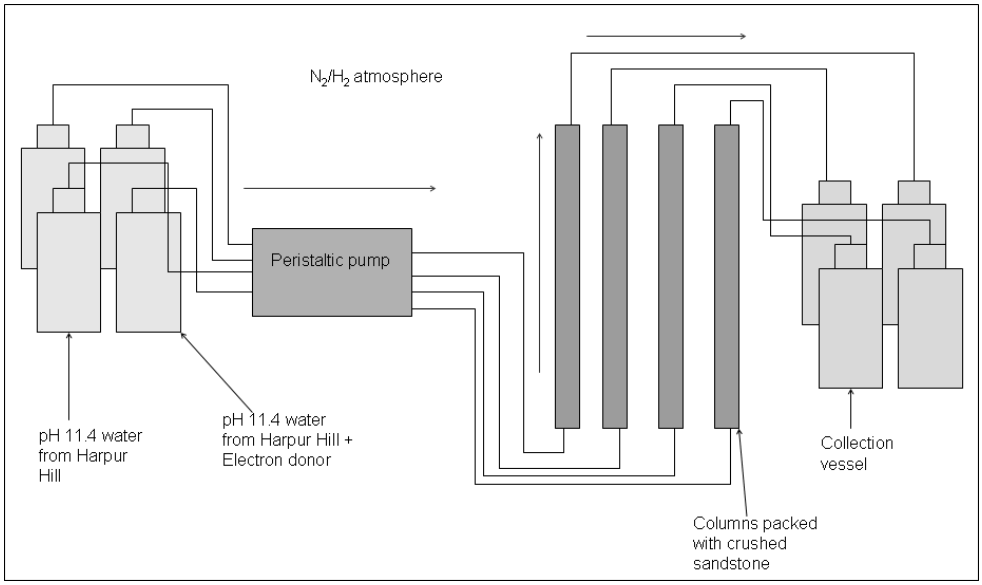

Figure 2 Schematic diagram showing the experimental set-up of the column experiments. Surface water collected from the Harpur Hill site was pumped through the columns via a peristaltic pump, with collection vessels collecting the outlet fluids, under anaerobic conditions (95%N_2/5%H_2).

4.3.4 DNA extraction, PCR amplification and sequencing

DNA was extracted from the five slurry samples collected from the pH variable sites at Harpur Hill, using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc) following the manufacturer's protocol, and also from each of the sediment samples taken from along the columns after operation. DNA was stored at -20° C until needed. Ribosomal Intergenic Spacer Analysis (RISA; Cardinale *et al*. 2004) was performed on the DNA extracts to give an indication of microbial diversity in the samples, and along the columns. Primer set ITSF/ITSFReub consisted of 5'-GTCGTAACAAGGTAGCCGTA-3' (forward primer) and 5'-GCCAAGGCATCCACC-3' (reverse primer). PCR conditions were described previously by Cardinale *et al*. (2004). PCR products were visualised on a 3% agarose gel. A pyrosequencing methodology was then applied to investigate the microbial community composition within the samples. DNA extracts were subjected to a 16S rRNA PCR targeting a 311 bp region of the gene. Primers used were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane, 1991) and 338 (5'- GCTGCCTCCCGTAGGAGT-3') (Daims *et al*. 1999). 454 Life Sciences adaptor primers were included in the forward primer, along with a 10 bp sample-specific multiplex identifier (MID). PCR products were verified on an agarose gel, and then purified with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Positive and negative controls were also subjected to PCR to ensure amplification of contaminants was minimal prior to pyrosequencing; the negative control

used was sterile purified water, and no amplification was noted from this sample. The positive control was DNA extracted from *Geobacter sulfurreducens*. Pyrosequencing on a GS Junior platform (454 Life Sciences, Roche Diagnostics, UK) was used to sequence amplicons according to the manufacturer's instructions. Pyrosequencing data were processed using the QIIME software pipeline (Caporaso *et al*. 2010) for quality control and primer removal. Sequences less than 300 bp and more than 400 bp were then removed.

4.3.5 Phylogenetic analysis

The QIIME software pipeline (Caporaso *et al*. 2010) was used to pick and assign OTUs (operational taxonomic units) using usearch (Edgar, 2010). Phylogeny was assigned using the Ribosomal Database Project (Cole *et al*. 2009), with a minimum confidence of 80%. Blastn nucleotide search was then used to identify representative sequences for each OTU.

4.3.6 Geochemical and mineralogical analyses

pH measurements were carried out using a Mettler Toledo pH probe, calibrated with pH 7 and pH 10 buffer solutions. Redox potential was measured with a HANNA ORP probe. Alkalinity measurements were carried out with acid titration (Burden and Cave, 1999). Trace metal analysis was carried out using an ICP-MS (Agilent 7500cx), anions and cations were quantified with IC (Dionex DX-600). Acetate, lactate and propionate were quantified using capillary ion exchange chromatography on a Dionex BioLC with a Dionex ICE AS1 column, as previously described by Bassil *et al*. (2015). Standards were prepared using Spex Certiprep 1000 mgL^{-1} Acetate solution, Accuspec 1000 ppm Lactate solution and Sigma Gold Label Propionic Acid. Quantitative X-ray diffraction analysis was carried out on the crushed sandstone before and after the experiments. Samples were finely ground with the addition of amyl acetate, and slides prepared. Slides were scanned with Bruker D8Advance X-Ray Diffractometer. Pore water and 0.5N HCl extractable Fe(II) was quantified using 2,2'-Bipyridyl.

4.3.7 Microbial imaging and cell counts

For bacterial cell counts, samples were fixed in 1% glutaraldehyde, and stored at 4°C until analysed. Direct cell counts were carried out using epifluorescence microscopy. Cells were stained with acridine orange, and slides were viewed with a Zeiss universal microscope with a Zeiss III RS epi-fluorescence head, filter set 09 (40-490 nm). For viewing with a confocal laser scanning microscope (CLSM), approximately 0.1 g of sediment was transferred to a petri dish. Stains from a LIVE/DEAD *bac*Light™ Bacterial Viability Kit (Life Technologies) were diluted 1:200 with deionised water; 10 µl of each of the dilute stains were applied directly to the sediment. The sample was then immersed in deionised water. Images were collected on a Leica TCS SP5 AOBS upright confocal microscope using a 63 x HCX Apo L Objective and 4 x zoom. Images were collected using the following settings: *FITC 494-530 nm* and *Texas Red 602-665 nm*, using the 488 nm and 594 nm laser lines respectively. Images collected using the confocal microscope were analysed using ImageJ (Abramoff *et al*. 2004).

4.4 Results

4.4.1 Harpur Hill microbial community analysis

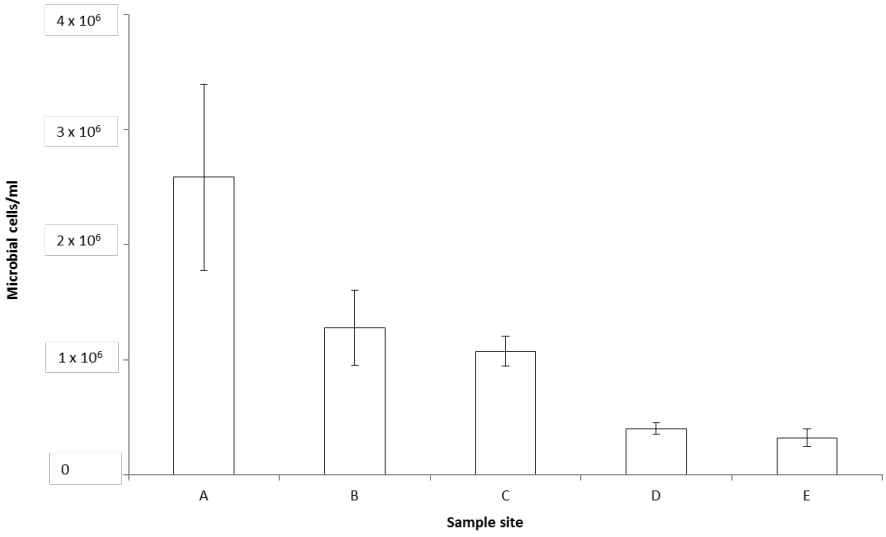

Figure 3 Total cell counts found under varying pH sample sites at Harpur Hill. A pH 7.5; B pH 8.9; C pH 11.4; D pH 12; E pH 13 Error bars show standard error calculated from ten fields of view.

To investigate the molecular ecology of the hyper-alkaline spring at Harpur Hill, samples were taken from across the site, at a range of pH values from 7.5-13. Total cell numbers decreased with increasing pH from approximately 2.5 x 10^6 cells mL^{-1} at pH 7.5 to approximately 3.0 x 10^5 cells mL^{-1} at pH 13 (Figure 3).

A pyrosequencing approach was utilised to characterise the composition of microbial populations found at the site. A total of 45,013 partial 16S rRNA gene sequences were obtained from the five Harpur Hill sampling sites. OTUs were assigned using usearch to cluster sequences to the genus level. The largest number of OTUs were observed in the pH 8.9 sample, (2090); the pH 13 sample site contained the smallest number of OTUs, with 390 observed. Rarefaction curves (not shown) confirmed that the pH 13 site contained a much lower diversity of bacterial species than the pH 7.5-12 sites.

Table 1 shows the relative abundance of bacterial genera from the five sampling sites. The sequence libraries from the site were dominated by members of the bacterial classes alphaproteobacteria, betaproteobacteria and gammaproteobacteria. The sequence library obtained from the pH 7.5 site was dominated by organisms affiliated with the families *Xanthomonadaceae* and *Comamonadaceae* (21.1%). Other families made up smaller percentages of the library, for example organisms affiliated with *Acidobacteria* (4.3%), and *Koribacter* (4.2%). The sequence libraries obtained from the pH 8.9 and pH 12 sites contained complex bacterial communities, with no single family dominant. Significant bacterial families in the pH 8.9 sequence library included *Acidobacteria* (3.6%), *Chitinophagaceae* (2.9%), *Bradyrhizobiaceae* (2.5%), *Sphingomonadaceae* (3%) and *Sinobacteriaceae* (2.9%). A total of 1276 OTUs were observed, although most families made up a very small percentage of the library. At pH 12 organisms affiliated with *Paenbacillaceae* (9.4%) and *Cyclobacteriaceae* (7.5%) were dominant; other significant families include *Chitinophagaceae* (5.6%), *Xanthomonadaceae* (5.4%), *Acidobacteria* (4.7%), *Oxalobactereaceae* (3.1%) and *Hydrogenophaga* (2.7%). The sequence library obtained from the sample collected at pH 11.4 was dominated by organisms affiliated with the families *Comamonadaceae* (24.4%), *Xanthomonadaceae* (12.7%), *Paenbacillaceae* (22.3%), *Sphingobacteriales* (4.3%), and *Acidobacteria* (3.1%). *Pseudomonadaceae* dominated the pH 13 community (25.9%), with other families highly abundant, including *Enterobacteriaceae* (10%), *Comamonadaceae* (7.7%), *Sphingomonadaceae* (4.9%), *Flavobacteriaceae* (3.6%), *Propionobacteriaceae* (3.4%), and *Methylobacteriaceae* (1.5%).

Table 1 Relative abundance of bacterial genera in samples collected from Harpur Hill. HH 7.5: pH 7.5, HH 8.9: pH 8.9, HH 11.4: pH 11.4, HH 12: pH 12, HH 13: pH 13. Only genera comprising more than 1% of the population are shown.

Phylum	Family	Genus	HH 7.5	HH 8.9	HH 11.4	HH 12	HH 13
AD3	Unknown	Unknown	4.70%	3.70%	2.50%	4.60%	
	Unknown	Unknown	1.50%	1.90%		1.60%	
Acidobacteria	Unknown	Unknown	4.30%	3.60%	3.10%	4.70%	
	Koribacteraceae	Unknown	4.20%	3.30%	3.00%	3.80%	
	Koribacteraceae	*Candidatus Koribacter*		1.30%			
	Unknown	Unknown		1.20%			
	Other	Other				1.00%	
	Solibacteraceae	*Candidatus Solibacter*	2.50%	3.90%	1.30%	2.00%	
Actinobacteria	Other	Other		2.20%			
	Propionibacteriaceae	*Microlunatus*					3.30%
Bacteriodetes	Flavobacteriaceae	*Flavobacterium*					3.60%
	Other	Other		1.10%			
	Unknown	Unknown		2.90%			
	Chitinophagaceae	Unknown		2.90%		5.60%	
	Cyclobacteriaceae	Unknown			4.30%	7.50%	
	Flammeovirgaceae	*A4*		1.10%			
	Flexibacteraceae	*Arcicella*		1.10%			
Firmicutes	Alicyclobacillaceae	*Alicyclobacillus*					1.20%
	Paenibacillaceae	*Cohnella*			12.10%		
	Paenibacillaceae	*Paenibacillus*			10.20%	8.90%	
Gal 15	Unknown	Unknown	1.10%			1.10%	
Other	Other	Other		1.60%			
Proteobacteria	Beijerinckiaceae	Unknown					1.20%
	Bradyrhizobiaceae	*Bradyrhizobium*					1.70%
	Bradyrhizobiaceae	Other	1.00%	2.50%	1.10%	1.60%	1.80%
	Hyphomicrobiaceae	*Rhodoplanes*	1.10%	2.10%	1.00%	1.20%	
	Methylobacteriaceae	*Methylobacterium*					1.50%
	Other	Other		1.10%		1.60%	
	Acetobacteraceae	*Roseococcus*	1.00%		1.20%		
	Rhodobacteraceae	*Rhodobacter*				2.10%	
	Rhodosporillaceae	Unknown		1.00%			
	Erthtobacteraceae	Other				1.00%	
	Sphingomonadaceae	Unknown		3.00%			
	Sphingomonadaceae	Other				1.20%	
	Sphingomonadaceae	*Sphingomonas*					4.90%
	EB1003	Unknown		1.20%			
	Comamonadaceae	*Hydrogenophaga*				2.70%	
	Comamonadaceae	Other	21.10%	2.10%	24.40%	3.80%	7.70%
	Comamonadaceae	*Rubrivivax*				1.60%	
	Comamonadaceae	*Variovorax*					1.60%
	Oxalobacteraceae	Other	2.10%	2.60%	1.10%	3.10%	
	Other	Other	1.10%	1.10%	1.10%		
	Unknown	Unknown		1.00%			1.40%
	Enterobacteriaceae	*Escherichia*					1.80%
	Enterobacteriaceae	Other					7.30%
	211ds20	Unknown		1.00%			
	Pseudomonadaceae	*Pseudomonas*	1.90%	2.60%	1.40%	2.10%	25.90%
	Sinobacteraceae	Unknown	1.40%	2.90%	1.00%	1.40%	1.20%
	Sinobacteraceae	Other		1.20%			
	Xanthomonadaceae	Other	26.00%	1.50%	12.70%	5.40%	
	Other	Other		1.10%			

4.4.2 Microcosm experiments

Microcosm experiments were assembled to investigate activity of the microbial communities found under varying pH conditions, especially metal-reducing bacteria that have been identified at this site (Rizoulis *et al*. 2012) and have the potential to reduce and precipitate a range of redox active priority radionuclides. Increases in Fe(II) concentrations were observed only in non-autoclaved microcosms at pH 7.5, 8.9 and 11.4 (Figure 4), and attributed to the microbial reduction of Fe(III) in the sandstone, the Harpur Hill sediment or the Harpur Hill fluid (containing approx. 5 ppb Fe by ICP-MS analyses).

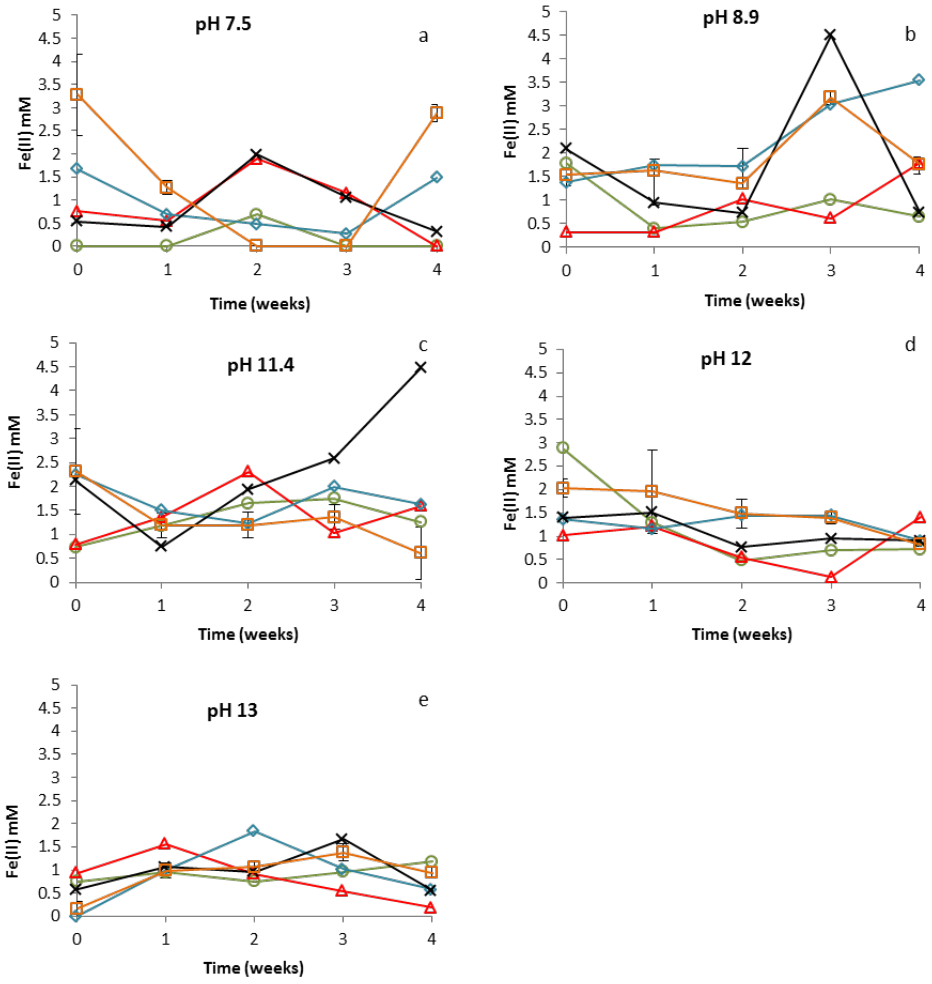

Figure 4 a-e: Fe(II) measurements in anaerobic microcosms assembled with crushed sandstone, sediment and fluid from sampling sites at Harpur Hill poised at a range of pH values, with additions of acetate and lactate as carbon sources. ○: sterile control (autoclaved microcosm); Δ: sterile medium (Harpur Hill fluid); x: Harpur Hill microcosm containing sediment and fluid from the site, with no sandstone; ◊: Unamended control; □: Carbon amended microcosms. Error bars show standard error calculated from three replicate values.

In these microcosms there were concomitant increases in total cell counts consistent with respiration of Fe(III) or other electron acceptors in the microcosms that were used under anoxic conditions. Increases in Fe(II) concentrations were not observed throughout the experiment in the pH 12 and pH 13 microcosms, suggesting that Fe(III) was not respired under these conditions. Increases in Fe(II) were, however, observed in the pH 7.5, 8.9 and 11.4 microcosms over the course of the experiment. The data, however, are noisy, and in some cases higher concentrations of Fe(II) are observed where there has been no carbon amendment meaning the data are difficult to interpret. This is possibly a result of the complex nature of the microcosms, as they contain an environmental microbial consortia, along with potential carbon substrates present in the sediment from Harpur Hill.

Microbial processes are most likely responsible for Fe(III) reduction in these microcosms, and at these pH values, increases in total cell counts were observed, consistent with energy conservation coupled to Fe(III) respiration during the experiment. The highest Fe(II)concentrations were observed in the pH 8.9 microcosms, reaching over 4 mM Fe(II) after 3 weeks (Figure 4). In autoclaved microcosms, no increases in Fe(II) were observed. The pH values of the microcosms, initially poised at pH 7.5 and pH 8.9, remained relatively constant throughout the experiment; in the microcosms with starting fluids of pH 11.4, 12 and 13, rapid pH buffering occurred. The pH 11.4 and 12 microcosms buffered to around pH 8.5 after approximately 1 week, whereas the pH 13 microcosms remained at around pH 10 (data not shown).

Lactate was utilised in biotic microcosms containing crushed sandstone (Figure 5a), although it was utilised more slowly in the microcosms containing fluid from the pH 13 sample site (and propionate production occurred at a slower rate (Figure 5c)) compared to the microcosms assembled with fluids of a lower pH. It would appear that acetate consumption occurred most extensively in the pH 12 and 13 microcosms (Figure 5b), although acetate production as a result of lactate fermentation may have been a factor that increased the acetate concentrations in the lower pH microcosms. Decreases in sulfate concentration were observed in the microcosms poised initially at pH 7.5, 8.9 and 11.4 and containing crushed sandstone, and decreases in nitrate concentration were observed in microcosms containing crushed sandstone at all pH values (Figure 5 (d and e)).

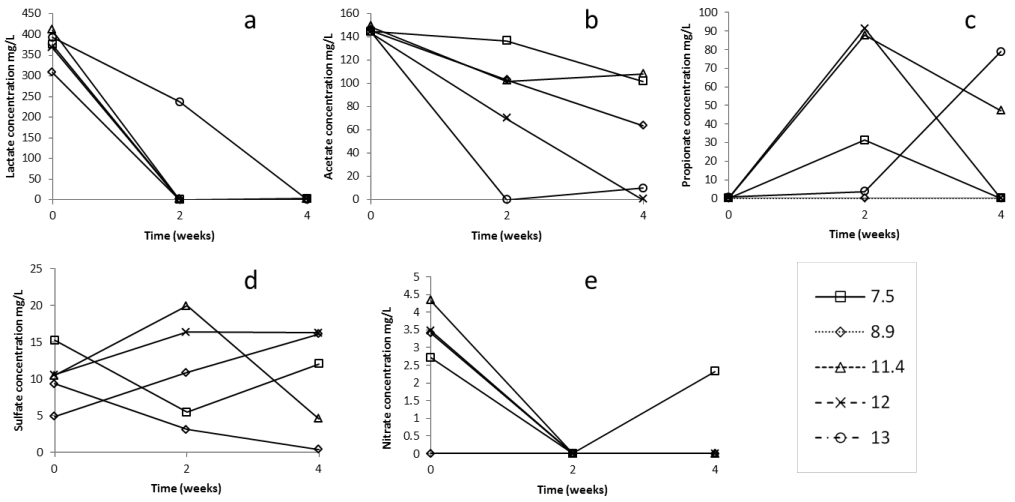

Figure 5 Lactate (a), acetate (b), propionate (c), sulfate (d) and nitrate (e) concentrations in fluids collected from carbon amended microcosms (starting pH values of microcosms are shown in the legend).

4.4.3 Column experiments

Both Fe(III) reduction and sulfate reduction was observed in microcosms containing fluid from the pH 11.4 sample site, so fluid from this sample site was subsequently used in column experiments. These experiments investigated the ability of alkaliphilic and alkalitolerant microorganisms from the site to colonise sandstone, and helped gain an understanding of the processes that they are capable of carrying out under high pH conditions, including biofilm formation and organic acid utilisation. Column experiments ran for a total of six weeks. Initially, a rapid drop in the pH of the outlet fluid (from 11.5 to 8) was observed in both carbon (lactate/acetate) amended and control (no added carbon) experiments (Figure 6). The pH of the control column outlet fluid (Figure 6) gradually increased throughout the experiment to 11.5, whereas the pH of the carbon amended column outlet fluid remained at around pH 8.5. A rapid increase in Eh (Figure 6) was observed initially in the outlet fluids compared to the Eh of the starting fluid in both carbon amended and control experiments; Eh then gradually decreased to approximately -200 mV. Total cell counts in the outlet fluid remained at around 1 x 10^5 cells mL^{-1} in the control experiments, whereas in the carbon amended columns total cell counts increased after 5 weeks to almost 1 x 10^7 cell mL^{-1} (Figure 7).

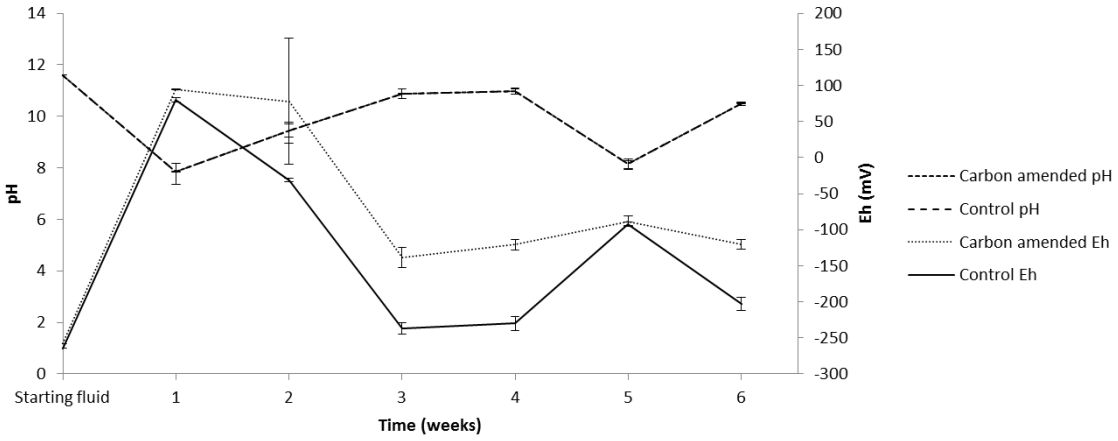

Figure 6 pH and Eh of outlet fluids of carbon amended and control columns. Error bars show standard error calculated from duplicate values. The starting fluid pH was around 11.6 and the Eh was around -260mV.

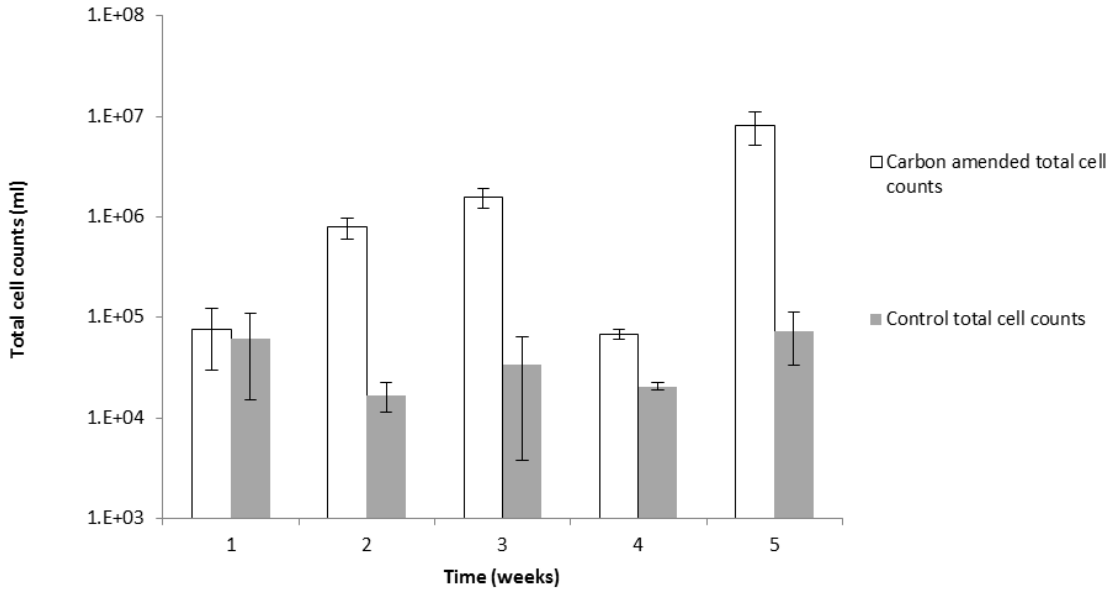

Figure 7 Total cell counts over the course of the column experiments. Error bars show standard error calculated from ten fields of view.

Acetate concentration in the outlet fluids from the biotic columns remained relatively constant at around 350 mgL^{-1}. Lactate concentration decreased rapidly, reaching 13 mgL^{-1} after 4 weeks. Propionate was observed in the outlet fluid after 2 weeks, eventually reaching 192 mgL^{-1} (Figure 8).

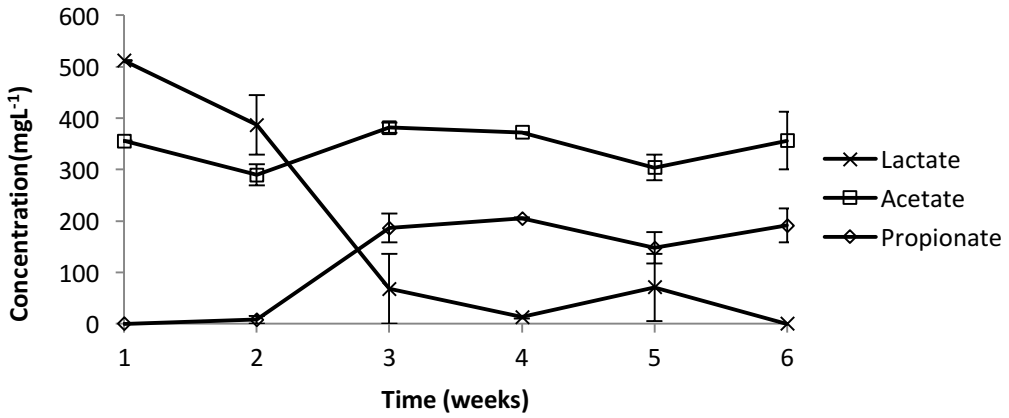

Figure 8 Lactate, acetate and propionate concentration in carbon amended columns outlet fluid. Error bars show standard error calculated from duplicate values.

RISA of sediment samples collected from intervals along the length of carbon amended and control columns (Figure 9) demonstrated differences in microbial community composition between carbon amended and control columns, with microbial biomass decreasing (indicated by decreasing band intensity) moving from the inlet towards the outlet ends of columns.

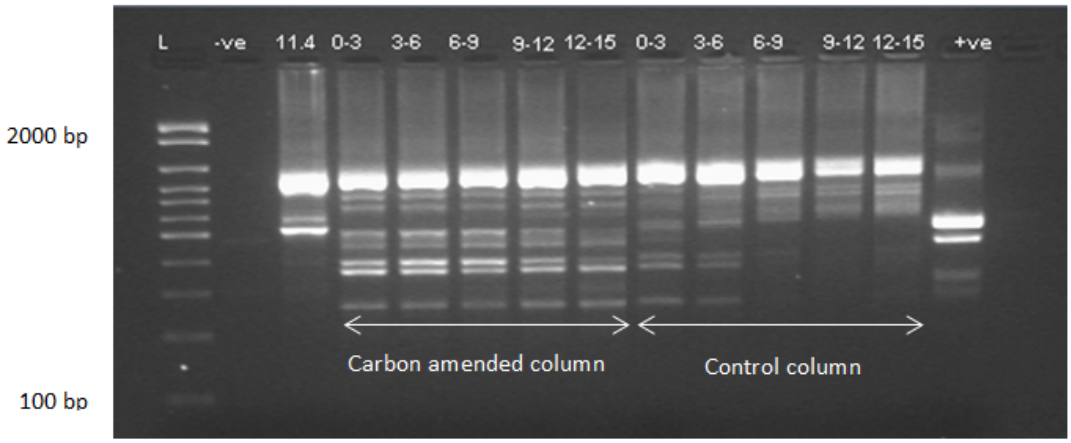

Figure 9 Ribosomal Intergenic Spacer Analysis (RISA) of DNA extracts from the column staring fluid and from sediment samples collected from along a carbon amended and control column. DNA was extracted from 3cm intervals along the columns and subject to PCR-based analysis by RISA. Lane 11.4: Microbial community in the starting fluid before being pumped through the columns. Distance from the inlet (in cm) is shown at the top of the lanes. Positive (*E.coli* genomic DNA extract) and negative (sterile deionised water) controls are labelled +ve and –ve respectively.

Figure 9 shows the presence of distinct microbial communities within the columns that differ from the community present in the Harpur Hill fluid supplied to the columns

throughout the experiment. Colonisation of the carbon amended columns occurred to a greater extent than the control columns, with a higher intensity banding pattern (indicating increased biomass) along the whole length of the column.

The bacterial community composition of samples taken from the inlet and outlet ends of both carbon amended and control columns were investigated, to gain an insight into community differences both spatially, and under carbon amended and control conditions. Genomic DNA extraction was attempted on the starting material (crushed sandstone) although when this extract was run on a 1% agarose gel, no product was observed, suggesting that biomass associated with the sandstone was low. Table 2 demonstrates the differences between the carbon amended and control columns. Members of the family *Clostridiaceae* dominated the inlet end of the carbon amended columns (45.8% of the sequence library), along with organisms affiliated with the family *Comamonadaceae* (35.2%). The relative abundance of *Clostridiaceae* decreased along the column, making up 20.8% of the sequence library at the outlet. There was a slight increase in the relative abundance of organisms affiliated with the family *Comamonadaceae* towards the end of the column to 38.3%. Towards the outlet end of the control column, *Comamonadaceae* made up 52.2% of the sequence library, while the proportion of organisms that were relatively abundant at the inlet end decreased significantly. For example, members of the family *Spingomonadaceae* decreased from 11.8% to 0.3%; *Oxalobactereaceae* decreased from 8.2% to 0.3%, and *Pseudomonadaceae* decreased from 10.9% to 4.9%. Microbial cells were clearly capable of colonising the surface of the sandstone under carbon amended conditions as demonstrated by CLSM (Figure 10).

Table 2 Relative abundance of bacterial genera at inlet and outlet ends of a carbon amended and control column. Only genera comprising more that 1% of the population are shown.

Phylum	Family	Genus	C. amended inlet	C. amended outlet	Control inlet	Control outlet
Acidobacteria	Koribacteriaceae	Unknown				1.00%
	Solibacteriaceae	*Candidatus Solibacter*				1.00%
Bacteriodetes	Other	Other		9.70%		1.00%
Firmicutes	Unknown	Unknown	7.80%	19.80%		3.10%
	Clostridiaceae	Other	45.80%	20.80%		
	Clostridiaceae	Unknown	1.50%			
	Peptostreptococcaceae	*Clostridium*	1.00%			
	Erysipelotrichaceae	*PSB-M-3*	7.50%	9.50%		1.00%
Proteobacteria	Acetobacteraceae	*Roseococcus*			1.50%	2.80%
	Sphingomonadaceae	Other			11.80%	
	Comamonadaceae	Other	31.50%	38.30%	35.20%	57.20%
	Oxalobacteraceae	Other			3.40%	
	Oxalobacteraceae	Unknown			8.20%	
	Enterobacteriaceae	Other			5.90%	2.10%
	Pseudomonadaceae	*Pseudomonas*	1.00%		10.90%	4.90%
	Xanthomonadaceae	Other			2.80%	5.20%

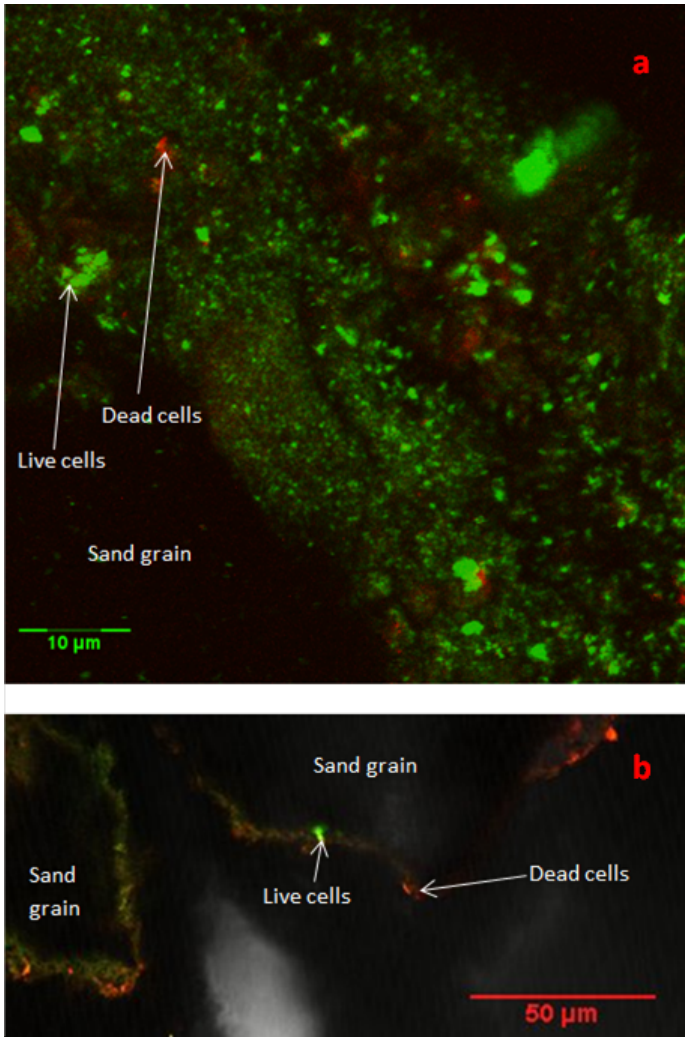

Figure 10 CLSM micrograph showing bacterial cells coating sand grains collected from the inlet end of a carbon amended column. Cells were stained with a LIVE/DEAD *backlight* Bacterial Viability Kit. Green: live cells, Red: dead cells. a: An image showing one view of a 3D projection of a stack comprised of 62 images of a sand grain collected over a depth of 18.08 µm. b: A single image of depth 0.3 µm showing live and dead cells attached to the surfaces of two sand grains.

4.5 Discussion

This study investigated the bacterial communities present in and around a hyper-alkaline spring at Harpur Hill near Buxton, Derbyshire, and assessed colonisation and the potential impacts of microbial growth on the fluid transport characteristics of crushed sandstone. The study provides insights into the types of communities that may be relevant in relation to a cement-based GDF for ILW after the formation of a hyper-alkaline plume, and the processes that they are able to carry out under such conditions.

4.5.1 Harpur Hill molecular ecology

Total cell counts carried out on samples collected from points with pH varying from 7.5-13 indicated that total cell numbers decreased as pH increased. Previous studies investigating microbial communities found in high pH environments have reported comparable levels of planktonic biomass, for example 10^5 cells mL^{-1} were observed in fluid from a hyper-alkaline spring in Maqarin at pH 12.9 (Pedersen *et al*. 2004). This is significantly lower than predictions of total numbers of cells (1.3×10^8 cells mL^{-1}) thought to be able to be supported in the hyper-alkaline waters based on availability of nutrients and energy sources present at the site (West *et al*. 1995). In this study, cells were observed at pH 13 (as shown by acridine orange staining), although the potential for metabolism and growth under these conditions is unlikely; the upper pH limit for bacterial growth is not thought to extend above 12 (Sorokin 2005; Rizoulis *et al*.2012). Neutraphilic bacteria are able to grow up to an external pH of 9 because of their ability to maintain cytoplasmic pH at around 7.5 via a range of mechanisms including up-regulation of "key cation-proton antiporters" (Krulwich *et al*. 2011), while obligately alkaliphilic species are thought to better tolerate increased cytoplasmic pH (Padan *et al*. 2005). The composition of the bacterial communities found at the varying pH sampling sites at Harpur Hill was investigated to determine which organisms may be carrying out geochemically important processes, and to determine the key differences in the communities living under different pH conditions.

Bacterial community composition from each of the sampling sites appeared to be distinct (Table 1). PCR-based pyrosequencing analyses indicated that although the communities differed between sampling sites, some families were common to several sites. For example, *Acidobacteria* spp were present in both neutral and hyper-alkaline samples. Previous studies have also observed *Acidobacteria* in alkaline environments (e.g. Tiago and Veríssimo, 2013; Castro-Silva *et al*. 2013; Keshri *et al*. 2013). Several genera in the family *Comamonadaceae* were also identified in samples taken from across the site up to pH 13. Members of this family have been found to include denitrifying bacteria (Khan et al, 2002) and have been found in other studies of hyper-alkaline environments (Burke *et al*, 2012). Genera in the family *Comamonadaceae* found at the site include *Hydrogenophaga*, which are capable of oxidising hydrogen (Willems *et al*, 1989). *Hydrogenophaga* spp have be found previously in hyper-alkaline environments, for example Tiago and Veríssimo (2013) found that in samples collected from Cabeço de Vide of pH 11.4, 35.1% of their clone library was closely affiliated with *Hydrogenophaga flava*. *Hydrogenophaga* spp were present in travertine springs in

Winter House Canyon at pH 12 (Brazelton *et al*, 2012), and were dominant in alkaline groundwater present in the Leka ophiolite (Daae *et al*. 2013).

Harpur Hill represents a highly alkaline environment where freshwater inputs and rainfall significantly alter the range of pH values found across the site over time. Indeed the presence of a diverse bacterial community at pH 13 may have been linked to heavy rainfall at the time of sampling, causing runoff from the surrounding grassland, as the dominant members of the sequence library obtained from this site were not affiliated with known alkaliphiles, and may not be adapted to life at high pH. Surface waters of pH> 13 have been observed at the site, particularly at the base of the limestone waste, but hyper-alkaline values have been observed across the whole site (pH 12-13) at times of low rainfall. At the time of sampling (April 2012), heavy rainfall had caused an influx of freshwater from the culvert (HH 7.5, Figure 1) at a pH of 7.5, and flow rates of fluid out of the culvert of up to 1.1 ms^{-1} were recorded here. The input of water of a circum-neutral pH value caused significant changes in the pH values of fluids found across the site, ranging from pH 7.5-13. Bacterial cell counts collected at this time reflect these changes in pH, as total cell numbers decrease with increasing pH (Figure 3). The predicted evolution of the ADZ will also deliver pH variations in and around a GDF over time, in this case with increasing distance from the waste, suggesting there may be distinct differences in microbial activity in this scenario also. Lower pH niche environments may also occur within the ADZ, perhaps resulting from factors such as variations in groundwater flow velocity, or heterogeneity related to organic inputs providing more favourable conditions for microbial activity to occur. The results from this investigation suggest that a number of bacterial groups are able to tolerate highly-alkaline conditions, although they may only be active when the pH decreases to more favourable levels in and around the GDF. The occurrence of microbial activity in column experiments carried out under highly-alkaline (pH 11.5) conditions may suggest that microbial activity could be significant in a GDF where the pH conditions are slightly more favourable.

4.5.2 Microcosm experiments

Microcosm experiments were conducted to further explore the functional diversity within communities in the Harpur Hill samples, focusing especially on anaerobes with the potential to impact on radionuclide speciation via enzymatic or sulfide/Fe(II)-mediated reduction mechanisms. Results from microcosm experiments containing crushed sandstone and microbial inocula from Harpur Hill indicated that the microorganisms

from the pH 11.4 site were capable of reducing Fe(III) and sulfate, whereas no increases in Fe(II) were seen in pH 12 and pH 13 microcosms. These findings are consistent with results from previous experiments carried out at hyper-alkaline pH, where anaerobic metabolism (including Fe(III) reduction) was limited at pH 12 (Rizoulis *et al*, 2012). It should be noted that pH buffering of the alkaline microcosms occurred during incubation; pH 11.5 microcosms eventually buffered to around pH 8.5, potentially creating a more favourable environment for biological activity. Metal reduction by bacteria under alkaline conditions has been observed in several other studies, for example Pollock *et al* (2007) isolated a strain similar to *Bacillus agaradhaerens* capable of Fe(III)- reduction up to pH 11. Williamson *et al*. (2013) observed microbial Fe(III) reduction in microcosm experiments at pH 10; with enhanced Fe(III)-reducing conditions observed in the presence of electron shuttles including humics. Fe(III)-reducing bacteria have been found previously to have a key role in controlling radionuclide mobility, as Fe(III)-reducing bacteria have the ability to reduce a broad range of redox active radionuclides (e.g. Tc (VII), U(VI), Np(V)) via enzymatic and Fe(II)- mediated mechanisms (Lloyd *et al.* 2002). Organic acid utilisation and nitrate reduction was also observed in biotic (non-autoclaved) microcosms containing crushed sandstone at all pH values; the pH 13 microcosms were buffered to around pH 10 during the experiment, allowing more favourable conditions for microbial activity to occur.

4.5.3 Column experiments

Sequence libraries from the inlet and outlet ends of the carbon amended and control (non-carbon amended) columns were compared to determine which organisms from the original community were able to colonise the sandstone in flow-through experiments, and how the communities differ when they are stimulated with the addition of carbon sources. Lactate and acetate were added as a surrogate for the cellulose degradation products that may be present in an ILW GDF, and the naturally occurring organic matter that may be present in the pore water of the host rock environment. RISA banding patterns on DNA extracts (Figure 9) from samples taken from 3 cm intervals along the columns demonstrated gradual changes in community composition along the columns, and also indicated that more DNA was extracted from the inlet ends of the columns (suggesting more biomass was present here); other studies have witnessed similar decreases in microbial attachment with distance travelled, even in field studies (Ginn *et al*. 2005). This behaviour may be a result of cell surface properties ensuring microorganisms that are more capable of attaching to surfaces are deposited close to the column inlet (Tong *et al*. 2005). The presence of more biomass at the carbon

amended column inlet is also potentially because of greater utilisation of lactate and acetate at the column inlet; the sequence library obtained from sediment from the carbon amended column inlet is dominated by members of the family *Clostridiaceae*, which became less dominant towards the column outlet. Several studies have demonstrated the changes to microbial community composition that take place as a result of variations in organic carbon availability, which could account for the variation observed along the columns. As an example, soil microbial populations are known to vary vertically along a soil profile as a result of declines in soil carbon availability with increasing depth (Fierer *et al*. 2003). Carbon availability is also known to impact the mechanisms of bioclogging; as an example Hand *et al* (2008) found that grain size impacted upon bioclogging, until carbon concentration decreased below a certain threshold, at which point grain size no longer had significant impacts on biomass build up.

Other environmental characteristics are thought to lead to spatial variations in microbial community composition, including flow heterogeneity through porous media (Besemer *et al*. 2009), and variations in dispersal mechanisms of microorganisms and nutrients (Lin *et al*. 2012). It is likely that during these column experiments, a combination of these factors led to the spatial variation in community composition, perhaps with lower pH niches at the column inlet occurring as a result CO_2 generation from organic acid utilisation. The data shown in Figure 7 could suggest that biomass builds up over time in carbon amended columns, and then processes such as clogging and dispersal occur. The spatial differences in microbial community composition may also be a result of the short timescale over which the experiment was carried out, and perhaps may have become more uniform over the length of the column over a greater timescale.

The sequence libraries obtained from the control column were dominated by *Comamonadaceae*, but far more phylogenetic groups were present in low abundance compared to the carbon amended columns. Previous studies have shown members of the family *Comamonadaceae* to be present in the mature stage of mixed biofilm development (Fernandez et al, 2008). Members of the family *Clostridiaceae* only made up 0.1% of the community in the control column, whereas they dominated the carbon amended column, suggesting the populations of these known fermentative bacteria were stimulated by the addition of a carbon source to the system.

The bacterial populations present in the control columns may be representative of the starting population in the Harpur Hill fluid collected from the pH 11.4 sample site. Although the starting fluids were collected on different sampling days, sequence

libraries obtained from both the pH 11.4 sample site, and the control column are dominated by members of the family *Comamonadaceae.*

Microbial communities in the biotic columns were capable of fermenting lactate to propionate; members of the bacterial family *Clostridiaceae* were dominant in the carbon amended column sequence libraries, and have previously been shown to carry out this fermentation process (e.g. Kuchta and Abeles, 1985). As the acetate concentration remained relatively constant throughout the experiment, this may suggest that acetate was not utilised, or more likely that it was both further oxidised and produced (e.g. via lactate metabolism) at similar rates. Fermentation of lactate to acetate and propionate can occur via several different pathways including the methylmalonly-CoA pathway and the acrylyl-CoA pathway (Seeliger *et al*. 2002). Other alkaliphilic/ alkalitolerant bacteria have been known to produce acetate and propionate as fermentation products under high pH conditions (Zhilina *et al*. 2004). This process may have implications for a GDF, as production of carbon dioxide gas can occur during fermentation (Seeliger *et al*. 2002).

Microbial CO_2 generation in a cementitious GDF may lead to changes in the host rock transport properties as $CaCO_3$ precipitation will occur if the Ca-rich cement pore waters come into contact with CO_2 (Ranaivomanana *et al*, 2013), potentially blocking pore throats. Indeed, the decreased calcium concentration (data not shown) in the outlet fluids of the carbon amended columns throughout the course of the experiment may be a result of organic acid fermentation (Figure 8) leading to the production of carbon dioxide gas, facilitating $CaCO_3$ precipitation. Another potential mechanism for the removal of calcium from solution is the microbially mediated precipitation of $CaCO_3$; it is thought that under appropriate conditions, most bacteria are capable of precipitating calcite crystals (Boquet *et al*. 1973).

In summary, diverse bacterial communities were identified at a highly alkaline spring, demonstrating the ability to tolerate highly alkaline conditions that could be representative of some of the conditions likely to be found in the ADZ around a cement-based GDF for ILW. The bacterial genera dominating the site have been observed previously in other hyper-alkaline environments. The microbial community present at pH 11.5 at the field site were able to reduce Fe(III) in microcosm experiments, and were capable of colonising sandstone in column experiments under alkaline conditions. The microbial communities within the columns also demonstrated the ability to utilize lactate, a surrogate for cellulose degradation products that will be present in a GDF, suggesting that these processes may be able to occur in a host rock environment. Fermentation resulting in the production of CO_2 could potentially cause the precipitation

of calcium carbonate in a cement-based GDF environment, potentially altering porosity and inhibiting radionuclide transport through the geosphere, and this could be accentuated by the bioreduction of radionuclides under anoxic conditions. Although these experiments demonstrate that microbial processes impact on the transport properties of sandstone under high pH conditions over relatively short spatial and temporal scales, the importance of these processes over the lifetime of a GDF remain uncertain. Because of the nature of the hyper-alkaline plume development, it is likely that during ILW repository evolution, microbial activity will be limited to localities where a pH decrease in the plume has occurred. The presence of a carbon source (in the form of cellulose degradation products) may stimulate microbial activity in a GDF host rock environment, although the extent to which alkaline degradation reactions generating these products will occur, before reaching conditions that will favour competing microbial degradation processes (< pH 12; Rizoulis et al., 2012) is uncertain, and will depend on parameters such as the rate of evolution of the hyper-alkaline plume. There also remain uncertainties regarding the evolution of microbial populations over the lifetime of a GDF, because the rapid changes that occurred during the short-term experiments carried out in this study are not representative of the timescales relevant to a GDF. Clearly further research is necessary to ensure that the microbial impacts over the lifetime of a GDF for ILW are better understood. Longer term flow-through experiments under conditions more representative of a potential GDF (with regards to environmental factors including temperature, groundwater velocity, pH, organic carbon availability and changes in the porosity and permeability of the media over time) may provide further insights into the possible impacts of microbial activity on GDF evolution and radionuclide transport.

4.6 Acknowledgements

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results presented. This paper is also published with the permission of the Executive Director, BGS (NERC).

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Chapter 5

Research paper

Microbial impacts on ^{99m}Tc migration through sandstone columns under highly alkaline conditions relevant to radioactive waste disposal

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5.1 Abstract

Geological disposal of intermediate level radioactive waste in the UK is planned to involve the use of cementitious materials, facilitating the formation of an alkali-disturbed zone within the host rock. The biogeochemical processes that will occur in this environment, and the extent to which they will impact on radionuclide migration, are currently poorly understood. This study investigates the impact of biogeochemical processes on the mobility of the radionuclide technetium, in column experiments designed to be representative of aspects of the alkali-disturbed zone. Results indicate that microbial processes were capable of inhibiting ^{99m}Tc migration through columns, and X-ray radiography demonstrated that extensive physical changes had occurred to the material within columns where microbiological activity had been stimulated. The utilisation of organic acids under highly alkaline conditions, generating H_2 and CO_2, may represent a mechanism by which microbial processes may alter the hydraulic conductivity of a geological environment. Column sediments were dominated by obligately alkaliphilic H_2-oxidising bacteria, suggesting that the enrichment of these bacteria may have occurred as a result of H_2 generation during organic acid metabolism. The results from these experiments show that microorganisms are able to carry out a number of processes under highly alkaline conditions that could potentially impact on the properties of the host rock surrounding a geological disposal facility for intermediate level radioactive waste.

5.2 Introduction

The UK's concept for the disposal of intermediate level radioactive waste (ILW) will involve a deep geological disposal facility (GDF), with numerous engineered barriers potentially containing cementitious materials, as, for example a backfill material (NDA, 2010). Movement of groundwater through a cementitious repository will cause an alkaline plume (pH≈13) to develop, which will interact with components of the host rock forming an alkali-disturbed zone (ADZ). This ADZ will impact on microbial populations and processes within the host rock; these biogeochemical processes may in turn play a defining role in controlling the migration of key radionuclides through the geosphere, although it is thought that the upper pH limit for microbial activities is approaching pH 12 (Rizoulis *et al*. 2012). Biofilm formation, for example, may lead to changes in the physical properties of the host rock (Coombs *et al*. 2010), by causing a decrease in pore volume (Taylor *et al*. 2008) and therefore retarding radionuclide migration. Most microorganisms are capable of colonising surfaces, with several environmental cues

thought to play a role in promoting biofilm formation including oxygen availability and pH (Babauta *et al*. 2013). Microbial cells secrete extracellular polymeric substances (EPS) comprising polysaccharides, nucleic acids, proteins and lipids which immobilise cells. The composition of the extracellular matrix is influenced by the microbial community composition, shear forces imposed on the biofilm structure, and other environmental factors (Flemming and Wingender, 2010). Microbial mineral precipitation, including microbially induced calcite formation may have similar impacts (DeJong *et al*. 2006, Cuthbert *et al*. 2012), particularly under the calcium-rich conditions that will be present in a cementitious repository. These processes may lead to alterations of pore space geometries, altering the hydraulic conductivity of the medium, and potentially resulting in a reduction in flow velocity (Hand *et al*. 2008).

The mechanisms by which microbial processes may impact on transport pathways in a GDF environment are wide-ranging and will depend on the local conditions. For example, processes that result in gas production, including organic matter utilisation, may cause pressure increases with the potential to damage waste containing barriers (Bonin *et al*. 2000). Depending on the rate at which gas generation occurs in a GDF, the consequences for fluid transport within a GDF host rock may be varied. Gas accumulation in a porous medium may lead to pore blockages (Taylor *et al*. 1990); the extent to which microbial gas generation occurs in a GDF host rock will depend on factors such as the availability of cellulose degradation products for microorganisms to ferment/ oxidise for example. The UK's ILW inventory contains 2,800 tonnes of cellulosic materials which will degrade under the hyper-alkaline conditions generated in a GDF (NDA, 2014), although there remain uncertainties regarding the concentrations of cellulose degradation products that will be available in a GDF host rock when the pH conditions are suitable for microbial metabolism (Humphreys *et al*. 2010).

As well as physically blocking transport pathways, microbial metabolism may also control radionuclide movement through the geosphere via a range of other mechanisms. These include the alteration of mineral surfaces, for example, by forming biofilms that coat grain surfaces decreasing the availability of sorption sites, or by causing mineral dissolution that may impact the ability of radionuclides to sorb to mineral surfaces (Brookshaw *et al*. 2012). Microbe-radionuclide interactions may also directly impact on the transport of radionuclides, via mechanisms including biosorption to ligands such as carboxyl, amine and phosphate groups associated with the cell surface, bioaccumulation within cells, and direct redox transformations e.g. bioreduction (Lloyd and Macaskie, 2002). Several Fe(III)-reducing microorganisms have demonstrated the ability to directly reduce redox active radionuclides to insoluble species, for example the

reduction of U(VI), Np(V) and Tc(VII) to insoluble tetravalent forms under circum-neutral conditions (Lloyd 2003). However, few studies have addressed microbial influences on radionuclide mobility under highly alkaline conditions representative of aspects of a GDF for ILW. The work that has been carried out investigating these high pH systems has suggested that microorganisms may play a role in immobilising some radionuclides up to a certain pH limit. As an example, Williamson *et al*. (2014) demonstrated that microbial U(VI) reduction occurred in microcosm experiments at pH 10.5, with most of the U(IV) formed associated with the solid phase.

Fe(II)-bearing minerals including biogenic siderite and vivianite resulting from the microbial reduction of Fe(III) may reduce radionuclides abiotically (Brookshaw *et al*. 2012). This mechanism has the potential to retard radionuclide migration through the geosphere, as several radionuclides including Tc are immobile in their reduced state (Lloyd *et al*. 2000). ^{99}Tc (a fission product of U-235 and Pu-239) is one of the UK's priority radionuclides (Walke *et al*. 2012) because of its long half-life (2.13 x 10^5 years; Zachara *et al*. 2007), and high mobility in its oxidised form [Tc(VII)] (Lear *et al*. 2010). Previous studies have investigated the mechanisms by which biogenic Fe(II) is capable of reducing Tc(VII) to Tc(IV), forming a poorly soluble precipitate (Zachara *et al*. 2007, McBeth *et al*. 2011). However, little is known about this process under the highly alkaline conditions that will be present in a GDF for ILW in the UK. A study carried out by Liu *et al*. (2008) demonstrated that the rate at which reductive immobilisation of Tc(VII) by Fe(II) occurs becomes slower as pH increases. Thorpe *et al*. (2014) found similar results when investigating Tc(VII) reduction with pre-reduced Fe(II)-bearing minerals, although a similar rate of Tc(VII) reduction was observed in microcosms at both circum-neutral and at pH 9, when Tc(VII) and Fe(III) reduction was concurrent.

This paper presents results of column experiments that aimed to investigate impacts of microbial processes under highly-alkaline conditions relevant to a GDF for ILW on transport in sandstone, and the resulting implications for Tc mobility. To investigate these processes, high pH surface waters (pH≈ 12.1) were collected from a hyper-alkaline spring which has formed at a legacy lime workings site in the Peak District, UK. This fluid was pumped through sandstone columns with and without acetate and lactate (proxies for cellulose degradation products that will be present in a GDF as a result of the cellulosic materials present in ILW). The impact of microbial colonisation on the mobility of ^{99m}Tc was assessed using a multidisciplinary approach including geochemical, mineralogical and molecular ecological analyses, coupled with radionuclide imaging techniques.

5.3 Methods

5.3.1 Field sampling

A hyper-alkaline spring is present at Harpur Hill, Buxton, which formed as a result of percolation of rainwater though lime kiln waste (Rizoulis *et al*.2012). Surface waters (pH ≈12.1, calcium hydroxide dominated) were collected from a hyper-alkaline spring at Harpur Hill, Buxton in sterile bottles. Bottles were filled completely to ensure no headspace remained, and were stored at 4°C.

5.3.2 Column experiments

Four polyether ether ketone (PEEK) columns (L=15 cm; ID=0.75 cm; Applied Research Europe, Berlin, Germany) were packed with approximately 10 g crushed sandstone (grain size <500 µm). Surface waters collected from the field site were distributed between two sterile polypropylene vessels. Half of the fluid was amended with carbon (5 mM acetate, 5 mM lactate and 50 mgL^{-1} yeast extract). Columns were assembled in duplicate, with two receiving carbon-amended fluid and two receiving unamended fluid. Prior to experiment assembly, all components were sterilised by soaking in Virkon for several hours and flushed through with sterile deionised water. Pressure transmitters (Danfoss MBS3000 0-4bar 4-20 mA transmitter, M&M controls, Manchester UK) were fitted at the inlet and outlet of all columns; pressure was logged in mA every 2 minutes and a calibration curve was prepared to convert mA to bar. Column experiments were carried out under anaerobic conditions (95% N_2, 5% H_2) for 247 days. A peristaltic pump was used to continuously upflow fluid through the columns at a flow rate of 100 μLhr^{-1}.

Outlet fluid samples were collected every two weeks, and fluid samples were collected from the starting fluid to monitor changes in fluid chemistry and biological activity approximately every five weeks. Samples were collected and preserved accordingly.

5.3.3 Geochemical analyses

pH measurements of fluid samples were collected using a Mettler Toledo pH probe which had been calibrated with pH 7 and pH 10 buffer solutions. A HANNA ORP probe was used to measure redox potential. Anions and organic acids were quantified by ion chromatography using a Dionex ICS5000 Dual Channel Ion Chromatograph. Weak acid extractable Fe(II) and total bioavailable Fe(III) was quantified spectrophotometrically as follows: sediment samples (approx. 0.1 g) were weighed and then digested in 4.9 mL

0.5 M hydrochloric acid for 1 h prior to performing a Ferrozine assay (Lovley and Phillips, 1986). Total biologically available Fe was quantified by reducing all bioavailable Fe within the sample using 6.25 M hydroxylamine hydrochloride for 1 h, and then quantified in duplicate after reaction with Ferrozine.

5.3.4 Cell counts

Microbial cells were enumerated in starting and outlet fluids from columns throughout the experiment. A direct counting method was applied using acridine orange staining; samples were prepared by first fixing 1 mL of sample in 10 ml 1% glutaraldehyde. A 5 mL aliquot of the fixed sample was filtered on to a 0.22 µm Millipore membrane filter and stained for two minutes with acridine orange, before rinsing with isopropyl alcohol. Slides were viewed under a Zeiss universal microscope using a Zeiss III RS epi-fluorescence head with filter set 09 (40-490 nm).

5.3.5 Gamma camera imaging of ^{99m}Tc migration through columns

At the end of the experiment, columns were transferred to the Nuclear Medicine department of the Manchester Royal Infirmary for ^{99m}Tc imaging. 25-40 mBq of ^{99m}Tc (as pertechnetate) was injected through one port of a three way tap attached to column inlets. N_2 sparged alkaline groundwater was then pumped through the columns at a flow rate of 6 mLh^{-1} for approximately 12 h using a peristaltic pump. Gamma camera images were taken at 15 minute intervals throughout the course of the experiment using a dual-headed Siemens Symbia T6 gamma camera. Once imaging was complete, columns were stored for one week at 4°C to allow decay of the ^{99m}Tc to background levels. Data were processed in Xeleris (GE Healthcare). For image analysis, the column images were divided into thirds (inlet, mid-section and outlet). Geometric means of counts were calculated from both heads of the gamma camera, and were normalised to account for the variation in activity injected into each of the columns.

5.3.6 X-ray radiography

At the end of the experiment, one carbon-amended and one control column were subjected to X-ray radiography to image the internal structure of the crushed sandstone within the packed columns. Columns were imaged using a GE Isovolt 320 kV unit (GE, Brisbane, Australia) at Intertek Non-Destructive Testing, Derby. Images were collected of

the length of the columns (0°), and then the columns were rotated to image at 90°. Images were collected at a distance of 1200 mm from the focal spot in the detector head and imaging was carried out at a 120 kV-4 mA setting for 45 seconds.

5.3.7 Scanning electron microscopy

Sediment samples were collected from along the columns under anaerobic conditions and rinsed with 100 % isopropyl alcohol to remove traces of the experimental fluid. For SEM imaging of sediment samples, samples were first dried and then transferred to an SEM stub. Samples were carbon coated before viewing using an FEI Quanta 600 ESEM.

5.3.8 Destructive sampling of columns

Following imaging, columns were cut into 3 cm sections by first cutting grooves to a depth of 2.5 mm into the columns using a lathe. Columns were then transferred to anaerobic conditions (95% N_2, 5% H_2), and cut all the way through using a hacksaw. The sediment at the exposed ends of the sections was removed with a sterile spatula to ensure no material was sampled that was contaminated by cutting tools.

5.3.9 DNA extraction, PCR amplification and sequencing

DNA was extracted from sediment samples collected from 3 cm intervals along the columns, and some key outlet fluid samples using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc) following the manufacturer's instructions, and stored at -20°C until analysed. A 16S rRNA PCR amplification was carried out on the DNA extracts to assess whether enough DNA was present to carry out 454 pyrosequencing on the samples. A pyrosequencing methodology was applied to some DNA extracts (with samples where PCR products were detected), targeting a 311 bp region of the 16S rRNA gene. The primer sets used were as follows: 27F (5'- AGAGTTTGATCCTGGCTCAG-3') (Lane, 1991) and 338R (5'- GCTGCCTCCCGTAGGAGT-3') (Daims *et al*. 1999). Sequencing was carried out using a Roche 454 Life Science GS Junior system at the University of Manchester. Phylogenetic analysis was carried out using the QIIME software pipeline (Caporaso *et al*. 2010). Operational taxonomic units (OTUs) were picked using usearch (97 % sequence similarity; Edgar, 2010). The Ribosomal Database Project (RDP; Cole *et al*. 2009) was used to assign phylogeny with a minimum confidence of 80 %. Representative sequences for each OTU were identified using a Blastn search.

Ribosomal Intergenic Spacer Analysis (RISA, Cardinale *et al*. 2004) was also carried out on DNA extracts from the outlet fluids of the column experiments to investigate population changes over time. Primer set ITSF/ITSFReub was utilised and consisted of 5'-GTCGTAACAAGGTAGCCGTA-3' (forward primer) and 5'-GCCAAGGCATCCACC-3' (reverse primer). PCR conditions are described in Cardinale *et al*. (2004). A 3 % agarose gel was use to visualise RISA PCR products.

5.4 Results and Discussion

Potential microbial impacts on the transport properties of porous media are wide ranging and are documented in numerous studies conducted at circumneutral pH. To address the limited amount of complementary data available on high pH systems, relevant to the UK's concept for geological disposal of ILW, flow-through experiments were conducted using columns containing sandstone material, and an aqueous phase consisting of surface water collected from a high pH lime-kiln waste impacted field site. Control experiments containing no added carbon were compared to experimental systems supplemented with the electron donors acetate and lactate.

5.4.1 Transport properties

During our 247 day experiments, injection pressure increases were observed in all columns at approximately 18- 20 weeks (Figure 1). After these pressure increases were observed, a decrease to pressures comparable to starting injection pressures was seen. After approximately 23 weeks, steady increases in injection pressures in the carbon-amended columns were observed, reaching almost 0.1 bar (g). Although these pressure increases are relatively small, the medium used was crushed sandstone with a high porosity and permeability, and a slow flow rate (100 μLh^{-1}) was used. Consequently, these pressure increases could be of significance.

Abiotic processes that may have caused these initial increases in injection pressure could include clogging as a result of the movement of fines (Oliveira *et al*. 2014), or mineralogical alterations as shown in Figure 2. Calcium silicate hydrate (CSH) phases formed a web like precipitate over quartz grains (a) and gelatinous coatings (f), while grains of potassium feldspar coated in calcite (b) and a gel like silicate hydrate were also noted. These reaction products have also been observed in other studies of hyper-alkaline experimental systems (e.g. Hodgkinson and Hughes, 1999). Hexagonal plates

of kaolinite were shown to have expanded (also shown by Xie *et al*. 2007), and were coated with a gel like phase at the edge of the plates (c), and were also observed with a fluffy gel like silicate hydrate coating (e). Debris was shown to adhere to grain surfaces by clumping together with reaction products (g).

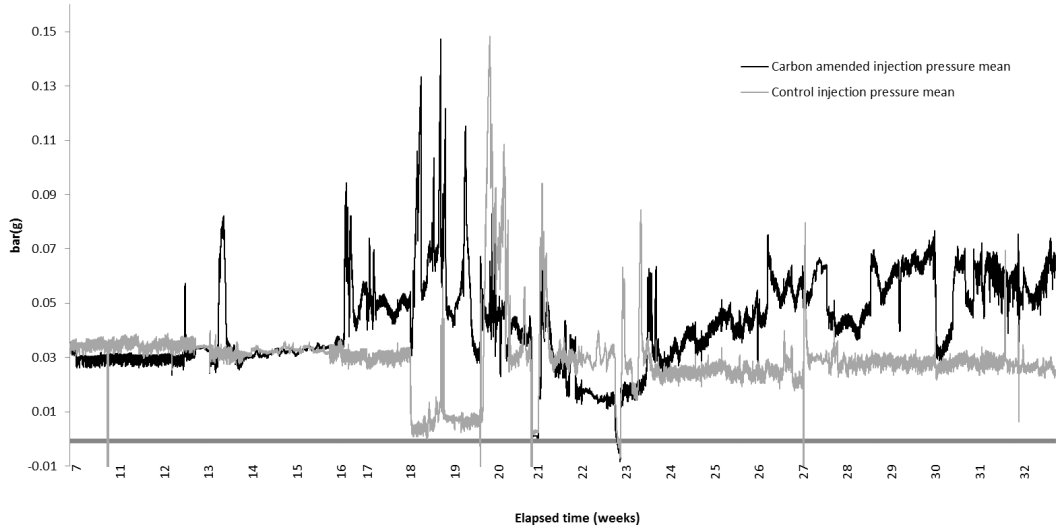

Figure 1 Mean injection pressures of carbon amended and control columns.

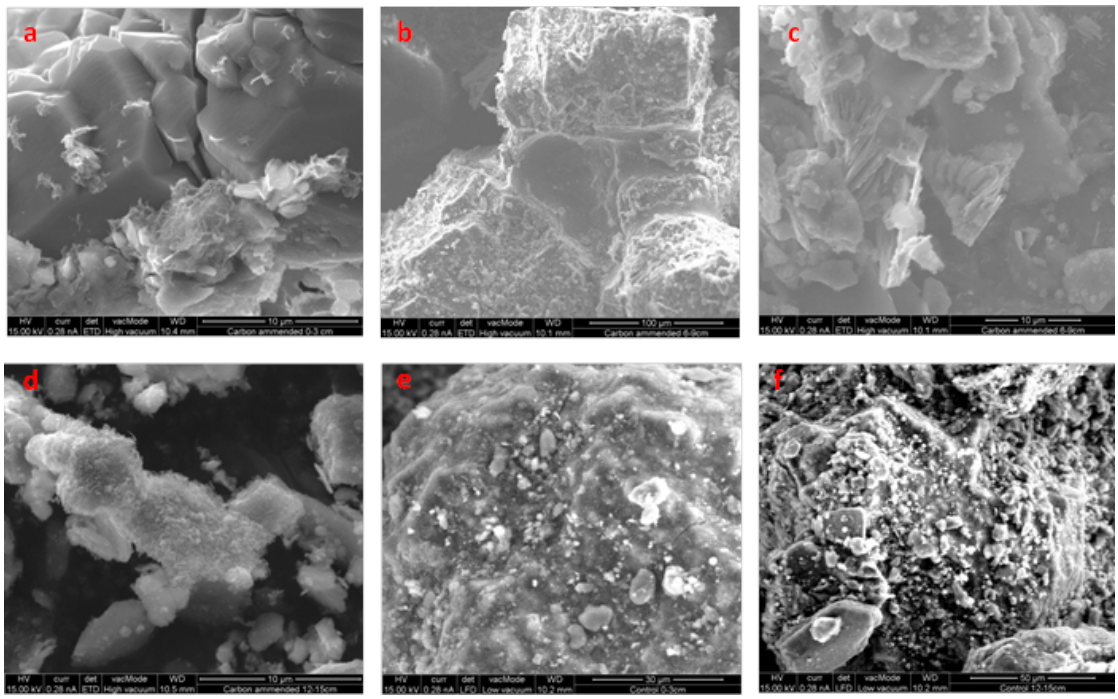

Figure 2 Scanning electron micrographs of sediments collected from columns at the end of the experiment. (a) carbon amended 1 column 0-3 cm, (b) carbon amended 1 column 6-9 cm, (c) carbon amended 1 column 6-9 cm (d) carbon amended 1 column 12-15 cm, (e) control 1 column 0-3 cm, (f) control 1 column 12-15 cm.

The decreases in pressure could perhaps be a result of processes such as the breakthrough of fines (e.g. Gravelle *et al*. 2011). Injection pressure increases towards the end of the carbon-amended experiments (after approximately 23 weeks) appear to be more sustained, and remained at approximately 0.1 bar (g), perhaps implying a microbial role in these increases in injection pressure. These processes could include bioclogging, although sediment samples were subjected to confocal laser scanning microscopy using nucleic acid stains to visualise live and dead cells, epifluorescence microscopy and ESEM (data not shown), and biomass on the sediments appeared to be very low.

At the end of the experiment, columns were subjected to additional analyses to further investigate how microbial activity had impacted the transmissive properties of the sandstone. As biogeochemical profiles had suggested that microbial processes had been stimulated at high pH in the carbon-amended columns, the impact of these stimulated microbial communities on Tc mobility was assessed by applying a spike of ^{99m}Tc (25-40 mBq initial activity) pumped through the columns at a rate of 6 mL h^{-1} and monitoring its mobility using a gamma camera (Figure 3).

A much faster flow rate of 6mL h^{-1} was used to ensure enough pore volumes of high pH fluid flushed the short (6 hr) half-life tracer through the columns. The use of a faster flow rate may have altered the transport properties of the crushed material, as sudden changes in fluid velocity have been shown to alter permeability of porous media (Mays and Hunt, 2005). Nevertheless, Figures 3 and 4 demonstrate that the migration of the ^{99m}Tc occurred much more slowly through the carbon-amended columns compared to the controls.

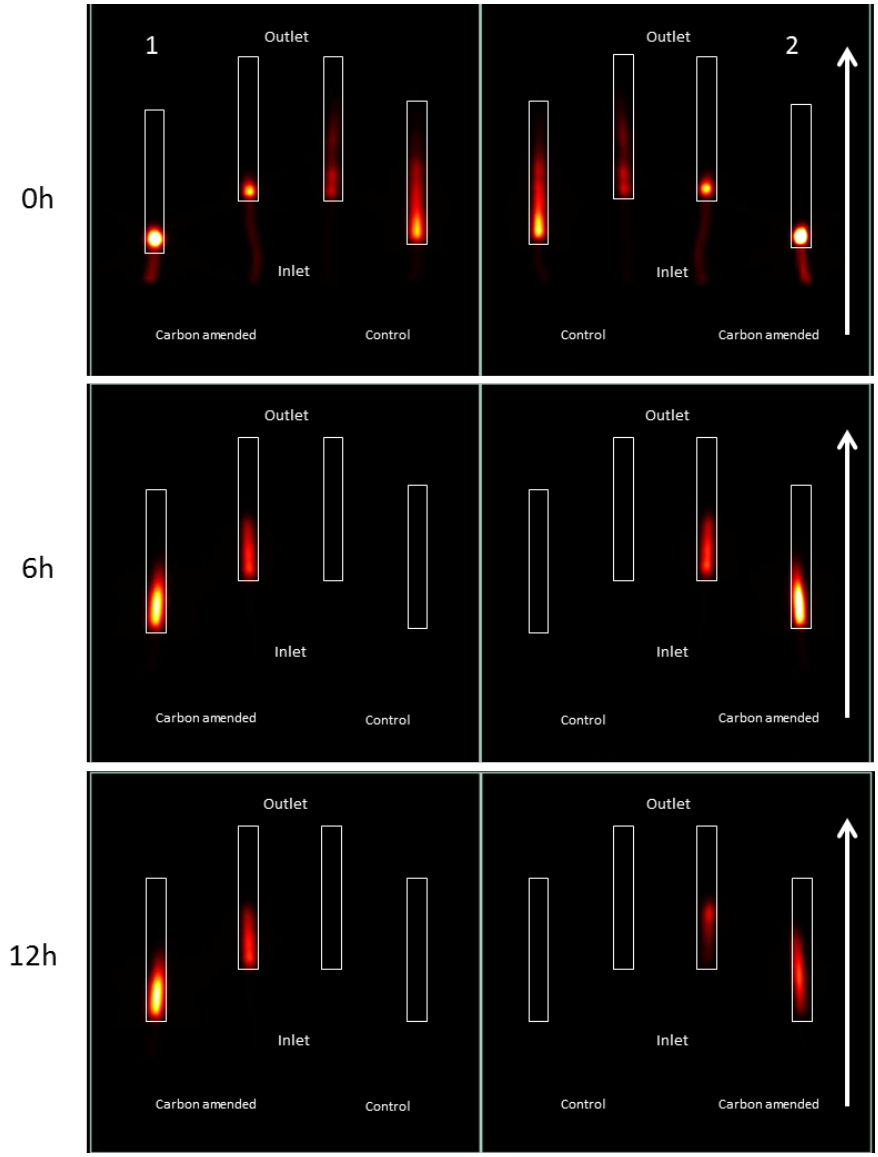

Figure 3 γ-camera images of columns (outlined) at 0 h, 6 h and 12 h. Images on the left were collected with the left hand side γ-camera and images on the right were collected with the right hand side γ-camera. Carbon amended and control columns are labelled in each of the images, and the white arrows show the direction of flow.

Figure 4 shows the location of the activity within the columns throughout the tracer test, indicating that at the end of the 12 h imaging period, the ^{99m}Tc was still retained in the carbon-amended columns; no activity was detected in the control columns after 1 h. There are a number of possible explanations for the inhibition of the ^{99m}Tc migration through the carbon-amended columns. At the end of the tracer test it was noted that the expected amount of fluid had travelled through the control columns (approximately 70 ml), whereas only a few mL of fluid passed through the carbon-amended columns (no leaks were detected throughout the tracer test). An increase in back pressure in the carbon-amended columns as a result of the faster flow rate, could explain the clogging

effect that was observed, due to processes such as shearing of biofilm, and movement of fines towards the outlet. X-ray radiography revealed extensive physical changes in the carbon-amended column that may have impacted the transmissive properties of the crushed sandstone. Gas generation as a result of some of these processes could be responsible for the extensive spherical voids present in carbon-amended columns. Spherical voids (presumed to be gas bubbles) were observed in both columns (Figure 5), though to a much greater extent in the carbon-amended column. The transport of gas bubbles to the column outlet could have also contributed to the clogging effect that was noted in the carbon-amended columns; previous experiments investigating the impacts of microbial gas generation on transport have shown that biogenic H_2 gas stopped fluid flow through columns (Gu *et al*. 1999). Biogenic gas generation has been known to lead to significant reductions in hydraulic conductivity, when entrapment of bubbles in a porous medium may occur in pore spaces, inhibiting transport of fluid through the pore (Baveye *et al*. 2010).

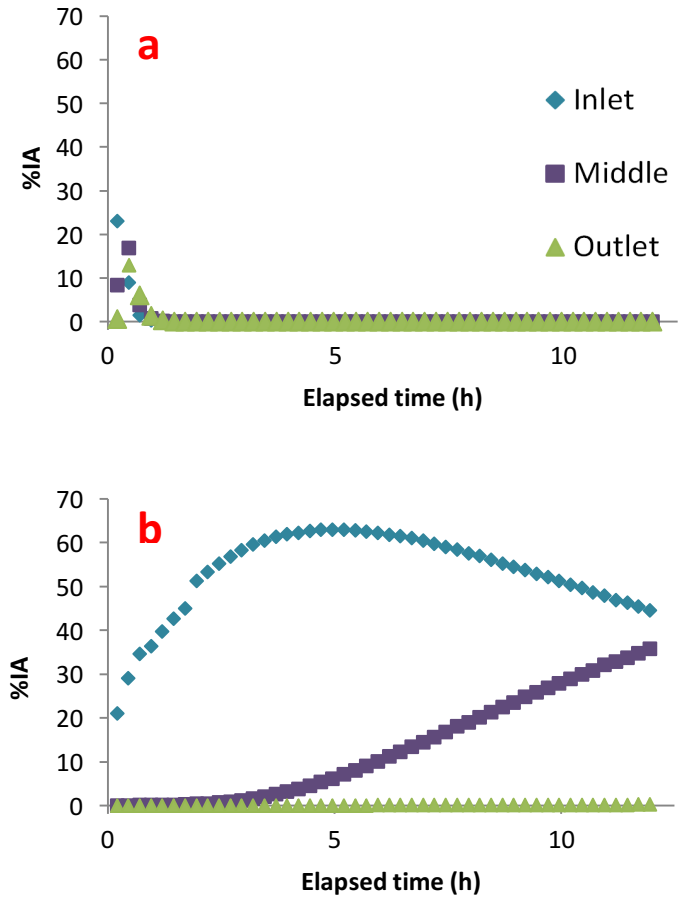

Figure 4 Mean decay corrected counts of Tc^{99m} in (a) control columns (b) carbon amended columns.

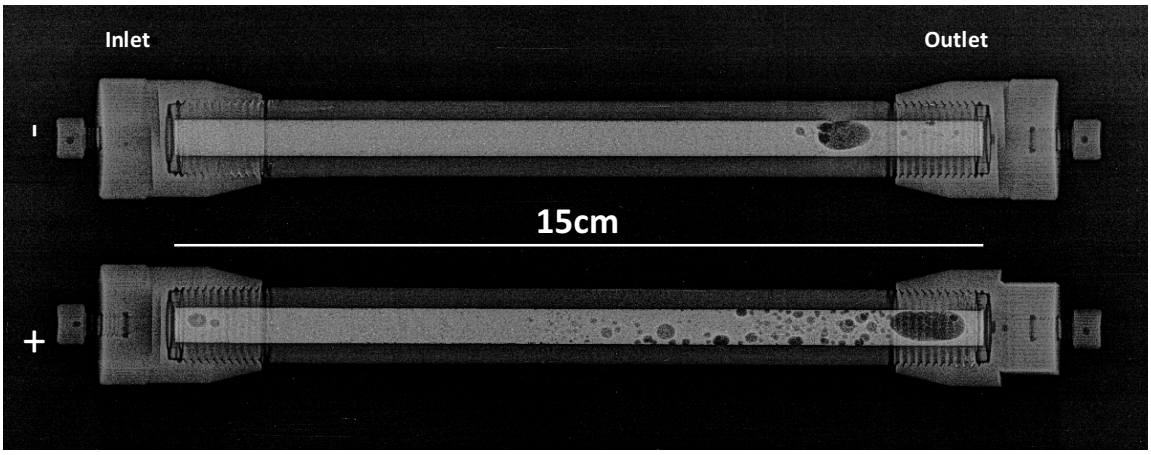

Figure 5 X-ray radiographs of carbon amended 2 and control 2 columns. During the experiment, flow occurred from the inlet to outlet. (-) control; (+) carbon amended.

The formation of gas bubbles in porous media is dependent on a number of factors, including the rate at which gases are being generated and utilised (Amos and Mayer, 2006), pressure (Hunt Jr and Berry Jr, 1956), and flow rate (Garstecki *et al*. 2005). Gas generation in sandy sediments has previously been shown to form spherical bubbles (as seen in Figure 5), with the sand responding fluidly as a result of stresses imposed by gas build up (Boudreau *et* al. 2005). Gas bubbles are known to impact on the hydraulic conductivity of porous media (Amos and Mayer, 2006), and have also been shown to be able to accumulate fine particles, further impacting on hydraulic conductivity (Goldenberg *et al*. 1989). It is possible that the gas bubbles noted in the columns were generated near the column inlet via microbial metabolism, and were transported to the outlet end of the columns in the direction of flow as a result of the upflow experimental design. The slow flow rate used in this experiment, coupled with the large porosity of the crushed material, may have allowed gases to accumulate, leading to the formation of bubbles. As the flow rate through the columns was slow, the presence of gas bubbles may not have been enough to impact on flow through the columns during the 8 month experiment (as no real decrease in flow rate was observed), but could have contributed to the pressure increases.

5.4.2 Geochemical analyses

During these experiments the starting fluid had an approximate pH of 12.2 and Eh of -12 mV. After being pumped through the columns, a significant decrease in pH was

observed initially, after which the pH of the outlet fluid increased to values comparable to the starting fluids (Figure 6). Although the pH of the starting fluids remained relatively constant throughout the experiment, variations in outlet fluid pH appeared to be closely related to variations in the rate and extent of microbial activity. Organic acid utilisation (Figure 7) occurred to the greatest extent when the outlet fluids were pH 9-10. pH increases above these values were accompanied by a sudden decrease in organic acid utilisation. A decrease in Eh was observed in the outlet fluids of all columns compared to the starting fluid, although the rate of decrease appeared to be more rapid in the carbon-amended columns than the control columns. Generally, throughout the experiment, the redox potential was lower in the carbon-amended columns compared to the controls.

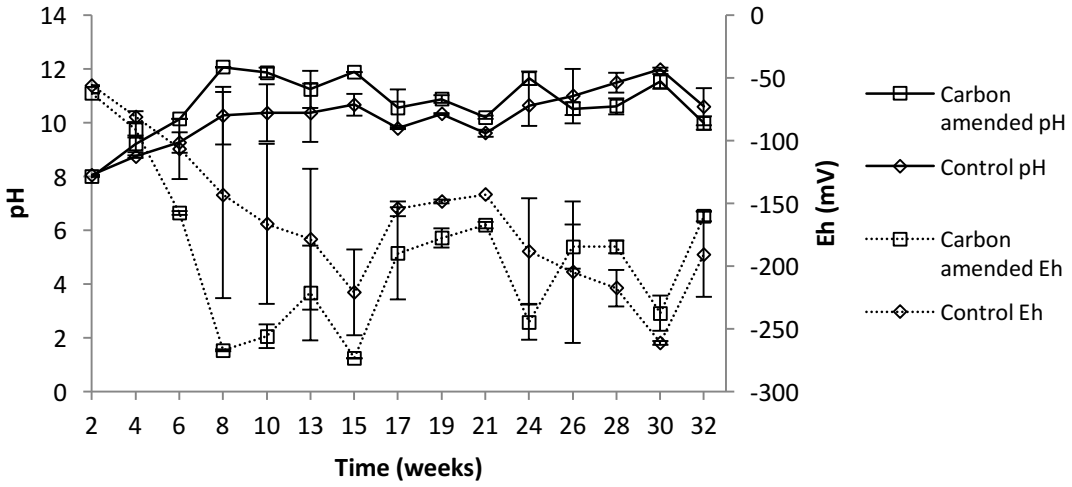

Figure 6 pH and Eh of column outlet fluids. Error bars show standard error calculated from duplicate values.

The extensive spherical voids that are present in the carbon-amended column (Figure 5) could be a result of the generation of gases during fermentative processes. Lactate and acetate were added to the fluid (560 mgL^{-1} and 410 mgL^{-1} respectively) collected from the field site, although formate was already present, indicating the presence of organic acids at the field site. The Acetate concentrations in the outlet fluids remains relatively constant throughout the carbon-amended experiments. At 4 weeks, lactate is absent from the outlet fluids of the carbon-amended columns, and at this time point there were concurrent increases in propionate, formate and butyric acids, which is consistent with metabolic gases including CO_2 and H_2 being generated within the columns. For example, butyric acid is generated from lactate via fermentation, with *Clostridum* species known to be able to carry out this process (He *et al*. 2005). The H_2 gas generated during this process (Chong *et al*. 2009) may have stimulated the H_2-utilising bacteria present in the

sediments at the end of the experiment. *Clostridia* are also known to be able to produce propionate by fermenting lactate via the acrylate pathway or the succinate-propionate pathway; this process generates CO_2 (White, 1995). As the pH of the outlet fluids increased above 10, lactate utilisation stopped (and generally the generation of lactate breakdown products stopped as well), until 15 weeks, when gradual decreases in lactate were observed. The decrease in lactate and acetate in the outlet fluids from carbon-amended columns towards the end of the experiment, coupled with the lack of breakdown products could suggest that they are being completely oxidised to CO_2, or that fermentation products/ oxidation intermediates are being utilised so quickly that they are not detected in the outlet fluids. Acetate and lactate were generated within the control columns, perhaps as a result of the breakdown of cellulosic materials present in the starting fluids, although without further analyses to investigate the compounds that acetate and lactate may have been metabolised from, it would be difficult to state the mechanisms responsible for their formation.

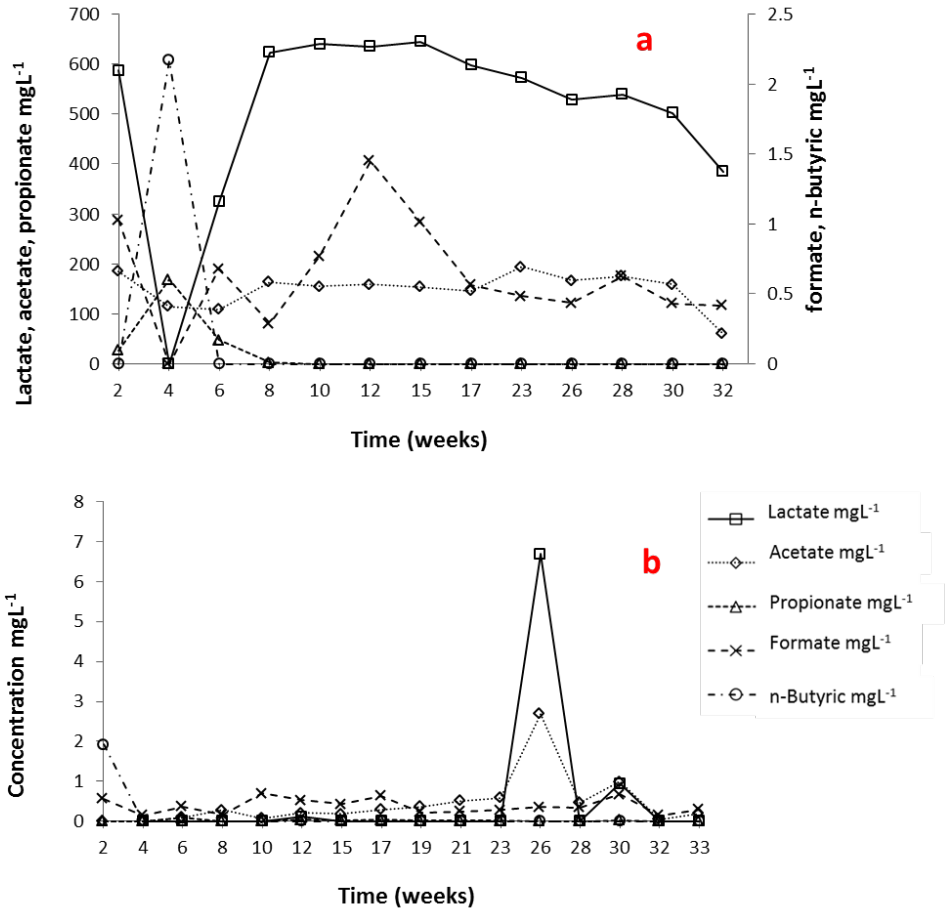

Figure 7 Volatile fatty acid concentrations in the outlet fluids of carbon (5 mM acetate and lactate) amended column 1 (a) and control column 1 (b). Data are representative of duplicate columns, with similar trends and concentrations seen in both.

Other processes may have played a role in the inhibition of ^{99m}Tc mobility through carbon-amended columns, including the reductive immobilisation of Tc(VII) by Fe(II)-bearing minerals. Weak acid extractable Fe(II) concentrations in sediments collected from a carbon-amended column were higher than observed in control sediments (Figure 8), implying the occurrence of microbial Fe(III) reduction at alkaline pH. Fe(II) concentrations in sediment samples collected over the length of the columns confirmed that Fe(III) reduction was stimulated at the carbon-amended inlet, with concentrations of approximately 4.5 mmol Fe(II) kg slurry^{-1} observed, while the concentration decreased over the length of the column. Lower concentrations of Fe(II) were observed in the control column (approximately 1.5 mmol Fe(II) kg slurry^{-1}), and variation was minimal over the length of the column.

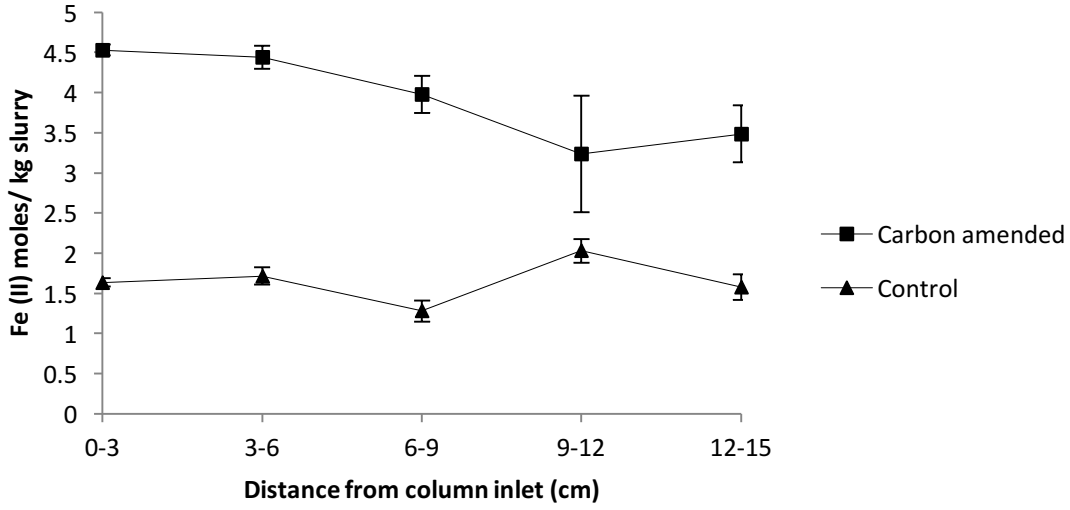

Figure 8 Fe(II) concentration in sediments collected from 3 cm intervals along a carbon amended and control column. Error bars show standard error calculated from triplicate measurements.

Several studies have documented the ability of microorganisms to carry out this process at high pH (e.g. Williamson *et al*. 2013; Pollock *et al*. 2007). Numerous studies investigating the biogeochemical behaviour of Tc(VII) under circum-neutral conditions have suggested reduction of Tc(VII) by Fe(II)-bearing minerals as a potential mechanism for the immobilisation of Tc in microcosm studies (e.g. McBeth *et al*. 2011), and in flow-through column experiments (e.g. Lear *et al*. 2010). Only a few studies have investigated this process under alkaline conditions; Thorpe *et al*. (2014) demonstrated that Tc(VII) reduction by Fe(II)-bearing minerals occurred at pH 9, whereas Williamson *et al*. (2014) demonstrated the occurrence of this process at pH 10. Direct enzymatic Tc(VII) reduction by microorganisms is also known to occur under alkaline conditions; as

an example Khijniak *et al*. (2003) described the ability of soda-lake isolates to reduce Tc(VII) at pH 10. Although it is clear that microbial processes impacted on the transport of ^{99m}Tc through the crushed sandstone columns in these experiments, the precise mechanism of Tc retention clearly warrants further investigation.

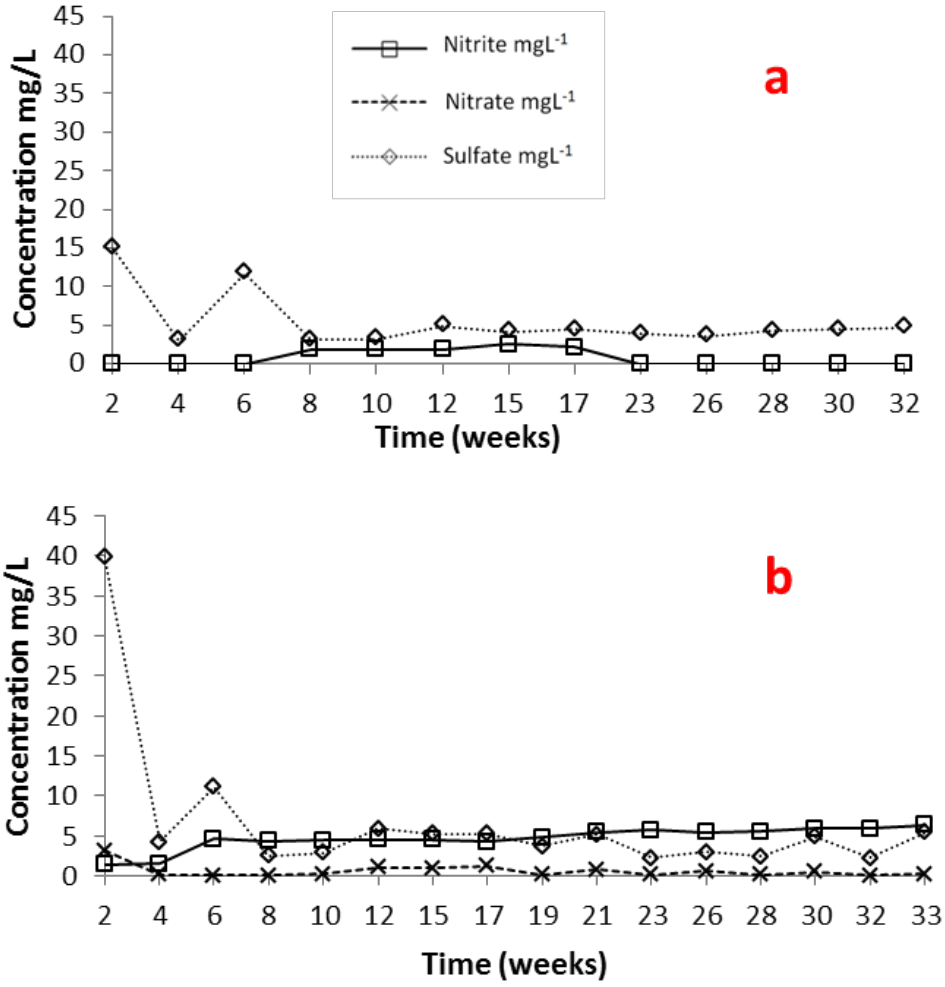

Figure 9 Nitrite, nitrate and sulfate concentrations in the outlet fluids of carbon amended column 1 (a) and control column 1 (b). Starting concentrations of nitrate were approximately 2 mgL^{-1} in the carbon amended fluid and approximately 4 mgL^{-1} in the control fluid. No nitrite was detected in the starting carbon amended fluid; approximately 0.8 mgL^{-1} was detected in the control starting fluid. Approximately 2 mgL^{-1} sulfate was detected in both of the starting fluids. Data are representative of duplicate columns, with similar trends and concentrations seen in both.

5.4.3 Microbiological and molecular ecological analyses

Cell numbers in column starting and outlet fluids were monitored throughout the experiment, along with the molecular ecology of outlet fluids and reacted sediments to aid in understanding the spatial and temporal changes to biogeochemical processes occurring within these column systems, and to identify some of the organisms facilitating these processes.

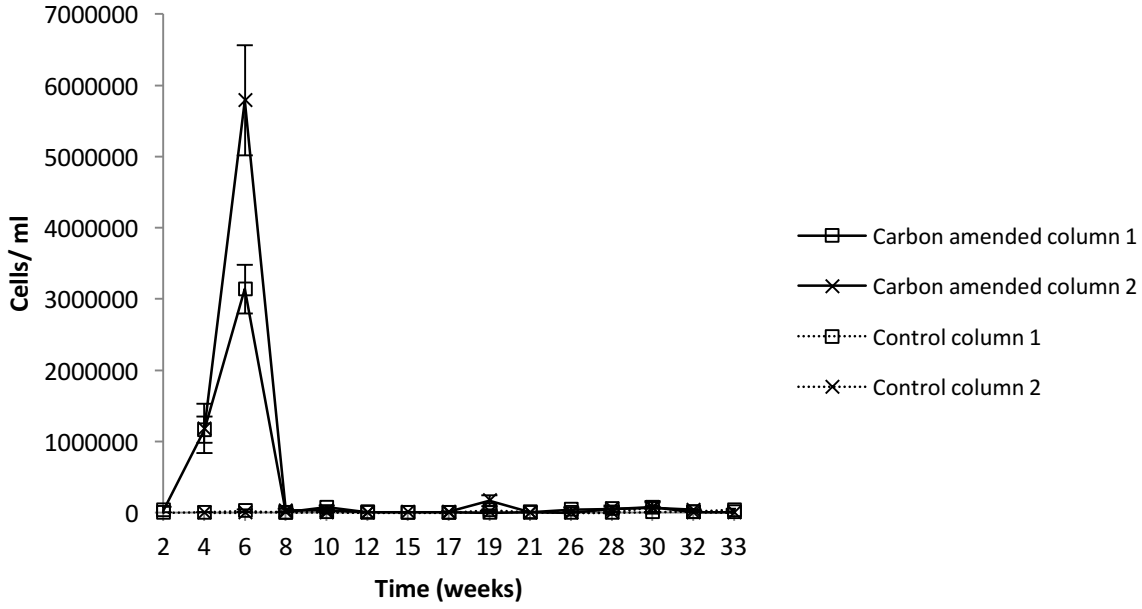

Figure 10 Total cell numbers in column outlet fluids. Error bars show standard error calculated from 5 replicates.

Organic acid utilisation over the first few weeks of the experiment was extensive, and was implicated in supporting biomass growth, increases in outlet fluid bacterial community diversity and significant quantities of gases generation within the carbon-amended columns. After the decrease in cell numbers in the outlet fluids of carbon-amended columns (after week 6; Figure 10), organic acid utilisation declined, pH increased, and the apparent diversity of the outlet fluid community decreased as demonstrated by RISA (Figure 11). Towards the end of the experiment, lactate and acetate concentrations declined, but cell numbers in the outlet fluids remained low, perhaps suggesting that most of the cells were being retained within the columns.

The bacterial communities in the outlet fluids of the column experiments varied between the control and carbon-amended columns, and also varied over time in the carbon-

amended columns as demonstrated by RISA (Figure 11). It would appear that in the starting fluids, bacterial biomass was very low, as only two very faint bands were present on the RISA gel. The community in the outlet fluid of the control column remained consistent throughout the experiment, with only one distinct band present.

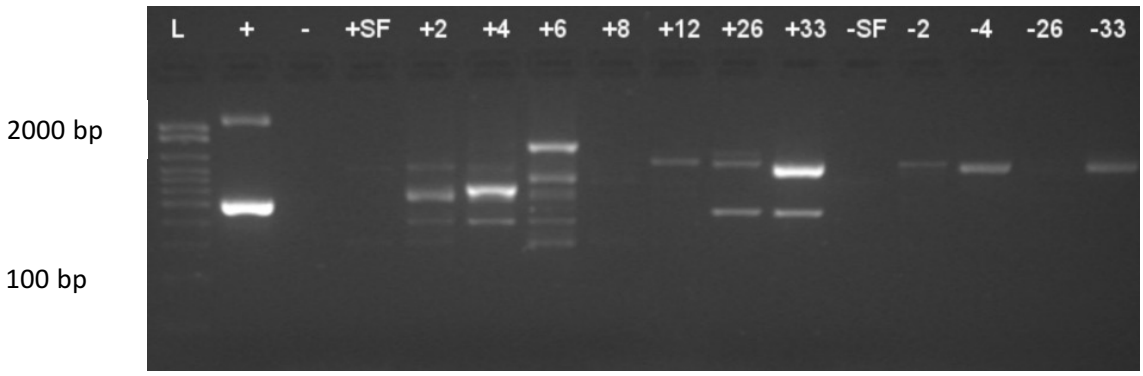

Figure 11 Ribosomal Intergenic Spacer Analysis (RISA) PCR products from outlet fluids of column experiments. (L) ladder, (+) positive control (*Geobacter sulfureducens*), (-) negative control (sterile DIW), (+SF) carbon amended starting fluid, (+2, 4, 6, 8, 12, 26, 33) outlet fluids from carbon amended 1 column at 2, 4, 6, 8, 12, 26 and 33 weeks, (-SF) control starting fluid, (-2, 4, 26, 33) outlet fluids from control column 1 at 2, 4, 26 and 33 weeks.

The community in the outlet fluids of the carbon-amended column, however, varied over the course of the experiment, with the diversity increasing at week 6, corresponding to the time at which the highest cell counts occurred in the outlet fluids (at a time of more favourable pH for microbial metabolism). A 454-pyrosequencing approach suggested that the microbial community at this time point was dominated by an organism affiliated with *Rhizobium selenitrireducens B1* (96 % sequence similarity). Other bacteria that were dominant in the sequence library included an organism closely affiliated with *Noviherbaspirillum aurantiacum SUEMI08* (97 % sequence similarity, 10.05 % of the sequence library), and organisms closely affiliated with *Bacillus* spp. These bacteria that dominated the sequence library are not known to be alkaliphilic, and have been isolated from a range of non-alkaliphilic environments including volcanic mountain soil (Carro *et al*. 2012), Arctic marine sediments (Zhang *et al*. 2014), and Guaymas Basin hydrothermal sediments (Dick *et al*. 2006).

The bacterial community present (Figure 12) in outlet fluids of the carbon-amended column at the end of the experiment appears to be well adapted to the highly alkaline

conditions, and the sequence library was dominated by obligately alkaliphilic bacteria, as revealed by 454-pyrosequencing. The sequence library obtained from this sample was dominated (91.47 % of the sequence library) by an organism closely affiliated with *Comamonadaceae bacterium B1* (99 % sequence similarity). 5.42 % of the sequence library was comprised of an organism closely affiliated (99 % sequence similarity) with an uncultured clone *CVCloAMPh135* belonging to the class *Clostridia.*

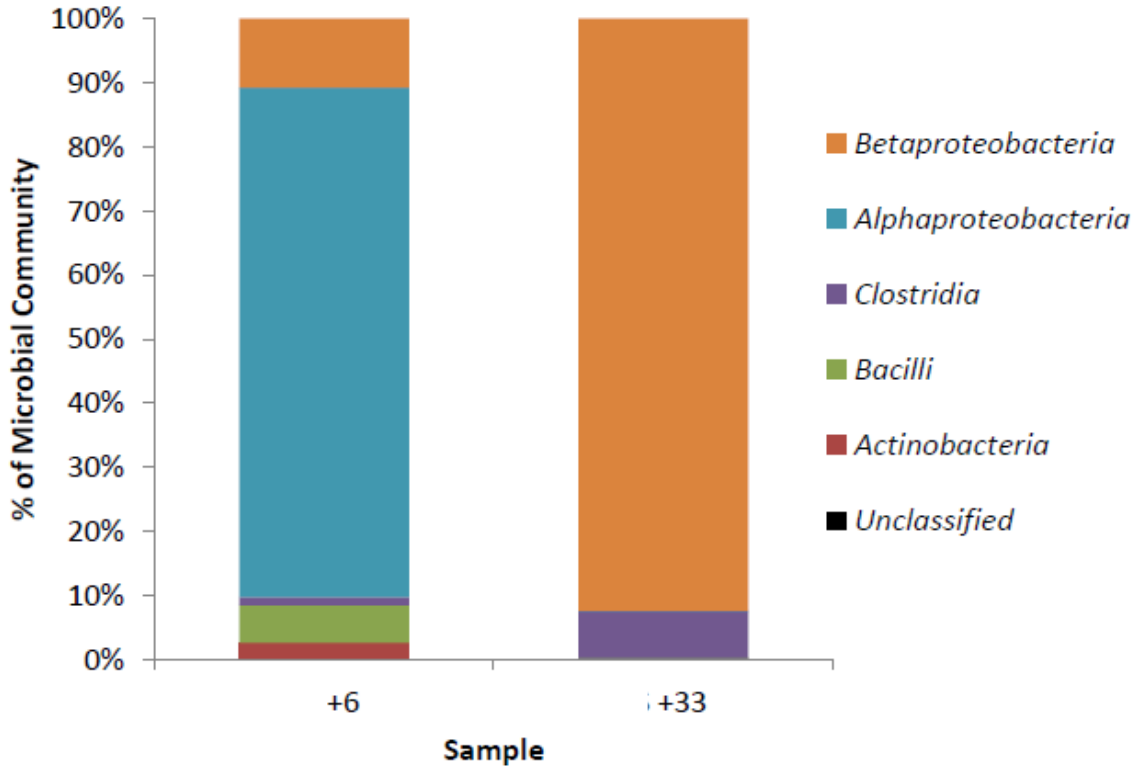

Figure 12 Bacterial community composition (class level) of communities present in the outlet fluids of a carbon amended column after 6 weeks (+6) and after 33 weeks (+33).

In addition, 454-pyrosequencing of key samples revealed spatial variations in bacterial community composition in column sediments collected at the end of the experiment. DNA was extracted from sediments collected from every 3 cm along a carbon-amended and control column, and subjected to 16S rRNA PCR; products were only obtained from DNA extracted from the inlet (0-3 cm) of the carbon-amended column, and from 0-3 cm and 6-9 cm from the control column. The DNA extracted from these samples was subsequently subjected to 454- pyrosequencing analysis (Figure 13).

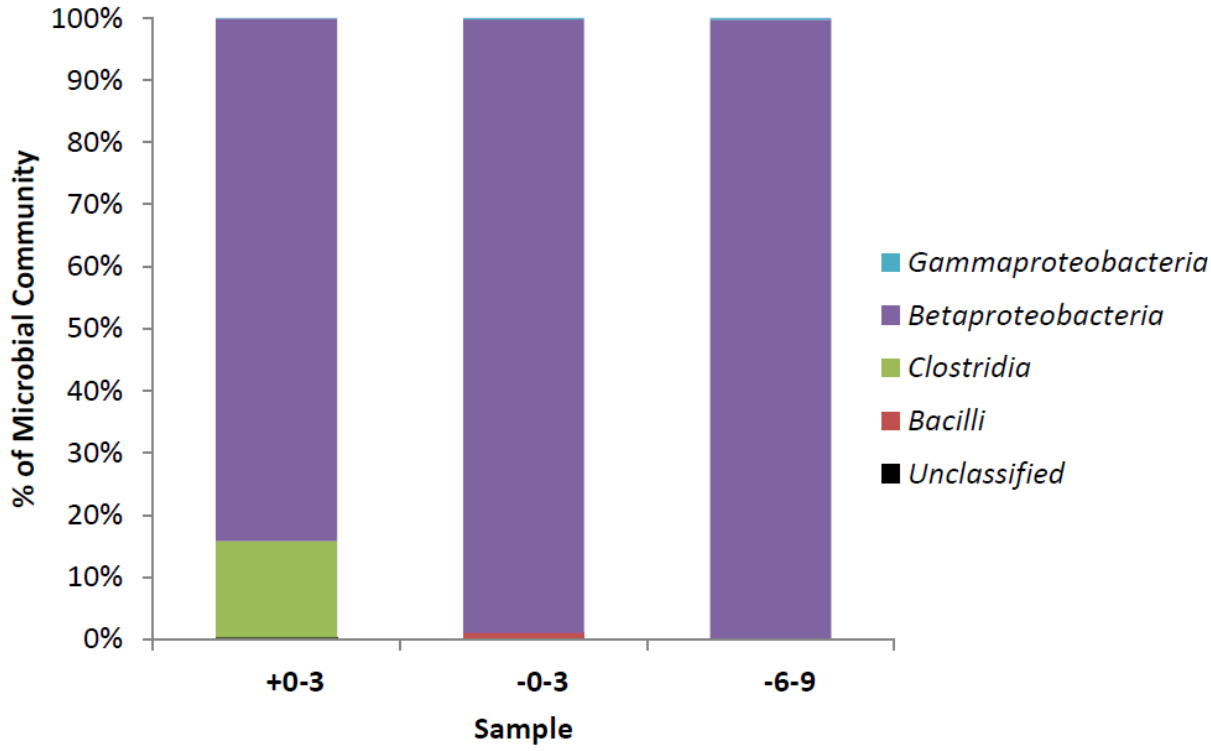

Figure 13 Composition of bacterial communities (class level) on sediments collected from column experiments. (+0-3) sediment collected from 0-3 cm from the inlet of a carbon amended column, (-0-3) from 0-3 cm from inlet of a control column, (-6-9) from 6-9 cm from inlet of a control column.

The bacterial communities present in the sediments of both the carbon-amended and control columns at the end of the experiment were dominated by a genus recently described as *Serpentinomonas* (Suzuki *et al*. 2014). The sequence library obtained from the carbon-amended column was dominated (80.35 % of the sequence library) by a strain with 99 % sequence similarity to *S. mccroryi*, as were the libraries from the control column (65.63 % at 0-3 cm and 83.41 % at 6-9 cm), with a strain with 99% sequence similarity to *S. raichei* also a significant component of the community (31.59 % at 0-3 cm and 14.39 % at 6-9 cm). These strains are obligately alkaliphilic, with optimum growth observed at pH 11, and have recently been isolated from serpentinising systems (Suzuki *et al*. 2014). As a result of culture- independent sequencing, Crespo-Medina *et al*. (2014) found that sequences with 100 % identity to *S. mccroryi* dominated a community found in fluids with pH>12 from subsurface samples from the Coast Range Ophiolite Microbial Observatory, a site of active serpentinization. The two dominant *Serpentinomonas* strains observed in column sediments in these experiments can use $CaCO_3$ as a carbon source (as well as acetate and lactate), and are capable of utilizing H_2 as an electron donor; two gene clusters are present in their genomes which code for [Ni-Fe]-hydrogenases, which are thought to be of importance during autotrophic growth

of the strains, enabling energy production via hydrogen oxidation (Suzuki *et al*. 2014). The active serpentinization at these sites generating significant quantities of H_2 and highly alkaline pore fluids, has likely selected for these *Serpentinomonas* strains, and it is possible that during the column experiments described in this study, the enrichment of these strains in column communities could perhaps be a result of H_2 generation during organic acid utilisation, or the presence of H_2 in the atmospheric composition of the anaerobic cabinet. Towards the end of the experiment, organic acid utilisation was minimal, perhaps suggesting that under the highly alkaline conditions that present in these experiments (apart from at the beginning when the pH was slightly lower and organic acid utilisation was extensive), autotrophic growth with H_2 as the electron donor was the dominant mechanism for bacterial growth. This process represents a potential mechanism for the prevention of over-pressurisation in a GDF that may occur as a result of H_2 generation during steel corrosion under highly alkaline conditions (NDA, 2010).

A strain with 98 % sequence identity to *Alkaliphilus crotonatoxidans* was also present in the carbon-amended column (comprising 11.75 % of the sequence library). This bacterium is known to dismutate crotonate (an intermediate in the oxidation of butyrate) to acetate and butyrate (Cao *et al*. 2003). Growth does not occur with lactate, butyrate or acetate, suggesting that during these experiments, crotonate may have been generated as a result of butyrate utilisation. Sulfate, nitrate and nitrite concentrations were also monitored in outlet fluids throughout the experiment. Initially, after the fluid was pumped through the columns, a sulfate spike was observed (much greater concentrations than the ≈2 mgL^{-1} present in the starting fluids), after which concentrations returned to values comparable to those of the starting fluids. A strain with 95 % sequence similarity to the obligately alkaliphilic (pH optimum of 9.5) *Dethiobacter alkaliphilus AHT1* was present in the sediments from the carbon-amended column (comprising 3.79 % of the sequence library). This organism is capable of utilizing H_2 as an electron donor, and elemental sulfur and polysulfide as electron acceptors (Sorokin *et al*. 2008). In microcosm studies carried out with fluids from serpentinising sites amended with thiosulfate and sulfide (Crespo-Medina *et al*. 2014), the resulting microbial community was dominated by sequences similar to this organism, and it may have been involved in sulfur metabolism in these experiments.

Nitrate disappeared from all starting fluids in samples collected throughout the course of the experiment (Figure 9); a nitrite build up was observed in the starting fluids (data not shown), suggesting nitrate reduction may have been occurring in the starting fluids, rather than in the columns themselves. The strain closely related to *S.mccroryi* present in both the carbon-amended and control column sediments is capable of utilising nitrate

as an electron acceptor (Suzuki *et al*. 2014), and may play a role in nitrate reduction within these experiments.

5.5 Conclusions

The results from this study indicate that microbial communities can impact on the transport properties of a porous medium under highly alkaline conditions representative of conditions that will be present in a GDF for ILW in the UK. Previous studies have demonstrated the ability of microorganisms to facilitate processes that may impact on radionuclide mobility in a GDF for ILW, for example Rizoulis *et al*. (2012) showed that microorganisms were capable of respiring a range of electron acceptors at pH 11. Bassil *et al*. (2014) also showed that microorganisms were capable of degrading ISA (a molecule that can form as a result of alkaline cellulose degradation), under highly alkaline conditions, indicating the potential for microorganisms to utilise cellulose degradation products as carbon sources in a GDF environment. Column experiments suggest that microorganisms existing under these conditions play a role in the biogeochemical cycling of Fe, nitrate, and sulfate, and are capable of metabolising organic acids under such conditions (as proxies for some of the cellulose degradation products that may be present as a result of alkaline degradation of cellulosic materials that will be present in ILW). Some of these processes are responsible for gas generation, possibly impacting on the hydraulic conductivity of these column experiments, resulting in a decrease in mobility of ^{99m}Tc through the columns. Other processes such as bioclogging and (bio) mineral precipitation may have also played a role in controlling fluid transport. The bacterial communities observed in sediments from these column experiments were dominated by obligately alkaliphilic bacteria capable of utilising H_2 as an electron donor. Overall the results from this study underline the potential importance of microorganisms in biogeochemical and physical processes of relevance to geological disposal. Further work is required to fully understand the extent to which each of these processes contribute to the alteration of the transport properties of the sandstone in these experiment, and other host rocks in relevant geological formations.

5.6 Acknowledgements

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Chapter 6

Research paper

The use of a biological flow apparatus (BFA) to quantify the impact of microbial processes; relevance to intermediate level radioactive waste geodisposal

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6.1 Abstract

The UK's concept for intermediate level radioactive waste (ILW) disposal will involve a multi-barrier system with the extensive use of cementitious materials, generating an alkali-disturbed zone in the host rock. Microorganisms may impact on the key characteristics of the geological disposal facility (GDF). For example processes including biofilm formation, gas generation and mineral alteration and precipitation can impact on the transmissive properties of the host rock. Direct enzymatic transformations can also impact on radionuclide speciation and hence mobility. However, the extent to which the hyper-alkaline environment will impact on microbial metabolism remains to be determined. To address this question, pressurized flow-through experiments were undertaken, with hyper-alkaline fluids (from a high pH spring containing microorganisms adapted to the alkaline conditions) pumped through sandstone cores, which aimed to investigate biogeochemical processes under conditions of relevance to the geological disposal of ILW. One core was amended with acetate and lactate (as surrogates for cellulose degradation products that will be present in a GDF for ILW) to stimulate microbial activity, whilst the other core was left as an unamended (no added carbon) control. X-ray radiographic analyses revealed physical differences between the carbon-amended and control core, whilst quantification of Fe(II) in the core sediments suggested that these differences could be a result of microbial Fe(III) reduction. The acetate and lactate concentrations in outlet fluids were much lower than in the starting fluids, although no degradation products were detected. Organic acids were generated in both experiments (before the addition of lactate and acetate in the case of the carbon-amended experiment). Some biogeochemical processes appeared to be impacted by increases in pore water pH (for example as the pH increased, cells in the outlet fluid increased, as did nitrite concentrations), suggesting the microorganisms present in these systems favoured alkaline conditions, which is of importance when considering the evolution of the hyper-alkaline plume over the lifetime of a GDF. Results from these experiments demonstrate that microbial processes may have a number of impacts in a host rock environment of a GDF for ILW that could potentially impact on radionuclide movement through the geosphere.

6.2 Introduction

The impact of microbial processes on the transport properties of subsurface environments are widely recognised, and may include the clogging of pore spaces as a result of biomass accumulation (e.g. Coombs *et al*. 2010; Surasani *et al*. 2013), the alteration of rock surface properties (e.g. the alteration of adsorption capacity (Anderson *et al*. 2007)), gas generation resulting in the alteration of hydraulic conductivity (e.g. Davey *et al*. 2009), or mediating the precipitation of mineral phases (e.g. Vasconcelos and McKenzie, 1997; Cuthbert *et al*. 2013). These processes may impact on the transport properties of a host rock environment during the geological disposal of radioactive waste.

The UK's concept for the disposal of intermediate level radioactive waste (ILW) in the UK considers the extensive use of cementitious materials, for example as a material to backfill the geological disposal facility (GDF). The hyper-alkaline plume that will be generated from the interactions between pore fluids and cement based materials will impact on the host rock in a number of ways. Interactions between the hyper-alkaline plume and the host rock may lead to the dissolution/ precipitation of minerals, potentially altering the porosity and permeability (e.g. Hodgkinson and Hughes, 1999; Savage *et al*. 1992), it may reduce the solubility of key radionuclides (NDA, 2010), and may also impact on microbial community composition and function.

Numerous studies of highly alkaline field sites with components analogous to an ILW GDF have found diverse microbial communities, often largely comprised of non-alkaliphilic organisms (Rizoulis *et al*. 2014). For example several studies have demonstrated the presence of members of the genera *Hydrogenophaga* (Brazelton *et al*. 2013; Rizoulis *et al*. 2014; Tiago and Veríssimo, 2013) in highly alkaline serpentinising systems, and a number of investigations have identified the presence of common environmental bacteria at high pH sites, including *Acidobacteria* (e.g. Keshri *et al*. 2013), *Bacillus* (e.g. Joshi *et al*. 2008; Tiago *et al*. 2006), *Sphingomonas* (e.g. Tiago *et al*. 2004; Tourova *et al*. 2014), and *Pseudomonas* (e.g. Valenzuela-Encinas *et al*, 2009; Rizoulis *et al*. 2014).

Previous studies have demonstrated that microorganisms can impact significantly on the transport properties of sandstone, although most of these studies have been carried out at circumneutral pH (e.g. Mitchell *et al*. 2009; Torbati *et al*. 1986). This study aimed to investigate some of these processes under highly alkaline conditions of relevance to the UK's concept for ILW disposal. An array of microorganisms will be present in a GDF; they will be associated with the waste forms, while there will be an indigenous population associated with the host rock, and contamination from the surface will also occur during the repositories' operational period. Microorganisms are known to adapt relatively quickly

to highly alkaline conditions (e.g. Pedersen *et al*. 2004 investigated the microbial populations within a highly alkaline spring, Maqarin, Jordan, and suggest that as a result of the lack of deeply-branching bacteria it can be assumed the population adapted to the conditions rapidly). It is thought that because of these rapidly occurring adaptations, it is likely that microorganisms will impact on the performance of a host rock during ILW disposal, although the extent to which these processes will occur, and the implications for radionuclide migration are uncertain.

This study aimed to investigate the impact of microbial activity in the transmissive properties of sandstone under high pH conditions, using pressurized flow-through experiments designed to be representative of aspects of a GDF for ILW.

6.3 Methodologies

6.3.1 Field sampling

Surface water samples were collected from a highly alkaline spring near Harpur Hill, Buxton, UK (previously described in Williamson *et al*. 2013). Water samples were taken from the locality at pH ≈ 12.45; autoclaved bottles were completely filled to minimise interactions between the fluid and air, and were stored in the dark at 4°C until needed.

6.3.2 Experimental design

Experiments were carried out using biological flow apparatus (BFA) to investigate microbial impacts on transport at high pH. Cores (diameter 3.7 cm, length 10 cm) were cut from a block of Sherwood sandstone and stored at 4°C in sealed plastic bags until needed. The experiment was assembled as follows (experimental setup shown in Figure 1): sterile porous frits were placed on either end of the cores, on top of which were placed plattens (end caps which allow fluid into and out of the core). The core was encased in heat-shrink Teflon, and then plumbed into a pressure vessel. The system was constructed with 1/8 inch Swagelok tubing and fittings. Syringe pumps (1000D syringe pump, Teledyne Isco, Lincoln, NE) were used to apply a confining pressure (4000 kPa) to the cores, and to pump fluid through the core at a constant flow rate of 200µl h^{-1}. Prior to starting the experiment, fluid collected from the field site was first vacuum filtered to approximately 10 µm to ensure removal of sediment from the fluid, and was then transferred to the syringe pumps. Outlet fluids were collected in sterile 50 ml syringes. Initially, all experiments were run without the addition of electron donors, to ensure that

cores were fully saturated before the stimulation of biological activity. After seven weeks, one experiment was amended with 5 mM of both acetate and lactate, as analogues for cellulose degradation products generated in ILW, to stimulate microbial activity. The other experiment continued to run as an unamended control. Pressure transducers (Gauge pressure sensor (either 40 or 100 bar) Gems Sensors and Controls, Plainville, US) monitored the confining pressure and injection pressure throughout the experiment. Thermocouples monitored ambient temperature, as well as the external temperatures of the syringe pumps and pressure vessels. Syringes were changed weekly, and samples were preserved accordingly.

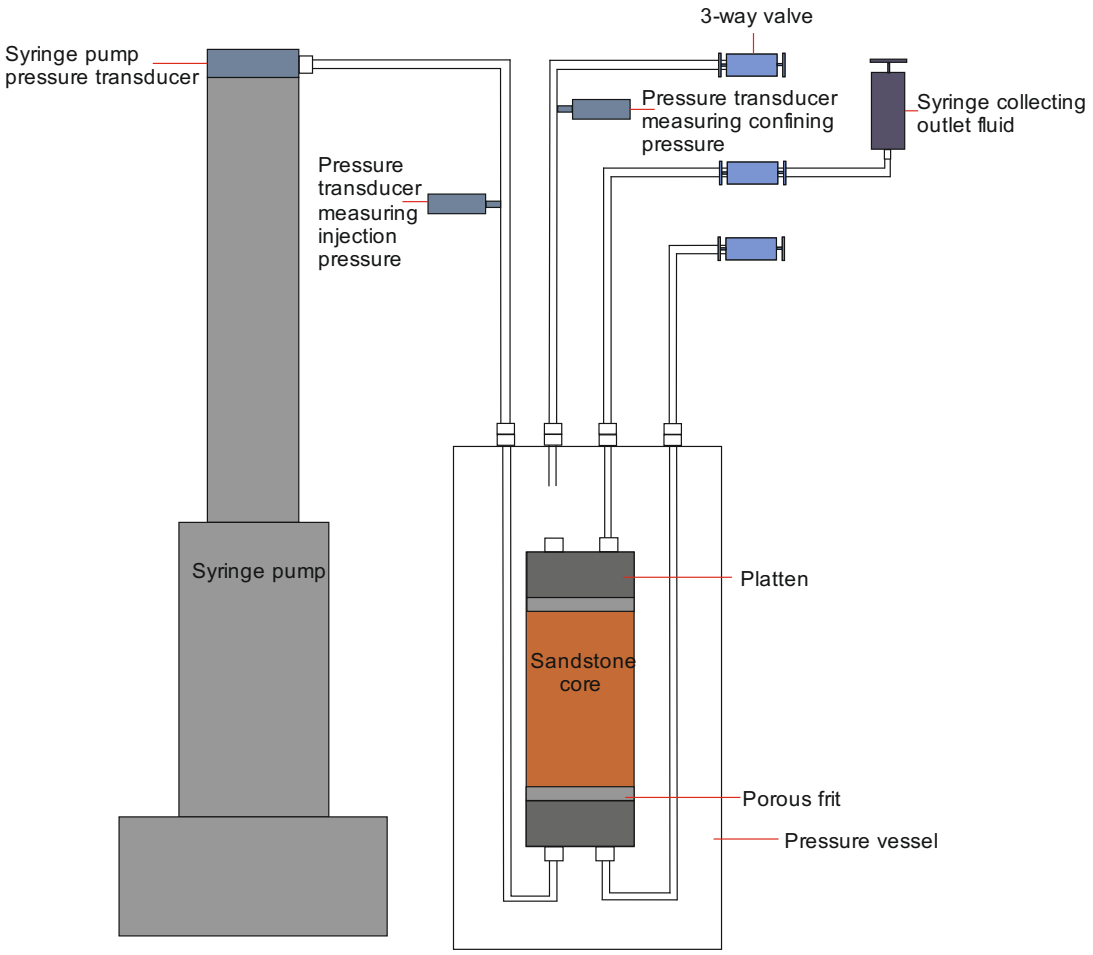

Figure 1 Schematic diagram of experimental setup.

6.3.3 Analytical geochemistry

pH was measured using a Mettler Toledo pH probe, calibrated to pH 7 and pH 10. Eh measurements were collected using a HANNA ORP probe. For ion chromatography analyses, fluid samples were first filtered to 0.22 µm. Volatile fatty acids, sulfate, nitrate, nitrite, phosphate and chloride quantification was carried out with a Dionex ICS5000 Dual Channel Ion Chromatograph as described in Bassil *et al*. (2014). 0.5 M HCl extractable Fe(II) and total Fe in sandstone samples (collected at the end of the experiment) were quantified using the Ferrozine Assay (Lovley and Phillips, 1986).

6.3.4 Destructive sampling of cores

Following X-ray radiography, the cores with the sealed plattens still attached, were transferred to an anaerobic cabinet (95 % N_2/ 5% H_2) to be sampled. A Dremel rotary tool was used to cut a longitudinal groove in the core approximately 3 mm deep. A hammer and chisel were then used to apply pressure to the groove, and split the core in half. Cores were then divided into inlet, mid, and outlet sections, which were subsampled and preserved accordingly.

6.3.5 Microbiological analyses

Total cells in fluid samples were enumerated with direct acridine orange counts. 1 ml of sample was fixed in 10 ml 1% glutaraldehyde solution. 5 ml of fixed sample was filtered onto a 0.22 µm Millipore membrane filter and stained with acridine orange, before rinsing with isopropyl alcohol. Slides were prepared by first air drying the filters, and then placing half a filter on a glass slide. Cover slips were fixed to the slides with a mounting solution (1:2 mixture of VECTASHIELD mounting medium (Vector Laboratories, Peterborough, UK) and TE buffer). Slides were viewed using a Zeiss universal microscope with a Zeiss III RS epi-fluorescence head, and filter set 09 (40-490 nm). Cells were counted in 5 fields of view. Most probable number counts were carried out on outlet fluids to enumerate culturable iron, sulfate and nitrate reducers. Media compositions are outlined in Table 1.

Table 1 Composition of media used for most probable number counts

Reagent	**Method (all values are in gL^{-1} unless otherwise stated)**
Trace Mineral Mix (Balch *et al*. 1979)	Nitrilotriacetic acid (1.5), $MgSO_4.7H_2O$ (3.0), $MnSO_4.2H_2O$ (0.5), NaCl (1.0), $FeSO_4.7H_2O$ (0.1), $CoSO_4$ (0.1), $CaCl_2.2H_2O$ (0.1), $ZnSO_4$ (0.1), $CuSO_4$. $5H_2O$ (0.01), $AlK(SO_4)_2$ (0.01), H_3BO_3 (0.01), $Na_2MoO_4.2H_2$ (0.01)
Trace Vitamin Mix (Balch *et al*. 1979; values in mgL^{-1})	Biotin (2), Folic acid (2), Pyridoxine hydrochloride (10), Thiamine hydrochloride (5), Riboflavin (5), Nicotinic acid (5), DL-calcium-pantothenate (5), Vitamin B_{12} (0.1)
Postgate B (sulfate-reducing microorganisms)	To tap water, add KH_2PO_4 (0.5), $CaSO_4$ (1.0), NH_4Cl (1.0), $MgSO_4.7H_2O$ (2.0), sodium lactate (3.5), yeast extract (1.0), $FeSO_4.7H_2O$ (0.5), ascorbic acid (0.1), sodium thioglycollate (0.1). Adjust pH to 7-7.5 with concentrated sodium hydroxide solution
Medium for Fe(III)-reducing microorganisms	Ferric citrate (13.7), Vitamin mix (10 ml), Mineral Mix (10 ml), $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.6), KCl (0.1), Sodium acetate (0.82). Adjust pH to 6.8 with concentrated sodium hydroxide solution
Medium for nitrate-reducing microorganisms	$NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.6), KCl (0.1), Mineral Mix (10 ml), Vitamin Mix (10 ml), Sodium acetate (0.82), KNO_3

In brief, under anaerobic conditions (95% N_2/ 5% H_2) 900 µl media was added to a 10 ml serum bottle, with 100 µl sample (in triplicate). A decimal dilution series was prepared (down to 1:1000), bottles were sealed with a butyl septa held in place with an aluminium crimp cap. Bottles were incubated in the dark at 21°C for two weeks. Most probable number estimates were calculated using MPN tables published in de Man (1983).

6.3.6 Imaging

6.3.6.1 X-ray radiography

After cores were removed from the pressure vessels, the ports were quickly sealed with Swagelok fittings to ensure minimal ingress of air into the cores. The sealed cores were then subjected to X-ray radiography using a GE Isovolt 320 kV unit (Brisbane, Australia)

at a distance of 1200 mm from the focal spot in the detector head to the imaging plate, using the following settings: 80 kV, 9 mA for 1 minute. Images were processed using ImageJ (Abramoff *et al*. 2004). Firstly, a variance filter was applied with a radius of 0.5 pixels to increase the contrast between the different phases within the cores by highlighting edges. The threshold was then adjusted to select for the densest materials within the core (other studies have identified dense bright patches on radiographs as Fe-bearing minerals (Gingras *et al*. 2014)). A mask was then created, and then a selection was created to highlight the selected particles, which could then be quantified as a percentage of the total area.

6.3.6.2 Scanning electron microscopy

The core was sampled under anaerobic conditions, and sandstone samples measuring approximately 5 x 5 x 5 mm were rinsed thoroughly with 100 % isopropyl alcohol to ensure the removal of the hyper-alkaline pore water for imaging using SEM. Samples were air dried prior to imaging, and then transferred to an SEM stub before viewing using an FEI Quanta 600 ESEM. EDX spectra were collected using an Oxford Instruments INCA 450 EDXA system.

6.4 Results

Pressurized flow-through experiments were assembled to investigate the impact of stimulating microbial activity using organic acids (proxies for the cellulose degradation products that will be present in a GDF for ILW) on the transport properties of sandstone core under highly alkaline conditions. Although measurements of several parameters indicated similarities between the carbon-amended and control experiments, some differences were revealed which could potentially be of significance to the evolution of a GDF for ILW, and could impact on radionuclide migration through a GDF.

6.4.1 Geochemical measurements

Geochemical parameters were monitored throughout the experiment, including outlet fluid pH and Eh (Figure 2). Measurements revealed that in both experiments, a decrease in pH was observed as the fluid was initially pumped through the cores, dropping from 12.5 to 8.1-8.5, after which it increased back to 12.5 over a period of 13 weeks. Eh increased as the fluid was initially pumped through the core, dropping to approximately -280 mV after 12-13 weeks.

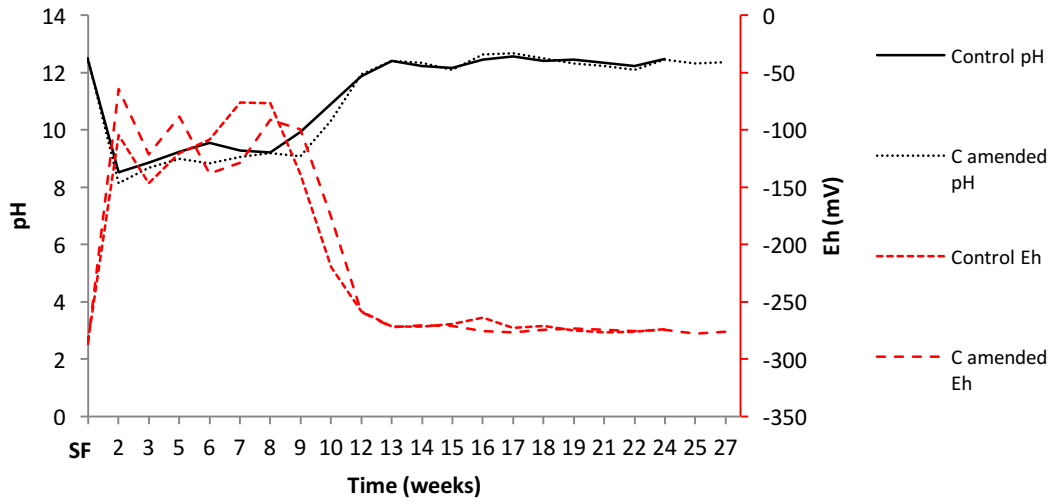

Figure 2 pH and Eh of outlet fluids over the course of the experiments. SF: starting fluids.

At approximately 7 weeks (when the outlet fluid pH began to increase, and the Eh decrease (Figure 2)), changes in numerous other parameters were observed as well. Figure 3 shows that at 8 weeks, increased nitrate concentrations were observed in the outlet fluids, after which nitrate reduction was observed with sustained nitrite increases. This time point corresponded to an increase in total cell numbers in the outlet fluids of both cores, reaching approximately 1.4 x 10^6 cells mL^{-1} and 6.8 x 10^6 cells mL^{-1} in the control and carbon-amended cores respectively.

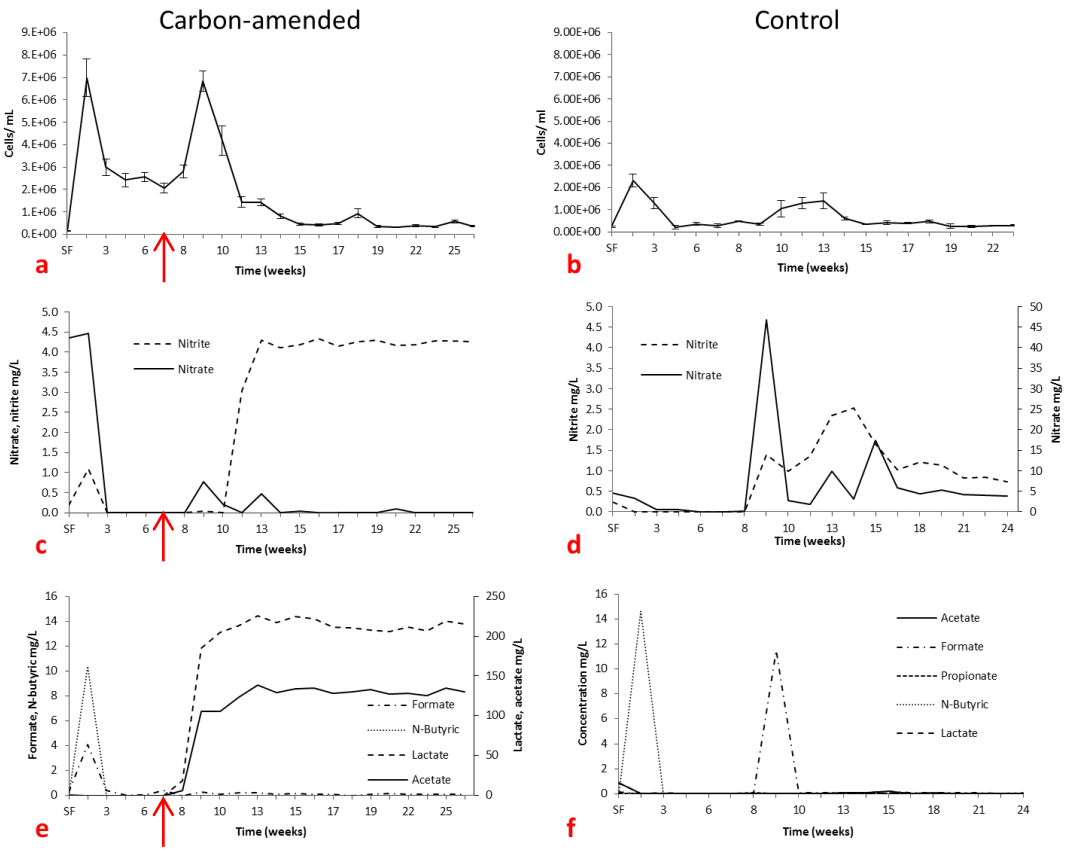

Figure 3 a,b: cells mL^{-1} in outlet fluids of the carbon-amended and control experiment respectively. c,d: nitrate and nitrite in outlet fluids of the carbon amended and control experiment respectively. e,f: organic acid concentration in the outlet fluids of the carbon-amended and control experiment respectively. Arrows indicate the time point at which carbon-amendment took place.

Lactate and acetate were added to the inlet fluid destined for the carbon-amended core after 7 weeks. As the starting fluid could not be monitored over the course of the experiment, a sample was taken from the pump at the end of the experiment. In this sample, lactate, acetate and formate concentrations were 509 mgL^{-1}, 190 mgL^{-1} and 11.6 mgL^{-1} respectively. Outlet fluid concentrations were significantly lower, and remained relatively constant, but no breakdown products were detected. Some organic acids were produced in both experiments (in the case of the carbon-amended experiment, this was before the addition of acetate and lactate). For example, in the starting fluids, N-butyric acid was not detected, however in the first outlet fluid samples, N-butyric acid concentrations were detected at approximately 10.4 mgL^{-1} and 14.6 mgL^{-1} in the outlet fluids of the carbon-amended and control cores respectively. Observable increases in formate concentration were also observed at various time points in the outlet fluids of

both the carbon-amended and control core, along with acetate generation within the control core.

To further investigate the microbial processes occurring within these experiments, 0.5 M HCl extractable Fe(II) present in the core sediments at the end of the experiment was quantified, and results are shown in Figure 4. Generally, higher Fe(II) concentrations were observed in the carbon-amended core compared to the control, and the highest 0.5M HCl extractable Fe(II) concentration was observed at the outlet of the carbon-amended column, with a concentration of 6.75 mM Fe(II) kg sandstone^{-1} seen.

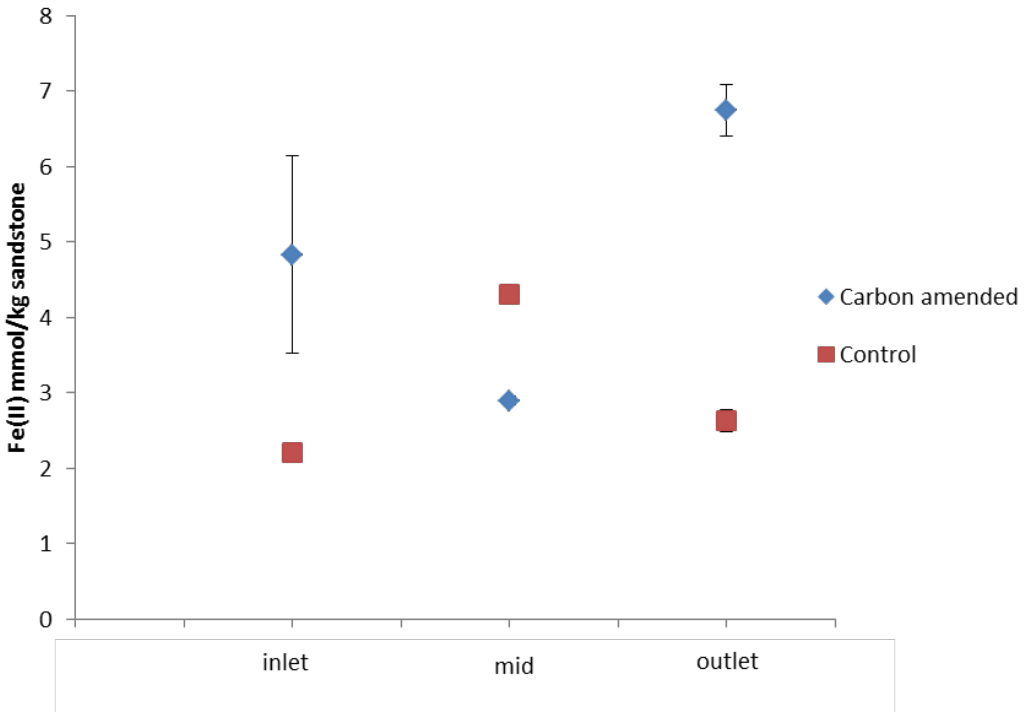

Figure 4 Fe(II) in sediment samples collected from along the length of the carbon-amended and control cores at the end of the experiment.

6.4.2 Physical properties

The physical impacts of the interactions between microorganisms, hyper-alkaline pore waters, and the sandstone core were investigated throughout the experiment by monitoring injection pressures into the cores, and at the end of the experiment using imaging techniques. Injection pressures were monitored throughout the experiment and no significant pressure increases were observed (increases from approximately 0.5- 1.1

bar (g) were noted, although it was difficult to determine what pressure increases occurred as a result of backpressure from the syringe collecting the outlet fluids). At the end of the experiment, the sealed intact cores were subjected to X-ray radiography, which revealed physical differences between the carbon-amended and control cores (Figure 5). Variations in the % area of the most dense material (the brightest patches) within the core were quantified, and revealed that the carbon-amended core image was comprised of 2.29 % of this dense material, whereas the in the control core it was 6.30 %. Although it appears that the percentage area of the dense material differs between the two cores, there are a number of sources of error associated with this quantification. For example, as images of the cores were not taken before the experiment was started, it is not known whether these differences occurred during the experiment, or are just natural variation in the rock, and as no replicate experiments could be carried out, these differences cannot be verified. There are also sources of error associated with the image analysis itself, for example, when selecting the bright patches in ImageJ, the masking technique can often lead to the smallest patches not being selected. Also, as the grey scale varies over the images, this could also have contributed to the masking technique missing some patches which should have been selected.

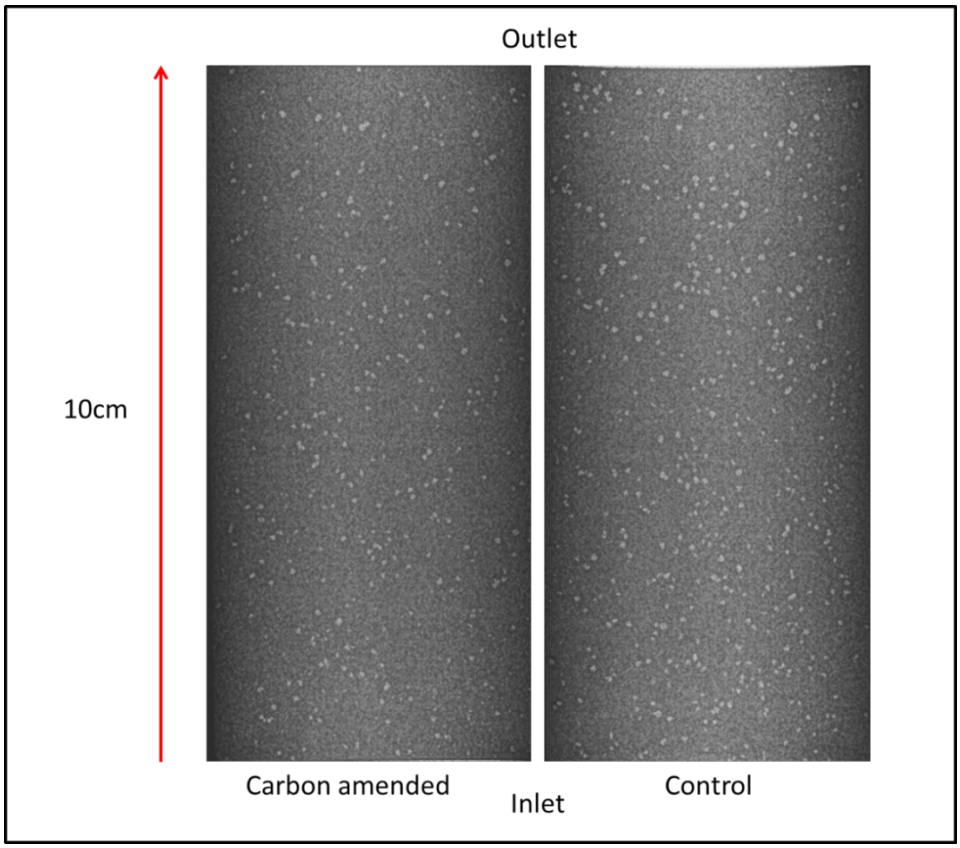

Figure 5 X-ray radiographs of the carbon-amended and control cores at the end of the experiment.

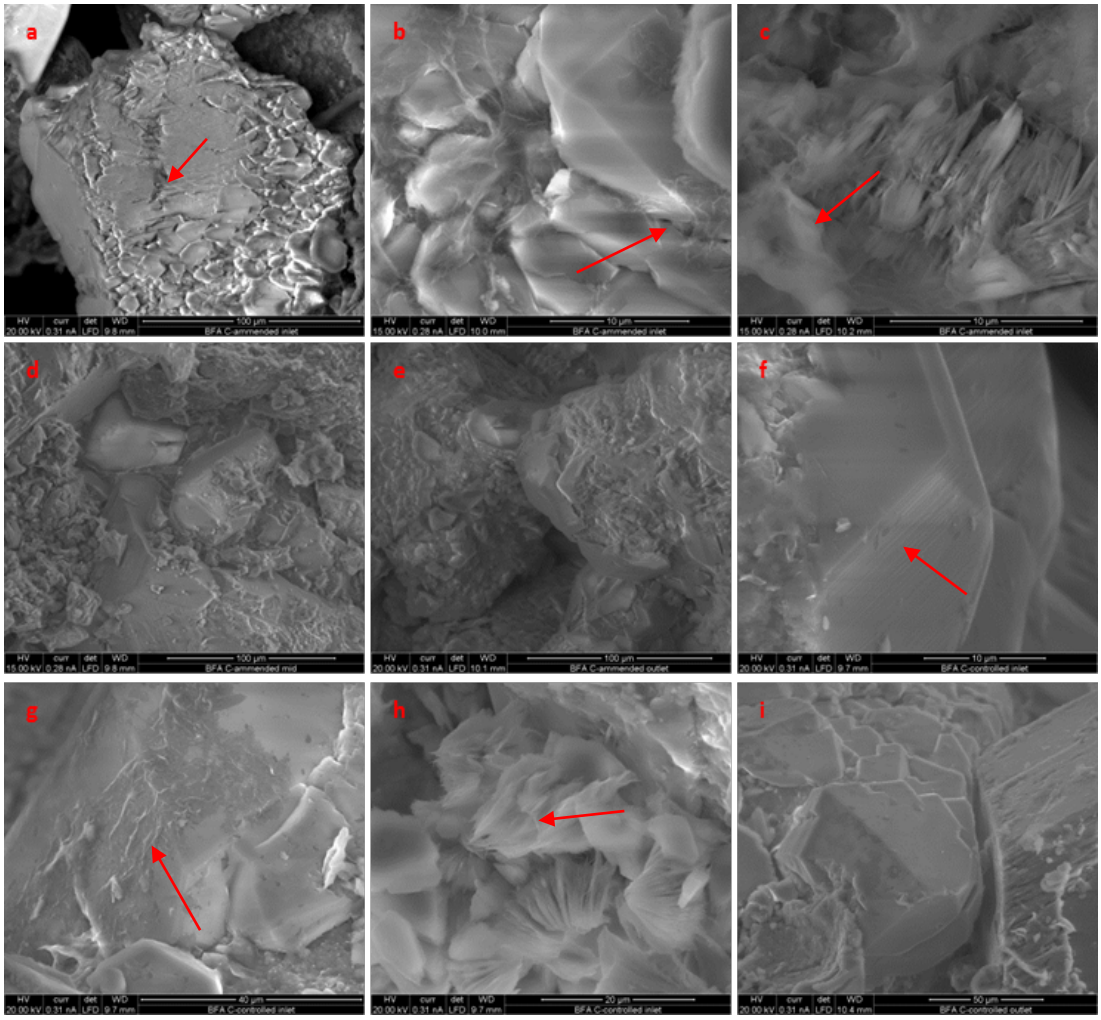

Figure 6 Scanning electron micrographs of core sediments. a,b,c: carbon-amended core inlet; d: carbon-amended mid-section; e: carbon-amended core inlet; f,g,h: control core outlet; i: control core outlet. The arrows point to the features described in the text below.

Scanning electron microscopy of sediment samples collected from along the cores at the end of the experiment revealed the presence of reaction products mainly at the inlet of the cores, with almost no reaction products seen at the core outlets. Significant etching of quartz surfaces was observed at the core inlets (Figure 6a and 6f; carbon-amended and control cores respectively), whereas quartz grains lacked etching at the core mid-sections and outlets (Figure 6d and e (carbon-amended core) and Figure 6i (Control core). Reaction products were observed to be coating quartz grains (Figure 6b) and kaolinite (Figure 6c) at the inlet of the carbon-amended core. Energy-dispersive X-ray (EDX) spectra revealed that these phases were thin filamentous calcium-aluminium-silicate-hydrate (C(A)SH) phases. Reaction products were also observed at the inlet of the control core, with EDX spectra indicating that C(A)SH phases were present on the quartz grains shown

in Figure 6g and very thin reaction products coated the edges of kaolinite grains shown in Figure 6h.

6.4.3 Most probable number counts

Most probable number counts of nitrate-, sulfate- and Fe(III)-reducing microorganisms in the outlet fluids of experiments were carried out, and suggested that no culturable Fe(III)-reducing microorganisms were present in the outlet fluids. Culturable nitrate reducers were quantified in the outlet fluids of the control experiment at 15 and 18 weeks, and the carbon-amended experiment at 15 weeks. Culturable sulfate reducers were observed at 15 weeks in the outlet fluid of the control experiment, and at 9 weeks in the carbon-amended experiment (Table 2).

Table 2 Most probable number counts mL^{-1} of nitrate, sulfate and iron reducers in the outlet fluids of the carbon-amended and control experiments. Upper and lower confidence limits for the values shown in this table are shown in Table 3.

	Weeks	Control	Carbon-amended
Nitrate reducers	**7**	<0.30	<0.30
	9	<0.30	<0.30
	12	<0.30	<0.30
	15	0.36	0.74
	18	1.1	<0.30
	25	<0.30	<0.30
Sulfate reducers	**7**	<0.30	<0.30
	9	<0.30	0.74
	12	<0.30	<0.30
	15	0.92	<0.30
	18	<0.30	<0.30
	25	<0.30	<0.30
Iron reducers	**7**	<0.30	<0.30
	9	<0.30	<0.30
	12	<0.30	<0.30
	15	<0.30	<0.30
	18	<0.30	<0.30
	25	<0.30	<0.30

Table 3 Upper and lower confidence limits for MPN values detected as a part of this research (de Man, 1983).

	Lower limit ≥ 95%	Upper limit ≥ 95%	Lower limit ≥ 99%	Upper limit ≥ 99%
<0.30	0.00	0.94	0.00	1.40
0.36	0.02	1.70	0.01	2.50
0.74	0.13	2.00	0.06	2.70
0.92	0.15	3.50	0.07	4.60
1.1	0.40	3.50	0.20	4.60

6.5 Discussion

Pressurized flow-through experiments were carried out using a bespoke biological flow apparatus over a period of 27 weeks under highly-alkaline conditions which aimed to be representative of certain aspects of the UK's concept for ILW disposal. Results from these experiments suggest that several processes, both biotic and abiotic, may occur under highly alkaline conditions, which could potentially impact on the transport characteristics of a GDF host rock, and subsequently radionuclide transport through the geosphere.

6.5.1 Transport properties

Some characteristics of the sandstone cores were altered during these experiments as a result of interactions with the hyper-alkaline pore waters, and potentially as a result of microbial activity. Alterations to components of the sandstone core as a result of interactions with the hyper-alkaline pore water include the swelling of clay minerals, quartz dissolution, and precipitation of C(A)SH phases, all of which have been shown previously to impact on transport properties (Bateman *et al,* 1999; Savage *et al*, 2006). It is possible that as a result of the highly porous nature of the sandstone these processes did not impact upon injection pressure, but the precipitation of C(A)SH phases has been shown to significantly impact on hydraulic conductivity in lower porosity rocks such as granite (Soler and Mäder, 2007). It was noted that extensive alterations to components of the sandstone occurred at the core inlets, after which point, minimal alteration to the material occurred. This feature is perhaps of importance to GDF processes, indicating that there may be extreme spatial variation in the precipitation of alteration products in a GDF host rock, as a result of the loss of calcium from the fluid as the plume migrates. This has been noted in numerous other experimental studies of hyper-alkaline plume migration. For example, Bateman *et al* (1999) demonstrated that in experiments that flowed hyper-alkaline fluid through quartz columns, most of the quartz dissolution occurred within the first half of the column.

Although numerous studies have noted the importance of biofilm formation on the transmissive properties of rock (e.g. Vandevivere and Baveye, 1992; Arnon *et al*, 2005; Lianfang *et al*. 2009), with increases in injection pressure as a result of bioclogging observed in some experiments (e.g. Hand *et al*. 2008), this did not appear to be the case in these highly alkaline experiments, as minimal changes in injection pressures were observed throughout the course of the experiment. It would also appear that the biomass associated with the sediment phase during the experiment was very low, as DNA

extraction from the core sediments at the end of the experiment proved to be unsuccessful. It is possible that the highly alkaline nature of the pore fluids may have been too high to facilitate any significant microbial growth, as microbial metabolism is thought to be limited at pH values >12 (Rizoulis *et al*. 2012). Growth may have only been possible in lower pH micro-niches within the core, or in the early stages of the experiment where pore water pH was slightly lower. Initially, a significant number of cells were present in the outlet fluids of both cores, presumably as a result of them being flushed from the cores, after which a decrease in cell numbers in the outlet fluids was apparent. A further increase in outlet fluid cell counts was seen at approximately 8-10 weeks, at which time changes in numerous other pore water characteristics were observed. At this time point, pore fluid pH increased and decreases in Eh were noted. A number of mechanisms may be responsible for the increase in cell numbers, including the detachment of cells that may have been attached to the rock surface, or increased cellular growth at this time point. It is possible that the addition of organic acids in the carbon-amended experiment resulted in an increase in cell numbers, but as an increase was also observed in the control experiment, it could also suggest that a shift in conditions resulted in increased cell growth, or increased detachment from the core. An increase in nitrate reduction as the pH increased, coupled with the increase in culturable nitrate reducers may suggest that as the pH increases, the pore water becomes a more favourable environment for the alkaliphilic and alkalitolerant microorganisms that are known to be present in the fluid (Bassil *et al*, 2015; Rout *et al*. 2015), stimulating growth, and as the pH increases further, the pH may become too high to facilitate any further microbial activity.

It is however possible that the increase in outlet fluid cell numbers at this time point, and the lack of bioclogging is a result of the experimental design, specifically that the experiment was carried out under constant flow conditions. Radu *et al*. (2012) suggest that under constant flow rates, channels become narrower resulting in localised flow velocity increases, leading to increased shear stress within the channels and subsequently increased biofilm removal.

6.5.2 Biogeochemistry

Results from flow-through experiments suggest that although some differences between the carbon-amended and control experiments were observed, some similar characteristics are also seen. This could perhaps mean that as biological activity is still occurring within the control core without the addition of organic acids autotrophic microorganisms could be active in these systems.

Results revealed differences in Fe(II) associated with core sediments between the carbon-amended and control experiment, suggesting that microbial Fe(III) reduction had occurred to a greater extent in the carbon amended experiments, consistent with the findings of other studies. Numerous studies have demonstrated the ability of some microorganisms to reduce Fe(III) under alkaline conditions (Thorpe *et al*, 2011; Rizoulis *et al*, 2012; Williamson *et al*. 2013). These results are supported by X-ray radiographs which indicate differences in the densities of the carbon amended and control cores at the end of the experiment. Other studies which have carried out X-ray imaging of sediments suggest that iron phases present as bright patches as a result of their high density (Gingras *et al*. 2014). It is possible that microbial activity resulted in the reductive dissolution of Fe(III) minerals to a greater extent in the carbon amended core compared to the control, resulting in the smaller percentage of bright dense patches on the radiographs, although without identification of this phase, this cannot be confirmed. In a GDF environment, microbial Fe(III) reduction is of importance as this process can impact on radionuclide mobility. Some Fe(III)-reducing microorganisms are known to be able to directly reduce radionuclides, for example the Fe(III)-reducing bacterium *Geobacter metallireducens* has been shown to reduce U(VI) enzymatically (Lovley *et al*. 1991). Fe(II) bearing minerals are also known to reductively precipitate some radionuclides, for example Tc(VII) reduction to the poorly soluble Tc(IV) (Brookshaw *et al*. 2012).

Lactate and acetate were added to one of the experiments as proxies for the cellulose degradation products that will be present in a GDF for ILW. Lactate and acetate concentrations in the outlet fluid were much lower than concentrations in the starting fluid, suggesting they were utilized within the core, although no breakdown products were detected. This could suggest that breakdown products were further utilized within the core, potentially generating methane, CO_2 and H_2 gas. Results suggest that organic acids were generated over the course of both experiments. N-butyric acid was observed (approximately 10-14 mgL^{-1}) in the outlet fluids of both experiments after 2 weeks, (before acetate and lactate were added to the amended column), although it was not present in the starting fluids for either core. A study carried out by Rout *et al* (2015) demonstrated the presence of Isosaccharinic acid (ISA) in fluids collected from the Harpur Hill field site. It is therefore possible that other organic substrates were present in the starting fluids, and were subsequently degraded throughout the experiment. Several studies have demonstrated the ability of microorganisms to degrade ISA under hyper-alkaline conditions (e.g. Bassil *et al*. 2015; Rout *et al*, 2015), and Rout *et al*. (2015) demonstrated the presence of a bacterium *Alkaliphiluis crotonatoxidans* in microcosm experiments when ISA was degraded. This organism is known to dismutate crotonate to acetate and butyrate (Cao *et al*. 2003), and may have played a role in the generation of butyric acid

during these experiments. In fact, this organism has been identified in sediments collected from previous experiments that we have carried out with fluid from the same field site, which could suggest that it is indeed responsible for the generation of butyric acid during these experiments, although pyrosequencing analyses have yet to be carried out to confirm this. Acetate was also generated within the control experiment, and a number of processes could be responsible for this. It is possible that other organic substrates present in the starting fluids were degraded by microorganisms to acetate, which has been shown to occur under hyper-alkaline conditions, for example from the degradation of ISA (Bassil *et al*, 2015; Rout *et al*, 2015). It is also possible that acetogenic microorganisms were responsible for the generation of acetate during these experiments, as previous studies have shown that numerous microorganisms are capable of acetogeneis under alkaline conditions (Zhilina *et al*, 1998; Zhilina *et al*, 2012).

Nitrate and nitrite behaviour in the outlet fluids of these experiments appears to be closely related to shifts in other characteristics of the fluids including changes in cell numbers, along with pH and Eh. Initially, nitrate and nitrite were not detected in outlet fluids (even though trace amounts were present in the inflow), suggesting they were both being reduced, either biotically, or abiotically. Nitrate concentration in the outlet fluid increased in both experiments at week 8, at which time pH increases were occurring, although the mechanism for this increase in nitrate is currently unknown. After these nitrate increases were observed, culturable nitrate reducers were detected in the outlet fluids, suggesting that the pH may be more favourable for nitrate reduction by alkaliphilic/ alkalitolerant microorganisms.

6.5.3 Microbiology and molecular ecology

The addition of organic acids to one experiment aimed to stimulate microbial activity, although some similar processes were observed in the control experiment, including increases in cell numbers, and nitrate reduction. This could suggest that autotrophic processes played a role in these control experiments. Numerous studies of highly alkaline systems have identified the presence of autotrophic hydrogen-utilizing bacteria (e.g. Brazelton *et al*, 2013; Suzuki *et al*, 2013), and have noted their potential importance in ILW GDF processes (e.g. Rizoulis *et al*. 2014). In previous experiments (unpublished work) we demonstrated the presence of close relatives to hydrogen-utilizing bacteria (*Serpentinomonas raichei* and *Serpentinomonas mccroryi*) in sediments and fluids when organic acids were no longer being extensively utilized, which could suggest that in these hyper-alkaline systems, a shift from heterotrophic to autotrophic processes occurs as the

pH increases. Suzuki *et al*. (2014) describe these bacteria as being capable of utilizing hydrogen as an electron donor, and calcium carbonate as a carbon source, but they are also able to utilize acetate and lactate. Microbial utilization of H_2 gas has previously been recognised as a potentially important process in ILW disposal, as it may help to mitigate over-pressurization of a GDF that could occur as a result of H_2 generation during steel corrosion (Rizoulis *et al*. 2014). Further work will characterise the bacterial communities present in these experiments.

In summary, we present results from experiments that investigated microbial processes in sandstone under hyper-alkaline conditions. These experiments aimed to explore some of the mechanisms by which microbial activity can impact on the physical and geochemical characteristics of an ILW GDF host rock, and infer some of the potential implications for radionuclide mobility. Host rock pore water pH is likely to remain above 12 for tens of thousands of years in the near-field of a GDF, suggesting that in the early stages of repository evolution, microbial activity may be confined to the far-field where pore water pH is lower. Results from these experiments suggest that clogging by biofilm may not significantly impact on the transmissive properties of sandstone under high pH conditions, even though this process has shown to be of significance under circum-neutral conditions (Vandevivere and Baveye, 1992). Future work should clearly focus on other rock types relevant to geodisposal scenarios in the UK and overseas, where biofilm development may have a more obvious impact e.g. fracture networks in grantie or mudstones. X-ray radiographs did however reveal physical differences between the carbon-amended and control cores, and Fe(II) quantification in the sediment phase suggests that this may be a result of microbial Fe(III) reduction. This process could have potential implications for a GDF as some Fe(III)-reducing microorganisms are known to be able to reduce some priority radionuclides, altering their solubility (Lloyd, 2003), and Fe(II)-bearing minerals are also able to reductively precipitate some radionuclides (Brookshaw *et al*. 2012). Cell counts over the course of the experiment suggest that cell numbers vary temporally and that they are sensitive to changes in geochemical characteristics of the pore fluids, such as pH, although cells are still present at elevated pH values, and microbial processes are clearly still occurring. As the upper pH limit for microbial processes is thought to be approaching pH 12, these results could suggest that microbial activity is occurring in lower pH micro-niches within the cores.

Acetate and lactate were utilized in the carbon-amended experiment, although there was evidence biological processes in the control experiment (for example increases in cell numbers), suggesting the occurrence of autotrophic processes in these systems. Previous experiments carried out with fluid collected from the same field site revealed that

microorganisms capable of utilizing H_2 and $CaCO_3$ were present in the pore fluids when organic acids were not being utilized. Microbial H_2 utilization has been identified as process of importance to GDF evolution, as it may provide a mechanism by which the over-pressurization of a GDF (resulting from H_2 generation during steel corrosion) could be mitigated (Rizoulis *et al*. 2014). Further work will characterise the microbial communities in these experiments.

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Chapter 7

Conclusions and future work

7.1 Summary

This thesis focuses on the potential impacts of microbial processes during the geological disposal of intermediate level radioactive waste. An experimental approach was adopted to investigate biogeochemical processes under highly alkaline conditions representative of an alkali-disturbed zone. The project also aimed to investigate the potential impacts on the physical properties of the host rock environment, and to characterise the structure and function of microbial communities involved in these processes.

Chapter 4 aimed to investigate the molecular ecology of a hyper-alkaline spring, and to assess how the community composition varied at sample sites over a range of pH values. Microcosm and column experiments were also undertaken using crushed sandstone and fluid and/ or sediments collected from the hyper-alkaline spring at Harpur Hill. These experiments aimed to investigate the biogeochemical processes that occur when alkaliphilic and alkali-tolerant microorganisms interact with crushed sandstone, and to examine these processes in a more realistic flow-through setup to elucidate the impacts on transport properties. Results revealed diverse bacterial communities were present at the hyper-alkaline spring, and community composition varied with pH, but generally the site was dominated by Proteobacteria, with bacteria belonging to the genera *Pseudomonas*, *Xanthomonas*, and to the family *Comamonadaceae* dominant. Microbial communities present in fluid samples collected from the field site demonstrated the ability to reduce Fe(III) in microcosm experiments initially posited at pH 11.5. The microbial communities appeared to be active in all microcosm experiments initially poised at pH values ranging from 7.5-13.

In column experiments amended with acetate and lactate (proxies for the cellulose degradation products that may be generated in a GDF for ILW), the microbial communities that were present demonstrated the ability to ferment lactate to propionate under alkaline conditions, and confocal laser scanning micrographs suggest that they are also capable of colonising sandstone grains. The bacterial communities present within the sandstone columns varied between carbon-amended and control columns, and they also varied spatially within the columns. 454 pyrosequencing of 16S rRNA genes amplified from DNA extracted from sediment (from one carbon-amended and one control column) revealed that the carbon-amended column was dominated by members of the family *Clostridiaceae*, but were less dominant at the column outlet, perhaps suggesting a decrease in the availability of carbon substrate towards the end of the column. The sequence library obtained from the control column was far more

diverse than the carbon-amended column, and was dominated by members of the family *Comamonadaceae*.

Chapter 5 aimed to investigate the impact of microbial processes in column experiments under highly alkaline conditions, on the transport properties of crushed sandstone. These experiments followed on from the column experiments described in Chapter 4, and were carried out at a much slower flow rate, aiming to provide a more realistic representation of microbial interactions with sandstone in a flow-through system. Microbial activity in the carbon-amended columns appeared to occur to the greatest extent when the pH of the outlet fluids ranged between 9 and 10. When the pH of the outlet fluids increased to values comparable to the starting fluids, microbial activity (measured by organic acid utilisation and cell numbers) slowed dramatically. At the end of the experiment, the columns were subjected to a tracer test using Tc^{99m} and were imaged over a 12 h period using a gamma camera. Results revealed that the Tc^{99m} migrated much more slowly through carbon-amended columns compared to the controls (no added carbon). To investigate the processes that may have been responsible for this result, columns were subjected to X-ray radiography, which revealed that extensive generation of gas had occurred within the carbon-amended columns, resulting in clogging of the columns. 454 pyrosequencing of 16S rRNA genes amplified from DNA extracted from sediments collected from columns (one carbon-amended and one control column) revealed that both columns were dominated by members of the genus *Serpentinomonas*; the sequence library obtained from the carbon-amended column also contained members of the class *Clostridia*. The dominating bacteria in these experiments are obligately alkaliphilic and are capable of utilizing H_2 as an electron donor. As organic acid utilization slowed after the initial burst of activity, this could suggest that towards the end of the experiment when pore fluid pH was elevated, autotrophic processes dominated the system.

Chapter 6 involved an experimental approach, which aimed at investigating microbial impacts on transport in intact sandstone cores rather than in columns packed with crushed sandstone, aiming to provide data more representative of microbial impacts on the physical properties of the host rock environment. This experiment was carried out at a slightly higher pH than the previous experiments, as when the field sampling was carried out, this was the lowest pH fluid that could be found at the site.

At this pH, organic acids were utilised in the carbon-amended experiment, but no breakdown products were detected in the outlet fluids, suggesting further utilisation of these products. Microbial processes were carried out in the control experiment (with no added carbon), perhaps suggesting the occurrence of autotrophic processes when

carbon is limiting. A number of processes occurred during these experiments, though, that could be of importance to the containment ability of the host rock environment. Although the pore water pH of these experiments was slightly higher than in previous experiments, cells were still present, and numbers appeared to be influenced by changing pH values. Clogging of the experiment as a result of microbial activity (as seen in chapter 5) did not occur during these experiments. A number of causes may be responsible for this, for example, the pH was slightly higher during these experiments, meaning that microbial activity may have been confined to lower pH micro-niches. It is also possible that the experimental design contributed to the variation in transport properties between the experiments in chapter 5 and chapter 6. The experiments in chapter 6 were carried out under constant flow conditions, which are known to encourage biofilm shear (Radu *et al*. 2012). It is also possible that as the experiments were carried out with intact material, this may not have allowed for an accumulation of gases.

7.2 Overarching conclusions in the context of radioactive waste disposal

To summarise, the studies described in this thesis highlight some of the biogeochemical processes and subsequent physical impacts that microbial activity may have during the geological disposal of ILW in a cementitious repository. The potential implications for a GDF are wide ranging and some of these processes could lead to increases or decreases in the rate of radionuclide migration through the host rock.

7.2.1 Biogeochemical processes relevant to a GDF for ILW

During the experiments described in this thesis, it was clear that pore water pH significantly impacted on microbial activity. Over the lifetime of a GDF, the pore water pH will evolve, with a gradual decrease over time from hyper-alkaline to circum-neutral conditions (Figure 1).

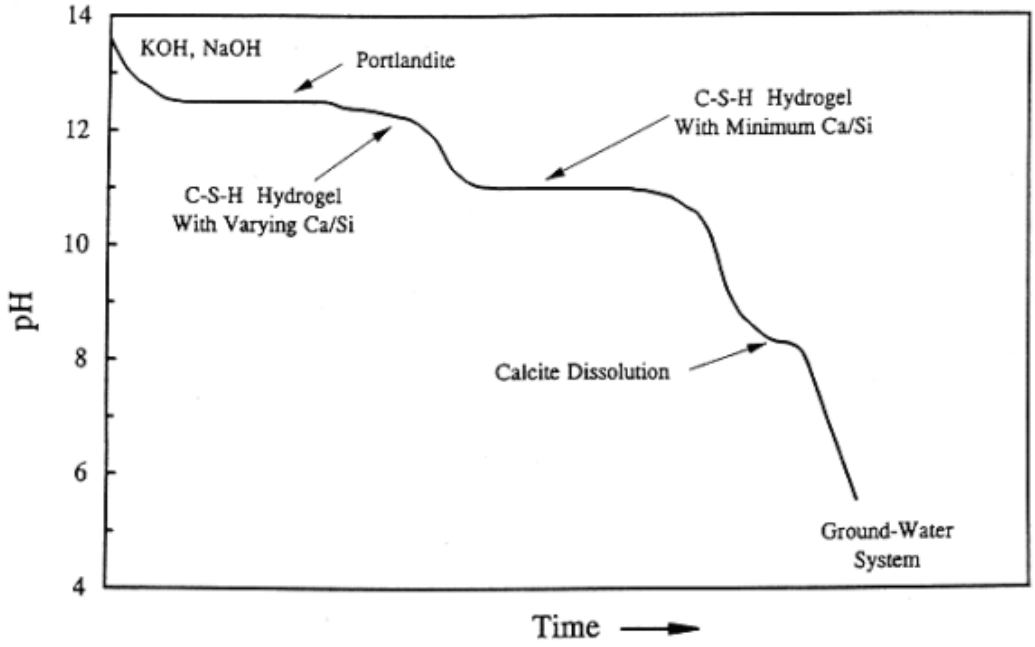

Figure 1 Pore fluid pH evolution over time (Wmsym, 2015)

A range of experiments were carried out representative of numerous stages of GDF evolution, and revealed that the rate of certain microbial processes were limited at higher pH values, for example column experiments suggested that the highest rates of organic acid utilisation occurred when pore water had a pH of 9-10, and organic acid utilisation slowed significantly after the pH increased above these values. The bacterial community at the end of this experiment was dominated by H_2-utilising bacteria, perhaps suggesting that as the pH increases, a shift in the community to obligately alkaliphilic H_2-utilising microorganisms occurred (perhaps as a result of H_2 generation during the initial utilisation of organic acids). These findings could be of importance to a GDF for ILW (particularly given the uncertainties regarding the availability of cellulose degradation products over the lifetime of a GDF) as they suggest that key microbial processes may be of importance at varying time points in the lifetime of a repository, and imply that the microbial communities present in the alkaline systems studied here can rapidly adapt to the local conditions and the available electron donors and acceptors.

Numerous studies have documented the potential impacts of microbial activity on the chemical containment of the host rock environment, and include alterations to pore water pH/ Eh evolution (impacting on radionuclide solubility; Krupka and Serne, 1998), microbial utilisation of organic acids (including the radionuclide complexant ISA; Bassil

et al. 2014) and may cause mineralogical alterations potentially altering the host rocks affinity for radionuclides. Previous investigations (e.g Rizoulis *et al*. 2012; Williamson *et al*. 2013; Williamson *et al*. 2014) have investigated microbial Fe (III) reduction under alkaline conditions and some of the resulting implications for radionuclide mobility. In this study Fe(III) reduction was investigated in flow-through systems; results demonstrated that Fe(III) reduction varies spatially within column sediments, probably because of variations in organic acid availability and utilisation with distance travelled. This variation in microbial Fe(III) may also occur in the host rock of a GDF as a result of spatial and temporal variations in organic acid availability, and will also be influenced by other aspects of pore water chemistry including pH. Furthermore, Fe(II) is known to play a role in the immobilisation of key radionuclides. Few studies have investigated the mobility of ^{99}Tc under hyper-alkaline conditions, and they have revealed that Fe(II)-bearing minerals are capable of reducing Tc(VII) to a poorly soluble precipitate (e.g. Thorpe *et al*. 2014). Additionally, some Fe(III)-reducing microorganisms are known to directly reduce certain radionuclides (e.g. Lloyd *et al*. 2000), decreasing their solubility and presenting a mechanism by which they may be immobilised, or their mobility significantly decreased.

7.2.2 Impacts on physical properties

Alterations to the physical properties of the host rock environment could potentially impact significantly on the migration of radionuclides through the geosphere. Numerous studies have characterised the impacts on the physical properties of rock as a result of interactions with hyper-alkaline pore waters, and demonstrate some of the impacts and changes that will occur over the lifetime of a GDF (e.g. Hodgkinson and Hughes, 1999; Savage *et al*. 2007; Moyce *et al*. 2014). Results from chapters 5 and 6 demonstrate alterations to sandstone as a result of interactions with the hyper-alkaline pore water, including the swelling of clays, accumulation of fines on reaction products, and the precipitation of products such as C(A)SH phases. These processes may have impacted on injection pressures into experiments, but these pressure increases do not appear to be sustained. The impact of these processes may vary under repository conditions, and will be influenced by certain conditions in the host rock environment, such as pore water velocity for example.

Microbial processes are known to significantly impact on the physical properties of geological environments (e.g. Baveye *et al*. 1998), but little is known about their impact under the highly alkaline conditions relevant to ILW disposal. In this thesis, experiments

revealed a number of processes which could potentially impact on the transmissive properties of the host rock, and subsequently impact on radionuclide transport through the geosphere. Results presented by Harrison *et al*. (2011) suggest that biofilm formation can significantly impact on the transport properties of geological media under circum-neutral conditions, whereas results from these experiments suggest that under hyper-alkaline conditions, there does not appear to be enough biomass build up to impact directly on transport under the conditions and time-frames studied.

Generation of gases, either via biological mechanisms or abiotic processes such as the corrosion of steel, does, however, have the potential to cause appreciable alterations to the transport characteristics of the host rock. This study highlights the importance of gas generation in a GDF, some of the resulting implications for radionuclide transport, and some of the potential mechanisms for the mitigation of over-pressurisation in a repository. Microbial metabolism of organic acids can result in the generation of gases including H_2 and CO_2, which could potentially be problematic in a GDF for a number of reasons. These could include the over-pressurization of a GDF host rock, which could potentially lead to structural damage (Williams *et al*. 2012). Results from experiments carried out as part of this thesis indicate that gas generation may have had a clogging effect in column experiments. However, in the geosphere it is unknown if microbial gas generation will have similar implications, as a build-up of gas bubbles may not occur, and is dependent on numerous factors. These include the rate of gas generation and groundwater velocity. Results from this thesis demonstrate that under highly alkaline conditions in the presence of hydrogen, that the microbial community may become dominated by microorganisms that can utilise H_2 gas, with $CaCO_3$ as a carbon source, potentially helping prevent over-pressurisation of a GDF, and at higher pH values this process may be carried out preferentially compared to heterotrophic processes. Clearly, further research is warranted to investigate the relationship between these processes in the subsurface in further detail.

7.2.3 Microbial ecology of hyper-alkaline systems of relevance to a GDF for ILW

The microbial ecology of a GDF may significantly impact on the biogeochemical processes that are able to occur, and subsequently impact on radionuclide migration through the geosphere. This thesis investigated the molecular ecology of a field site with components analogous to a GDF for ILW, and also of experimental systems aiming to represent aspects of a cementitious GDF. Analyses suggest that diverse bacterial communities are present at a hyper-alkaline spring, and community composition varies

with surface water pH, however probably because of the regular influx of water of circum-neutral pH, the site was not dominated by bacteria affiliated with known alkaliphilic species. Despite these findings, the results suggest that numerous neutrophilic bacteria may be capable of tolerating highly alkaline conditions. However, bacterial communities in column experiments appeared to rapidly adapt to the evolving conditions, with obligately alkaliphilic microorganisms dominant in column experiments after 33 weeks. Numerous studies have documented the potential significance of microbial activity during ILW disposal (e.g. Rizoulis *et al*. 2014). These results could be of importance when considering the evolution of a GDF for ILW as microorganisms will be present in a GDF and will arrive from a number of sources including contamination during the operational period of a GDF, and some will be associated with the subsurface and the waste-forms. The communities may adapt readily to the hyper-alkaline conditions that will be generated, suggesting that microbial processes may be of significance over the lifetime of a GDF, providing there are lower pH micro-niche environments when the alkalinity of the pore water is too high to facilitate microbial activity.

Comparisons of the bacterial communities present in column experiments suggest that pore water pH may significantly impact on the composition of bacterial communities that will be present in a GDF. In column experiments that ran for 6 weeks with an experimental fluid of pH 11.6, the bacterial community composition in sediments at the end of the experiment was diverse and not dominated by obligately alkaliphilic bacteria. The differences in community composition between carbon-amended and control columns may suggest that the communities in this experiment may be more heavily influenced by organic acid utilisation, rather that pH, which was relatively low compared to subsequent experiments. In longer term experiments carried out at a slightly higher pH, a similar result was seen when a community in the outlet fluid was investigated when organic carbon utilisation was high, and the pH was slightly lower than the starting fluid. This community was dominated by non-alkaliphilic heterotrophic bacteria, whereas at the end of the experiment, when the pH was elevated and organic carbon utilisation was low, the community present in the sediment and outlet fluid was dominated by obligately alkaliphilic bacteria (*Serpentinomonas* sp. which are capable of utilising H_2 as an electron donor and $CaCO_3$ as a carbon source, but are also capable of heterotrophic growth). This may also be the case in the control experiments with intact sandstone core; organic acids were not added to this experiment but biological processes were still occurring, suggesting that autotrophic microorganisms capable of utilising H_2 as an electron donor may have been present in this system. Overall, these results suggest that bacterial communities can adapt rapidly to the extreme conditions that will be present

over the lifetime of a GDF for ILW, and can carry out processes under these conditions that could potentially impact on radionuclide migration through the geosphere.

7.2.4 Limitations of experimental designs used in this thesis for investigations of GDF processes

Although experimental approaches provide valuable insights into some of the biogeochemical processes that could occur during the geological disposal of ILW, the limitations of these approaches must be considered, especially when thinking about extrapolating results to GDF spatial and temporal scales.

Microcosm experiments have been carried out extensively to investigate ecosystem processes; experiments aim to reconstruct certain aspects of an ecosystem (Haag and Matschonat, 2001). Simplifications of a natural ecosystem aid in understanding processes that occur in the natural environment (Fraser and Keddy, 1997), although these types of experiments have limitations that in some cases may mean results cannot be extrapolated to natural ecosystems. Previous studies have found that results from microcosm experiments mirror those from field scale experiments (Drenner and Mazumder, 1999), although the authors state the need for a range of approaches to fully understand the functioning of an ecosystem. Other studies recognise the need to understand ecosystem processes on a microscale to aid in understanding microbial processes in the field (Haag and Matschonat, 2001). Oksanen (2003) suggests that microcosm experiments are an appropriate starting point when first investigating a hypothesis on a particular ecosystem function, to make a more informed assessment on whether the hypothesis is worth pursuing (Oksanen, 2003). Advantages of microcosm experiments include the ability to control environmental variables, and the fact that experiments can include large numbers of replicates and can easily be repeated (Fraser and Keddy, 1997), these experiments also enable the manipulation of spatial heterogeneity (Drake *et al*. 1996).

Microcosm experiments do however have several limitations which may make them inappropriate for certain applications. For example, the small spatial scale that microcosm experiments are carried out on, are unrealistic (Benton *et al*. 2007); the spatial scale properties of an ecosystem may "influence even fundamental principles of community structure" (Oksanen, 2003), and experiments may be over simplified to such an extent that they are no longer representative (Fraser and Keddy, 1997). Carpenter (1996) suggests that due to the rapid rate of microbial processes in microcosm experiments, the population densities that occur are unrepresentative of the natural

environment. Natural ecosystems are far more complex than can be represented in microcosm experiments, therefore when extrapolating the results of laboratory studies to the field scale there is need for caution (Drenner and Mazumder, 1999). Previous studies have argued that several characteristics of microcosm experiments make them inappropriate for inferring what processes will occur on the field scale; for example the limited number of samples collected from microcosm experiments may not represent the heterogeneous nature of a natural ecosystem (Haag and Matschonat, 2001). The complexities of microbial community dynamics mean that if microbial processes are sensitive to minor variations in conditions, processes and community composition present in microcosm experiments may differ enormously from those that occur in natural ecosystems (Drake *et al*. 1996).

Column experiments provide an opportunity to carry out investigations on transport in porous media under controlled conditions, (Köhne and Mohanty, 2005) although the highly complex nature of geological materials may mean certain experimental factors have to be simplified to ensure accurate interpretation of results. Microbial impacts on transport characteristics involve highly dynamic processes, which cannot be investigated thoroughly using batch experiments. The nature of batch microcosm experiments means that the same fluid reacts with the porous medium throughout the course of the experiment, whereas the use of flow-through column experiments means that there is a constant input of fresh unreacted fluid, proving a more realistic view of what would actually occur in a geological environment with groundwater flow, enabling the incorporation of hydrologic effects (USEPA, 2010). Column experiments are of benefit as they allow the observation of effects including colloid transport and dispersion (USEPA, 2010) which are of interest in the field of geological disposal due to the potential influences of these factors on radionuclide transport, and may be of particular benefit when investigating the attachment mechanisms of biological materials to porous media. For example, Schijven (2000) found that sorption of viruses to soil particles were more realistic in flow-through column experiments, compared to batch and recirculating column experiments. Other studies have found that column experiments provide more realistic results regarding kinetics compared to batch experiments (Bermúdez-Couso *et al*, 2012).

Limitations of column experiments include the variation in results, which can occur due to experimental design factors such as column length and internal diameter. For example different transport mechanisms are found to be dominant with column length and flow rates; it has previously been shown that the use of a slow flow rate in short columns can increase the influence of diffusion as a transport mechanism (Relyea,

1982). The selection of an appropriate flow rate for column experiments may significantly impact on transport processes, and may be considered a limitation of these types of experiments. For example, groundwater velocities during column experiments investigating bacterial impacts on transport found that bacterial adsorption to porous media was influenced by flow rate (Hendry *et al*, 2005). The flow rates for the flow-through experiments carried out as part of this project were selected due to the amount of sample needed for analysis on a weekly basis, and was also quite high due to the short term nature of the experiments. Other limitations include the impact of experimental setup on the results; for example Bi *et al*. (2010) state that differences in column packing are known to impact on the quality and reproducibility of column experiments.

As a result of some of these limitations, there is a need for experimental work that investigates microbial impacts on GDF processes in underground research laboratories. The use of such facilities has proven to be useful for the Swedish geodisposal concept for studying microbial processes including gas metabolism, and to investigate the indigenous microbial populations under *in situ* conditions, and the implications for GDF processes (Pedersen, 2012).

7.3 Future directions

Results from this thesis demonstrate some of the potential processes that could impact on GDF performance including:

- Microbial processes that can impact on transport properties including gas generation, along with abiotic processes including the precipitation of C(A)SH phases.
- The presence of H_2-utilizing microorganisms, presenting a potential mechanism for mitigating GDF over-pressurization.
- Microbial Fe(III) reduction- presenting direct and indirect mechanisms for inhibiting radionuclide migration.

Although this thesis has explored certain aspects of potential microbial impacts in an alkaline host rock environment, clearly further work is needed to understand some of the mechanisms involved in these processes, and to understand their importance over the lifetime of a GDF. Some of these ideas are discussed below.

7.3.1 Modelling

Although the data collected as part of this thesis may provide an understanding of some of the microbial processes that could impact on the transport characteristics of a GDF host rock under hyper-alkaline conditions, to understand the impact of these processes over the lifetime of a GDF a modelling approach may be required; it is clearly unfeasible to carry out experimental work representative of every possible scenario that could occur over the very long lifetime of a GDF. Numerous models have attempted to describe microbial transport coupled with the physical and geochemical properties of subsurface environments. Some of the approaches that could be applied to data collected during this project are discussed below.

Numerous models have been developed that aim to simulate the changes that occur to the hydraulic properties of rock as a result of biomass accumulation and microbial activity. For example, Thullner *et al*. (2004) described a model that incorporates transport, biochemistry, chemistry and clogging to predict the impact of biofilm formation on hydraulic conductivity. Other models (such as the one described by Rockhold *et al*. 2005) take into account the impacts of gases generated and utilized by microbial metabolism to predict changes to hydraulic characteristics of porous media. Various models exist to describe microbial impacts on the redox chemistry of radionuclides, and the subsequent results of radionuclide migration in subsurface environments. For example, a model described by Wang and Papenguth (2001) couples the redox chemistry of radionuclides with subsurface biogeochemical processes, including Fe(III) and sulfate reduction, along with methanogenesis. Further models go on to describe the relationship between substrate utilisation, and attached (within a biofilm) and planktonic biomass, including the one describe by Taylor and Jaffé *et al*. (1990); such models could be useful when trying to predict attached biomass in an experimental system, as methods which attempted to image and quantify biofilm on sediment samples during this project proved to be relatively unsuccessful.

7.3.2 Gas generation and utilisation

This research has highlighted the importance of microbial gas generation and utilisation under GDF conditions, and some of the potential impacts on both physical processes and microbial ecology. Several other studies (e.g. Rizoulis *et al*. 2014) have recognised the potential of microbial H_2 utilisation as a mechanism to prevent GDF host rock over-pressurization from H_2 generation during steel corrosion/ microbial metabolism (Williams, 2012). Further research may be required to investigate the impacts of

microbial gas generation on the transport characteristics of the host rock, although much work has already been carried out studying the fate of gases generated within a GDF (e.g. Shaw, 2015). Work may be required to study the specific amounts of gas that could be generated as a result of the microbial metabolism of cellulose degradation products, taking into account the UK's inventory of cellulosic material in ILW. It is also recognised that when considering gas migration through a host rock, specific characteristics of the geology are a major influence (Environment Agency, 2008), so it may be of importance to carry out experiments investigating microbial gas generation/ utilisation in systems that are more representative (e.g. in terms of porosity and groundwater flow) of a future host rock, when it is decided upon.

The presence of potential H_2-utilising microorganisms in these experiments, and their dominance in sediments and fluids at the end of the long-term PEEK column experiments could suggest that they could survive and even thrive under ILW GDF conditions. This could suggest that, although cellulose degradation products may not be available over the lifetime of a GDF for microbial utilization, H_2 gas could potentially be used as an electron donor during respiration, and may be coupled to the reduction of radionuclides. Research has shown that H_2 can be used by some bacteria as an electron donor during the reduction of radionuclides (e.g. technetium as demonstrated by Lloyd *et al*. 1999), although little is known about these processes under hyper-alkaline conditions.

7.3.3 Transitions in conditions over a GDF lifetime

Results from this thesis have highlighted the sensitive nature of the microbial community composition and function in these hyper-alkaline systems. For example, shifts in bacterial community composition appear to take place rapidly when pore water pH changes. This has implications for a GDF as numerous environmental changes, both at the macro and micro scales will occur over the lifetime of a GDF, and will potentially have huge impacts on the microbial community composition and function. These changes will include the evolution of pore water pH over time, changes in concentrations of cellulose degradation products available for microbial metabolism, and also rapid changes in the initial post closure period as the repository transitions from oxic to anoxic conditions. On a larger scale, future climatic changes could also impact on repository performance, for example the presence of permafrost, or alterations in groundwater velocity or recharge chemistry (SKB, 2001). These alterations in environmental conditions are likely to impact significantly on microbial activity, which

may in turn have subsequent impacts on radionuclide mobility, illustrated, for example in chapter 5 of this thesis. Although Tc mobility was addressed in this thesis, further work could consider the impact of microbial processes on the transport of other redox active radionuclides, including U(VI) and Np(V). Experimental and modelling approaches to investigate some of these changes may be important to predict the impact of microbial activity on radionuclide migration in situ over the lifetime of a GDF.

7.4 References

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Methods to investigate biomass accumulation on grain surfaces

As part of this research project, numerous techniques were applied to attempt to image microbial cells and biofilm growing on grain surfaces, with varying degrees of success. Some of these techniques are discussed here, which could potentially be useful for investigating biomass accumulation on geological material, but as the methodologies did not contribute any significant data during this study, further development of the techniques may be required.

Experimental protocols were applied to investigate the formation of biofilm on sandstone under highly-alkaline conditions. Experiments utilising a Modified Robbins Device (MRD) were carried out (Figures 1 and 2). Full details of the experimental protocol are discussed in the methodologies chapter.

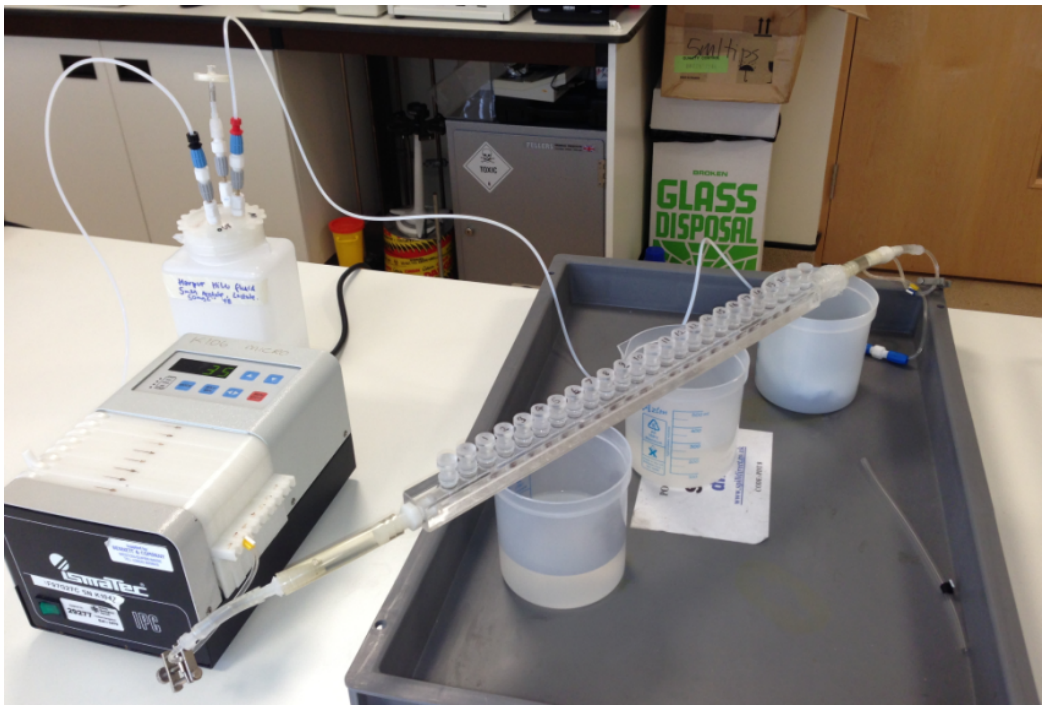

Figure 1 Setup of Modified Robbins Device (MRD) experiments

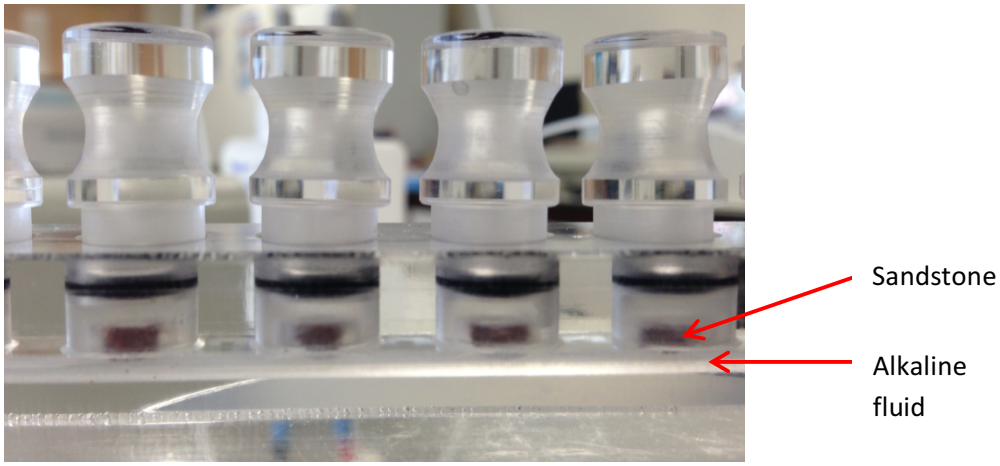

Figure 2 MRD plugs showing attached sandstone chips in contact with alkaline fluids

Some examples of microbial cells growing on the surface of the sandstone were observed (Figure 3) using ESEM, but the use of CLSM proved more difficult on intact material compared to imaging individual grains (as seen in chapter 4). It was difficult to remove residual fluorescent stain from the intact material, and was therefore difficult to determine the difference between microbial cells and the fluorescence from the residual stain, making quantification of biomass accumulation difficult.

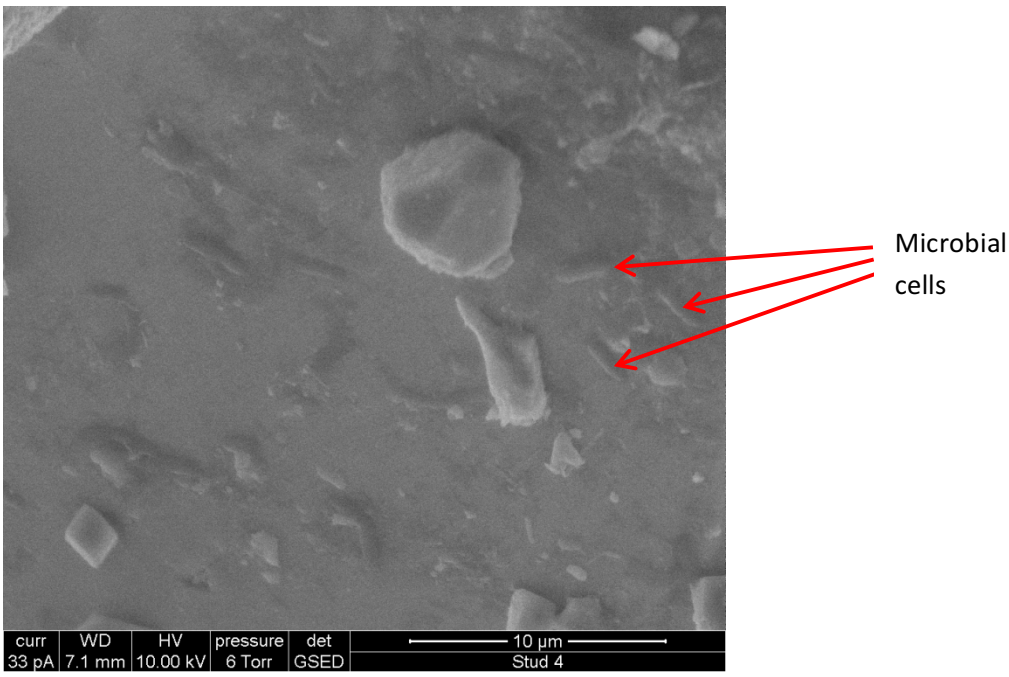

Figure 3 Environmental scanning electron micrograph showing microbial cells on a grain surface

At the end of the column experiments in Chapter 4, one of the carbon-amended columns was subjected to X-ray microtomography as described in Chapter 3. This

technique is use to visualise macro-scale structures, although imaging biofilm associated with the sediments in these experiments proved to be unsuccessful. This was probably a result of the lack of contrast between the liquid phase and any biomass present, although the use of contrast agents such as 1-chloronapthalene has been used successfully to increase this contrast in microtomographic applications (du Roscoat *et al*. 2014). Future work could involve the utilisation of such a contrast agent to visualise the spatial distribution of biofilm in flow-through systems relevant to radioactive waste disposal, and may provide useful insights into the impact of biofilm formation on the transport properties of porous material, and potential impacts for a GDF host rock.

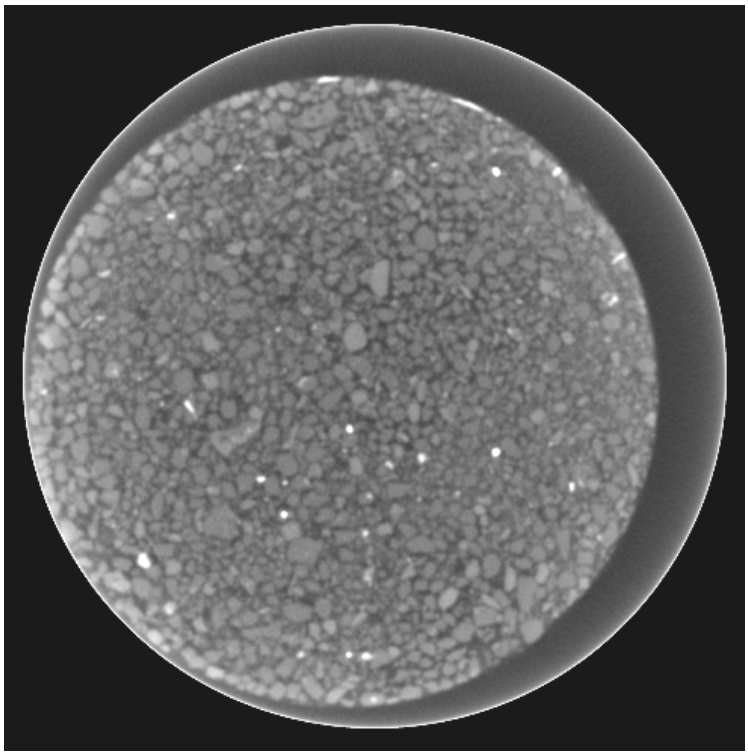

Figure 4 An example of an ortho-slice through a carbon-amended column from the experiments carried out in Chapter 4.

Other imaging techniques were applied to intact material collected at the pilot experiment using the BFA apparatus described in Chapter 3. Attempts were made to visualise the spatial variation in biofilm accumulation along the cores using a fluorescent carbohydrate binding stain (Concanavalin A (ConA) conjugated to FITC; Sigma-Aldrich, Dorset, UK). FITC has an excitation wavelength of 490 nm (range and emission wavelength of 525 nm).

At the end of the pilot flow-through experiment described in Chapter 3, section 3.7.1 the core was halved longitudinally under anaerobic conditions. One half of the core was

used for fluorescence scanning. The stain was firstly prepared by dissolving in a 0.9 % sodium chloride solution (1 mg mL^{-1}), and then diluting to 5 µg mL^{-1}. 20 mL of the dilute stain was placed in a petri dish, and the core was placed flat side down in the stain, and then incubated in the dark at room temperature for 30 minutes. The stain was rinsed from the core using autoclaved PBS. The core was then imaged twice using a GE Amersham Biosciences STORM 860 (GE, CT, USA), using excitation wavelengths 450 nm and 635 nm.

As fluorescence was detected from the core with both excitation wavelengths, some controls were prepared to visualise biofilm on membrane filters based on the method described by Strathmann *et al*. (2002). 10 mL of liquid *P. aeruginosa* culture was filtered on to an Isopore membrane filter (0.2 µm, Merck Millipore, MA, USA); the filter was then placed on a nutrient agar plate and incubated at 36°C for 24 h. This filter, along with 2 clean filters were stained with 50 µL ConA-FITC, and incubated in the dark for 30 minutes. The filters were then rinsed with PBS and then imaged in the same way as the cores.

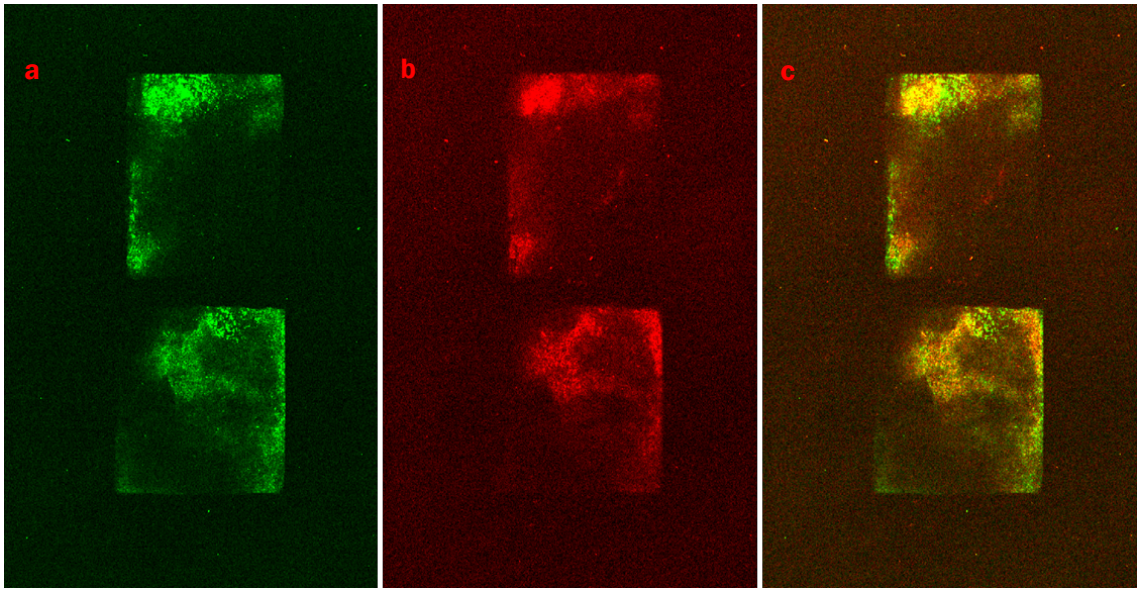

Figure 5 Fluorescence scans of a core stained with Concanavalin A. a: fluorescence scan taken in Blue-excited mode (excitation 450 nm, emissions longer than 520 nm), (b): Red-excited mode (excitation 635 nm, emissions longer than 650 nm), c: an image created by merging Figures 5(a) and 5(b).

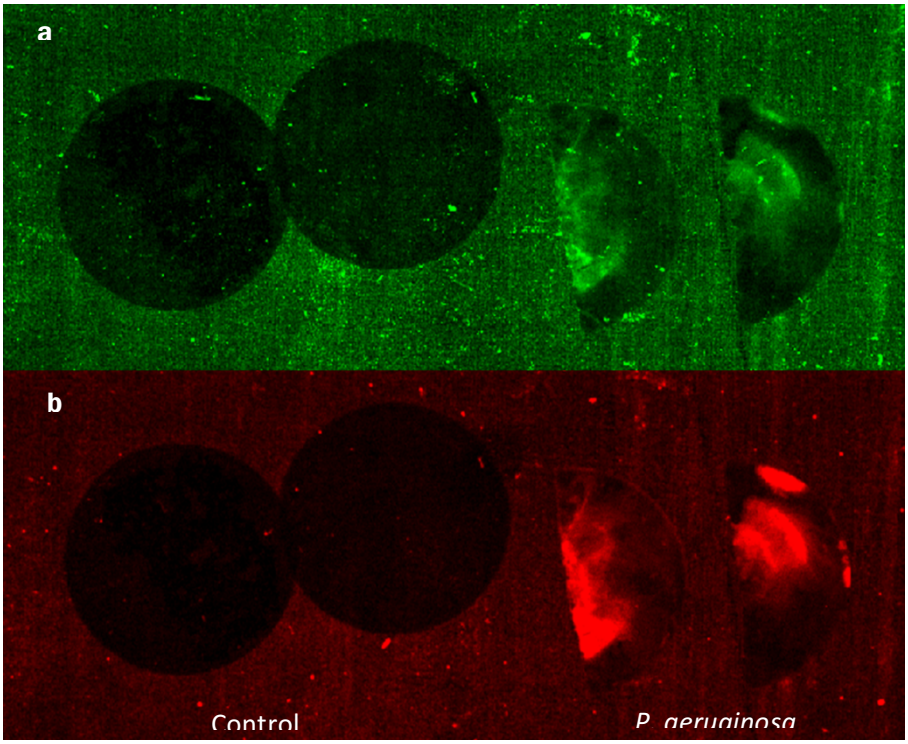

Figure 6 Fluorescence scans of sterile filters (control) and filters with *P. aeruginosa* at excitation wavelengths 450 nm (a) and 635 nm (b).

When the filtered were scanned, again fluorescence was present with excitation wavelengths of 450 nm and 635 nm on the filters where *P. aeruginosa biofilm* was present. This could perhaps suggest that factors such as bleed-through artefacts, or autofluorescence. Further work could involve the use of more controls, for example staining and fluorescence scanning of sterilized core as a control, to quantify any autofluorescence from the core.

References

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